

**Psychosocial stress and cardiovascular disease: The role of the
hypothalamic-pituitary-adrenal axis**

Amy Ronaldson

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Student Declaration

I, Amy Ronaldson, confirm that the work presented in this thesis is my own. Where information has been derived by other sources, I confirm that this has been indicated in the thesis.

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Date: _____

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Abstract

There is evidence to suggest that dysregulation of the HPA axis might be one of the biological pathways linking psychosocial stress with cardiovascular disease (CVD). This PhD consisted of three studies that aimed to assess the role of HPA axis dysregulation in CVD, and to examine potential biological mechanisms that might be involved in stress-related HPA axis dysregulation.

Study 1 assessed the utility of pre-surgical diurnal cortisol rhythm in predicting adverse outcomes in advanced heart disease using data from an observational clinical cohort study. The results showed that patients with flatter pre-surgical cortisol slopes were at increased risk of experiencing an adverse event in the years following coronary revascularisation. This finding provides evidence for the clinical relevance of HPA axis dysregulation in CVD.

Study 2 and 3 sought to garner more information about the biological mechanisms underlying stress-related HPA axis dysregulation using data from a randomised controlled trial involving the administration of pharmacological probes to healthy volunteers.

In Study 2 the effects of six-day administration of beta-blockers and SSRIs on diurnal cortisol secretion were examined. Results indicated that women taking SSRIs had significantly steeper diurnal cortisol slopes compared to placebo. Mechanistically, these results support the notion that the serotonergic system exerts substantial effects on the HPA axis, potentially through modulation of the serotonergic or corticosteroid receptors. Therapeutically, these results suggest that SSRIs might be a plausible intervention for female CHD patients with flatter cortisol slopes

In Study 3 the effects of seven-day administration of beta-blockers and SSRIs on cortisol stress reactivity and corticosteroid receptor sensitivity in the laboratory were investigated. The results indicated that generally, acute stress brought about a decrease in corticosteroid receptor sensitivity. SSRIs enhanced this decrease and also blunted the cortisol stress response. These results suggest that SSRIs may enhance adaptive stress-related changes in HPA axis function, thereby having therapeutic implications for stress-related illness such as CVD.

Together this body of work indicates that alterations in HPA axis function play a role in CVD and that the serotonergic system likely plays a role in stress-related dysregulation of the HPA axis.

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List of Abbreviations

11β-HSD	11 β -hydroxysteroid dehydrogenase
5-HTP	5-hydroxytryptophan
ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
ACTH	Adrenocorticotrophic hormone
AP-1	Activator protein-1
ARCS	Adjustment and recovery after cardiac surgery
AUC	Area under the curve
AVP	Arginine vasopressin
BDI	Beck Depression Inventory
BMI	Body mass index
BP	Blood pressure
CABG	Coronary artery bypass graft
CAR	Cortisol awakening response
CBG	Corticosteroid-binding globulin
CI	Confidence interval
CHD	Coronary heart disease
CNS	Central nervous system
CRH	Corticotropin releasing hormone
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DEX	Dexamethasone

DST	Dexamethasone suppression test
ERI	Effort-reward imbalance
ESSI	ENRICHD Social Support Instrument
EuroSCORE	European System for Cardiac Operative Risk Evaluation
GAD	Generalised anxiety disorder
GHQ	General Health Questionnaire
GLM	General linear model
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HADS	Hospital Anxiety and Depression Scale
HPA	Hypothalamic-pituitary-adrenal
HSP-90	Heat shock protein 90
IC₅₀	Inhibitory concentration 50%
IFN-γ	Interferon- γ
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
LC-NE	Locus coeruleus-norepinephrine
LPS	Lipopolysaccharide
M	Molar
MACE	Major adverse cardiac event
MCP-1	Monocyte chemotactic protein 1
MI	Myocardial infarction
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NF-κB	Nuclear factor-kappa B
OC	Oral contraception
p23	Protein 23

PANAS	Positive and negative affect scale
PBMC	Peripheral blood mononuclear cells
PHA	Phytohaemagglutinin
PRED	Prednisolone
PSS	Perceived stress scale
PST	Prednisolone suppression test
PTSD	Post-traumatic stress disorder
RCT	Randomised controlled trial
SAM	Sympatho-adrenal-medullary
SBP	Systolic blood pressure
SES	Socioeconomic status
SNP	Single nucleotide polymorphism
SNS	Sympathetic nervous system
SRO	Social reorganisation stress
SNRI	Selective norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
T2DM	Type 2 diabetes
TNF-α	Tumour necrosis factor- α
TSST	Trier Social Stress Test
WC	Waist circumference
WHO	World Health Organisation

Chapter 1

Literature review: Stress and cardiovascular disease

1.1 Introduction

This chapter will describe the literature relating to the role of stress in cardiovascular disease (CVD). Firstly, the pathophysiology of CVD will be described. Following this, evidence for the role of psychosocial stress in the aetiology of CVD will be provided with a particular focus on external stressors, such as work stress, financial stress, and caregiver stress; negative emotional disorders, such as depression and anxiety; and acute stress triggers, such as natural disasters, war and terrorism, and periods of intense emotion. Additionally, this chapter will describe the literature on the effects of psychosocial stress on prognosis in those already diagnosed with CVD. The aim of this chapter is to highlight the importance of psychosocial stress in CVD progression and prognosis, while highlighting some of the limitations of the work to date.

1.2 Cardiovascular disease: Pathogenesis and prevalence

CVD is an umbrella term referring to a group of diseases affecting the circulatory system. The most common forms of CVD are coronary heart disease (CHD) and stroke. Atherosclerosis is the primary pathological process underlying the development of CHD. It is a lifelong process whereby fatty deposits lead to the progressive narrowing of the coronary arteries due to the formation of atheromatous plaques. The lipid hypothesis of atherosclerosis holds that it is primarily a cholesterol storage disease. However, it is now understood that atherosclerosis is also an inflammatory disorder which can affect all middle- and large-sized blood vessels in the circulatory system (Hansson & Libby, 2006; Libby, Ridker, & Hansson, 2011). Atherosclerosis begins in childhood, with atherosclerotic change and development occurring during adolescence and young

adulthood (McGill et al., 2000; Williams et al., 2002). Across the lifespan, the cumulative effect of known cardiovascular risk factors accelerates the progression of atherosclerosis. These risk factors include clinical, biological, behavioural, and social factors. The clinical factors include hypertension, dyslipidaemia, type 2 diabetes, and overweight/obesity. Biological factors include genetic predisposition, older age, and being male. Behavioural factors include smoking, sedentary lifestyle, excessive alcohol intake, and poor diet. Low socioeconomic status (SES) and low education comprise the social factors. The majority of people who develop advanced atherosclerosis will be in an asymptomatic disease state for many years.

The human artery contains three layers (See Figure 1.1, Box 1). The inner layer is called the tunica intima and is lined by a layer of endothelial cells. The next layer is the media, followed by the adventitia which contains nerve endings, microvessels, mast cells, and fibroblasts. Dyslipidaemia, hypertension, and the presence of pro-inflammatory cytokines can cause irritation to the endothelial cells lining the tunica intima. These endothelial cells then express adhesion molecules which capture leukocytes on their surface, when ordinarily white blood cells stream past without attaching. These leukocytes, which are primarily monocytes, then migrate into the intima where they mature into macrophages. The macrophages become resident in the artery wall and engulf lipoprotein molecules thus becoming foam cells (See Figure 1.1, Box 2). They also have a number of pro-inflammatory functions producing high levels of cytokines such as IL-1 β and tumour necrosis factor. The development of atheromatous plaques also involves the migration of smooth muscle cells (the endogenous cells of the artery wall) from the media into the tunica intima where they proliferate forming a complex extracellular matrix through the release of macromolecules such as collagen and proteoglycans (See Figure 1.1, Box 3). This extracellular matrix forms a fibrous cap that covers the plaque. Underneath this cap,

the macrophages in the plaque begin to die via apoptosis thus releasing the lipids they have engulfed. The cellular debris and lipid molecules form a lipid-rich centre referred to as the necrotic core of the plaque. As cells and lipids accumulate the plaque enlarges and bulges into the lumen of the artery. Over time, the fibrous cap becomes thin and can fracture. If the plaque ruptures, the necrotic core of the plaque can leak into the lumen triggering the development of a thrombus (See Figure 1.1, Box 4).

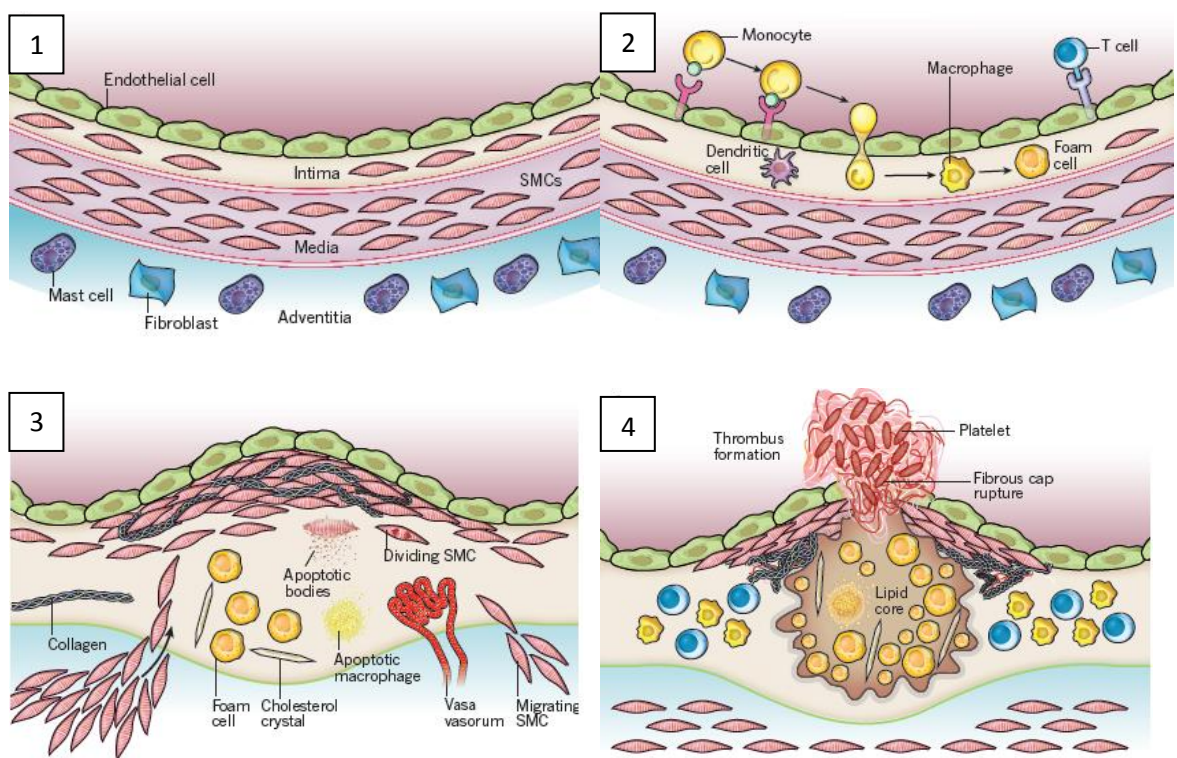


Figure 1.1. The stages of atherosclerosis. Box 1 shows the cell structure of a healthy human artery. Boxes 2, 3, and 4 show the gradual progression of atherosclerosis culminating in plaque rupture.

Adapted from Libby, Ridker, & Hansson (2011)

Plaques can cause clinical manifestations of CVD by either bringing about stenoses that limit blood flow to certain tissues leading to ischaemia, or by creating thrombi that lodge in arteries and interrupt blood flow. These clinical manifestations of CVD include acute coronary syndromes (ACS), namely myocardial infarction (MI) and unstable angina, as

well as stable angina. MI occurs when one of the coronary arteries is occluded by a thrombus following the rupture of an atheromatous plaque. The resulting ischaemia can lead to damage or death of cardiac tissue. Stable angina is a chronic condition characterised by chest pain on exertion caused by a lack of oxygen supply to the heart due to stenosis brought about by atherosclerosis. Unstable angina is distinct from stable angina in that chest pain occurs more frequently and for longer, and is not necessarily triggered by exertion. Unlike stable angina, unstable angina is caused by a thrombus partially occluding a coronary artery.

Recent estimates from the World Health Organisation (WHO) revealed that CVD is the leading cause of death worldwide (WHO, 2015). In 2012 an estimated 17.5 million people died from CVD, accounting for 31% of all global deaths. Roughly 7.4 million of these deaths were caused by CHD and 6.7 million were due to stroke. Recent statistics from the UK have revealed that CVD is the second main cause of death after cancer with these diseases causing 27% and 29% of all deaths in 2014 respectively (Townsend, Bhatnagar, Wilkins, Wickramasinghe, & Rayner, 2015). In 2014, CVD accounted for around 155,000 deaths in the UK – approximately 69,000 deaths were due to CHD, and 39,000 were due to stroke (*ibid*). This makes CHD the biggest single cause of death in the UK accounting for 15% of male and 10% of female deaths (*ibid*). In the UK, CVD mortality rates have been in decline since the 1970s. Recent statistics from the British Heart Foundation show that between 1974 and 2013 CHD mortality rates have declined by 73% in those dying at any age, and 81% in those dying before 75 years (*ibid*). This reduction in mortality is thought to be attributable to a combination of reductions in major risk factors such as smoking, as well as improved hospital treatment and better clinical management of hypertension and dyslipidaemia (O’Flaherty, Buchan, & Capewell, 2013; Smolina, Wright, Rayner, & Goldacre, 2012). Despite the reduction in CVD mortality

rates, the economic costs of the disease are vast. In 2013/2014, the CVD healthcare expenditure within the UK amounted to approximately £5.9 billion (Townsend et al., 2015). Moreover, the total cost of CVD to the UK economy was estimated to be £15.2 billion in 2014 with this figure being attributable to direct healthcare costs, productivity losses, and informal care of CVD patients (*ibid*). A recent report by the Centre for Economics and Business Research predicts that the total costs of CVD to the UK will rise to £18.7 billion by 2020 (Centre for Economics and Business Research, 2014).

1.3 Stress and cardiovascular disease: Introduction

As mentioned in the previous section, there are a number of well-established clinical, biological, behavioural, and social risk factors for CVD. Recently, there has been emerging interest in psychological risk factors for CVD with a particular focus on psychosocial stress. There has been accumulating evidence that psychosocial stress plays a role in the pathogenesis of CVD (Dimsdale, 2008; Hjemdahl, Rosengren, & Steptoe, 2011; Neylon et al., 2013; Steptoe & Kivimäki, 2013). Systematic reviews are in agreement that psychosocial stress predicts CVD incidence in initially healthy populations independent of standard risk factors (Everson-Rose & Lewis, 2005; Kuper, Marmot, & Hemingway, 2005). For the purposes of this literature review, psychosocial stress will be divided into three distinct categories: external stressors, negative emotional disorders, and acute stress triggers. Additionally, in this literature review I will also evaluate the evidence for the role of psychosocial stress in the prognosis of those already diagnosed with CVD.

1.4 Stress and cardiovascular disease: External stressors

In this section I will seek to review the literature looking at associations between external stressors and CVD risk. Firstly, I will describe studies that have focused on broad composite measures of perceived life stress and chronic stress burden. Following this, I will describe associations between more specific types of psychosocial stress and CVD risk. These include caregiver stress, financial and work stress, and social isolation and loneliness. Where possible, results of systematic reviews and meta-analyses will be reported.

1.4.1 External stressors: Perceived stress and chronic stress burden

The INTERHEART Study (Yusuf et al., 2004) examined the association between psychosocial stress over the previous 12 months and MI incidence in a standardised case-control study. Psychosocial stress was a composite self-report measure comprising stress at work and home, financial stress, the occurrence of major life events, lack of perceived control, and depression. This association was assessed in 15,152 MI cases and 14,820 CHD-free matched controls in 52 countries representing every inhabited continent. Results indicated that higher levels of psychosocial stress increased the risk of MI almost threefold after controlling for a range of traditional CVD risk factors, as well as geographic region. This association was seen in both men and women of all ages in all regions of the world.

Andersen and colleagues (Andersen, Diderichsen, Kornerup, Prescott, & Rod, 2011) prospectively examined the association between major life events across the lifespan and incident CHD in 8,738 participants from the Copenhagen City Heart Study. There was no significant association between major life events and incident CHD. The authors put the lack of association down to the measurement of major life events, arguing that it may not

be a measure of chronic stress. They also argue that the 16-year follow-up period was too wide a timespan for the stress to have a meaningful effect on cardiac health.

A meta-analysis carried out in 2012 examined the association of perceived stress and incident CHD (Richardson et al., 2012). Six (n=118,696) of the 23 potentially relevant articles met the criteria for selection indicating that many of the studies examining this association were not of adequate quality. Meta-analysis revealed that high levels of perceived stress were associated with a moderately increased risk of incident CHD. However, the studies included in the meta-analysis differed in terms of covariates included in the models. All studies controlled for age, blood pressure, smoking, and cholesterol. Only three studies controlled for social factors such as SES, and only one study controlled for psychological factors such as depression and anxiety.

A recent study prospectively examined the independent effects of individual-level stressors and neighbourhood-level stressors on incident CHD in a large sample from the Multi-Ethnic Study of Atherosclerosis with a 10.2 year follow-up period (Kershaw et al., 2015). Individual-level stressors included financial, work, relationship, and health-related stress. Neighbourhood-level stressors included neighbourhood safety and violence, social cohesion, and aesthetic quality. Higher individual-level stressors were linearly associated with incident CHD (n=6678). However, neighbourhood-level stressors were non-linearly associated with incident CHD, with medium levels of neighbourhood stress having a higher CHD risk (49%) than high levels of neighbourhood stress (27%). The authors find this result difficult to interpret and put it down to a stress measurement issue.

Associations specifically between psychosocial stress and stroke incidence have also been described. Truelsen and colleagues (Truelsen, Nielsen, Boysen, & Grønbaek, 2003) prospectively examined associations between self-reported stress and stroke incidence

and fatality 13 years later in 12,574 men and women from the Copenhagen City Heart Study. Self-reported stress was measured in terms of stress intensity and frequency pertaining to feelings of tension, nervousness, impatience, anxiety, or sleeplessness. Results indicated that high stress frequency and intensity were associated with almost a doubled risk of fatal stroke compared to low stress after controlling for a number of traditional risk factors. But, self-reported stress was not associated with non-fatal stroke after adjustment for these covariates. The authors posit that the lack of significant association between stress and non-fatal stroke may be in part due to differences in CVD risk profiles amongst participants.

A number of studies have also examined associations between psychosocial stress and both CHD and stroke incidence combined. Iso and colleagues looked at associations between perceived stress measured at baseline and stroke and CHD mortality in 73,424 initially disease-free Japanese men and women, with a follow-up of 580,378 person-years (Iso et al., 2002). Japanese women with high levels of perceived stress had a two-fold higher risk of death from stroke and CHD compared to those who reported low stress after adjusting for known cardiovascular risk factors. However, the same association was not observed in Japanese men.

Stressful life events and social strain were measured at baseline in 82,000 women from the Women's Health Initiative (Kershaw et al., 2014). After a follow-up period of 18 years, higher levels of stressful life events and social strain were associated with higher incident CHD and stroke. These associations were attenuated and became non-significant after adjustment for behavioural (e.g. smoking, dietary intake) and biological (e.g. hypertension, diabetes) CVD risk factors. The lack of association reported here lends support to the argument put forward by Andersen and colleagues (2011) that there was

too wide a timespan (18 years) between the stress exposure and the cardiovascular event for stress to have a meaningful effect on cardiovascular health.

In the Hispanic Community Health Study, chronic stress burden, but not perceived or traumatic stress, was associated with a higher prevalence of CHD and stroke prevalence in 5313 men and women of mixed Hispanic/Latino ethnic backgrounds (Gallo et al., 2014). Additionally, chronic stress burden was associated with a higher prevalence of known CVD risk factors such as type 2 diabetes and hypertension in those free from CVD. Associations between stress and subclinical CVD have also been reported. Life stress (a composite measure of childhood trauma, negative life events, daily hassles, and job strain) was found to be associated with increased arterial stiffness, but not carotid atherosclerosis, in 650 participants from the Netherlands Study of Depression and Anxiety after controlling for many cardiovascular risk factors (Bomhof-Roordink et al., 2015).

The studies outlined above focused on associations between broad composite measures of stress or perceived stress and CVD risk. Strengths of these studies include large sample sizes, with a number of studies being carried out across different cultures and ethnicities. On the whole these studies controlled for a large number of biological and behavioural cardiovascular risk factors. Overall, the evidence from these studies suggests that psychosocial stress is a significant risk factor for CVD incidence and mortality. However, a number of studies did not find such associations. How stress was conceptualised in these studies may be partially responsible for the lack of significant findings. The two studies that reported non-significant associations measured stress in terms of stressful or major life events (Andersen et al., 2011; Kershaw et al., 2014) whereas the other studies used either measures of perceived stress, or composite measures of a number of stress factors. What both these studies also have in common is the long follow-up length. Andersen and

colleagues (2011) argue that major life events may have a short-term effect on CVD risk, and if the life events occurred many years before the cardiovascular event this would explain why there is only a very weak association reported.

1.4.2 External stressors: Caregiver stress

I will now describe research which has focused on specific types of external chronic stressors and their associations with cardiovascular risk. The chronic stress of caregiving for an elderly, ill, or disabled loved-one has been found to be associated with poor health and premature mortality (Schulz & Beach, 1999). Lee and colleagues examined 54,412 CVD-free women from the Nurse's Health Study (Lee, Colditz, Berkman, & Kawachi, 2003). Information about caregiver stress was measured at baseline, and reports of incident CHD were collected throughout the four year follow-up period. Caregiving for an ill or disabled spouse for ≥ 9 hours per day was associated with an increased risk of incident CHD after adjusting for numerous cardiovascular risk factors. Interestingly, caregiving for an ill parent or other relative was not associated with higher CHD risk. This indicates that the high level of care required when taking care of a spouse may be more of a stressor and therefore increase CHD risk in women.

Another study examining the effects of spousal caregiving strain on CVD risk found that high strain was associated with a 23% higher covariate-adjusted Framingham stroke risk score in both male and female caregivers (n=716) (Haley, Roth, Howard, & Safford, 2010). However, there was no association between caregiving strain and Framingham CHD risk scores (n=607). Capistrant and colleagues (Capistrant, Moon, Berkman, & Glymour, 2012) examined the association between spousal caregiving stress and CVD risk in 8,472 CVD-free participants from the Health and Retirement Study. Long-term spousal caregiving, defined as ≥ 14 hours of care per week measured in two consecutive

biennial questionnaires, was associated with a two-fold risk (hazard ratio=1.95) of CVD onset, but only in white individuals.

Caregiving stress has also been associated with known CVD risk factors. Roepke and colleagues (Roepke et al., 2012) found that the duration of care in caregivers of those with Alzheimer's disease was associated with increased carotid intima-media thickness independent of risk factors. This indicates that caregiving stress may increase CVD risk through atherosclerotic burden. Dementia caregivers have also been found to have higher levels of plasma IL-6 and D-dimer compared to sex-matched non-caregiving controls (von Känel et al., 2006).

In terms of caregiving stress, the evidence does suggest that this type of chronic stress increases overall CVD risk, as well as levels of known CVD risk factors. Research indicates that the spousal caregiving is linked with increased CVD risk suggesting that caring for a spouse is a larger stressor than caring for another relative with a disability or illness. Studies in this area have largely focused on spousal caregiving. Further research should focus on CVD risk in other types of caregiver stress, such as CVD risk in carers/parents of sick children, or caregiver burden in mental illness. Interestingly, research in this area also indicates that ethnicity is an important factor in the association between caregiving stress and CVD risk, and therefore should be adjusted for in studies of this kind. Duration of care also appears to be important, indicating a cumulative effect of this type of chronic stress on CVD risk.

1.4.3 External stressors: Work stress, financial stress, and social isolation

Work stress is the most widely studied external stressor. The work stress literature has been largely dominated by the 'demand-control' or 'job strain' model in which a combination of highly demanding work and low control conditions elicits stress in the

workplace (Karasek & Theorell, 1990). A systematic review examining work-related psychosocial factors and development of CHD found that there was moderate evidence that high demand, and a combination of high job strain and low social support at work (iso-strain), were associated with increased CHD risk in men (Eller et al., 2009). This finding was in men only as studies involving women were too few at the time to draw any meaningful conclusion. Pejtersen and colleagues (Pejtersen, Burr, Hannerz, Fishta, & Hurwitz Eller, 2015) updated this systematic review and meta-analysis with the results of 11 new studies examining work-related psychosocial factors and the development of CHD. The main result of this meta-analysis was that the 'control' element of job strain explained excess risk for MI amongst the selected studies (44 studies in total). However, results also revealed that a large amount of the selected studies (42/44) lacked sufficient power to detect a meaningful excess risk of MI. The authors also posit that the overwhelming focus on psychosocial stress models such as the job strain model make it difficult to paint a clear picture of what psychosocial factors at work are affecting CVD risk. A recent overview of systematic reviews carried out in this field confirmed the overwhelming focus on the job strain model in psychosocial stress research. Based on the evidence to date, the authors of this overview concluded that there is modest to moderate evidence for an association between psychosocial work stress and CVD risk in men (Fishta & Backé, 2015).

The most compelling evidence for an association between work stress and CVD risk comes from a recent systematic review of the evidence from 27 studies from Europe, Asia, and the United States (n= >600,000) (Kivimäki & Kawachi, 2015). Results from this review found that work stress, with a focus on job strain and long working hours, was associated with a 10-40% excess risk of incident CHD and stroke, independent of conventional risk factors such as age, sex, and SES. They also reported associations

between work stress and type 2 diabetes, but not with cancer or chronic pulmonary obstructive disorder, which suggests outcome specificity in terms of work stress effects on health. A recent meta-analysis of the same magnitude (25 studies, $n > 600,000$) found that long working hours (≥ 55 hours per week) compared to standard working hours (35-40 hours per week) were associated with an increased risk of CHD and stroke incidence (Kivimäki et al., 2015), after controlling for age, sex, and SES. The association between longer working hours and stroke was stronger than the association between working hours and CHD and demonstrated a dose-response association.

Although distinct from work stress, financial stress has also been associated with CVD risk. A Swedish study reported that men without a cash margin (i.e. the ability to raise approximately £1000 in one week if an unexpected situation were to occur) had an increased risk of incident CVD after adjusting for relevant covariates (Carlsson et al., 2014). This link between financial strain and incident CVD was not present for women.

Thus, we see that there is evidence suggesting that both work and financial stress are associated with increased CVD risk. However, these associations have been largely reported in men. Further research is needed in female samples to elucidate the effects work stress in this population. Also, overuse of the job strain model in studies assessing associations between work stress and CVD risk may be hampering our ability to assess what other elements of work stress are important. Future research should include other work-related variables, such as long working hours.

Social isolation is another external stressor that has been associated with CVD progression. A meta-analysis of nine prospective cohort studies in CHD-free populations revealed that social isolation and loneliness were associated with a 50% excess risk of CHD on average (Steptoe & Kivimäki, 2012). A more recent meta-analysis of 11

longitudinal CHD and eight longitudinal stroke studies found that poor social relationships, defined as social isolation or loneliness, were associated with a 29% increase in the risk of incident CHD and a 32% increase in the risk of stroke (Valtorta, Kanaan, Gilbody, Ronzi, & Hanratty, 2016).

1.4.4 External stressors: Summary

In sum, many external stressors have been associated with increased CVD risk, incident CVD, and CVD mortality. However, there are a number of issues to consider when interpreting the evidence. There are differences in the way stress is conceptualised across studies which potentially affect the associations reported with CVD. For example, measuring stressful life events rather than measuring broad composite measures of stress, or focusing on the job strain model rather than taking a wider approach to work stress, seems to attenuate the association between stress and CVD. Additionally, the timing between measurement of stress and measurement of cardiovascular health appears to be of importance to results. Studies with longer durations between these measurements have reported null findings (Andersen et al., 2011; Kershaw et al., 2014). In support of this, Nielsen and colleagues (Nielsen et al., 2006) reported that significant associations between high levels of perceived stress and CHD were attenuated as follow-up continued. This implies that psychosocial stress may have a relatively short-term effect on CVD incidence. Another prevalent issue in studies measuring associations between psychosocial stress and CVD is choice of covariates included in analyses. Most studies tend to adjust for well-established cardiovascular risk factors such as age sex, smoking, cholesterol, hypertension, etc. However, some fail to adjust for known social and behavioural cardiovascular risk factors. Therefore, the extent to which the stress-CVD link is associated with different behavioural, clinical, and social risk factors is difficult to interpret. Despite the problems listed above, most studies examining the associations

between external stressors and CVD tend to report at least modest to moderate associations after controlling for traditional risk factors, providing support for the role of these types of stressors in the development of CVD.

1.5 Stress and cardiovascular disease: Negative emotional disorders

In this section I will review the literature examining associations between negative emotional disorders and CVD risk. Firstly, I will describe studies that have focused on psychological distress as measured by the 12-item General Health Questionnaire (GHQ) (Goldberg, 1992). The GHQ provides quite a comprehensive measure of psychological distress consisting of items that capture depressive symptoms, anxiety, social dysfunction, and loss of confidence. Following this, I will describe studies that have focused specifically on associations between depressive symptoms and CVD risk. Studies examining associations between anxiety and CVD risk will then be outlined.

1.5.1 Negative emotional disorders: Psychological distress

Hamer and colleagues (Hamer, Molloy, & Stamatakis, 2008) examined data from 6,576 healthy men and women from the Scottish Health Study and revealed associations between baseline psychological distress and CVD events 7.2 years later. However, this association was only significant when adjusting for age and sex and did not survive the addition of behavioural cardiovascular risk factors including smoking, alcohol intake, and physical activity. A meta-analysis of 10 large prospective cohort studies from the Health Survey for England revealed an association between psychological distress and CVD mortality in 68,222 people who were initially disease-free (Russ et al., 2012). This association remained after adjustment for a number of relevant covariates including SES, body mass index (BMI), smoking status, alcohol intake, physical activity, blood pressure, and diabetes status. Another study using data from the Health Survey for England

examined associations between psychological distress and CVD mortality in 66,500 initially disease-free men and women and found that a one category increase in GHQ scores predicted increased stroke mortality (hazard ratio=1.18) and CHD mortality (hazard ratio=1.24) at a median follow-up time of 7.9 years (Lazzarino, Hamer, Stamatakis, & Steptoe, 2013). These associations were found to be strongest in the lowest SES categories. Similar covariates were adjusted for as in the meta-analysis carried out by Russ and colleagues (2012), with the absence of certain behavioural factors such as physical activity and alcohol intake.

1.5.2 Negative emotional disorders: Depression and anxiety

There are a number of well-conducted systematic reviews and meta-analyses that show that depression is an independent risk factor for CVD (Dhar & Barton, 2016), and evidence suggests that as depressive symptoms worsen risk of developing CHD increases (Glassman & Shapiro, 1998). Nicholson and colleagues (Nicholson, Kuper, & Hemingway, 2006) carried out a meta-analysis of 21 aetiological studies examining associations between depression and future CVD. Together, these 21 studies comprised 124,509 participants and 416 cardiac events. Over a mean follow-up period of 10.8 years, results revealed an 80% higher risk of developing or dying from CHD in those with depression at baseline. Adjusting for other cardiovascular risk factors resulted in marginal reductions in relative risk.

A later meta-analysis examining 28 studies confirmed the findings of Nicholson and colleagues (Van der Kooy et al., 2007). Sixteen of these studies examined CVD-free populations at baseline and found an increased risk of CVD in those who reported depressive symptoms at baseline (risk estimate = 1.57). The authors reported that clinically diagnosed major depression showed the greatest risk for the development of

CVD, equalling the risk of smoking and diabetes. A recent meta-analysis of 30 prospective cohort studies (n=893,850) revealed a pooled relative risk of 1.30 for both CHD and MI incidence in those with depression (Gan et al., 2014). Interestingly, the pooled relative risk for both CHD and MI was stronger with a follow-up period of less than 15 years (relative risk = 1.36) compared with follow-up periods of 15 years or longer (relative risk = 1.09).

The most recent studies examining the role of depression in the aetiology of heart disease are in agreement with results from previous meta-analyses. In a study of 3572 men and women who had experienced an acute MI, 48% of the women and 25% of the men reported a lifetime history of depression (Smolderen et al., 2015). In a recent study using data from the Netherlands Study of Depression and Anxiety, there was a significant association between depression and new-onset CVD over a six year period in 2,510 initially CVD-free participants (Seldenrijk et al., 2015). Cox regression models revealed that having depression more than doubled the risk of developing new onset CVD (hazard ratio = 2.30).

Anxiety has also been found to be an independent risk factor for CHD. Roest and colleagues carried out a meta-analysis of 20 studies reporting on anxiety and incident CHD over a mean follow-up period of 11.2 years in 249,846 individuals (Roest, Martens, de Jonge, & Denollet, 2010). The authors found people high in anxiety were at an increased risk of CHD (hazard ratio = 1.26) and cardiac death (hazard ratio = 1.48) independent of biological, social, and behavioural cardiovascular risk factors. There was no association between anxiety and nonfatal MI. A recent large meta-analysis of 37 studies (n=1,565,699) examining associations between anxiety and new onset CVD found that anxiety was associated with a 52% increase in risk of CVD (Batelaan, Seldenrijk, Bot, van Balkom, & Penninx, 2016). Anxiety was also associated with an increased risk

of MI of 38%, and a 74% increased risk for stroke. Adjustment for publication bias reduced the strength of all the reported associations. Although they remained significant the authors do not provide the attenuated hazard ratios. Therefore, results of this meta-analysis should be interpreted with that in mind.

The latest meta-analysis examining anxiety and CVD risk included 46 cohort studies (n=2,017,276) and found that anxiety was associated with an increased risk of CVD mortality (41%), CHD (41%), stroke (71%), and heart failure (35%) (Emdin et al., 2016). However, in concurrence with Roest and colleagues (2010), anxiety was not associated with MI. The most recent study examining the role of anxiety in new onset CVD has reported that anxiety is a unique risk factor for stroke and MI in older primary care patients initially CVD-free (Stewart, Hawkins, Khambaty, Perkins, & Callahan, 2016). The authors examined the predictive value of anxiety and depression screening in 2,041 older primary care patients with a follow-up of eight years. Cox proportional hazards models revealed that a positive anxiety screen at baseline, but not a positive depression screen, was associated with a 54% increased risk of a CVD event in the first three years of follow up, after controlling for demographic and biological cardiovascular risk factors. However, after three years of follow-up this association disappeared. Conversely, a recent study found that depression, and comorbid depression and anxiety, was associated with new onset CVD, but anxiety alone was not (Seldenrijk et al., 2015). This indicates that inclusion of depressive symptoms as a covariate in research examining associations between anxiety and CVD is important.

1.5.3 Negative emotional disorders: Summary

In sum, the evidence does seem to suggest that negative emotional disorders are associated with increased CVD risk, incidence, and mortality. However, as with research

on external stressors, some studies failed to adjust for known behavioural, social, and clinical risk factors. Failure to include some of these well-established risk factors could inflate the magnitude of associations reported between negative emotional disorders and CVD and this should be taken in to account when interpreting results. Interestingly, as seen with some external stressor types, the duration of follow-up in studies examining associations between depression and anxiety and CVD risk seems to be of importance to results. The relative risk of CVD in those with depression was 27% higher with a follow-up period of less than 15 years (Gan et al., 2014), and association between anxiety and CVD risk disappeared after three years of follow-up (Stewart et al., 2016). These findings support the notion that psychosocial stress may have a relatively short-term effect on CVD risk.

1.6 Stress and cardiovascular disease: Acute stress triggers

Episodes of acute emotional stress have been shown to trigger adverse cardiovascular events in individuals with underlying CVD. In this section I will describe the literature examining associations between these acute stress triggers and cardiac events. Evidence for emotional triggering of cardiac events comes from both population-based studies and patient studies.

Population-based studies have revealed that major events such as natural disaster, war, terrorist attacks, and major sporting events can trigger cardiac events in those with underlying CHD (Steptoe & Brydon, 2009). Natural disasters such as large-scale earthquakes, tsunamis, and hurricanes are recognised as acute stressors. A number of studies have described associations between natural disasters and increased rates of cardiac events and cardiac mortality. In the week following the Northridge Earthquake in California in 1994, hospital admissions for acute MI in the surrounding areas increased

by 35% (Leor & Kloner, 1996). This same earthquake was also found to increase rates of sudden cardiac death from the normal daily average of 4.6 (± 2.1) to 24 on the day of the earthquake (Leor, Poole, & Kloner, 1996). The Hanshin-Awaji earthquake in 1995 resulted in a 3.5 fold increase in hospital admissions for acute MI in the four weeks following the earthquake (Suzuki et al., 1997). Hospital admissions for ACS also increased in the three week period following the Great Eastern Japan Earthquake and tsunami in 2011 (Nozaki et al., 2013). Additionally, the incidence of sudden cardiac and unexpected deaths doubled in the four week period following this earthquake (Niiyama et al., 2014). The authors also reported significant associations between the rates of sudden death and seismic activity following the earthquake, indicating that the acute stress associated with fear of a repeated earthquake may have been a causal factor in these sudden deaths.

Taken together, the studies provide evidence that acute stress brought about by an earthquake can trigger cardiac events. However, following the Loma Prieta earthquake in San Francisco in 1989, there was no increase in hospital admission for ACS (Brown, 1999). Steptoe and Brydon (2009) suggest that the timing of the earthquakes may provide an explanation for this disparity in findings. The Hanshin-Awaji and Northridge earthquakes struck in the early morning in winter, whereas the Loma Prieta earthquake occurred on an afternoon in the autumn. Susceptibility to acute MI is known to be raised in winter months, and in the early mornings (Elliott, 2001). The Great Eastern Japan Earthquake occurred on a spring afternoon, which is not in line with this argument. The subsequent occurrence of a large-scale tsunami and nuclear emergency may be the reason for this disparity.

Large-scale natural disasters have also been shown to affect long-term cardiac health. In the three years following the Hanshin-Awaji earthquake, mortality from acute MI

increased by 14% (Nakagawa et al., 2009). In the six years following Hurricane Katrina, there was a more than three-fold increase in admissions for acute MI (Peters et al., 2014). Together, these studies suggest that as well as being acute stress triggers, natural disasters can result in chronic stress that affects the cardiovascular risk profile. This chronic stress may be to do with fear of recurrence of the disaster, bereavement, financial loss, forced migration, and general social upheaval.

Research into acute stress triggers for cardiac events have also focused on the effects of war and terrorist attacks. During the Gulf War in 1991 incidences of acute MI and sudden cardiac death increased in response to Iraqi missile attacks in an area of Israel that was not hit by missiles, but was within hearing range of the explosions (Meisel et al., 1991). A number of studies have examined the cardiovascular effects of the terrorist attacks on the World Trade Centre in New York in 2001 (9/11). In the 60 days following these attacks, hospital admissions for acute MI increased by 49% in 16 New Jersey hospitals (Allegra, Mostashari, Rothman, Milano, & Cochrane, 2005), and increases in MI admissions were also observed in a Brooklyn hospital (Feng, Lenihanx, Johnson, Karri, & Reddy, 2006). However, a study of eight New York City hospitals found no acute increases in hospitalisation for cardiac events in the week following 9/11 (Chi, Speakman, Poole, Kandefer, & Kloner, 2003). Examining mortality data also found that there was no significant increase in cardiac deaths in New York in the months following the 9/11 attacks (Chi, Poole, Kandefer, & Kloner, 2003). These results are rather mixed. Holman and colleagues found that people who made subjective reports of high acute stress responses to the 9/11 attacks had a 53% increased incidence of cardiovascular events over the following three years, indicating that the degree to which the person found 9/11 stressful may account for the varying results across studies (Holman et al., 2008).

Although nowhere near as severe or traumatic as natural disasters or terrorist attacks, sporting events have also been found to have acute effects on cardiac health. During the 1998 World Cup, hospital admissions for acute MI increased by 25% in England on the day the English team lost to Argentina in a penalty shoot-out (Carroll, Ebrahim, Tilling, Macleod, & Smith, 2002). This effect extended to two days after the football match. A retrospective study examining the effects of football matches in England from 1994 to 1998 found that acute MI and stroke mortality was significantly increased (relative risk = 1.28) in men when the local football team lost at home (Kirkup & Merrick, 2003). Similar results have been reported in Germany with the incidence of acute cardiovascular emergencies increasing 2.66 times on World Cup match days involving the German team (Wilbert-Lampen et al., 2008). More specifically, research has shown that cardiovascular risk is increased in football fans only when the team in question loses. When Los Angeles played in the Superbowl and lost, deaths from CHD and acute MIs increased significantly (Kloner, McDonald, Leeka, & Poole, 2009). However, when Los Angeles played in the Superbowl and won all-cause mortality rates were reduced (*ibid*).

Patient studies in acute stress trigger research have revealed that acute periods of intense, anger, stress, depression, and sadness can trigger coronary events (Steptoe & Brydon, 2009). A meta-analysis of five case-crossover studies (the gold standard of research in this area) revealed that the pooled relative risk of an ACS being preceded by a period of anger, sadness, or stress was 2.48 (Steptoe & Kivimäki, 2013). A recent meta-analysis examined nine independent case-crossover studies looking at associations between periods of intense anger and adverse cardiac outcomes. The authors concluded that there was an elevated risk of ACS, ischaemic and haemorrhagic stroke, and arrhythmia in the two hour period following an outburst of intense anger (Mostofsky, Penner, & Mittleman, 2014). However, these findings were more pronounced in people with higher underlying

cardiovascular risk who experienced frequent outbursts of anger in general. The most recent study examining the effects of anger on cardiac events is in keeping with the results of Mostofsky and colleague's (2014) meta-analysis. Buckley and colleagues (Buckley et al., 2015) report results of a case-crossover study that revealed an increased relative risk (8.6) of experiencing an MI within 2 hours of experiencing very intense anger. Acute grief has also been shown to elevate risk of cardiac events. In a UK-based matched cohort study, the rate of MI and stroke in older adults who had recently lost their partners was increased almost two-fold, but only in the 30 days following the bereavement (Carey et al., 2014).

In sum, the evidence supports the idea that intense emotional stress brought about by large-scale events or personal emotional experience can increase rates of cardiac events, in particular acute MI. But, as Steptoe and Brydon (2009) point out in their review, it is difficult to rule out alternative explanations for cardiac events following large-scale natural disasters, acts of terrorism, and sporting events. It is quite possible that disruption of health services at the time, or perhaps physical trauma or exertion, or even drinking too heavily at a football match, could have brought about the cardiac events in question. Although the evidence from patient studies indicates that intense emotions can trigger cardiac events, it is also possible that other factors are involved.

1.7 Stress and cardiovascular disease: Prognosis in those already affected

As well as playing a role in the aetiology of CVD and the triggering of acute cardiac events, psychosocial stress can also worsen prognosis in those who already have CVD. In this section I will first describe literature examining the role of external stressors in CVD prognosis, with a particular focus on perceived stress, work stress, and the role of

social support. I will then discuss the role of negative emotional disorders in disease progression.

1.7.1 The role of external stressors in CVD prognosis

In a large study of 4,204 acute MI patients, levels of perceived stress over the month preceding the MI were measured during hospitalisation. Patients with moderate to high perceived stress had increased 2-year all-cause mortality (hazard ratio = 1.42) compared with patients low in perceived stress after adjusting for conventional risk factors (Arnold, Smolderen, Buchanan, Li, & Spertus, 2012). Furthermore, patients with high/moderate perceived stress levels also had worse angina-specific quality of life one year after their initial MI. Similarly, high perceived stress scores measured during hospitalisation in 3,572 acute MI patients were associated with worse angina-related quality of life one month after the MI (Xu et al., 2015). The role of perceived stress in heart failure prognosis has also been examined but the authors reported that it was not significantly associated with event free survival in 81 heart failure patients (Alhurani et al., 2014).

A recent systematic review and meta-analysis identified five papers derived from four different prospective cohort studies that examined associations between work stress and recurrent events in patients following their first cardiac event (Li, Zhang, Loerbroks, Angerer, & Siegrist, 2014). Meta-analysis (n=2,578) revealed that work stress increased the risk of future cardiac events by 65%. One of the studies included in the meta-analysis failed to find a significant association between work stress and further cardiac events in 292 female ACS patients in Sweden (Orth-Gomér et al., 2000). Interestingly, in these women marital stress was associated with a 2.9 fold increase in future cardiac events, even after adjusting for a large number of known cardiovascular risk factors. This may be because about a third of the sample was not in employment when baseline data were

collected. The authors also posit that women generally perceive spousal relationships as less supportive than men which may explain why marital stress, rather than work stress, was a predictor of future cardiac events in this study. Financial strain has also been associated with poor CHD prognosis. Financial strain over the previous year was measured in women who had been hospitalised for an ACS and was found to be associated with an almost threefold (hazard ratio = 2.76) risk of recurrent cardiac events after controlling for numerous potential confounders (Georgiades, Janszky, Blom, László, & Ahnve, 2009).

Social support appears to have a protective role when it comes to CVD prognosis. Barth and colleagues carried out a systematic review and identified 20 prognostic papers examining associations between social support and CVD mortality suitable for inclusion in a meta-analysis (Barth, Schneider, & von Känel, 2010). Results indicated that patients with low functional support had an increased risk of both cardiac and non-cardiac mortality after adjustment for relevant risk factors (pooled hazard ratio = 1.59). High social support and strong social relationships have also been associated with better cardiovascular prognosis (Holt-Lunstad, Smith, & Layton, 2010).

Thus, we see that as well as playing a role in CVD risk, external stressors play a role in CVD prognosis. However, this body of prognostic research is beset by similar issues seen in the CVD risk literature. Firstly, the way stress is conceptualised may be problematic – particularly in the prognostic literature relating to work stress. All the studies included in Li et al.'s (2014) meta-analysis conceptualised stress using the job strain model which means other psychosocial factors pertaining to work stress, and specifically the stress of returning to work after a cardiac event, were not considered. Secondly, the effects of some external stressors on CVD prognosis have not been examined. For example, the role of caregiver stress in CVD sufferers is yet to be explored. Associations between both marital

stress and financial strain and poor cardiovascular prognosis have been reported only in women, meaning that the relevance of these types of external stressors in men is as of yet unknown. Thirdly, studies measuring associations between external psychosocial stressors and CVD prognosis differ in terms of covariates adjusted for. A number of studies failed to control for important clinical and biological variables. Therefore, the extent to which stress affects prognosis may have been inflated in these studies.

1.7.2 The role of negative emotional disorders in CVD prognosis

Negative emotional disorders have been associated with worse prognosis in CVD patients. Depression is prevalent and persistent in CHD patients and a comprehensive review has shown that 19.8% of acute MI survivors meet the criteria for major depression, while approximately 30% have mild-to-moderate depressive symptoms (Thombs et al., 2006). A number of meta-analyses have provided evidence for the link between depressive symptoms and worse prognosis in CVD patients. Van Melle and colleagues included 22 papers examining associations between depressive symptoms in acute MI patients and long-term cardiovascular prognosis in a meta-analysis (n=6,367) (Van Melle et al., 2004). The results indicated that MI patients with depression had more than a 2.5-fold increase in cardiac mortality, and an almost two-fold risk for new cardiovascular events. Interestingly, neither follow-up duration nor method of measuring depression significantly affected the association between depression and mortality. A meta-analysis of 29 papers published in the same year also reported a two-fold increase of mortality in depressed patients in the two years after initial assessment (Barth, Schumacher, & Herrmann-Lingen, 2004). This association weakened after two years, but remained significant long-term.

In a 2006 meta-analysis of 34 prognostic studies, the pooled relative risk of all-cause or CHD mortality associated with depression was 1.80 (Nicholson et al., 2006). Interestingly, left ventricular function was only adjusted for in a small number of studies and inclusion of this covariate attenuated the relative risk by 48%. Although depression plays a role in CVD prognosis, this led the authors to suggest that depression was not yet an established independent risk factor for poor CHD prognosis as many studies failed to adjust for relevant risk factors. Meijer and colleagues identified 29 studies for inclusion in a meta-analysis examining the relationship between depression following the occurrence of an MI and cardiac prognosis (n=16,889) (Meijer et al., 2011). Similar to both meta-analyses carried out in 2004, the authors reported a 2.7-fold increased risk of cardiac mortality and a 1.6-fold increased risk of cardiac events in patients with post-MI depression. However, the strength of the association between depression and cardiac events decreased as follow-up duration increased – a finding also reported in Barth et al.'s (2004) meta-analysis. A recent meta-analysis sought to ascertain whether the cognitive/affective or somatic/affective symptoms of depression were more relevant for cardiovascular prognosis (de Miranda Azevedo, Roest, Hoen, & de Jonge, 2014). Thirteen prospective studies of 11,128 participants were included in the meta-analysis. In the fully adjusted analysis, somatic/affective depression symptoms, but not cognitive/affective symptoms, were associated with poor prognosis in CVD patients (hazard ratio = 1.19).

There is evidence that anxiety is also associated with poorer prognosis in CVD patients. A 2010 meta-analysis of 12 studies comprising 5,750 MI patients reported associations between anxiety and cardiac mortality as well as new cardiac events independent of clinical variables, including depression (Roest, Martens, Denollet, & de Jonge, 2010). Roest and colleagues followed up this meta-analysis with a study examining associations

between generalised anxiety disorder (GAD) and adverse cardiac outcomes in MI patients with a 7-10 year follow-up period (Roest, Zuidersma, & de Jonge, 2012). Results from simple age and sex adjusted models showed that GAD was associated with an almost twofold risk of adverse events. Adjustment for various other clinical factors, including depression, did not affect the magnitude of the association greatly. However, the authors did not adjust for any social or behavioural factors.

A systematic review of studies examining the role of worry and GAD in cardiovascular health found that three studies had reported associations between GAD and poorer prognosis in CHD patients, even after adjusting for depression (Tully, Cosh, & Baune, 2013). However, a year later Tully and colleagues carried out a meta-analysis on five studies examining the role of GAD in CHD patients and reported no significant associations (Tully, Cosh, & Baumeister, 2014). The latest meta-analysis in the area of anxiety and CVD prognosis included 44 articles examining prospective associations between anxiety and mortality in CHD patients (n=30,527) (Celano et al., 2015). After adjusting for a number of covariates, anxiety was not associated with mortality or poorer outcomes in CHD patients. The authors performed sensitivity analyses and found that when they separated the samples into post-ACS patients and stable CHD patients, the risk of poorer outcomes in anxious stable CHD patients was significantly elevated after adjusting for a number of relevant covariates. There were no significant increases in outcome risk in anxious post-ACS patients.

In summary, the evidence suggests that negative emotional disorders play a role in prognosis in those already with CVD. Three meta-analyses to date have reported 2 to 2.5-fold increases in risk of future cardiac events and mortality in CHD patients with depression (Barth et al., 2004; Meijer et al., 2011; Van Melle et al., 2004). However, the largest meta-analysis carried out so far (Nicholson et al., 2006) found that many studies

failed to adjust for relevant risk factors such as smoking and BMI, leading to inflated associations between depression and prognosis in CVD patients. More than 50% of patients suffering from depression or anxiety will also suffer from a comorbid depressive or anxiety disorder (Hirschfeld, 2001). Therefore, failure to adjust for symptoms of anxiety in many of these studies could also lead to inflated risk estimates. Adjusting for symptoms of depression seems to be more commonplace in prognostic studies measuring anxiety in CHD patients. This may be why the results of meta-analyses in this field are a little more mixed. Another reason for the mixed results seen in the prognostic meta-analyses related to anxiety may be failure to define samples correctly, i.e. separate stable CHD patients from post-ACS patients who are likely more symptomatic (Celano et al., 2015). Nevertheless, the literature suggests that both depression and anxiety play a significant role in CVD prognosis, but more work is needed with both well-adjusted statistical models and well-defined patient samples.

1.8 Chapter summary

Overall the evidence suggests that psychosocial stress contributes significantly to the aetiology of CVD, CVD mortality, and CVD prognosis in those already affected. External life stressors, depression and anxiety, and intense periods of acute stress all seem to play a role in cardiovascular health. However, all studies in this area of research have been either cross-sectional or longitudinal prospective observational studies, meaning that these studies provide evidence for associations between stress and CVD, but are not able to establish causality.

Results in this research area have been mixed and this is probably due to a number of methodological factors. On the whole, studies in this field tend to be well-powered and well-designed. But, there are issues with how stress is conceptualised and measured that

may affect the results of these studies. As mentioned earlier in this literature review, measuring life events rather than how people perceive life events as stressful, or focusing on the 'job strain' model rather than taking a wider approach to measuring work stress has likely affected results in the external stressor literature. Additionally, failure to adjust for covariates relevant to the development of CVD may have resulted in inflated associations between psychosocial stress and CVD outcomes. In general, most studies tend to adjust for traditional risk factors. But, health behaviours, social, and psychological factors known to be relevant to cardiovascular risk are often not controlled for.

One interesting issue that emerges from the stress-CVD literature is duration of time between measurement of stress and cardiovascular event. Longer follow-up durations seem to weaken associations between psychosocial stress and cardiovascular risk and this is seen in external stressor research (Andersen et al., 2011; Kershaw et al., 2014), and both depression (Barth et al., 2004; Gan et al., 2014; Meijer et al., 2011) and anxiety (Stewart et al., 2016) research. What this suggests is that psychosocial stress likely has cumulative effects that lead to biological alterations that increase CVD risk over time, and that stress needs to be sustained in order to have a long-term effect. The lack of significant findings in the studies with long follow-up durations implies that perhaps the stress had dissipated (i.e. major life events), or the depression or anxiety symptoms had been dealt with or had waned.

Nevertheless, this body of research does support the role of psychosocial stress in cardiovascular disease. The next step is to increase our understanding of the underlying biological mechanisms and pathways that link psychosocial stress with CVD. Then we may be able to devise targeted interventions to prevent psychosocial stress from developing into disease. In the next chapter I will discuss the role of a specific biological

pathway, the hypothalamic-pituitary-adrenal axis, in the link between psychosocial stress and CVD.

Chapter 2

Literature review: The role of the hypothalamic-pituitary-adrenal axis and the corticosteroid receptors

2.1 Introduction

In this chapter I will define and describe the hypothalamic-pituitary-adrenal (HPA) axis and its role in the stress response. I will then provide evidence for associations between chronic stress and dysregulation of the HPA axis, and associations between dysregulation of the HPA axis and CVD risk and prognosis. By doing this, I hope to show how dysregulation of the HPA axis might be one of the biological pathways linking psychosocial stress and CVD. I will then introduce the corticosteroid receptors and define and describe their role in the stress response. I will argue that stress-related modulation of these receptors, resulting in reduced glucocorticoid sensitivity, might be one mechanism through which HPA axis dysregulation is brought about. Thus, the aim of this chapter is to highlight the role of stress-related HPA axis dysregulation in CVD, and to provide evidence for the role of the corticosteroid receptor in HPA axis dysregulation.

2.2 Potential pathways linking psychosocial stress and CVD

There are a number of pathways through which psychosocial stress may contribute to the pathophysiology of CVD. One possibility is that the relationship between stress and CVD may be mediated through behavioural pathways. Psychosocial stress can influence CVD risk indirectly by increasing more adverse health behaviours (Stephoe & Kivimäki, 2012). A prospective cohort study (n=7,066) examining stress-related changes in health behaviours found that individuals with high levels of perceived stress were less likely to quit smoking over time, more likely to be sedentary, and less likely to keep alcohol consumption within the recommended limits (Rod et al., 2010). Psychosocial stress and

particularly depression has been associated with poorer adherence to medication in CHD patients (Gehi, Haas, Pipkin, & Whooley, 2005). Additionally, psychological distress has been associated with poor cardiac rehabilitation attendance in CHD patients (Glazer, Emery, Frid, & Banyasz, 2002).

There is substantial evidence from both observational and laboratory studies suggesting that there are direct pathophysiological links between psychosocial stress and CVD. Psychosocial stress factors have been associated with increases in autonomic and endothelial dysfunction, increased systemic inflammation, upregulated cellular adhesion, and also promotion of a pro-thrombotic state (von Känel, 2012). Of particular relevance to this PhD is the association between psychosocial stress and alterations in HPA axis activity. The HPA axis is the major neuroendocrine system in humans that is activated during times of stress and incorporates a major part of the stress response. Before describing associations between psychosocial stress, alterations in HPA axis function, and the development of CVD, I will provide a brief overview of the stress system and the stress response.

2.3 The stress system and the stress response

When homeostasis is threatened, or perceived to be so, the stress response is initiated. The stress response is an adaptive response that brings about changes in the sympatho-adrenal-medullary (SAM) system and the HPA axis which then go onto induce cardiovascular, metabolic, and immune changes that serve to protect the body from stress (Brotman, Golden, & Wittstein, 2007). The neural circuitry that initiates the stress response is mainly located in the hypothalamus and the brain stem. This circuitry includes corticotropin releasing hormone (CRH) neurons of the paraventricular nucleus of the

hypothalamus, and the locus coeruleus-norepinephrine (LC-NE) system in the pons and medulla (Tsigos & Chrousos, 2002).

The SAM system is comprised of the sympathetic nervous system (SNS) and the adrenal medulla. During times of stress, the SAM system is activated by the LC-NE system. Firstly, the LC-NE system releases epinephrine into the brain which results in heightened alertness and a decrease in functions such as sleeping and eating (Brotman et al., 2007). This system also stimulates the hypothalamus which activates the SNS and results in the secretion of epinephrine and norepinephrine from the adrenal medulla. The release of these catecholamines results in increased heart rate, blood pressure, blood viscosity, and inflammation (Brotman et al., 2007). That is, in times of acute stress the SAM system is responsible for initiating the 'fight or flight' response, readying the body for any injury that may occur.

During times of stress (see Figure 2.1), the HPA axis is activated by CRH from the paraventricular nucleus of the hypothalamus, which then leads to the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH then stimulates the release of glucocorticoids from the adrenal cortices, as well as some mineralocorticoids and androgens. Cortisol is the neuroendocrine end-point of the HPA axis and is the main circulating glucocorticoid in humans. Cortisol is a pleiotropic hormone. It has central energy-conserving effects as well as regulatory effects on the metabolism of protein, glucose, and fat for energy release. Cortisol exerts an immunomodulatory effect inhibiting the stress-related release of a number of inflammatory cytokines such as interleukin (IL) -6, IL-1, and tumour necrosis factor- α (TNF- α) (Kaltsas, Zannas, & Chrousos, 2012). Cortisol also increases blood pressure in times of stress via vasoconstriction (Girod & Brotman, 2004). In addition, cortisol exerts

a regulatory effect on itself suppressing the release of its biological precursors (CRH and ACTH) thus forming a negative feedback loop.

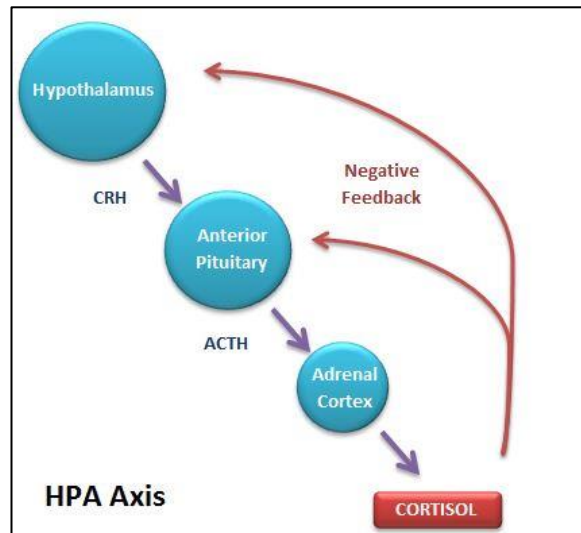


Figure 2.1. The hypothalamic-pituitary-adrenal axis

During times of stress, there is a lot of cross-talk between the SAM system and the HPA axis. For example, while catecholamines released by the adrenal medulla serve to stimulate secretion of IL-6 (März et al., 1998), the HPA axis serves to inhibit the release of pro-inflammatory cytokines (Kaltsas et al., 2012). Moreover, IL-6 has also been found to stimulate activation of the HPA axis independent of CRH release (Bethin, Vogt, & Muglia, 2000). In sum, both systems serve to regulate each other, and a number of inflammatory mediators. However, when these systems become dysregulated, there can be adverse cardiovascular consequences.

Although both systems are interrelated, this PhD will predominantly focus on the causes of HPA axis dysregulation and its implications for cardiovascular health.

2.4 Stress-related HPA axis activity

HPA axis activity increases in response to stress, resulting in increased levels of cortisol in humans, in order to exert the metabolic, cardiovascular, and anti-inflammatory effects required to maintain what Sterling and Eyer referred to as ‘allostasis’ (Sterling & Eyer, 1988). Allostasis is the process of achieving stability through a number of short-term adaptive physiological changes (McEwen & Wingfield, 2003). Alterations in HPA axis activity in response to stress is just one example of allostasis. Other examples include alterations in catecholamine levels, cytokine levels, heart rate, and blood pressure (McEwen & Wingfield, 2010). When allostasis is called upon too often, or is not managed efficiently, the demand of all these adaptive processes on the body can take its toll. This is referred to as ‘allostatic load’ or ‘allostatic overload’ (McEwen, 2000). Allostatic load can be interpreted as the ‘wear and tear’ of certain biological systems when faced with either too much stress, or failure to adapt to stress biologically, i.e. not ‘turning off’ the stress response when it is no longer required (McEwen, 2007). As the stress response is sustained over time, the biological ‘wear and tear’ on the body and brain can lead to the development of pathology and illness (McEwen, 2008).

Figure 2.2 illustrates four different conditions that lead to allostatic load (McEwen, 1998). For the purposes of this PhD the ‘physiological response’ referred to in the figure represents the cortisol stress response. Box (1) illustrates the normal cortisol response to stress, where cortisol increases, the increase is sustained for an appropriate amount of time in order for cortisol to exert its effects, and then the response is turned off. Box (2) represents repeated stress ‘hits’ from different stressors meaning that the cortisol stress response is frequently being triggered. Box (3) represents repeated stress hits from the same type of stressor and failure to habituate to that stressor. Box (4) represents a prolonged cortisol response to stress due to a delayed or failed shut down of the stress

response. Finally, Box (5) represents an inadequate or blunted cortisol response to stress. The prolonged (Box 4) and inadequate (Box 5) cortisol responses are likely what occurs after many repeated stress ‘hits’ (Box 2), or failure to adapt to stress (Box 3). A prolonged increase in cortisol after stress indicates that the hormone is unable to exert its self-regulatory function. An inadequate cortisol response to stress means that cortisol cannot exert its regulatory effects on inflammation, metabolism, and the cardiovascular system, potentially leading to hyperactivity of other stress-related mechanisms (e.g. increased inflammation).

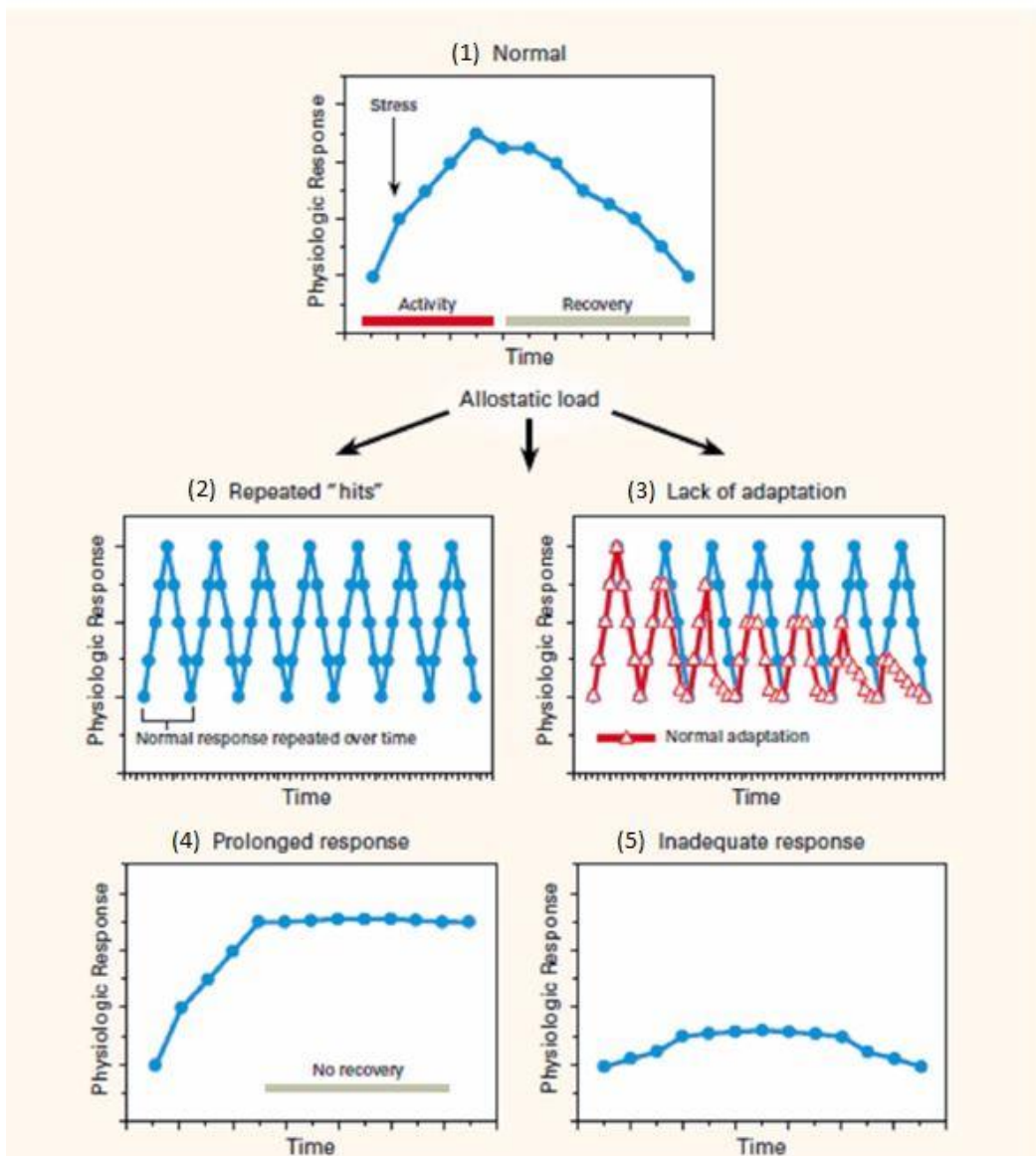


Figure 2.2. Four types of allostatic load. Box (1) represents the normal cortisol stress response. Box (2) represents cortisol responses to repeated different stress hits. Box (3) represents lack of adaptation of cortisol to a similar stressor. Box (4) represents a prolonged cortisol response due to a delayed shutdown. Box (5) represents an inadequate cortisol stress response.

Adapted from McEwen (1998).

2.5 Diurnal HPA axis activity

Under basal, unstressed conditions, the HPA axis shows marked diurnal patterning and levels vary substantially throughout the day (Figure 2.3). These patterns can be observed with repeated plasma samples, but are more commonly assessed noninvasively with assays of free cortisol in saliva. The circadian rhythm of the HPA axis is regulated by the suprachiasmatic nucleus in the hypothalamus, which responds to levels of light in the environment, and then goes on to stimulate the release of CRH from the neurons in the paraventricular nucleus (Spiga, Walker, Terry, & Lightman, 2014). Cortisol is at high levels on waking, followed by a rise that reaches a peak approximately 30 minutes after waking. This is referred to as the cortisol awakening response (CAR). There is then a subsequent decline across the day (the cortisol slope), with cortisol reaching its nadir at around midnight (Adam & Kumari, 2009).

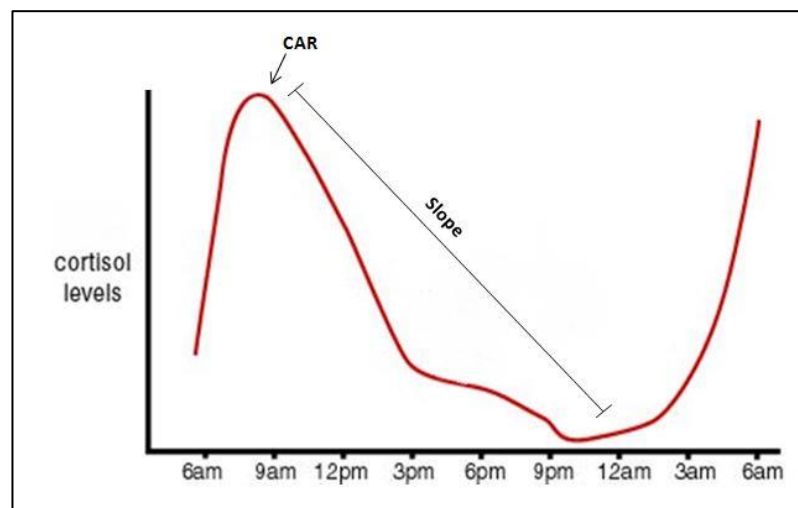


Figure 2.3. The diurnal nature of HPA axis function. Under basal (unstressed) conditions, cortisol secretion is characterised by a circadian rhythm. The decline of cortisol across the day is referred to as the cortisol 'slope'.

As well as dysregulation of the HPA axis manifesting as prolonged or inadequate cortisol responses to stress, dysregulation also occurs at the basal, unstressed level. Dysregulation of basal HPA axis function can cause alterations in the diurnal cortisol rhythm that take the form of blunted or heightened CARs, as well as flatter or steeper cortisol slopes (Adam & Kumari, 2009). These flatter cortisol slopes can be driven by lower waking cortisol levels, higher evening cortisol levels, or both. Another proxy for HPA axis dysregulation is area under the curve (AUC) which is an estimate of average cortisol exposure across the day (Adam & Kumari, 2009).

2.6 Psychosocial stress and dysregulation of diurnal HPA axis function

A growing body of evidence suggests an association between psychosocial stress and dysregulation of diurnal HPA axis function. A number of different types of psychosocial stress have been shown to be associated with alterations in diurnal HPA axis indices to date including acute psychosocial stress, a number of types of chronic stress, as well as stress-related disorders such as depression (Collomp et al., 2016).

2.6.1 Psychosocial stress and the CAR

The CAR can be determined by either calculating simple change scores between cortisol levels at waking and levels at 20-45 minutes after waking, or by calculating the AUC, or the overall volume, of cortisol released over this waking period (Clow, Thorn, Evans, & Hucklebridge, 2004). Chida and Steptoe carried out a meta-analysis of 62 studies that examined associations between different psychosocial stress factors and the CAR (Chida & Steptoe, 2009). These psychosocial stress factors included job stress, general life stress (including perceived stress), depression, anxiety, posttraumatic stress disorder (PTSD), fatigue or burnout, as well as positive psychological factors such as positive affect and optimism. The authors separated studies into those that examined the CAR calculated

using simple change scores (CAR_i) and those that examined the CAR by calculating the AUC over the waking period (CAR_{AUC}).

Meta-analysis revealed that the CAR_i was positively associated with levels of job stress and general life stress. Interestingly there were negative associations between the CAR_i and fatigue or burnout. Similarly, the CAR_{AUC} was found to be associated with higher general life stress. When the authors limited the meta-analysis to include only studies with high methodological quality scores, the positive association between the CAR_{AUC} and general life stress remained, but a negative association between the CAR_{AUC} and PTSD also emerged. These results indicate that different psychosocial stress factors have differing effects on the CAR. More recent research has replicated the negative associations between the CAR and people with burnout. Oosterholt and colleagues reported a blunted CAR in clinical and non-clinical burnout patient groups compared to healthy controls (Oosterholt, Maes, Van der Linden, Verbraak, & Kompier, 2015). Negative associations between the CAR and PTSD have also been replicated with a blunted CAR being reported in 24 adolescent girls with diagnoses of PTSD (Keeshin, Strawn, Out, Granger, & Putnam, 2014).

Although Chida and Steptoe's meta-analysis reported associations between enhanced CAR and general life and job stress, some research carried out since the meta-analysis reports contradictory associations. Academic stress was found to be associated with a reduced CAR in 42 healthy young men compared to 21 age-matched men not undergoing examinations (Duan et al., 2013). These reductions in CAR were negatively correlated with perceived stress and anxiety levels. Cropley and colleagues reported blunted CAR in school teachers with high levels of work-related rumination compared to teachers with low levels (Cropley, Rydstedt, Devereux, & Middleton, 2015). High levels of perceived stress have also been associated with blunted CAR in 64 healthy men and women

(Gartland, O'Connor, Lawton, & Bristow, 2014). Conversely, enhanced CAR has been reported in 24 healthy adults who had experienced early life trauma (Lu et al., 2013). This finding was later replicated in 58 people who had experienced childhood trauma (Lu, Gao, Huang, Li, & Xu, 2016).

Collectively, what these results indicate is that different types of stress potentially have differing effects on the CAR. Other factors may come into play such participant age, sex, or clinical status. Additionally, the timing and duration of the type of stress is probably important. Whether or not participants were going through a particularly stressful period at the time of data collection may have affected results. Acute anticipatory stress has been shown to result in an increase in cortisol levels after awakening (Wetherell, Lovell, & Smith, 2015). Working mothers reporting high job strain and high parenting stress have been found to have enhanced CAR increases on workdays compared with non-workdays (Hibel, Mercado, & Trumbell, 2012). What these results suggest is that stress-related situational factors affect the CAR. Therefore, future studies should seek to measure and adjust for these factors.

Interestingly, depression has also been found to have a varying association with the CAR. There has been a large amount of research carried out on CAR profiles in depression. A recent systematic review of the literature concluded that depression is associated with both a heightened and a blunted CAR (Dedovic & Ngiam, 2015). The authors suggest that this discrepancy might be related to depression severity. In fact, one study examining basal HPA axis function in depression described an inverted U-shaped association between depression and CAR_{AUC} (Veen et al., 2011). This non-linear association has been replicated in a much larger sample from the Netherlands Study of Depression and Anxiety (Wardenaar et al., 2011). It is possible that this non-linear association was the reason why there was no association between depression and the CAR in Chida and Steptoe's (2009)

meta-analysis. However, a recent case-controlled study examining associations between depression and morning cortisol in older adults did not observe this U-shaped association between morning HPA axis function and depression (Rhebergen et al., 2015).

2.6.2 Psychosocial stress and the cortisol slope

The cortisol slope is a measure of cortisol decline across the day and can be calculated in a number of ways. Typically, where a number of cortisol samples have been provided across the day a line of best fit is applied to each individuals' data points using linear regression and the slope of this line is used as an estimate of the cortisol slope across the day (Adam & Kumari, 2009). The CAR sample (waking +30 minutes) is generally not included in these calculations and the slope is based on the first sample of the day taken upon waking.

Flatter cortisol slopes have been associated with a number of different stressors and negative emotional disorders. In a 2007 meta-analysis, Miller and colleagues examined the effects of chronic stress on a number of diurnal cortisol parameters (Miller, Chen, & Zhou, 2007). They identified 119 papers (n=8,521) studying a number of different types of chronic stress including combat/war experience, abuse/assault, bereavement, caregiving stress, natural disasters, and job loss. Meta-analysis revealed that exposure to chronic stress was significantly associated with a flatter diurnal rhythm, as well as significantly lower morning cortisol levels and higher afternoon/evening levels which were likely resulting in the flattened slope.

Research carried out in this area since this meta-analysis has largely corroborated this result. In a large study (n=1,694) of men and women from the National Study of Daily Experiences, a greater frequency of daily stressors was associated with a flatter diurnal cortisol slope (Stawski, Cichy, Piazza, & Almeida, 2013). This association remained

robust after adjustment for negative affect, and clinical factors also. Perceived stress in the home has also been associated with a flatter cortisol slope in men, but not in women (Sjörs, Ljung, & Jonsdottir, 2014). Work stress has been associated with flatter cortisol slopes. In a large occupational cohort (n=2,126), effort-reward imbalance was found to be related to flatter cortisol rhythm throughout the day (Liao, Brunner, & Kumari, 2013). However, this association was modest after adjustment for a number of demographic factors. Family-to-work spillover, i.e. the extent to which work infringes on your family life, has also been associated with flatter cortisol slopes (Zilioli, Imami, & Slatcher, 2016).

In a study of 98 older adults from the Brain Health Substudy of the Baltimore Experience Corps Trial, those deemed as socioeconomically disadvantaged had flatter cortisol slopes across the day (Agbedia et al., 2011). Early life adversity has also been associated with flattened diurnal cortisol rhythms in children and adolescents (Koss, Mliner, Donzella, & Gunnar, 2016; McLachlan et al., 2016). However, a previous study reported no difference in cortisol slope between women who had experienced early life adversity and matched controls who had not (Gonzalez, Jenkins, Steiner, & Fleming, 2009).

Negative emotional disorders, in particular depression, have also been associated with aberrant cortisol rhythm throughout the day. In a sample of 257 Swedish men and women, depression was found to be associated with flatter diurnal cortisol slopes (Sjögren, Leanderson, & Kristenson, 2006). Flatter cortisol slopes have also been reported in women with major depressive disorder (Jarcho, Slavich, Tylova-Stein, Wolkowitz, & Burke, 2013), as well as in adolescents who have had recent episodes of major depressive disorder (Doane et al., 2013). Negative emotions such as sadness, loneliness, and high reports of general distress were also associated with flattened rhythms in this adolescent sample (*ibid*). More severe depressive symptoms have also been associated with more

pronounced flattening of the cortisol slope (Hsiao et al., 2010). In disagreement with previous research, a recent study reported no difference in cortisol slope between depressed and non-depressed individuals (Booij et al., 2015). It is possible that this study may have been underpowered to detect significant differences between groups (n=15 per group).

Conversely, associations between positive psychosocial factors and steeper cortisol declines across the day have been reported, lending support for the associations between negative stress factors and flatter cortisol slopes. Social support and positive coping styles were associated with steeper cortisol rhythms across the day (Sjögren et al., 2006). There have also been associations reported between high levels of positive affect, such as feelings of alertness and activeness, and steeper diurnal cortisol slopes (Hoyt, Craske, Mineka, & Adam, 2015).

2.6.3 Psychosocial stress and cortisol AUC

The cortisol AUC is not a measure of the circadian variation of cortisol but instead reflects the average levels of cortisol secreted throughout the day (Adam & Kumari, 2009). Nevertheless, associations between cortisol AUC and psychosocial stress factors have also been reported. As with research looking at the CAR, results from AUC research have been varied. Miller and colleagues (2007) meta-analysis cited earlier also elicited significant associations between exposure to chronic stress and a higher daily volume of cortisol output. However, more recent research has produced mixed results. Examination stress has been linked with reduced cortisol AUC in healthy young men (Duan et al., 2013). Job strain has also been linked with altered cortisol AUC. In 104 healthy adults, higher job strain was associated with higher cortisol AUC (Maina, Bovenzi, Palmas, & Filon, 2009). Conversely, a more recent study reported lower total cortisol AUC in older

adults with higher levels of job strain from the Multi-Ethnic Study of Atherosclerosis Stress Study (Rudolph et al., 2016). The authors posit that the age of the participants may be the reason for the discrepancy between these two studies. Interestingly in a sample of 68 healthy younger adults there was no association between job strain and cortisol AUC (Maina, Palmas, & Filon, 2007). However the cortisol AUC was significantly higher on working days compared with non-working days. This indicates that, like the CAR, situational factors have influence on the AUC. Temporality may also be an issue affecting results in these studies. In their meta-analysis, Miller and colleagues (2007) showed that as time since the stress exposure increased the strength of the association between stress and cortisol AUC decreased.

Alterations in cortisol AUC have also been reported in depression. In 45 female caregivers of stroke survivors, higher levels of depressive symptoms were associated with lower cortisol levels across the day (Saban, Mathews, Bryant, O'Brien, & Janusek, 2012). In a study of 401 men and women in Canada, lower cortisol concentrations across the day were associated with symptoms of depression, psychological distress, and burnout (Marchand, Durand, Juster, & Lupien, 2014). Conversely, elevated cortisol AUC has been reported in 57 depressed individuals compared to healthy controls (Dienes, Hazel, & Hammen, 2013). In a meta-analysis of 20 studies examining salivary cortisol in depression, the results suggested that salivary cortisol levels are generally increased in patients with a depressive disorder (Knorr, Vinberg, Kessing, & Wetterslev, 2010). However, using meta-regression the authors found that the difference in salivary cortisol levels observed was probably associated with age and intra-assay variability of the cortisol kits, rather than depression scores. Low social support has been associated with higher cortisol AUC in healthy students (Heaney, Phillips, & Carroll, 2010), whereas in older adults low social support was associated with a reduced cortisol AUC (Piazza,

Charles, Stawski, & Almeida, 2013). In this same older sample, higher levels of negative affect were positively associated with cortisol AUC. What these results suggest is that, like CAR, alterations in cortisol AUC related to depression are mixed and are possibly related to other factors like age, temporality, and other psychosocial factors such as social support and affect.

2.7 Psychosocial stress and dysregulation of stress-related HPA axis activity

Acute psychosocial stress induced in the laboratory leads to activation of the HPA axis and a subsequent increase in cortisol levels (Dickerson & Kemeny, 2004). There is a body of evidence suggesting that exposure to chronic stressors can bring about alterations in the magnitude of cortisol responses to acute stress.

2.7.1 Exposure to chronic stress and early life adversity

The most comprehensive meta-analysis to date examining the effects of chronic psychosocial factors on cortisol responses to acute stress in the laboratory revealed that positive psychological traits, i.e. openness, spirituality, self-esteem, and positive coping style, were associated with reduced cortisol stress reactivity in the laboratory (Chida & Hamer, 2008). However, there were no significant associations between negative stress-related factors and laboratory-induced cortisol stress responses due to inconsistency between studies. This lack of association may have been down to the nature of the chronic stressor or the duration between stress exposure and acute stress testing (Miller et al., 2007). Perhaps the positive psychological factors were associated with cortisol responses in the laboratory because they are stable traits rather than transient stress factors that can dissipate over time.

Much of the research looking at the effects of chronic stress exposure on acute cortisol stress reactivity has focused on early life adversity. In healthy adults with no

psychopathology, those with a history of moderate to severe childhood maltreatment exhibited blunted cortisol responses to psychosocial stress in the laboratory compared to those with no experience of maltreatment (Carpenter et al., 2007). In a follow-up study the authors replicated these findings in a non-clinical sample of women and found that those who had experienced childhood physical abuse had blunted cortisol responses to laboratory stress compared to those who had not (Carpenter, Shattuck, Tyrka, Geraciotti, & Price, 2010). Similarly, in a study of 80 healthy men and women exposed to a psychosocial laboratory stress, those who had high exposure to adverse childhood events (n=33) had significantly blunted cortisol responses to the stress tasks compared to those with no exposure to adverse events (Elzinga et al., 2008). Pre-stress cortisol values did not differ between groups.

In a highly cited study, Heim and colleagues compared four different groups of women (n=49) on cortisol and ACTH responses to acute psychosocial stress in the laboratory (Heim, Ehlert, & Hellhammer, 2000). One group had current major depression and had experienced childhood abuse, one group was free from depression but had experienced childhood abuse, one group had current major depression and had not experienced childhood abuse, and the control group had experienced neither depression nor abuse in childhood. The results indicated that after the acute stress protocol, abused women with current major depression exhibited significantly higher cortisol responses to stress compared with the other three groups. In terms of ACTH responses, both groups of abused women, regardless of depression status exhibited significant increases compared to non-abused depressed women and controls. These findings are in contrast with those of Suzuki and colleagues who found that cortisol responses to stress were blunted in those who had experienced childhood trauma, regardless of depressive status (Suzuki, Poon, Papadopoulos, Kumari, & Cleare, 2014).

Goldman-Mellor and colleagues compared three different groups of healthy men and women from the Whitehall II cohort (n=543) (Goldman-Mellor, Hamer, & Steptoe, 2012). Two of the groups had experienced early life stress whereas one had not (control group). Of the early life stress groups, one had a history of recurrent psychological distress over the previous 20 years, whereas the other group did not. Following an acute stress laboratory protocol, those who had experienced both early life stress and recurrent psychological distress had blunted cortisol responses to stress compared with the control group. Conversely, similar to the findings of Heim and colleagues, those who had experienced early life stress with little or no history of ongoing distress had elevated baseline cortisol levels and prolonged cortisol responses to stress compared to the control group. These results differ from the earlier studies mentioned above. This may be because in these earlier studies the ‘healthy’ participants who had experienced early childhood adversity may have had underlying depressive symptomatology or psychological distress that was not taken into account. The discrepancies between results in this area could also have to do with the way early childhood adversity is defined. Stress involving threat to the physical self or trauma are known to elicit different HPA axis responses compared to stress that threatens the social self (Miller et al., 2007).

2.7.2 The effects of depression

Cortisol stress reactivity has been found to be dysregulated in depression. In a small meta-analysis of seven studies (n=196), those with major depressive disorder were found to have prolonged cortisol responses compared to non-depressed individuals indicating delayed shutdown of the stress response (Burke, Davis, Otte, & Mohr, 2005). However, within this meta-analysis, older patients and more severely depressed patients were found to have blunted cortisol reactivity to acute stress, particularly when the laboratory session was in the afternoon. The results of a more recent study partially mirror those of this meta-

analysis. Amongst 351 adolescents from the TRAILS cohort, Booij and colleagues found that adolescents with recent-onset major depressive problems had prolonged responses to a laboratory-based stress protocol (Booij, Bouma, de Jonge, Ormel, & Oldehinkel, 2013). However, those who had persistent or recurrent depression throughout adolescence had blunted cortisol responses to the same stress protocol. These results suggest that initially, depressive symptoms might enhance (prolong) the cortisol stress response, but over time responsivity diminishes possibly due to repeated stress hits. This may be why blunted cortisol responses to stress were seen in older and more severely depressed patients in Burke et al.'s (2005) meta-analysis.

Amongst a sample of older people (n=68, >55y) with elevated cardiovascular risk, those who were clinically depressed were found to have blunted cortisol responses to acute laboratory stress compared to their non-depressed counterparts (Taylor et al., 2006). Similarly, a recent study showed that in a large older sample (n=725, 50-65y) from the Dutch Famine Birth Cohort Study, higher symptoms of depression and anxiety were associated with blunted cortisol stress reactivity in the laboratory (de Rooij, 2013). This finding is in support of the notion that older age, and therefore perhaps longer exposure to depression and anxiety throughout the lifespan, results in diminished cortisol reactivity to stress. A recent study examining cortisol stress reactivity in youth depression (n=115, 9–16y) found that depressive symptoms were associated with higher cortisol responses to a socially evaluated cold-pressor test, but only in boys (Lopez-Duran et al., 2015). This lends further support that age, and exposure to depression, plays a role in the association between depression and cortisol stress reactivity.

Overall, the evidence suggests that psychosocial stress factors and negative emotional disorders are associated with dysregulation of both basal and stress-related HPA axis function. Different stress types seem to exert different effects on the direction of

dysregulation of the HPA axis. For example, both heightened and blunted CARs have been reported across different types of stress. This also applies to cortisol AUC as well as cortisol stress reactivity. Factors that appear to influence the direction of dysregulation are age, temporal issues, and also the severity of the stressor. However, in terms of cortisol slope, flatter cortisol slopes seem to be uniformly associated with stress-related factors.

As mentioned previously, cortisol is a pleiotropic hormone that exerts regulatory effects on energy release, cardiovascular function, and the release of a number of pro-inflammatory cytokines, as well as regulating its own release via a negative feedback loop. Stress-related dysregulation of the HPA axis may then have further reaching biological implications that could promote the development of a number of diseases, including CVD. In the next section I will provide evidence for the role of HPA axis dysregulation in CVD.

2.8 HPA axis dysregulation and CVD

In a comprehensive review, Girod and Brotman lay out the ways in which the HPA axis is important for cardiovascular function and reduction of CVD risk (Girod & Brotman, 2004). Firstly, they note that a normally functioning HPA axis ‘primes’ the body for stress by preparing the metabolic, cardiovascular, haemostatic and autonomic components of the stress response required for the experience of everyday stress. Secondly, they outline the ‘suppressive’ role of cortisol in that it prevents inflammation and tissue repair processes from exceeding required levels and resulting in damage to the self. Thirdly, cortisol is known to play a role in insulin sensitivity, lipid production, and fat accumulation (Peckett, Wright, & Riddell, 2011). Based on these three roles of the HPA axis outlined above, dysregulation of the axis and abnormal cortisol secretion could therefore negatively alter cardiovascular risk (Girod & Brotman, 2004).

2.8.1 Diurnal HPA axis function in CVD

Standard observational methods have revealed associations between dysregulation of basal diurnal HPA axis activity and progression of CVD. High levels of cortisol reactivity in the hour after waking have been found to be positively associated with intima media thickness of the artery carotis communis in women (Eller, Netterstrøm, & Allerup, 2005; Eller, Netterstrøm, & Hansen, 2001). Morning levels of cortisol have been found to be elevated in men who have moderate to severe coronary atherosclerosis (Troxler, Sprague, Albanese, Fuchs, & Thompson, 1977). In the CARDIA study, there was a significant cross-sectional association between a flatter cortisol slope across the day and higher levels of coronary artery calcification in 718 healthy middle-aged adults (Matthews, Schwartz, Cohen, & Seeman, 2006). In the Multi-Ethnic Study of Atherosclerosis Stress Study, a unit increase in coronary calcium was associated with a 1.77% flatter decline in cortisol in 464 older men and women (Hajat et al., 2013). In 1,866 healthy participants from the Rotterdam Study, higher cortisol AUC values were associated with an increased number of atherosclerotic plaques in the carotid arteries (Dekker et al., 2008).

Dysregulated diurnal HPA axis function has been reported in clinical cohorts also. Patients with CHD have been found to have flatter diurnal cortisol slopes compared to healthy controls (Nijm, Kristenson, Olsson, & Jonasson, 2007). However, this finding was not replicated by Bhattacharyya and colleagues who examined cortisol slopes in patients with CAD compared to those without (Bhattacharyya, Molloy, & Steptoe, 2008). The CAR has also been found to be blunted in CVD patients (Vreeburg et al., 2009). Interestingly, dysregulation of the CAR seems to vary according to disease severity. CHD patients who had a history of MI had a more blunted CAR compared to CHD patients who had no previous MI (Merswolken, Deter, Siebenhuener, Orth-Gomér, & Weber, 2013).

Thus, the evidence suggests that dysregulation of basal HPA axis function is associated with markers of cardiovascular risk, as well as being characteristic of CHD itself. This implies that HPA axis dysregulation may be one of the biological pathways through which psychosocial stress causes the development of CVD. The impact of stress on the pathophysiology of CVD is also likely to be mediated in part by mild chronic systemic inflammation (Steptoe & Kivimäki, 2013). The role of inflammation in atherosclerosis is well established (Hansson & Hermansson, 2011) and markers of low grade inflammation have been associated with higher risk of CVD (Danesh et al., 2004). Seeing as glucocorticoids serve to regulate inflammation, it is likely that dysregulation of the HPA axis contributes to chronic systemic inflammation characteristic of CVD. In fact, in the cross-sectional study where Nijm and colleagues showed that flatter cortisol slopes were seen in CHD patients compared to healthy controls, they also reported that levels of evening cortisol (which were the driving force behind the flattened cortisol rhythm) were strongly correlated with serum levels of IL-6 and C-reactive protein (CRP) (Nijm et al., 2007).

An important study has shown that dysregulation of the HPA axis not only plays a role in the development of CVD, but is also associated with cardiovascular mortality. Kumari and colleagues examined diurnal cortisol patterns in 4,047 civil servants from the Whitehall II cohort and assessed mortality data over a follow-up period of 6.1 years (Kumari, Shipley, Stafford, & Kivimaki, 2011). The results showed that flatter cortisol slopes were associated with increased risk of all-cause mortality, but that this association was mainly driven by an increased risk of cardiovascular death. These results indicate that dysregulation of diurnal cortisol secretion is related to CVD mortality in originally disease-free individuals. To date, no one has examined the role of diurnal HPA axis dysregulation in the prognosis of those who already have advanced CVD.

Therefore, the first study of this PhD presented in Chapter 3 will examine whether pre-surgical diurnal cortisol profiles can predict adverse clinical outcomes in patients with advanced heart disease.

2.8.2 Cortisol stress reactivity in CVD

Evidence for the role of HPA axis dysfunction in CVD also comes from laboratory studies of cortisol stress reactivity. Dysregulated cortisol responses to stress have been associated with elevated CVD risk factors. As mentioned before, the role of systemic inflammation in atherosclerosis is well established. Inflammation increases in response to acute stress challenges (Steptoe, Hamer, & Chida, 2007). In a laboratory-based acute stress study healthy middle-aged participants were divided into cortisol responders and cortisol non-responders. Following the stress protocol, cortisol non-responders had higher levels of plasma IL-6 and a greater IL-1 receptor antagonist (IL-1Ra) response to stress compared with cortisol responders (Kunz-Ebrecht, Mohamed-Ali, Feldman, Kirschbaum, & Steptoe, 2003). This suggests that an adequate cortisol response to stress is required to regulate the inflammatory stress response. Those with blunted cortisol stress reactivity (i.e. the non-responders) had both increased systemic inflammation (IL-6), an increased inflammatory stress response (IL-1Ra), as well as lower heart rate variability, which are all factors associated with the development of CVD (Kunz-Ebrecht et al., 2003).

Interestingly, cortisol responders to acute laboratory stress have been found cross-sectionally to have increased levels of significant coronary artery calcification (Agatston score ≥ 100) after adjustment for a number of traditional risk factors (Hamer, O'Donnell, Lahiri, & Steptoe, 2010). Since interpretation of causality in cross-sectional data can be problematic, the authors decided to carry out a prospective follow-up of this study. They examined coronary artery calcification progression over the three year follow-up period

and found an association between higher, or more prolonged, cortisol stress reactivity and rate of calcification progression (Hamer, Endrighi, Venuraju, Lahiri, & Steptoe, 2012). In older initially healthy men and women, cortisol stress reactivity in the laboratory was found to be associated with higher incident hypertension at three year follow-up after adjusting for a number of clinical factors (Hamer & Steptoe, 2012).

The results of the four aforementioned studies provide conflicting results. On the one hand blunted cortisol stress reactivity is associated with a number of CVD risk factors, and on the other, heightened cortisol stress reactivity is associated with increased coronary artery calcification (a sub-clinical marker of atherosclerosis), and incident hypertension. It is possible that age is a factor in the discrepancy between these results. In the earlier study by Kunz-Ebrecht and colleagues, the sample was comprised of healthy middle-aged participants. In the studies carried out by Hamer and colleagues, the samples were comprised of healthy older adults. Age is known to be a strong regulatory factor of cortisol secretion (Veldhuis, Sharma, & Roelfsema, 2013). Nevertheless, the results of these studies provide evidence that dysregulation of the cortisol stress response, regardless of direction, is associated with adverse cardiovascular and atherosclerotic factors.

Cortisol responses to acute laboratory stress have also been measured in CHD patients. Thirty patients who had recently experienced a first-time ACS underwent a psychosocial stress protocol comprising anger recall and arithmetic. Compared with age-matched healthy controls, the CHD patients had blunted cortisol responses to stress, even after adjusting for confounding factors such as smoking or medication use (Nijm et al., 2007). A very recent study has replicated these findings. In 91 participants who underwent the Trier Social Stress Test (TSST) in the laboratory, those who had CHD (n=46) had blunted cortisol stress reactivity compared to those who were CHD-free (Waller et al., 2016). This

group difference remained significant even after adjustment for cardiovascular medication use. These findings are in line with the results of a population based study which showed that Lithuanian men had significantly lower cortisol responses to acute psychosocial stress in the laboratory compared to men from Sweden (Kristenson et al., 1998). Men from Lithuania have been shown to have a four-fold risk for CHD mortality, more atherosclerotic plaques, increased intima-media thickness, and higher levels of carotid artery stiffness compared to men from Sweden (Kristenson et al., 2000).

Taken together, these observational and laboratory-based studies suggest that dysregulation of the HPA axis, through changes in both the diurnal cortisol profile and cortisol stress reactivity, may increase CVD risk and progression. The evidence suggests that psychosocial stress factors and negative emotional disorders can bring about dysregulation of the HPA axis. Therefore, it is possible that dysregulation of the HPA axis may be one of the biological pathways through which psychosocial stress ‘gets under the skin’ and affects the pathophysiology of CVD. It is therefore important that we establish *how* psychosocial stress might bring about sustained changes in HPA axis function. One possible course is via changes in the sensitivity of the corticosteroid receptors.

2.9 The role of the corticosteroid receptors

Cortisol exerts its effects by binding to its receptors – the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). GRs are ubiquitously expressed around the body, whereas MRs are expressed only in selected tissues such as the kidney, colon, heart, and central nervous system (CNS). In their inactivated state, both receptors reside within the cell cytoplasm anchored in place by chaperone molecules. Once bound to cortisol, the receptor sheds its chaperone molecules and translocates into the cell nucleus (see Figure

2.4). Within the cell nucleus there are two distinct mechanisms of action through which the 'activated' receptor exerts effects on gene transcription. Firstly, the ligand-receptor complex can directly bind to glucocorticoid response elements (GREs) in target genes in order to enhance gene transcription. The activated receptor can also bind with negative GREs in order to inhibit gene transcription. Binding to GREs represents the classic model of corticosteroid receptor action (Bamberger, Schulte, & Chrousos, 1996) and allows cortisol to exert its regulatory effects. The second mechanism of action is largely of relevance to the anti-inflammatory effects of cortisol. A number of immune genes (e.g. IL-6, IL-2) do not have GREs yet their expression is suppressed by cortisol (Bamberger et al., 1996). This is because cortisol can also exert its effects by binding directly with transcription factors within the cell nucleus, such as Nuclear factor- κ B (NF- κ B) or activator protein-1 (AP-1), in order to down-regulate inflammatory gene transcription (Girod & Brotman, 2004).

The MRs are referred to as Type I receptors. They have a high affinity for endogenous glucocorticoids (i.e. cortisol in humans, corticosterone in rats) and aldosterone (salt and water regulation) and are therefore thought to regulate basal activity of the HPA axis as well as the onset of the stress response (de Kloet, 1998). The GRs are referred to as Type II receptors. They have a high affinity for dexamethasone (a synthetic glucocorticoid) but a low affinity for endogenous glucocorticoids. Therefore, they are thought to be important in the regulation of the stress response when levels of endogenous glucocorticoids are high, and the subsequent shutdown of the cortisol stress response via the negative feedback loop of the HPA axis (Carvalho & Pariante, 2008). One explanation of HPA axis dysregulation may be diminished sensitivity of the corticosteroid receptors. With reduced receptor sensitivity, cortisol is no longer able to exert its regulatory effects successfully leading to a breakdown in the HPA axis negative feedback loop, and an

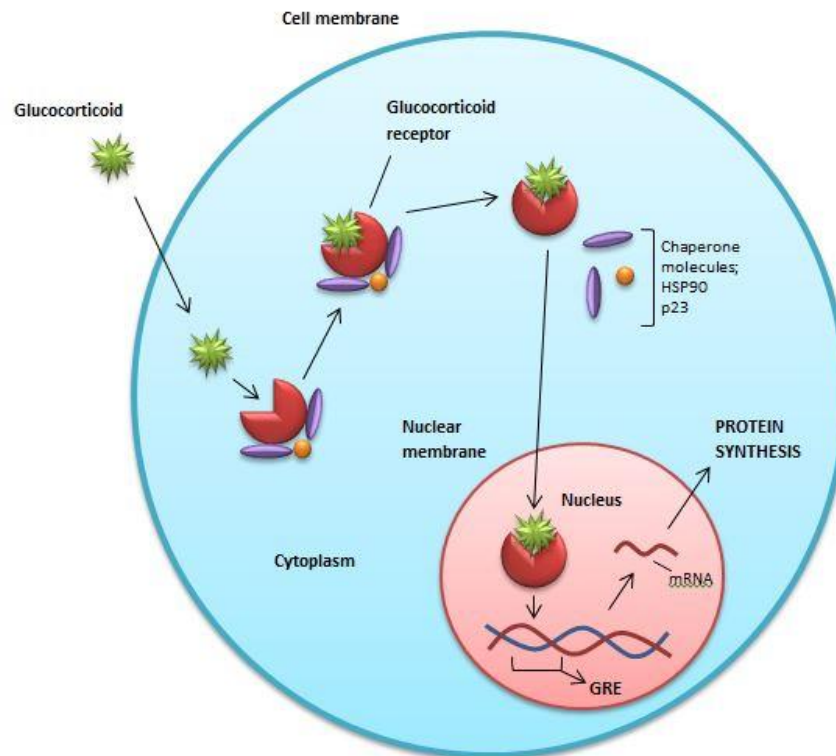


Figure 2.4. Translocation of the corticosteroid receptor into the cell nucleus. Once a glucocorticoid binds to its receptor, the receptor sheds its chaperone molecules (HSP90, p23) and translocates into the cell nucleus where it binds to GREs on target genes, or directly with transcription factors, in order to activate or repress gene transcription.
HSP90: heat shock protein 90; p23: protein 23; GRE: glucocorticoid response element

increase in the intensity of the inflammatory response (Cohen et al., 2012). An increase in the intensity or duration of the inflammatory response has consequences for the development and progression of chronic inflammatory diseases such as CVD (Danesh et al., 2004).

2.9.1 What causes modulation of corticosteroid receptor sensitivity?

There is a substantial amount of difference between individuals in corticosteroid receptor sensitivity (Quax et al., 2013) and there are a number of factors that modulate this sensitivity. Firstly, the extracellular and intracellular bioavailability of glucocorticoids will affect sensitivity of the corticosteroid receptors. For example, patients on long term exogenous treatment with synthetic glucocorticoids will quite often develop tissue-

specific glucocorticoid resistance (Oakley & Cidlowski, 2013). Circulating levels of corticosteroid-binding globulin (CBG) may influence the bioavailability of glucocorticoids (Bamberger et al., 1996). CBG is the major transporter protein for glucocorticoids and binds to approximately 80-90% of all circulating cortisol. The remaining 20% is comprised of albumin-bound and free cortisol. Only cortisol not bound to CBG is biologically active (Lewis, Bagley, Elder, Bachmann, & Torpy, 2005). Therefore, the amount of CBG in circulation will influence the amount of cortisol available to act on intracellular corticosteroid receptors. CBG levels are under complex regulatory control and exposure to inflammatory cytokines such as IL-6 and IL-1 β have been shown to influence CBG secretion and messenger RNA (mRNA) levels (Emptoz-Bonneton, Crave, LeJeune, Bréban, & Pugeat, 1997). CBG release is also known to be affected by psychosocial stress (Kumsta, Entringer, Hellhammer, & Wüst, 2007).

Variations in the levels of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) may also affect receptor sensitivity. 11 β -HSD is an enzyme that can convert cortisol both to its active and inactive forms. 11 β -HSD-1 converts cortisone, which is biologically inactive, to cortisol. 11 β -HSD-2 oxidises cortisol into the inactive metabolite cortisone. Changes in the levels of these enzymes within the cell exert effects on the bioavailability of cortisol, thereby affecting corticosteroid receptor sensitivity (Oakley & Cidlowski, 2013). Increased levels of 11 β -HSD-1 have been associated with an increase in GR sensitivity (Whorwood, Donovan, Wood, & Phillips, 2001). Interestingly, increased levels of 11 β -HSD-2 have been found in the offspring of maternal Holocaust survivors who underwent severe trauma (Bierer et al., 2014).

Corticosteroid receptor sensitivity may also be affected by the number of receptors in the cell, or the 'hormone binding capacity' of the cell (Bamberger et al., 1996). Lower cell receptor concentrations have been found in patients with depression (Pariante & Miller,

2001), and depression has been associated with decreased glucocorticoid sensitivity (Pace, Hu, & Miller, 2007). Glucocorticoids themselves have been shown to bring about significant downregulation of corticosteroid receptors (Bamberger et al., 1996). This may be an adaptive function preventing tissue damage from overexposure to glucocorticoids, which may over time become maladaptive. The hormone binding affinity of the receptors also likely plays an important role in modulation of receptor sensitivity. Every receptor has a ligand-binding domain which is the area to which glucocorticoids bind. Co-incubation with IL-2 and IL-4 has brought about alterations in the ligand-binding domains of human lymphocytes leading to reduced hormone binding affinity of the GR (Kam, Szeffler, Surs, Sher, & Leung, 1993). What this indicates is that increased inflammation may bring about reduced corticosteroid receptor sensitivity through reducing hormone binding affinity.

Differing ratios of splice variants of the corticosteroid receptors may also affect receptor sensitivity. The GR gene NR3C1 consists of nine exons which are subject to splicing, which gives rise to a number of splice variants of the gene, two of which are the GR α and GR β isoforms (Quax et al., 2013). In isolation, GR α facilitates the action of glucocorticoids, whereas GR β is inactive. However, when GR β is co-expressed with GR α , GR β inhibits the action of GR α which suggests that a higher GR β :GR α ratio may lead to glucocorticoid resistance (Oakley & Cidlowski, 2013). The GR β isoform is present in many cells, but is usually found in lower levels than the GR α isoform. However, cytokines have been found to influence the expression of GR splice variants. IL-2 and IL-4 were found to increase the expression of GR β isoforms in peripheral blood mononuclear cells (PBMCs) by more than 100% (Leung et al., 1997). The pro-inflammatory cytokines TNF- α and IL-1 β were shown to increase GR α expression by 150% while increasing GR β by 350% in HeLA cells which express both isoforms

endogenously (Webster, Oakley, Jewell, & Cidlowski, 2001). The results of these studies indicate that inflammatory cytokines could bring about a decrease in corticosteroid receptor sensitivity through upregulation of the GR β splice variant of the receptor.

Individual variation in GR sensitivity may also be influenced by genetic difference. Functional polymorphisms of the GR gene have been shown to influence the effects of glucocorticoids. Individuals with the ER22/23EK polymorphism of the GR gene have been found to demonstrate glucocorticoid resistance, whereas individuals with the N363S single nucleotide polymorphism (SNP) have demonstrated enhanced GR sensitivity (Manenschijn, Van Den Akker, Lamberts, & Van Rossum, 2009).

2.9.2 How do we measure corticosteroid receptor sensitivity?

Corticosteroid receptor sensitivity can be indirectly assessed both *in vivo* and *in vitro*. Assessment involves measuring associations between a specific input (e.g. different concentrations of synthetic glucocorticoids) and suppression of a specific output, such as ACTH or cortisol production, mitogen-induced lymphocyte proliferation, or lipopolysaccharide (LPS)–induced inflammatory cytokine production (Rohleder, Wolf, & Kirschbaum, 2003). These associations allow us to examine glucocorticoid sensitivity in peripheral blood cells thus providing us with a proxy measure of corticosteroid receptor sensitivity. Note that from now on the terms ‘glucocorticoid sensitivity’ and ‘corticosteroid receptor sensitivity’ will be used interchangeably.

In vivo, the most widely used method to examine glucocorticoid sensitivity is the dexamethasone suppression test (DST) (Rohleder et al., 2003). This test involves peripheral administration (usually oral) of a low dose of the synthetic glucocorticoid dexamethasone which in theory should then suppress the release of ACTH from the pituitary via negative feedback. In turn, the release of cortisol should also be suppressed.

The results of the DST can be interpreted as an index of glucocorticoid sensitivity with non-suppression of cortisol release being indicative of diminished GR sensitivity (Ebrecht et al., 2000).

In vitro assays have also been developed in order to examine glucocorticoid resistance within immune cells. In this assay the effect of dexamethasone on lymphocyte proliferation or production of inflammatory cytokines, both of which should be inhibited by glucocorticoids, is used as an index of GR sensitivity (Carvalho & Pariante, 2008; Rohleder et al., 2003). The most common assay used today was developed by DeRijk and colleagues who use LPS to stimulate the release of inflammatory cytokines in whole blood, or PBMCs isolated from whole blood, (DeRijk, Petrides, Deuster, Gold, & Sternberg, 1996). LPS is an endotoxin produced by gram-negative bacteria known to induce an inflammatory immune response from cells (Raetz & Whitfield, 2002). The inhibition of LPS-stimulated secretion of inflammatory cytokines by different concentrations of dexamethasone is used as an index of glucocorticoid sensitivity. Failure to inhibit, or partial inhibition, indicates reduced *in vivo* GR sensitivity and non-suppression in the DST has been correlated with reduced dexamethasone-induced inhibition of lymphocyte proliferation *in vitro* (Carvalho & Pariante, 2008). Within this thesis, these *in vitro* assays will be referred to as glucocorticoid sensitivity assays.

As dexamethasone has a high binding affinity for the GR, the DST and glucocorticoid sensitivity assays outlined above only provide a proxy measure of sensitivity of this specific receptor. The prednisolone suppression test (PST) has been developed which allows the evaluation of both the GR and the MR. Prednisolone is a synthetic glucocorticoid which is more similar than dexamethasone to cortisol and therefore binds to both the GR and the MR (Pariante et al., 2002). Thus, the inhibition of LPS-stimulated secretion of inflammatory cytokines by different concentrations of prednisolone provides

an indirect measure of GR and MR sensitivity. Prednisolone can also be used *in vitro* in glucocorticoid sensitivity assays.

In vitro glucocorticoid sensitivity assays are usually performed using whole blood or using PBMCs isolated from whole blood. Whole blood allows for rapid measurement of peripheral glucocorticoid sensitivity in white blood cells. However, it does not account for differences in cell population ratios within the white blood cells which could influence variability within the sample being measured (Burnsides et al., 2012). Therefore, it is preferable to carry out these assays using specific isolated PBMCs such as lymphocytes or monocytes.

One flaw of glucocorticoid sensitivity assays is that they are not tissue specific. These assays are carried out using whole blood or PBMCs meaning that the results give an indication of peripheral corticosteroid receptor sensitivity and cannot be extended to other tissues of interest, such as cardiac or brain tissue (Carvalho & Pariante, 2008). Also, measuring a small number of specific outcomes, such as LPS-induced IL-6 or TNF- α levels, means we are not examining all the wider effects of glucocorticoids (Quax et al., 2013). Therefore results of these assays should be interpreted with these issues in mind.

There are other *in vitro* methods used to measure corticosteroid receptor sensitivity and receptor function. The number of receptors within cells and the hormone binding affinity of the receptors can be measured directly using a glucocorticoid binding assay (Chriguer et al., 2005). Corticosteroid receptor mRNA expression can be assessed in PBMCs and receptor protein levels can be measured directly using Western blot techniques and indirectly using cytosol binding (Carvalho & Pariante, 2008). This provides an indication of the number of receptors within the cells. Measuring the number of corticosteroid receptors is an indicator of glucocorticoid sensitivity, but does not provide information

about the biological effectiveness of the receptor (Quax et al., 2013). Examining the rate of translocation of the corticosteroid receptors into cell nuclei also provides another proxy of receptor function.

2.10 Psychosocial stress and the corticosteroid receptors

To date, a number of studies have examined the effects of both chronic and acute stress on corticosteroid receptor sensitivity. Before describing this body of literature it is worth noting that the majority of research has focused on the sensitivity of the GR, with very little attention paid to the MR. I will first describe studies that have looked at associations between chronic stress and corticosteroid receptor sensitivity, and then move on to describe studies of the effects of acute stress on receptor sensitivity in the laboratory.

2.10.1 Chronic stress and corticosteroid receptor sensitivity

Many types of chronic stressors, including negative emotional disorders, have been found to affect the sensitivity of GRs (See Table 2.1). Associations between job strain and glucocorticoid resistance have been reported. In a study measuring vital exhaustion in male industrial employees, those who were highly exhausted had reduced GR sensitivity compared to non-exhausted employees (Wirtz et al., 2003). Highly exhausted employees also had elevated levels of CRP. However, a recent study reported increased GR sensitivity and function in 12 men suffering from job-related exhaustion compared to 12 matched healthy controls (Menke et al., 2014). In 46 healthy school teachers, those who reported high levels of effort-reward imbalance at work had reduced GR sensitivity compared to those with low effort-reward imbalance (Bellingrath, Rohleder, & Kudielka, 2013). In a study assessing the effects of academic stress on glucocorticoid resistance in 11 healthy students, the authors compared glucocorticoid sensitivity in lymphocytes one hour before an examination and also on a control day during a holiday period (Sauer et

al., 1995). They found that academic stress resulted in a decrease in cortisol inhibition of lymphocyte IL-2 production, implying reduced lymphocyte sensitivity to cortisol. The authors posit that exposure of lymphocytes to increased cortisol levels during the pre-exam stress period may have resulted in a loss of GR sensitivity. This reduction in sensitivity could be an adaptive response to short-term hypercortisolism. However, the small sample size means that results should be interpreted with caution.

Reduced lymphocyte sensitivity to cortisol has also been reported in elderly caregivers of dementia patients compared to elderly non-caregivers (Bauer et al., 2000). Miller and colleagues reported decreased dexamethasone suppression of LPS-induced IL-6 production in whole blood of parents of children with cancer compared to parents of healthy children, indicating reduced GR sensitivity to dexamethasone (Miller, Cohen, & Kim, 2002). These same parents also reported high levels of psychological distress, and had flatter cortisol slopes across the day. However, in a recent study there were no significant differences in GR protein levels or hydrocortisone suppression of LPS-induced IL-6 production from monocytes in adult caregivers of family members with glioblastoma compared with controls whose lives were free of major stressors (Miller et al., 2014). The authors posit that hydrocortisone could be acting on the MR which may be why there were no significant differences in the caregiver sample.

Reduced GR sensitivity has also been reported in those suffering from emotional disorders. Women with major depressive disorder were shown to have diminished GR sensitivity compared to healthy controls, and this diminished sensitivity was associated with flatter diurnal cortisol slopes (Jarcho et al., 2013). A systematic review of 34 studies examining associations between early life stress, depression, and GR and MR sensitivity found that early life stress leads to reduced inhibitory feedback of the HPA axis via

Table 2.1. Studies examining the effects of chronic stress on corticosteroid receptor function

Author/date	Sample	Study design	Chronic stress type	GR/MR measurement protocol	Statistical test and covariates	Main findings
Sauer et al. (1995)	11 healthy students (6 female), mean age 19y	Differences in glucocorticoid sensitivity during examination period and holiday period	Academic stress	Cortisol suppression of PHA-induced lymphocyte proliferation in isolated PBMCs	Spearman's rank correlations; no covariates	Academic stress was associated with reduced glucocorticoid sensitivity, implying reduced sensitivity of the corticosteroid receptors.
Bauer et al. (2000)	49 spousal caregivers of dementia patients (24 female), mean age 72y, 67 matched non-caregiver controls	Differences in glucocorticoid sensitivity between elderly caregivers and non-caregivers	Caregiver stress	DEX and cortisol suppression of PHA-induced lymphocyte proliferation in isolated PBMCs	ANOVA; no covariates	Caregivers had reduced glucocorticoid sensitivity compared to non-caregivers, implying reduced sensitivity of the corticosteroid receptors.
Miller et al. (2002)	25 parents of children undergoing cancer treatment (mean age 36y), 25 matched controls with healthy children	Differences in glucocorticoid sensitivity between both groups of parents	Chronic psychological stress of having a child who is undergoing treatment for cancer	DEX suppression of LPS-induced IL-6, IL-1 β , and TNF- α production in whole blood	ANOVA; baseline cytokine values	Parents of children with cancer had reduced GR sensitivity compared to parents of medically healthy children. They also had flatter cortisol slopes.
Wirtz et al. (2003)	325 healthy adults (280 male), mean age 40y	Difference in glucocorticoid sensitivity between those who are non-exhausted, and highly exhausted	Vital exhaustion in industrial employees	DEX suppression of LPS-induced IL-6 production in whole blood	ANOVA; no covariates	Men who were highly exhausted had reduced GR sensitivity compared to those who were non-exhausted, but not those who were moderately exhausted.

DEX = dexamethasone; DST = dexamethasone suppression test; ERI = effort-reward-imbalance; GLM= general linear model; GR = glucocorticoid receptor; IL-1 β = interleukin-1 β ; IL-6 = interleukin-6; LPS = lipopolysaccharide; PHA = phytohaemagglutinin (stimulates lymphocyte proliferation); PBMC = peripheral blood mononuclear cell; TNF- α = tumour necrosis factor - α ; TSST = Trier Social Stress Test; WC = waist circumference.

Table 2.1 (continued) Studies examining the effects of chronic stress on corticosteroid receptor function

Author/date	Sample	Study design	Chronic stress type	GR/MR measurement protocol	Statistical test and covariates	Main findings
Bellingrath et al. (2013)*	46 healthy adults (29 female), mean age 50y.	Associations between chronic stress and GR responses to the TSST in the lab	ERI at work and the TSST	DEX suppression of LPS-induced IL-6 production in whole blood	ANCOVA; gender, BMI, depression scores	High levels of ERI were associated with decreased GR sensitivity both before and after acute stress, compared to low levels of ERI.
Menke et al. (2014)	12 men suffering from job-related exhaustion (mean age 45y), and 12 matched healthy controls	Difference in GR sensitivity and function between exhausted and non-exhausted men	Job-related exhaustion	GR sensitivity: DST <i>in vivo</i> , GR function: DEX induced gene expression	GLM, linear regression; age, BMI	Enhanced GR sensitivity and function in those suffering from exhaustion.
Miller et al. (2014)	33 caregivers of relatives with cancer (21 female, mean age 54y) and 47 non-caregiving matched controls	Difference in GR sensitivity between caregivers and non-caregivers	Caregiver stress	GR sensitivity: hydrocortisone suppression of LPS-induced IL-6 production in monocytes; monocyte expression of GR protein levels measured using flow cytometry	Generalised estimating equations; age, sex, ethnicity, education, smoking, alcohol intake, physical activity, WC.	Both groups had similar levels GR protein levels. No difference in GR sensitivity between groups.

DEX = dexamethasone; DST = dexamethasone suppression test; ERI = effort-reward-imbalance; GLM= general linear model; GR = glucocorticoid receptor; IL-1 β = interleukin-1 β ; IL-6 = interleukin-6; LPS = lipopolysaccharide; PHA = phytohaemagglutinin (stimulates lymphocyte proliferation); PBMC = peripheral blood mononuclear cell; TNF- α = tumour necrosis factor - α ; TSST = Trier Social Stress Test; WC = waist circumference.

*Although study includes an acute psychosocial stress measure, the relevant associations are between a measure of chronic stress and GR sensitivity

changes in GR and MR sensitivity, and the subsequent development of depression (von Werne Baes, de Carvalho Tofoli, Martins, & Juruena, 2012). However, within this review there was large methodological variation between studies which may have affected the conclusions drawn. Instead of measuring sensitivity of receptors, some studies have examined the number of corticosteroid receptors within each cell as a proxy for their sensitivity. Calfa and colleagues found that depressed patients had reduced GR numbers in PBMCs compared to healthy controls (Calfa et al., 2003).

Together, this body of research indicates that chronic stress, including depression, results in decreased sensitivity of the GR. Pariante and colleagues posit that this diminished GR sensitivity brings about impaired feedback inhibition of the HPA axis, thus explaining the enhanced cortisol stress reactivity seen in major depression (Pariante, Thomas, Lovestone, Makoff, & Kerwin, 2004). As well as shutting down the cortisol stress response, the GR are also responsible for regulating the magnitude of the response. This means that diminished GR sensitivity could also explain the blunted cortisol stress reactivity observed in older and more severely depressed patients (Burke et al., 2005; Taylor et al., 2006). Interestingly, the MR appears to be slightly oversensitive in depressed patients (Young, Lopez, Murphy-Weinberg, Watson, & Akil, 2003). As the MR regulates basal activity of the HPA axis, altered MR sensitivity may have implications for the dysregulation of diurnal HPA axis activity brought about by depression. It is therefore likely that depression is characterised by an imbalance of both GR and MR sensitivity (de Kloet, DeRijk, & Meijer, 2007). This imbalance in sensitivity likely has consequences for levels of inflammation in the body also. In support of this, a number of the studies outlined above reported higher levels of inflammation and signs of HPA axis dysregulation (i.e. flatter diurnal cortisol slopes) within samples experiencing stress-related loss in GR sensitivity. This all lends support to the notion that over time,

chronic stress brings about dysregulation of the HPA axis and increased systemic inflammation via diminished sensitivity of the corticosteroid receptors.

2.10.2 Acute stress and corticosteroid receptor sensitivity

Looking at the effects of acute stress on GR and MR sensitivity may shed some light on how stress-related loss of receptor sensitivity comes about. The effects of acute stress on corticosteroid receptor function have been examined in a number of studies.

2.10.2.1 Acute exercise stress and GR sensitivity

Most of the early studies used exercise paradigms to examine acute stress-induced changes in receptor sensitivity to cortisol. DeRijk and colleagues examined the effects of dexamethasone on LPS-induced production of IL-6 in whole blood in healthy men exposed to graded exercise on a treadmill (DeRijk et al., 1996). Following exercise, more dexamethasone was required to inhibit the LPS-induced release of IL-6 indicating a reduction in GR sensitivity. The effects of dexamethasone on LPS-induced release of IL-6, TNF- α , IL-10 and interferon (IFN)- γ in whole blood were examined in nine well-trained oarsmen who underwent strenuous exercise for a 15-20 minute period (Smits, Grünberg, Derijk, Sterk, & Hiemstra, 1998). Similar to the results of DeRijk and colleagues, following exercise, the inhibitory effect of dexamethasone on IL-6 and TNF- α secretion was reduced indicating reduced GR sensitivity. However, dexamethasone effects on IL-10 and IFN- γ release were not altered by exercise.

In contrast to the results of these studies, Duclos and colleagues looked at the effects of an acute bout of exercise on sensitivity to cortisol in the isolated cultured monocytes of endurance-trained men (n=6) and found an exercise-induced increase in GR sensitivity (Duclos et al., 1999). Similarly, in a more recent study, an acute resistance exercise

protocol in resistance-trained men and women (n=15) brought about increased GR expression in lymphocytes (Fragala et al., 2011). The reason for the discrepancy in results between exercise studies may be the use of different culture conditions across studies (Rohleder et al., 2003). As mentioned previously, performing glucocorticoid sensitivity assays in whole blood, as opposed to isolated PBMCs, does not take into account individual variability in white blood cell population ratios.

2.10.2.2 Acute psychosocial stress and GR sensitivity: Murine studies

The effects of acute psychosocial stress on GR sensitivity have largely been investigated in animals. Sheridan and colleagues subjected mice to social reorganisation (SRO) and measured GR sensitivity using a synthetic glucocorticoid suppression test on proliferation of splenocytes (Sheridan, Stark, Avitsur, & Padgett, 2000). SRO stress involves randomly housing groups of male mice separately for two weeks in order for stable social hierarchies to form. The dominant mouse from each group is then transferred to a different cage where it is perceived as an aggressive intruder. This is stressful for both the resident mice and the intruder. The authors found that proliferation of splenocytes was inhibited in a dose-dependent manner by glucocorticoids in control mice, whereas proliferation of splenocytes in the SRO mice was resistant to glucocorticoid suppression. This indicates reduced GR sensitivity in the SRO mice brought about by acute psychosocial stress.

Similarly, Stark and colleagues demonstrated that the splenocytes of SRO mice were resistant to the antiproliferative effects of corticosterone compared to control mice, suggesting a decrease in GR sensitivity following bouts of acute psychosocial stress (Stark et al., 2001). SRO exposure in mice has also been shown to downregulate the expression of GR mRNA (Quan et al., 2001). In all studies, resistance to glucocorticoids developed following repeat, but not acute, exposures to SRO, and the resistance persisted

for 10 days after the stress exposure ended (Avitsur, Stark, Dhabhar, Padgett, & Sheridan, 2002). Similarly, Bauer and colleagues showed that repeated exposure to restraint stress induced a slight increase in glucocorticoid resistance, i.e. decreased GR sensitivity (Bauer, Perks, Lightman, & Shanks, 2001). However, acute exposure did not induce any significant changes in GR sensitivity.

2.10.2.3 Acute psychosocial stress and corticosteroid receptor sensitivity:

Human studies

To date, five studies have assessed the effects of acute psychosocial stress on corticosteroid receptor sensitivity in humans. In these studies, participants were exposed to a number of behavioural tasks known to induce activation of the HPA axis stress response. In all studies (see Table 2.2) receptor sensitivity was measured using dexamethasone suppression of LPS-induced cytokine production in whole blood (see *Section 6.8.2* for a more detailed description of this procedure). The first study measured sex differences in GR sensitivity following acute psychosocial stress in healthy young men and women (Rohleder, Schommer, Hellhammer, Engel, & Kirschbaum, 2001). Twenty-seven men and 18 women in the luteal phase of their menstrual cycle were exposed to the TSST (Kirschbaum, Pirke, & Hellhammer, 1993). Men and women did not differ in their salivary cortisol responses to acute stress. However, GR sensitivity showed marked gender differences. Examination of the inhibitory concentration 50% (IC₅₀) of dexamethasone revealed that one hour after stress GR sensitivity had significantly increased in men, whereas sensitivity had decreased in women, although this change failed to achieve statistical significance. In agreement with these findings, the authors report that IL-6 levels one hour post-stress had significantly decreased in men but remained unchanged in women.

The second study measured age and sex-steroid related differences in GR sensitivity following acute psychosocial stress in healthy elder men (n=14), healthy young men (n=14), and healthy elder men who had received a testosterone injection five days prior to testing (n=12) (Rohleder, Kudielka, Hellhammer, Wolf, & Kirschbaum, 2002). All participants underwent the TSST. An hour after the stress protocol there were no differences between groups in terms of stress-induced increases in cortisol. However, GR sensitivity as indexed by the IC₅₀ of dexamethasone was significantly increased in the younger men, and significantly decreased in the older men. Interestingly, testosterone-treated older men showed the same significant increase in GR sensitivity as the healthy younger men. These findings provide further evidence that acute stress modulates GR sensitivity. Furthermore, they indicate that GR sensitivity in response to stress changes with age and that these changes are associated with the presence of sex steroids.

The third study examined the effects of oral contraception on GR sensitivity after acute psychosocial stress (Rohleder, Wolf, Piel, & Kirschbaum, 2003). Previous research has shown that women taking oral contraceptives have blunted cortisol responses to stress (Kirschbaum, Kudielka, Gaab, Schommer, & Hellhammer, 1999). HPA axis activation and GR sensitivity were measured in 14 women using oral contraception and 11 women in the luteal phase of the menstrual cycle that underwent the TSST. Following stress, luteal phase women showed an increase in cortisol whereas the contraceptive users showed blunted cortisol stress responses. Luteal phase women exhibited a non-significant decrease in GR sensitivity. Women taking oral contraceptives displayed an increase in GR sensitivity following acute stress. The authors posit that this increase in GR sensitivity is an adaptive response to the blunting of the cortisol stress reactivity which may protect women using oral contraceptives from the inflammatory stress response.

Table 2.2. Studies examining the effects of acute stress on corticosteroid receptor function

Author/date	Sample	Study design	Stress paradigm	GR/MR measurement protocol	Statistical test and covariates	Main findings
Rohleder et al. (2001)	45 healthy adults (18 women), mean age 25y	Difference between men and women in GR sensitivity following acute stress	TSST	DEX suppression of LPS-induced IL-6 and TNF- α production in whole blood	ANOVA; no covariates	Basal GR sensitivity lower in men. Increase in GR sensitivity in men, and a non-sig decrease in women 60 mins after acute stress
Rohleder et al. (2002)	40 healthy men, 14 young (mean age 25y), 14 elderly (mean age 67y), 12 elderly + testosterone treatment (mean age 68y)	Difference between young men, elderly men, and elderly men + testosterone in GR sensitivity following acute stress	TSST	DEX suppression of LPS-induced IL-6 and TNF- α production in whole blood	ANOVA; no covariates	Basal GR sensitivity lower in younger men. Increase in GR sensitivity in young and testosterone-treated elderly men, non-sig decrease in elderly men, 60 mins after acute stress.
Rohleder et al. (2003)	25 healthy women , 14 taking OC (mean age 22y), 11 OC-free (mean age 25y)	Difference between women taking OC, and women not, in GR sensitivity following acute stress	TSST	DEX suppression of LPS-induced IL-6 and TNF- α production in whole blood	ANOVA; no covariates	No difference in basal GR sensitivity Increase of GR sensitivity in OC users, no sig. change in women not taking OC.

DEX = dexamethasone; GLM= general linear model; GR = glucocorticoid receptor; IL-6 = interleukin-6; LPS = lipopolysaccharide; MR = mineralocorticoid receptor; OC = oral contraception; PRED = prednisolone; TNF- α = tumour necrosis factor – α ; TSST = Trier Social Stress Test.

Table 2.2. (Continued) Studies examining the effects of acute stress on corticosteroid receptor function

Wirtz et al. (2008)	42 healthy men (mean age 43y)	Association between BMI and GR sensitivity following acute stress	TSST	DEX suppression of LPS-induced TNF- α production in whole blood	ANCOVA, GLM; baseline GR sensitivity, age, mean arterial pressure	Basal GR sensitivity not associated with BMI. Higher BMI associated with decrease in GR sensitivity after acute stress
Carvalho et al. (2015)	74 older adults, 37 with T2DM (mean age 64y), 32 healthy controls (mean age 67y)	Difference between adults with T2DM and healthy controls in GR and MR sensitivity following acute stress	2x 5 min behavioural tasks	DEX and PRED suppression of LPS-induced IL-6 production in whole blood	GLM; BMI, time of session	T2DM group had higher GR sensitivity at baseline, but not MR sensitivity. Decrease in GR and MR sensitivity in healthy controls, no change on T2DM

DEX = dexamethasone; GLM= general linear model; GR = glucocorticoid receptor; IL-6 = interleukin-6; LPS = lipopolysaccharide; MR = mineralocorticoid receptor; OC = oral contraception; PRED = prednisolone; T2DM = type 2 diabetes; TNF- α = tumour necrosis factor - α ; TSST = Trier Social Stress Test.

The fourth study investigated whether BMI affected changes in GR sensitivity following acute psychosocial stress (Wirtz, Ehlert, Emini, & Suter, 2008). Forty-two men underwent the TSST. BMI was not associated with either diurnal or stress-induced cortisol secretion. However, results indicated that a higher BMI was associated with a more pronounced loss of GR sensitivity following acute stress. The authors suggest that this could be a pathway through which BMI might alter the stress response in ways that are detrimental to cardiovascular health.

The fifth study carried out by our group examined both GR and MR sensitivity to acute stress in 37 people with type 2 diabetes and 37 healthy controls (Carvalho et al., 2015). People with type 2 diabetes have an increased risk of CVD as well as impairments of the HPA axis (Bruehl et al., 2007; Hackett, Steptoe, & Kumari, 2014). MR sensitivity was measured using prednisolone suppression of LPS-induced cytokine production in whole blood (see *Section 6.8.2* for a full description of the procedure). Prednisolone is a synthetic glucocorticoid that binds to both the GR and the MR. Mental stress was induced using two 5-minute behavioural tasks. Following stress, the healthy controls (mean age = 67.5 years) exhibited a decrease in both GR and MR sensitivity, which is in line with the previous finding that GR sensitivity decreases in healthy older men (Rohleder et al., 2002). However, there was no change in GR or MR sensitivity in those with type 2 diabetes. The diabetic patients also had blunted stress responses in terms of systolic blood pressure, heart rate, and levels of IL-6. The authors suggest that the impaired stress responsiveness in type 2 diabetes is in part due to a lack of stress-induced alterations in GR and MR sensitivity.

Apart from this fifth study, very little work has been done examining the effects of acute stress on the MR. Studies have shown that MR antagonists such as spironolactone result in increased basal cortisol levels and increased cortisol responses to exercise stress

(Heuser, Deuschle, Weber, Stalla, & Holsboer, 2000; Wellhoener, Born, Fehm, & Dodt, 2004). A common polymorphism in the MR gene has been associated with higher cortisol responses to acute stress (DeRijk et al., 2006). As the MR and GR work in concert to regulate the cortisol and inflammatory stress response, future stress research should examine both GR and MR sensitivity in order to gain further understanding of the link between stress and CVD.

Therefore, the third study of this PhD presented in Chapter 6 will examine the effects of an acute psychosocial stress paradigm on both GR and MR sensitivity in healthy volunteers.

To summarise, results from studies examining the effects of acute stress on corticosteroid receptor sensitivity have been mixed. Murine studies suggest that acute stress brings about a decrease in GR sensitivity. However, it could be argued that these studies adopt a sub-chronic stress paradigm as the effects on GR sensitivity are only seen after repeated exposures to the stressor. Human studies have provided varied data on the effects of both exercise and psychosocial stress on GR sensitivity. The main conclusion that can be drawn from results so far is that acute stress modulates GR sensitivity. There is rather large variability in corticosteroid receptor sensitivity in humans with regards to sex, age, sex steroid hormone status, BMI, as well as diabetes status.

2.11 Chapter summary

Although the direction of results is mixed, psychosocial stress factors and negative emotional disorders appear to be associated with dysregulation of both basal and stress-related HPA axis function. Dysregulation of both basal and stress-related HPA axis function has been associated with markers of cardiovascular risk and have been seen in CVD patients. This evidence suggests that dysregulation of the HPA axis is likely one of

the biological pathways through which psychosocial stress contributes to the pathophysiology of CVD.

The evidence also suggests that alterations in the sensitivity of the corticosteroid receptors may be one of the mechanisms through which psychosocial stress brings about sustained changes in HPA axis function. The studies cited in the previous sections provide support for the notion that stress modulates corticosteroid sensitivity. Reduced GR sensitivity has been reported in depression. Chronic life stressors, such as job stress and caregiver stress, have been shown to reduce GR sensitivity also. Repeated stress ‘hits’ over time may result in a loss of receptor sensitivity, thereby leading to dysregulated cortisol secretion, and increased systemic inflammation. For example, in CHD patients, 24-hour cortisol secretion is higher than healthy controls and this is accompanied by higher levels of CRP and IL-6 (Nijm et al., 2007). This implies diminished corticosteroid receptor sensitivity in these patients.

Data from studies assessing the effects of acute stress on corticosteroid receptor sensitivity are more mixed. There is a large amount of variability in GR sensitivity following stress in humans with regards to sex, age, and BMI. Moreover, work examining the effects of acute stress on MR sensitivity is scarce. Nevertheless, results of these studies show that acute stress does modulate corticosteroid receptor sensitivity. Taken together, the evidence suggests that dysregulation of the HPA axis, via stress-related modulation of the corticosteroid receptors, is one of the biological pathways linking psychosocial stress and CVD.

Chapter 3

Study 1 - Diurnal cortisol rhythm and adverse clinical outcomes in patients with advanced CVD: The ARCS Study

3.1 The Adjustment and Recovery after Cardiac Surgery (ARCS) Study

Coronary artery bypass graft (CABG) surgery is used to relieve symptoms and improve life-expectancy in those suffering from advanced coronary heart disease. The ARCS Study was designed to investigate the causes and consequences of poor physical and emotional wellbeing following CABG surgery, and the implications for patient quality of life and physical recovery. Five sets of factors potentially relevant to emotional and physical quality of life post-CABG surgery were the focus of the study: (1) clinical factors, e.g. existing heart problems and illness as well as factors pertaining to the surgery itself, (2) cognitive factors, e.g. cognitive function as well as the patients' ability to understand health information, (3) social factors, e.g. social support, (4) emotional factors, e.g. depression and anxiety, (5) biological factors, e.g. inflammatory markers measured in the blood and salivary cortisol measured across the day.

The ARCS Study used a prospective longitudinal design with a number of assessment periods spanning up to 2.68 years after the CABG procedure. Patients were recruited at their surgical pre-assessment clinic and were assessed approximately one month prior to their surgery (T1), 4-5 days after their surgery while still in hospital care (T2), 8-10 weeks after surgery (T3), and 12 months after surgery (T4). At each time point, participants were asked to complete a questionnaire pack and provide saliva samples across the day for measurement of diurnal cortisol profiles (saliva was not provided at the visit 4-5 days after surgery). Blood measures were taken prior to surgery and in the days following surgery in order to measure markers of inflammation. Approximately 2.5 years following

the procedure, long term clinical outcomes for each patient were collected from electronic and paper medical records (T5). This included mortality data, development of post-surgical infections, any cardiac or non-cardiac related hospital readmissions, adverse cardiac events, occurrence of other cardiac procedures or tests (e.g. angiogram, percutaneous coronary intervention), occurrence of new onset depression or anxiety, and diagnoses of any other major illnesses.

3.2 My contribution to the ARCS Study

As part of a team of several ARCS Study researchers, I was involved in study recruitment and data collection at all time-points. I recruited a large number of patients at their surgical pre-assessment. As well as explaining the study to the patient and obtaining informed consent, this also involved administering a short cognitive examination and health literacy test, as well as organising blood sample collections for each patient. In terms of data collection, I carried out a large number of on-ward structured interviews with patients approximately 4-5 days after surgery. I also sent questionnaire and saliva-collection packs to patients at the 8-10 week and 12 month follow-up points. Additionally, I was responsible for prompting patients over the telephone who may have forgotten to return their questionnaire packs in the post.

My largest contribution to the ARCS Study was the collection of the long-term clinical outcomes which I was responsible for. In the early stages of my PhD, I spent a number of months on site at St. George's hospital collecting long-term clinical outcome data for each individual patient from electronic and paper medical records.

Additionally, I was largely involved in ARCS Study data entry as well as maintenance of the dataset. Furthermore, I have been involved in data analysis. To date, I have produced two first-author publications using ARCS data, and have contributed to several other

ARCS Study publications. (Kidd et al., 2014; Kidd, Poole, Leigh, et al., 2016; Kidd, Poole, Ronaldson, et al., 2016; Poole et al., 2015; Poole, Kidd, et al., 2014a, 2014b, 2016; Poole, Leigh, et al., 2014; Poole, Ronaldson, et al., 2016; Ronaldson et al., 2014, 2015; Steptoe et al., 2015).

3.3 Differentiating my PhD from the ARCS Study

The ARCS Study is a multidisciplinary study involving several researchers. This study has produced a rich dataset containing information pertaining to the five sets of factors outlined previously. Accordingly, many issues have been and will be investigated that are beyond the scope of my PhD. In my PhD, I used pre-surgical data from T1 of the ARCS Study to examine the association between pre-surgical diurnal cortisol rhythm and major adverse cardiac events (MACE) and death (T5 data) in patients with advanced heart disease undergoing CABG surgery. I also used T1 data to cross-sectionally explore what psychosocial stress factors may be affecting diurnal HPA axis function. Results from this study have been published in the *Journal of Clinical Endocrinology & Metabolism* (Ronaldson et al., 2015).

3.4 Introduction

As mentioned previously in this thesis, there is growing evidence that the HPA axis plays a role in the progression of CVD. Elevated 24h urinary cortisol has been found to predict cardiovascular death in older people both with and without CVD (Vogelzangs et al., 2010). Higher serum cortisol has also been associated with cardiovascular mortality in a cohort of patients with mood disorder (Jokinen & Nordström, 2009). However, the role of the HPA axis in patients with advanced CVD is less clear. Higher serum cortisol levels have been found to predict both mortality risk and risk of future cardiac events in chronic heart failure (Güder et al., 2007; Yamaji et al., 2009) and ischaemic stroke (Barugh, Gray,

Shenkin, MacLulich, & Mead, 2014). However, results from studies of cortisol in acute coronary syndrome have been less consistent (Jutla, Yuyun, Quinn, & Ng, 2014; Reynolds et al., 2010).

One difficulty in interpreting this evidence is that cortisol is typically measured with a single serum sample. Inconsistencies in associations between cortisol and CVD may be because the diurnal nature of cortisol is not being taken into account. More detailed measurement of the diurnal cortisol profile would allow for a more in depth investigation of the associations between cortisol and clinical endpoints in CVD patients. Dysregulation of the HPA axis can result in a reduction in the amplitude of the diurnal pattern, or a flatter slope across the day. As mentioned earlier in Chapter 2, flatter cortisol slopes have been associated with higher levels of coronary artery calcification (Hajat et al., 2013; Matthews et al., 2006), and increased cardiovascular mortality in nonclinical populations (Kumari et al., 2011).

There is a paucity of studies examining the effects of variations in diurnal cortisol rhythms on future cardiac events and mortality in patients with established CVD. This study therefore sought to examine the relationship between pre-surgical diurnal cortisol and clinical outcomes in patients undergoing CABG surgery.

3.4.1 Hypotheses

Based on previous research, I hypothesised that a flatter diurnal cortisol slope before surgery would be associated with higher rates of future cardiac events and mortality in the years following CABG. I also examined associations between the cortisol awakening response (CAR) and total cortisol output across the day, and adverse clinical outcomes. However, in keeping with previous research I did not expect to find significant associations with these cortisol parameters (Kumari et al., 2011; Matthews et al., 2006).

As a flatter diurnal slope could reflect a negative psychosocial stress profile I carried out exploratory analyses examining cross-sectional associations between pre-surgical cortisol slopes and psychosocial stress variables, namely stressful life events, depression, anxiety, and social support, in order to garner information about stress-related factors that may bring about dysregulation of the HPA axis. I hypothesised that flatter cortisol slopes would be associated with more depressive symptoms, higher levels of anxiety, more stressful life events, and low social support.

3.5 Materials and methods

3.5.1 Participants

The data we used in this analysis were collected as part of the ARCS Study, involving patients undergoing first-time elective CABG surgery or CABG plus valve replacement. CABG surgery in a single centre (Steptoe et al., 2015) included both on-pump and off-pump procedures. All procedures were carried out with written informed consent of the participants. Ethical approval was obtained from the National Research Ethics Service.

Participants were 262 prospective CABG patients who were recruited from a pre-surgical assessment clinic at St. George's Hospital, London. Eligible participants had to be at least 18 years of age and had to be able to complete questionnaires in English. Long term recovery outcomes were collected from electronic and paper patient records on average 2.68 years ($SD = 0.40$) after surgery. We carried out analyses on 250 patients with complete data on clinical outcomes and cortisol slope. There were no significant associations between the use of steroid medications and cortisol output, outcome variables or covariates (all p values > 0.05). Therefore patients taking steroid medications ($n = 8$) were included in the analyses.

There were no significant differences between patients included in and excluded from the analyses in terms of age, sex, BMI, smoking status, length of hospital stay, the occurrence of major adverse cardiac events (MACE), chronic disease burden, diabetes status, or whether or not the person had on-pump surgery (all p values < 0.05). However, European System for Cardiac Operative Risk Evaluation (EuroSCORE) was higher in the 12 patients without cortisol data ($F(2, 345) = 5.23, p = 0.006$) indicating poorer prognosis on average. Patients included in the analyses did not differ from those excluded in terms of any of the psychosocial stress variables (all p values < 0.05).

3.5.2 Biological and clinical measures

Diurnal salivary cortisol

At the pre-surgical assessment clinic (T1) participants received a saliva collection kit and were given instructions for collection at home. The kit included seven pre-labelled ‘salivette’ collection tubes (Sarstedt, Leicester, UK) and a cortisol diary. The cortisol diary contained instructions on how and when to give samples (Appendix A). These diaries were also used to record information on factors likely to introduce variation in cortisol samples such as mood, exercise, and daily stressors. Participants were instructed to choose one day prior to surgery on which to provide seven saliva samples at set time points: on waking, 30 minutes after waking (30+), 10am, 12pm, 4pm, 8pm, and bedtime. Participants stored their samples in the refrigerator before returning them to the clinic. The samples were obtained an average 30.6 days ($SD = 36.9$) prior to surgery and were stored at -20°C for analysis at a later date. Cortisol levels were assessed from saliva using a time resolved immunoassay with fluorescence detection at the University of Dresden, Germany. The intra- and inter-assay coefficients of variation were less than 4%.

We computed three different cortisol measures. Total cortisol output over the day was assessed by calculating the cortisol AUC with respect to ground (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). The CAR was calculated by measuring the difference between the sample taken on waking and the 30+ sample. In line with other work produced by Steptoe's group, participants who reported giving their first sample more than 15 minutes after waking were excluded from the analyses. Previous research has shown that a long delay between waking and providing the 'waking' sample can produce misleading CAR results, but a delay of less than 15 minutes between waking and providing the sample does not seem problematic (Dockray, Bhattacharyya, Molloy, & Steptoe, 2008). An expert panel recently recommended that CAR data should be excluded if the waking sample is provided with a delay of 5 minutes or more. However, the same expert panel also stated that this tight accuracy margin would result in substantial data loss (26-46%) and therefore researchers need to choose between scientific precision and practical feasibility (Stalder et al., 2016). A number of previous studies have selected an accuracy margin of <15 minutes (DeSantis, Adam, Mendelsohn, & Doane, 2010; Dockray et al., 2008; Okun et al., 2010). In order to see if the accuracy margin would affect results obtained, CAR was also calculated using an accuracy margin of <5 minutes. The implications of selecting these accuracy margins for the current study will be addressed in the Discussion (Section 3.7). The cortisol slope was calculated in nmol/l/h by regressing cortisol on sample collection time, with 30+ excluded (Messerli-Bürge et al., 2012); higher values indicate a steeper decrease in cortisol over the day. Waking and evening (the average of 8pm and bedtime) values were also calculated.

Participants were to be excluded from analysis if any cortisol value exceeded 70 nmol/L. No participants had cortisol values that exceeded this limit. Cortisol slope was calculated if the participant had at least four available cortisol measures (excluding the 30+ morning

sample). 250 patients provided sufficient saliva samples for the calculation of cortisol slope. The CAR was calculated for 179 patients as 70 patients reported providing their waking sample more than 15 minutes after waking, and one patient failed to provide a waking sample. Cortisol AUC was calculated only for those who provided all seven saliva samples. Therefore, cortisol AUC was calculated for 220 patients as 30 failed to provide all samples.

Long term clinical outcomes

Long term clinical outcomes included in this study were occurrence of a MACE and death (all-cause mortality) and were collected up to 2.68 years after surgery (T5). Post-operative MACE included admissions for myocardial infarction, unstable angina, stroke, and/or heart failure. Occurrence of MACE was treated as a binary variable where either no MACE occurred or ≥ 1 MACE occurred. Mortality and MACE data were gathered by reviewing in-hospital electronic and paper patient records.

3.5.3 Psychosocial stress variables

A number of measures from the ARCS study were selected for use in this thesis in order to assess cross-sectional associations between pre-surgical psychosocial stress variables and pre-surgical diurnal salivary cortisol. The stress variables included in the analyses were depressive symptoms, anxiety, stressful life events, and social support. These measures were completed an average 29.1 ($SD = 29.7$) days prior to surgery.

Depressive symptoms

Depressive symptoms were measured using the Beck Depression Inventory (BDI) (Beck, Steer, & Carbin, 1988). The BDI can be used to measure depressive symptoms in both psychiatric and non-psychiatric healthy individuals, and has been found to be preferable

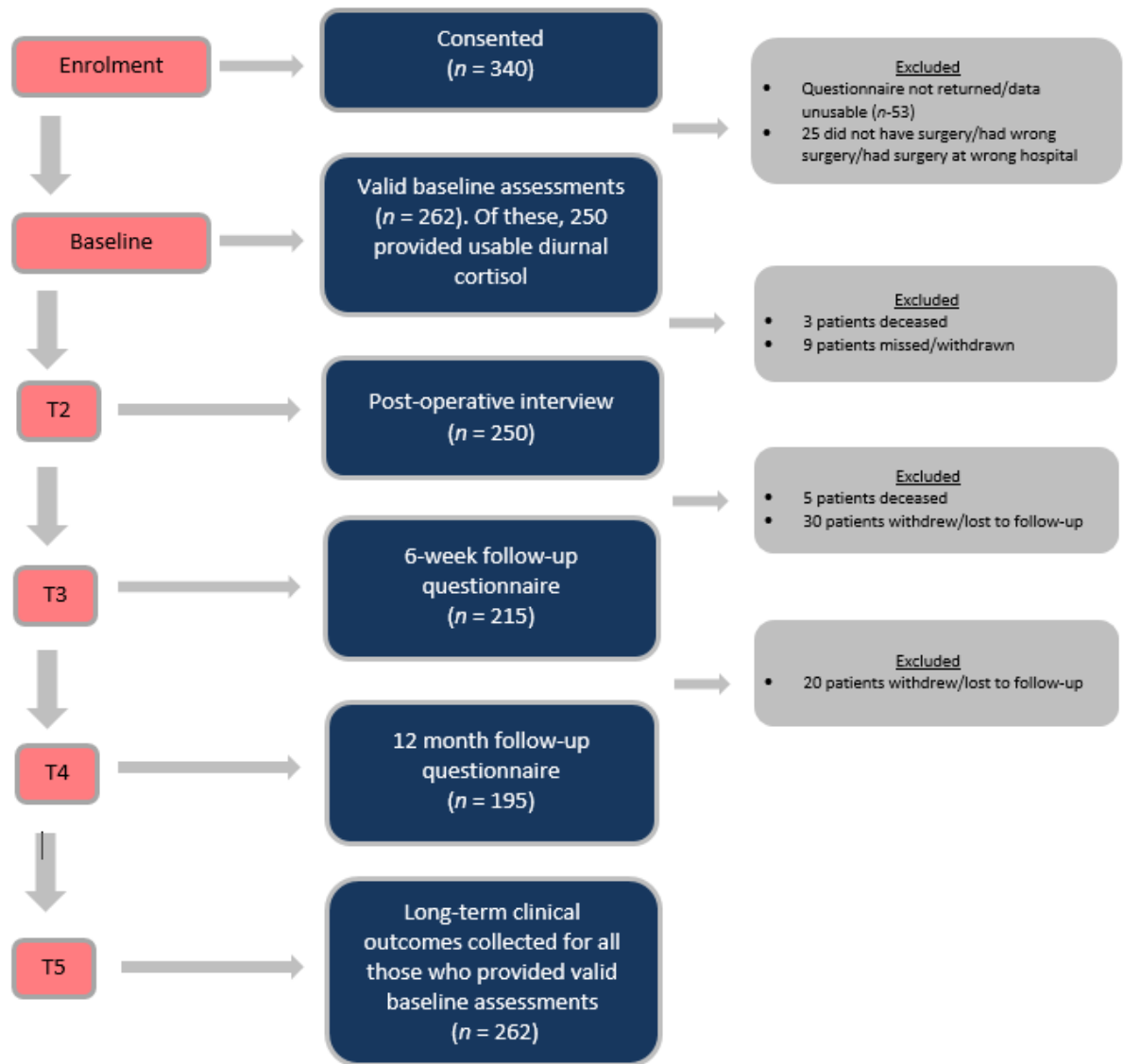


Figure 3.1. Flow diagram of participant recruitment and attrition from the ARCS Study

to the Hospital Anxiety and Depression Scale (HADS) for measuring depressive symptoms in cardiac patients (Thombs et al., 2006). It comprises 21 items that are scored on a scale ranging from 0-3, with total scores ranging from 0-63. Higher scores indicate greater emotional disturbance. Respondents are asked to provide answers that best describe the way they have been feeling over the past two weeks. The Cronbach's alpha for the BDI in this sample was 0.85.

Anxiety

Anxiety was measured using the seven-item anxiety subscale of the HADS (Zigmond & Snaith, 1983). This subscale was favoured over other anxiety questionnaires due to its brevity. Each item is scored on a scale ranging from 0-3, with total scores ranging from 0-21. Items are summed to generate a total score, with reverse coding on item 4 (*'I can sit at ease and feel relaxed'*). The anxiety subscale of the HADS has been shown to be suitable for use in cardiac patients (Roberts, Bonnici, Mackinnon, & Worcester, 2001). The Cronbach's alpha for the HADS anxiety subscale in this sample was 0.88.

Stressful life events

A modified version of the chronic burden scale used in the Multi-Ethnic Study of Atherosclerosis was used to measure stressful life events in the current sample (Diez Roux et al., 2006). The chronic burden scale comprises five items that ask respondents to report ongoing difficulties or stress in five areas of life: health of self, health of others, job or ability to work, financial strain, and relationships. In the ARCS study two extra items were added in order to measure ongoing difficulties relating to grief or bereavement, and living conditions. Patients were coded as having difficulty or stress in one of the areas of life if they reported a moderately stressful or severely stressful ongoing problem that had been present for six months or more. The stressful life events score was the number of items a patient reported having difficulty with (range 0-7). Associations between pre-surgical cortisol and individual items pertaining to each area of life were also examined.

Social support

Social support was measured using the ENRICH Social Support Instrument (ESSI). The ESSI is a validated seven-item scale used to assess the quality of social support and was

developed specifically for use in the ENRICHHD study of cardiac patients (Mitchell et al., 2003). The items relate to structural (partner), instrumental (tangible), and emotional (caring) support. Items are scored on a five-point Likert scale ranging from 1 'None of the time' to 5 'All of the time'. Responses to item 7 ('Are you currently married or living with a partner?') were scored 4 'Yes' or 2 'No' in accordance with scoring guidelines. Total scores range from 8-34 with higher scores indicating greater social support. The Cronbach's alpha for this sample was 0.93.

3.5.4 Covariates: clinical and sociodemographic factors

Cardiovascular history and clinical factors during admission and management (length of hospital stay, whether the patient had on-pump surgery) were obtained from clinical notes. Clinical risk was assessed using the EuroSCORE (Roques, Michel, Goldstone, & Nashef, 2003). EuroSCORE is a combined measure of procedural mortality risk based on 17 factors comprising patient-related factors (e.g. age, sex), cardiac-related factors (e.g. unstable angina, recent MI), and surgery-related factors (e.g. surgery on thoracic aorta). Items were scored in accordance with the 'logistic EuroSCORE' method to generate a percentage mortality risk estimate; further details of the scoring method can be found on the EuroSCORE website (www.euroscore.org/logisticEuroSCORE.htm). In addition, we recorded whether a patient underwent cardiopulmonary bypass. History of diabetes was taken from medical notes, categorising patients as diabetic or non-diabetic.

Participants were asked to report any longstanding illnesses apart from heart disease prior to surgery (e.g. cancer, thyroid disorder); responses were summed to compute a chronic illness burden variable. Smoking was measured as a binary variable (current smoker/non-smoker). BMI was assessed at the pre-operative clinic appointment and calculated using the standard formula (kg/m^2).

3.5.5 Statistical analyses

A composite outcome was created combining MACE and mortality. Cox proportional hazards models were used to determine relationships between cortisol before surgery and clinical outcome; when a patient experienced more than one MACE, the earliest time interval from baseline was analysed. Separate models were fitted for the cortisol slope over the day, cortisol AUC, CAR, and waking and evening cortisol values.

Because of the low number of clinical events ($n = 18$), only three covariates were included in the Cox regression models in order to avoid over-fitting. Therefore we included those covariates deemed most clinically relevant: EuroSCORE, whether the patient underwent cardiopulmonary bypass, and chronic illness burden. Age and sex were not adjusted for separately in the Cox regression models as both age and sex are included in the EuroSCORE.

In order to garner information about psychosocial factors which may influence diurnal cortisol measures, cross-sectional associations between pre-surgical psychosocial stress variables and cortisol were examined using Pearson's correlations. Statistically significant correlations were then entered into simple age and sex-adjusted linear regression models, with the psychosocial variable acting as the predictor and the cortisol variable as the outcome.

Associations between pre-surgical cortisol and covariates were examined using Pearson's correlations for continuous data and independent t-tests for categorical variables. Differences between mean cortisol values between patients who died or experienced a MACE and patients who experienced no event were examined using independent t-tests.

The significance level was set to $p < 0.05$ for all analyses, with precise p values reported for all test results. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA).

3.6 Results

Table 3.1 summarises the characteristics of the patients. The sample had an age range of 44-90 years, was predominantly male (86.4%), and overweight (BMI>25 = 81.6%). Just under a quarter of the patients were diabetic (24%). The majority had on-pump cardiopulmonary bypass surgery (79.2%). In the years following surgery ($M = 2.68$ years, $SD = 0.40$) nine patients (3.6%) experienced a MACE and 10 patients (4%) died, with one individual experiencing both outcomes.

Table 3.1. Demographic, cortisol, and clinical characteristics of the sample (n=250)

<i>Characteristic</i>	<i>Mean ± SD or n(%)</i>
Age (years)	68.1±8.9
Female	34(13.6)
BMI (kg/m ²)	28.8±4.4
Smoker	20(8.0)
Ethnicity (white)	219(87.6)
<i>Co-morbidities</i>	
Diabetes	60(24.0)
<i>Chronic illness burden</i>	
No other chronic illness	156(62.4)
1 other chronic illness	74(29.6)
2 other chronic illnesses	20(8.0)

Table 3.1. (continued) Demographic, cortisol, and clinical characteristics of the sample (n=250)

<i>Characteristic</i>	<i>Mean ± SD or N(%)</i>
<i>Pre-surgical measures of cortisol</i>	
Slope (nmol/L/hr)	1.67±1.31
Area under the curve (nmol/L.hr)	147.9±46.2
Waking cortisol (nmol/L)	19.4±8.7
Time of waking (hh:mm)	06:56±01:12
Average evening cortisol (nmol/L)	4.37±3.81
<i>Clinical factors</i>	
Logistic EuroSCORE (%)	4.49±3.06
Number of grafts	2.97±1.13
On-pump	198(79.2)
<i>Long-term recovery</i>	
Major adverse cardiac event	9(3.6)
Deceased	10(4.0)
<i>Psychosocial stress variables</i>	
Depressive symptoms	8.54±6.55
Anxiety	5.87±4.33
Stressful life events	1.32±1.27
Social support	28.7±5.7

Figure 3.2 depicts the mean cortisol profiles across the day of patients who either died or experienced a MACE, and patients who experienced no events in the years following bypass surgery. Cortisol slope, cortisol AUC, CAR, and waking cortisol levels were not significantly associated with EuroSCORE, cardiopulmonary bypass, or chronic illness burden. Evening cortisol levels were associated with EuroSCORE ($r = 0.14$, $p = 0.030$)

but not with cardiopulmonary bypass or chronic illness burden. Occurrence of death or MACE following surgery was associated with EuroSCORE ($r = 0.24, p < 0.001$), and chronic illness burden ($r = 0.19, p = 0.003$).

In terms of psychosocial stress variables, patients scored relatively low on measures of depression and anxiety and had experienced roughly one stressful life event in the previous six months (Table 3.1). Social support as measured by the ESSi appeared to be relatively high in this sample ($M = 28.7, SD = 5.7$).

3.6.1 Pre-surgical cortisol and clinical outcomes

Diurnal cortisol slope predicted the occurrence of death or MACE following CABG surgery (hazard ratio = 0.73, 95% CI = 0.56 – 0.96, $p = 0.023$) (see Table 3.2). Patients with a steeper cortisol decline over the day were at reduced risk of experiencing adverse clinical outcomes (Table 3.2). More specifically, these results indicate that for every 1 nmol/l/h increase in cortisol slope the risk of death or MACE fell by 27%. Chronic illness burden ($p = 0.035$) and EuroSCORE ($p = 0.002$) also predicted death or MACE following surgery.

These results indicate that higher illness burden and a worse EuroSCORE were associated with negative outcomes in the years following surgery. These analyses were repeated after excluding immediate events (3 events) that occurred in the 5 day post-operative period. A steeper pre-surgical cortisol slope remained predictive of reduced risk of adverse clinical outcomes (hazard ratio = 0.70, 95% CI = 0.52 – 0.94, $p = 0.017$). For every nmol/l/hr increase in cortisol slope, the risk of death or MACE after the 5-day post-operative period fell by 30%. These survival analyses were carried out treating cortisol slope as a continuous variable, but for descriptive purposes participants were split into two equal groups based on cortisol slope using a median split. Cortisol changes over the

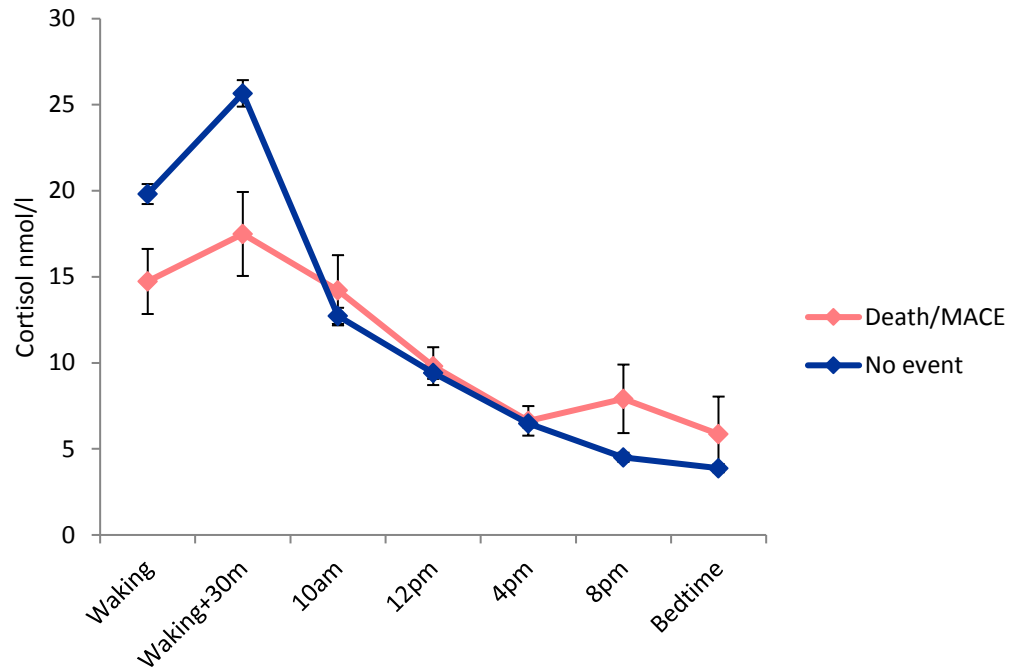


Figure 3.2. Mean salivary cortisol values sampled on waking, waking+30mins, in the morning (10am), at noon, in the afternoon (4pm), evening (8pm), and at bedtime, in patients who experienced death or MACE in the years following surgery (pink line) and in patients who experienced no events (blue line). Error bars represent SEM.

day ≤ 1.68 nmol/l/h were considered indicative of ‘flatter’ slopes. Kaplan-Meier survival plots of the two groups are shown in Figure 3.3. This plot reveals that divergence in survival/occurrence of MACE as a function of cortisol slope emerges very soon after CABG surgery.

Table 3.2. Results of Cox regression analysis; showing predictive effects of cortisol slope and covariates on the occurrence of MACE and/or death in the years following CABG surgery*

Variable	Coefficient (B)	SE	Wald χ^2	p	HR	95% CI
Cortisol slope	-0.31	0.14	5.16	.023	0.73	0.56–0.96
Chronic illness burden	0.67	0.32	4.46	.035	1.96	1.05-3.65
EuroSCORE	0.18	0.06	9.27	.002	1.20	1.07-1.35
Bypass ^a	-0.28	0.67	0.18	.670	0.75	0.20-2.79

*This model includes MACE/mortality cases that occurred within the 5 day post-operative period

^aWhether the patient underwent cardiopulmonary bypass (on pump/off pump)

A flatter cortisol slope across the day can be due to low cortisol output on waking and/or higher evening cortisol values. We therefore examined associations between both waking and evening cortisol and clinical outcome. Waking cortisol was inversely associated with clinical outcome (hazard ratio = 0.93, 95% CI = 0.88 – 0.98, $p = 0.011$) suggesting that higher cortisol output on waking is linked to event-free survival. Evening cortisol levels were also significantly associated with clinical outcome (hazard ratio = 1.09, 95% CI = 1.01 – 1.17, $p = 0.019$) indicating that higher evening cortisol is linked to MACE or death in the years following surgery. So the relationship between cardiac morbidity and flatter slope appeared to result both from lower cortisol on waking and higher cortisol in the evening.

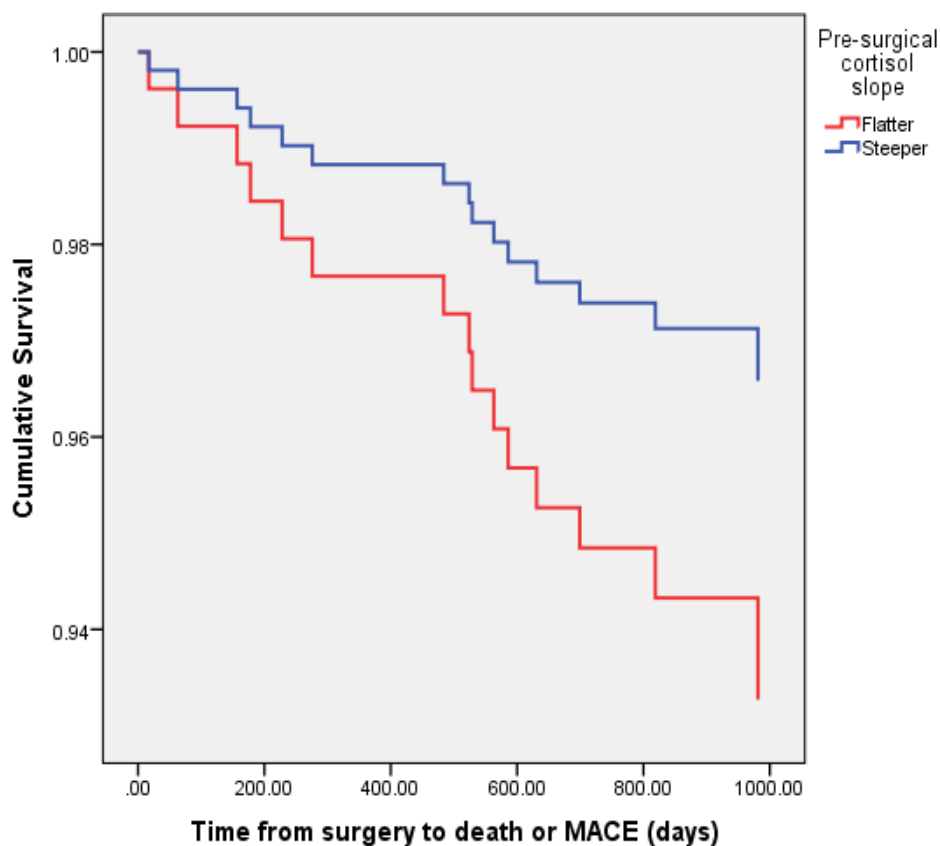


Figure 3.3. Kaplan-Meier survival curves for patients split into two equal groups at the median diurnal cortisol slope. This median split was performed only for illustrative purposes.

We also examined the association between cortisol AUC and adverse clinical outcomes (Table 3.3). Pre-surgical AUC did not predict survival or the occurrence of a MACE in

Table 3.3. Results of Cox regression analysis, showing predictive effects of cortisol AUC and covariates on the occurrence of MACE and/or death in the years following CABG surgery*

<i>Variable</i>	<i>Coefficient (B)</i>	<i>SE</i>	<i>Wald χ^2</i>	<i>p</i>	<i>HR</i>	<i>95% CI</i>
Cortisol AUC	0.00	0.00	0.32	.575	1.00	1.00-1.00
Chronic illness burden	0.49	0.36	1.90	.169	1.63	0.81-3.29
EuroSCORE	0.18	0.06	7.47	.006	1.19	1.05-1.35
Bypass ^a	-0.04	0.80	0.00	.962	0.96	0.20-4.58

*This model includes MACE/mortality cases that occurred within the 5 day post-operative period

^a Whether the patient underwent cardiopulmonary bypass (on pump/off pump)

Table 3.4. Results of Cox regression analysis, showing predictive effects of CAR and covariates on the occurrence of MACE and/or death in the years following CABG surgery*

<i>Variable</i>	<i>Coefficient (B)</i>	<i>SE</i>	<i>Wald χ^2</i>	<i>p</i>	<i>HR</i>	<i>95% CI</i>
CAR (<15m delay)	-0.01	0.03	0.03	.871	0.99	0.94-1.05
CAR (<5m delay)	-0.01	0.04	0.06	.813	0.99	0.91-1.07
Chronic illness burden	0.48	0.42	1.33	.249	1.62	0.71-3.69
EuroSCORE	0.18	0.08	5.03	.025	1.53	1.02-1.39
Bypass	0.43	1.07	0.16	.690	1.19	0.19-12.6

*This model includes MACE/mortality cases that occurred within the 5 day post-operative period

the years following bypass surgery ($p = 0.27$). Excluding death or MACE that occurred in the 5 day post-operative period did not change these results. Similarly, pre-surgical CAR did not predict survival or MACE occurrence in the years following bypass surgery regardless of the accuracy margin used (<15 min delay: $p = 0.87$, <5 min delay: $p = 0.81$) (Table 3.4). This association also remained non-significant after excluding death or MACE that occurred in the 5 day post-operative period.

As mentioned in *Section 3.5.5* age and sex were not adjusted for separately in the Cox regression models as both age and sex are accounted for in the EuroSCORE. However, exploratory analysis revealed that after simple adjustment for age and sex, cortisol slope no longer significantly predicted survival or MACE occurrence ($p = 0.07$), whereas sex did (hazard ratio = 3.91, 95% CI = 1.47 – 10.4, $p = 0.006$). This will be discussed as a limitation in *Section 3.7*.

3.6.2 Pre-surgical cortisol slope and psychosocial stress variables

Since pre-surgical cortisol slope was associated with the occurrence of death or MACE, one possibility is that it reflects a negative psychosocial stress profile, and that this in turn might be related to cardiovascular morbidity. I therefore computed cross-sectional associations between pre-surgical cortisol slope and psychosocial stress variables; I hypothesised that steeper cortisol slopes would be related to fewer depressive symptoms, lower anxiety, fewer stressful life events, and greater social support. These results are summarised in *Table 3.5*. Interestingly, cortisol slope was not associated with any of the

Table 3.5. Cross-sectional associations between pre-surgical cortisol slope, and waking and evening levels, and psychosocial stress variables

	Depressive symptoms		Anxiety		Stressful life events		Social support	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Cortisol slope	-0.83	.193	-0.01	.822	-0.09	.131	0.04	.555
Waking cortisol	-0.13	.040	-0.03	.638	-0.09	.170	0.10	.113
Evening cortisol	0.04	.523	0.02	.751	0.01	.863	-0.03	.646

psychosocial stress variables (all p values > 0.05). We also examined associations between waking and evening cortisol, and the psychosocial stress variables. Waking

cortisol was not correlated with anxiety symptoms ($r = -0.05$, $p = 0.64$), stressful life events ($r = -0.09$, $p = 0.17$), or social support ($r = 0.10$, $p = 0.11$). However, there was a significant correlation between depressive symptoms and waking cortisol ($r = -0.13$, $p = 0.040$). This indicates that higher levels of waking cortisol were associated with lower depressive symptoms. As mentioned previously, higher waking cortisol levels were also linked to event-free survival. However, the association between depressive symptoms and waking cortisol levels became non-significant in a simple age- and sex-adjusted linear regression ($p = 0.13$). There were no significant associations between evening cortisol levels and any of the psychosocial stress variables (all $p > 0.05$).

3.7 Discussion

3.7.1 Summary of results

As hypothesised, the results of the study suggest that a flatter diurnal cortisol slope prior to surgery predicts the occurrence of MACE or mortality in CABG patients. Cortisol was sampled a month before surgery, so does not reflect acute anticipatory stress responses prior to surgery. Our findings suggest that a flatter cortisol slope is related to poorer long-term outcomes in a patient sample with advanced CVD following bypass surgery, and that this association is being driven by alterations in both waking and evening cortisol. These associations were independent of EuroSCORE, whether or not the patient underwent cardiopulmonary bypass, and chronic illness burden. There was no association between pre-surgical CAR or cortisol AUC and adverse clinical outcomes in the years following surgery. Contrary to expectation, there were no significant relationships between cortisol slope and depressive symptoms, anxiety, stressful life events, or social support. Higher waking cortisol was associated with lower depression scores. However, this association did not survive a simple age- and sex-adjusted linear regression.

3.7.2 Comparison to previous research

To my knowledge, this is the first study to examine the association between diurnal cortisol and the occurrence of MACE or mortality in the years following CABG surgery. These findings are in line with research which has reported associations between flatter diurnal slopes and adverse clinical events in other serious illnesses. For example, a flatter cortisol slope has been found to predict worse prognosis and mortality in metastatic breast cancer, lung cancer, and epithelial ovarian cancer (Schrepf et al., 2015; Sephton et al., 2013; Sephton, Sapolsky, Kraemer, & Spiegel, 2000). Patients with heart disease have been found to have a flatter cortisol rhythm compared with healthy controls (Nijm, Kristenson, Olsson, & Jonasson, 2007). However, as mentioned previously Bhattacharyya and colleagues found no significant difference in cortisol slope between patients with CAD and healthy controls (Bhattacharyya et al., 2008). What the authors did find was that cortisol slopes were flatter in CAD patients who were depressed (*ibid*). In the current study we found no association between depressive symptoms and cortisol slope. Therefore, the association between cortisol rhythm and mortality and adverse events in the current sample indicates that HPA axis dysregulation may increase with disease progression.

Our results indicate that both waking and evening cortisol levels predicted adverse outcomes for CABG patients, so the adverse effects of flatter profiles are not the result only of reduced waking concentration or elevated evening values. Kumari and colleagues found that an association between flatter cortisol slope and CVD mortality in a nonclinical sample was driven primarily by changes in evening levels of cortisol only (Kumari et al., 2011). One reason for this discrepancy may be that HPA axis dysregulation has progressed further in individuals with advanced CVD. Fatigue and vital exhaustion are associated with cortisol output, as well as being risk factors for the occurrence of adverse

cardiac events over time (Nicolson & van Diest, 2000; Williams et al., 2010). Associations between lower levels of waking cortisol and fatigue have been reported in older adults and coronary artery disease patients (Bunevicius et al., 2012; Kumari et al., 2009). Breast cancer survivors suffering from fatigue have been found to have flatter diurnal cortisol slopes than survivors without fatigue (Bower et al., 2005). It is possible that fatigue or exhaustion may be a factor influencing the association between lower waking cortisol and adverse outcomes in the current study.

3.7.3 Potential mechanisms explaining the link between diurnal cortisol rhythm and adverse outcomes

It is likely that one of the factors contributing to the link between cortisol rhythm and MACE/mortality in these patients is inflammation. As mentioned before, cortisol exerts an immunomodulatory effect on inhibiting the release of pro-inflammatory cytokines. Therefore, dysregulation of the HPA axis in these patients may lead to sustained high levels of inflammation (Nijm & Jonasson, 2009). A flattened cortisol slope has been associated with higher levels of circulating IL-6 in CHD (Nijm et al., 2007), epithelial ovarian cancer (Schrepf et al., 2015) and metastatic colorectal cancer (Rich et al., 2005).

CABG surgery leads to substantial increases in cortisol concentration that decline over the post-operative period, and is coupled with alterations in sensitivity to ACTH (Gibbison et al., 2015). Mechanistically, dysregulation of the HPA axis in these patients was likely caused in part by diminished sensitivity of the GR and MR. Diminished GR sensitivity has been associated with a flatter diurnal cortisol slope (Jarcho et al., 2013). It is possible that the association between diurnal cortisol slope and MACE observed in the current study reflects reduced sensitivity of the corticosteroid receptors. Future research

should simultaneously assess both diurnal cortisol rhythm and GR and MR function in CVD patients.

3.7.4 CAR: Lack of association

In this study I found no association between the CAR and the overall cortisol output (AUC) and MACE or mortality following CABG surgery. One possibility regarding the CAR is that the chosen accuracy margin of <15 minutes between waking and providing the waking sample was too wide which went on to attenuate CAR estimates (Stalder et al., 2016). However, using a smaller accuracy margin of <5 minutes also produced non-significant results. This indicates that the CAR was not associated with adverse clinical outcomes in this patient sample. Interestingly, there is evidence that the CAR is under a regulatory cycle distinct from the other diurnal cortisol parameters (Clow et al., 2004) which may in part explain why associations were seen between cortisol slope and adverse outcomes, but not the CAR. A previous study examining associations between diurnal cortisol rhythm and cardiovascular mortality in a non-clinical sample also found no significant associations with the CAR (Kumari et al., 2011). Similarly, cortisol AUC also was not associated with coronary artery calcification in a non-clinical population (Matthews et al., 2006). A study of lung cancer survival also found that cortisol AUC had no predictive value in terms of mortality (Sephton et al., 2013). This adds support to the notion that measuring cortisol slope across the day is likely to be a more useful prognostic tool than the awakening response or total cortisol output. Inflammatory cytokines exhibit distinct diurnal patterns that are inversely related to the diurnal rhythm of cortisol (Petrovsky, McNair, & Harrison, 1998). Diurnal cortisol rhythm may then be a more useful prognostic tool as it reflects dysregulation of inflammation, whereas the CAR or total cortisol output does not.

3.7.5 Psychosocial stress: Lack of association

Flatter diurnal cortisol rhythms have been associated with a number of psychosocial stress factors, such as depression, chronic stress, and work stress (Bhattacharyya et al., 2008; Liao et al., 2013; Miller et al., 2007). Many of these factors are associated with CHD incidence (Steptoe & Kivimäki, 2012), and also contribute to recurrent events and mortality in patients with advanced CVD (Meijer et al., 2011). However, in the current study we found no associations between flatter cortisol slopes and any of the psychosocial stress variables measured. When examining waking and evening levels of cortisol separately, we did find that depressive symptoms were negatively correlated with levels of waking cortisol. In the current study, higher waking cortisol levels were also linked to event-free survival, meaning that depressive symptoms may play a role in this link. However, the correlation did not survive a simple age- and sex-adjusted regression indicating that the association between depressive symptoms and cortisol profiles in these patients is tenuous at best. Previous research has shown that waking cortisol levels are increased in depressed individuals and in people at risk of depression (Bhagwagar, Hafizi, & Cowen, 2005; Mannie, Harmer, & Cowen, 2007) which may be why the association reported in the current study disappeared after adjustment for age and sex.

One of the reasons why we may not have found cross-sectional associations between the psychosocial stress variables and diurnal cortisol rhythm in this study is that our patient sample was relatively unstressed. That is, they had a rather low number of stressful life events, low depression and anxiety scores, and high levels of social support. It may be that the association between stress and diurnal cortisol profiles in the current study were too weak to detect. Future research of this kind should seek to include more stress-related measures such as early childhood adversity, and measures of perceived stress. Another possibility is that because patients all had advanced coronary artery disease, cortisol was

driven more by the physiological dysregulation associated with the disease itself rather than psychosocial factors.

3.7.6 Strengths and limitations

A strength of this study is that cortisol was measured repeatedly across a day several weeks before surgery. The pattern of output may therefore represent habitual profiles rather than being affected by acute anticipation of hospitalisation. Had cortisol been measured the day before surgery, for example, there may have been alterations in the normal diurnal cortisol profiles of the patients. Anticipation of a stressful event has been shown to result in an elevated CAR (Wetherell et al., 2015) and acute stress has also been found to affect cortisol slope and evening levels (Hulme, French, & Agrawal, 2011).

The study had a prospective design and attrition was low, with ascertainment of clinical outcomes in more than 95% of participants. However, the sample size was relatively small, with MACE and death occurring in only 18 participants (19 events). The ARCS study was not specifically designed to investigate cortisol and cardiac outcomes, and this limited statistical power and reduced the number of covariates that could be included in the analyses. Larger studies of patients with advanced cardiac disease are needed to establish the robustness of the findings. Our sample was largely composed of white men of European origin. Additionally, this sample also appeared to be particularly well-adjusted in terms of psychosocial stress factors. Thus, the results may not be readily generalizable to other groups. We were unable to access information about specific causes of death for all patients who died. Therefore, specific associations between dysregulation of the HPA axis and CVD mortality could not be assessed. It is possible that a number of these patients died from non-cardiac causes. Nevertheless, since all these patients had advanced CHD, this is likely to have been the cause of the majority of deaths.

Another issue is that cortisol was measured over a single day meaning that the diurnal rhythm may have been affected by situational factors related to that particular day, rather than long-term factors. Measurement over several days might provide a more robust estimate of stable individual differences in the diurnal cortisol profile. Previous research has shown that measurement of CAR and AUC data over a single day is affected more by situational rather than trait factors (Hellhammer et al., 2007). However, these same authors argue that measures of CAR and AUC are predominantly affected by trait factors on weekdays, and situational factors on weekend days. In the current study, cortisol was measured over the course of a weekday which may help counteract the effects of single-day sampling. Furthermore, previous studies of the stability of cortisol across days have been predominantly based on younger people (students or working adults) in which factors related to demands on different days are accentuated. Our sample had an average age of 68 years, and most patients were retired. Their habits may be less variable across days, meaning that a single day could be more representative than in younger people. Nonetheless, these measurement issues should be borne in mind while interpreting these results. Another factor which may have influenced cortisol secretion in this sample was the inclusion of patients who use steroid medications. Synthetic corticosteroids are known to affect the negative feedback regulation of the HPA axis and also potentially affect the immunoassays used to measure cortisol (Granger, Hibel, Fortunato, & Kapelewski, 2009). Consequently inclusion of these patients may have influenced the results of this study. However, the use of steroid medications was not associated with any of the cortisol parameters. Therefore these participants were included in the analysis in order to increase statistical power.

Additionally, night-time cortisol was not measured so it was not possible to assess total 24 hour cortisol exposure. Cortisol levels reach their nadir at about midnight (Adam &

Kumari, 2009) and increase slowly throughout the night remaining at low levels at waking. In the current study there was an association between low levels of waking cortisol and adverse long-term clinical outcomes. Therefore, examining the gradual night-time increase in these patients would have been of interest. Sleep complaints have been shown to play a role in adverse recovery following CABG surgery (Poole, Kidd, et al., 2014b) indicating that poor sleep quality in these patients may be a relevant factor in their night-time/waking cortisol profiles.

Furthermore, these data do not provide direct evidence of a causal connection between diurnal cortisol slope and MACE or mortality in these patients; although we included important clinical covariates, there might be unmeasured factors influencing diurnal cortisol rhythms that also increased risk of adverse outcomes. For example, both BMI (Champaneri et al., 2013) and smoking (Badrack, Kirschbaum, & Kumari, 2007) have been shown to affect diurnal cortisol profiles, as well as adverse clinical outcomes in CVD patients (Critchley & Capewell, 2003). However, I was unable to control for these factors in the main analysis due to the relatively low number of events in the study. Additional exploratory analyses were carried out in order to see if other factors might influence adverse clinical outcomes in these patients. Adding BMI, smoking, sleep disturbance, depression, or anxiety to the model did not affect the association between cortisol slope and adverse clinical outcomes. Furthermore, none of these factors were significantly associated with adverse outcomes in these patients.

However, additional exploratory analysis revealed that after simple adjustment for age and sex, cortisol slope was no longer significantly associated with MACE or mortality, whereas sex was. This may be because women who undergo coronary revascularisation generally experience greater complications and mortality post-surgically (Kim, Redberg, Pavlic, & Eagle, 2007) due to older age at diagnosis, increased thrombotic complications,

and anatomical mechanisms such as smaller coronary arteries compared to men (Swaminathan et al., 2016). Future research in CABG patients should seek to have a more balanced gender ratio.

3.7.7 Conclusion

In conclusion, these results indicate that a flatter diurnal cortisol slope prior to surgery is associated with poorer long-term outcomes in patients undergoing coronary revascularisation. They provide evidence for a possible role of HPA axis dysregulation in CVD and indicate that more pronounced dysregulation may be a marker of more advanced disease progression in CVD patients. On the other hand, the results tell us little about what brings about HPA axis dysregulation in these patients. In the following chapter I will introduce the Stress Pathways Study which was designed to examine the effects of pharmacological blockade on a number of stress-related biological factors including HPA axis function. The results of the Stress Pathways Study may tell us more about how dysregulation of HPA axis functioning comes about.

Chapter 4

The Stress Pathways Study: Introduction and methods

4.1 The Stress Pathways Study

As outlined in Chapter 2, to date, evidence for the role of HPA axis dysregulation in the stress-CVD link has been provided by both observational studies and psychophysiological laboratory stress testing. One way in which more in-depth information about stress-related biological pathways can be derived is through the use of pharmacological blockade experiments. In these experiments, putative pathways are pharmacologically blocked and effects on stress-related biological systems are measured.

The Stress Pathways Study was set up in order to investigate the psychobiological mechanisms through which psychosocial stress is thought to contribute to the development of CVD. This study was a randomised controlled trial (RCT) which assessed the effects of pharmacological blockade on a number of biological and psychological responses to acute laboratory stress. More specifically, healthy volunteers were randomised to receive short-term doses of escitalopram (a selective serotonin reuptake inhibitor (SSRI)), propranolol (a beta-blocker), or placebo, and the effects of these drugs on responses to acute psychosocial stress in the laboratory were measured.

The Stress Pathways Study was primarily designed to assess the effects of pharmacological intervention on inflammatory responses to acute stress. SSRIs and beta-blockers were selected mainly due to their relevance to inflammation. In principle, the inflammatory stress response may be partially regulated by processes at two levels – the central and peripheral. SSRIs induce changes in central neurotransmitter function. In addition to their ability to alter the reuptake of serotonin, SSRIs exert anti-inflammatory

actions and it is thought that this is one of the ways in which they impact upon depressed mood (Walker, 2013). Therefore, SSRIs were selected for inclusion in the study as it is plausible that SSRI-induced changes in the CNS may attenuate the inflammatory stress response. Beta-blockers were chosen in order to assess effects of changes in the peripheral nervous system, and the impact of sympathetic activation on the inflammatory stress response. Both short- and long-term administration of beta-blockade have been shown to reduce basal levels of inflammatory cytokines in murine models (Nguyen et al., 2008), as well as in CHD, heart failure, and cardiomyopathy patients (Aronson & Burger, 2001; Jenkins, Keevil, Hutchinson, & Brooks, 2002; Ohtsuka et al., 2001). Therefore, beta-blockade may plausibly reduce inflammatory responses to acute stress.

The inflammatory markers measured in the Stress Pathways Study included IL-6, IL-1Ra, and monocyte chemoattractant protein 1 (MCP-1). Markers of endothelial dysfunction were also analysed. Secondary aims of the study also included assessment of the effects of pharmacological interventions on cardiovascular parameters, neuroendocrine parameters, and a number of psychosocial factors. Cardiovascular parameters included blood pressure, heart rate, and cardiac index. Neuroendocrine parameters included cortisol stress reactivity and corticosteroid receptor function. The effects of the pharmacological probes on diurnal cortisol profiles were also assessed prior to the stress laboratory visit. A number of psychosocial factors were measured via self-report. These included depressive symptoms, anxiety, perceived stress, affect, sleep quality, optimism, and a number of health behaviours including smoking, alcohol intake, and physical activity.

4.2 Differentiating my PhD from the Stress Pathways Study

The Stress Pathways Study was a multidisciplinary study involving several researchers that produced a rich dataset containing information about a number of stress-related

biological and psychological parameters. As mentioned above, the primary focus of the Stress Pathways Study was to assess the effects of SSRIs and beta-blockers on inflammatory responses to acute psychosocial stress in a laboratory setting. My PhD study was an extension of this larger study that had related but different aims. Primarily, I sought to use the Stress Pathways Study as an opportunity to investigate the impact of acute psychosocial stress on corticosteroid receptor sensitivity and cortisol stress reactivity. I also sought to assess the effects of the pharmacological probes on both corticosteroid receptor sensitivity and cortisol stress reactivity in order to gain insight into some of the biological mechanisms involved. Additionally, I also examined the effects of SSRIs and beta-blockers on diurnal cortisol rhythm outside of the laboratory in order to learn more about what might bring about dysregulation of the HPA axis. The focus of the following chapters (Chapters 5 and 6) will be on the effects of the study medications on these three aspects of HPA axis function. Detailed reviews of the literature examining the effects of beta-blockers and SSRIs on basal/diurnal HPA axis function, and cortisol stress reactivity and corticosteroid receptor function will be provided in Chapters 5 and 6 respectively.

4.3 The pharmacological probes

We chose to assess the effects of seven-day administration of SSRIs and beta-blockers on biological responses to acute psychophysiological stress. Originally these drug types were chosen to further explore their known effects on inflammatory aspects of the stress response. Therefore, these pharmacological interventions may not be the most suited to eliciting information about the biological pathways involved in dysregulation of the HPA axis, and the link with CVD. The effects of these drugs on certain aspects of HPA axis function have been investigated previously and detailed literature reviews will be provided in Chapters 5 and 6. However, overall the effects of these drugs on stress-related

and basal HPA axis function are not very well-known and therefore relatively exploratory. The implications of these drug choices for this PhD thesis will be dealt with in detail in the final Discussion chapter (Chapter 7).

Specifically, we chose to assess the effects of the beta blocker propranolol and the SSRI escitalopram in the Stress Pathways Study for reasons outlined below.

4.3.1 Beta-blocker: Propranolol

The SNS is one of the major systems activated during the stress response. Its effectors epinephrine and norepinephrine are released from the adrenal glands via stimulation from the brain stem during times of stress. These catecholamines serve to initiate the ‘fight or flight’ response resulting in increased heart rate, respiratory rate, as well as energy mobilisation within cells throughout the body. Beta-blockers are antihypertensive agents also used to treat tremors and anxiety. Their mechanism of action is believed to be through blocking the effects of catecholamines on β -adrenoreceptors, thereby reducing cardiac output, and attenuating the pressor response to catecholamines during times of stress and anxiety (Ripley & Saseen, 2014).

Beta-blockers are widely prescribed in the treatment of hypertension and have a wide survival benefit for those with CHD and heart failure (Gorre & Vandekerckhove, 2010). Beta-blockers may partially exert their therapeutic effect by altering the biological stress response in a way that is beneficial for cardiac health. As mentioned previously, beta-blockers have been shown to reduce basal levels of inflammation in CHD patients (Jenkins et al., 2002). Beta-blockers also attenuate cardiovascular responses to acute stress (Mills & Dimsdale, 1991), and have been shown to mitigate the natural killer cell stress response (Benschop et al., 1994). The release of natural killer cells is activated by a number of pro-inflammatory cytokines. A decrease in natural killer cell release

following stress indicates that beta-blockade may be attenuating the release of these cytokines. For these reasons we chose to incorporate beta-blockade into the study. Additionally, there is evidence to suggest that using beta-blockers to attenuate sympathetic activation will have effects on HPA axis function, and corticosteroid receptor function. This evidence will be described in detail in Chapters 5 and 6.

Specifically, we chose to use the beta-blocker propranolol in the Stress Pathways Study. Propranolol was chosen as it is a widely prescribed non-selective beta-blocker. This means that it blocks the effects of catecholamines at both β 1- and β 2-adrenoceptors, rather than selectively binding to one or the other. This is useful as both of these receptors are involved in the biological stress response (β 1 – cardiac output; β 2 – *fight or flight*). Propranolol was also chosen as it does not bind to the α -adrenoceptors. This means that any observed effects of propranolol on biological responses to stress in the Stress Pathways Study can be ascribed to blockade of the β -adrenoceptors only.

As propranolol is rapidly metabolised with a plasma elimination half-life of approximately 4 hours it normally has to be administered 2-4 times per day (Leahey, Neill, Varma, & Shanks, 1980). Therefore, in order to reduce burden we gave participants 80mg of sustained-release propranolol once a day after breakfast. Participants took this every morning for seven days. The 80mg dosage was decided on by a clinical research fellow involved in the development of the Stress Pathways Study. This dosage is the minimum recommended clinical dosage and therefore was chosen for use in a sample of healthy volunteers in order to minimise the likelihood of possible side effects. The seven day duration period was chosen to keep participant burden to a minimum.

4.3.2 SSRI: Escitalopram

Serotonin is a neurotransmitter that is synthesised from its chemical precursor the essential amino acid tryptophan in the raphe nuclei of the brain. From these nuclei, serotonergic neurons spread to almost all parts of the CNS making the serotonergic system one of the most diffuse neurochemical systems in the body (Lanfumeey, Mongeau, Cohen-Salmon, & Hamon, 2008). A wide variety of functions are controlled in part by serotonin such as cardiovascular function and endocrine regulation. SSRIs are the most widely prescribed antidepressant drug type. They serve to block the reuptake of serotonin resulting in an increase in neurotransmitter availability at the synaptic cleft (Stahl, 1998). Depression is one of the most common stress-related disorders, and depression is a known independent risk factor for CHD (Nicholson et al., 2006; Van der Kooy et al., 2007). CVD patients are also known to have higher levels of depression compared to the general population (Hare, Toukhsati, Johansson, & Jaarsma, 2014).

Seeing as SSRIs are the treatment of choice for depression, and many patients with comorbid CVD and depression take SSRIs (Shapiro, 2015), we chose to include this medication type in the Stress Pathways Study in order to assess its effects on biological responses to stress. These effects on the stress response may be clinically relevant for CVD patients. SSRIs also have known influences on cardiovascular responses to stress as mentioned in *Section 4.1* (Golding et al., 2005; Hanson, Outhred, Brunoni, Malhi, & Kemp, 2013). It has been proposed that SSRIs may exert their therapeutic effect via reduction in levels of inflammatory cytokines (Hannestad, DellaGioia, & Bloch, 2011). Additionally, there is evidence that SSRIs alter HPA axis function in ways that are relevant to the therapeutic action of the medication (Pariante, Thomas, et al., 2004). This evidence will be described in more detail in both Chapter 5 and 6. Seeing as SSRIs appear to affect a

number of the systems involved in the stress response we decided to include this type of medication in the study.

Specifically, we chose to use the SSRI escitalopram in the Stress Pathways Study. Escitalopram was chosen based on results from a study which showed that this particular SSRI significantly reduced mental stress-induced myocardial ischaemia in patients with stable CHD, as well as reducing blood pressure and heart rate responses to stress (Jiang, Velazquez, Kuchibhatla et al., 2013).

Additionally, escitalopram was chosen as it is a fast-acting antidepressant with a rapid onset of action compared to other SSRIs (Kasper, Spadone, Verpillat, & Angst, 2006). Steady-state concentrations of this medication are achieved within seven days of administration (Rao, 2007) which fits with the Stress Pathways Study protocol. In adults the dosage for escitalopram may vary from 5 to 20mg once daily. We gave participants 10mg escitalopram once daily to take after breakfast. We chose this dose for a number of reasons. Firstly, previous research where escitalopram has been administered to healthy participants have prescribed a 10mg dose (Arce, Simmons, Lovero, Stein, & Paulus, 2008; Bui et al., 2013; Knorr et al., 2012). Secondly, clinically meaningful effects of 10mg escitalopram have been seen after one week in patients with major depressive disorder (Burke, Gergel, & Bose, 2002; Montgomery, Loft, Sánchez, Reines, & Papp, 2001; Nierenberg et al., 2007). Thirdly, as we were administering these drugs to non-depressed healthy volunteers we chose the 10mg dosage to minimise the chance of adverse drug effects.

4.4 Method

4.4.1 Study design

The Stress Pathways Study was a randomised, double-blind, placebo-controlled, parallel-groups trial. Participants were randomised to receive 10mg/day escitalopram, 80mg/day propranolol, or placebo, for seven days in a double-blind manner using simple random allocation. This was carried out using a random number generator and was stratified by sex to ensure an equal amount of men and women in each condition. The allocation sequence was generated using computer software by a member of departmental staff not involved in the Stress Pathways Study. On the sixth day of medication, participants provided seven saliva samples across the day for measurement of diurnal cortisol secretion. On the seventh day of medication, participants underwent psychophysiological stress testing in the laboratory.

4.4.2 Sample size

To date, no study has assessed the effects of SSRIs on inflammatory responses to acute mental stress. Therefore, the sample size for the Stress Pathways Study was calculated with reference to a previous study looking at the efficacy of aspirin and propranolol in attenuating the inflammatory response (IL-6) to stress (von Känel, Kudielka, Metzenthin, et al., 2008). In this previous study, propranolol had a small effect size of 0.2 (n = 17 in the propranolol group). Due to time and laboratory constraints, the Stress Pathways Study aimed to have a sample of 90 participants (n=30 per medication group). We performed statistical power analyses using G*Power software which revealed that with a sample of 90 participants we would have >65% power to detect the effects of propranolol on levels of the inflammatory cytokine IL-6. As no previous studies have reported effect sizes for associations between beta-blockers, SSRIs and cortisol stress reactivity in the laboratory,

power calculations directly relevant to neuroendocrine parameters, and therefore this PhD, could not be carried out.

4.4.3 Participants

As mentioned in the previous section, we planned to recruit a sample of 90 participants (n=30 per medication group). Participants were recruited in and around UCL campus via email and poster advertisements. Participants were then contacted via telephone to screen them based upon the inclusion and exclusion criteria detailed below. Participants had to be generally healthy, aged between 18-65 years and not taking any medications regularly (excluding the contraceptive pill). Specific exclusion criteria included any haematological, pulmonary, liver, renal, gastrointestinal, heart, cerebrovascular, and psychiatric disease; any history of thromboembolism, and any current infection. Participants who suffered from asthma, who had known allergies to any of the study medications, previous gastrointestinal bleedings, or who were currently pregnant or breastfeeding were excluded. Only patients with blood pressure in the normal range were included (90/60mmHg to 140/90mmHg). Recruitment began in October 2014 and was completed in August 2015.

We recruited 104 healthy men and women in total. Eight participants were excluded leaving 96 remaining participants with either complete or partial data. Out of these eight participants, four dropped out due to side effects potentially related to the study medications, two failed to turn up to their second study appointment, one participant lost their medications, and one participant took cold medication while taking the study medication and therefore had to be excluded from the trial. Of the remaining 96 participants, 94 provided saliva samples for the measurement of diurnal cortisol rhythm, 91 provided saliva samples in the laboratory for measurement of cortisol stress reactivity,

and 85 provided full or partial blood samples in the stress laboratory for the analysis of corticosteroid receptor sensitivity, inflammatory cytokines, and markers of endothelial dysfunction. Full or partial cardiovascular data from the stress laboratory was gathered for 90 participants. A more detailed flow diagram of participant data collection and attrition is provided in Figure 4.1. All data were collected with the written informed consent of the participants. Ethical approval for the Stress Pathways Study was obtained from the UCL Research Ethics Committee.

4.4.4 Study protocol

Day 1: Participants came to UCL for a short session where they provided consent to take part in the study and had the opportunity to ask any questions. Following this, body composition was measured (weight, height, waist circumference, waist-to-hip ratio, Bodystat®) and blood pressure was measured to ensure it was within the normal range. Female participants were asked whether or not they were taking oral contraception and were also asked to provide the date of the first day of their last period. Participants were asked to fill out a questionnaire containing demographic information and measures of depression, anxiety, affect, perceived stress, sleep quality, optimism, and health behaviours. Following this, participants received a bottle containing 12 capsules of the study medication (five extra in case the participant needed to reschedule the stress testing appointment unexpectedly). All capsules were identical to ensure the protocol was double-blind. Participants were instructed to take one capsule every morning after breakfast for seven days, the last one on the day of stress testing. Participants were advised not to take any other form of medication or herbal remedy while taking the study medications, and to avoid alcohol and vigorous physical activity. Participants were also provided with a saliva sampling kit with instructions for collection at home.

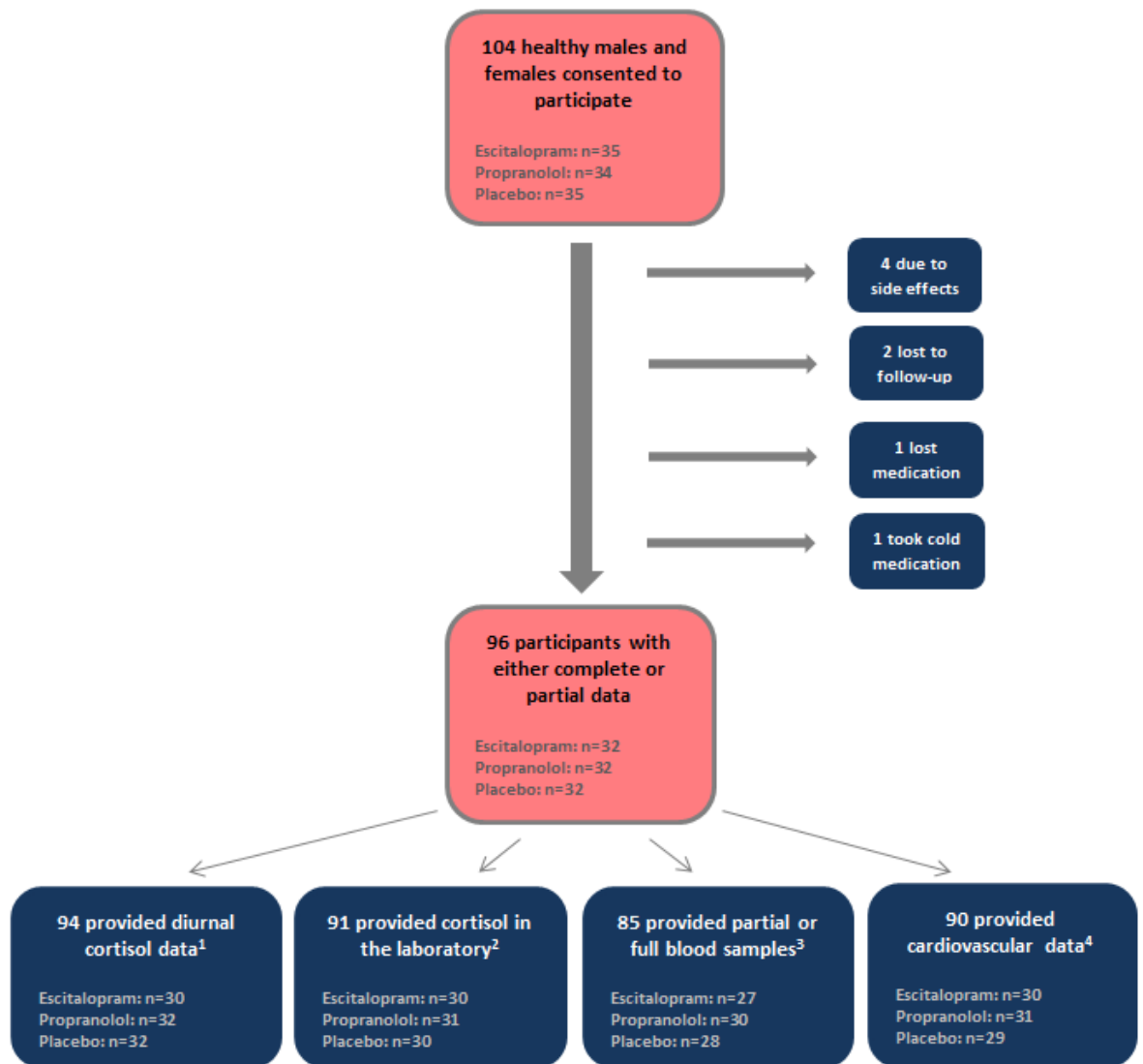


Figure 4.1. Flow diagram of participant data collection and attrition from the Stress Pathways Study

¹Two participants failed to provide diurnal cortisol samples. One failed to provide the samples due to exams. One forgot to provide the samples on the required day.

²Five participants failed to provide cortisol samples during the laboratory session. Two participants fainted during the session and were excluded. One participant smoked an e-cigarette during the session and was excluded. One participant missed the last dose of their medication and did not undergo the laboratory session as a result. One participant was unable to attend the laboratory session.

³Eleven participants failed to provide blood samples. The reasons why five participants could not provide blood samples are outlined in footnote ². Of the six remaining, four of the participants were unable to have a cannula inserted, and a phlebotomist was unavailable to take blood samples from two participants. Of the 85 people who provided blood samples, six of these provided partial blood samples: five participants provided blood at baseline and +45m. One participant provided blood at baseline, immediately post-stress, and +75m.

⁴Six participants failed to provide cardiovascular data in the laboratory. The reasons why five participants could not provide cardiovascular data are outlined in footnote ². One other participant failed to provide cardiovascular data as the Finometer failed to calibrate correctly.

Day 2: Participants began taking the study medication.

Day 7: Saliva sampling for the measurement of diurnal cortisol secretion took place on the day six of the medication. Participants were recruited in a manner which ensured saliva sampling always took place on a weekday. Participants used the saliva sampling kit they were provided with on Day 1. The kit included seven pre-labelled 'salivette' collection tubes (Sarstedt, Leicester, UK) and a cortisol sampling diary (Appendix F). The cortisol diary contained instructions on how and when to give samples. These diaries were also used to record information on factors likely to introduce variation in cortisol samples such as mood, exercise, and daily stressors. Participants provided seven saliva samples over the course of a week day, on waking, 30 minutes after waking (30+), 10am, 12pm, 4pm, 8pm, and bedtime. Participants stored their samples in the refrigerator before returning them to the researcher at their stress laboratory appointment the following day.

Day 8: Participants returned to the psychobiology laboratory at UCL to undergo psychophysiological stress testing either in the morning (9am-12pm) or the afternoon (1.30-4.30pm). They were instructed to refrain from engaging in any physical exercise prior to the session, from drinking any alcohol the night before the testing session, and from consuming any caffeine on the morning of the testing day. They were told to eat a light breakfast and/or lunch. Participants returned the remaining medication to the experimenter at this laboratory session and the experimenter then performed a pill count in order to ensure adherence to the protocol.

The laboratory protocol: On arrival at the laboratory participants completed a physical symptoms form in order to check health status. The experimenter noted the content and time of the participant's last meal and noted if anything unusual happened to them on their way to the testing session. The schedule for the stress testing session is summarised

in Figure 4.2. A questionnaire was given to the participant measuring depression, anxiety, sleep quality, affect, and any adverse effects from the study medications. The participant gave the initial practice saliva sample (S1) using a ‘salivette’ (Sarstedt, Leicester, UK). They were then brought to the stress protocol laboratory where they were fitted with an intravenous cannula and given 25 minutes to relax before a baseline blood sample (B1; approximately 35ml) and a baseline saliva sample (S2) were taken. The participant was attached to a Finometer in order to measure cardiovascular parameters (e.g. heart rate, blood pressure) continually throughout the testing session. Once the baseline blood and saliva samples were taken, the Finometer had been correctly calibrated, and the participant had completed a baseline subjective stress questionnaire (Appendix G) the psychophysiological stress protocol began.

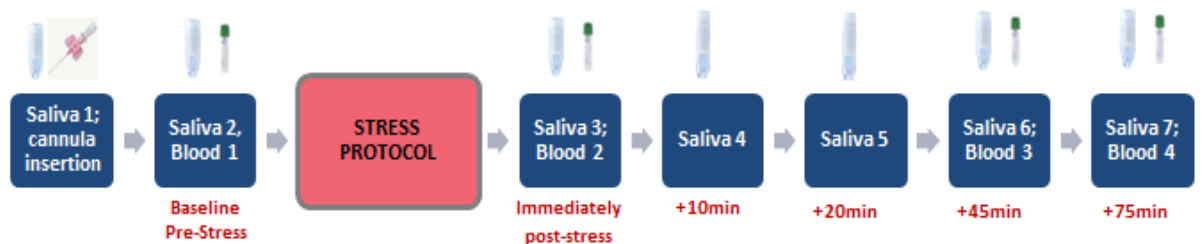


Figure 4.2. Study protocol: blood and saliva sampling

A modified version of the TSST (Kirschbaum et al., 1993) was used in this study in order to facilitate data collection. This modified version comprised three tasks.

1. Socially evaluative public speaking task: The participant sat facing a video camera. They were told that they should speak to the video camera as if it was a person and that all images recorded would be analysed and rated for content. The experimenter then read out a difficult interpersonal scenario to the participant who was told that after a 2-minute preparation period they would be given a free speech period of 3 minutes in which to tackle this interpersonal scenario. Each participant had to tackle the same interpersonal

scenario which involved being falsely accused of pickpocketing. The experimenter remained in the room for the duration of the task. If the participant stopped speaking within the free speech period the experimenter prompted them to continue by saying aloud the time remaining.

2. Mirror tracing task: The participant traced around a copper star with a metal stylus while only being allowed to see the mirror image of the star. Going off the star outline produced a loud error sound. Participants were instructed to trace around the star as many times as possible in 5 minutes while making as few errors as possible.

3. Arithmetic Task: The participant was asked to serially subtract the number 13 from 1,022 as fast and as accurately as possible for 5 minutes (Kirschbaum et al., 1993). On every failure the participant had to restart at 1,022 with the experimenter interfering 'Stop, 1,022'.

Between each individual stress task participants were given a moment to complete a task impact questionnaire in order to measure subjective stress ratings (Appendix G). Mean subjective stress ratings for each task were calculated from a single item on each questionnaire. This item asks participants to rate how stressed they felt during the task on a scale ranging from 1 'Not at all' to 5 'Very'. Subjective stress ratings were also measured prior to the stress protocol (resting), and 20 minutes after the protocol (recovery). Mean subjective stress ratings for each task and for the resting and recovery periods are provided in Figure 4.3. Following completion of all three tasks, the participant provided a saliva sample (S3 – immediately after stress) and a blood sample (B2). The experimenter left the room for 10 minutes to allow the participant to relax with some neutral reading material. At 10 minutes post-task the participant provided another saliva sample (S4) and again at 20 minutes post-task (S5). They were then allowed to relax for

25 minutes. At 45 minutes post-task the participant provided a saliva sample (S6) and blood sample (B3). This was also repeated at 75 minutes post-task (S7 and B4). Following the protocol, the cannula was removed from the participant's arm and they were detached from the Finometer. The experimenter debriefed the participant about the goal of the study and informed them that no video analysis of the speech task would be performed.

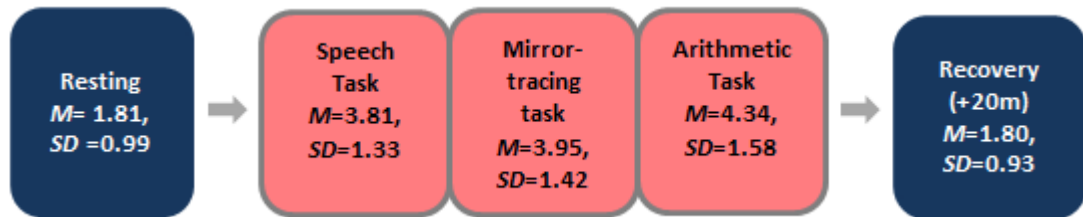


Figure 4.3. Flow diagram of the stress protocol with mean subjective stress ratings. Ratings were provided before the tasks, immediately after each task, and 20 minutes after the stress protocol had been completed.

4.4.5 Psychosocial measures

We chose to include a number of stress-related psychosocial measures in the Stress Pathways Study. At baseline we measured depression, anxiety, perceived stress, and positive affect. At follow-up (Day 8: testing session) we measured depression, anxiety, and positive affect. This was in order to examine whether the study medications affected any of these stress-related psychosocial factors. All questionnaire measures were researched and selected based on several criteria. First, measures that had been validated in healthy non-clinical populations were given preference. Second, validated brief or shortened versions were chosen over full versions to reduce response burden. Third, where measures were being administered twice, measures that have been shown to be valid over repeated time-points were chosen. Details of the individual measures used are

provided below and full versions of the study questionnaires are provided in Appendix D and E.

Demographic information

Demographic information was gathered from all participants. This included age, sex, marital status, ethnicity, employment status, level of education, and level of both mother's and father's education. As the majority of participants were students, parental education was used as an indicator of SES. The highest educational qualification of either parent was chosen as the SES indicator and based upon this participants were classified as having a high, medium, or low SES. Low SES was categorised as those who had less than a high school education, medium SES was categorised as those who had a high school education, and high SES included those who had an undergraduate university degree, or higher. Participants were asked to specify their marital status by selecting from the following options: single, married, living as married, separated, divorced, widowed, other. They were also asked to specify their ethnicity by choosing from the following options: white, black or black British, mixed, Chinese, Asian or Asian British, other ethnic group. As the majority of participants were white (62%) we subsequently created a binary ethnicity variable where participants were classified as 'white' or 'non-white'. Employment status was measured with the following options: employed full-time, employed part-time, self-employed, student, unemployed, volunteer, disabled. Participants were asked to provide their own level of education as well as the level of both their mother and father using the following options: school certificate, GCSEs/O-levels/CSEs, A-levels, undergraduate degree, postgraduate degree, none, don't know, other.

Smoking status was measured as a binary variable (current smoker/non-smoker). If participants were current smokers they were asked to indicate how many cigarettes they

smoked per day and also how long they had been a smoker for in years and months. If participants were non-smokers they were asked if they had ever been a smoker in the past. If they responded yes to this participants were asked when they quit smoking and also if they were currently taking any nicotine replacement therapy.

Depression

Depressive symptoms were measured using the BDI-II (Beck, Steer, & Brown, 1996). The BDI-II has demonstrated high internal reliability and high test-retest reliability among both clinical and non-clinical populations, and adequate validity has been established (Dozois, Dobson, & Ahnberg, 1998; Wang, & Gorenstein, 2013). The BDI-II comprises 21 items that are scored on a scale ranging from 0-3, with total scores ranging from 0-63. Participants are asked to provide answers that best describe the way they have been feeling during the past two weeks. However, due to the Stress Pathways Study duration we amended this to avoid confusion and asked participants to describe the way they have been feeling over the past week. A psychometric evaluation of the BDI-II recommended the use of the following cut-off criteria which we adopted in the current study: 0-12, non-depressed; 13-19, dysphoric; 20-63, dysphoric or depressed (Dozois et al., 1998). Participants were asked prior to recruitment if they had ever received a clinical diagnosis of depression. If not, they were recruited into the study. The Cronbach's alpha for the BDI-II in this sample (n=104) at baseline was 0.87. The Cronbach's alpha at follow-up (n=92) was 0.88.

Anxiety

Symptoms of anxiety were measured using the seven-item anxiety subscale of the HADS (Zigmond & Snaith, 1983). This subscale was favoured over others due to its brevity. The HADS anxiety subscale has also been found to be sensitive to changes across time in

response to therapeutic intervention and has performed well in the assessment of anxiety symptoms in the general population as well as patient groups (Bjelland, Dahl, Haug, & Neckelmann, 2002).

The seven items of the HADS anxiety subscale are answered on a Likert scale ranging from 0 to 3, to indicate the extent to which the symptom has been experienced over the past two weeks. We amended this duration to one week to reflect the Stress Pathways Study protocol. Items are summed to generate a total score (0-21), with reverse coding on item 4 (*I can sit at ease and feel relaxed*). The recognised cut-off for moderate anxiety is a score of ≥ 11 with higher scores indicating higher anxiety (Snaith, 2003). Participants were asked prior to recruitment if they had ever received a clinical diagnosis of anxiety. If not, they were recruited into the study. The Cronbach's alpha for the HADS anxiety subscale in this sample (n=104) at baseline was 0.83. The Cronbach's alpha at follow-up (n=92) was 0.86.

Perceived stress

Perceived stress was measured using the Perceived Stress Scale (PSS) (Cohen, Kamarck, & Mermelstein, 1983). We chose to use the PSS in this study as it poses an advantage over life events scales that are usually used to measure stressful experiences. Life events scales usually measure the number of life events and the difficulty adjusting to these events without taking into account the personal and contextual factors that influence the degree to which a person perceives a situation as stressful. The PSS measures the extent to which an individual appraises aspects of one's life as stressful. The PSS has been found to be a reliable and valid self-report measure of perceived stress in a non-clinical student sample (Roberti, Harrington, & Storch, 2006) and a psychiatric sample (Hewitt, Flett, & Mosher, 1992). The 10-item PSS is scored on a five-point Likert scale ranging from 0

'Never' to 4 'Very often' and includes items such as 'In the last month, how often have you felt nervous and stressed?' and 'In the last month how often have you felt that you were unable to control the important things in your life?'. Participants are asked to indicate to what extent they have felt or thought a certain way in the past month. Items are summed to generate a total score (0-40) with higher scores indicative of greater perceived stress. Items 4, 5, 7, and 8 are reverse scored. The Cronbach's alpha for the PSS in this sample (n=104) was 0.86.

Affect

Affect was measured using the 10-item positive subscale of the Positive and Negative Affect Scale (PANAS) (Watson, Clark, & Tellegen, 1988). Only the positive affect subscale was included as both the BDI-II and HADS anxiety subscale provide adequate information about negative mood states. There is a great deal of evidence to suggest that positive affect and negative affect as conceptualised in the PANAS are distinct constructs (Watson, Wiese, Vaidya, & Tellegen, 1999). Therefore, independent inclusion of the positive affect subscale is acceptable. The positive affect subscale was chosen for its brevity. Also, this subscale is used to measure aspects of positive activation such as high energy, enthusiasm, and alertness. These are factors likely to be affected by administration of the medications in this study. Both PANAS scales have been shown to demonstrate adequate psychometric properties in non-clinical populations (Crawford & Henry, 2004) and have also been shown to be sensitive to change over time (Watson, 1988).

The 10 items of the positive affect subscale are as follows: *interested, excited, strong, enthusiastic, proud, alert, inspired, determined, attentive, and active*. Participants are asked to indicate what extent they have felt this way in the past week. A number of

different time-frames have been used with the PANAS in previous research, but ‘in the past week’ was chosen for the purposes of the Stress Pathways Study. Each item of the positive affect subscale is scored on a scale ranging from 1 ‘*Never*’ to 5 ‘*Always*’. Scores range from 10-50 with higher scores indicating higher positive affect. The Cronbach’s alpha for the positive affect subscale in this sample (n=104) was 0.83. The Cronbach’s alpha at follow-up (n=92) was 0.90.

4.4.6 Adverse events and drug effects

We included the following open-ended questions at the end of the questionnaire the participants completed at the laboratory session (Day 8): ‘*Did you have any significant symptoms or medical problems since the last study visit?*’, ‘*Would you say that any medical problem experienced in the last week was due to the study medication?*’, and ‘*If you can, please indicate below which medication you think you have been taking for the last 7 days*’. Space was provided after all of these questions for participants to provide any extra details or information about their responses.

4.4.7 Biological measures

Cortisol sampling

In the Stress Pathways Study we measured both diurnal cortisol secretion (Day 7), and salivary cortisol stress reactivity in the laboratory (Day 8). Cortisol can be assessed in a number of biological specimens including saliva, blood, urine, and hair. We chose to measure salivary cortisol for a number of reasons. Salivary cortisol provides a reliable measure of unbound or ‘free’ cortisol, that is the biologically active cortisol in the body, and there are generally high correlations between salivary cortisol levels and levels of unbound cortisol in plasma and serum (Hellhammer, Wüst, & Kudielka, 2009;

Kirschbaum & Hellhammer, 2000). Salivary cortisol also has advantages over measuring cortisol in blood or urine in terms of ease of measurement. Saliva sampling is a non-invasive, relatively inexpensive way of measuring cortisol and is especially ideal for ambulatory assessment in naturalistic settings where participants are responsible for collecting their own samples (Kirschbaum & Hellhammer, 2000). Therefore, we chose to measure salivary cortisol, rather than serum or plasma levels, in order to ensure consistency between diurnal ambulatory measures and measures taken in the laboratory during stress testing. Additionally, cortisol is stable in saliva and is therefore unaffected by storage conditions and transport for analysis.

All saliva samples were collected using ‘salivettes’ (Sarstedt, Leicester, UK). On Day 1 participants were provided with a cortisol sampling kit for collection of saliva samples at home on Day 7, and were shown how to provide a saliva sample. The sampling protocol is detailed earlier in this chapter in *Section 4.4.4*. Once the samples were returned they were stored at -20°C for analysis at a later date. Salivary cortisol levels were measured using a time-resolved immunoassay with fluorescence detection at the University of Dresden. Inter- and intra-assay variability was below 4%. Following analysis, the cortisol data were cleaned and four different indices of HPA axis function were computed: CAR, total cortisol output (AUC), cortisol slope across the day, and the difference between waking and bedtime values. A more detailed account of how these indices were computed will be provided in *Section 5.5* of this thesis.

During the laboratory session on Day 8 participants provided seven saliva samples across the session. Details of the timings of these samples are provided earlier in this chapter in *Section 4.4.4*. These samples were also stored at -20°C for analysis at a later time. Following analysis at the University of Dresden, the cortisol data was cleaned and a

number of indices were computed relating to cortisol stress reactivity. A more detailed account of these computations will be provided in *Section 6.8.1* of this thesis.

Corticosteroid receptor sensitivity

GR and MR sensitivity was measured by lipopolysaccharide (LPS) stimulation of whole blood cultures co-incubated with different concentrations of glucocorticoids and subsequent determination of IL-6 production. IL-6 production was measured using a commercially available Luminex technology kit for IL-6 from Bio-RAD®. GR and MR sensitivity was measured at each blood-collection time point, i.e. pre-stress, immediately post-stress, +45m, and +75m. A more detailed description of the corticosteroid sensitivity assay protocol will be provided in *Section 6.8.2* of this thesis.

Cardiovascular measures

Blood pressure (BP), heart rate, and cardiac output were continuously measured during the laboratory session on Day 8 using a Finometer® PRO (Finapres Medical Systems, Amsterdam, the Netherlands). The Finometer® PRO uses the Modelflow approach developed by Wesseling et al. (Wesseling, Jansen, Settels, & Schreuder, 1993) in order to estimate cardiac output. Modelflow estimates stroke volume via a three-element model using arterial compliance, aortic flow, and systemic vascular resistance (Shibasaki et al., 2011). All cardiovascular measures were averaged into mean readings taken from five-minute intervals. There was a five-minute baseline interval (pre-stress), as well as two five-minute recovery period intervals (+40-45m, and +70-75m). Cardiovascular measures during the stress protocol were averaged across tasks. Cardiac index (L/min/m²) was calculated by dividing cardiac output (L/min) by the body surface area (m²).

4.5 Data storage

All Stress Pathways Study data were collected and stored in line with UCL policy and adhered to strict ethical guidelines. The project was registered with the UCL Data Protection Office. All data were treated as strictly confidential and were anonymised using unique study IDs. Participant consent forms and personal details were stored separately from all questionnaire and biological data; all paper data was stored in a locked filing cabinet in locked offices at UCL. All electronic data were stored in password-protected computer files on password-protected computers. Prior to analyses, all biological samples collected were stored in a secure code-protected laboratory within the Department of Epidemiology & Public Health. All saliva samples were recoded to ensure anonymity before being transported to the University of Dresden for analysis via secure international courier. Following analysis, these samples were destroyed in Dresden. The results of the saliva analyses were returned to UCL electronically in password-protected spreadsheets and are currently stored in password-protected computer files. Blood samples from each participant are currently being securely stored in -80°C freezers in a code-protected laboratory. All data from the Stress Pathways Study may be kept in the secure manner described above for up to 20 years prior to being destroyed.

4.6 Statistical analyses

All statistical analyses were performed using SPSS version 22.0 software (SPSS Inc., Chicago, Illinois, USA). The significance level was set to $p < 0.05$ for all analyses. Specific details of statistical analyses carried out are included in chapters 5 and 6 which deal with diurnal cortisol, cortisol stress reactivity, and corticosteroid receptor function.

4.7 My contribution to the Stress Pathways Study

I was directly involved in the development, design, organisation, and running of the Stress Pathways Study, under guidance from my supervisors Professor Andrew Steptoe and Dr Livia Carvalho. I was responsible for drafting the application for ethical approval for the study. In February 2014 I obtained ethical approval for the Stress Pathways Study from the UCL Research Ethics Committee. I helped select and compile the measures included in the study questionnaires and was very involved with the design of the study. I was responsible for the development and creation of all the study materials and was solely responsible for study recruitment. Due to a slight delay with the manufacture of the study medications, study recruitment began in October 2014. From October 2014 – August 2015 I conducted the Stress Pathways Study with the assistance of a research nurse and research assistant from the department. I ran all 104 laboratory stress sessions and was solely responsible for saliva sampling and collection. I also carried out corticosteroid receptor sensitivity assays on the blood samples of all 85 participants who were successfully cannulated during the laboratory session. I was responsible for the recoding of all the saliva samples collected (both diurnal and laboratory) and organised their transport to the University of Dresden for cortisol analysis. I created the dataset for the study and undertook all of the data entry. I conducted all the statistical analyses myself, with help from my PhD supervisors.

Chapter 5

Study 2 – The Stress Pathways Study results: The effect of pharmacological blockade on diurnal cortisol secretion in healthy volunteers

5.1 Introduction

The results of Study 1 in Chapter 3 of this thesis provided support for the role of dysregulation of basal HPA axis function in CVD. However, the results told us very little about what brought about this dysregulation. In this chapter I will present results from the Stress Pathways Study concerning the effects of beta-blockade and SSRIs on diurnal cortisol secretion in healthy volunteers. These results may tell us more about the mechanisms and different biological systems involved in dysregulation of diurnal HPA axis functioning. Furthermore, these results may highlight potential therapeutic interventions for impaired diurnal cortisol rhythms.

5.2 Literature Review: Beta-blockers and basal HPA axis function

In Chapter 2 of this thesis I described both the HPA axis and the SAM system and how these systems are connected. As well as interacting with each other, these systems are also anatomically linked. Within the CNS, there are fibres linking norepinephrine releasing neurons in the brainstem with CRH releasing neurons in the hypothalamus (Ulrich-Lai & Herman, 2009). Norepinephrine levels have been shown to stimulate the release of CRH from the hypothalamus, thereby affecting the regulation of the HPA axis (Pacak, 2000; Pacak, Palkovits, Kopin, & Goldstein, 1995). As mentioned in Chapter 4, beta-blockers exert their anti-hypertensive and anxiolytic effects by reducing cardiac output via antagonism of the β -receptors, reducing norepinephrine release, and attenuating the pressor response to catecholamines (Ripley & Saseen, 2014).

5.2.1 Acute administration of beta-blockers

Due to the interplay between the HPA axis and SAM system, the effects of beta-blockade on basal unstressed cortisol secretion have been investigated. However, these studies have largely focused on assessing the effects of acute doses of beta-blockers on single plasma measures of cortisol. Results of these studies have been mixed, with some reporting acute increases in circulating cortisol levels and some reporting no significant changes. In an early study, an acute 80mg dose of propranolol given to seven healthy men resulted in significant increases in levels of circulating plasma cortisol measured throughout the night (Lewis, Groom, Barber, & Henderson, 1981). Using a placebo-controlled crossover design, administration of acute doses of metoprolol (100mg) or propranolol (80mg) to six men and women with insulin-dependent diabetes resulted in an increase in plasma cortisol levels for up to four hours after receiving the medication (Popp, Tse, Shah, Clutter, & Cryer, 1984). A single dose of 30mg pindolol did not bring about any significant changes in cortisol levels in twelve healthy men (Meltzer & Maes, 1994). However, this same dose administered to 23 healthy men was found to increase circulating cortisol levels over the next three hours (Meltzer et al., 1994).

More recently, Kizildere and colleagues administered either 10mg propranolol or placebo to 28 healthy men and women (Kizildere, Glück, Zietz, Schölmerich, & Straub, 2003). Both groups then underwent a CRH test designed to stimulate cortisol secretion. The group that received the acute propranolol dose prior to the CRH test had significantly higher levels of serum cortisol between 40 and 120 minutes after the test compared to the placebo group. However, Nonell and colleagues found that intravenous administration of propranolol (13µg/minute) to 10 healthy young men over five hours resulted in no significant changes in plasma levels of cortisol measured over the same time period (Nonell et al., 2004).

5.2.2 Long-term administration of beta-blockers

The effects of more long-term beta-blockade on basal levels of cortisol have also been examined. Dart and colleagues examined the effects of six week treatment with 80mg propranolol twice daily on night time cortisol secretion in eight healthy male volunteers. They found that propranolol brought about increases in plasma cortisol levels in the first six hours of the night (9pm-3am), but subsequently decreased cortisol levels in the second period (3am-9am). The authors interpreted this as an alteration in the ‘diurnal variation’ of plasma cortisol levels brought about by beta-blockade (Dart, Lewis, Groom, Meek, & Henderson, 1981). In the same year, Golub and colleagues found that one month of treatment with propranolol (120-240mg/day) in eight patients with essential hypertension did not affect plasma cortisol secretion significantly (Golub, Tuck, & Fittingoff, 1981).

Similarly, a study examining the effects of seven-day treatment with beta-blockers on sexual function in 30 healthy male volunteers found that none of the administered drugs (atenolol, metoprolol, pindolol, and propranolol) had any significant effects on morning serum cortisol levels (Rosen, Kostis, & Jekelis, 1988). More recently, Ahmed and colleagues sought to assess the effects of four weeks administration of atenolol (50mg/day) on cortisol secretion in 21 healthy men (Ahmed et al., 2010). Compared to baseline measures, plasma levels of cortisol after drug treatment were significantly lowered.

Taken together, these studies examining effects of acute beta-blockade on basal HPA axis function suggest that acute suppression of the SNS, via its β -adrenergic receptors, seems to enhance cortisol secretion. The effects of long-term administration have been more mixed. However, the heterogeneity between studies means that this suggestion is made with caution. Samples differed widely in terms of sample size, sex, and also health status,

with some studies assessing clinical samples (hypertension, diabetes). The studies also differed widely in terms of drug type (selective versus non-selective beta blocker), dosage, treatment duration, and also route of administration. Additionally, in some cases the drugs were co-administered with other substances (e.g. insulin) so it is difficult to tease apart the actual effects of beta-blockade. Additionally, in one study a CRH test was used to enhance cortisol secretion making the comparison of the results of this study with others difficult (Kizildere et al., 2003). All these methodological differences across studies likely account for the mixed results, and prevent us from making any reasonable assertions about the effects of beta-blockade on basal HPA axis function.

Probably the biggest difficulty in interpreting evidence from these studies is that cortisol is measured either using a single plasma sample taken at different times, or a number of plasma samples taken over a short period. The diurnal nature of cortisol secretion is not being taken into account. In 1997, Gudbjörnsdóttir and colleagues assessed effects of long-term administration of beta-blockers on 'diurnal' plasma cortisol levels in seven mildly hypertensive men and women (Gudbjörnsdóttir et al., 1997). Participants were randomised to receive six weeks treatment with metoprolol (100mg twice daily) or placebo in a double-blind crossover trial. Levels of plasma cortisol were measured using continuous blood sampling over a 24 hour period at the end of the six weeks. Plasma concentrations of cortisol remained unchanged by metoprolol treatment. However, although the authors measured cortisol output across the day, they failed to take into account its unique diurnal patterning and did not calculate the CAR, AUC, or slope across the day.

In sum, evidence from acute studies, and some long-term studies, suggests that beta-blockers do exert effects on cortisol secretion. Despite the heterogeneity across studies and the problems surrounding cortisol measurement, the evidence to date suggests that

SNS suppression by beta-blockade enhances HPA axis activation. Therefore, beta-blockade likely has consequences for diurnal cortisol secretion.

5.3 Literature Review: SSRIs and basal HPA axis function

Depression is one of the most common stress-related disorders. As mentioned in Chapter 1, depression is a known independent risk factor for CVD (Nicholson et al., 2006; Van der Kooy et al., 2007) and also affects prognosis in those already with CVD (Meijer et al., 2011). The neurobiology of depression is defined by a deficit in serotonergic activity (Owens & Nemeroff, 1994). Hyperactivity of the HPA axis has also been widely reported in major depression (Pariante & Lightman, 2008). There is evidence that the abnormalities of the serotonergic system and the HPA axis are linked and this interaction may be an important mechanism involved in the development of depression, particularly at the level of the serotonergic receptors 5-HT_{1A} and 5-HT_{2A} (Porter, Gallagher, Watson, & Young, 2004).

5.3.1 Acute administration of SSRIs

Evidence for interactions between the serotonergic system and the HPA axis come from studies assessing effects of both acute and chronic administration of SSRIs on basal HPA axis function in healthy volunteers and depressed patients. Acute administration of SSRIs to healthy participants appears to result overall in an increase in basal levels of cortisol. The majority of research in this field has used the SSRI citalopram as a neuroendocrine probe. Seifritz and colleagues administered an intravenous infusion of 20mg citalopram over a 30 minute period to nine healthy men (Seifritz et al., 1996). The citalopram infusion resulted in an increase in plasma cortisol levels which reached a peak approximately an hour after administration. The same dose administered orally induced increases in plasma cortisol levels about two hours after intake in 48 healthy men

(Henning & Netter, 2002). At lower doses citalopram has also been found to induce increases in basal cortisol levels. Twelve healthy men and women received 10mg citalopram via intravenous infusion which brought about increases in plasma cortisol levels in the 150 minute period following infusion (Bhagwagar, Hafizi, & Cowen, 2002). Lotrich and colleagues examined the acute effects of four separate IV doses of citalopram (10mg, 20mg, 40mg, and 0.33mg/kg) and placebo on cortisol output in 75 healthy subjects (Lotrich et al., 2005). Citalopram produced a dose-dependent increase in basal cortisol levels. Increases in plasma cortisol levels have also been brought about by oral administration of 40mg citalopram in healthy men (Hawken, Owen, Van Vugt, & Delva, 2006; Mattos, Franco, Noel, Segenreich, & Gonçalves, 2006). Berardelli and colleagues found that two hour intravenous infusion of 20mg citalopram brought about increases in levels of both ACTH and cortisol, suggesting that increases in serotonin stimulate activity of the HPA axis at the pre-pituitary level (Berardelli et al., 2010). These increases were found to be more pronounced in middle-aged and elderly men compared to young adults suggesting that the influence of serotonin on the HPA axis becomes more pronounced with age.

More recent research has examined acute effects of the SSRI escitalopram on neuroendocrine function. A single 10mg dose of escitalopram increased basal levels of salivary and plasma cortisol in 15 healthy men and women in a randomised placebo-controlled cross-over study (Nadeem, Attenburrow, & Cowen, 2004). Single 10mg and 20mg oral doses of escitalopram have also brought about increases in salivary cortisol in healthy men and women (Kuepper, Bausch, Iffland, Reuter, & Hennig, 2006). Interestingly, women tended to show significantly more pronounced increases in salivary cortisol compared to men, regardless of menstrual cycle phase. Using a repeated measures counter-balanced design, Hawken and colleagues found that single oral doses of 20mg

escitalopram given to eight healthy men resulted in significant increases in plasma cortisol levels in the 240 minutes following drug administration (Hawken, Owen, Hudson, & Delva, 2009).

The SSRI paroxetine has also been found to induce increases in basal cortisol secretion. In twenty healthy participants 20mg of paroxetine brought about significant increases in ACTH and cortisol levels (Kojima et al., 2003). Five of these participants also received a 4mg dose of cyproheptadine which is a 5-HT_{2A} receptor antagonist. These participants had attenuated cortisol responses to paroxetine which indicates that the neuroendocrine response to paroxetine is mediated in some way by the 5-HT_{2A} receptors. However, cyproheptadine failed to attenuate the increase in cortisol levels brought about by an acute dose of citalopram, suggesting that cortisol response to citalopram are not mediated by these receptors (Attenburrow, Mitter, Whale, Terao, & Cowen, 2001).

5.3.2 Long-term administration of SSRIs

The evidence unanimously suggests that acute administration of SSRIs brings about increases in HPA axis function in healthy volunteers, albeit with differences between men and women in some studies. However, the results from studies assessing longer-term administration of SSRIs are more difficult to interpret. Most of these studies have been carried out in patient samples with depression. As mentioned previously, hyperactivity of the HPA axis has been widely reported in major depression. A number of studies have shown that SSRI treatment decreases basal cortisol levels, suggesting 'normalisation' of HPA axis function. Inder and colleagues gave 27 patients with depression six weeks treatment with fluoxetine, which brought about significant reductions in plasma ACTH, but not cortisol (Inder, Prickett, Mulder, Donald, & Joyce, 2001). The authors interpret this as evidence for SSRI-induced restoration of glucocorticoid negative feedback on

ACTH levels. Seven months treatment with fluoxetine brought about reductions in urinary free cortisol in 22 depressed patients, but it did not affect basal plasma cortisol levels (Vythilingam et al., 2004). However, eight weeks treatment with fluoxetine (20mg/day) brought about significant decreases in plasma cortisol levels in 14 men and women with major depressive disorder (Jazayeri et al., 2010).

Escitalopram has also been shown to bring about reductions in cortisol secretion. Ahmed and colleagues gave 26 depressed male patients six week treatment with escitalopram (Ahmed et al., 2011). Following treatment, basal plasma cortisol concentrations were significantly lower, as were depression scores. The authors also measured cortisol in single fasting urine samples, but found no significant reduction in urinary cortisol. In a recent study, 51 patients with major depressive disorder were given four weeks treatment with escitalopram (10-20mg/day) (Park, Lee, Jeong, Han, & Jeon, 2015). Regardless of depression scores following treatment, both responders and non-responders had significant reductions in basal plasma cortisol levels.

Dziurkowska and colleagues examined the collective effects of a number of different SSRIs on cortisol secretion in 40 depressed men and women (Dziurkowska, Wesolowski, & Dziurkowski, 2013). The results showed that SSRIs brought about overall reductions in salivary cortisol levels. Similarly, Hernandez and colleagues assessed the collective effects of SSRIs (fluoxetine, paroxetine, sertraline) on urinary cortisol levels in 31 patients with major depressive disorder (Hernandez et al., 2013). After 52 weeks treatment, urinary cortisol levels were significantly reduced compared to baseline values. Wedekind and colleagues examined the effects of 10 weeks treatment with paroxetine (20-40mg/day) on urinary cortisol levels in men and women with panic disorder (Wedekind et al., 2008). Paroxetine treatment had no significant effects on urinary

cortisol. However, when analysed separately, men receiving paroxetine displayed a trend towards lower basal HPA axis function after the treatment period.

The results of these studies provide support for the notion that SSRIs may exert their therapeutic effect via ‘normalisation’ of HPA axis function. However, a number of studies have also found that long-term treatment with SSRIs brings about no significant changes in basal plasma cortisol (Kauffman, Castracane, White, Baldock, & Owens, 2005; Mück-Seler, Pivac, Sagud, Jakovljević, & Mihaljević-Peles, 2002) or salivary cortisol values (Deuschle et al., 2003). One study has even found that treatment with SSRIs brings about increases in basal cortisol values in depressed patients. Four weeks treatment with sertraline (42.5mg/day) increased plasma cortisol levels in 15 female patients with major depression (Sagud et al., 2002). Increases in cortisol values following SSRI treatment have also been reported in healthy men. Ljung and colleagues examined the effects of six months treatment with citalopram (10-20mg/day) on basal HPA axis function in 16 healthy men with moderate abdominal obesity (Ljung et al., 2001). Prior to beginning treatment, morning cortisol levels were low in these men indicating dysregulation of the HPA axis. Following treatment with citalopram, there was a significant increase in morning cortisol levels. However, urinary cortisol levels remained unchanged.

Taken together, the evidence suggests that acute administration of SSRIs increases cortisol secretion. Longer-term administration appears to reduce cortisol secretion in depressed patients indicating ‘normalisation’ of HPA axis function brought about by increased serotonergic activity. However, the results of these studies have been mixed. This is largely to do with methodological heterogeneity between studies. The majority of studies examined the effects of SSRIs in depressed individuals, meaning that the decreases in cortisol secretion may have been to do with symptom remission rather than the direct biological effects of SSRIs on the HPA axis. In fact, in healthy individuals

longer-term SSRIs brought about increased basal cortisol levels. Studies also differ in terms of specimen used to measure cortisol. Some studies reported alterations in plasma cortisol levels and no change in urinary cortisol levels, whereas some studies reported the opposite pattern. As with the beta-blocker studies, the biggest difficulty in interpreting the evidence from these SSRI studies is that cortisol is measured using single plasma, salivary or urinary cortisol samples. These samples are taken at different times across studies also making it very difficult to compare results. The diurnal nature of cortisol secretion is not being taken into account which makes it difficult to make inferences about the effects of SSRIs on HPA axis function.

5.3.3 SSRIs and diurnal HPA axis function

To date, a number of studies have assessed the effects of SSRIs on diurnal cortisol secretion. The majority of these studies have been on depressed patients and have yielded mixed results. Rota and colleagues assessed the effects of six week treatment with fluvoxamine (200mg/day) on circadian cortisol rhythm in 20 patients with major depressive disorder (Rota et al., 2005). The authors measured circadian cortisol rhythm by calculating the ratio between salivary cortisol measured at 8pm and salivary cortisol measured at 8am. They found at baseline, patients with major depression had increased ratios compared to controls, which was indicative of a flattened cortisol rhythm. By day 14 of fluvoxamine treatment the patients had significantly decreased cortisol ratios, implying a correction in the circadian rhythm, or a steepening of the slope.

Three week treatment with escitalopram (10-20mg/day) in major depressive disorder patients (n=52) was found to significantly reduce cortisol AUC calculated from four salivary cortisol measures taken over the course of a day (Hinkelmann et al., 2012). By the end of treatment cortisol AUC in the patient group was reduced to the levels of age

and gender matched healthy controls. Furthermore, reduction in cortisol AUC in the patient group correlated with improvements in depressive symptoms.

Similarly, 12 week treatment with paroxetine (varying dosages) brought about significant decreases in cortisol AUC in 70 patients with depression (Ruhé et al., 2015). Moreover, over the treatment course, waking cortisol levels decreased and the CAR was significantly increased in patients who experienced symptom remission. Reduced cortisol AUC and reduced waking cortisol levels have also been reported in eight healthy first degree relatives of depressed patients who received four weeks treatment with escitalopram (10mg/day) (Knorr et al., 2012). In a large-scale study the association between antidepressant use and diurnal cortisol secretion was investigated in 1526 participants from the Netherlands Study of Depression and Anxiety (Manthey et al., 2011). Those who took SSRIs (n=309) were found to have significantly higher evening cortisol levels compared to non-users. However, one study examining the effects of ten weeks open-labelled treatment with paroxetine (10mg/day) in patients with depression reported no changes in diurnal cortisol rhythm (Tucker et al., 2004). Similarly, long-term treatment with SSRIs (sertraline, escitalopram) did not alter cortisol rhythm across the day in children with problem behaviour (Hibel, Granger, Cicchetti, & Rogosch, 2007). However, only eight children were taking SSRIs meaning that the study may have been underpowered to detect effects.

Lenze and colleagues administered 12 weeks treatment with escitalopram (10-20mg/day) or placebo to 60 adults with GAD (Lenze et al., 2011). Similar to results seen in depressed patients, treatment significantly reduced cortisol AUC and decreased anxiety levels. Furthermore, treatment with escitalopram also reduced the CAR. Diurnal cortisol parameters were calculated using salivary cortisol measures taken at six daily time points for two consecutive days. However, the authors did not report data on the cortisol slope.

Overall, these results indicate that SSRI treatment does affect diurnal cortisol secretion. Despite differences across studies in terms of drug type, duration of treatment, and dosage, a number of studies reported reduced cortisol AUC and reduced waking levels of cortisol. However, results are mixed regarding the CAR and diurnal cortisol rhythm. There is also a paucity of studies assessing the effects of SSRIs on diurnal cortisol secretion in healthy volunteers. This means that we cannot distinguish whether observed changes in cortisol secretion are due to symptom remission or direct biological effects of serotonergic alterations on HPA axis function. More work is needed examining the effects of SSRIs on diurnal cortisol parameters in healthy volunteers in order to find out more about how serotonergic activity may affect diurnal HPA axis function.

5.4 Aims and hypotheses

The aim of this study was to assess the effects of six-day administration of beta-blockers and SSRIs on diurnal cortisol parameters using data from the Stress Pathways Study. As detailed in previous chapters, research suggests that dysregulation of these diurnal cortisol parameters are associated with chronic stress, negative emotional disorders, heightened cardiovascular risk, and poor prognosis in those who have CVD. Examining the effects of these pharmacological probes on diurnal cortisol secretion may tell us more about what biological systems are involved in this dysregulation, and also may identify potential therapeutic interventions for ‘normalisation’ of HPA axis function.

It should be emphasised that although participants were randomised into three groups, this was done as an efficient way to compare beta-blockers with placebo, and SSRIs with placebo. No hypotheses were generated concerning the differences between beta-blocker and SSRI groups, and the data were analysed as two parallel comparisons: beta-blockade versus placebo, and SSRIs versus placebo.

Acute doses of beta-blockers appear to enhance cortisol secretion, whereas the results of studies looking at more long-term administration of these drugs are mixed. To date, no study has assessed the effects of beta-blockers on specific diurnal cortisol parameters, i.e. the CAR, the cortisol slope, waking cortisol levels, evening cortisol levels, and cortisol AUC. Therefore, based upon the increases in cortisol levels brought about by acute beta-blockade, and based on the notion that SNS suppression brings about increased HPA axis activation, **I hypothesise that beta-blockers will increase diurnal cortisol secretion, i.e. the CAR and AUC will be larger, leading to flatter cortisol slopes.**

Quite a number of studies have assessed the effects of SSRIs on diurnal cortisol secretion and the results suggest that SSRIs do affect cortisol across the day. Four studies to date have reported reduced cortisol AUC in those taking SSRIs. Three studies also report either reduced waking levels or reduced CAR. Therefore, **I hypothesise that six-day administration of escitalopram will reduce the cortisol AUC and reduce the CAR. In line with these reductions, I also hypothesise that SSRIs bring about steeper cortisol slopes.**

Daily cortisol secretion is known to be affected by sex (Veldhuis et al., 2013). Sex has also been shown to influence cortisol responses to SSRI administration (Kuepper et al., 2006; Wedekind et al., 2008). Therefore, in this study we will also examine how sex influences the effects of the study drugs on diurnal HPA axis function.

5.5 Calculation of diurnal cortisol parameters

A detailed description of the cortisol sampling procedure is provided in Chapter 4 of this thesis. Four different indices of HPA axis function were calculated for each participant: CAR, cortisol AUC, cortisol slope across the day, and the difference between waking and bedtime values. The CAR was calculated by subtracting the waking from the waking +

30 min values. When calculating the CAR, we omitted individuals who reported a delay of >15 min between waking and taking the 'waking' sample leaving a sample of 73. As mentioned in Chapter 3, a long delay between waking and providing the 'waking' sample can produce misleading CAR results, but a delay of less than 15 minutes does not seem problematic (Dockray et al., 2008). The cortisol AUC over the day was computed according to the methods described by Pruessner and colleagues (Pruessner et al., 2003). The slope of decline in cortisol across the day was estimated using two methods. First, we regressed the samples across the day against time. Second, the difference between waking and bedtime values was computed and divided by the time elapsed between the two samples. Both are expressed in nmol/L/min. The two slope values correlate 0.91, so reflect very similar processes. The average evening cortisol concentration was also calculated (mean of 8pm and bedtime values). The exact number of participants available for each analysis is detailed in Table 5.2.

Participants were to be excluded from the analysis if any of the cortisol values exceeded 70 nmol/L. Of the 94 participants in the study, there were some missing cortisol samples as follows: two on waking, one on waking+30 min, three at 10am, and one at 4pm. Cortisol slope was calculated if the participant had at least four available cortisol measures (excluding the 30+ morning sample). 92 patients had sufficient data for the calculation of cortisol slope (two participants provided less than four samples). The CAR was calculated for 73 participants as 16 participants reported providing their waking sample more than 15 minutes after waking, two participants failed to provide waking samples, one participant failed to provide one waking+30 min, and two had cortisol sample values that exceeded 70 nmol/L. Cortisol AUC was calculated only for those who provided all seven saliva samples. Therefore, cortisol AUC was calculated for the 87 participants who provided all saliva samples successfully.

5.6 Statistical Analyses

All data were analysed as two parallel comparisons: beta-blockers versus placebo, and SSRIs versus placebo.

Kolmogorov-Smirnov tests were performed in order to test for normality of the distribution in diurnal cortisol parameters. These normality tests revealed that all diurnal cortisol parameters were normally distributed (all p values > 0.05) apart from bedtime cortisol levels, and average evening cortisol levels. However, parametric tests were performed on the data as they are the preferred method of analysing data from randomised trials (Vickers, 2005). Two-way ANOVAs and chi-square tests were used to compare the study medication groups on all demographic characteristics. Where possible, sex was included as a between-person factor alongside experimental condition. Changes in stress-related psychological factors were assessed using two-way ANOVAs, with experimental condition (propranolol versus placebo and escitalopram versus placebo) and sex being included as the main fixed factors.

The diurnal cortisol parameters were also analysed using two separate pairwise analyses; propranolol versus placebo, and escitalopram versus placebo. Differences between the two conditions were analysed using two-way ANOVAs, with experimental condition and sex being included as the main fixed factors. We examined the main effects of experimental condition as well as the interactive effect of sex. Where there were significant interaction effects of sex on diurnal cortisol parameters, we split the data by sex and ran one-way ANOVAs in order to determine the effects of the experimental condition in men and women separately. Where there were significant differences between experimental conditions on any of the demographic characteristics, ANCOVAs were run where the demographic variable of interest was included as a covariate.

The significance level was set to $p < 0.05$ for all analyses, with precise p values reported for all test results. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA).

5.7 Results

5.7.1 Participants

Of the 94 participants who provided saliva samples, 32 were taking escitalopram, 30 were taking propranolol, and 32 were taking placebo. Table 5.1 summarises the characteristics of the participants. The sample had an age range of 18-48 years, were almost two-thirds women (62.8%), and were mostly normal weight (79.8% BMI<25). Over half of the sample were white (58.5%) and the majority of participants had a high SES based upon parental education (81.9%).

Scores on the BDI-II at baseline ranged from 0-31 indicating the presence of depression in some participants. Frequency analysis revealed that two participants had BDI-II scores greater than 19 indicating the presence of clinical depression. Scores on the HADS anxiety subscale at baseline ranged from 0-15 indicating the presence of anxiety in some participants. Frequency analysis revealed that nine participants had scores of 11 or greater indicating the presence of anxiety. Sensitivity analyses were carried out with these participants removed (n=10). Exclusion of these participants did not affect results obtained. Scores on the PSS ranged from 2-31, indicating a good level of variability in perceived stress within the sample.

The propranolol group did not differ significantly from the placebo group in terms of age ($F(1, 58) = 2.18, p = 0.15$), sex ($\chi^2 = 0.52, df = 1, p = 0.47$), BMI ($F(1, 58) = 1.33, p = 0.25$), smoking status ($\chi^2 = 0.10, df = 1, p = 0.76$), ethnicity ($\chi^2 = 3.66, df = 4, p = 0.45$),

or SES ($\chi^2 = 0.60$, $df = 2$, $p = 0.74$). There were also no significant differences between groups in baseline depression scores ($F(1, 58) = 0.04$, $p = 0.85$), anxiety scores ($F(1, 58) = 0.01$, $p = 0.96$), perceived stress scores ($F(1, 58) = 0.26$, $p = 0.53$), or positive affect scores ($F(1, 58) = 0.17$, $p = 0.68$). Amongst female participants there was no significant difference between drug groups in terms of hormonal contraception use ($\chi^2 = 3.33$, $df = 3$, $p = 0.34$).

The escitalopram group did not differ significantly from the placebo group in terms of age ($F(1, 60) = 0.16$, $p = 0.69$). However, there was a significant interaction between experimental group and sex with respect to age ($F(1, 60) = 5.60$, $p = 0.021$). There was a significant difference in age between the two groups in men ($F(1, 22) = 5.34$, $p = 0.031$) but not women ($p = 0.15$). Men in the escitalopram group were younger ($M = 20.1$ years, $SD = 0.6$ years) than men receiving placebo ($M = 22.4$ years, $SD = 0.8$ years). The escitalopram group did not differ significantly from the placebo group in terms of sex distribution ($\chi^2 = 2.40$, $df = 1$, $p = 0.12$), BMI ($F(1, 60) = 0.10$, $p = 0.76$), smoking status ($\chi^2 = 2.41$, $df = 1$, $p = 0.12$), ethnicity ($\chi^2 = 0.67$, $df = 4$, $p = 0.96$), or SES ($\chi^2 = 0.32$, $df = 2$, $p = 0.85$). There were also no significant differences between groups in baseline depression scores ($F(1, 60) = 0.17$, $p = 0.69$), anxiety scores ($F(1, 60) = 0.11$, $p = 0.74$), perceived stress scores ($F(1, 60) = 1.14$, $p = 0.29$), or positive affect scores ($F(1, 60) = 0.36$, $p = 0.55$). Amongst female participants there was no significant difference between experimental conditions in terms of hormonal contraception use ($\chi^2 = 3.63$, $df = 3$, $p = 0.30$).

5.7.2 Study medication effects on stress-related psychological factors

We investigated the effects of the study medications on depression scores, anxiety scores, and positive affect on Day 7 of administration. This was in order to clarify that any

Table 5.1. Demographic characteristics of the sample (n = 94)

Characteristic	Propranolol (n=30)	Escitalopram (n=32)	Placebo (n=32)	Propranolol vs. placebo		Escitalopram vs. placebo	
	Mean ± SD or N(%)	Mean ± SD or N(%)	Mean ± SD or N(%)	Group <i>p</i> value	Group*sex <i>p</i> value	Group <i>p</i> value	Group*sex <i>p</i> value
Age (years)	24.5±6.35	22.1±3.9	22.2±3.0	0.145	0.552	0.692	0.021*
Female	19(63.3)	17(53.1)	23(71.9)	0.472	-	0.121	-
BMI (kg/m ²)	22.5±2.3	23.3±4.2	23.2±4.0	0.254	0.506	0.757	0.267
Smoker	3(10.0)	9(28.1)	4(12.5)	0.756	-	0.120	-
Ethnicity (White)	21(70.0)	17(53.1)	17(53.1)	0.454	-	0.955	-
SES (n=93)				0.741	-	0.853	-
<i>Low</i>	4(13.3)	3(9.4)	3(9.4)	-	-	-	-
<i>Medium</i>	3(10.0)	1(3.1)	2(6.3)	-	-	-	-
<i>High</i>	23(76.7)	27(84.4)	27(84.4)	-	-	-	-
Hormonal Contraception (n=59)	6(31.6)	7(41.2)	5(21.7)	0.343	-	0.304	-
Depressive symptoms	7.03±6.40	5.91±6.59	6.41±5.07	0.845	0.504	0.685	0.463
Anxiety symptoms	4.97±4.21	4.75±2.81	5.28±4.03	0.963	0.595	0.739	0.945
Perceived stress	14.2±6.8	13.2±5.6	15.4±7.2	0.611	0.879	0.290	0.407
Positive Affect	33.7±5.6	35.2±5.7	35.0±4.8	0.648	0.244	0.548	0.128

differences in diurnal cortisol parameters between drug groups on Day 6 were not caused by changes in stress- or mood-related factors. The propranolol group did not differ from the placebo group in depression scores ($F(1, 56) = 0.81, p = 0.37$), anxiety scores ($F(1, 56) = 0.32, p = 0.57$), or positive affect ($F(1, 56) = 0.65, p = 0.42$). The escitalopram group did not differ from the placebo group in depression scores ($F(1, 57) = 0.01, p = 0.93$), anxiety scores ($F(1, 57) = 3.11, p = 0.08$), or positive affect ($F(1, 57) = 0.04, p = 0.85$). There were also no main or interactive effects of sex on any of these factors (all p values > 0.05).

5.7.3 Study medication effects on diurnal cortisol parameters

Propranolol versus placebo

The analyses of cortisol over the day in relation to experimental condition are summarised in Table 5.2. A graphical representation of mean cortisol values across the day in both the propranolol and placebo groups is provided in Figure 5.1. In terms of cortisol AUC, there was no main effect of experimental condition ($F(1, 54) = 0.57, p = 0.46$) and no main ($p = 0.31$) or interactive effects of sex ($p = 0.65$). Similarly, the CAR was unaffected by experimental condition ($F(1, 43) = 0.07, p = 0.79$) and there was no interactive effect of sex ($F(1, 43) = 0.63, p = 0.43$). However, there was a main effect of sex on the CAR ($F(1, 43) = 4.73, p = 0.035$). The means indicate that female participants had a more pronounced CAR than male participants, regardless of experimental condition (Women: $M = 15.9, SD = 2.6$; Men: $M = 6.5, SD = 3.5$) (see Figure 5.2). Regarding cortisol slope, there was no main effect of experimental condition ($F(1, 57) = 0.94, p = 0.34$) and no main ($p = 0.42$) or interactive effect of sex ($p = 0.98$). Similar findings emerged relating to the difference between waking and bedtime values. There was no main effect of experimental condition

($F(1, 55) = 0.72, p = 0.40$) and no main ($p = 0.31$) or interactive effect of sex ($p = 0.63$) on wake-bedtime difference.

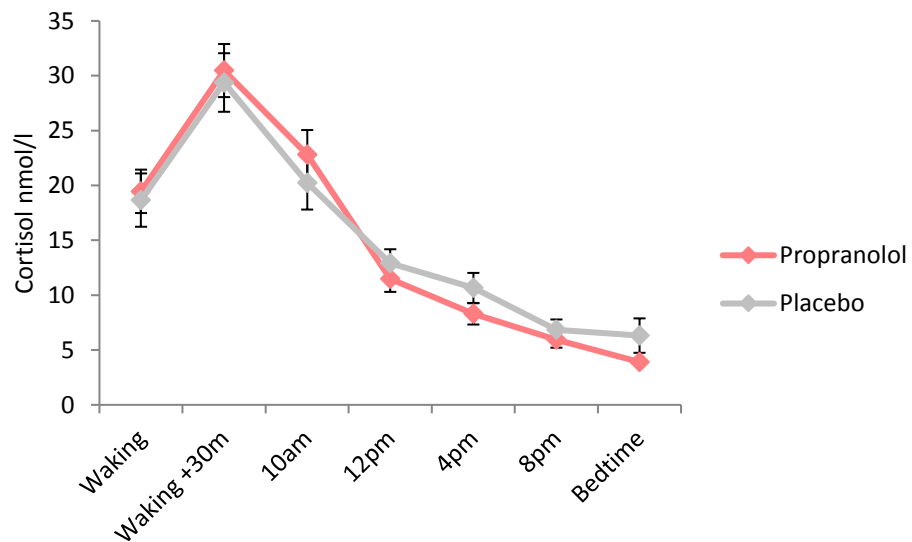


Figure 5.1. Propranolol versus placebo: Mean salivary cortisol values across the day averaged across men and women. Saliva samples were taken on waking, waking+30mins, 10am, noon, 4pm, 8pm, and at bedtime in healthy volunteers who received six day treatment with propranolol (pink line), or placebo (grey line). Error bars represent SEM.

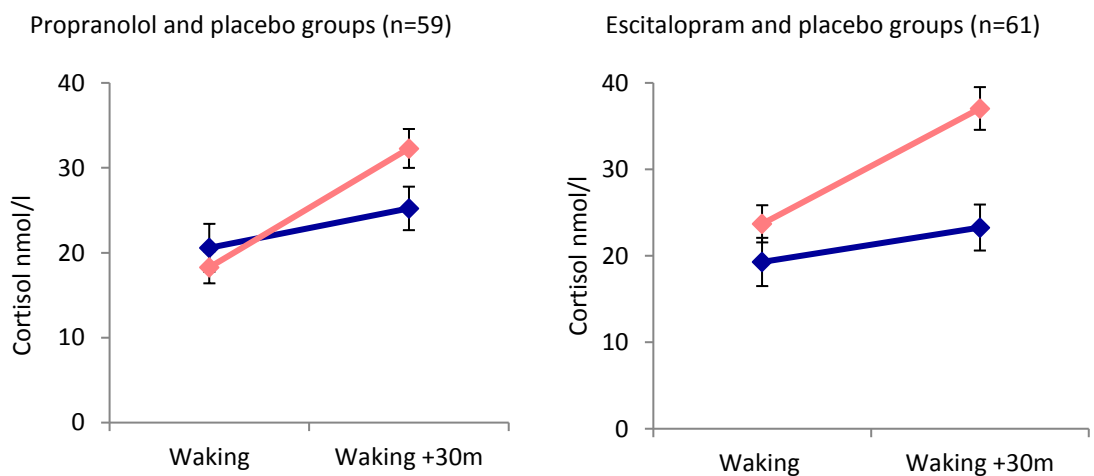


Figure 5.2. Sex differences in the CAR across groups in both sets of pairwise analyses. Blue line = male CAR; Pink line = female CAR. Error bars represent SEM.

Table 5.2. Mean cortisol parameter values and *p* values from ANOVAs comparing the effects of escitalopram and propranolol to placebo

	<i>Propranolol</i> (<i>n</i> =30)	<i>Escitalopram</i> (<i>n</i> =32)	<i>Placebo</i> (<i>n</i> =32)	<i>Propranolol vs. placebo</i>	<i>Escitalopram vs. placebo</i>		
<i>Diurnal cortisol parameters</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Group</i> <i>p value</i>	<i>Group*sex</i> <i>p value</i>	<i>Group</i> <i>p value</i>	<i>Group*sex</i> <i>p value</i>
Waking cortisol (nmol/L) (<i>n</i> =90)	19.5±10.7	25.2±12.8	18.7±13.3	0.719	0.585	0.071	0.104
<i>Mean difference±SE</i>	<i>0.8±3.1</i>	<i>6.5±3.4</i>					
Bedtime cortisol (nmol/L) (<i>n</i> =92)	3.91±2.79	5.99±9.68	6.31±8.76	0.332	0.428	0.836	0.092
<i>Mean difference±SE</i>	<i>-2.40±1.65</i>	<i>-0.32±2.34</i>					
Average evening cortisol (nmol/L) (<i>n</i> =92)	4.92±2.83	6.41±5.87	6.58±5.29	0.184	0.962	0.904	0.085
<i>Mean difference±SE</i>	<i>-1.66±1.08</i>	<i>-0.17±1.42</i>					
Cortisol AUC (nmol/L.hr) (<i>n</i> =87)	170.5±55.8	209.6±82.5	188.3±71.8	0.455	0.652	0.225	0.676
<i>Mean difference±SE</i>	<i>-17.9±16.8</i>	<i>21.3±20.2</i>					
CAR (nmol/L) (<i>n</i> =73)	12.5±14.2	7.6±12.3	13.1±14.9	0.790	0.432	0.440	0.221
<i>Mean difference±SE</i>	<i>0.2±3.9</i>	<i>-3.9±3.8</i>					
Cortisol slope (nmol/L/min) (<i>n</i> =92)	0.0209±0.0157	0.0219±0.0239	0.0153±0.0224	0.338	0.976	0.378	0.023*
<i>Mean difference±SE</i>	<i>0.0056±0.0274</i>	<i>0.0066±0.0328</i>					
Cortisol slope (wake/bedtime)(nmol/L/min) (<i>n</i> =90)	0.0152±0.0124	0.0193±0.0153	0.0123±0.0137	0.401	0.312	0.089	0.024*
<i>Mean difference±SE</i>	<i>0.0029±0.0185</i>	<i>0.0070±0.0205</i>					

Mean difference is calculated by subtracting placebo values from each experimental condition. The SE of the mean difference was calculated as the square root of the sum of the squares of the SE for each group.

Escitalopram versus placebo

There was no main effect of experimental condition ($F(1, 55) = 1.51, p = 0.23$) and no main ($p = 0.40$) or interactive effect of sex ($p = 0.68$) on cortisol AUC. There was also no main effect of experimental condition ($F(1, 48) = 0.61, p = 0.44$) or interactive effect of sex ($p = 0.22$) on the CAR. However, there was a main effect of sex on the CAR ($F(1, 48) = 4.62, p = 0.037$). The means indicate that women had more pronounced CARs than men, regardless of experimental condition (Women: $M = 13.2, SE = 2.4$; Men: $M = 5.0, SE = 3.0$) (see Figure 5.2).

There was no main effect of experimental condition ($F(1, 58) = 0.79, p = 0.38$) or sex ($p = 0.12$) on cortisol slope. However, the ANOVA revealed a significant condition by sex interaction effect on cortisol slope ($F(1, 58) = 5.49, p = 0.023$) (see Figure 5.3). Splitting the file by sex revealed no effect of experimental condition on cortisol slope in men ($F(1, 22) = 0.75, p = 0.40$). However, there was an effect of experimental condition in women ($F(1, 36) = 7.54, p = 0.009$). The means indicate that women taking escitalopram had steeper cortisol slopes ($M = 0.0331, SD = 0.0173$) compared with women receiving placebo ($M = 0.0140, SD = 0.0233$).

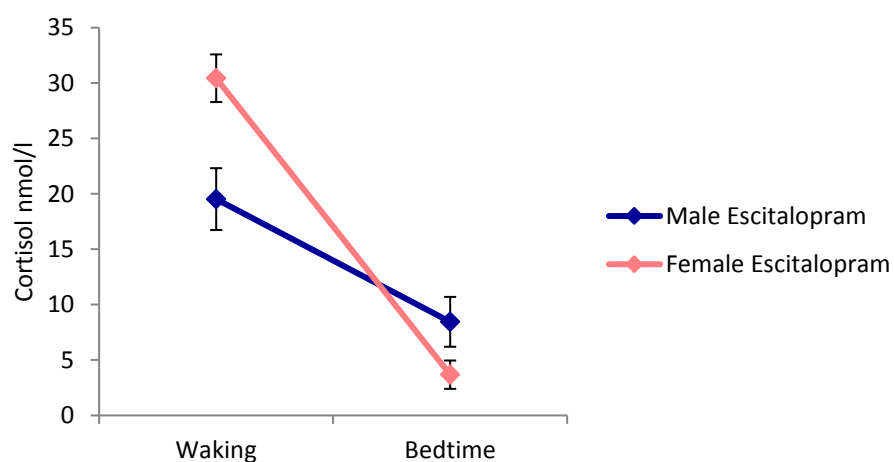


Figure 5.3. The interactive effect of sex on cortisol slope (wake-bedtime difference) within participants receiving escitalopram. Error bars represent SEM.

Similar findings emerged relating to the difference between waking and bedtime values. There was no main effect of experimental condition ($F(1, 57) = 2.99, p = 0.09$) or sex ($p = 0.08$) on wake-bedtime difference. There was a significant condition by sex interaction effect on wake-bedtime difference ($F(1, 57) = 5.38, p = 0.024$). Splitting the file by sex revealed no effect of experimental condition in men ($F(1, 24) = 0.12, p = 0.75$), but did reveal a main effect of condition in women ($F(1, 35) = 13.5, p = 0.001$). Examining the means revealed that women taking escitalopram had a greater wake-bedtime difference ($M = 0.0267, SD = 0.0106$) compared to women taking placebo ($M = 0.0118, SD = 0.0133$). These results indicated that women taking escitalopram had a steeper rate of cortisol decline across the day compared to those taking placebo. See Figure 5.4 for a graphical representation of salivary cortisol profiles across the day in men and women receiving escitalopram compared to placebo.

Alterations in cortisol slope can be driven by levels of cortisol at waking and in the evening. Therefore, due to its effects on cortisol slope in women, we examined the effect of escitalopram on waking and evening cortisol levels in female participants. There was a significant main effect of drug on cortisol waking values ($F(1, 35) = 9.21, p = 0.005$). Looking to the mean waking cortisol values indicates that levels were higher in female participants taking escitalopram ($M = 30.4, SD = 9.4$) compared to placebo ($M = 18.6, SD = 13.3$). There was no main effect of drug on cortisol evening values ($F(1, 36) = 2.47, p = 0.13$). I also examined the effects of the drugs on cortisol values at 10am, 12pm, and 4pm. However, there were no significant main effects of drug, sex, or interactive effects of sex on any of these cortisol values (all p values > 0.05). What these findings suggest is that the alterations in cortisol slope seen in the female escitalopram group were being driven by increases in waking cortisol levels.

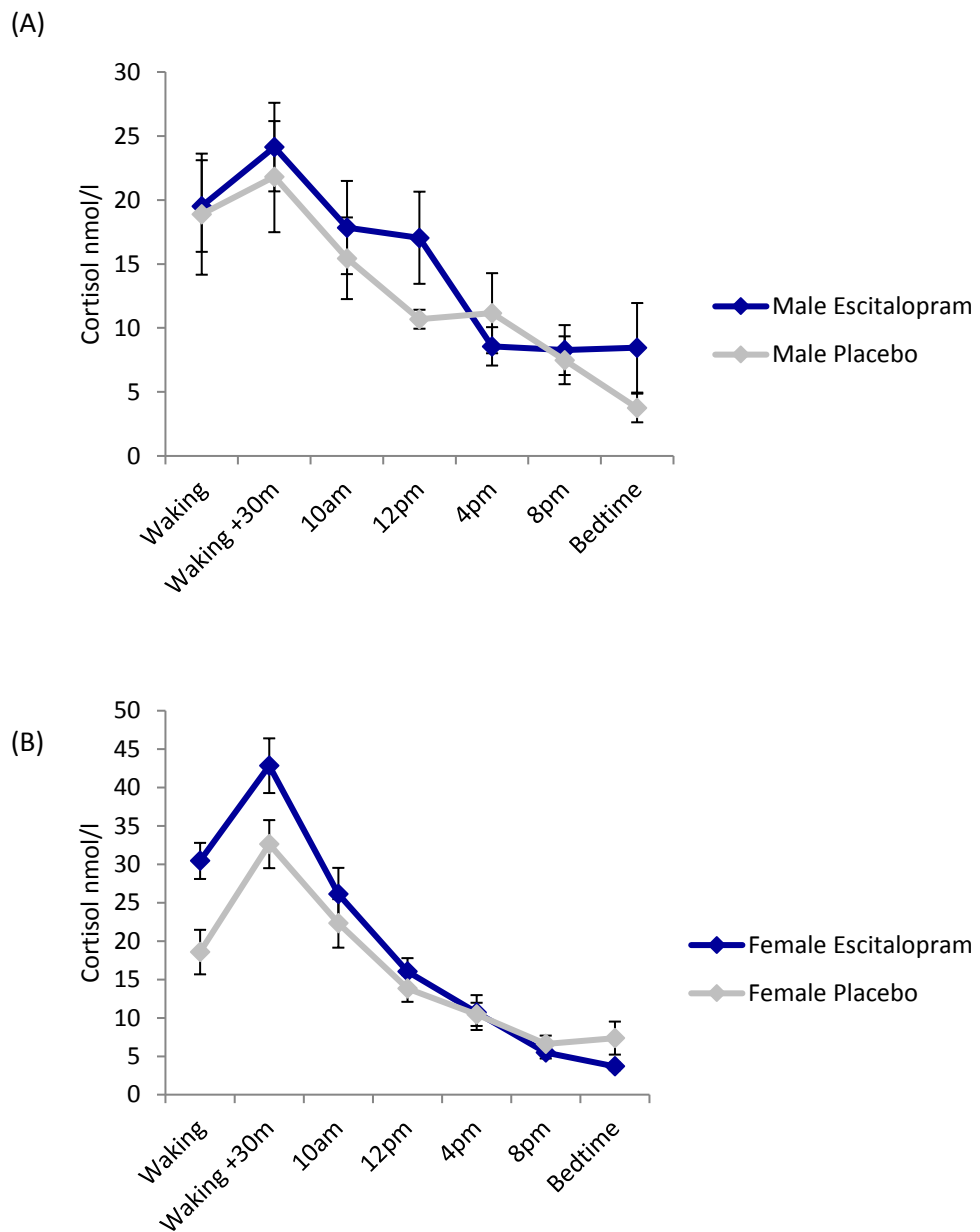


Figure 5.4. Escitalopram versus placebo: Mean salivary cortisol values across the day in (A) men and (B) women. Saliva samples were taken on waking, waking+30mins, 10am, noon, 4pm, 8pm, and at bedtime in healthy volunteers who received six day treatment with escitalopram (blue line), or placebo (grey line). Error bars represent SEM.

Seeing as men receiving escitalopram were significantly younger than men receiving placebo, sensitivity analyses were carried out with age included as a covariate in the male-only analyses. ANCOVA revealed no significant main effects of drug on any of the cortisol parameters (all p values > 0.05).

5.8 Discussion

5.8.1 Summary of results

The aim of this study was to assess the effects of six-day administration of beta-blockers and SSRIs on several different indices of diurnal HPA axis function. We hypothesised that both drugs would bring about changes in diurnal cortisol secretion. More specifically, we hypothesised that beta-blockers would increase diurnal cortisol secretion, in that the CAR and cortisol AUC would be enhanced leading to flatter cortisol slopes. We hypothesised that SSRIs would lead to reduced cortisol output with lower waking cortisol levels, a reduced CAR and cortisol AUC, and steeper cortisol slopes. We also postulated that sex would play a role in the effects of the study drugs on cortisol secretion over the day. The results of this study provide limited support for these hypotheses. There were no effects of beta-blockade on cortisol dynamics. Compared with placebo, women taking SSRIs had significantly steeper cortisol slopes across the day. This observed difference in cortisol slope was independent of any differences in stress- or mood-related factors, suggesting that the observed results were due to direct biological effects of SSRIs on HPA axis function. The group taking SSRIs did not differ significantly on any other cortisol parameter.

5.8.2 Comparison to previous research

Our results are in line with those of Rota et al. (2005) who found that 14 days administration of fluvoxamine to 20 depressed individuals resulted in a steepening of the cortisol slope. However, our findings are not in line with those of Hibel and colleagues and Tucker and colleagues who found that SSRIs had no significant effect on cortisol rhythm across the day in children with problem behaviour and depressed patients respectively (Hibel et al., 2007; Tucker et al., 2004).

It is difficult to compare the results of our study to others. This is largely because previous research has assessed effects of SSRIs on HPA axis function in clinical samples. As outlined previously, long-term treatment with SSRIs has been shown to reduce the cortisol AUC, as well as reduce waking levels of cortisol in patient samples with depression and generalised anxiety disorder (Hinkelmann et al., 2012; Knorr et al., 2012; Lenze et al., 2011; Ruhé et al., 2015). Cortisol AUC is known to be altered in depression and anxiety (Heaney et al., 2010; Marchand et al., 2014). Therefore, the effects of SSRI treatment on cortisol AUC may be more pronounced in those who have these stress-related illnesses. We examined the effects of SSRIs in healthy volunteers which may explain why we did not observe any significant effects on overall daily cortisol output.

In the current study, we found that women taking escitalopram had higher waking levels of cortisol compared to women taking placebo. This alteration in morning cortisol likely drove the significant changes in cortisol slope in this group. This finding is in line with that of Harmer and colleagues who found that six days administration of citalopram (20mg/day) brought about significant increases in waking cortisol in healthy volunteers (Harmer, Bhagwagar, Shelley, & Cowen, 2003). Conversely, in depressed patients, SSRIs have been found to lower levels of waking cortisol (Knorr et al., 2012; Ruhé et al., 2015). Waking levels of cortisol have been found to be increased in depression (Bhagwagar et al., 2005; Mannie et al., 2007). Therefore, the direction of the effect of SSRIs on waking cortisol may be related to mental health status – in depressed patients SSRIs ‘normalise’ elevated levels of waking cortisol, and in healthy individuals SSRIs increase waking levels. More research is needed to confirm this.

It is possible that SSRI dosage (10mg/day) used in the current study was not sufficient to elicit changes in certain diurnal cortisol parameters in healthy volunteers (i.e. cortisol AUC, CAR, evening cortisol levels). Previous studies assessing the effects of

escitalopram initially prescribed 10mg per day to participants but then titrated up to 20mg as the study progressed (Hinkelmann et al., 2012; Park et al., 2015). Up-titration was not practicable in the current study due to the study duration. Perhaps if we had increased the dosage we would have observed significant effects of escitalopram on other diurnal cortisol parameter also. Additionally, we may have observed significant effects if we increased the duration of treatment.

Previous studies have shown that 12 weeks treatment with paroxetine and escitalopram have significantly reduced the CAR in patients with major depressive disorder and generalised anxiety disorder respectively (Lenze et al., 2011; Ruhé et al., 2015). In this study SSRIs had no effect on the CAR. This may be for reasons to do with using a healthy sample as the CAR has been shown to be altered in depression and anxiety (Dedovic & Ngiam, 2015; Veen et al., 2011; Wardenaar et al., 2011), or it may be a power issue. As mentioned in Chapter 3, the accuracy margin of <15 minutes which we chose for the calculation of the CAR may have attenuated CAR estimates (Stalder et al., 2016). Another factor that may account for the lack of effect of SSRIs on the CAR is that the CAR may be under a regulatory cycle distinct from the cortisol slope (Clow et al., 2004).

5.8.3 Potential mechanisms explaining SSRI effects on the HPA axis

Mechanistically, there are a number of ways in which escitalopram could have altered HPA axis function in women in the current study. As mentioned previously, the serotonergic system and HPA axis are reciprocally linked (Porter et al., 2004). The serotonergic system has been found to exert substantial effects on HPA axis function. 5-HT_{1A} receptors agonists are known to induce cortisol secretion in healthy volunteers (Pitchot, Wauthy, Legros, & Ansseau, 2004). Escitalopram administration leads to rapid desensitisation of the 5-HT_{1A} receptor (Zhong, Haddjeri, & Sánchez, 2012). Therefore,

six day administration of escitalopram could lead to changes in 5-HT_{1A} receptor sensitivity, therefore leading to changes in cortisol secretion. In fact, immunohistochemical studies have shown that 5-HT_{1A} receptors are present on the paraventricular nucleus of the hypothalamus which is responsible for the release of CRH – the initial effector of the HPA axis (Lanfumeey et al., 2008).

As well as changes at the level of the serotonergic receptors, escitalopram may have exerted non-serotonergic effects on HPA axis function. A growing body of research suggests that SSRIs may exert effects on the HPA axis via modulation of the corticosteroid receptors. Four days treatment with citalopram has been shown to increase both GR and MR sensitivity in healthy humans (Pariante et al., 2012; Pariante, Papadopoulos, et al., 2004). Flatter cortisol slopes have been associated with reduced GR sensitivity (Jarcho et al., 2013). It is possible that the steeper cortisol slope seen in women taking escitalopram in the current study is a result of increased sensitivity of the corticosteroid receptors. I will investigate this in the next chapter of this thesis. SSRIs are also known to inhibit P-glycoprotein which is a protein that expels glucocorticoids from cells (Ruhé et al., 2015). Therefore, SSRIs may result in increases in levels of intracellular cortisol. As mentioned in Chapter 2, intracellular bioavailability of cortisol affects corticosteroid receptor sensitivity (Oakley & Cidlowski, 2013). However, escitalopram has little effect on the inhibition of P-glycoprotein (Zhong et al., 2012) so it is unlikely that this particular mechanism is of relevance to the results of the current study.

5.8.4 Sex differences in SSRI effects and HPA axis function

Steeper slopes were only observed in women taking escitalopram. There are a number of reasons why this might be. Firstly, there are known sex differences in HPA axis function. Women have been shown to have increased diurnal cortisol secretion (Carpenter,

Grecian, & Reynolds, 2015), and higher oestrogen levels have also been associated with higher morning cortisol peaks (Wolfram, Bellingrath, & Kudielka, 2011). This may be why we observed higher CARs in women compared to men in the current study, independent of the effects of the study medications. Male steroidal sex hormones also appear to play a role in cortisol secretion. For example, testosterone is known to decrease corticosterone in rats (Panagiotakopoulos & Neigh, 2014).

Secondly, the sex difference observed in the current study may be related to the 5-HT_{1A} receptor. As mentioned earlier, stimulation of the 5-HT_{1A} receptor increases cortisol secretion. It may be that the female HPA axis response is more responsive to increased levels of serotonin due to more enhanced stimulation of these receptors. According to Goel and colleagues, oestrogen potentiates 5-HT_{1A} receptor stimulation of the HPA axis, whereas testosterone decreases it (Goel, Workman, Lee, Innala, & Viau, 2014). In further support of this, research has shown that the level of 5-HT_{1A} receptor mRNA in the pituitary gland is almost seven times higher in women (Goel & Bale, 2010). This notion is also backed up by studies outlined earlier in this chapter where acute oral doses of SSRIs brought about significantly higher cortisol increases in women compared to men (Kuepper et al., 2006), and long-term doses brought about lower levels of basal cortisol secretion in men (Wedekind et al., 2008).

Thirdly, and on a more general level, women with depression are known to have better responses to SSRIs compared to men. A review of 15 RCTs (n=332) revealed that female depressed patients on the whole are more responsive to SSRI treatment than male patients in terms of symptom remission (Khan, Brodhead, Schwartz, Kolts, & Brown, 2005). A more recent review provides evidence that oestrogen likely plays a role in the sex differences seen in therapeutic responses to SSRIs (Damoiseaux, Proost, Jiawan, & Melgert, 2014).

5.8.5 Therapeutic implications

Flatter slopes are characteristic of depression (Doane et al., 2013; Jarcho et al., 2013; Sjögren et al., 2006). Although no changes in stress or mood factors were observed in the current study, the steeper cortisol rhythm observed in women taking SSRIs may be one of the mechanisms through which these drugs exert their therapeutic effects. Flatter slopes are also characteristic of CHD (Nijm et al., 2007). In Chapter 3 of this thesis I showed how flatter cortisol rhythms, and lower waking cortisol levels, were associated with adverse outcomes in patients with advanced CHD (Ronaldson et al., 2015). The results of the current study suggest that SSRIs may be a potential therapeutic intervention for female CHD patients with flattened diurnal cortisol slopes, seeing as they steepen the cortisol slope and increase waking cortisol levels. Many patients with comorbid depression and CVD take SSRIs (Shapiro, 2015). In 1834 patients (female n = 849) with comorbid CHD and depression, SSRI use was associated with a significantly lower risk of death or recurrent cardiac event in the 29 months following the occurrence of an MI (Taylor, Youngblood, Catellier, et al., 2005). It is possible that alterations in HPA axis function may be one of the ways in which SSRIs exert their protective effects in CHD patients. However, a recent study examining the effect of 18 months treatment with escitalopram on all-cause mortality in heart failure patients with depression found that escitalopram did not significantly reduce mortality or hospitalisation rates compared to placebo (Angermann, Gelbrich, Störk, et al., 2016). Only a quarter of the sample was comprised of women which may be why no effects were detected. Another reason for this lack of effect might be age. Age has been shown to influence the effectiveness of SSRIs (Olivier, Blom, Arentsen, & Homberg, 2011) with older people (>50 years) having poorer responses to SSRIs (Thase, Entsuah, Cantillon, & Kornstein, 2005). The current study was carried out in a relatively young sample, meaning that the results may not be

replicable in older patient sample, like in those with CVD. This area warrants further investigation, and future studies should take age and sex differences into consideration.

5.8.6 Beta-blockers: Lack of effect

In the current study, beta-blockers had no significant effect on any of the diurnal cortisol parameters. This is in line with previous findings where long-term administration of beta-blockers brought about no changes in plasma cortisol levels, although these studies did not assess diurnal cortisol patterns (Golub et al., 1981; Rosen et al., 1988). As mentioned in Chapter 2, the SAM system and the HPA axis are the major biological systems involved in the stress response, and these systems interact during times of stress. There is also evidence to suggest that these two systems interact under basal, unstressed conditions. In both healthy and depressed patients diurnal variations cerebrospinal fluid levels of norepinephrine and plasma cortisol levels are very highly correlated (Wong et al., 2000). Therefore it is puzzling that under unstressed conditions beta-blockade did not bring about alterations in diurnal cortisol secretion in this study.

It is possible that the propranolol dosage used in the current study was not sufficient to elicit changes in diurnal cortisol parameters in healthy volunteers. An 80mg dose failed to bring about changes in the current study. However, 80mg of propranolol was found to be sufficient to induce change in basal plasma levels of night-time cortisol in healthy men (Dart et al., 1981). These alterations in night-time cortisol levels were observed after six-week administration of propranolol. It is possible that the treatment duration of the current study (six days) was not sufficient to elicit changes in indices of diurnal HPA axis function. Although, administration of acute doses of propranolol (ranging from 10-80mg) have brought about increases in circulating cortisol levels in healthy and diabetic volunteers (Kizildere et al., 2003; Lewis et al., 1981; Popp et al., 1984). Propranolol is a

rapidly metabolised drug that exerts effects on the β -adrenergic receptors quickly (Leahey et al., 1980). Propranolol has a half-life of about three to four hours and there is large variability in bioavailability across individuals due to rapid metabolism of propranolol in the liver (Gomeni, Bianchetti, Sega, & Morselli, 1977). In the current study, participants were instructed to take propranolol every morning after their breakfast. However, there is no guarantee participants took the drug at the same time every day, or with food which is known to affect its bioavailability (Liedholm, Wåhlin-Boll, & Melander, 1990). Therefore, although participants received sustained-release propranolol, it is possible that there may have been a high rate of variability between participants in the current study regarding the bioavailability of propranolol. This may be why there were no significant effects on diurnal HPA axis function. Future studies should determine blood concentrations of propranolol at the end of the treatment period in order to adjust for this factor.

As mentioned in Chapter 4, propranolol was chosen to assess the effects of changes in the peripheral nervous system on HPA axis function. However, as well as exerting effects on the beta-adrenergic receptors, there is evidence that propranolol also might act as an antagonist of the serotonin receptors 5-HT_{1A} and 5-HT_{1B} (Davids & Lesch, 1996; Hoyer et al., 1994). Therefore, propranolol may not have been the most appropriate pharmacological probe for assessing the effects of sympathetic activation on HPA axis function. Future research should seek to use beta-blockers that do not act on serotonin receptors, such as the selective beta-blocker metoprolol.

5.8.7 Strengths and limitations

A strength of this study is that it was a randomised placebo-controlled double-blind trial. We adopted a parallel group design meaning that participants receiving placebo acted as

the control comparison group for both experimental medications. The three groups in the study did not differ significantly in demographic or stress-related factors. As we did not employ a crossover design it is possible that the treatment groups were unbalanced on some covariates that were not measured in the study. However, adopting a parallel groups design allowed us to avoid problems relating to order and carry-over effects to do with the study medications. It also meant that participants were unable to become habituated to the stress protocol.

This study had a retention rate of 88.5% with 94 participants providing usable data on some parameters of diurnal cortisol secretion. However, it is possible that this study was underpowered to detect certain effects. There were also more women than men in the current study, meaning that we may have lacked sufficient statistical power to detect drug effects in men. Additionally, our sample was largely composed of healthy university students from high socioeconomic backgrounds. Therefore the results may not be readily generalizable to other groups, or to clinical groups with depression or CVD.

Cortisol was measured over a single day meaning that the diurnal secretion may have been affected by situational factors rather than long-term factors. As mentioned previously in Chapter 3 of this PhD, diurnal indices of HPA axis function are predominantly affected by trait factors on weekdays as most people have established weekday routines (Hellhammer et al., 2007). In the Stress Pathways Study we measured cortisol over the course of a weekday which may help counteract the effects of single-day sampling. However, the majority of participants were students meaning that routine may have been quite variable across weekdays. Therefore the diurnal cortisol profiles of participants may have reflected state-like properties rather than trait-like influences. This measurement issue should be borne in mind while interpreting results.

One further limitation of this study was the use of multiple testing. Within each comparison group the effects of experimental condition on seven different cortisol parameters were measured simultaneously. This means that the probability of observing a significant result due to chance was increased. Use of the Bonferroni correction would have set the significance cut-off at $p < 0.007$, thereby rendering the effects of SSRIs on cortisol in females non-significant. However, the Bonferroni correction has a tendency to be too conservative (Narum, 2006), and the replication of the significant effects of SSRIs in both cortisol slope and wake/bedtime difference in women implies that this result was not down to chance.

5.8.8 Conclusion

In conclusion, the results of this study indicate that six day treatment with the SSRI escitalopram may bring about a steepening of cortisol slopes in healthy women, via increases in waking cortisol levels. Flattened cortisol rhythms have been associated with chronic stress, depression, and CHD. This finding suggests that flattening of the cortisol slope in women may be related to alterations in the serotonergic system. It also implies that SSRIs may exert their therapeutic effects in women via correction of a flattened diurnal cortisol rhythm. However, due to the various methodological limitations of this study future research is needed to replicate this result. In the following chapter I will examine the effects of these study medications on cortisol stress reactivity, corticosteroid receptor sensitivity, and other stress-related factors which may help to further explain the results of the current study.

Chapter 6

Study 3 – The Stress Pathways Study results: The effect of pharmacological blockade on cortisol stress reactivity and corticosteroid receptor sensitivity in healthy volunteers

6.1 Introduction

In this chapter I will present results from the Stress Pathways Study concerning the effects of beta-blockade and SSRIs on cortisol secretion in response to acute psychosocial stress. I will also present results regarding the effects of acute psychosocial stress on corticosteroid receptor sensitivity. Moreover, I will examine the effects of the study medications on baseline corticosteroid receptor sensitivity, and changes in receptor sensitivity after acute stress.

In order to paint a broader picture of how the study medications are affecting the biological stress response, I will examine the effects of the medications on cardiovascular stress reactivity.

Together with the results from Chapter 5 of this thesis, these results may shed light on the biological mechanisms involved in dysregulation of HPA axis, and highlight medications that might be suitable for the treatment of impaired HPA axis function.

6.2 Literature review: Beta-blockers and cortisol stress reactivity

In Chapter 5 I described how the HPA axis and the SAM system are anatomically linked and how they interact with each other in order to regulate a number of stress-related functions, including the release of pro-inflammatory cytokines. However, under basal, unstressed conditions I found that six-day treatment with propranolol brought about no significant changes in diurnal HPA axis function. It is possible that propranolol may affect

stress-related cortisol levels. A number of studies have investigated the effects of beta-blockade on cortisol responses to a number of types of stress which will be outlined below.

6.2.1 Acute administration of beta-blockers

Early investigations into the effects of beta-blockade on stress-related HPA axis function examined cortisol responses to exercise. MacDonald and colleagues administered either a single dose of metoprolol (100mg), propranolol (80mg), or placebo in a crossover design to 10 men with essential hypertension (Macdonald, Bennett, Brown, Wilcox, & Skene, 1984). The men then underwent a prolonged exercise protocol. The single dose of propranolol, but not metoprolol, brought about significant increases in plasma cortisol and adrenaline levels during exercise compared to placebo. Similar increases in cortisol responses to submaximal exercise have been observed in healthy untrained men receiving acute intravenous pre-treatment with propranolol (Jezová, Vígás, Klimes, & Jurcovicová, 1983). In agreement with these earlier studies, a more recent study reported increases in plasma cortisol levels in ten healthy men undergoing maximal exercise to exhaustion after a dose of 80mg propranolol compared to placebo (Virtanen et al., 2007). However, single doses of 150mg metoprolol and 120mg propranolol brought about no significant changes in cortisol secretion following exercise (30m cycle) in seven healthy men compared to placebo (Uusitupa et al., 1982).

Acute effects of beta-blockers on cortisol reactivity to psychosocial stress have also been examined. Andrews and Pruessner, in what they called the 'propranolol suppression test' administered a single dose of propranolol (80mg) or placebo to 30 healthy men (n=15 in each group) (Andrews & Pruessner, 2013). Following this, participants underwent the TSST. Results showed that those who received propranolol had significantly increased

cortisol responses to acute stress alongside significantly decreased heart rate responses, compared to the placebo group. A single 80mg dose of propranolol given to 14 healthy men who underwent a psychosocial stress protocol resulted in higher salivary cortisol stress responses compared to placebo-treated men (Maheu, Jooper, & Lupien, 2005). In six young Type A men, a single dose of propranolol attenuated heart rate responses and increased cortisol responses to mental arithmetic stress (Williams, Lane, Kuhn, Knopes, & Schanberg, 1988). However, Dreifus and colleagues administered 60mg propranolol or placebo to 48 healthy women prior to undergoing the TSST and found that both groups experienced stress-induced cortisol increases that did not differ significantly (Dreifus, Engler, & Kissler, 2014). It is possible that the 60mg dose administered by Dreifus and colleagues (2014) was not adequate to bring about changes in stress-related HPA axis function, seeing as the majority of studies reporting cortisol increases administered 80mg propranolol.

More novel stress paradigms have been used to investigate the effects of acute beta-blockade on stress-related HPA axis function. Benschop and colleagues report that 160mg (4x40mg doses) propranolol administered over 1.5 days to 16 healthy men undergoing their first-time parachute jump elicited no effects in cortisol stress reactivity compared to placebo (Benschop et al., 1996). However, the men receiving propranolol did have more pronounced ACTH responses to the jump than those receiving placebo (Oberbeck et al., 1998). Cortisol responses to cold water immersion stress were also increased by a 40mg dose of propranolol in eight healthy young men (Šimečková, Janský, Lesna, Vybiral, & Šrámek, 2000). Khan and colleagues examined the effects of an acute intravenous dose of propranolol (0.2mg/kg) on cortisol responses to pentagastrin administration in 16 healthy men and women (Khan, Liberzon, & Abelson, 2004). Pentagastrin is a substance that produces symptoms of anxiety and panic, and brings about strong activation of the

HPA axis. Compared to placebo, those who received propranolol had a delayed but enhanced cortisol response to pentagastrin. ACTH and adrenaline responses were also enhanced in the propranolol group. The heart rate acceleration usually brought about by pentagastrin was virtually eliminated by propranolol also.

Together, what these results suggest is that acute beta-blockade brings about enhanced cortisol stress reactivity. At the same time, beta-blockade also attenuates heart rate responses to stress. This implies an inverse relationship between the HPA axis and the SAM system in that suppression of the SNS stress response by beta-blockade brings about an increase in HPA axis function. In support of this, a study that combined the DST with the TSST found that those who had received dexamethasone the night before stress testing had lower cortisol stress responses in combination with an elevated heart rate compared to the placebo group (Andrews, D'Aguiar, & Pruessner, 2012).

6.2.2 Long-term administration of beta-blockers

The effects of more long-term administration of beta-blockers on cortisol stress reactivity have also been examined, but to a lesser extent than acute administration. Two early studies examined the effects of long-term beta-blockade on cortisol responses to exercise. In the first study, 10 men with essential hypertension received 28 day treatment with propranolol (80mg/day), metoprolol (100mg/day), and placebo in a crossover design (Macdonald et al., 1984). Following this, the men underwent a prolonged exercise protocol. Both the propranolol and metoprolol treatment brought about increased cortisol responses to exercise compared to placebo. Similarly, in 18 healthy young men 100mg metoprolol (twice daily) and 10mg timolol (twice daily) for five days resulted in significantly increased cortisol responses to exercise compared to placebo (Gullestad, Dolva, Kjeldsen, Eide, & Kjekshus, 1989).

To date, only one study has examined the effects of prolonged beta-blockade on cortisol responses to psychosocial stress. Kudielka and colleagues administered propranolol (80mg/day) to 19 healthy men and women for five days (Kudielka et al., 2007). Participants then underwent the TSST. Neither pre-stress cortisol levels nor cortisol responses to the TSST in the propranolol group differed significantly from placebo. Similar null findings have been reported in 20 healthy male volunteers undergoing a bungee jump who received three day pre-treatment with propranolol (3 x 40mg/day) (van Westerloo et al., 2011).

6.2.3 Summary

Taken together, the studies examining effects of acute beta-blockade on cortisol stress reactivity suggest that acute suppression of the SNS via beta-blockade seems to enhance HPA axis stress reactivity. This effect appears to hold despite heterogeneity between studies in terms of sample size, stress paradigm, route of drug administration, and biological specimen used for cortisol measurement (saliva/plasma). However, results from studies examining more long-term effects of beta-blockade on cortisol stress reactivity have been mixed. Five-day and 28-day administration of beta-blockers appear to increase cortisol secretion following exercise paradigms. However, using a psychosocial stress paradigm and a more novel bungee jump paradigm has produced null findings. This is peculiar considering acute doses appear to bring about enhanced cortisol responses to the TSST. Interestingly, the studies that report these enhanced responses do so only in male samples (Andrews & Pruessner, 2013; Maheu et al., 2005; Williams et al., 1988). Dreifus and colleagues reported null findings following the TSST in a sample comprised of women (Dreifus et al., 2014). The inclusion of women in the sample may have been one of the reasons Kudielka and colleagues (2007) report null findings following longer-term administration of propranolol. Neither study include the use of

contraceptive pill as a covariate – something that is known to affect cortisol stress reactivity (Rohleder, Wolf, Piel, & Kirschbaum, 2003). This may also have affected results obtained.

In sum, the results of this body of work suggest that suppression of the SNS brings about increases in stress-related HPA axis function. However, further work is needed examining effects of longer-term beta-blocker administration.

6.3 Literature review: SSRIs and cortisol stress reactivity

In Chapter 5 I reported results from the Stress Pathways Study showing that SSRIs brought about steeper diurnal cortisol rhythms in women. Flatter slopes are known to be characteristic of depression (Doane et al., 2013; Jarcho et al., 2013; Sjögren et al., 2006) and the results from the Stress Pathways Study suggest that SSRI treatment may normalise the diurnal cortisol rhythm in women. Cortisol stress reactivity is also known to be dysregulated in depression with both enhanced and blunted cortisol responses to stress being reported depending on the severity and duration of depressive symptoms. It is possible that SSRIs may affect the cortisol stress response also. A number of studies have examined these effects.

6.3.1 Acute administration of SSRIs

Using a crossover design, Ahrens and colleagues administered single doses of either the SSRI sertraline (100mg) or placebo to 12 healthy men (Ahrens, Frankhauser, Lederbogen, & Deuschle, 2007). They then examined neuroendocrine responses to exercise stress. Baseline pre-exercise cortisol levels in the sertraline group were higher, and the cortisol responses to exercise stress were enhanced in this group also. The effects of acute SSRI administration on cortisol response to psychosocial stress have also been examined.

Healthy men were randomised to receive single doses of 10mg escitalopram (n=17), 20mg escitalopram (n=14), or placebo (n=12) (Garcia-Leal, Del-Ben, Leal, Graeff, & Guimarães, 2010). Following this, they underwent a simulated public speaking protocol, similar to the public speaking component of the TSST. Escitalopram did not bring about any significant alterations in cortisol stress reactivity compared to placebo. However, this is probably because the public speaking protocol did not elicit a cortisol stress response in either group.

6.3.2 Long-term administration of SSRIs

A number of studies have examined the effects of longer-term SSRI administration on cortisol stress reactivity, with mixed results. Ljung and colleagues administered six months treatment with citalopram (20-40mg/day) or placebo to 16 healthy men with moderate abdominal obesity in a crossover trial (Ljung et al., 2001). Following the treatment period, the men underwent an arithmetic stress test. Citalopram brought about increases in baseline morning cortisol values, and following stress cortisol secretion was enhanced in the citalopram group. Duncko and colleagues examined the effects of SSRIs in an all-male sample also. Thirty-one healthy men were randomised to receive either tianeptine (37.5mg/day), citalopram (20mg/day), or placebo for six days. Following this, the men underwent a stress protocol comprised of a short intelligence test and the Stroop colour interference test. The antidepressant drugs brought about no changes in cortisol stress reactivity compared to placebo. However, after seven days administration, antidepressant treatment in the same male sample brought about enhanced ACTH responses to insulin-induced hypoglycaemia compared to placebo (Jezová & Duncko, 2002). Cortisol remained unaffected.

Kotlyar and colleagues have investigated the effects of SSRIs on cortisol stress reactivity in a mixed healthy sample. Using a crossover design, 62 men and women received one month treatment with paroxetine (10-20mg/day) and placebo (Kotlyar et al., 2013). Following treatment participants underwent a modified version of the TSST. Paroxetine brought about a significant overall increase in cortisol levels. However, there was no significant difference in the cortisol stress response between the conditions.

Cortisol stress reactivity in depressed patients undergoing treatment with SSRIs has also been examined. Patients with major depression who had been treated with bupropion (200-450mg/day, n=17), or paroxetine (10-50mg/day, n=17) for at least two months were compared to 15 non-depressed controls (Straneva-Meuse, Light, Allen, Golding, & Girdler, 2004). All participants underwent a modified version of the TSST. Those taking bupropion and paroxetine had blunted cortisol stress reactivity compared to healthy controls.

6.3.3 Summary

Only one study to date has assessed the effects of acute SSRI treatment on cortisol responses to psychosocial stress (Garcia-Leal et al., 2010). The pharmacological effects of SSRIs are known to be delayed (Frazer & Benmansour, 2002) meaning that longer-term administration may be required to see the effects of SSRIs on cortisol stress reactivity. However, the results from these longer-term studies have been mixed. As with the longer-term beta-blocker studies, SSRIs only seem to enhance stress-related neuroendocrine activity in all-male samples (Jezová & Duncko, 2002; Ljung et al., 2001). Healthy and depressed samples report null findings or blunted cortisol reactivity respectively (Kotlyar et al., 2013; Straneva-Meuse et al., 2004). To date, there have been too few studies carried out on the effects of SSRIs on the cortisol stress response, and

amongst the studies that do exist there has been much variability in terms of sample characteristics (e.g. healthy versus depressed versus abdominally obese), treatment duration, and stress protocol used. The evidence suggests that SSRIs affect basal/diurnal cortisol secretion, and that they likely also affect secretion during times of stress. More work is needed to clarify these effects.

6.4 Cortisol stress reactivity: Aims and hypotheses

The aim of this study was to examine the effects of seven-day administration of beta-blockers and SSRIs on cortisol stress reactivity in the laboratory using data from the Stress Pathways Study. As discussed in Chapter 2, chronic stress and depression are associated with changes in the cortisol stress response. Altered cortisol stress reactivity has also been associated with cardiovascular risk factors, and has been seen in CHD patients. Looking at how beta-blockers and SSRIs might alter cortisol secretion after acute laboratory stress in healthy volunteers may provide information about the biological systems involved in stress-related HPA axis dysregulation and may also identify potential therapeutic interventions.

Beta-blockers

Based on results from studies outlined above, I hypothesise that seven-day treatment with propranolol will bring about increased cortisol stress reactivity in the laboratory.

SSRIs

Based on results from studies outlined above in healthy volunteers free from depression, I hypothesise that seven-day treatment with escitalopram will bring about increases in cortisol stress reactivity in the laboratory.

Moreover, in studies examining longer-term administration of beta-blockers and SSRIs on cortisol stress reactivity, significant enhancement of cortisol secretion has only been reported in all-male samples. Therefore, I will also examine how sex influences the effects of the study medications.

6.5 Literature review: Beta-blockers and corticosteroid receptor sensitivity

As mentioned previously, studies using beta-blockers have provided evidence for the notion that suppression of the SNS brings about increased HPA axis activity. It is possible that beta-blockade may modulate HPA axis activity via the corticosteroid receptors. For example, epinephrine and norepinephrine have been shown to affect GR transactivation, and GR binding to GREs within the cell nucleus (Schmidt, Holsboer, & Spengler, 2001). However, there is a dearth of research assessing the effects of beta-blockade on corticosteroid receptor function. To date, one study has assessed the effects of drugs commonly used to treat CHD on GR protein levels, and one of the drugs included was the beta-blocker metoprolol. Measuring GR protein levels gives an indication of GR gene function. Eighty hospitalised CHD patients were enrolled in the study. Twenty of these patients received metoprolol (50mg/day) and GR protein levels in lymphocytes were measured before and one month after administration of the drug (Ji, Guo, Yan, Li, & Lu, 2010). Results indicated that those taking metoprolol had increased GR protein levels following one month of treatment, compared with baseline levels. This result provides support for modulation of the corticosteroid receptors by beta-blockade. However, much more work is needed, particularly in healthy volunteers where the drug effects cannot be ascribed to symptom remission. Examining both the effects of beta-blockade on basal corticosteroid receptor sensitivity and on how the receptors respond to acute stress would provide information on how suppression of the SNS brings about increases in HPA axis function.

6.6 Literature review: SSRIs and corticosteroid receptor sensitivity

As mentioned previously, depression is known to be characterised by dysregulation of the HPA axis. One explanation of this dysregulation is an imbalance in both GR and MR sensitivity. There is evidence to suggest that SSRIs directly modulate corticosteroid receptor sensitivity. This may be one of the mechanisms through which SSRIs serve to ‘normalise’ HPA axis activity (Anacker, Zunszain, Carvalho, & Pariante, 2011). The effects of SSRIs on both corticosteroid receptor function and sensitivity have been examined in both murine and human studies.

6.6.1 Murine studies

Pariante and colleagues examined the effects of 24-hour co-incubation of LMCAT murine cells with dexamethasone and the SSRIs paroxetine, citalopram, and fluoxetine on GR function (Pariante et al., 2001; Pariante, Kim, Makoff, & Kerwin, 2003). GR function was measured by looking at rates of GR-mediated gene transcription. Citalopram, paroxetine, and fluoxetine, were all found to enhance GR function in this cell line. They also found that SSRIs increased GR function by inhibiting the LMCAT cell membrane steroid transporter (a protein, like p-glycoprotein, that expels glucocorticoids from cells). This idea was later disproven when Mason and colleagues showed that the effects of the tricyclic antidepressant desipramine on glucocorticoid accumulation did not differ between wild-type and p-glycoprotein knockout mice (Mason, Thomas, Lightman, & Pariante, 2011).

Lai and colleagues assessed the effects of four-day incubation with fluoxetine on GR and MR mRNA expression in rat hippocampal cells (Lai et al., 2003). In line with Pariante’s findings, fluoxetine significantly increased GR mRNA expression. However, MR mRNA expression was unaffected. The authors suggest this shows that GR and MR are

differentially regulated by short-term exposure to increased serotonin levels. Interestingly, nine-day incubation with fluoxetine brought about a decrease in MR mRNA expression in rat hippocampal cells (Yau, Noble, Hibberd, & Seckl, 2001). However, this differential regulation in GR and MR function seems to even out following 14-day treatment with SSRIs. In rat hippocampal cells, 14-day incubation with paroxetine brought about increases in GR mRNA expression (Okugawa et al., 1999), and incubation with citalopram for the same time period also brought about increases in MR mRNA expression (Seckl & Fink, 1992). This indicates that longer-term incubation with SSRIs brings about increased GR and MR function.

6.6.2 *Human in vitro studies*

The effects of fluoxetine on GR function has been measured in the lymphocytes of healthy volunteers (Okuyama-Tamura, Mikuni, & Kojima, 2003). In this study GR function was measured by looking at the rate of translocation of the GR into the cell nuclei. Following one hour incubation, fluoxetine induced rapid translocation of the GR into the cell nuclei, meaning this SSRI enhanced GR function. Carvalho and colleagues investigated the effects of a number of different types of antidepressants on GR sensitivity in whole blood drawn from healthy volunteers (Carvalho, Garner, Dew, Fazakerley, & Pariante, 2010). GR sensitivity was measured using dexamethasone inhibition of LPS-stimulated IL-6 production in whole blood. Whole blood was co-incubated for 24 hours with dexamethasone and two different tricyclic antidepressants, one serotonin and norepinephrine reuptake inhibitor (SNRI) and two SSRIs (sertraline and paroxetine). The results indicated that all the antidepressant types brought about reduced GR sensitivity. This finding is in disagreement with previous research which indicates that antidepressants seem to increase GR function in murine and human *in vitro* studies.

6.6.3 Human *in vivo* studies

As mentioned in *Section 2.9.2* of this thesis, the DST is the most widely used method to measure GR-mediated negative feedback of the HPA axis in humans *in vivo* (Rohleder, Wolf, & Kirschbaum, 2003). The dexamethasone/CRH (dex/CRH) test is another version of the DST which is said to be more specific, and have more utility when it comes to diagnosing mood disorders (Watson, Gallagher, Smith, Ferrier, & Young, 2006). The dex/CRH test is essentially a DST followed by a CRH infusion which is supposed to induce the release of ACTH from the pituitary. Like the DST, the dex/CRH test is considered an indirect way to measure corticosteroid receptor sensitivity as dexamethasone administration will modulate the HPA axis via interaction with the corticosteroid receptors. Some argue that the dex/CRH test can detect subtle changes in HPA axis function that the DST cannot (Watson et al., 2006). However, others claim that the dex/CRH test simply measures GR-mediated negative feedback of the HPA axis at both the level of the adrenal and the pituitary glands (Pariante & Miller, 2001).

Studies that have investigated the effects of antidepressants on glucocorticoid sensitivity *in vivo* have almost exclusively used the dex/CRH test. The majority of studies have been carried out in depressed patients. Nickel and colleagues provided six weeks treatment with paroxetine to 22 depressed men and women (Nickel et al., 2003). These patients underwent the dex/CRH test at baseline and at the end of the treatment period. Paroxetine administration resulted in decreases in ACTH and cortisol levels following the dex/CRH tests indicating SSRI-induced increases in GR sensitivity. In a similar study, 20 depressed patients underwent the dex/CRH test following one week of receiving placebo, and after two, four, and 16 weeks of receiving treatment with citalopram (40mg/day) (Nikisch et al., 2005). There was a time-dependent reduction in the ACTH and cortisol responses to the test over the 16-week treatment period indicating an increase in corticosteroid receptor

sensitivity. Moreover, the magnitude of the decrease in cortisol responsivity (increase in receptor sensitivity) at four weeks was significantly associated with a reduction in depressive symptoms at 16 weeks.

Bschor and colleagues gave 30 patients with depression four weeks SSRI therapy with citalopram (20-40mg/day) (Bschor et al., 2012). The patients underwent the dex/CRH test before and after treatment. Citalopram reduced the amount of ACTH released following the dex/CRH test indicating increased GR sensitivity. Cortisol levels remained unaffected. This implies that citalopram effects took place at the pituitary, but not the adrenal, level of the HPA axis. Reductions in ACTH and cortisol responses to the dex/CRH test have also been observed in 30 female patients with borderline personality disorder receiving 12-week treatment with fluvoxamine (150mg/day) (Rinne et al., 2003). Interestingly, those who had a history of sustained childhood abuse showed the strongest reduction in responses, and they also had the lowest GR sensitivity at baseline. This indicates that SSRI treatment increased GR sensitivity in these patients, particularly in those who had experienced chronic stress in early life.

However, some studies have reported contradictory results in depressed patients. In a recent study, 28 patients with major depression received five weeks treatment with escitalopram (10mg/day) (Sarubin, Nothdurfter, Schmotz, et al., 2014). The dex/CRH test was carried out at baseline, and after one and five weeks of treatment with the SSRI. Interestingly, escitalopram led to an increase in cortisol responses to the dex/CRH test after week one, whereas levels of suppression at baseline and five weeks were comparable. What this implies is that treatment with escitalopram brought about a transient decrease in GR sensitivity, but overall had no significant long-term effect. An observational study has also reported decreased GR sensitivity in SSRI users. Manthey and colleagues examined cross-sectional associations between SSRI use and responses to

the DST in 1526 patients from the Netherlands Study of Depression and Anxiety (Manthey et al., 2011). Compared to non-users (n=1068), those who used SSRIs (n=309) had decreased cortisol suppression after dexamethasone ingestion. This implies that they had reduced GR sensitivity. The authors controlled for a number of relevant factors including duration of SSRI use, and severity of depression. They posit that treatment resistance, a factor they did not consider in their analysis, may explain their incongruous result. Treatment resistance has been associated with impaired responses to glucocorticoid suppression tests (Juruena et al., 2009).

The effects of SSRIs on glucocorticoid sensitivity have also been examined using healthy samples. Carpenter and colleagues administered six weeks treatment with either sertraline (100mg/day) or placebo to 22 healthy men and women (Carpenter et al., 2011). Participants underwent the dex/CRH test at baseline and following the treatment period. The results showed that those who received sertraline had increased cortisol levels following the dex/CRH test compared with placebo. This implies that SSRI treatment in healthy people led to a decrease in GR sensitivity, leading to impaired feedback inhibition of the HPA axis. Pariante and colleagues examined the effects of shorter-term administration of SSRIs in healthy volunteers (Pariante et al., 2004). Eight healthy men and women were given four days administration of citalopram (20mg/day). Participants underwent the PST at baseline and after the four days treatment. Citalopram increased cortisol suppression by prednisolone indicating that this SSRI brought about increased corticosteroid receptor sensitivity (prednisolone binds to both the GR and MR). However, this increase in suppression was only observed in the morning, and not in the early or late afternoon, implying that the diurnal rhythm of HPA axis activity (regulated by the MR) may be an influencing factor here.

6.6.4 Summary

In murine samples it appears that SSRIs bring about increases in corticosteroid receptor function. Similarly, *in vitro* examination of GR function in human lymphocytes reveals that SSRIs increase the rate of translocation of the GR into the cell nuclei. However, GR sensitivity, measured using *in vitro* glucocorticoid sensitivity assays, appears to be decreased in human whole blood incubated with antidepressants. Directly measuring GR function (translocation of receptors into cell nuclei) is different to assessing sensitivity using glucocorticoid sensitivity assays as they provide only a proxy measure of biological receptor ‘function’. This may be a reason for the discrepancy in results.

Within depressed patients, the research suggests that SSRI treatment does bring about increases in GR receptor sensitivity as measured by the dex/CRH test. In one case the magnitude of the increase in sensitivity was associated with symptom improvement. These results are in support of the notion that SSRIs may help to ameliorate symptoms of depression by ‘normalising’ dysregulated HPA axis function. Two studies reported decreases in receptor sensitivity in depressed patients (Manthey et al., 2011; Sarubin, Nothdurfter, Schmotz, et al., 2014). However, Manthey and colleague’s study did not adopt an experimental design meaning a number of factors could not be controlled for. In Sarubin and colleague’s study, the participants were also randomised to undergo a yoga intervention (Sarubin, Nothdurfter, Schüle, et al., 2014). Yoga is known to affect cortisol levels (Field, 2011), and this factor was not adjusted for in this study meaning the yoga intervention could have influenced how the SSRIs interacted with the HPA axis.

To date, only two studies have assessed the effect of SSRIs on corticosteroid receptor sensitivity in healthy people, eliciting mixed results. Long-term treatment was found to decrease GR sensitivity whereas short-term treatment was found to increase both GR and

MR sensitivity in the morning only. Due to the difference in treatment durations, and the use of different suppression tests (dex/CRH versus PST), it is difficult to draw conclusions from the results of these studies. More work is needed examining the effects of SSRIs on corticosteroid receptor function in healthy individuals. Additionally, examining the effects of SSRIs on how corticosteroid receptors sensitivity changes in response to acute stress may shed light on how SSRIs affect cortisol stress reactivity.

6.7 Corticosteroid receptor sensitivity: Aims and hypotheses

The aim of this study was to assess the effects of seven-day administration of beta-blockers and SSRIs on corticosteroid receptor sensitivity both before and after acute psychosocial stress in the laboratory using data from the Stress Pathways Study. As outlined in Chapter 2, chronic stress and depression are associated with alterations in corticosteroid receptor sensitivity. This implies that stress-related HPA axis dysregulation is brought about via diminished sensitivity of these receptors. Examining how beta-blockers and SSRIs might alter pre-stress baseline receptor sensitivity, and how these drugs affect receptor sensitivity responses to acute stress, may provide more information about how stress-related HPA axis dysregulation comes about.

Placebo

Acute receptor reactivity: Earlier in this thesis I outlined the both human and animal studies carried out to date examining the effects of acute psychosocial stress in the laboratory on corticosteroid receptor sensitivity (See Chapter 2, Table 2.2). The results of these studies suggest that the effects of acute stress on GR sensitivity vary according to sex, age, BMI, and health status. Overall, there is a lack of studies investigating the effects of acute stress on GR and MR sensitivity, and the observed effects of covariates are yet to be replicated. Due to the variability of results from these studies, and the lack of studies

examining the effects of acute stress on the MR, in this study I will examine the effects of acute psychosocial stress on both GR and MR sensitivity in unmedicated healthy volunteers who have received placebo. Based on the findings of human and murine studies outlined in Chapter 2 (*Section 2.10.2*) and based on the findings from previous work carried out by our group (Carvalho et al., 2015) **I hypothesise that acute stress will lead to a decrease in corticosteroid receptor sensitivity in young unmedicated healthy volunteers.**

Beta-blockers

Baseline receptor sensitivity: As beta-blockade induced increases in GR protein levels in CHD patients (Ji et al., 2010), this suggests that beta-blockers increase GR sensitivity. However, the effects of beta-blockers on GR sensitivity directly are not known. To date there have been no studies assessing the effects of beta-blockers on MR sensitivity. **I hypothesise that baseline GR and MR sensitivity will be increased in healthy volunteers receiving beta-blockers compared with placebo.**

Acute stress receptor sensitivity: In Section 6.4 I hypothesise that seven-day treatment with propranolol will bring about increased cortisol stress reactivity in the laboratory. **I therefore hypothesise that seven-day treatment with propranolol will bring about enhanced changes in GR and MR sensitivity in response to acute stress compared with placebo.**

SSRIs

Baseline receptor sensitivity: Although results from healthy volunteers have been mixed, the results from murine models and depressed patients seem to suggest that SSRI administration brings about increased GR sensitivity. The results regarding the MR are a

little more mixed, but the results of Pariante and colleague's (2004) study suggest that short-term SSRI administration increases both GR and MR sensitivity, albeit in the morning. Therefore, **I hypothesise that seven-day treatment with escitalopram will bring about increases in baseline GR and MR sensitivity compared with placebo.**

Acute stress receptor sensitivity: In Section 6.4 I hypothesise that seven-day treatment with escitalopram will bring about increased cortisol stress reactivity in the laboratory. **Therefore, I hypothesise that seven-day treatment with escitalopram will bring about enhanced changes in GR and MR sensitivity in response to acute stress compared with placebo.**

It should be noted that I had no hypotheses concerning differences between propranolol and escitalopram. The study was analysed as two parallel comparisons with placebo, rather than contrasting the two active medication conditions.

Sex

Sex has not been considered in the studies assessing the effects of beta-blockers and SSRIs on corticosteroid receptor functions in humans. However, as I am examining the effects of how sex influences the effects of the study medications on cortisol stress reactivity, I will also explore how sex influences the medication effects on baseline and stress-related corticosteroid receptor sensitivity. Sex differences in how the corticosteroid receptors respond to acute stress have been previously reported (Rohleder et al., 2001).

6.8 Biological measures

Data from the Stress Pathways Study were used to test the hypotheses of this study. To reiterate, participants were randomised to receive seven-day administration of either propranolol (80mg/day), escitalopram (10mg/day), or placebo. Following this, they

underwent acute psychosocial stress testing in the laboratory. Saliva and blood samples were taken throughout the session for the measurement of cortisol and corticosteroid receptor sensitivity respectively. A detailed account of the methodology is provided in Chapter 4 of this thesis.

6.8.1 Calculation of cortisol parameters in the laboratory

A detailed description of the cortisol sampling procedure is provided in Chapter 4, *Section 4.4.4* and *Section 4.4.7*. To reiterate, during the laboratory stress testing session, one saliva sample was taken prior to the stress protocol to allow measurement of baseline cortisol levels (25 minutes after cannulation). A sample was then taken immediately post-stress, and at 10, 20, 45, and 75 minutes post-stress to measure cortisol stress reactivity. Cortisol values for each time-point were calculated as well as the overall cortisol AUC in the laboratory (post-stress – 75 minute sample). The cortisol AUC was calculated with respect to ground (Pruessner et al., 2003). Although 91 participants provided complete samples during the laboratory session, not all time-points were included for each participant. Participants were excluded from the analysis if any cortisol value in the laboratory exceeded 50 nmol/L. Therefore, at baseline five values were removed, post-stress two values were removed, at 10m post-stress three values were removed, at 20m post-stress and 45m post-stress one value was removed, and at 75m post-stress four values were removed. Cortisol AUC was calculated only for those who provided six usable saliva samples. Therefore, after the removal of outliers from the sample, cortisol AUC was calculated for 85 participants. Different sample sizes for each cortisol time-point are detailed in Table 6.4.

Counter to expectations, cortisol levels decreased following the acute stress protocol in every experimental condition (see Figure 6.6, *Section 6.10.4*). The possible reasons for

this will be discussed in *Section 6.11.6*. Therefore, I categorised the participants according to whether or not they responded to the stress protocol. In accordance with Hamer et al. (2010), participants were considered responders if an increase of ≥ 1 nmol/L cortisol was detected immediately after the stress protocol, 10 minutes, or 20 minutes post-stress relative to baseline. The number of responders in each medication group is provided in Table 6.4.

6.8.2 Corticosteroid receptor sensitivity

Reagents

RPMI 1640 medium (Sigma), 500ml, sterile, R8758; foetal calf serum (Gibco 10270); penicillin/streptomycin (Sigma), 500ml, sterile, P4458; LPS (Sigma), 10mg, L2630; dexamethasone (Sigma), D4902; prednisolone (Sigma), P-6004.

Protocol

Corticosteroid receptor sensitivity was measured using an *in vitro* glucocorticoid sensitivity assay (see Figure 6.1). Sensitivity was assessed by measuring dexamethasone (GR) and prednisolone (MR) suppression of LPS-induced IL-6 production in whole blood. Whole blood was diluted ten-fold using RPMI 1640 medium supplemented with 10% foetal calf serum, 100IU/ml penicillin, and 100mg/ml streptomycin. LPS and either dexamethasone or prednisolone were added into each well of two 48-well FALCON cell culture plates. The following concentrations of dexamethasone and prednisolone were used: 0M, 5.4×10^{-6} M, 1.8×10^{-6} M, 5.4×10^{-7} M, 1.8×10^{-7} M, and 5.4×10^{-8} M. Subsequently, 540ml of diluted blood was added to each well. Samples were incubated for 24 hours in a humidified atmosphere containing 5% CO₂. After incubation, plates were centrifuged (1000 x g, 4°C, 10mins) and the cell culture supernatant was carefully

collected. The samples were then stored at -80°C before being analysed for IL-6 production.

IL-6 production analysis was carried out using a commercially available Luminex technology kit for IL-6 from Bio-RAD®. The inter- and intra-assay coefficient of variation (CV) for IL-6 analysis was 13.3% and 7% respectively, and the detection limit was 2.6 pg/ml. Dexamethasone suppression of IL-6 production was assessed using the following concentrations: 0M, $5.4 \times 10^{-6}\text{M}$, $1.8 \times 10^{-6}\text{M}$, $5.4 \times 10^{-7}\text{M}$, $1.8 \times 10^{-7}\text{M}$, and $5.4 \times 10^{-8}\text{M}$ dexamethasone. Prednisolone suppression of IL-6 production was assessed using the following concentrations: 0M, $5.4 \times 10^{-6}\text{M}$, $1.8 \times 10^{-6}\text{M}$, $5.4 \times 10^{-7}\text{M}$, and $1.8 \times 10^{-7}\text{M}$ prednisolone. IL-6 suppression by $5.4 \times 10^{-8}\text{M}$ prednisolone was not assessed as prednisolone had a higher IC_{50} and $5.4 \times 10^{-8}\text{M}$ of prednisolone is not associated with any biological function.

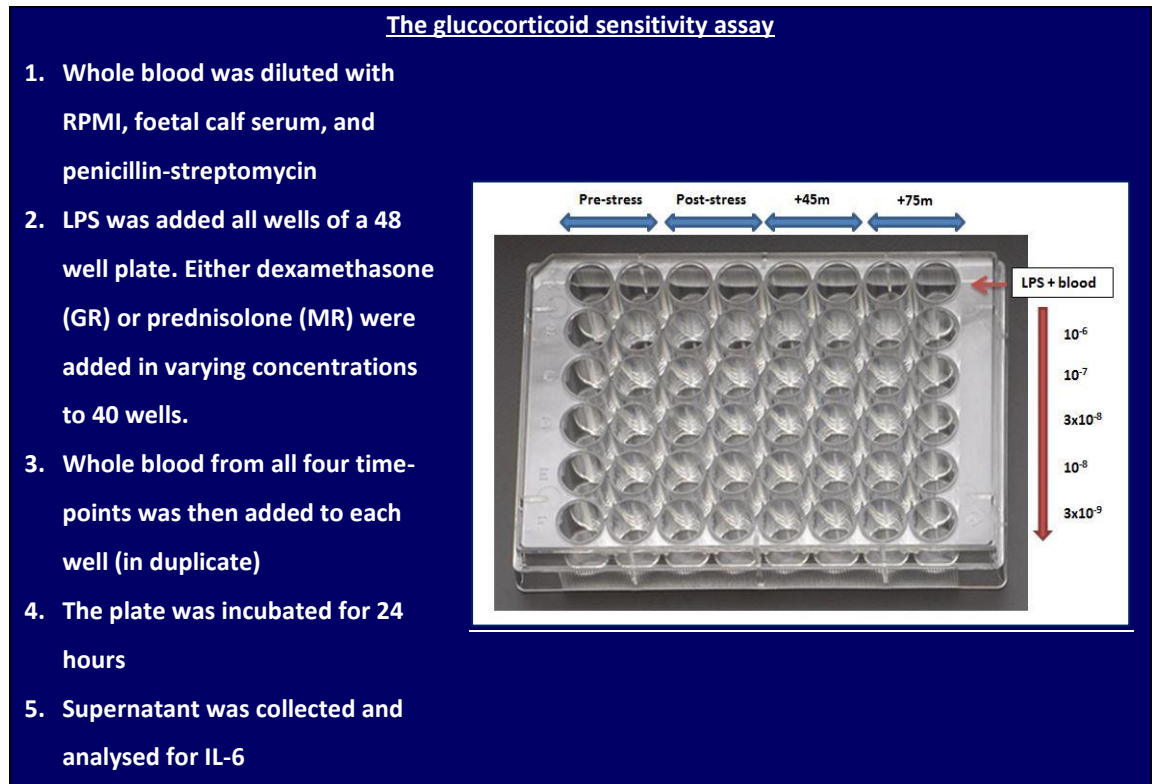


Figure 6.1. The glucocorticoid sensitivity assay

Glucocorticoid suppression of IL-6 was calculated by considering LPS-stimulated levels of IL-6 in the absence of either dexamethasone or prednisolone as 100%. Percentage inhibition of IL-6 by the glucocorticoids was then calculated using the following equation:

$$\left(\frac{\text{LPS-induced IL-6 levels in the presence of glucocorticoids}}{\text{LPS-induced IL-6 levels in the absence of glucocorticoids}} \times 100 \right) = \% \text{ inhibition}$$

Percentage inhibition for each concentration of dexamethasone and prednisolone was then entered into GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) in order to calculate the log inhibitory concentration 50% (IC₅₀) values of the dose-response curve of dexamethasone and prednisolone suppression of IL-6 production at each time-point. The IC₅₀ is the measure of how effective a substance is at inhibiting a specific biological function. It represents the concentration of substance or drug required to bring about 50% inhibition *in vitro*. Figure 6.2 provides an example of how the IC₅₀ is calculated. Log IC₅₀ values are inversely proportional to glucocorticoid sensitivity. Higher log IC₅₀ values indicate that more dexamethasone or prednisolone was required to suppress IL-6 production by 50%, and this implies that GR and/or MR sensitivity is decreased.

6.8.3 Cardiovascular measures

A detailed description of the cardiovascular data measured in the stress laboratory is provided in Chapter 4, *Section 4.4.7*. To reiterate, all participants were attached to a Finometer® PRO in order to measure BP, heart rate, and cardiac output continuously during the laboratory session. All cardiovascular measures were averaged into mean readings taken from five-minute intervals. There was a five-minute baseline interval (pre-stress), as well as two five-minute recovery period intervals (+40-45m, and +70-75m).

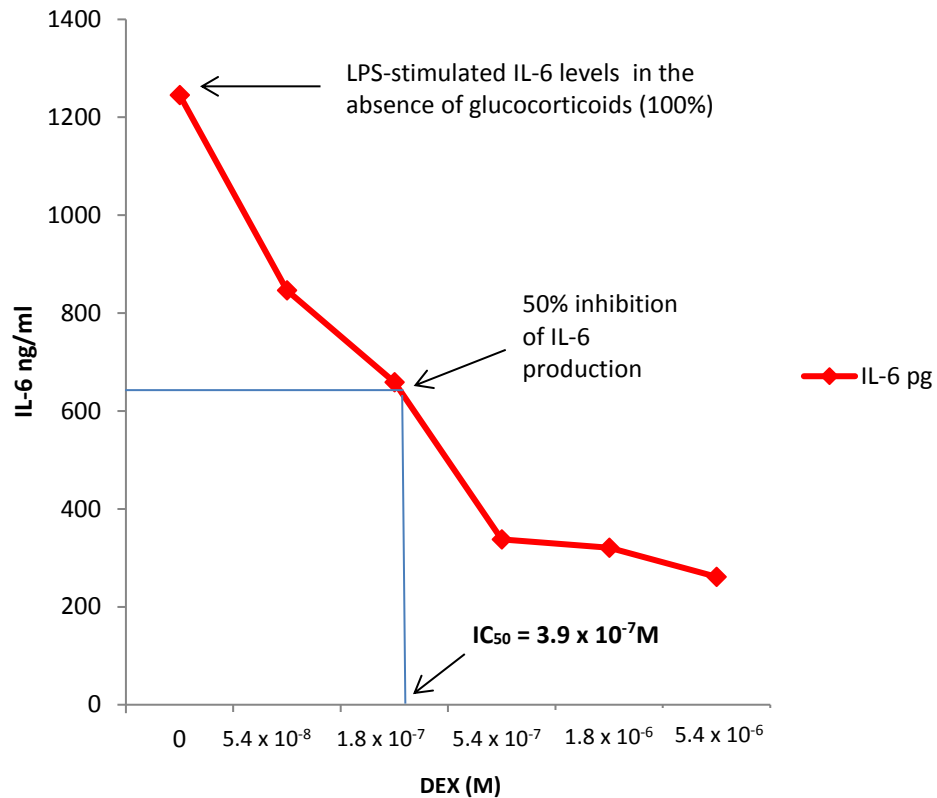


Figure 6.2. Sample calculation of the IC_{50} from one participant

Cardiovascular measures during the stress protocol were averaged across tasks. Cardiac index ($L/min/m^2$) was calculated by dividing cardiac output (L/min) by the body surface area (m^2).

6.9 Statistical analyses

Kolmogorov-Smirnov tests were performed to test for normality of the distribution in measures of cortisol, corticosteroid receptor sensitivity (IC_{50} values), and cardiovascular measures in the laboratory. These normality tests revealed that all corticosteroid receptor sensitivity and cardiovascular measures were normally distributed (all p values > 0.05). However, all measures of salivary cortisol in the laboratory were not normally distributed. Log transformation (base 10) normalised the distributions.

As in Chapter 5, the data were analysed using two parallel statistical analyses: propranolol versus placebo, and escitalopram versus placebo. Two-way ANOVAs and chi-square tests were used to compare the three study medication groups on all demographic characteristics. Where possible, sex was included as a between-person factor alongside experimental condition. Paired t-tests were used to assess differences between subjective stress ratings at rest and following the acute stress protocol in the overall sample. One-way ANOVAs were used to assess the effects of the study medications on subjective stress ratings at rest, and ANCOVAs were used to assess the effects of the study medications on subjective stress ratings following the stress protocol, adjusting for rest ratings.

All biological stress measures in the laboratory were analysed using two separate pairwise analyses; propranolol versus placebo, and escitalopram versus placebo. Repeated measures ANOVAs were used to examine stress-related changes over time in cortisol, corticosteroid receptor sensitivity, and cardiovascular measures. Paired t-tests were used to explore significant within-subject contrasts. Where necessary, differences between the two experimental conditions in biological stress parameters at each time-point were analysed using two-way ANOVAs, with experimental condition and sex being included as the main fixed factors. Logistic regression was used to assess the effects of experimental condition on the cortisol responder category. Where there were significant differences between experimental conditions on any of the demographic characteristics, repeated measures ANCOVAs were run where the demographic variable of interest was included as a covariate. Pearson's R correlations were used in exploratory analysis to ascertain what factors were associated with pre-stress salivary cortisol levels in the laboratory.

The significance level was set to $p < 0.05$ for all analyses, with precise p values reported for all test results. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA).

6.10 Results

6.10.1 Participants

As mentioned previously, 91 participants provided saliva samples for cortisol measurement in the laboratory. Blood used for the measurement of corticosteroid receptor sensitivity was drawn successfully from 85 participants, and cardiovascular measures were gathered from 90 participants. Participants with at least one of these biological measures (cortisol, receptor sensitivity, or cardiovascular measures) were included in the main sample of this study ($n = 91$). Of the 91 participants in this main sample, 30 were taking escitalopram, 31 were taking propranolol, and 30 received placebo. Table 6.1 summarises the characteristics of the participants in each experimental condition. The sample had an age range of 18-48 years ($M = 22.8$, $SD = 4.8$), were almost two-thirds women (63.7%), and were mostly normal weight (78.0% BMI<25). Over half of the sample were white (58.2%) and the majority of participants had a high SES based upon parental education (80.2%). Smokers comprised 16.5% of the sample. Baseline scores (measured on Day 1 of the study) on the BDI-II ranged from 0-31 indicating the presence of severe depressive symptoms in some participants. Frequency analysis revealed that four participants had BDI-II scores greater than 19 suggesting the presence of clinical depression. Baseline scores on the HADS ranged from 0-15 indicating anxiety in some participants. Nine participants had scores of 11 or greater, suggesting the presence of clinical anxiety in these individuals. Sensitivity analyses were carried out with these participants removed ($n = 12$; propranolol $n = 7$, escitalopram $n = 1$, placebo $n = 4$).

Exclusion of these participants did not affect results obtained. Scores on the PSS ranged from 2-32 indicating a good level of variability in perceived stress within the sample.

The propranolol group did not differ significantly from the placebo group in terms of age ($F(1, 57) = 1.82, p = 0.18$), sex ($\chi^2 = 0.36, df = 1, p = 0.85$), BMI ($F(1, 57) = 0.90, p = 0.30$), smoking status ($\chi^2 = 0.13, df = 1, p = 0.72$), ethnicity ($\chi^2 = 3.28, df = 1, p = 0.51$), or SES ($\chi^2 = 0.77, df = 1, p = 0.68$). Amongst female participants there was no difference between the propranolol and placebo group in terms of hormonal contraception use ($\chi^2 = 1.12, df = 1, p = 0.29$). The propranolol and placebo groups also did not differ in time of study session (morning versus afternoon) ($\chi^2 = 0.11, df = 1, p = 0.92$), or in the arm used for cannulation (dominant versus non-dominant) ($\chi^2 = 0.25, df = 1, p = 0.62$). Groups also did not differ significantly in baseline depression scores ($F(1, 57) = 0.06, p = 0.81$), anxiety scores ($F(1, 57) = 0.01, p = 0.93$), positive affect ($F(1, 57) = 0.49, p = 0.49$), or perceived stress ($F(1, 57) = 0.17, p = 0.68$).

The escitalopram group did not differ significantly from the placebo group in terms of age ($F(1, 56) = 1.14, p = 0.75$). However, there was a significant interaction between experimental group and sex with respect to age ($F(1, 56) = 5.29, p = 0.03$). There was a significant difference in age between the escitalopram and placebo group in men ($F(1, 21) = 4.94, p = 0.037$) but not in women ($p = 0.16$). Men in the escitalopram group were slightly younger ($M = 20.1$ years, $SD = 1.6$) than men in the placebo group ($M = 22.4$ years, $SD = 3.5$). The escitalopram group did not differ from the placebo group in terms of sex ($\chi^2 = 1.76, df = 1, p = 0.18$), BMI ($F(1, 56) = 1.26, p = 0.74$), smoking status ($\chi^2 = 2.78, df = 1, p = 0.10$), ethnicity ($\chi^2 = 1.03, df = 1, p = 0.91$), or SES ($\chi^2 = 0.32, df = 1, p = 0.85$). Amongst female participants there was no difference in hormonal contraception use ($\chi^2 = 2.65, df = 1, p = 0.10$). The escitalopram and placebo groups also did not differ

in time of study session ($\chi^2 = 0.00$, $df = 1$, $p = 1.00$), or in the arm used for cannulation ($\chi^2 = 0.32$, $df = 1$, $p = 0.57$). Groups also did not differ in baseline depression scores ($F(1, 56) = 6.86$, $p = 0.66$), anxiety scores ($F(1, 56) = 2.86$, $p = 0.63$), positive affect ($F(1, 56) = 15.0$, $p = 0.47$), or perceived stress ($F(1, 56) = 1.19$, $p = 0.28$).

I also investigated the effects of the study medications on changes in stress-related psychological factors on Day 7 of administration. Paired t-tests revealed that there was no significant change in depression scores ($p = 0.76$), anxiety scores ($p = 0.78$), or positive affect ($p = 0.29$) over the seven day study period in the placebo group. There was also no significant change in anxiety scores ($p = 0.57$) or positive affect ($p = 0.22$) in the propranolol group. However, propranolol appeared to bring about a significant decrease in depression scores ($t(30) = 2.62$, $p = 0.014$). In the escitalopram group there were no changes in depression ($p = 0.95$) or anxiety ($p = 0.10$) scores, but there was a significant decrease in positive affect ($t(29) = 2.37$, $p = 0.025$). Exploratory analysis revealed that those who reported experiencing adverse effects within the escitalopram group had lower levels of positive affect ($M = 28.7$, $SD = 8.1$) on Day 7 compared to those who had not reported experiencing adverse effects ($M = 35.4$, $SD = 7.3$) ($t(28) = 2.35$, $p = 0.028$). This suggests that the lowering of positive affect in the escitalopram group was related to experiencing adverse effects.

6.10.2 Subjective stress ratings

In the overall sample, the stress protocol used in the current study brought about a significant increase in subjective stress ratings ($t(90) = -15.8$, $p < 0.001$). Prior to the stress protocol, the mean subjective stress rating of the sample was 1.81 ($SD = 0.99$). This increased to 4.04 ($SD = 0.10$) following acute stress. There was no difference between the propranolol group or the placebo group in terms of subjective stress ratings at rest

Table 6.1. Demographic characteristics of the sample (n = 91)

	Propranolol (n=31)	Escitalopram (n=30)	Placebo (n=30)	Propranolol vs. placebo		Escitalopram vs. placebo	
<i>Characteristic</i>	<i>Mean ± SD or N(%)</i>	<i>Mean ± SD or N(%)</i>	<i>Mean ± SD or N(%)</i>	<i>Group p value</i>	<i>Group*sex p value</i>	<i>Group p value</i>	<i>Group*sex p value</i>
Age (years)	24.5±6.4	22.1±3.9	22.2±3.1	0.183	0.504	0.751	0.025
Female	21(67.7)	16(53.3)	21(70.0)	0.849	-	0.184	-
BMI (kg/m ²)	22.6±2.5	23.3±4.3	23.3±4.1	0.346	0.498	0.795	0.283
Smoker	4(12.9)	8(26.7)	3(10.0)	0.722	-	0.095	-
Ethnicity (White)	21(67.7)	17(56.7)	15(50.0)	0.511	-	0.905	-
SES (n=93)				0.681	-	0.854	-
<i>Low</i>	5(16.1)	3(10.0)	3(10.0)	-	-	-	-
<i>Medium</i>	3(9.7)	1(3.3)	2(6.7)	-	-	-	-
<i>High</i>	23(74.2)	25(83.3)	25(83.3)	-	-	-	-
Hormonal Contraception (n=58)	7(33.3)	7(43.8)	4(19.0)	0.292	-	0.103	-
Depressive symptoms baseline	7.16±6.65	5.60±6.54	6.10±4.99	0.814	0.526	0.658	0.492
Depressive symptoms follow-up	5.13±4.96	5.67±5.19	6.20±6.98	0.385	0.485	0.901	0.573
Anxiety symptoms baseline	5.10±4.20	4.70±2.87	5.40±4.04	0.926	0.689	0.633	0.812
Anxiety symptoms follow-up	4.81±4.17	3.90±2.87	5.60±4.39	0.522	0.979	0.088	0.320
Positive Affect baseline	33.1±6.1	35.5±5.8	35.0±4.9	0.489	0.131	0.470	0.146
Positive affect follow-up	33.9±6.1	32.7±8.2	34.1±6.0	0.712	0.184	0.789	0.063

Table 6.1 continued

	Propranolol (n=31)	Escitalopram (n=30)	Placebo (n=30)	Propranolol vs. placebo		Escitalopram vs. placebo	
<i>Characteristic</i>	<i>Mean ± SD or N(%)</i>	<i>Mean ± SD or N(%)</i>	<i>Mean ± SD or N(%)</i>	<i>Group p value</i>	<i>Group*sex p value</i>	<i>Group p value</i>	<i>Group*sex p value</i>
Perceived stress	14.5±6.9	12.9±5.5	15.1±7.3	0.377	0.719	0.280	0.415

($F(1, 57) = 0.93, p = 0.34$), or following the stress protocol ($F(1, 56) = 0.95, p = 0.34$) (Table 6.2). There was also no difference between the escitalopram group and the placebo group in subjective stress ratings at rest ($F(1, 56) = 0.67, p = 0.42$), or following the stress protocol ($F(1, 56) = 0.04, p = 0.85$) (Table 6.2).

6.10.3 Cardiovascular measures

Overall sample

In the overall sample, repeated measures ANOVA revealed a significant main effect of time on heart rate levels across the testing session ($F(1, 87) = 129.6, p < 0.001$) (see Figure 6.3). Pairwise comparisons revealed that heart rate during stress was significantly higher than baseline heart rate ($p < 0.001$). Heart rate values during stress were also significantly higher than post-stress heart rates at 45 minutes ($p < 0.001$) and 75 minutes ($p < 0.001$).

There was a significant main effect of time on systolic blood pressure (SBP) levels across the testing session in the overall sample ($F(1, 87) = 101.9, p < 0.001$). Pairwise comparisons showed that SBP values during stress were significantly higher than baseline levels ($p < 0.001$), and levels at 45 minutes ($p < 0.001$) and 75 minutes ($p < 0.001$) post-stress.

There was a significant main effect of time on diastolic blood pressure (DBP) levels across the testing session in the overall sample ($F(1, 87) = 146.1, p < 0.001$). DBP values during stress were significantly elevated compared to baseline ($p < 0.001$), and 45 ($p < 0.001$) and 75 minutes ($p < 0.001$) post-stress.

There was a main effect of time on measures of cardiac index across the testing session in the overall sample ($F(1, 87) = 56.8, p < 0.001$). Cardiac index values during stress were

Table 6.2. Subjective stress ratings at baseline, post-stress, and 20 minutes after stress

	Propranolol (n=31)	Escitalopram (n=30)	Placebo (n=30)	Propranolol vs. placebo		Escitalopram vs. placebo	
<i>Characteristic</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Group p value</i>	<i>Group*sex p value</i>	<i>Group p value</i>	<i>Group*sex p value</i>
Baseline subjective stress	1.68±0.80	1.73±1.05	2.03±1.10	0.340	0.319	0.417	0.093
Post-task subjective stress	3.80±0.98	4.19±0.98	4.13±0.98	0.335	0.812	0.846	0.364
Recovery subjective stress	1.65±0.80	1.80±0.99	1.97±0.99	0.321	0.491	0.723	0.153

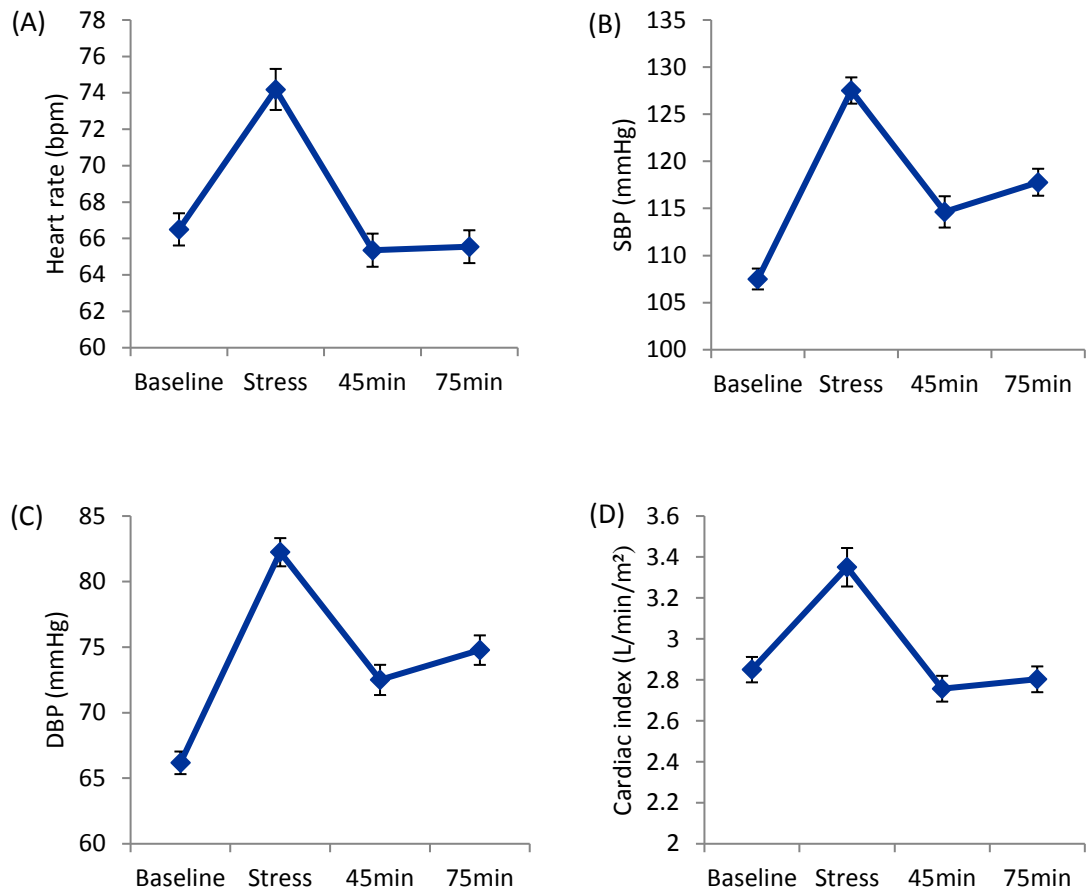


Figure 6.3. Heart rate (A), SBP (B), DBP (C), and cardiac index (D) values across the testing session in the overall sample. Error bars represent SEM.

significantly elevated compared to baseline values ($p < 0.001$), cardiac index values at 45 minutes ($p < 0.001$) and 75 minutes ($p < 0.001$) post-stress.

These cardiovascular results indicate that the tasks did induce substantial blood pressure and heart rate responses in the overall sample. SBP increased around 18%, DBP by 24%, and heart rate by 11% during the stress protocol across experimental conditions. There was complete recovery to baseline levels in heart rate and cardiac index, while SBP and DBP remained somewhat elevated during the post-stress recovery period.

Table 6.3. Mean values on cardiovascular measures in the laboratory in each experimental condition

	Propranolol (n=31)	Escitalopram (n=30)	Placebo (n=30)	Propranolol vs. placebo	Escitalopram vs. placebo		
<i>Cardiovascular Measure</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Group p value</i>	<i>Group*sex p value</i>	<i>Group p value</i>	<i>Group*sex p value</i>
<i>Heart rate baseline (bpm)(n=90)</i>	61.2±6.8	68.7±6.7	70.2±8.7	<0.001*	0.422	0.714	0.726
<i>Mean difference±SE</i>	<i>-9.0±2.0</i>	<i>-1.5±2.0</i>					
<i>Heart rate stress (bpm)(n=90)</i>	66.6±9.1	78.1±8.9	78.3±9.3	<0.001*	0.372	0.817	0.850
<i>Mean difference±SE</i>	<i>-11.7±2.4</i>	<i>-0.2±2.4</i>					
<i>Heart rate 45m post-stress (bpm)(n=89)</i>	60.5±7.4	66.3±6.8	69.3±8.9	0.001*	0.375	0.330	0.506
<i>Mean difference±SE</i>	<i>-8.8±2.1</i>	<i>-3.0±2.1</i>					
<i>Heart rate 75m post-stress (bpm) (n=89)</i>	60.6±7.4	67.4±6.3	69.5±9.1	0.001*	0.198	0.710	0.123
<i>Mean difference±SE</i>	<i>-8.9±2.2</i>	<i>-2.1±2.1</i>					
<i>SBP baseline (mmHg)(n=90)</i>	104.9±9.5	109.6±10.5	108.2±10.8	0.064	0.077	0.909	0.825
<i>Mean difference±SE</i>	<i>-3.3±2.6</i>	<i>1.4±2.8</i>					
<i>SBP stress (mmHg)(n=90)</i>	124.8±11.9	129.9±13.6	127.7±13.7	0.266	0.420	0.776	0.702
<i>Mean difference±SE</i>	<i>-2.9±3.3</i>	<i>2.2±3.6</i>					
<i>SBP 45m post-stress (mmHg)(n=89)</i>	112.2±14.2	119.9±15.9	111.3±15.8	0.690	0.096	0.167	0.351
<i>Mean difference±SE</i>	<i>0.9±3.9</i>	<i>8.6±4.2</i>					
<i>SBP 75m post-stress (mmHg)(n=89)</i>	112.9±13.5	123.1±13.1	117.8±11.8	0.142	0.754	0.194	0.655
<i>Mean difference±SE</i>	<i>-4.9±3.3</i>	<i>5.3±3.3</i>					

bpm = beats per minute; mmHg = millimetre of mercury. Mean difference is calculated by subtracting placebo values from each experimental condition. The SE of the mean difference was calculated as the square root of the sum of the squares of the SE for each group

Table 6.3. continued

<i>Cardiovascular Measure</i>	Propranolol (n=31)	Escitalopram (n=30)	Placebo (n=30)	Propranolol vs. placebo		Escitalopram vs. placebo	
	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Group p value</i>	<i>Group*sex p value</i>	<i>Group p value</i>	<i>Group*sex p value</i>
<i>DBP baseline (mmHg)(n=90)</i>	63.5±8.6	68.5±5.5	66.9±8.9	0.051	0.121	0.661	0.546
<i>Mean difference±SE</i>	-3.4±2.3	1.6±1.9					
<i>DBP stress (mmHg)(n=90)</i>	80.2±9.0	83.6±6.4	82.7±13.6	0.228	0.259	0.973	0.702
<i>Mean difference±SE</i>	-2.5±2.9	0.9±2.8					
<i>DBP 45m post-stress (mmHg)(n=89)</i>	69.6±11.2	76.5±9.2	71.1±11.0	0.253	0.082	0.168	0.332
<i>Mean difference±SE</i>	-1.5±2.9	5.4±2.7					
<i>DBP 75m post-stress (mmHg)(n=89)</i>	70.6±10.3	78.3±7.5	75.7±12.0	0.081	0.675	0.429	0.985
<i>Mean difference±SE</i>	-5.1±2.9	2.6±2.6					
<i>Cardiac index baseline (L/min/m²)(n=90)</i>	2.60±0.48	3.08±0.48	2.94±0.71	0.133	0.170	0.244	0.346
<i>Mean difference±SE</i>	-0.34±0.16	0.14±0.16					
<i>Cardiac index stress (L/min/m²)(n=90)</i>	2.97±0.72	3.78±0.64	3.38±1.06	0.341	0.063	0.027*	0.149
<i>Mean difference±SE</i>	-0.41±0.24	0.4±0.23					
<i>CI 45m post-stress (L/min/m²)(n=89)</i>	2.54±0.47	3.01±0.45	2.76±0.74	0.423	0.221	0.093	0.475
<i>Mean difference±SE</i>	-0.22±0.16	0.25±0.16					
<i>CI 75m post-stress (L/min/m²)(n=89)</i>	2.56±0.49	3.13±0.49	2.78±0.69	0.479	0.094	0.010*	0.068
<i>Mean difference±SE</i>	-0.22±0.16	0.35±0.16					

bpm = beats per minute; mmHg = millimetre of mercury. Mean difference is calculated by subtracting placebo values from each experimental condition. The SE of the mean difference was calculated as the square root of the sum of the squares of the SE for each group

Propranolol versus placebo

There was a significant main effect of experimental condition on heart rate across the testing session ($F(1, 56) = 16.0, p < 0.001$). The propranolol group had significantly lower measures of heart rate at baseline ($F(1, 56) = 14.8, p < 0.001$), during stress ($F(1, 56) = 17.7, p < 0.001$), 45 minutes after stress ($F(1, 56) = 12.7, p = 0.001$), and 75 minutes after stress ($F(1, 56) = 12.0, p = 0.001$), compared with the placebo group (see Figure 6.4). Mean heart rate values for each experimental condition are provided in Table 6.3. There were no significant effects of sex on any of the heart rate measures (all p values > 0.05). This indicates that propranolol was biologically active in these participants as heart rate was significantly reduced in this experimental condition at all time-points.

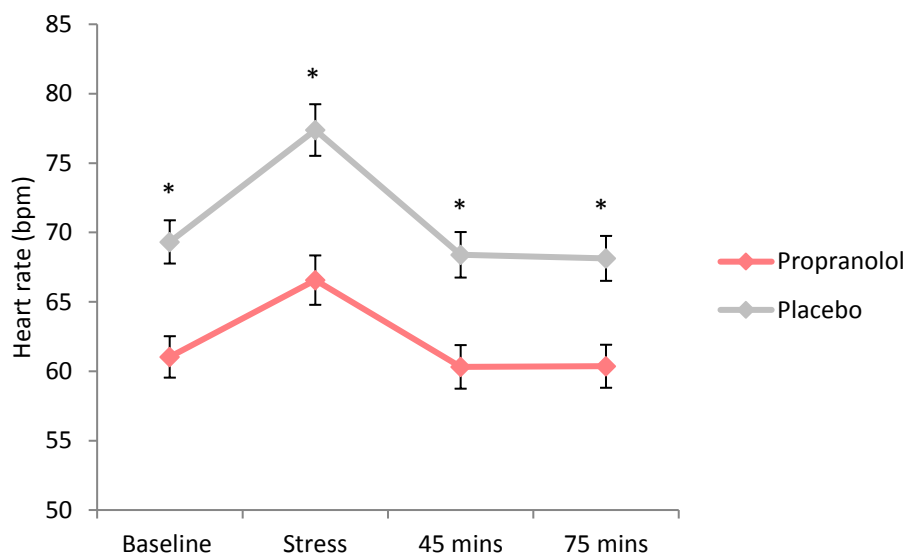


Figure 6.4. Mean heart rate values across the session in the propranolol (pink line) and placebo (grey line) groups. Error bars represent SEM.

The propranolol group and the placebo group did not differ significantly in SBP, DBP, or cardiac index values at any time-point (all p values > 0.05 , see Table 6.3). There was also no main or interactive effect of sex on these cardiovascular parameters at any time-point (all p values > 0.05 , see Table 6.3).

Escitalopram versus placebo

The escitalopram and placebo groups did not differ significantly on any measure of heart rate, SBP, or DBP, throughout the testing session (all p values > 0.05 , see Table 6.3). There was also no main or interactive effect of sex on heart rate, SBP, or DBP values at any time-point (all p values > 0.05). There was no significant difference between experimental conditions on cardiac index at baseline and 45 minutes after stress (all p values > 0.05). However, the escitalopram group had a higher cardiac index during stress compared to those who had taken placebo ($F(1, 55) = 5.13, p = 0.027$) (see Figure 6.5).

This was also the case 75 minutes after stress ($F(1, 54) = 7.22, p = 0.010$). There were no significant main interactive effects of sex on any of the cardiac index measures (all p values > 0.05). Cardiac index is calculated by multiplying heart rate by stroke volume. As cardiac index was elevated during stress and 75 minutes post-stress in the escitalopram condition this indicates that escitalopram increased stroke volume during stress, seeing as heart rate was not affected.

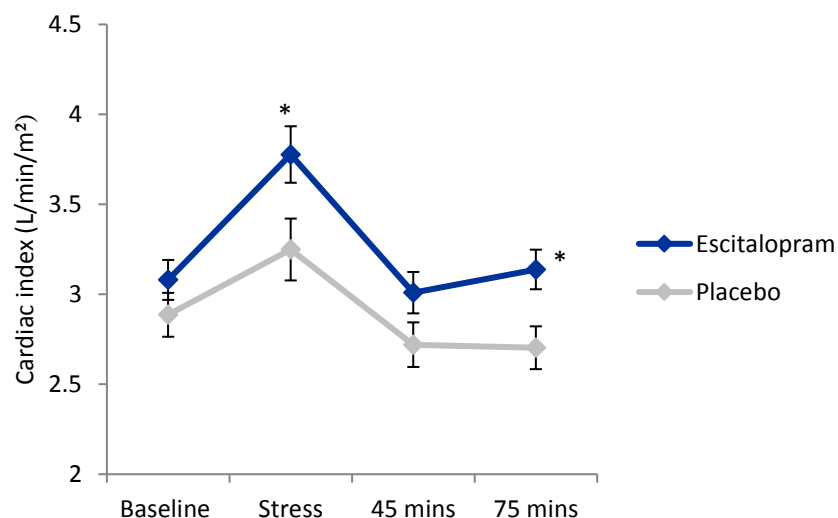


Figure 6.5. Mean cardiac index values across the session in the escitalopram (blue line) and placebo (grey line) groups. Error bars represent SEM.

Taken together, these cardiovascular results indicate that both propranolol and escitalopram were biologically active in these participants as both medications altered heart rate and cardiac index respectively.

6.10.4 Cortisol stress reactivity

Overall sample

In the overall sample, there was a significant linear main effect of time on cortisol levels across the testing session ($F(1, 82) = 84.3, p < 0.001$). Pairwise comparisons revealed that there was a significant difference in cortisol levels between each time-point (p values range from $< 0.001 - 0.027$). However, looking to the mean values indicated that contrary to expectation cortisol levels steadily decreased from baseline values irrespective of the acute stress protocol (see Figure 6.6).

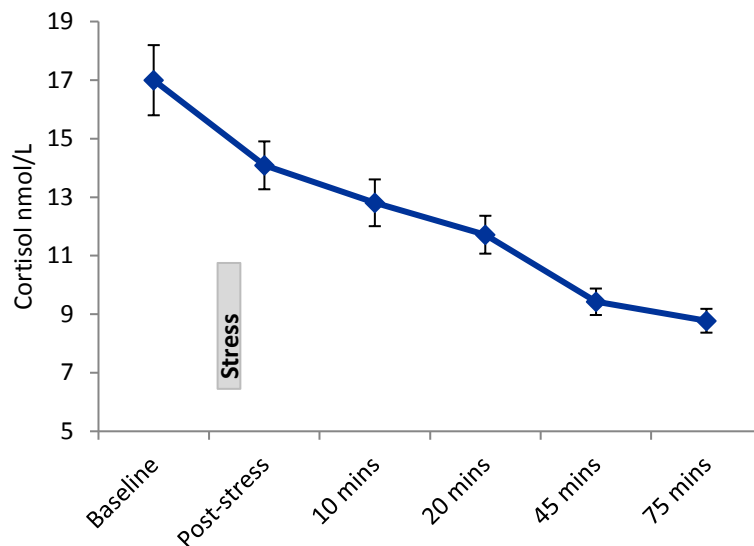


Figure 6.6. Mean cortisol values (not log-transformed) at each time-point across the testing session in the overall sample. Error bars represent SEM.

Table 6.4. Mean (raw) cortisol values across the laboratory session in each experimental condition (*p* values from analyses with log transformed cortisol values).

	Propranolol (n=31)	Escitalopram (n=30)	Placebo (n=30)	Propranolol vs. placebo	Escitalopram vs. placebo		
<i>Cortisol stress reactivity (raw scores)</i>	<i>Mean ± SD or N(%)</i>	<i>Mean ± SD or N(%)</i>	<i>Mean ± SD or N(%)</i>	<i>Group p value</i>	<i>Group*sex p value</i>	<i>Group p value</i>	<i>Group*sex p value</i>
<i>Baseline cortisol (nmol/L)(n=86)</i>	18.0±12.1	17.7±10.9	15.4±10.0	0.255	0.763	0.597	0.279
<i>Mean difference±SE</i>	2.6±2.9	2.3±2.7					
<i>Post-stress cortisol (nmol/L)(n=89)</i>	13.5±7.5	14.9±8.3	13.9±6.7	0.642	0.322	0.630	0.239
<i>Mean difference±SE</i>	-0.4±2.3	1.0±2.0					
<i>Cortisol 10m post-stress (nmol/L)(n=88)</i>	12.4±6.7	12.0±5.6	13.9±9.1	0.845	0.619	0.235	0.433
<i>Mean difference±SE</i>	-1.5±2.3	-1.9±2.1					
<i>Cortisol 20m post-stress (nmol/L)(n=90)</i>	11.4±4.9	11.1±5.7	12.6±7.0	0.540	0.413	0.140	0.399
<i>Mean difference±SE</i>	-1.2±2.31	-1.5±1.84					
<i>Cortisol 45m post-stress (nmol/L)(n=90)</i>	9.63±3.52	9.27±4.59	9.38±4.31	0.214	0.319	0.642	0.756
<i>Mean difference±SE</i>	0.25±1.60	-0.11±1.46					
<i>Cortisol 75m post-stress (nmol/L)(n=87)</i>	8.87±2.96	8.68±4.21	8.78±3.95	0.194	0.578	0.990	0.704
<i>Mean difference±SE</i>	0.09±1.05	-0.10±1.09					
<i>Cortisol AUC (nmol/L)(n=85)</i>	833.7±342.4	774.3±367.1	818.2±384.6	0.798	0.871	0.479	0.293
<i>Mean difference±SE</i>	15.5±95.6	-43.9±100.5					
<i>Cortisol responders (n=86)</i>	4(12.9)	7(23.3)	10(33.3)	0.084 [†]	-	0.359 [†]	-

nmol = nanomole. Mean difference is calculated by subtracting placebo values from each experimental condition. The SE of the mean difference was calculated as the square root of the sum of the squares of the SE for each group.

[†]*p* value for logistic regression

Propranolol versus placebo

Repeated measures ANOVA revealed no significant main effect of drug ($p = 0.98$) or time x drug interaction ($p = 0.21$) on cortisol stress reactivity. The propranolol and placebo groups did not differ significantly on baseline levels of cortisol, post-stress levels of cortisol, or levels of cortisol at 10, 20, 45, and 75 minutes after stress (all p values > 0.05 , see Table 6.4). Additionally, there was no difference between groups on overall cortisol output in the laboratory (AUC) ($p = 0.80$). There was no interactive effect of sex on cortisol levels across the session (all p values > 0.05). Logistic regression was used to test associations between drug type and whether participants were cortisol responders or non-responders. There was no association between experimental condition and cortisol response ($p = 0.08$).

Escitalopram versus placebo

Repeated measures ANOVA revealed a significant time x drug quadratic effect ($F(1, 52) = 4.49, p = 0.039$) which indicates that the slope of change in cortisol across the testing session differed across experimental conditions (see Figure 6.7). Paired t-tests were used to explore this effect further. In the escitalopram group, baseline cortisol values were significantly higher than values during stress ($p = 0.001$), and at 10 ($p < 0.001$), 20 ($p < 0.001$), 45 ($p < 0.001$), and 75 minutes ($p < 0.001$) post-stress. In the placebo group, baseline cortisol values did not differ significantly from values during stress ($p = 0.90$), values 10 minutes ($p = 0.59$), or values 20 minutes post-stress ($p = 0.23$). Cortisol values at 45 ($p < 0.001$) and 75 minutes ($p = 0.001$) post-stress were significantly lower than baseline values in the placebo group. In the escitalopram group, cortisol values during stress were significantly higher than values at 10 ($p = 0.004$), 20 ($p < 0.001$), 45 ($p < 0.001$), and 75 ($p < 0.001$) minutes post-stress. In the placebo group, cortisol values during

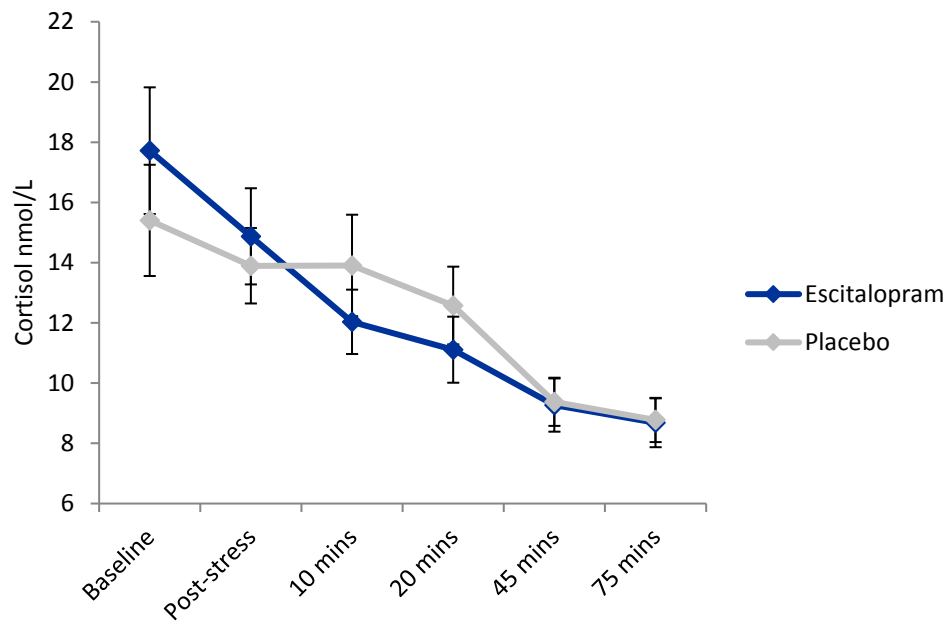
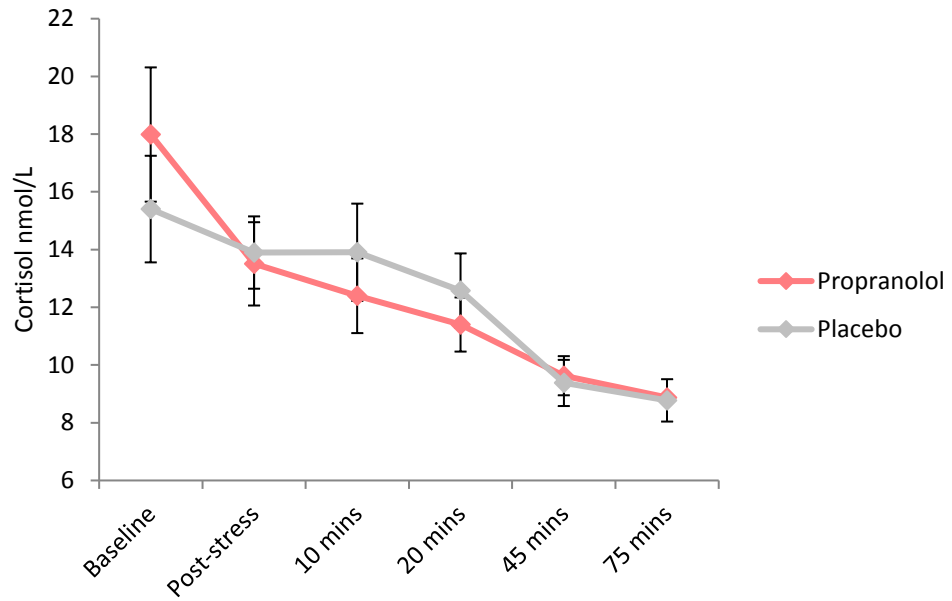


Figure 6.7. Mean cortisol values (not log-transformed) at each time-point across the testing session in the propranolol (pink line), escitalopram (blue line), and placebo (grey line) groups. Error bars represent SEM.

stress did not differ from values 10 minutes post-stress ($p = 0.37$). Values at 20 ($p = 0.012$), 45 ($p < 0.001$), and 75 minutes ($p < 0.001$) were significantly lower than stress values in the placebo group. What these results show is that decreases in cortisol values

across the testing session were more pronounced in the escitalopram group indicating a blunting of the cortisol stress response in this group.

In sum, although the stress tasks did not elicit an increase in cortisol in any of the experimental conditions, a steeper slope of decline was present in those taking escitalopram. Propranolol did not significantly affect cortisol values across the testing session.

6.10.5 Corticosteroid receptor sensitivity

Overall sample

In the overall sample, there was a significant main effect of time on LPS-stimulated IL-6 release across the testing session ($F(1, 77) = 20.2, p < 0.001$) (see Table 6.5 for LPS-stimulated IL-6 values). Pairwise comparisons revealed that IL-6 release post-stress, and at 45 and 75 minutes after stress was significantly higher than baseline IL-6 release (all p values < 0.001). This indicates that the stress protocol brought about changes in immune function. There was also a significant main effect of time on dexamethasone suppression of LPS-induced IL-6 release in the overall sample ($F(1, 77) = 6.82, p = 0.001$). Compared to baseline, GR sensitivity significantly decreased immediately following stress ($p < 0.001$), at 45 minutes post-stress ($p < 0.001$), and at 75 minutes post-stress ($p = 0.003$). There was a significant main effect of time on prednisolone suppression of LPS-induced IL-6 release ($F(1, 77) = 13.3, p < 0.001$). Results indicate that MR sensitivity also decreased significantly immediately post-stress ($p = 0.011$), at 45 minutes ($p = 0.001$), and at 75 minutes post stress ($p < 0.001$).

Together, these results show that the stress protocol brought about changes in immune function, and GR and MR sensitivity in the overall sample.

Placebo group

As mentioned in Chapter 2, one of the main aims of this study was to examine the effects of acute psychosocial stress on both GR and MR function in healthy medication-free volunteers. Within the placebo group, repeated measures ANOVA showed a significant quadratic effect of time on dexamethasone IC₅₀ values across the testing session ($F(1, 22) = 5.37, p = 0.030$). Individual pairwise comparisons revealed that compared to baseline, GR sensitivity decreased immediately post-stress ($p = 0.007$), and remained decreased at 45 minutes post-stress ($p = 0.045$), but had returned towards baseline levels by 75 minutes ($p = 0.54$ in comparison with baseline) post-stress (see Figure 6.8).

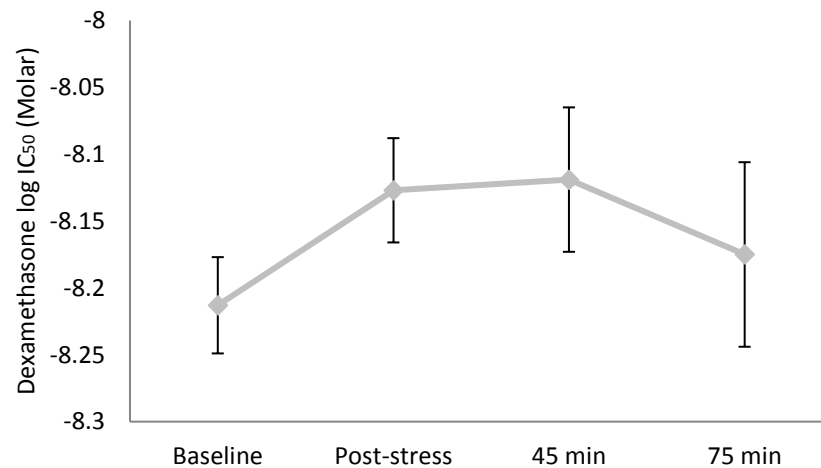


Figure 6.8. Mean log IC₅₀ values for dexamethasone in the placebo group. Error bars represent SEM.

There was also a significant linear effect of time on prednisolone IC₅₀ values across the testing session ($F(1, 22) = 5.06, p = 0.035$) (see Figure 6.9). As can be seen, receptor sensitivity decreased across the session, with individual pairwise comparisons showing a significant difference between prednisolone IC₅₀ values at baseline and at 75 minutes after stress ($p = 0.010$).

There was no main or interactive effect of sex on dexamethasone or prednisolone IC₅₀ values across the testing session in the placebo group.

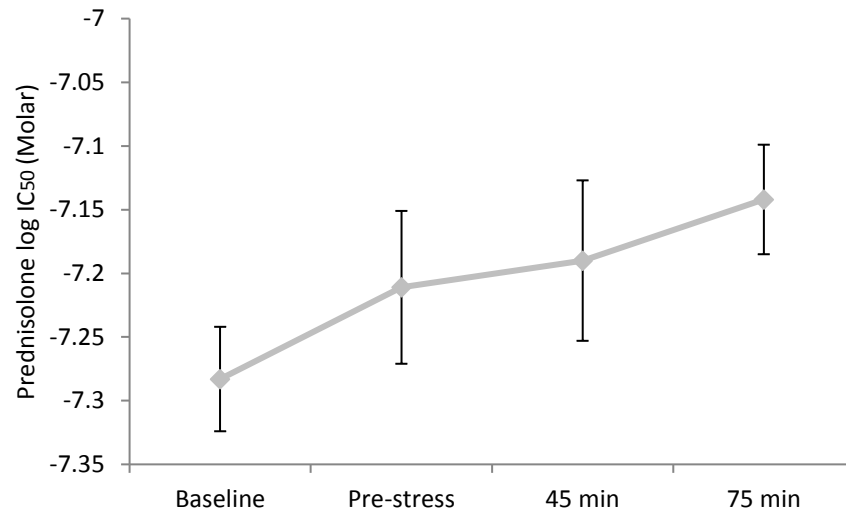


Figure 6.9. Mean log IC₅₀ values for prednisolone in the placebo group. Error bars represent SEM.

In sum, within unmedicated healthy volunteers, acute stress brought about a transient decrease in GR sensitivity that had normalised to baseline levels by 75 minutes post-stress. Acute stress also brought about a decrease in MR sensitivity that was more sustained and most pronounced at 75 minutes post-stress.

Propranolol versus placebo

Repeated measures ANOVA revealed that the propranolol and placebo conditions did not differ significantly on measures of LPS induced IL-6 production across the testing session ($p = 0.25$). Similarly, the propranolol and the placebo group did not differ significantly on measures of GR sensitivity (dexamethasone IC₅₀) across the testing session ($p = 0.35$) (see Figure 6.10). Although the groups did not differ significantly, there appears to be a different pattern of change in GR sensitivity in each group (see Figure 6.10). This is supported by the significant linear effect of time on dexamethasone IC₅₀ values across the testing session in the propranolol group ($F(1, 27) = 4.61, p = 0.041$) in contrast with the

Table 6.5. Raw IL-6 values (LPS) and mean log IC50 values (dexamethasone and prednisolone) across the laboratory session in each experimental condition

	Propranolol (n=31)	Escitalopram (n=30)	Placebo (n=30)	Propranolol vs. placebo		Escitalopram vs. placebo	
<i>Corticosteroid receptor sensitivity</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	<i>Group p value</i>	<i>Group*sex p value</i>	<i>Group p value</i>	<i>Group*sex p value</i>
LPS only							
<i>Baseline (ng/ml)(n=85)</i>	1710.3±918.5	1820.3±1291.8	1581.5±616.5	0.761	0.537	0.531	0.674
<i>Post-stress(ng/ml)(n=80)</i>	2040.0±1029.1	2044.2±1102.4	1956.3±832.6	0.997	0.411	0.964	0.926
<i>45min (ng/ml)(n=84)</i>	2018.1±994.5	2122.7±1289.6	1871.3±821.6	0.743	0.605	0.549	0.694
<i>75min (ng/ml (n=79)</i>	2074.9±1106.3	2024.3±1268.2	1810.4±782.0	0.494	0.490	0.667	0.819
Dexamethasone							
<i>Baseline log IC₅₀ (M)(n=85)</i>	-8.17±0.26	-8.15±0.19	-8.18±0.17	0.435	0.112	0.284	0.250
<i>Post-stress log IC₅₀ (M)(n=80)</i>	-8.12±0.25	-8.08±0.20	-8.11±0.18	0.960	0.761	0.484	0.576
<i>45min log IC₅₀ (M)(n=84)</i>	-8.07±0.23	-8.04±0.18	-8.08±0.25	0.698	0.780	0.282	0.080
<i>75min log IC₅₀ (M)(n=79)</i>	-8.08±0.24	-7.98±0.35	-8.19±0.31	0.189	0.932	0.058	0.758
Prednisolone							
<i>Baseline log IC₅₀ (M)(n=85)</i>	-7.26±0.25	-7.29±0.18	-7.24±0.23	0.745	0.178	0.747	0.296
<i>Post-stress log IC₅₀ (M)(n=80)</i>	-7.22±0.27	-7.24±0.21	-7.19±0.28	0.639	0.969	0.642	0.696
<i>45min log IC₅₀ (M)(n=84)</i>	-7.18±0.27	-7.21±0.24	-7.16±0.28	0.895	0.994	0.686	0.307
<i>75min log IC₅₀ (M)(n=79)</i>	-7.15±0.27	-7.07±0.32	-7.16±0.20	0.966	0.850	0.367	0.554

IC₅₀ = inhibitory concentration 50%; ng/ml = nanogram/milliliter; M = molar concentration

significant quadratic effect of time on dexamethasone IC₅₀ values across the testing session in the placebo group ($F(1, 22) = 5.37, p = 0.030$).

The propranolol and placebo group did not differ significantly in MR sensitivity (prednisolone IC₅₀) across the testing session ($p = 0.81$) (see Figure 6.11). There was no main or interactive effect of sex on GR or MR sensitivity (all p values > 0.05).

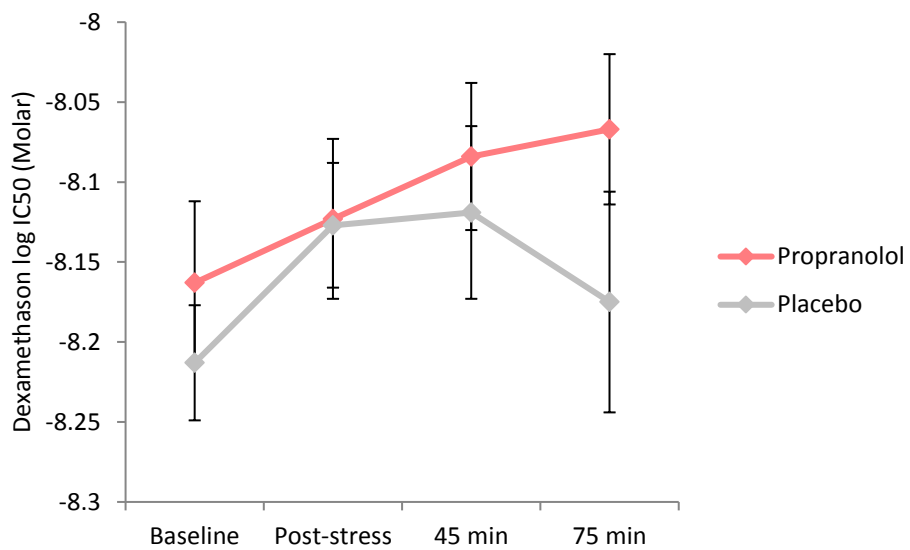


Figure 6.10. Mean dexamethasone log IC₅₀ values in the propranolol (pink line) and placebo (grey line) groups. Error bars represent SEM.

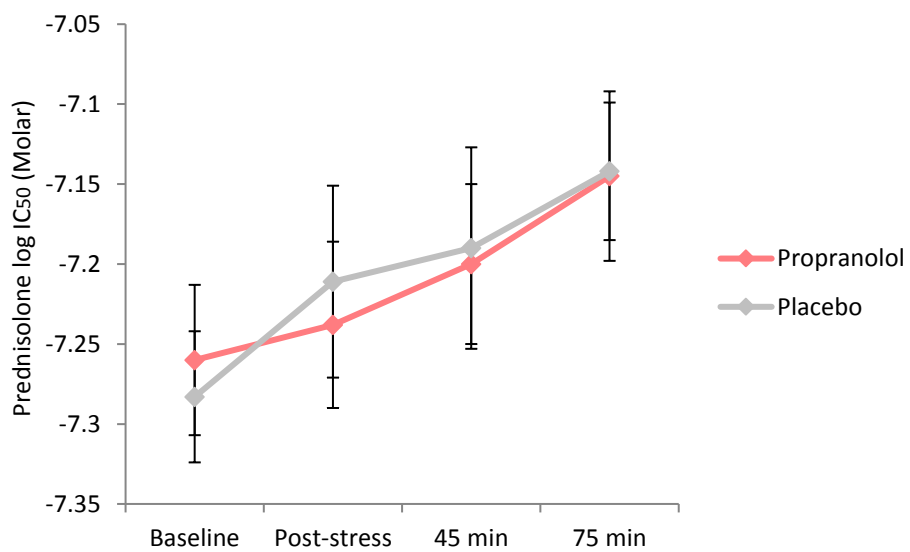


Figure 6.11. Mean prednisolone log IC₅₀ values in the propranolol (pink line) and placebo (grey line) groups. Error bars represent SEM.

Over all time points, GR sensitivity was lower in the escitalopram group. Additionally, the escitalopram group exhibited a marked decrease in GR sensitivity 75 minutes post-stress, whereas the receptor sensitivity of the placebo group returned towards baseline.

Escitalopram versus placebo

The escitalopram and placebo conditions did not differ significantly on measures of LPS induced IL-6 production across the testing session ($p = 0.40$). There was a main effect of drug on GR sensitivity across the testing session ($F(1, 45) = 4.18, p = 0.048$) indicating that overall the groups differed in GR sensitivity across the testing session (mean values provided in Table 6.5, see Figure 6.12). Compared with placebo, GR sensitivity was reduced in the escitalopram group. Moreover, at 75 minutes post-stress there was a marked decrease in GR sensitivity in the escitalopram group, whereas GR sensitivity returned towards baseline levels in the placebo group. This difference reached borderline significance ($F(1, 46) = 3.79, p = 0.058$). In order to further explore this difference I created change scores by subtracting baseline log IC₅₀ values from log IC₅₀ values at 75 minutes. An independent samples t-test revealed a significant difference between conditions ($t(48) = -2.02, p = 0.049$) indicating a more pronounced change in the escitalopram group ($M = 0.17, SD = 0.29$) compared with the placebo group ($M = 0.01, SD = 0.29$). The escitalopram and placebo conditions did not differ significantly on MR sensitivity (prednisolone IC₅₀) across the testing session ($p = 0.83$) (see Figure 6.13); instead, both groups showed a progressive reduction in MR sensitivity across samples. There was no main or interactive effect of sex on GR or MR sensitivity (all p values > 0.05).

To summarise, these results show that propranolol did not bring about any significant changes in stress-related GR and MR sensitivity compared with placebo. Although not

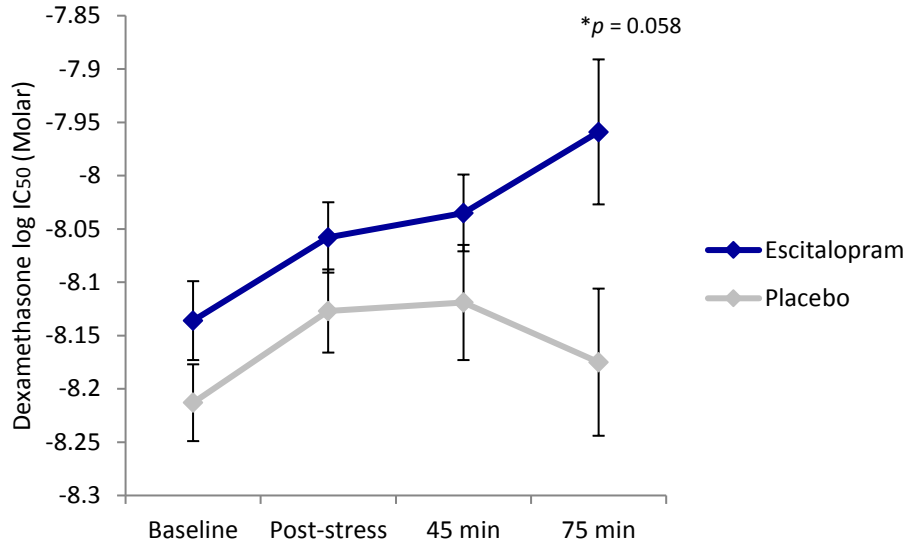


Figure 6.12. Mean dexamethasone log IC₅₀ values in the escitalopram (blue line) and placebo (grey line) groups. Error bars represent SEM.

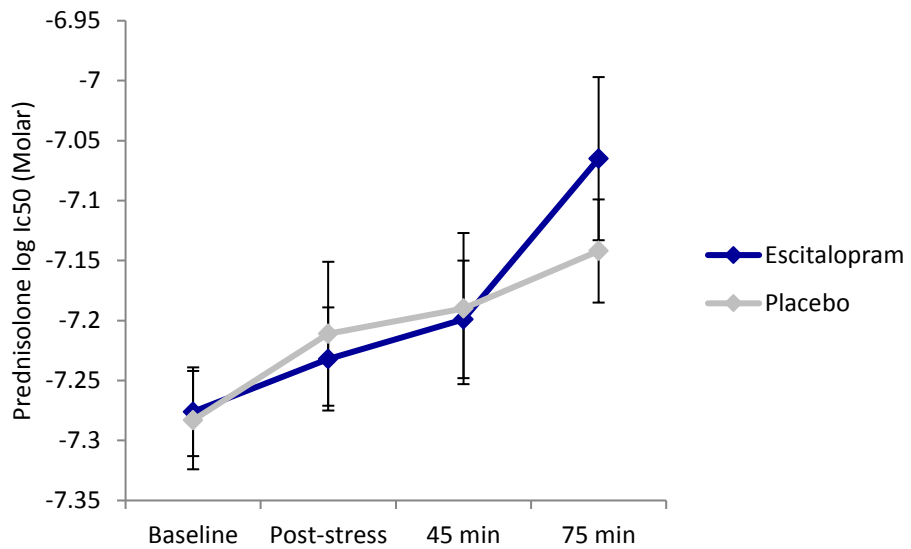


Figure 6.13. Mean prednisolone log IC₅₀ values in the escitalopram (blue line) and placebo (grey line) groups. Error bars represent SEM.

statistically significant, examining Figure 6.10 indicates that the propranolol group experienced a linear decrease in GR sensitivity after stress compared to the quadratic pattern seen in the placebo group. Compared with the placebo group, escitalopram brought about significant changes in GR sensitivity that were observed throughout the

session. Over all time points, GR sensitivity was lower in the escitalopram group. Additionally, the escitalopram group exhibited a marked decrease in GR sensitivity 75 minutes post-stress, whereas the receptor sensitivity of the placebo group returned towards baseline.

6.10.6 Sensitivity Analyses

Counter to expectation, cortisol values exhibited a distinct decline across the testing session, without any experimental condition exhibiting a cortisol stress response. Therefore, I ran some exploratory analysis examining factors that might explain the unusually high baseline cortisol values in participants. In the current study pre-stress cortisol values were measured from saliva collected 25 minutes after cannulation. Therefore, it is possible that the number of cannulation attempts was associated with baseline cortisol levels. However, there was no significant correlation between these factors ($r = 0.47, p = 0.08$). Nevertheless, since the correlation was quite high, I created a binary variable in order to compare participants who experienced one cannulation attempt with those who experienced two or three attempts. An independent t-test revealed no significant difference between groups ($p = 0.51$).

Cortisol levels at baseline may also have been elevated due to anticipatory stress. Therefore, I examined associations between pre-stress subjective stress and anxiety levels. There was no association between baseline cortisol levels and responses to the item '*How stressed do you feel?*' ($p = 0.63$). However, there was significant negative correlation between baseline cortisol levels and responses to the item '*How relaxed do you feel?*' ($r = -0.32, p = 0.003$). There was also a significant correlation between pre-stress cortisol values and baseline scores on the HADS anxiety subscale ($r = 0.28, p = 0.009$).

6.11 Discussion

6.11.1 Aims and hypotheses

The aim of this study was to assess the effects of seven-day administration of beta-blockers and SSRIs on cortisol secretion in response to acute psychosocial stress in the laboratory in healthy volunteers. This study also aimed to assess the effects of these drugs on baseline corticosteroid receptor sensitivity and corticosteroid receptor responses to acute psychosocial stress. I also sought to examine the effects of acute psychosocial stress on both GR and MR sensitivity in unmedicated participants.

In terms of cortisol stress reactivity, I hypothesised that both seven-day treatment with propranolol and escitalopram would enhance cortisol stress reactivity and that sex would be an influential factor on the effects of the medications. In the placebo group, I hypothesised that acute stress would lead to a decrease in both GR and MR sensitivity. Moreover, I hypothesised that both propranolol and escitalopram would enhance these changes in GR and MR sensitivity in response to acute stress. Regarding baseline GR and MR sensitivity, I hypothesised that both propranolol and escitalopram would increase sensitivity. I also posited that sex would be an influential factor on the stress and medication effects on receptor sensitivity. The results of this study provided limited support for these hypotheses.

6.11.2 Summary of results

Following the acute stress protocol there were significant increases in subjective stress ratings in the overall sample. During the stress tasks there were also significant increases in cardiovascular responses. Heart rate, SBP, DBP, and cardiac index all increased in response to the stress tasks in the overall sample. What this indicates is that the stress protocol used in the current study was successful in eliciting a subjective and a biological

stress response. Moreover, propranolol was found to decrease baseline heart rate and stress-related heart rate compared to placebo indicating that beta-blockade had been successfully achieved. Similarly, escitalopram also exerted effects on cardiac index during and at 75 minutes after stress indicating that this SSRI was also biologically active. Propranolol brought about significant decreases in depression scores and escitalopram brought about significant decreases in positive affect. Exploratory analysis revealed that the decrease in positive affect was likely due to experiencing adverse effects over the seven days.

In terms of cortisol stress reactivity, in the overall sample there was a significant effect of stress on cortisol values over the testing session. However, counter to expectations, cortisol values declined steadily throughout the session. I will discuss possible explanations for this pattern of results later in the Discussion section. Propranolol did not have an effect on cortisol stress reactivity. In the escitalopram group there was a steeper slope of change over the testing session, which is in disagreement with the study hypothesis. Instead of the interrupted decrease in cortisol across the session seen in the placebo group in response to tasks (Figure 6.7), the escitalopram group showed a continuous decline over time.

In the overall sample LPS-induced IL-6 release was increased following the stress protocol. This suggests that the stress protocol led to an increase in immune activity. The stress protocol also elicited changes in GR and MR sensitivity in the overall sample. Within the placebo group, I hypothesised that acute stress would bring about decreases in both GR and MR sensitivity. In support of this hypothesis, there was a transient decrease in GR sensitivity after stress that had normalised to baseline values by 75 minutes post-stress. Acute stress brought about a linear decrease in MR sensitivity in unmedicated healthy volunteers that was sustained at 75 minutes post-stress.

There was no effect of propranolol on baseline or stress-related changes in GR and MR sensitivity. Compared to the placebo group, escitalopram brought about significant changes in GR sensitivity that were observed throughout the session. Over all time points, GR sensitivity was lower in the escitalopram group. Additionally, 75 minutes after the stress protocol, the escitalopram group exhibited a marked decrease in GR sensitivity whereas GR sensitivity in the placebo group had returned towards baseline. I hypothesised that SSRI administration would result in enhanced changes in GR sensitivity in response to acute stress. This prolonged decrease in sensitivity suggests that escitalopram is serving to enhance the GR decrease in response to stress thereby providing support for this hypothesis. There was no effect of escitalopram on MR sensitivity at 75 minutes post-stress.

6.11.3 Stress-related changes in cardiovascular measures

In the current study propranolol decreased heart rate at all time-points and did not affect blood pressure or cardiac index. These findings are in line with the results of a review of 59 studies which summarised that beta-blockade significantly reduces heart rate, but not blood pressure following acute psychosocial stress (Mills & Dimsdale, 1991). These results are also similar to those of von Känel and colleagues who found that five-day administration of propranolol (80mg/day) to healthy volunteers also did not bring about alterations in blood pressure after an acute stress paradigm (von Känel, Kudielka, Helfricht, et al., 2008).

I also found that the escitalopram group had increased cardiac index immediately following stress, and at 75 minutes after stress, compared to the placebo group. To reiterate, cardiac index is a measure of cardiac output that takes the body surface area into account thus relating cardiovascular performance to the size of the person. Cardiac output

is calculated by multiplying heart rate by stroke volume. As heart rate was unaffected by escitalopram in the current study, the changes in cardiac output were likely driven by alterations in stroke volume. SSRI use within the Multi-Ethnic Study of Atherosclerosis has been associated with increased right ventricular stroke volume in those free of CVD (Ventetuolo et al., 2012). However, Straneva-Meuse and colleagues found that depressed patients taking the SSRI paroxetine had reduced cardiac output after acute stress compared to healthy controls (Straneva-Meuse et al., 2004). This implies that health status plays a role in SSRI effects on cardiac output.

6.11.4 Cortisol stress reactivity

In the current study, propranolol had no effect on cortisol stress reactivity. This is in line with the results of Kudielka and colleagues who found that five-day treatment with propranolol (80mg/day) had no effect on pre-stress cortisol levels or cortisol stress reactivity in healthy men and women (Kudielka et al., 2007). Null cortisol findings in 20 healthy men undergoing a bungee jump who had received three-day administration of propranolol (40mg/day) also corroborate the findings of the current study (van Westerloo et al., 2011). Similarly, in agreement with our findings, acute doses of propranolol have been found to have no effect on cortisol stress reactivity (Benschop et al., 1996; Dreifus et al., 2014; Uusitupa et al., 1982). Taken together, our results and the results of these previous studies do not provide support for the supposed inverse association between the HPA axis and the SAM system. In the Introduction of this chapter (*Section 6.2.3*) I highlight the fact that acute beta-blockade only affects cortisol stress reactivity in all-male samples, and that this might be the reason for the null findings reported by Kudielka and colleagues (2007) who used a sample comprised of men and women. In the current study sex did not influence propranolol effects on cortisol stress reactivity providing no

evidence for the role of sex in the interaction between beta-adrenergic receptors and cortisol stress reactivity.

Escitalopram resulted in a steeper slope of change across the session compared with placebo. This finding is in disagreement with previous studies that have found that longer-term SSRI administration increases cortisol stress reactivity in healthy volunteers (Kotlyar et al., 2013; Ljung et al., 2001). However, the results of the current study are in line with those of Straneva-Meuse and colleagues (2004) who found that depressed patients receiving SSRIs had blunted cortisol stress reactivity compared to controls. This was unexpected considering the sample in the current study comprised healthy volunteers. The results of the current study indicate that seven-day administration of SSRIs results in down-regulation of stress-related HPA axis function. In the studies cited above using healthy volunteers, SSRIs were administered for one month (Kotlyar et al., 2013) and six months (Ljung et al., 2001). Perhaps in the current study seven-day treatment brought about a transient blunting of the cortisol stress response that would correct itself with longer-term administration.

6.11.5 Stress-related changes in corticosteroid receptor sensitivity in healthy unmedicated volunteers

In healthy unmedicated volunteers acute stress brought about a significant decrease in GR sensitivity immediately after stress, and at 45 minutes after stress, with GR sensitivity returning to baseline levels by 75 minutes. A number of previous studies have found that acute stress brings about decreases in GR sensitivity in both murine and human models. Acute SRO exposure in mice has been found to bring about reduced GR sensitivity (Sheridan et al., 2000; Stark et al., 2001). SRO exposure has also reduced GR mRNA expression in mice (Quan et al., 2001). In humans, acute exercise paradigms have also

been found to bring about transient reductions in GR sensitivity (DeRijk et al., 1996; Smits et al., 1998). Similar to this study, acute psychosocial stress in the laboratory has brought about transient decreases in GR sensitivity in young women (Rohleder et al., 2001), healthy older men (Rohleder et al., 2002), and healthy older men and women (Carvalho et al., 2015). However, acute stress in the laboratory has been found to bring about increases in GR sensitivity in healthy young men (Rohleder et al., 2001), healthy older men who had received a testosterone injection (Rohleder et al., 2002), and healthy young women on the oral contraceptive pill (Rohleder et al., 2003).

In the placebo group, acute stress also brought about a decrease in MR sensitivity which became significant at 75 minutes post-stress. Decreased MR sensitivity following acute stress has been reported in healthy older adults (Carvalho et al., 2015). However, in Carvalho and colleague's sample, this decrease was transient and MR sensitivity had returned to baseline levels by 75 minutes post-stress.

To reiterate, in the current study I found that GR sensitivity transiently decreased following acute stress in the placebo group, but returned to normal baseline levels by 75 minutes post-stress. MR sensitivity also decreased following acute stress in this group and this linear decrease became even more pronounced at 75 minutes post-stress.

It is plausible that this transient decrease in GR sensitivity after stress may serve to temporarily prevent cortisol from exerting its anti-inflammatory effects. This would allow the immune system to mount its inflammatory response to stress. This adaptive inflammatory immune response serves to protect against injury and infection potentially brought about by the 'stressor' (Segerstrom & Miller, 2004). Seventy-five minutes after stress, GR sensitivity returned to pre-stress levels. Restored GR sensitivity might then allow cortisol to exert its regulatory function, shutting down the inflammatory response

in order to prevent tissue damage. Furthermore, cortisol itself is known to downregulate corticosteroid receptor function (Bamberger et al., 1996). Therefore, this stress-induced decrease in GR sensitivity might be an adaptive function preventing tissue damage from overexposure to cortisol, which may over time become maladaptive. However, the results of the current study provide little evidence for this hypothesis seeing as the transient decrease in GR sensitivity occurred alongside a steady decrease in cortisol levels.

This is the first study to report a linear decrease in MR sensitivity following acute stress. The decrease in MR sensitivity was most pronounced at 75 minutes post-stress. By this time GR sensitivity had returned to pre-stress levels. As mentioned previously, the GR and the MR both work in concert to regulate the cortisol and inflammatory stress response. It is possible that the prolonged decrease in MR sensitivity at 75 minutes facilitated the return of GR sensitivity to baseline levels. More research is needed to confirm this.

6.11.6 Stress-related changes in corticosteroid receptor sensitivity in those receiving propranolol

Propranolol had no effects on baseline corticosteroid receptor sensitivity or stress-related changes in sensitivity. This finding is at odds with those of Ji and colleagues who found that treatment with metoprolol increased GR protein levels in CHD patients (Ji et al., 2010). However, these findings are not easily comparable as GR protein levels provide a measure of the number of GR whereas the *in vitro* glucocorticoid sensitivity assay carried out in the current study provides a measure of GR function. Furthermore, Ji and colleagues (2010) examined effects in a CHD patient sample and therefore the changes in GR protein levels may have been related to symptom remission. The null findings in the current study are in line with a murine study which found that propranolol had no

effect on GR mRNA levels in rat hippocampal cells (Lai et al., 2003). Together, the lack of effect of beta-blockade on cortisol stress reactivity and corticosteroid receptor sensitivity in the present study provide no support for the notion that there is an inverse relationship between the SAM system and the HPA axis, i.e. blocking the beta-adrenergic receptors does not affect cortisol stress responsivity via modulation of the corticosteroid receptors. There was no effect of propranolol on baseline or stress-related changes in GR and MR sensitivity.

6.11.7 Stress-related changes in corticosteroid receptor sensitivity in those receiving escitalopram

Escitalopram brought about significant changes in GR sensitivity that were observed throughout the session. Compared with placebo, GR sensitivity was lower in the escitalopram group over all time points. Furthermore, 75 minutes after acute stress, the escitalopram group exhibited a prolonged marked decrease in GR sensitivity compared to the placebo group where GR sensitivity had returned towards baseline levels. To date, no one has examined the effects of SSRI treatment on corticosteroid receptor responses to stress. However, this finding is in line with previous studies which have found that SSRI administration brings about decreased receptor sensitivity. The only previous study to also measure GR sensitivity using LPS-stimulation of IL-6 production in whole blood found that 24-hour treatment with various SSRIs brought about a reduction in GR sensitivity in healthy volunteers (Carvalho et al., 2010). In healthy adults six-week SSRI treatment has been found impair feedback inhibition of the HPA axis, which is considered a proxy for GR sensitivity (Carpenter et al., 2011). This is in agreement with the findings of the current study where the overall and prolonged decrease in GR sensitivity indicates a protracted reduction in stress-related HPA axis feedback in those taking SSRIs. Also in line with the findings of the current study were results reported by Sarubin and colleagues

(2014) who found that 10mg escitalopram per day for one week brought about a transient decrease in glucocorticoid sensitivity in depressed patients, implying a decrease in GR sensitivity.

Seven day administration of escitalopram altered both stress-related cortisol secretion and corticosteroid receptor sensitivity. Compared to placebo, the slope of decline in the escitalopram group was steeper. Due to the linear decrease in cortisol secretion seen in all experimental conditions over the testing, this steepening is difficult to interpret. However, this pattern of cortisol stress reactivity in the escitalopram group suggests a blunting of the cortisol stress response. Escitalopram administration also resulted in an enhanced and prolonged decrease in GR sensitivity following acute stress compared to placebo. This decrease could be considered to be an enhancement of stress-related changes in GR function brought about by SSRI use. As well as influencing the onset of the cortisol stress response, the GR is also responsible for the magnitude of the cortisol stress response (de Kloet, 1998). Therefore, this decrease in GR sensitivity might be accountable for the blunting of the cortisol stress response in the escitalopram group.

A possible mechanism through which SSRIs may have decreased stress-related GR sensitivity is through inhibition of the nuclear transcription factor NF- κ B. NF- κ B is an important transcription factor that plays a crucial role in mediating the production of pro-inflammatory cytokines such as IL-6 and TNF- α (McKay & Cidlowski, 1999). Stress activates NF- κ B, which translocates to the nucleus where it binds to its response elements leading to the stress-related production of pro-inflammatory cytokines (Pace et al., 2007). Acute psychosocial stress in the laboratory has been found to increase levels of NF- κ B in healthy volunteers (Bierhaus et al., 2003).

NF- κ B is known to interact with the GR (McKay & Cidlowski, 1999) and has been shown to inhibit GR function (Pace et al., 2007). This increase in NF- κ B after stress and the subsequent inhibition of GR function likely facilitates the mounting of the inflammatory stress response. In this sense, stress-related increases in NF- κ B might be seen as adaptive. However, over time, prolonged exposure to stress might lead to sustained increases in both NF- κ B and inflammation. In fact, high levels of NF- κ B have been found in stress-related diseases such as diabetes and depression (Bierhaus et al., 2001; Miklowitz et al., 2016), and there is evidence to suggest a role for NF- κ B in inflammatory heart disease (Bangert et al., 2016).

SSRIs have been found to reduce both NF- κ B activity (Daniele, Da Pozzo, Zappelli, & Martini, 2015; Roumestan et al., 2007) and levels of inflammatory cytokines (Strawbridge et al., 2015). In the current study it is possible that administration of escitalopram resulted in a reduction in the NF- κ B and inflammatory stress response leading to enhanced GR function, i.e. an enhanced and prolonged decrease in GR sensitivity in response to stress. NF- κ B activity was not measured in this study so this suggestion is speculative. Future work is needed to examine the simultaneous effects of SSRIs on stress-related NF- κ B activity and GR function.

One of the mechanisms that might explain the prolonged decrease in GR function in the escitalopram group is increased nuclear translocation of the GR at 75 minutes post-stress. In line with this timing, GR nuclear translocation rates have been shown to peak approximately one hour after exposure to corticosterone (Nishi, Tanaka, Matsuda, Sunaguchi, & Kawata, 2004). However, in the current study cortisol did not increase following the acute stress paradigm in the escitalopram group meaning that if the pronounced decrease in GR sensitivity following stress was due to increased rates of nuclear translocation, this was caused by a cortisol-independent mechanism (such as

alterations in NF- κ B activity). SSRIs and TCAs have been found to enhance GR nuclear translocation in the absence of any steroids *in vitro* (Okuyama-Tamura et al., 2003; Pariante, Pearce, Pisell, Owens, & Miller, 1997) meaning that escitalopram may have caused enhanced stress-related GR nuclear translocation in the current study in the absence of an increase in cortisol. However, as we did not measure the rate of nuclear translocation in the current study, we cannot confirm this was the case.

These changes in stress-related cortisol secretion and GR sensitivity might be one of the ways in which SSRIs exert their therapeutic effects. Perhaps altering the way in which the body responds to stressful situations is one of the ways in which antidepressants serve to ameliorate symptoms of depression. Future research should seek to measure how long these alterations in GR sensitivity are sustained for following stress.

6.11.8 Cortisol stress reactivity: Lack of response

In the current study there was a lack of effect of acute stress on cortisol secretion. There are a number of possible explanations for why this occurred. Firstly, the stress protocol used in the current study may not have been sufficient to bring about a biological stress response in the overall sample. However, subjective stress ratings, cardiovascular measures, and changes in corticosteroid receptor sensitivity suggest that the participants experienced both a psychological and a biological response to the stress protocol. It is possible that the stress protocol was not sufficient to elicit changes in HPA axis function. In a large meta-analysis of 208 laboratory studies, psychological stress paradigms overall were found to induce cortisol increases (Dickerson & Kemeny, 2004). However, the stress effects varied widely across studies with a number of studies reporting no changes in cortisol (*ibid*).

Although previously in this thesis I mentioned that there are generally high correlations between salivary cortisol levels and levels of unbound cortisol in plasma and serum (*Section 4.4.7*), there is evidence to suggest that this might not necessarily be the case during and after stress. Some study participants have been found to have an absent or blunted cortisol stress response in saliva, with marked cortisol increases in plasma (Kirschbaum & Hellhammer, 2000). As cortisol was measured in saliva in the current study it may be that the cortisol stress response was missed due to failure to measure cortisol in plasma.

Due to the unusually high levels of pre-stress cortisol in the current study it is more probable that anticipatory effects of attending the laboratory session masked the effects of the stress protocol. The baseline cortisol value was calculated from saliva collected 25 minutes after venepuncture. Venepuncture is thought to elicit a rise in cortisol levels (Kirschbaum & Hellhammer, 1989). The 25 minute rest period may not have been sufficient time for cortisol levels to return to unstressed levels. However, exploratory analysis revealed no significant association between venepuncture attempts and pre-stress cortisol levels.

On recruitment to the study, all participants were aware that during the laboratory session they would undergo a battery of ‘challenging mental tasks’ while regularly providing biological samples. It is possible that participants were anxious or nervous prior to attending the laboratory session. Exploratory analysis showed that high baseline cortisol values were associated with lower levels of subjective feelings of relaxation, and higher levels of baseline anxiety. This indicates that feelings of nervousness or anxiety affected baseline cortisol levels. Perhaps to tackle this future studies should alter the language used during the study recruitment process. Omitting terms or phrases such as ‘stress’ or

‘challenging mental tasks’ could have reduced anxiety levels at the beginning of the laboratory session.

6.11.9 Strengths and limitations

A strength of the current study is that it was a randomised placebo-controlled double blind trial. We used a parallel group design meaning that participants receiving placebo acted as the control comparison for both experimental medications. Adopting a parallel groups design allowed us to avoid problems relating to order and carry-over effects to do with study medications. It also meant that participants were unable to become habituated to the stress protocol. The three groups did not differ significantly on demographic or stress-related factors. However, as we did not adopt a crossover design where participants act as their own placebo comparison, it is possible that the experimental groups were unbalanced on some covariates that were not measured in the study. For example, we did not assess menstrual cycle phase in female participants. Menstrual cycle phase has been shown to affect cortisol responses to acute stress (Kirschbaum et al., 1999), although the evidence is mixed (Kirschbaum, Klauer, Filipp, & Hellhammer, 1995).

This study had a retention rate of 87.5% with 91 participants providing usable stress-related cardiovascular, cortisol, or corticosteroid receptor data. However, the sample sizes for individual analyses were less than this (see Table 6.3, 6.4, and 6.5). It is possible that this study was underpowered to detect certain effects of the medications. Additionally, our sample was composed mostly of young healthy university students from high socioeconomic backgrounds. Therefore the results of this study may not be readily generalizable to other groups.

The lack of effect of propranolol observed in the current study may be related to issues surrounding the study medications. Propranolol elicited the expected changes on stress-

related cardiovascular function. This implies that the medication did have a biological effect. However, it is possible that the dosage used, and the treatment duration, were not sufficient to induce changes in the HPA axis, or the corticosteroid receptors. Previously, five day administration of 80mg propranolol per day (Kudielka et al., 2007) and three day administration of 120mg per day (van Westerloo et al., 2011) did not affect cortisol stress reactivity in healthy volunteers. This suggests that beta-blocker dosage was probably not an issue in the current study. In terms of treatment duration, propranolol is a rapidly metabolised drug that exerts its effects rapidly (Leahey et al., 1980). Therefore, it is difficult to say whether increasing the treatment duration in the current study would have affected the propranolol group. Furthermore, as mentioned in *Section 5.8.6*, propranolol may not have been the most appropriate probe to use to assess the effects of sympathetic activation on HPA axis function as this drug is known to act as an antagonist of the serotonin receptors 5-HT1A and 5-HT1B. The use of a cleaner probe such as metoprolol may have elicited different results.

Although escitalopram did bring about changes in cortisol stress reactivity and stress-related corticosteroid receptor sensitivity it is possible that increasing the study treatment duration might have made these changes more pronounced. Many SSRIs take at least two weeks to elicit any beneficial response from depressed patients (Kasper et al., 2006). However, escitalopram has been shown to be the most fast-acting SSRI on the market exerting clinically meaningful effects within seven days (Burke et al., 2002; Montgomery et al., 2001; Nierenberg et al., 2007). Nevertheless, future research should seek to extend the duration of administration of escitalopram in healthy individuals to explore whether the effects on the HPA axis and its receptors are more pronounced.

It is possible that some participants did not adhere to the study protocol and missed doses of medications on certain days. There is also no guarantee that participants took the drugs

at the same time every day with food as directed. Therefore, there may have been some variability between participants in the current study regarding the bioavailability of the medications. However, pill counts were performed in order to ensure adherence to the protocol and changes in cardiovascular parameters across the medication groups indicated that the participants did take the medications as required. Nevertheless, future studies should measure blood concentrations of pharmacological probes in order to measure the bioavailability of the drug and confirm adherence.

The stress protocol used in the current study elicited changes in subjective stress levels, cardiovascular parameters, and corticosteroid receptor sensitivity. Therefore the lack of stress-related effects on cortisol stress reactivity in the current study is likely not related to the stress protocol used. Issues with the measurement of cortisol may partly explain the null findings. As mentioned previously, there can be discrepancies between levels of salivary and plasma levels of cortisol during and after stress. Therefore, the stress protocol may have brought about increases in plasma but not salivary cortisol in the current study, although this is unlikely. ACTH, but not cortisol, levels also may have been altered by the stress protocol in the current study. Failure to measure ACTH in the current study means that we could not assess these effects at the level of the pituitary. Future studies should endeavour to measure both plasma levels of cortisol and ACTH.

In terms of corticosteroid receptor sensitivity, performing glucocorticoid sensitivity assays in whole blood rather than in isolated PBMCs may have affected results obtained. It is preferable to carry out these tests in isolated lymphocytes or monocytes as analysis in whole blood means that individual differences in cell population ratios are not being taken into account. For example, in studies looking at the effects of exercise on GR sensitivity in healthy volunteers, the glucocorticoid sensitivity assay carried out in whole blood shows that sensitivity decreases in response to exercise (DeRijk et al., 1996; Smits

et al., 1998) whereas the assay carried out in isolated PBMCs shows that sensitivity increases in response to exercise (Duclos et al., 1999; Fragala et al., 2011). Future studies using *in vitro* glucocorticoid sensitivity assays to examine receptor sensitivity should carry out these tests in isolated PBMCs. Moreover, these assays only provide a proxy measure of GR and MR sensitivity. In the current study we measured glucocorticoid suppression of LPS-induced IL-6 release meaning we did not examine all the wider effects of glucocorticoids. Perhaps it would have been useful to directly measure receptor mRNA levels, or measure the rate of translocation of the corticosteroid receptors into the cell nuclei using Western blot analysis or a DNA binding ELISA for activated corticosteroid receptors. Future studies should try to measure both receptor levels and sensitivity in order to gain a more in depth understanding of what is occurring at the cellular level.

In the current study, pre-stress cortisol levels were high and steadily declined across the testing session despite participants undergoing a modified version of the TSST. Exploratory analysis indicated that this was probably due to high levels of anticipatory stress. This may be due to the language that was used in the recruitment material. As mentioned earlier, future studies should avoid using certain terminologies that might make participants anxious or nervous about attending the laboratory session.

A further limitation of the current study is that there were 12 participants who had high depression and/or anxiety scores. Prior to recruitment, participants were asked if they had ever received a clinical diagnosis of any psychiatric disorder. As a result, those participants who had never received a clinical diagnosis of anxiety or depression, but who did meet the depression/anxiety cut-offs on self-report measures were recruited into the study. Future studies should screen participants using brief measures of depression and

anxiety prior to recruitment into the study to ensure that the sample is comprised of healthy volunteers free of mental illness.

One additional limitation of this study was the use of multiple comparisons. Within each comparison group, the effects of experimental condition on a number of cortisol related parameters (stress-related secretion, changes in GR and MR sensitivity) were measured simultaneously. This means that the probability of observing a significant result due to chance may have been increased. The use of Bonferroni or Sidak corrections could have been applied to deal with multiple comparisons within the repeated measures analyses. However, these corrections have a tendency to be too conservative (Narum, 2006) which may have been problematic for results, particularly when dealing with minute changes at the cell receptor level. Nevertheless, this issue should be borne in mind when interpreting results.

6.11.10 Conclusion

To conclude, the results of this study showed that acute stress brings about decreases in corticosteroid receptor sensitivity that likely occur to facilitate the inflammatory stress response. This seems to occur independently of stress-related changes in cortisol secretion, which did not follow the expected pattern. Seven-day administration of SSRIs brought about a steeper slope of decline in the cortisol stress response and also enhanced and prolonged the stress-induced decrease in GR sensitivity. These changes might be brought about through alterations in cellular pathways known to influence inflammation. Altering the biological stress response using SSRIs might have implications for stress-related diseases such as depression and CVD. Future work is needed to confirm these findings and also delineate in more detail the possible mechanisms involved.

Chapter 7

Discussion

7.1 Overview

There is evidence to suggest that dysregulation of the HPA axis might be one of the biological pathways linking psychosocial stress with CVD. This PhD consisted of three studies that aimed to assess the role of HPA axis dysregulation in CVD, and to examine potential biological pathways that might be involved in basal and stress-related HPA axis dysregulation. Two different methods of investigation were used in this thesis to investigate these aims. Firstly, an observational clinical cohort study was used to assess the role of HPA axis dysregulation in patients with advanced CVD. Secondly, an RCT where healthy volunteers were randomised to receive different pharmacological probes was used to examine the role of certain biological pathways in basal and stress-related HPA axis function.

A body of evidence has highlighted the role of psychosocial stress in CVD and provided evidence for the role of the HPA axis in the stress-CVD link. However, gaps in the understanding remain which this PhD sought to address, particularly concerning the clinical relevance of HPA axis dysregulation in CVD, and the biological mechanisms through which psychosocial stress might cause this dysregulation.

In Study 1 (presented in Chapter 3) I examined the utility of pre-surgical diurnal cortisol rhythm in predicting the occurrence of post-surgical death and/or MACE in patients undergoing CABG surgery. In Study 2 (presented in Chapter 5) I examined the effects of six-day administration of beta-blockers and SSRIs on diurnal cortisol secretion in healthy volunteers. This was in order to find out more about what mechanisms and biological systems are involved in dysregulation of diurnal HPA axis functioning. In Study 3

(presented in Chapter 6) I examined the effects of seven-day administration of these same medications on cortisol stress reactivity and basal and stress-related changes in corticosteroid receptor sensitivity. Moreover, I examined the effects of acute stress on both GR and MR sensitivity in unmedicated healthy volunteers as there is a relative dearth of research in this area. In this Discussion chapter the hypotheses and findings of the three studies presented in this thesis will be briefly summarised and the contribution of these studies to the literature will be highlighted. Implications of the results, limitations of this thesis, and ideas for future research will also be discussed.

7.2 Main findings

7.2.1 Study 1: Diurnal cortisol rhythm and adverse clinical outcomes in patients with advanced CVD: The ARCS Study

In Study 1 (presented in Chapter 3) I examined the relationship between pre-surgical diurnal cortisol and adverse post-surgical outcomes in patients with advanced CHD undergoing CABG surgery. There is growing evidence (provided in Chapter 2) that the HPA axis plays a role in the progression of CVD. A number of studies have provided evidence for associations between single measures of plasma or serum cortisol and mortality or risk of future cardiac events in CVD (Güder et al., 2007; Jutla, Yuyun, Quinn, & Ng, 2014; Reynolds et al., 2010; Yamaji et al., 2009). However, the directions of these associations are mixed. In Chapter 3 I argue that this is because the diurnal nature of cortisol secretion is not being taken into account. One important large-scale study has shown an association between flatter cortisol slopes and increased risk of cardiovascular death in 4,047 originally healthy civil servants from the Whitehall II cohort (Kumari, Shipley, Stafford, & Kivimaki, 2011).

To date, no published studies have examined the role of diurnal HPA axis function in the prognosis of those who already have advanced CVD. In order to address this gap in the literature I measured several indices of diurnal cortisol secretion in 250 patients undergoing CABG surgery approximately 30 days prior to the procedure. I also collected data on long-term clinical outcomes (death/MACE) for these patients up to approximately 2-3 years after surgery. I hypothesised that a flatter diurnal cortisol slope pre-surgery would be associated with higher rates of adverse cardiac events and mortality in the years following revascularisation, but not CAR or cortisol AUC. I also hypothesised that poorer psychosocial stress profiles (high depressive symptoms, high anxiety symptoms, more stressful life events, low social support) would be cross-sectionally associated with a flatter cortisol rhythms.

As hypothesised, the results showed that patients with flatter pre-surgical cortisol slopes were at increased risk of experiencing death and/or MACE in the years following CABG surgery. The results showed that lower cortisol on waking and higher evening cortisol were also associated with increased risk of adverse clinical outcomes. This suggests that alterations in morning and evening levels were driving the association between slope and adverse outcomes. In agreement with the hypotheses, neither the CAR nor the cortisol AUC were associated with adverse clinical outcomes in these patients. Contrary to expectation, there were no robust cross-sectional associations between the psychosocial stress measures and cortisol slope.

This was the first study that examined the association between diurnal cortisol rhythm and prognosis in patients with CVD. The findings of this study add to a small but important body of work that show that flatter cortisol slopes also have prognostic value in other serious illnesses such as breast, lung, ovarian cancer, and renal cell carcinoma (Cohen et al., 2012; Schrepf et al., 2015; Sephton et al., 2013; Sephton, Sapolsky,

Kraemer, & Spiegel, 2000). Flatter cortisol slopes have also been observed in CHD patients compared to controls (Nijm et al., 2007). The association I report in this thesis implies variation of cortisol rhythm within patients who have CVD, which suggests that HPA axis dysregulation might worsen with disease progression. Furthermore, Kumari and colleagues found that alterations in evening cortisol drove the association between cortisol slope and cardiovascular mortality in originally healthy subjects, whereas I report that alterations in both morning and evening cortisol levels drive the association observed in the current study. This indicates that HPA axis dysregulation has progressed further in people with advanced CVD, again suggesting that dysregulation might worsen with disease progression. It is noteworthy that flatter slopes are also indicative of disease progression in other physical illnesses (Cohen et al., 2012; Schrepf et al., 2015; Sephton et al., 2013, 2000) meaning that these effects are not specific to CVD.

Counter to expectation I found no association between flatter cortisol slopes and any of the psychosocial stress variables. This implies that at this stage of advanced CHD, cortisol dysregulation might be driven more by physiological factors associated with the disease rather than psychosocial factors. The lack of association might also be due to our sample being a relatively unstressed sample. Psychosocial stress factors might not have been measured comprehensively or accurately. For example, in Chapter 1 I assert that life events, which were included as the main stress measure in the ARCS Study, might not provide a meaningful assessment of chronic stress levels.

Further limitations of this study also relate to the sample in that it was comprised largely of white males and thus the results cannot be readily generalised to other groups. A detailed discussion of the limitations of this study is provided in Chapter 3. However, it is important to reiterate here that the sample of this study was relatively small with only 19 adverse events occurring in the follow-up period. Therefore, statistical power was

limited and only a small number of covariates could be included in analyses. It is also important to mention that these results do not provide direct evidence of a causal association between cortisol slope and adverse outcomes in these patients as there may have been other unmeasured factors influencing diurnal cortisol rhythms and increasing risk of adverse outcomes.

The findings of this study need to be corroborated by further research with larger, more varied samples, and longer follow-up periods. Despite these considerations and others outlined in more detail in Chapter 3, the findings of Study 1 of this thesis are novel and have significantly added to the literature providing support for the role of HPA axis dysregulation in CVD prognosis.

7.2.2 Study 2: The effect of pharmacological blockade on diurnal cortisol secretion in healthy volunteers

In Study 2 (presented in Chapter 5) I sought to build on the findings presented in Chapter 3 by examining the effects of beta-blockade and SSRIs on diurnal cortisol secretion in healthy volunteers using data from the Stress Pathways Study described in Chapter 4. Using these pharmacological probes might tell us more about the mechanisms and different biological pathways involved in dysregulation of diurnal HPA axis functioning. There is a body of work that suggests that acute and longer-term administration of beta-blockers affects cortisol secretion. More specifically, SNS suppression by beta-blockade seems to enhance HPA axis activation. However, to date, no one has examined the effects of beta-blockade on diurnal cortisol parameters. On the other hand, a number of studies have examined the effects of SSRIs on diurnal cortisol secretion and these studies have shown that long-term treatment with SSRIs does alter diurnal cortisol rhythm. However, all studies to date have been carried out in depressed patients meaning we cannot

distinguish whether the observed effects are due to symptom remission of direct biological effects on HPA axis function.

In order to address these gaps in the literature, I examined the effects of six-day administration of beta-blockers and SSRIs on diurnal cortisol parameters in 94 healthy volunteers. Although no study to date has examined effects of beta-blockade on diurnal cortisol secretion, I hypothesised that beta-blockers would increase secretion leading to more enhanced CAR and cortisol AUC, and flatter cortisol slopes. Based on previous research in depressed patients, I hypothesised that SSRIs would reduce cortisol AUC and reduce the CAR. In line with these reductions I also hypothesised that SSRIs would steepen slopes. I also sought to examine how sex influences the effects of the medications.

The results presented in Chapter 5 provide limited support for the hypotheses. There was no effect of beta-blockade on any diurnal cortisol parameter. Similarly, SSRI administration did not affect cortisol AUC or the CAR. However, women taking SSRIs had significantly steeper cortisol slopes over the day compared to those taking placebo. These changes in HPA axis occurred independently of any change in mood which suggests that the observed results were due to direct biological effects of SSRIs on HPA axis function. Mechanistically, these results provide support for the notion that the serotonergic system exerts substantial effects on the HPA axis, potentially via desensitisation of the 5-HT_{1A} receptor. These results also provide evidence in support of the idea that SSRIs may directly exert effects on the HPA axis via modulation of the corticosteroid receptors (this issue was investigated in Study 3 presented in Chapter 6). The observed sex difference found in Study 2 is interesting and implies that oestrogen plays a role in how SSRIs affect the HPA axis.

Flatter cortisol slopes are characteristic of depression (Doane et al., 2013; Jarcho, Slavich, Tylova-Stein, Wolkowitz, & Burke, 2013; Sjögren, Leanderson, & Kristenson, 2006). The steeper cortisol rhythm brought about by brief SSRI treatment in women in Study 2 of this thesis implies that this might be one of the mechanisms through which these medications exert their therapeutic effect. Perhaps SSRIs might be a plausible therapeutic intervention for female CHD patients with flatter cortisol slopes. SSRI treatment has been associated with reduced mortality in CVD patients (Taylor, Youngblood, Catellier, et al., 2005).

There are a number of possible reasons why SSRI administration did not bring about alterations in other cortisol parameters. Health status appears to be an influential factor on results in that SSRI effects on HPA axis function seem to be more pronounced in depressed patients. Dosage may also be an issue and treatment duration might also be an issue for both the SSRI and beta-blocker group. This and other limitations are dealt with in detail in Chapter 5. Future research is needed to replicate the results of this study.

Despite methodological limitations, the findings of Study 2 are novel. It is the first time that the effects of beta-blockade on diurnal cortisol secretion have been measured. It is also the first time that the effects of SSRIs on several diurnal cortisol parameters have been measured in healthy people. These findings add to those of Study 1 as they indicate that SSRIs modulate diurnal cortisol slope – a cortisol parameter that is associated with worse prognosis in CVD.

7.2.3 Study 3: The effect of pharmacological blockade on cortisol stress

reactivity and corticosteroid receptor sensitivity in healthy volunteers

In Study 3 (presented in Chapter 6) I sought to extend existing knowledge on stress-related HPA axis function by examining the effects of seven-day administration of beta-

blockers and SSRIs on cortisol stress reactivity and stress-related corticosteroid receptor function. Furthermore, I examined the effects of these medications on basal corticosteroid receptor function. I also examined the effects of acute stress on GR and MR sensitivity in unmedicated healthy volunteers in order to garner more information on how these receptors respond to stress. Together with the results from Study 2, these results were intended to provide insight into the biological mechanisms involved in stress-related dysregulation of the HPA Axis, which is known to be a factor in CVD risk and prognosis.

There is a body of work that suggest that suppression of the SNS via beta-blockade enhances HPA axis stress reactivity. The main effectors of the SAM system, epinephrine and norepinephrine, have been shown to affect GR function and beta-blockade has also been shown to increase GR protein levels in CHD patients (Ji, Guo, Yan, Li, & Lu, 2010; Schmidt, Holsboer, & Spengler, 2001). However, to date no published study has assessed the effects of beta-blockade on corticosteroid receptor function in healthy people, or on acute stress-related changes in corticosteroid receptor sensitivity. Studies assessing the effects of SSRI administration on cortisol stress reactivity are scarce but the evidence suggests that this drug might enhance the cortisol stress response (Jezová & Duncko, 2002; Ljung et al., 2001). SSRIs are known to modulate corticosteroid receptor sensitivity (see Section 6.6) but, to date, no study has assessed the effects of SSRI administration on stress-related changes in corticosteroid receptor sensitivity.

Study 3 sought to address these gaps in the literature using data from the Stress Pathways Study described in Chapter 4. Firstly I examined the effects of acute stress on corticosteroid receptor sensitivity in unmedicated healthy volunteers. I hypothesised that acute stress would lead to a decrease in GR and MR sensitivity. I then assessed the effects of beta-blockade and SSRI administration on cortisol stress reactivity and baseline and stress-related corticosteroid receptor sensitivity. I hypothesised that beta-blockade would

bring about increased cortisol stress reactivity. Based on Ji and colleagues (2010) work I hypothesised that beta-blockade would increase basal GR sensitivity. I also hypothesised that stress-related changes in GR and MR sensitivity would be enhanced in the volunteers receiving beta-blockers. I hypothesised that SSRIs would bring about increased cortisol stress reactivity. I also hypothesised that SSRI treatment would increase baseline GR and MR sensitivity and enhance stress-related changes in GR and MR sensitivity compared to placebo.

The results of Study 3 provided limited support for the hypotheses. In the placebo group, acute stress brought about decreases in both GR and MR sensitivity as hypothesised. There was a transient decrease in GR sensitivity which had returned towards baseline by 75 minutes post-stress. Acute stress brought about a linear decrease in MR sensitivity which was most pronounced at 75 minutes post-stress. Propranolol had no significant effects on cortisol stress reactivity or corticosteroid receptor sensitivity. However, escitalopram administration resulted in a blunted cortisol stress response and a more enhanced and prolonged decrease in GR sensitivity throughout the stress testing session compared to placebo.

As discussed in Chapter 6, the transient decrease in GR sensitivity after acute stress in the placebo group is likely an adaptive function preventing tissue damage from overexposure to glucocorticoids while allowing the immune system to mount its inflammatory response to stress. The prolonged linear decrease in MR sensitivity following stress might serve to facilitate the return of GR sensitivity towards baseline levels. More work is needed to confirm this. Seven-day treatment with escitalopram enhanced this decrease in GR sensitivity across the testing session and also prolonged it. Additionally cortisol stress reactivity was blunted in those receiving escitalopram. It is possible that this enhanced decrease in GR sensitivity was accountable for the blunting of the cortisol stress response

in this group seeing as the GR is responsible for the magnitude of the cortisol stress response (de Kloet, 1998). Mechanistically, SSRI administration might have altered stress-related GR sensitivity through altering levels of the transcription factor NF- κ B which is known to play a role in the inflammatory stress response. This is described in detail in *Section 6.11.7* of Chapter 6. The prolonged decrease in GR sensitivity brought about by SSRIs might be associated with alterations in nuclear translocation rates. Future research should aim to measure stress-related changes in levels of NF- κ B and rates of nuclear translocation alongside changes in GR sensitivity in order to shed more light on the mechanisms involved. Although replication is needed, these results suggest that SSRIs alter HPA axis and corticosteroid receptor stress reactivity indicating that changing the way in which the body responds to stressful situations might be one of the ways in which antidepressants serve to ameliorate symptoms of depression.

There are a number of methodological issues which may have affected the results obtained. A detailed account of these is provided in Chapter 6. Measurement of cortisol and corticosteroid receptor sensitivity may have been problematic, and also the sample contained participants who had high scores on measures of depression and/or anxiety. Suggestions for improvement in future research were provided in Chapter 6 and will also be provided later in this chapter.

Despite the methodological shortcomings, the findings of Study 3 are novel. To date, the effects of acute stress on both GR and MR sensitivity in healthy younger volunteers had yet to be examined. Additionally, no published study had examined the effects of beta-blockers or SSRIs on stress related changes in corticosteroid receptor sensitivity. Although the results of this study provided limited support for the hypotheses, the results are still novel and will be discussed in detail later in the Discussion.

7.3 Overall summary of findings and implications

Firstly, this PhD has provided support for the clinical relevance of HPA axis dysregulation in advanced CVD by showing that flatter cortisol slopes were associated with adverse outcomes in the years following CABG surgery. The results of this PhD also indicate that changes in central neurotransmitter function brought about by SSRIs affect HPA axis function. Augmenting levels of serotonin resulted in a steepening of the cortisol slope in women. This may have therapeutic implications for both depression and CVD. Results from this PhD also show that in unmedicated individuals, acute stress brings about a decrease in GR and MR sensitivity. Augmenting levels of serotonin with SSRIs appears to have implications for cortisol levels and GR sensitivity after acute stress providing further evidence for the role of central neurotransmitters in HPA axis function. However, this study provided no evidence for the involvement of the peripheral nervous system in HPA axis function.

7.3.1 Study 1: Implications

The findings from Study 1 provide support for the clinical relevance of HPA axis function in CVD. HPA axis dysregulation was associated with poorer outcomes in CVD patients undergoing coronary revascularisation. Diurnal cortisol profiles can be obtained without difficulty because the measures are non-invasive and samples are stable for several days. Measuring diurnal cortisol rhythm in CVD patients may help to identify those at risk of adverse events or death allowing additional support and care to be provided. However, replication of the findings of Study 1 is required in order to confirm the clinical utility of measuring diurnal HPA axis function in CVD. Ideas for future research will be provided in *Section 7.5.1*.

Flattening of the diurnal cortisol slope is seen in CHD patients compared to healthy controls (Nijm et al., 2007) and in this study flattening of the cortisol slope was associated with adverse outcomes within a CHD patient group. This begs the question could modifying cortisol be beneficial for people with CVD? One intervention that has been used to modify cortisol secretion in patient cohorts is physical activity (Collomp et al., 2016). To date, studies have assessed the effects of both aerobic exercise and yoga on cortisol secretion in a number of patient groups. In breast cancer, a six month cardiovascular and diet intervention led to increases in morning cortisol levels, and decreases in depressive symptoms in 90 overweight patients (Saxton et al., 2014). However, it is difficult to ascertain whether it was the physical activity or the dietary intervention that was responsible for this change in cortisol secretion. Furthermore, the authors did not report whether or not patients experienced weight-loss – a factor that is known to affect HPA axis function (Seimon, Hostland, Silveira, Gibson, & Sainsbury, 2013).

Exercise has also been shown to alter cortisol secretion in the metabolic syndrome. Corey and colleagues randomised 136 people with the metabolic syndrome to undergo a stretching (n = 64) or a yoga (n = 72) intervention for six months (Corey et al., 2014). Salivary cortisol was measured at four time-points over three days at baseline and at the end of the intervention. Following the intervention, the stretching group had decreased waking and bedtime cortisol levels and increased GR sensitivity as assessed by the DST compared to those receiving the yoga intervention. Exploratory analysis revealed that these decreases were driven by an increase in social support experienced by the stretching group. Unfortunately, the authors do not report whether any of the symptoms of the metabolic syndrome were reduced or associated with changes in HPA axis function.

Yoga has also been found to affect cortisol secretion in patient groups. Vadiraja and colleagues randomly enrolled 88 breast cancer patients to either a six-week yoga programme (n=44) or brief therapy while undergoing radiotherapy (Vadiraja et al., 2009). After six weeks, those enrolled on the yoga programme had decreased depression and anxiety symptoms, decreased PSS scores, and increased morning cortisol levels. In a small RCT, 18 breast cancer patients were randomly assigned to attend yoga classes for 90 minutes twice weekly (n=9) or to a wait-list control group (Banasik, Williams, Haberman, Blank, & Bendel, 2011). After the yoga course, the patients reported better emotional well-being, lower fatigue, and lower morning and 5pm cortisol levels. However, this study was limited by a small sample of 18 which may have affected results.

Aside from cancer patients, the effects of yoga on cortisol changes in depression have been examined. Woolery and colleagues randomised 28 young volunteers with mild levels of depression to undergo a five-week yoga program or be enrolled into a wait-list control group (Woolery, Myers, Sternlieb, & Zeltzer, 2004). Compared with controls, those who underwent the yoga intervention had lower depression and anxiety scores following the five-week program, and also had higher levels of morning cortisol.

Overall, the evidence suggests that exercise and yoga can alter diurnal cortisol secretion in patient groups. However, the evidence largely comes from small sample sizes, and it is difficult to tease apart exactly how these physical activities might modulate HPA axis function. A number of factors might be relevant, such as weight loss, social support, and improvements in sleep brought about by the physical activity (Chen et al., 2009). Moreover, the relaxation element of yoga may be what is driving the alterations in cortisol secretion rather than the physical activity itself. To date, the effects of exercise or yoga on diurnal HPA axis function in CVD patients has yet to be examined. Based on evidence

from cancer and depression, the use of physical activity to alter HPA axis function in patient groups is an interesting prospect.

In recent years a large study has been carried out by Blumenthal and colleagues intending to examine the effects of stress management training on changes in biomarkers (including cortisol) in patients enrolled in traditional exercise-based cardiac rehabilitation (Blumenthal et al., 2010). Recent results from the ENHANCED trial indicate that patients who received the stress management training alongside the traditional cardiac rehabilitation experienced significant reductions in psychosocial stress measures (depression, anxiety, PSS) and markers of inflammation (CRP) (Blumenthal et al., 2016). However, results pertaining to cortisol have not yet been reported.

Another way of moderating HPA axis function in CVD is through pharmacological treatment. Results from Study 2 of this PhD provide limited evidence that serotonergic antidepressants may have clinical utility in altering HPA axis function in females. This will be discussed in the next section.

7.3.2 Study 2: Implications

Results from this PhD indicated that changes in central neurotransmitter function affected HPA axis function. Pharmacologically increasing levels of serotonin resulted in a steepening of the cortisol slope in women after six days. These changes in HPA axis function occurred independently of any alterations in mood indicating that they were a result of direct biological effects. This implies that increasing the bioavailability of serotonin alters the diurnal rhythm of the HPA axis. As well as increasing levels of serotonin, SSRIs are thought to exert anti-inflammatory effects which might have implications for HPA axis functioning (Walker, 2013). Nonetheless, these results provide further evidence that the CNS and HPA axis are functionally related. It is possible that

stress-related dysregulation of the HPA axis could be down to alterations in the CNS particularly related to serotonin. Chronic stress and depression are known to be associated with decreased levels of brain serotonin and increased levels of cortisol (Cowen, 2002; Tafet et al., 2001). The serotonergic system and the HPA axis are reciprocally linked (Porter, Gallagher, Watson, & Young, 2004) and altering levels of one has implications for levels of the other. In this PhD I have provided further evidence for this reciprocal association as SSRI administration in women brought about steeper cortisol slopes. However, there was no change in cortisol AUC showing that the diurnal rhythm was altered rather than overall cortisol levels.

These results suggest that the steeper cortisol rhythm observed in women taking SSRIs may be one of the mechanisms through which these drugs exert their therapeutic effects. Furthermore, these results also suggest that SSRIs may be particularly valuable for female CHD patients with flattened diurnal cortisol slopes. As mentioned before in this thesis, depression is prevalent and persistent in CHD patients (Thombs et al., 2006) and many patients with comorbid CHD and depression take SSRIs (Shapiro, 2015). A number of studies have assessed the effects of SSRI use in CHD patients who are depressed. In a recent systematic review of 40 studies assessing associations between antidepressant use and CHD, the authors concluded that SSRIs (compared to other types of serotonergic antidepressants) are cardio-protective in nature (Nezafati, Vojdanparast, & Nezafati, 2015). They posit that SSRIs may exert these protective effects by promoting optimal platelet activity, thus preventing the development of atherosclerotic plaques and thrombi (*ibid*). Within CHD patients with depression, SSRI use has also been found to be associated with lower risk of death and recurrent cardiac events in the 29 months following an MI (Taylor et al., 2005). Conversely, antidepressant use in CHD has been associated with increased mortality (Brouwers et al., 2016; Hansen et al., 2016).

However, these studies failed to take antidepressant type into account. In Study 1 of this PhD I provided evidence for an association between flattened cortisol slopes and adverse clinical outcomes in patients with advanced CHD. It is possible that direct effects of SSRIs on diurnal cortisol slope might be a pathway through which SSRIs exert their supposed protective effects in CHD patients with depression. However, I have only shown this association in healthy women. Replication of these findings in larger studies is required before any conclusions can be drawn. Ideas for future research will be provided in Section 7.5.

7.3.3 Study 3: Implications

Results from this PhD indicate that in unmedicated healthy volunteers acute stress brings about decreases in both GR and MR sensitivity. The decrease in GR appears to be transient returning towards baseline levels just a little over an hour after the stressor. It is possible that this transient decrease is an adaptive function allowing the immune system to mount its inflammatory response to stress by reducing the inhibitory effects of cortisol. Although adaptive, this stress-related decrease in corticosteroid receptors might signpost what happens in chronic stress. In Chapter 2 I outlined a number of studies that have shown that baseline GR sensitivity is decreased in those experiencing chronic stress including depression (Bauer et al., 2000; Bellingrath, Rohleder, & Kudielka, 2013; Calfa et al., 2003; Jarcho et al., 2013; Miller, Cohen, & Kim, 2002; Sauer et al., 1995; Wirtz et al., 2003). In some cases reduced GR sensitivity was associated with higher levels of circulating IL-6 and CRP (Jarcho et al., 2013; Wirtz et al., 2003). Although decreased corticosteroid receptor function following acute stress might be adaptive in healthy, unstressed individuals, it is possible that exposure to chronic stress results in a long-term reduction in GR sensitivity leading to a pro-inflammatory state. These changes in

corticosteroid receptor sensitivity therefore have implications for the aetiology of stress-related inflammatory disorders such as CVD.

In some of the studies cited above examining associations between chronic stress and basal GR sensitivity, decreased sensitivity was associated with flatter diurnal cortisol slopes (Jarcho et al., 2013; Miller et al., 2002). In Study 2 (Chapter 5), we found that six day SSRI administration resulted in steeper diurnal cortisol slopes in female participants. In the Discussion of that chapter I posited that the steeper slope seen in these women might be a result of increased sensitivity of the corticosteroid receptors. However, the results from Study 3 provided no support for this. This implies that SSRI-induced changes in diurnal cortisol slope occurred independently of alterations in basal corticosteroid receptor function. It is possible that changes in the serotonin receptors might be involved here seeing as escitalopram has been shown to desensitise the 5-HT_{1A} receptor which could lead to changes in cortisol levels (Zhong, Haddjeri, & Sánchez, 2012). However, there is a body of evidence outlined in Section 6.6 of this thesis suggesting that SSRI administration modulates basal corticosteroid receptor function. Therefore, more research is required in order to delineate the associations between SSRI induced changes in diurnal cortisol secretion and basal corticosteroid receptor function.

As well as showing that acute stress brings about a transient decrease in GR sensitivity, the results of this PhD also show that seven-day administration of escitalopram enhanced and prolonged this desensitisation of the GR. In Chapter 6 I argued that this might be one of the mechanisms through which SSRIs exert their therapeutic effects. This might seem counterintuitive seeing as decreased basal GR sensitivity has been associated with chronic stress and depression. However, in the current study we are reporting SSRI-induced alterations in *stress-related* GR sensitivity which is distinct from basal GR function.

SSRIs enhance and prolong the response which could be adaptive preventing tissue damage from overexposure to glucocorticoids (Bamberger, Schulte, & Chrousos, 1996).

What these results indicate is that increasing levels of serotonin results in a more pronounced GR desensitisation following stress. Altering how the body responds to stressful situations might be one of the ways in which antidepressants exert their therapeutic effects in depression. As mentioned previously a recent review of 40 studies concluded that SSRIs are cardio-protective in nature via promoting optimal platelet activity (Nezafati et al., 2015). Perhaps this cardio-protective effect is also exerted via modulation of the stress response at the level of the GR.

7.4 Methodological issues and limitations

The results presented in this thesis have to be interpreted with their limitations borne in mind. The short-comings of each individual study were provided at the end of each chapter. Therefore, in this section, only the most important limitations and issues will be discussed

7.4.1 The study samples

In this PhD two study samples were used. The sample from Study 1 was taken from the ARCS Study carried out by the Psychobiology Group at UCL. This sample comprised 250 men and women with advanced heart disease undergoing CABG surgery. In Study 2 and 3 the samples were taken from the Stress Pathways Study. These samples comprised 94 and 91 healthy volunteers respectively who were randomised to receive either beta-blockers or SSRIs for one week and then undergo acute psychosocial stress testing in the laboratory. In Study 1, one advantage of the sample used was that they were all undergoing CABG surgery. What this indicates is that all patients in the sample had received a diagnosis of CVD that was advanced enough to warrant coronary

revascularisation. In Study 2 and 3, an advantage of the sample was that each participant had to meet strict inclusion criteria (see *Section 4.4.3*) in order to take part in the study.

However, these participant samples were not without their limitations. The ARCS sample was largely comprised of white men of European origin recruited from a single hospital in South London (St. George's University hospital, Tooting). This hospital is located in an ethnically diverse area of South West London, with approximately 22% of the borough stating their ethnicity as non-white (Wandsworth Council, 2011). In Study 1, 12.4% of the sample were non-white. Ethnicity is known to be a factor that affects long-term recovery after CABG surgery (Deb et al., 2016; Rumsfeld et al., 2002) with ethnic minorities being less likely to be invited to attend or enrol in cardiac rehabilitation programmes in the UK (Bethell, Lewin, & Dalal, 2009). This means the results of Study 1 may not be readily generalizable to other groups. Additionally, the sample appeared to be fairly well-adjusted in terms of psychosocial stress factors. Depression and anxiety symptoms were relatively low in this patient group. Only 8.5% of the sample met the criteria for moderate depressive symptoms, and 15.4% met the criteria for high anxiety levels. These rates are lower than those presented in the literature which states that about 30% of CHD patients undergoing CABG surgery have mild-to-moderate depressive symptoms (Ravven, Bader, Azar, & Rudolph, 2013) and about 38.7% suffer from an anxiety disorder (Gallagher & McKinley, 2009). Additionally, this sample also had on average experienced only one stressful life event in the previous six months and had high levels of social support. Therefore, it may have been that the cross-sectional associations between psychosocial stress factors and diurnal cortisol profiles were too weak to detect seeing as at the time of collection the sample was relatively unstressed.

The Stress Pathways Study sample was largely comprised of healthy young students from high socioeconomic backgrounds. Therefore the results might not be generalizable to

other groups, or to clinical groups with depression or CVD. Furthermore, a number of participants in each sample group had high depression and anxiety symptoms. Prior to recruitment, participants were asked whether they had ever received a clinical diagnosis of a psychiatric disorder, but were not screened using measures of depression and anxiety. To account for this, in both Study 2 and Study 3 sensitivity analyses were performed excluding these participants from the main analyses. This did not have major repercussions for the results of Study 2 or Study 3. Future studies should screen participants using brief measures of depression and anxiety prior to recruitment into the study to ensure that the sample is comprised of healthy volunteers free of mental illness.

The recruitment strategies used may have introduced bias into the studies. The sample from Study 1 was recruited from pre-surgical assessment clinics. Potential participants were approached in the waiting room prior to their appointment to see if they wished to enrol on the study. Many participants who refused to participate were either not interested in the study or were too stressed or anxious to take part. This means that patients that may have been most relevant to the hypotheses of Study 1 were not recruited. Additionally, the fact that recruitment was limited to one hospital within one London borough means we only had access to patients from similar areas of London. This, as outlined above, had issues for the representativeness of the study sample.

The sample used in Study 2 and 3 was recruited in and around UCL campus via email and poster advertisements. This resulted in a sample largely comprised of students. The study advertisement specified that people would have a cannula inserted for blood sampling, would have to take medications for a week, and would have to undergo some 'challenging mental tasks' in the laboratory. Many prospective participants may have found this study daunting and therefore chose not to participate. This means the sample was only comprised of those that may not have felt unnerved or anxious by the protocol.

However, baseline cortisol levels from Study 3 suggest that the sample was anxious prior to the laboratory testing session. Nevertheless, the daunting protocol meant the study sample was probably not representative of the broader student body at UCL.

7.4.2 The HPA axis: Measurement issues

In Study 1 and Study 2, diurnal cortisol secretion was measured using seven saliva samples taken over the course of one weekday. This means that in both samples diurnal rhythm may have been affected by situational factors, rather than long-term factors. Diurnal cortisol secretion is primarily affected by trait rather than situational factors on a weekday as most people have established weekday routines (Hellhammer et al., 2007). However, the sample from Study 2 (chapter 5) was comprised mostly of university students. Students are likely to have a routine that is quite variable across weekdays meaning that it might have been preferable to take measures over the course of several days. In fact, diurnal cortisol parameters measured over the course of three days have shown considerable day-to-day fluctuation with little evidence for stable trait-like influences (Ross, Murphy, Adam, Chen, & Miller, 2014). The sample from Study 1 (chapter 3) was comprised of mostly older, retired CVD patients and their habits may be less variable across days meaning that a single day could be more representative than in younger people. Even so, it would have been preferable to measure diurnal cortisol secretion over several days in both the ARCS and the Stress Pathways Study. In both studies it was decided to measure diurnal cortisol secretion over the course of one day only in order to minimise participant burden and therefore facilitate recruitment.

In Studies 1, 2, and 3 cortisol was measured in saliva. Cortisol can be measured in a number of biological specimens including saliva, blood, urine, and hair. Cortisol was measured in saliva for a number of reasons. Saliva-sampling is a non-invasive, relatively

inexpensive way to measure cortisol and allows for ambulatory assessment in naturalistic setting where participants are collecting their own samples. Salivary cortisol also provides a reliable measure of unbound, biologically active cortisol. There are generally high correlations between salivary cortisol levels and levels of unbound plasma cortisol (Hellhammer, Wüst, & Kudielka, 2009). Therefore, we chose to measure cortisol in saliva to reduce participant burden, and to ensure that ambulatory measures were consistent with laboratory measures. Measurement of diurnal cortisol parameters also required the use of salivary cortisol as repeat blood samples across the day would be impractical and would likely affect cortisol levels. However, it is worth mentioning that during times of stress plasma cortisol can rise with no change at the salivary level (Kirschbaum & Hellhammer, 2000). Similarly, stress can also elicit changes in ACTH but not cortisol (Cacioppo et al., 1995; Malarkey, Kiecolt-Glaser, Pearl, & Glaser, 1994; van der Pompe, Antoni, & Heijnen, 1996). This is of particular relevance to Study 3 as not measuring plasma cortisol or ACTH levels means that stress effects in the laboratory may have been missed.

In Study 3 corticosteroid sensitivity at several time-points was measured using glucocorticoid sensitivity assays. Specifically, GR and MR sensitivity was measured by dexamethasone and prednisolone suppression of LPS induced IL-6 levels in whole blood. This assay provides a proxy measure of GR and MR sensitivity. Whole blood allowed for the rapid measurement of peripheral glucocorticoid sensitivity. For this reason the assay was carried out in whole blood in the Stress Pathways Study. However, measurement in isolated monocytes or lymphocytes would have allowed for a more focused measure of IL-6 suppression. This is because certain leukocyte subsets produce more inflammatory cytokines than others. For example, monocytes are the main source of LPS-stimulated pro-inflammatory cytokines such as IL-6 (Berczi, 1998). Additionally, only IL-6 suppression was measured to facilitate brevity in the Stress Pathways Study. Measuring

only a single outcome which is known to be affected by glucocorticoids (i.e. IL-6) does not allow for examination of the wider effects of glucocorticoids. The results of Study 3 should be interpreted with these issues borne in mind.

7.4.3 The study medications

Data from the Stress Pathways Study were used in Study 2 and Study 3. As mentioned in Chapter 4, the Stress Pathways Study was primarily designed to assess the effects of pharmacological probes on inflammatory responses to acute stress. SSRIs and beta-blockers were chosen mainly for their relevance to inflammation. My PhD sought to use this study as an opportunity to assess the effects of these pharmacological probes on HPA axis function. One of the main roles of the HPA axis is inhibition of the stress-related release of inflammatory cytokines (Kaltsas, Zannas, & Chrousos, 2012) meaning that the anti-inflammatory actions of SSRIs and beta-blockers may be related to alterations in HPA axis function. Literature which has provided evidence for the effects of beta-blockers and SSRIs on HPA axis function has been described in both Chapter 5 and 6. However, these drug types may not have been the ideal choices for garnering information about the biological mechanisms underlying stress-related changes in HPA axis function. Many studies described in Chapter 5 and 6 report null findings and in this PhD beta-blockade had no significant effects on HPA axis function. Reasons for this (e.g. dosage, treatment duration) are provided in the Discussion sections of each chapter. However, it could be that alternative pharmacological probes would have provided more information about HPA axis dysregulation. Suggested alternatives are provided in *Section 7.6.1*.

7.5 Suggestions for future research

Specific suggestions for future research have been provided in the Discussion section of each study. In this section I will outline more general ideas for future work in the area of stress-related HPA axis dysregulation.

7.5.1 The clinical utility of HPA axis dysregulation in CVD

Study 1 provided evidence for the clinical utility of measuring diurnal cortisol secretion, in that flatter cortisol slopes predicted adverse long-term outcomes in patients undergoing CABG surgery. Firstly, this study needs replication in a larger, more representative sample, recruited across several hospital sites. More psychosocial stress measures should be included also in order to fully explore associations between stress and HPA axis dysregulation in CHD. Additionally, diurnal cortisol secretion could be measured over several days to minimise the influence of situational factors.

In Chapter 3 I posit that HPA dysregulation of diurnal HPA axis function may worsen with CVD progression and that a more pronounced flattening of the cortisol slope might be associated with greater disease severity. In breast cancer patients, it has been shown that patients with more severe metastatic spread showed a tendency towards flatter cortisol slopes across the day (Abercrombie et al., 2004). Future research could seek to characterise diurnal cortisol profiles in CHD patients according to disease severity. Examining associations between cortisol profiles and long-term outcomes in these patient subgroups could also be interesting. This may allow for further investigation into the clinical utility of measuring diurnal cortisol secretion in CHD patients. Additionally, psychosocial factors that are associated with HPA axis dysregulation could also be measured in these patient subgroups. Examining basal corticosteroid receptor sensitivity in these patients might also be useful.

Study 1 examined associations between diurnal cortisol secretion and long-term outcomes in those with advanced CHD that required CABG surgery. Future research should also seek to assess associations between diurnal cortisol secretion and long-term outcomes in other CVD patients, such as those who have heart failure, or who have had a stroke or an MI. Measuring cortisol concentrations in the hair of these patients would provide a retrospective indicator of average cortisol exposure over the previous months (Stalder & Kirschbaum, 2012) thus allowing for a pre-event measure of cortisol. Retrospectively measuring psychosocial stress factors in these patients would allow for associations between pre-event levels of stress and exposure to cortisol to be examined. Hair cortisol levels have been found to associate with measures of chronic stress, depression, and anxiety (Herane Vives et al., 2015; Russell, Koren, Rieder, & Van Uum, 2012; Staufenbiel, Penninx, Spijker, Elzinga, & van Rossum, 2013).

7.5.2 Alternative pharmacological probes

In Study 2 and Study 3 the effects of beta-blockers and SSRIs on diurnal cortisol secretion, cortisol stress reactivity, and corticosteroid receptor sensitivity were assessed in healthy volunteers. In Study 2, six-day SSRI administration resulted in steeper cortisol slopes in women. In Study 3, seven-day SSRI treatment resulted in an enhanced GR response (decrease in sensitivity) to acute stress in the laboratory. It is possible that increasing the SSRI treatment duration may have resulted in more pronounced changes in HPA axis function in both studies. However, asking healthy volunteers to take serotonergic antidepressants for a considerable period may have ethical implications (Uher et al., 2009). One way in which future research could be carried out examining the effects of increasing the bioavailability of serotonin on HPA axis function in healthy volunteers is through the use of 5-hydroxytryptophan (5-HTP) (Turner, Loftis, & Blackwell, 2006). 5-HTP is an amino acid and is the immediate biological precursor of

serotonin. It is essentially a nutritional supplement that has been used in the treatment of depression and anxiety for over 30 years (Birdsall, 1998; Iovieno, Dalton, Fava, & Mischoulon, 2011). There has not been much work examining the effects of 5-HTP on cortisol but preliminary work suggests it modulates cortisol secretion (Meltzer & Maes, 1994; Schruers, van Diest, Nicolson, & Griez, 2002). This nutritional supplement may allow us to examine the effects of longer-term changes of serotonergic function on diurnal and stress-related HPA axis function.

As mentioned previously, SSRIs and beta-blockers were chosen as the pharmacological probes for the Stress Pathways Study mainly due to their relevance to inflammation. These probes may not have been the most appropriate choices for garnering information about the biological mechanisms underlying stress-related changes in HPA axis function. There are a number of medications that might be better suited to examining these mechanisms. Firstly, examining drugs that are known to directly modulate the HPA axis would be useful to gain knowledge about basal and stress-related function. Metyrapone is a cortisol synthesis inhibitor used in the treatment of adrenal insufficiency. Inhibition of cortisol with metyrapone reduces baseline cortisol levels and also reduces cortisol stress reactivity (Broadley et al., 2005). Reducing the cortisol stress response pharmacologically would likely have consequences for how GR and MR sensitivity changes in response to stress. Administration of metyrapone might tell us a little more about why corticosteroid receptor sensitivity decreases in response to acute stress. However, it has been found to elicit adverse effects in many people (Ducat et al., 2013). Mifepristone is a GR antagonist meaning that it inhibits the binding of GR agonists (such as cortisol) to the GR. It is known to increase numbers of GR rapidly thus restoring 'normal' HPA axis negative feedback. Therefore, mifepristone has been used in the treatment of depression and other neuropsychiatric disorders (DeBattista & Belanoff,

2006; Maric & Adzic, 2013). Examining how rapidly increasing GR numbers affects diurnal cortisol secretion and cortisol stress reactivity in the laboratory might tell us more about the role of the GR in HPA axis dysregulation. Mifepristone is well-tolerated with few side effects. However, it is used as a form of emergency contraception which may have reproductive implications for women taking it (von Hertzen et al., 2002).

It may also be useful to examine the effects of medications that are frequently prescribed to CVD patients that are known to have effects on HPA axis function also. Angiotensin-converting-enzyme (ACE) inhibitors are used to treat hypertension and heart failure. They decrease vasoconstriction and a number of hormones that play role in blood pressure (e.g. aldosterone, vasopressin). In murine models, ACE inhibitors have been found to attenuate HPA axis reactivity to a CRH injection (Raasch et al., 2006). A SNP in the ACE gene had also been associated with both depression and hypercortisolism (Baghai et al., 2006). Administering ACE inhibitors to healthy volunteers might tell us more about cortisol stress reactivity and stress-related GR and MR sensitivity.

Calcium channel blockers are also frequently prescribed to CVD patients for the treatment of hypertension. These drugs reduce hypertension by disrupting the movement of calcium ions through calcium channels. In a placebo-controlled study, seven-day administration of calcium channel blockers decreased serum cortisol levels in 12 hypertensive men (Beer, Jakubowicz, Beer, & Nestler, 1993). One month treatment with calcium channel blockers increased GR protein levels in 20 hospitalised patients with CHD (Ji et al., 2010). Therefore, calcium channel blockers might also be a potential pharmacological probe for use in future research assessing the biological pathways underlying stress-related HPA axis dysregulation.

7.5.3 The role of arginine vasopressin

Arginine vasopressin (AVP) is a hormone produced in the paraventricular nucleus of the hypothalamus which regulates osmotic homeostasis and blood pressure. AVP also works synergistically with CRH in the modulation of stress-related ACTH secretion (Rivier & Vale, 1983). AVP levels are responsive to changes in plasma levels of glucocorticoids and are under feedback inhibition by glucocorticoids making AVP part of the HPA axis feedback loop (Aguilera & Rabadan-Diehl, 2000). AVP levels are responsive to stress. Acute stress has been found to lead to increases in AVP levels and basal circulating AVP levels are increased in chronic stress (Aguilera, Subburaju, Young, & Chen, 2008).

AVP can be difficult to measure due to its small size and short half-life. Often, copeptin is measured as a surrogate marker for AVP. Copeptin is a byproduct of AVP production. It is easier to measure as it is a more stable peptide with a longer half-life than AVP. Copeptin levels have been found to correspond with individual stress levels. Katan and colleagues examined cortisol and copeptin at three stress levels – unstressed healthy controls, hospitalised medical patients with moderate stress, and surgical patients 30 minutes after extubation with maximal stress (Katan et al., 2008). They found that cortisol levels were significantly higher in the maximally stress surgical patients compared to the medical patients and healthy controls. Copeptin was significantly higher in the medical patients compared to the healthy controls, and even higher in the maximally stressed surgical patients. Furthermore, levels of cortisol and copeptin were highly correlated in all groups. This shows that copeptin, as an AVP surrogate marker, is a novel indicator of stress that might even be more sensitive to individual stress levels than cortisol.

AVP has also been linked to the emotional stress response. In a recent study, 166 men and women underwent the TSST (Moons, Way, & Taylor, 2014). Those with

polymorphisms in the AVP receptor gene (AVPR1A) had higher levels of post-stress AVP and also reported more post-stress anger than non-carriers of the polymorphism.

AVP also appears to play a role in CVD (Yalta, Yalta, Sivri, & Yetkin, 2013). Increased copeptin levels have been found to be associated with poor prognosis in patients with CHD. Khan and colleagues examined copeptin levels in 980 men and women who had been admitted to hospital with an MI. They found that increased copeptin levels were associated with future readmittance to hospital and mortality in the 60 day period following an MI (Khan et al., 2007). In a sample of 2700 CHD patients undergoing coronary angiography, von Haehling and colleagues found that initial increases in copeptin levels were associated with increased risk of all-cause and cardiovascular mortality, stroke, and reoccurrence of MI in the following three months (von Haehling et al., 2012).

AVP also seems to be associated with clinical factors associated with the development of CVD. Higher levels of circulating copeptin have been cross-sectionally associated with prevalent diabetes mellitus and insulin resistance in a large Swedish cohort of 4747 men and women (Enhörning et al., 2010). Copeptin levels were also predictive of new onset diabetes over 12 years of follow-up. In this same cohort of patients, higher levels of copeptin were associated with hypertension, abdominal obesity, obesity, higher circulating levels of CRP, and the metabolic syndrome (Enhörning et al., 2011).

Together, this research suggests that AVP plays a role in stress, and also plays a role in CVD development and prognosis. Therefore, it is possible that AVP may be a HPA axis hormone involved in the link between stress and CVD. To date, very little research has examined the effects of psychosocial stress on AVP and more work is needed on the role of AVP in the development of CVD. Future research should seek to clarify the role of

AVP in both chronic stress and CVD in order to further our understanding of HPA axis involvement in the stress-CVD link.

7.6 Final conclusion

This PhD aimed to assess the role of HPA axis dysregulation in CVD, and to examine potential biological pathways that might be involved in HPA axis dysregulation through the use of two pharmacological probes: beta-blockers and SSRIs. To conclude, the results of this PhD provide evidence for the clinical relevance of HPA axis dysregulation in CVD by showing that flatter cortisol slopes were associated with adverse outcomes in the years following coronary revascularisation. Augmenting levels of serotonin using SSRIs appeared to steepen diurnal cortisol slopes in women, which may have therapeutic implications for both depression and CVD. This finding provided further evidence for the functional relationship between central neurotransmission and HPA axis function.

At the cellular level, this PhD showed that acute stress brought about decreases in both GR and MR sensitivity that are likely adaptive in nature. Augmenting levels of serotonin with SSRIs appeared to enhance and prolong stress-related decreases in GR sensitivity, while blunting the cortisol stress response. This finding also provided further evidence for the role of central neurotransmitters in HPA axis function. SSRI-induced alterations in the biological stress response might have implications for stress-related diseases such as CVD.

Together this body of work provides support for the notion that alterations in HPA axis function play a role in CVD and that the serotonergic system likely plays a role in stress-related dysregulation of the HPA axis. Future work should seek to replicate these findings and describe in more detail the possible mechanisms involved through the use of different

pharmacological probes and more detailed measurement of other biological factors of relevance.

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List of Publications

1. Poole, L., **Ronaldson, A.**, Kidd, T., Leigh, E., Jahangiri, M., & Steptoe, A. (2016). Pre-surgical depression and anxiety and recovery following coronary artery bypass graft surgery. *Journal of Behavioral Medicine*, 1-10. [Impact factor 2.959]
2. Poole, L., Kidd, T., **Ronaldson, A.**, Leigh, E., Jahangiri, M., & Steptoe, A. (2016). Depression 12-months after coronary artery bypass graft is predicted by cortisol slope over the day. *Psychoneuroendocrinology*, 71, 155-158. [Impact factor 4.944]
3. Jackowska, M., **Ronaldson, A.**, Brown, J., & Steptoe, A. (2016). Biological and psychological correlates of self-reported and objective sleep measures. *Journal of Psychosomatic Research*, 84, 52-55. [Impact factor 3.268]
4. Poole, L., **Ronaldson, A.**, Kidd, T., Leigh, E., Jahangiri, M., & Steptoe, A. (2016). Pre-operative cognitive functioning and inflammatory and neuroendocrine responses to cardiac surgery. *Annals of Behavioral Medicine*, 1-9. [Impact factor 4.200]
5. **Ronaldson, A.**, Gazali, A. M., Zalli, A., Kaiser, F., Thompson, S. J., Henderson, B., Steptoe, A., & Carvalho, L. (2016). Increased percentages of regulatory T cells are associated with inflammatory and neuroendocrine responses to acute psychological stress and poorer health status in older men and women. *Psychopharmacology*, 1-8. [Impact factor 3.875]
6. Kidd, T., Poole, L., **Ronaldson, A.**, Leigh, E., Jahangiri, M., & Steptoe, A. (2016). Attachment anxiety predicts depression and anxiety symptoms following coronary artery bypass graft surgery. *British Journal of Health Psychology*. [Impact factor 2.895]

7. Kidd, T., Poole, L., Leigh, E., **Ronaldson, A.**, Jahangiri, M., & Steptoe, A. (2016). Health-related personal control predicts depression symptoms and quality of life but not health behaviour following coronary artery bypass graft surgery. *Journal of Behavioral Medicine*, 39(1), 120-127. [Impact factor 2.959]
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Conference Presentations

1. 'The effects of escitalopram and propranolol on cardiovascular stress reactivity and recovery in healthy volunteers'. Poster presentation at the 74th annual meeting at the American Psychosomatic Society at Denver Colorado (March 2016).
2. 'Optimism and recovery following acute coronary syndrome'. Citation poster presentation at the 73rd annual meeting of the American Psychosomatic Society at Savannah, Georgia (March 2015).

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Appendix A: Example page form the cortisol sampling diary (ARCS Study)

TUBE 1 : AS SOON AS YOU WAKE UP					
What is the time now?		_____ a.m. / p.m.			
What was the exact time you collected the sample?		_____ a.m. / p.m.			
Was there a delay between waking up and collecting your first sample? Yes No					
↳ If yes, how long? ____ hrs & ____ mins					
In the last 30 minutes how much did you feel....					
	<i>Not at all</i>				<i>Very much</i>
In control	1	2	3	4	5
Tired	1	2	3	4	5
Happy	1	2	3	4	5
Frustrated or angry	1	2	3	4	5
Sad	1	2	3	4	5
Stressed	1	2	3	4	5
Pain	1	2	3	4	5
If you talked with others, how pleasant was the interaction?					
<i>Not applicable</i> <input type="checkbox"/>	1	2	3	4	5
In the last 30 minutes, but before you collected your sample did you....					
Brush your teeth				No	Yes
Drink any tea, coffee or other caffeinated drinks				No	Yes
Take any medicines				No	Yes
Eat a meal				No	Yes
Drink any alcohol				No	Yes
Do any exercise?				No	Yes
Smoke any cigarettes?				No	Yes

Appendix B: Stress Pathways Study participant information sheet

THE STRESS PATHWAYS STUDY

You are being invited to take part in a research study. It is up to you to decide whether to take part or not; choosing not to take part will not disadvantage you in any way. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. If you do decide to take part you are still free to withdraw at any time and without giving a reason.

What is the purpose of the study?

Cardiovascular disease (CVD) is the leading cause of death in the UK. Effective prevention relies on the identification of those at risk. Psychological stress is a risk factor for CVD but research done so far has provided little information on how stress causes CVD. By studying biological responses to challenging tasks in the laboratory we can gather information about what biological pathways are most relevant in the stress-CVD link. Furthermore, by asking healthy volunteers to take certain medications that block biological pathways suspected to be involved we can then gain further insight into the stress-CVD link and may also identify suitable therapeutic interventions.

Who can take part?

Healthy men and women aged 18-65 years can take part in this study. However, there are some exclusion criteria, so please do read them carefully:

Please do not take part in this study if any of the following apply to you:

- If you are taking any medicines on a regular basis
- If you have any haematological, pulmonary, liver, renal, gastrointestinal, heart,

- cerebrovascular, or psychiatric disease
- If you have any history of thromboembolism
- If you suffer from asthma and/or have any known allergies to the study medication
- If you are currently pregnant or breastfeeding
- If you have low or high blood pressure

What will I have to do if I decide to join the study?

Day 1: You will be invited to an appointment at UCL (taking about 45 minutes) where you will fill out some questionnaires and have your body composition measured. At this appointment you will be given some bottles to take home which will be used to provide saliva samples in a non-invasive manner. You will also be given the study medication. You will either be given a 7-day supply of propranolol (a beta-blocker), escitalopram (a selective serotonin reuptake inhibitor), or placebo (an inert sugar pill). Because the researcher cannot know what medication you are given you won't be able to find this out until all the study data has been collected. If you have any current infection (e.g. common cold, flu, etc.) your appointment will be postponed.

Day 2: We will ask you to begin taking your study medication. You will be required to take one pill every morning for 7 days. While taking the medication we recommend that you do not take any other medication for any condition, any kind of herbal remedy, do any high-intensity physical activity, drive or operate machinery, and that you avoid excessive alcohol intake.

Day 7: We will ask you to provide 7 saliva samples at home which involves putting a cotton dental swab in your mouth for a couple of minutes several times over the course of one day and then returning it to a special storage tube which we will provide. This is so that we can measure a chemical called cortisol that we believe is relevant to the stress-CVD link.

Day 8: You will take your last pill on this morning and then be invited to an appointment at UCL which will take approximately 3 hours. At this appointment we will ask you to complete some questionnaires and, following this, we will fit your arm with a small butterfly needle which will remain in place for the duration of the testing session so we won't have to stick you several times. We will then ask you to carry out some challenging mental tasks. During the testing session we will take further saliva samples and 4 sets of blood samples from the needle fitted in your arm. At the end of the session you will receive a £50 honorarium as a token of our gratitude.

Why do we take blood samples?

We are taking your blood to check for different markers that we believe are relevant to the stress-CVD pathway. On each blood-draw we will take approximately 35ml (about 6 teaspoons). This is about 140ml in total across the testing session, which is below a third of what is taken when you go to donate blood (470ml).

What are the possible benefits of taking part?

By taking part in this study you will receive a complete and accurate body composition report with information like waist-to-hip ratio, amount of body fat, amount of muscle, body mass index, etc. This can be interesting as many people are routinely unaware of their body composition and this objective report will allow you to evaluate this. You will also receive a final report when the research data has been analysed describing the results of the study.

What are the possible disadvantages of taking part?

It is possible that some of the questionnaire items may be sensitive in nature – if there are any items you do not wish to answer it is ok to skip them. It is unlikely but possible that the study medications may cause some adverse effects. Details of the possible side-effects

are detailed on the study medications information sheet provided. Over the week where you are taking the study medication if any health problems become apparent that may require medical attention we will advise you to contact your GP so that you can seek medical advice as soon as possible. During the time you will be with us in the lab and blood is being taken, we will have a medically qualified professional who will be able to assist in the unlikely event anything happens to you. We realise that not everyone likes having blood samples taken, but this is a crucial part of the study. The procedure for obtaining the blood sample may cause a little discomfort or small bruising. Blood will be taken by a qualified research nurse and they will follow procedures and take appropriate precautions to minimise any discomfort.

What if there is a problem?

In the event of any health concerns that arise over the course of the study you will be asked to contact one of the researchers who can facilitate your withdrawal from the study without penalty if you so desire and invite you to consult your GP or mental health professional. **Complaints:** If you wish to complain about any aspect of the way you have been approached or treated as part of this study, you should initially contact the study researchers that will do their best to answer your questions. If you remain unhappy and wish to complain formally, please email the Chair of the UCL Committee for the Ethics of Non-NHS Human Research (gradschoolhead@ucl.ac.uk) or send a letter to The Graduate School, North Cloisters, Wilkins Building, UCL, Gower Street, London WC1E 6BT) quoting reference: 5203/001. All communication will be dealt in strict confidence.

Will my taking part be confidential?

All results obtained will be strictly confidential and will only be used for medical research purposes. All personal information will be coded and kept separately to your name and address so that you cannot be recognised from it. All paper questionnaires will be kept in

locked filing cabinets, in locked offices, accessible only to members of the research team. In compliance with UCL regulations all data will be stored in this way for up to 10 years before being destroyed. You may withdraw your data from the project at any time up until it is used in the final report (January 2015).

What will happen to the results of the research study?

The results will be statistically analysed and findings subsequently published in scientific journals and presented at scientific meetings and conferences. You will not be identified in any publication.

Contact for further information:

If you have any questions or concerns please contact the research team (Amy Ronaldson, Livia Carvalho, Argita Zalli) at the Department of Epidemiology and Public Health, University College London, 1-19 Torrington Place, London, WC1E 6BT. Telephone: 020 7679 1682; email: stresspathwaysstudy@gmail.com or a.ronaldson@ucl.ac.uk

Appendix C: Stress Pathways Study consent form

Confidential: Volunteer Informed Consent Form

THE STRESS PATHWAYS STUDY

PATIENT IDENTIFICATION NUMBER: _____

**PLEASE
INITIAL BOX**

1. I confirm that I have read and understood the participant information sheet dated 06/12/2013 (Version 1.1) for the above study. I have had the opportunity to consider the information, ask questions and have
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my legal rights being affected.
3. I understand that I am required to have a butterfly needle inserted into my arm and have four rounds of blood samples taken during the course of the study. Tissue samples will be used only as described in the information sheet and samples will be destroyed after the study.
4. I understand that I am required to provide saliva samples which will be used only as described in the information sheet.
5. I understand I must not take part if I meet any of the exclusion criteria detailed in the information sheet dated 06/12/2013 (Version 1.1)
6. I understand that I am being paid for my assistance in this research and that some of my personal details will be passed to UCL Finance for administration purposes.
7. I understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.
8. I agree to take part in the above study.

Participant:..... Date:..... Signature:.....

Researcher:..... Date:..... Signature:.....

Appendix D: Baseline questionnaire – Stress Pathways Study

Thank you very much for agreeing to be involved in the Stress Pathways Study. We would like to get information about your health and lifestyle in order to interpret the biological response data we will collect in the study. We should be most grateful if you could take the time to complete this booklet during your first appointment at University College London.

The answers to these questions will of course be kept strictly confidential. The information will be anonymised before being analysed, and it will not be possible to identify your responses from any reports or publications or from the database. None of the information will be made available to anyone else.

Most of the questions can be answered by ticking the appropriate answer.

For example:

‘I am relaxed’

- Strongly disagree
- Disagree
- Neither agree nor disagree
- Agree
- Strongly agree

Other questions ask you to circle a number on a scale to indicate **the extent to which** you agree with a statement, the lowest number indicating complete disagreement and the highest indicating complete agreement.

For example:

“Over the past two weeks I have been able to relax...”

0	1	2	3	4	5	6	7	8	9	10
None of the time										All of the time

Please be sure to read the instructions to each section carefully. After you have completed the questionnaire **please check through all the pages** to make sure you haven’t missed any out.

This questionnaire should take approximately 20 minutes to complete.

Once again, thank you very much for your cooperation

Section 1: About your personal details, education, and work history

1. Today's date: ____ / ____ / ____

2. Age: _____

3. Date of Birth: ____ / ____ / ____

4. Sex: Male Female

5. Marital status:

Single Married Living as Married Separated
 Divorced Widowed Other: (please specify) _____

6. Which category do you feel best describes your ethnic origin?

WHITE

White British
 White Irish
 Other White background (Please specify)

ASIAN or ASIAN BRITISH

Indian
 Pakistani
 Bangladeshi
 Other Asian background (Please specify)

MIXED

White and Black Caribbean
 White and Black African
 White and Asian
 Black and Asian
 Other mixed background (Please specify)

BLACK or BLACK BRITISH

Black Caribbean
 African
 Other Black background (Please specify) _____

CHINESE

OTHER ETHNIC GROUP (Please specify) _____

7. Country of birth: _____

8. What educational qualifications do you have? Tick all that apply.

School Certificate GCSEs /O-levels/CSEs A-levels
 Undergraduate degree Postgraduate degree None
 Other: _____

9. How would you best describe your current employment status? Please tick all that apply.

- Employed full-time Employed part-time Self-employed Student
 Unemployed Volunteer Disabled

10. If currently employed, what is your job title?

Job title: _____

11. What educational qualifications do your parents have? Tick highest that applies.

Father

- School Certificate GCSEs /O-levels/CSEs A-levels
 Undergraduate degree Postgraduate degree None
 Don't know
 Other: _____

Mother

- School Certificate GCSEs /O-levels/CSEs A-levels
 Undergraduate degree Postgraduate degree None
 Don't know
 Other: _____

Section 2: Your Health and Wellbeing

12. Please read each group of statements carefully and then pick out the **one** statement in each group that best describes the way you have been feeling during the ***past week, including today.***

a) Sadness

- 0 I do not feel sad
1 I feel sad much of the time
2 I am sad all the time
3 I am so sad or unhappy that I can't stand it

b) Pessimism

- 0 I am not discouraged about my future
1 I feel more discouraged about my future than I used to be
2 I do not expect things to work out for me
3 I feel my future is hopeless and will only get worse

c) Past Failure

- 0 I do not feel like a failure
1 I have failed more than I should have
2 As I look back, I see a lot of failures
3 I feel I am a total failure as a person

d) Loss of Pleasure

- 0 I get as much pleasure as I ever did from the things I enjoy
1 I don't enjoy things as much as I used to
2 I get very little pleasure from the things I used to enjoy
3 I can't get any pleasure from the things I used to enjoy

e) Guilty Feelings

- 0 I don't feel particularly guilty
- 1 I feel guilty over many things I have done or should have done
- 2 I feel quite guilty most of the time
- 3 I feel guilty all of the time

f) Punishment Feelings

- 0 I don't feel I am being punished
- 1 I feel I may be punished
- 2 I expect to be punished
- 3 I feel I am being punished

g) Self-Dislike

- 0 I feel the same about myself as ever
- 1 I have lost confidence in myself
- 2 I am disappointed in myself
- 3 I dislike myself

h) Self-Criticalness

- 0 I don't criticise or blame myself more than usual
- 1 I am more critical of myself than I used to be
- 2 I criticise myself for all of my faults
- 3 I blame myself for everything bad that happens

i) Suicidal Thoughts or Wishes

- 0 I don't have any thoughts of killing myself
- 1 I have thoughts of killing myself, but I would not carry them out
- 2 I would like to kill myself
- 3 I would kill myself if I had the chance

j) Crying

- 0 I don't cry any more than I used to
- 1 I cry more than I used to
- 2 I cry over every little thing
- 3 I feel like crying, but I can't

k) Agitation

- 0 I am no more restless or wound up than usual
- 1 I feel more restless or wound up than usual
- 2 I am so restless or agitated that it's hard to stay still
- 3 I am so restless or agitated that I have to keep moving or doing something

l) Loss of Interest

- 0 I have not lost interest in other people or activities
- 1 I am less interested in other people or things than before
- 2 I have lost most of my interest in other people or things
- 3 It's hard to get interested in anything

m) Indecisiveness

- 0 I make decisions about as well as ever
- 1 I find it more difficult to make decisions than usual
- 2 I have much greater difficulty in making decisions than I used to
- 3 I have trouble making any decisions

n) Worthlessness

- 0 I do not feel I am worthless
- 1 I don't consider myself as worthwhile and useful as I used to
- 2 I feel more worthless as compared to other people
- 3 I feel utterly worthless

o) Loss of Energy

- 0 I have as much energy as ever
- 1 I have less energy than I used to have
- 2 I don't have enough energy to do very much
- 3 I don't have enough energy to do anything

p) Changes in Sleeping Pattern

- 0 I have not experienced any change in my sleeping pattern

- 1a I sleep somewhat more than usual
- 1b I sleep somewhat less than usual

- 2a I sleep a lot more than usual
- 2b I sleep a lot less than usual

- 3a I sleep most of the day
- 3b I wake up 1-2 hours early and can't get back to sleep

q) Irritability

- 0 I am no more irritable than usual
- 1 I am more irritable than usual
- 2 I am much more irritable than usual
- 3 I am irritable all the time

r) Changes in Appetite

- 0 I have not experienced any change in my appetite

- 1a My appetite is somewhat less than usual
- 1b My appetite is somewhat greater than usual

- 2a My appetite is much less than before
- 2b My appetite is much greater than usual

- 3a I have no appetite at all
- 3b I crave food all the time

s) Concentration Difficulty

- 0 I can concentrate as well as ever
- 1 I can't concentrate as well as usual
- 2 It's hard to keep my mind on anything for very long
- 3 I find I can't concentrate on anything

t) Tiredness or Fatigue

- 0 I am no more tired or fatigued than usual
- 1 I get more tired or fatigued more easily than usual
- 2 I am too tired or fatigued to do a lot of the things I used to do
- 3 I am too tired or fatigued to do most of the things I used to do

u) Loss of Interest in Sex

- 0 I have not noticed any recent changes in my interest in sex
- 1 I am less interested in sex than I used to be
- 2 I am much less interested in sex now
- 3 I have lost interest in sex completely

13. Tick the reply which comes closest to how you have been feeling in the ***past week***:

a) I feel tense or 'wound-up':

- Most of the time
- A lot of the time
- Time to time, occasionally
- Not at all

c) Worrying thoughts go through my mind:

- A great deal of the time
- A lot of the time
- From time to time but not too often
- Only occasionally

b) I get a sort of frightened feeling as if something awful is about to happen:

- Very definitely and quite badly
- Yes, but not too badly
- A little, but it doesn't worry me
- Not at all

d) I can sit at ease and feel relaxed:

- Definitely
- Usually
- Not often
- Not at all

- e) I get a sort of frightened feeling like 'butterflies' in the stomach:
- Very often
 Quite often
 Occasionally
 Not at all
- g) I get sudden feelings of panic:
- Very often indeed
 Quite often
 Not very often
 Not at all

- f) I feel restless as if I have to be on the move:
- Very much indeed
 Quite a lot
 Not very much
 Not at all

14. The questions in this scale ask you about your feelings and thoughts **during the past week.**

- a) In the last month, how often have you been upset because of something that happened unexpectedly?
- Never
 Almost never
 Sometimes
 Fairly often
 Very often

- d) In the last month, how often have you felt confident about your ability to handle your personal problems?
- Never
 Almost never
 Sometimes
 Fairly often
 Very often

- b) In the last month, how often have you felt that you were unable to control the important things in your life?
- Never
 Almost never
 Sometimes
 Fairly often
 Very often

- e) In the last month, how often have you felt that things were going your way?
- Never
 Almost never
 Sometimes
 Fairly often
 Very often

- c) In the last month, how often have you felt nervous and 'stressed'?
- Never
 Almost never
 Sometimes
 Fairly often
 Very often

- f) In the last month, how often have you found that you could not cope with all the things you had to do?
- Never
 Almost never
 Sometimes
 Fairly often
 Very often

g) In the last month, how often have you been able to control irritations in your life?

- Never
- Almost never
- Sometimes
- Fairly often
- Very often

i) In the last month, how often have you been angered because of things that were outside of your control?

- Never
- Almost never
- Sometimes
- Fairly often
- Very often

h) In the last month, how often have you felt that you were on top of things?

- Never
- Almost never
- Sometimes
- Fairly often
- Very often

j) In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?

- Never
- Almost never
- Sometimes
- Fairly often
- Very often

15. Please read each item and then circle the number which is most appropriate. Indicate to what extent you have felt this way during the ***past week***:

a) Interested	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
b) Excited	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
c) Strong	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
d) Enthusiastic	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
e) Proud	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
f) Alert	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
g) Inspired	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
h) Determined	1	2	3	4	5
	<i>Never</i>				<i>Always</i>

i) Attentive				
1	2	3	4	5
<i>Never</i>				<i>Always</i>
j) Active				
1	2	3	4	5
<i>Never</i>				<i>Always</i>

16. In the ***past week***...

a) My sleep was restless...

- Not at all
 A little bit
 Somewhat
 Quite a bit
 Very much

b) I was satisfied with my sleep...

- Not at all
 A little bit
 Somewhat
 Quite a bit
 Very much

c) My sleep was refreshing...

- Not at all
 A little bit
 Somewhat
 Quite a bit
 Very much

f) I had trouble sleeping...

- Not at all
 A little bit
 Somewhat
 Quite a bit
 Very much

d) I had difficulty falling asleep...

- Not at all
 A little bit
 Somewhat
 Quite a bit
 Very much

g) I got enough sleep

- Not at all
 A little bit
 Somewhat
 Quite a bit
 Very much

e) I had trouble staying asleep

- Never
 Rarely
 Sometimes
 Often
 Always

h) My sleep quality was...

- Very poor
 Poor
 Fair
 Good
 Very good

Section 3: Your Lifestyle

About smoking...

17. Do you smoke cigarettes, cigars or pipes? Please specify.

- Yes No – **Go to question 20** Type: _____

If yes:

18. How many per day do you smoke? _____
19. How long have you smoked for? _____ years and _____ months
20. If not a current smoker, did you smoke in the past?
 Yes No – **Go to question 23**

If yes:

21. When did you quit smoking? _____
22. Are you currently taking nicotine replacement therapy? Yes No

About drinking...

23. Thinking of the **last 7 days**, how much of each of the following did you drink? (If it helps, think back over each day to this time last week). If none, please enter 0.

		Mon	Tues	Wed	Thurs	Fri	Sat	Sun
a) Beer, lager, cider	pints	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
b) Wine	glasses	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
c) Martini, sherry, port	glasses	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
d) Spirits	measures	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
e) Other alcoholic drinks	glasses	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>

24. In the last year how often have you had a hangover from drinking alcohol? Select one only.

- At least once a week
- 2-3 times a month
- Once a month
- Less than once a month
- Not at all in the last year

About exercise...

25. How often do you take part in sports or activities that are mildly energetic, moderately energetic or vigorous? See details below.

Tick one answer for each question

	Never or hardly ever	About 1-3 times a month	Once or twice a week	3 times a week or more
a. Mildly energetic (for example, walking, bicycle repair, playing darts, general housework)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
b. Moderately energetic (for example, scrubbing, dancing, golf, cycling, decorating, leisurely swimming)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c. Vigorous (for example, running, hard swimming, tennis, squash, cycle racing)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please give the average number of **hours per week** you spend in such sports or activities

d. Mildly energetic	<input type="text"/>
e. Moderately energetic	<input type="text"/>
f. Vigorous	<input type="text"/>

26. Thinking about the days of the **past week**:

On average, for how long did you **walk** outside your home/workplace?
(If you did not walk, please enter '0' in each box)

	hours	minutes
On each weekday	<input type="text"/>	<input type="text"/>
On each weekend day	<input type="text"/>	<input type="text"/>

On average for how long did you cycle?
(If you did not cycle, please enter '0' in each box)

	hours	minutes
On each weekday	<input type="text"/>	<input type="text"/>
On each weekend day	<input type="text"/>	<input type="text"/>

Thank you very much for taking the time to complete this questionnaire.

Please remember to return it to the experimenter before you leave this testing session

Appendix E: Follow-up questionnaire – Stress Pathways Study

1. Please read each group of statements carefully and then pick out the **one** statement in each group that best describes the way you have been feeling during the ***past week, including today.***

a) Sadness

- 0 I do not feel sad
- 1 I feel sad much of the time
- 2 I am sad all the time
- 3 I am so sad or unhappy that I can't stand it

b) Pessimism

- 0 I am not discouraged about my future
- 1 I feel more discouraged about my future than I used to be
- 2 I do not expect things to work out for me
- 3 I feel my future is hopeless and will only get worse

c) Past Failure

- 0 I do not feel like a failure
- 1 I have failed more than I should have
- 2 As I look back, I see a lot of failures
- 3 I feel I am a total failure as a person

d) Loss of Pleasure

- 0 I get as much pleasure as I ever did from the things I enjoy
- 1 I don't enjoy things as much as I used to
- 2 I get very little pleasure from the things I used to enjoy
- 3 I can't get any pleasure from the things I used to enjoy

e) Guilty Feelings

- 0 I don't feel particularly guilty
- 1 I feel guilty over many things I have done or should have done
- 2 I feel quite guilty most of the time
- 3 I feel guilty all of the time

f) Punishment Feelings

- 0 I don't feel I am being punished
- 1 I feel I may be punished
- 2 I expect to be punished
- 3 I feel I am being punished

g) Self-Dislike

- 0 I feel the same about myself as ever
- 1 I have lost confidence in myself
- 2 I am disappointed in myself
- 3 I dislike myself

h) Self-Criticalness

- 0 I don't criticise or blame myself more than usual
- 1 I am more critical of myself than I used to be
- 2 I criticise myself for all of my faults
- 3 I blame myself for everything bad that happens

i) Suicidal Thoughts or Wishes

- 0 I don't have any thoughts of killing myself
- 1 I have thoughts of killing myself, but I would not carry them out
- 2 I would like to kill myself
- 3 I would kill myself if I had the chance

j) Crying

- 0 I don't cry any more than I used to
- 1 I cry more than I used to
- 2 I cry over every little thing
- 3 I feel like crying, but I can't

k) Agitation

- 0 I am no more restless or wound up than usual
- 1 I feel more restless or wound up than usual
- 2 I am so restless or agitated that it's hard to stay still
- 3 I am so restless or agitated that I have to keep moving or doing something

l) Loss of Interest

- 0 I have not lost interest in other people or activities
- 1 I am less interested in other people or things than before
- 2 I have lost most of my interest in other people or things
- 3 It's hard to get interested in anything

m) Indecisiveness

- 0 I make decisions about as well as ever
- 1 I find it more difficult to make decisions than usual
- 2 I have much greater difficulty in making decisions than I used to
- 3 I have trouble making any decisions

n) Worthlessness

- 0 I do not feel I am worthless
- 1 I don't consider myself as worthwhile and useful as I used to
- 2 I feel more worthless as compared to other people
- 3 I feel utterly worthless

o) Loss of Energy

- 0 I have as much energy as ever
- 1 I have less energy than I used to have
- 2 I don't have enough energy to do very much
- 3 I don't have enough energy to do anything

p) Changes in Sleeping Pattern

- 0 I have not experienced any change in my sleeping pattern

- 1a I sleep somewhat more than usual
- 1b I sleep somewhat less than usual

- 2a I sleep a lot more than usual
- 2b I sleep a lot less than usual

- 3a I sleep most of the day
- 3b I wake up 1-2 hours early and can't get back to sleep

q) Irritability

- 0 I am no more irritable than usual
- 1 I am more irritable than usual
- 2 I am much more irritable than usual
- 3 I am irritable all the time

r) Changes in Appetite

- 0 I have not experienced any change in my appetite

- 1a My appetite is somewhat less than usual
- 1b My appetite is somewhat greater than usual

- 2a My appetite is much less than before
- 2b My appetite is much greater than usual

- 3a I have no appetite at all
- 3b I crave food all the time

s) Concentration Difficulty

- 0 I can concentrate as well as ever
- 1 I can't concentrate as well as usual
- 2 It's hard to keep my mind on anything for very long
- 3 I find I can't concentrate on anything

t) Tiredness or Fatigue

- 0 I am no more tired or fatigued than usual
- 1 I get more tired or fatigued more easily than usual
- 2 I am too tired or fatigued to do a lot of the things I used to do
- 3 I am too tired or fatigued to do most of the things I used to do

u) Loss of Interest in Sex

- 0 I have not noticed any recent changes in my interest in sex
- 1 I am less interested in sex than I used to be
- 2 I am much less interested in sex now
- 3 I have lost interest in sex completely

2. Tick the reply which comes closest to how you have been feeling in the ***last week, including today:***

a) I feel tense or 'wound up':

- Most of the time
- A lot of the time
- Time to time, occasionally
- Not at all

b) I get a sort of frightened feeling as if something awful is about to happen:

- Very definitely and quite badly
- Yes, but not too badly
- A little, but it doesn't worry me
- Not at all

c) Worrying thoughts go through my mind:

- A great deal of the time
- A lot of the time
- From time to time but not too often
- Only occasionally

d) I can sit at ease and feel relaxed:

- Definitely
- Usually
- Not often
- Not at all

e) I get a sort of frightened feeling like 'butterflies' in the stomach:

- Very often
- Quite often
- Occasionally
- Not at all

f) I feel restless as if I have to be on the move:

- Very much indeed
- Quite a lot
- Not very much
- Not at all

g) I get sudden feelings of panic:

- Very often indeed
- Quite often
- Not very often
- Not at all

3. In the ***past week...***

a) My sleep was restless...

- Not at all
- A little bit
- Somewhat
- Quite a bit
- Very much

c) My sleep was refreshing...

- Not at all
- A little bit
- Somewhat
- Quite a bit
- Very much

b) I was satisfied with my sleep...

- Not at all
- A little bit
- Somewhat
- Quite a bit
- Very much

d) I had difficulty falling asleep...

- Not at all
- A little bit
- Somewhat
- Quite a bit
- Very much

e) I had trouble staying asleep

- Never
- Rarely
- Sometimes
- Often
- Always

g) I got enough sleep

- Not at all
- A little bit
- Somewhat
- Quite a bit
- Very much

f) I had trouble sleeping...

- Not at all
- A little bit
- Somewhat
- Quite a bit
- Very much

h) My sleep quality was...

- Very poor
- Poor
- Fair
- Good
- Very good

4. Please read each item and then circle the number which is most appropriate. Indicate to what extent you have felt this way during the **past week**:

a) Interested	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
b) Excited	1		3	4	5
	<i>Never</i>	2			<i>Always</i>
c) Strong	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
d) Enthusiastic	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
e) Proud	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
f) Alert	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
g) Inspired	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
h) Determined	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
i) Attentive	1	2	3	4	5
	<i>Never</i>				<i>Always</i>
j) Active	1	2	3	4	5
	<i>Never</i>				<i>Always</i>

5. Did you have any significant symptoms or medical problems since the last study visit?

If your answer to this question is 'yes' please detail the symptoms/problems in the space provided below.

Yes No

6. Would you say that any medical problem experienced in the last week was due to the study medication?

If your answer to this question is 'yes' please provide us with the specific medical problem below.

Yes No

7. If you can, please indicate below which medication you think you have been taking for the last 7 days.

Please also provide a reason for your answer in the space below.

Escitalopram (antidepressant) Propranolol (beta-blocker) Placebo (inert)

Thank you very much for taking the time to complete this questionnaire.

Appendix F: Example page from the cortisol sampling diary (Stress Pathways Study)

TUBE 1 : AS SOON AS YOU WAKE UP					
What is the time now?		_____ a.m. / p.m.			
What was the exact time you collected the sample?		_____ a.m. / p.m.			
Was there a delay between waking up and collecting your first sample? Yes No					
↳ If yes, how long? ____ hrs & ____ mins					
In the last 30 minutes how much did you feel....					
	Not at all				Very much
In control	1	2	3	4	5
Tired	1	2	3	4	5
Happy	1	2	3	4	5
Frustrated or angry	1	2	3	4	5
Sad	1	2	3	4	5
Stressed	1	2	3	4	5
Pain	1	2	3	4	5
If you talked with others, how pleasant was the interaction?					
<i>Not applicable</i> <input type="checkbox"/>	1	2	3	4	5
In the last 30 minutes, but before you collected your sample did you....					
Brush your teeth			No		Yes
Drink any tea, coffee or other caffeinated drinks			No		Yes
Take any medicines			No		Yes
Eat a meal			No		Yes
Drink any alcohol			No		Yes
Do any exercise?			No		Yes
Smoke any cigarettes?			No		Yes

Appendix G: Subjective stress and task impact questionnaires

The STRESS PATHWAYS Study

Rest Questionnaire

Please answer the following questions by circling the number that best describes the way you feel

1. How relaxed do you feel at the moment?

Not at all
relaxed

Very
relaxed

1

2

3

4

5

6

7

2. How anxious do you feel at the moment?

Not at all
anxious

Very
anxious

1

2

3

4

5

6

7

3. How stressed do you feel at the moment?

Not at all
stressed

Very
stressed

1

2

3

4

5

6

7

The STRESS PATHWAYS Study

Task Impact Questionnaire

Please answer the following questions by circling the number that best describes the way you felt during the task

1. How difficult did you find the task?

Not at all difficult							Very difficult
1	2	3	4	5	6	7	

2. How involved in the task did you feel?

Not at all involved							Very involved
1	2	3	4	5	6	7	

3. How well do you think you performed the task?

Not at all well							Very well
1	2	3	4	5	6	7	

4. How stressed did you feel during the task?

Not at all stressed							Very stressed
1	2	3	4	5	6	7	

5. How much in control of the task did you feel?

Not at all in control							Very in control
1	2	3	4	5	6	7	

6. How relaxed did you feel during the task?

Not at all relaxed							Very relaxed
1	2	3	4	5	6	7	

The STRESS PATHWAYS Study

Recovery Questionnaire

Please answer the following questions by circling the number that best describes the way you feel

1. How relaxed do you feel at the moment?

Not at all
relaxed

Very
relaxed

1 2 3 4 5 6 7

2. How anxious do you feel at the moment?

Not at all
anxious

Very
anxious

1 2 3 4 5 6 7

3. How stressed do you feel at the moment?

Not at all
stressed

Very
stressed

1 2 3 4 5 6 7