

Depression and inflammation: implications for cardiovascular disease

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Student Declaration

I, Samantha Lawes, 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.

Signed: _____ Date: _____

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Abstract

There is extensive evidence linking inflammation with depression, but the nature of this relationship remains unclear. This PhD consisted of two studies that aimed to investigate the interaction of immune and neuroendocrine function in major depressive disorder (MDD) and examine the effects of depressive symptoms and inflammation on mortality risk.

Study 1 sought to garner more information about the relationship between immune activation and HPA-axis function using data from an observational, case-control study including people with MDD and healthy controls.

In Study 1a differences in inflammatory cytokines and HPA-axis function, and associations between them were examined. Results indicated increased inflammation, a flattened cortisol awakening response under the curve (CAR AUC) and impaired corticosteroid function in depressed people. Furthermore there was evidence of a negative association between inflammation and diurnal cortisol rhythm.

In Study 1b changes in depressive symptoms, inflammatory cytokines and HPA-axis function were investigated longitudinally in people with MDD. The results demonstrated an improvement in depressive symptoms and mineralocorticoid sensitivity. We also observed a trend towards an improvement in the CAR AUC and an apparent trend towards a negative pattern of association between change in inflammation and change in the CAR.

In Study 1c differences in Treg frequency were assessed between people with MDD and healthy controls. The findings showed no difference between the groups and no association with either inflammation or HPA-axis function.

Study 2 investigated the combined effects of depressive symptoms and inflammation on CVD and all-cause mortality risk in a longitudinal cohort study of older people. The results

showed that the combination of both factors confers a considerable increase in CVD mortality risk for men.

Together these studies indicate that there is a relationship between inflammation and HPA-axis dysfunction in MDD and that men with depressive symptoms and increased inflammation constitute a high-mortality risk phenotype.

Impact Statement

There are a number of ways in which the knowledge presented in this thesis could be/has been put to a beneficial use. These include:

- i. Future research: Depression affects over 300 million people worldwide, with the majority of patients failing to make a complete recovery, therefore it remains a key area of research. Efforts to identify the biological underpinnings of depression are aimed at identifying people who are at risk of developing the condition and finding more effective treatments. This is one of the first pieces of empirical work comprehensively investigating the relationship between immune and endocrine dysfunction in people with depression. By linking two areas of research that have been largely investigated separately, this research fills a critical gap in the literature and opens up new challenges and opportunities for academic enquiry.

- ii. Clinical practice: Current National Institute for Health and Clinical Excellence (NICE) guidelines recommend the use of the QRISK2 risk assessment tool to assess risk for the primary prevention of CVD. To date this has not included measures of circulating inflammatory markers. There has been some debate about whether inflammation should be included, with the main uncertainty being whether the modest increases in risk associated with higher inflammation have clinically meaningful effects on health. The findings from this PhD demonstrates that men with comorbid depressive symptoms and high inflammation are almost four times as likely to die from CVD and therefore do constitute a clinically meaningful risk category. In light of this, it might be worth considering inflammation as a cardiovascular risk factor in depressed men. This could help identify patients who may benefit from targeted preventative treatment, such as anti-inflammatories. It may also improve screening efficacy and cardiovascular outcomes.

- iii. I have presented my work on the ELSA study at the Psychoneuroimmunology Research Society conference (2016) and it was subsequently published in the journal *Psychological Medicine* (2018). During my PhD I also contributed to an article examining whether physical activity explained the association between elevated inflammation and subsequent depressive symptoms which was published in *Brain, Behaviour and Immunity* (2019). I hope to submit a paper from the Resist Study in 2020.

- iv. I have included the findings from my research into academic lectures given to Medical (MBBS BSc) students, Population Health Sciences (BSc) students and Health, Nutrition and Exercise (BSc) students. I have also incorporated this knowledge into non-academic presentations within the National Health Service (NHS), including community cardiac nurses and psychological well-being practitioners. I also co-led the Healthy Psychology module for the BSc in Population Health Sciences, assisting with curriculum design, and wrote exam questions for the Medical students.

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List of Abbreviations

ACS: Acute coronary syndrome

ACTH: Adrenocorticotrophic hormone

APA: American Psychological Association

AUC: Area under the curve

BDI: Beck Depression Inventory

BDNF: Brain-derived neurotrophic factor

BMI: Body Mass index

CABG: Coronary artery bypass graft

cAMP: Cyclic adenosine monophosphate

CAR: Cortisol awakening response

CBT: Cognitive behavioural therapy

CES-D: Center for Epidemiological Studies-Depression

CHD: Coronary heart disease

CI: Confidence interval

CISR: Clinical Interview Schedule-Revised

CNS: Central nervous system

CRH: Corticotropin-releasing-hormone

CRP: C-reactive protein

CSF: Cerebrospinal fluid

CVD: Cardiovascular disease

DA: Dopamine

DEX: Dexamethasone

DIGS: Diagnostic interview for genetic studies

DSH: Data Safe Haven

DST: Dexamethasone suppression test

DSM-V: Diagnostic and Statistical Manual of Mental Disorders

DST: Dexamethasone suppression test

ES: Effect size

ELISA: Enzyme-linked immunosorbent assay

ELSA: English Longitudinal Study of Ageing

FACS: Fluorescence-activated cell sorting

FMO: Fluorescence minus one

fMRI: functional magnetic resonance imaging

GAD: Generalised anxiety disorder

GR: Glucocorticoid receptor

GRE: Glucocorticoid response element

GC: Glucocorticoid

GHQ: General health questionnaire

GWAS: Genome wide association study

HRSD: Hamilton Rating Scale for Depression

HF: Heart Failure

HIV: Human immunodeficiency virus

HPA: Hypothalamic-pituitary-adrenal

HRV: Heart-rate variability

HS: High sensitivity

HSE: Health Survey for England

IAPT: Improving access to psychological therapies

ICD-10 International: International Classification of Diseases and Related Health Problems

IDO: Indoleamine 2,3-dioxygenase

IFN- α : Interferon-alpha

IFN- γ : Interferon-gamma

IL: Interleukin

IL-1 β : Interleukin-1 beta

IL-1 α : Interleukin-1 receptor alpha

LPS: Lipopolysaccharide

MDD: Major depressive disorder

MFQ: Mood and feelings questionnaire

MI: Myocardial infarction

MR: Mineralocorticoid receptor

MRI: Magnetic resonance imaging

mRNA: Messenger RNA

NA: Noradrenaline

NHS: National Health Service

NICE: National Institute for Health and Clinical Excellence

NSAID: Non-steroidal anti-inflammatory drug

PMBC: Peripheral blood mononuclear cell

PET: Positron emission tomography

pg/ml: Picograms/millilitre

PRED: Prednisolone

PST: Prednisolone suppression test

PSNS: Parasympathetic nervous system

PTSD: Post traumatic stress disorder

PVN: Paraventricular nucleus

RA: Rheumatoid arthritis

RR: Relative risk

SCD: Sudden cardiac death

SES: Socioeconomic status

SLC6A4: Serotonin transporter gene

SLE: Systemic lupus erythematosus

SMD: Standard mean difference

SNP: Single nucleotide polymorphism

SNS: Sympathetic nervous system

SNRI: Selective noradrenalin reuptake inhibitor

SSRI: Selective serotonin reuptake inhibitor

TCA: Tricyclic antidepressant

TGF- β : Tumour necrosis factor-beta

Tregs: Regulatory T-cells

TNF- α : Tumour necrosis factor-alpha

TRD: Treatment resistant depression

TRP: Tryptophan

TSST: Trier social stress test

WHO: World Health Organisation

WMD: Weighted mean difference

VSMCs: Vascular smooth muscle cells

5-HT: 5-hydroxytryptamine (Serotonin)

5-HTT: Serotonin transporter

5-HTTLPR: Serotonin-transporter-linked promoter region

1. Literature review: Depression and inflammation

1.1 Introduction

This chapter will describe the role of inflammation in the pathogenesis of depression. This will begin with a brief overview of the prevalence and mortality data regarding depression, and current guidelines for diagnosis. Following this evidence for the role of immune dysregulation in the pathogenesis of depression will be presented, with a particular focus on interaction between inflammatory activation and neuroendocrine dysfunction. Additionally, this chapter will describe the evidence for alterations in adaptive immunity in people with depression. The aim of this chapter is to demonstrate the complexity of the biology of depression and highlight some of the limitations of current work to date.

1.2 Depression: Prevalence and diagnosis

1.2.1 Prevalence

Depression is a common yet devastating mental disorder, characterized by low mood, a reduced ability to experience pleasure, complex psychological, physical and social symptoms and in severe cases, suicidal ideation. Depression affects over 300 million people, is the leading cause of disability worldwide and has become a major contributor to the overall global burden of disease (World Health Organisation, 2017b). In the UK, one in five people become depressed during their lifetime (Royal College of Psychiatrists, 2011). A number of socio-demographic correlates of depression are consistent across cultures. Women are twice as likely to develop depression compared with men and being separated, divorced or widowed is also associated with increased risk. The relationship between depression and age varies across countries. In high income countries, younger

people (18-34 years) are more likely to develop depression than older people (65+), however in low income countries being younger is associated with reduced risk. Low income is associated with depression in high income countries, however in low-middle income countries no significant association has been observed (Kessler & Bromet, 2013).

In addition to the psychological effects of depression, this disorder is associated with a wide variety of physical health conditions such as asthma, rheumatoid arthritis, inflammatory bowel disease, metabolic syndrome, coronary heart disease, diabetes mellitus and chronic pain, thereby further increasing its burden of disease (Slavich & Irwin, 2014). Furthermore, the presence of depressive symptoms worsens health status substantially more when comorbid with these diseases, than when the diseases occur alone (Moussavi et al., 2007). Depression has also been associated with infectious diseases. In patients with human immunodeficiency virus (HIV) infection, depression negatively affects disease progression, leading to increased risk of developing Acquired Immune Deficiency Syndrome (AIDS) as well as an increased likelihood of AIDS-related death (Leserman, 2008). The association between depression and physical health outcome also appears to be bi-directional. A systematic review and meta-analysis showed that the risk of depression is trebled for people with two or more chronic physical conditions compared to those with no physical condition (Read et al., 2017).

Epidemiological studies have consistently demonstrated an increased mortality associated with depression in both community and psychiatric samples (Cuijpers & Smit, 2002;Lasserre et al., 2016;L. A. Pratt et al., 2016;E. R. Walker et al., 2015;Wulsin et al., 2005;Wulsin et al., 1999) and in people suffering from stroke (Bartoli et al., 2013), coronary heart disease (CHD) (Barth et al., 2004;Fan et al., 2014;Q. Wu & Kling, 2016), chronic kidney disease (Palmer et al., 2013), cancer (Chida et al., 2008) and diabetes (Lin et al., 2009). Depression has also been specifically associated with mortality in studies of older adults (Geerlings et al., 2002;Schoevers et al., 2009;Teng et al., 2013).

However, there is some inconsistency in the literature, with some studies reporting null findings (Callahan et al., 1998;Cuijpers, 2001;Hybels et al., 2002;McCusker et al., 2006). A meta-analysis of 293 prospective studies, including 1,813,733 participants from 35 countries, associating depression at baseline with increased mortality at follow-up, was conducted by Cuijpers et al. (2014). The analysis included studies of general community samples as well as specific patient groups. The authors reported that the risk of mortality in depressed people was 1.52 times that in non-depressed people. Furthermore, the risk was similar between specific patient populations as well as healthy community-based samples. This finding suggests that the association between depression and mortality may be better explained by dysfunction in common biological pathways or lifestyle factors, as opposed to disease-specific mechanisms.

Differences in mortality according to symptom severity has also been explored. A meta-analysis of 22 studies, including 18,705 participants, was conducted by Cuijpers et al. (2013) and compared patients with MDD (major depressive disorder), also known as clinical depression, to individuals with subthreshold depression, identified by the presence of depressive symptoms using self-report questionnaires. The authors reported that both MDD and subthreshold depression were associated with mortality. Furthermore there was no significant difference in mortality risk between the two types of depression, suggesting that even mild symptoms of depression can increase the risk of death. The association between depressive symptoms and mortality across the full continuum of severity was investigated by White et al. (2015) using data from the English Longitudinal Study of Ageing (ELSA). Depressive symptoms were associated with increased mortality risk even when symptom severity was low. The duration of depressive symptoms has also been associated with mortality in a dose-response manner, using data from the same cohort (White et al., 2016).

It is still unclear exactly what mechanisms account for the increased mortality rate in depressed people, however it is likely to be partly explained by the mediating effects of

health behaviours. Compared with non-depressed individuals, people with depression are less likely to be physically active and more likely to be sedentary (Schuch et al., 2017), more likely to be smokers (Luger et al., 2014), more likely to engage in substance abuse (Davis et al., 2008), less likely to adhere to medication regimes for chronic illness (Grenard et al., 2011) and more likely to eat a pro-inflammatory diet, characterised by high levels of processed meats and trans fats (Lassale et al., 2018). Such general unhealthy lifestyles will inevitably contribute to the fact that depressed individuals are more at risk for adverse health outcomes, such as CHD, as these are also important risk factors for physical illness. However, health behaviours have been shown to explain only 62% of the mortality risk associated with depressive symptoms and 38.5% of the graded association between the duration of depressive symptoms and mortality risk (White et al., 2016; White et al., 2015). An alternative explanation is that depressive symptoms reflect physiological changes which increase the risk of mortality.

1.2.2 *Diagnosis*

In clinical practice, the most commonly used method for diagnosing MDD is the structured clinical interview. Interviews are based on diagnostic criteria from the *Diagnostic and Statistical Manual (DSM-V)* produced by the American Psychiatric Association (APA) (American Psychiatric Association, 2013) (see Table 1.1 for a summary of the DSM-V criteria for MDD) and the *International Statistical Classification of Diseases and Related Health Problems (ICD-10)* produced by the World Health Organization (WHO) (Montgomery, 2016).

According to the DSM-V, MDD is diagnosed by a combination of any five or more symptoms out of nine occurring during the same two-week period. One of these symptoms must be depressed mood or anhedonia (loss of interest or pleasure). Additional cognitive (worthlessness or guilt, concentration, indecisiveness, recurrent thoughts of suicide) and physiological (appetite, weight, sleep and movement) symptoms

need to be present and the symptoms need to disrupt normal social and occupational functioning. The frequency and duration requirements for each symptom during the two-week period vary but most have to be present “nearly every day”.

These criteria emerged out of a need to devise a common language for the purpose of both describing the disorder and providing treatment (B. A. Fischer, 2012). Whilst this system may appear to be largely broad and rather subjective, it has been useful and effective in clinical practice, in raising public awareness and in encouraging the development of specialized forms of psychotherapy (Goldberg, 2011; Sibille & French, 2013). This has resulted in the acceptance by many that depression is homogeneous in nature and that it is usually a ‘major’ disorder, an idea that is now being called into question.

Firstly, with the exception of the first symptom, all other eight symptoms contain sub-symptoms (e.g. fatigue or loss of energy). Secondly, three symptoms contain opposites (significant weight loss or gain, insomnia or hypersomnia and psychomotor agitation or retardation). This scoring method has the potential to create over 1,000 combinations of symptoms that would all qualify the individual for a diagnosis of MDD (Fried & Nesse, 2015a) resulting in four main symptom subtypes: melancholic, atypical, psychotic and anxious depression (Femke Lamers et al., 2018). Melancholic depression, also referred to as ‘endogenous’ or ‘typical’ depression, is characterised by loss of pleasure or nonreactive mood, plus three or more of the following symptoms: distinct quality of mood, mood that is worse in the morning, early morning awakening, psychomotor changes, weight loss or decreased appetite, excessive guilt and completed suicide. Atypical depression is characterised by mood reactivity plus two or more of the following symptoms: weight gain or increased appetite, hypersomnia, leaden paralysis, and interpersonal rejection sensitivity. Psychotic or ‘delusional’ depression is severe major depression with psychotic features, marked by delusions or hallucinations, as well as over-valued feelings of guilt and worthlessness, severe psychomotor disturbance and

cognitive symptoms, characterised by deficits in attention, psychomotor speed, executive functioning and memory (Harald & Gordon, 2012). Finally, anxious depression is usually described as major depression with a high level of anxiety, however it may also be identified as anxiety with depression to a lesser degree or depression caused by anxiety (Parker & Manicavasagar, 2005). Due to the fact that the other three subtypes are frequently comorbid with anxiety disorders, anxious depression may overlap with any of them. Furthermore, there is little evidence of any specific biological or psycho-social correlates of anxious depression other than an early age of onset, causing some to argue that it should not qualify as a specific subtype of depression (Harald & Gordon, 2012). In light of all this, it is hardly surprising that patients with depression exhibit a wide range of different symptoms.

It is also important to note that many mild cases of depression remit without any treatment at all, suggesting that they are a self-regulating, adaptive response to life stress (Wakefield, 1997). Depression may also be a response to drugs or may reflect hormonal dysfunction in physical illness such as seen in Cushing's syndrome (Goldberg, 2011). Symptoms may be confused with the depressed phase of bipolar disorder and there are five particular subtypes of depression that require a specific clinical approach (somatic, depression with panic attacks, depression in people with obsessional traits, depression accompanying known physical illnesses and pseudo-demented depression). Depression is also often co-morbid with other psychiatric disorders, such as anxiety and personality disorders.

Table 1.1 DSM-V diagnostic criteria for major depression Adapted from the *Diagnostic and statistical manual of mental disorders 5th edition. 2013.*

A. Five (or more) of the following symptoms present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) anhedonia
B. Symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning
C. Episode is not attributable to the physiologic effects of a substance or another medical condition
1. Depressed mood most of the day (e.g. feels sad, empty, hopeless)
2. Markedly diminished interest or pleasure in almost all activities nearly every day
3. Significant appetite changes or significant weight loss or gain
4. Insomnia or hypersomnia nearly every day
5. Psychomotor agitation or retardation
6. Fatigue or loss of energy
7. Feelings of worthlessness or excessive guilt
8. Diminished ability to think or concentrate or indecisiveness
9. Thoughts of death or suicidal ideation or attempt

Many studies use cut-off scores on validated questionnaires such as the Beck Depression Inventory (BDI) (A. T. Beck et al., 1996b) or the Hamilton Rating Scale for Depression (HRSD) (Hamilton, 1960) to establish the presence of depressive symptoms. Many of these patients will also meet the criteria for MDD and vice versa. Unlike the DSM there are no essential core symptoms and no requirement for symptoms to disrupt normal daily functioning (Fried & Nesse, 2015b). This adds further variability to depressed samples.

Whether or not these categorical models of depression map onto any biological substrate has yet to be determined. Needless to say, no biological test for depression is included in the DSM-V. It is also becoming increasingly clear that the clinical variations in MDD are hindering the identification of biomarkers and that different subtypes may reflect different pathophysiological mechanisms (Antonijevic, 2006). It is therefore unlikely that a disorder as complex, heterogeneous and widely diagnosed as depression will reflect a single biological process. Depression not only affects the brain but potentially many other peripheral organs (Halaris, 2009;McIntyre et al., 2007;Moulton et al., 2015). The notion of depression as a biological syndrome is supported by the fact that it often has a lifelong trajectory, mimicking the disease course of neuro-generative disorders which often include recurring episodes of increasing severity, reduced treatment response and shorter remission periods (Sibille & French, 2013). To date a clear, comprehensive, and coherent disease model has yet to emerge and it is most likely that developments in understanding the pathophysiology of depression will arise from the inter-disciplinary efforts of many different research fields

1.3 Depression: aetiology

For the last 50 years, the monoamine hypothesis has dominated neurobiological accounts of depression (Willner et al., 2013). This hypothesis states that the underlying pathophysiological basis of depression is a deficiency in monoamine levels, including serotonin (5-HT), dopamine (DA) and/or noradrenaline (NA), at functionally important receptor sites in the brain. This hypothesis is supported by evidence from animal studies (Krishnan & Nestler, 2011) and by the mechanism of action of antidepressants, which cause the short-term increase of monoamine synaptic concentrations, thereby correcting these deficiencies, and as such has remained the principle theoretical framework for antidepressant drug development.

However, studies of monoamine metabolites and post-mortem brain studies of depressed patients have yet to provide conclusive evidence for this deficiency (Ferrari & Villa, 2017). Furthermore the inability of this model to completely explain either the pathophysiology of depression or the mechanism of action of antidepressants was highlighted in a study by Miller et al. (1996). The study examined the effects of NA and 5-HT depletion in antidepressant-remitted individuals and healthy controls. The authors reported NA and 5-HT depletion led to relapse in patients who were in remission, however it did not lead to depressive symptoms in controls. Furthermore, a meta-analysis of 52 studies, demonstrated that monoamine depletion decreased mood in subjects with a family history of MDD and in antidepressant-free patients with MDD in remission, but did not decrease mood in healthy people (Ruhé et al., 2007). These findings suggest that monoamines are likely involved in the maintenance of antidepressant response and may potentially identify individuals who may be vulnerable to depressive episodes, however they fail to demonstrate a causal link. The intuitive appeal of this conclusion is strengthened by length of time required for the therapeutic action of antidepressant treatments to be induced, usually several weeks, despite the fact that monoamine levels increase within minutes (Ferrari & Villa, 2017).

Newer generation antidepressants have been developed over the years, primarily in order to reduce the frequency of troublesome, unwanted side effects. However whilst tolerability has improved, increases in clinical effect have been minimal (Willner et al., 2013). For approximately 60% of depressed patients, treatments including both psychotherapy and psychopharmacology do not result in disease remission (Papakostas & Ionescu, 2015) and of those patients who do respond, many of them will respond just as well to placebo (Kirsch et al., 2008). Furthermore, approximately 30% of patients do not respond to treatment at all despite multiple attempts and are considered to be suffering from treatment resistant (refractory) depression (TRD) (Mrazek et al., 2014). Although a universal definition of TRD has yet to be clarified, the general consensus is

that TRD can be identified by the failure to respond to two or more mechanistically dissimilar antidepressants (McIntyre et al., 2014).

The limited success of antidepressants may be a reflection of a wider, more complex neurobiology of depression including genetic effects, changes in neurocircuitry, neuroplasticity, structural brain alterations and changes in the main neurobiological systems that mediate the stress response such as the hypothalamic–pituitary–adrenal (HPA) axis, the autonomic nervous system and the immune system (Kupfer et al., 2012). I will provide a brief overview of potential aetiological explanations below, before focusing my attention on the role of the immune system and HPA-axis in the context of depression.

1.3.1 *Structural brain alterations*

Studies using neuroimaging techniques have reported structural abnormalities in brain volumes in depressed patients and have been reported across diverse brain networks (Shen et al., 2017). The most consistent evidence comes from structural magnetic resonance imaging (MRI) studies which demonstrate that hippocampal volume is reduced in patients with depression. A meta-analysis of 143 studies showed that when compared with a healthy brain, depression was associated with smaller grey matter volume of the basal ganglia, thalamus, hippocampus and various frontal regions (Kempton et al., 2011). Another meta-analysis by Schmaal et al. (2016) which used three-dimensional brain MRI data from 1,728 MDD patients and 7,199 controls from 15 research samples worldwide, showed that relative to controls, patients had significantly lower grey hippocampal volumes but did not show differences in any other subcortical structures. In addition, a meta-analysis of 193 voxel-based morphometry studies, demonstrated a depression-specific reduction in hippocampal volume (Goodkind et al., 2015).

Researchers have also investigated the connectivity between brain networks by examining changes in white matter structure. Altered connectivity in frontal-limbic circuits, which are involved in communication between brain regions that are involved in emotion processing, have been shown to significantly predict treatment outcome in patients with MDD (Korgaonkar et al., 2014). Studies have used diffusion tensor imaging to investigate white matter microstructure abnormalities in depressed patients. Meta-analyses of studies measuring fractional anisotropy, a technique used to infer connectivity between regions, have identified reductions in the left superior longitudinal fasciculus (M. L. Murphy & Frodl, 2011) and in the white matter fascicles connecting the prefrontal cortex within cortical (frontal, temporal and occipital lobes) and subcortical areas (amygdala and hippocampus) (Liao et al., 2013). A more recent study, using data from the UK Biobank investigated differences in both subcortical grey matter volume and white matter integrity between depressed individuals and controls in 8,590 participants (Shen et al., 2017). The authors reported no significant differences in grey matter but significant reductions were found global white matter integrity in depressed individuals versus controls. It should, however be noted that findings in this area are inconsistent and are often complicated by co-morbidities. Furthermore, any clear cause–effect relationships between these pathological changes and any cognitive aspects of depression have yet to be demonstrated (Leistedt & Linkowski, 2013).

1.3.2 *Neuroplasticity and neurogenesis*

A popular hypothesis for the aetiology of depression concerns reductions in neurotrophic factors which regulate brain plasticity (brain remodeling by forming new neural connections). Evidence exists for disrupted neuroplasticity and neurogenesis (generation of new neurons from neural stem or progenitor cells) (Otte et al., 2016). These studies have predominantly focused on the role of brain-derived neurotrophic factor (BDNF), a protein essential for neuronal development and survival, synaptic plasticity, and cognitive function, which is expressed abundantly in adult limbic structures

(Krishnan & Nestler, 2008). Reduced levels of BDNF has been observed in the serum of patients with MDD (Molendijk et al., 2014) and a reduction in BDNF messenger RNA (mRNA) levels have also been noted in the leukocytes of patients with MDD. A post-mortem study has also reported a decrease in BDNF levels in the brains of suicide victims compared with non-suicide controls (Karege et al., 2005). Furthermore, both pharmacological and non-pharmacological antidepressant therapies have also been shown to normalize BDNF levels (Molendijk et al., 2014). Another effect of many antidepressant treatments is the initiation of hippocampal neurogenesis. Blockade of hippocampal neurogenesis in murine models inhibits the effects of most antidepressant treatments (Sahay & Hen, 2007). However the exact mechanism involved in how new neurons improve mood are still unclear.

1.3.3 *Brain activity*

It seems reasonable to assume that changes in molecular brain structure as discussed above may be accompanied by changes in brain activity. In fact, experiments examining brain function such as functional magnetic resonance imaging (fMRI) and positron-emission tomography (PET), report that activity in the amygdala and prefrontal cortex is associated with feelings of dysphoria and is chronically increased in people with depression, returning to normal levels following successful antidepressant therapy (Drevets, 2001; Ressler & Mayberg, 2007). Complementing these findings, studies have investigated the effects of deep brain stimulation on people with TRD. Mayberg et al. applied stimulation (via surgical implantation of electrodes) to the white matter tracts adjacent to the subgenual cingulate gyrus and reported a prominent and sustained remission of symptoms (Mayberg et al., 2005). Similar results have been observed following deep brain stimulation of the nucleus accumbens, a region associated with reward and feelings of pleasure (Schlaepfer et al., 2008).

Evidence from neuroimaging studies also exists for abnormalities in neural pathways which suggest that people with depression may have an attentional bias to negative stimuli. Findings show increases in activity in the amygdala, ventral striatal and medial prefrontal cortex in response to fearful stimuli (Dannlowski et al., 2007;Fales et al., 2008;Fu et al., 2008;Keedwell et al., 2005;Surguladze et al., 2005) and reduced ventral striatal activity in response to positive stimuli (Epstein et al., 2006;Surguladze et al., 2005).

Studies have also used quantitative electroencephalographic measures of “spontaneous” brain activity to predict response to antidepressants. Bruder et al. showed that treatment responders had greater alpha activity compared with non-responders and healthy controls at occipital sites (2008). Increases in pre-treatment theta activity has also been observed in the rostral anterior cingulate cortex (rACC) of responders compared with non-responders (Mulert et al., 2007; Pizzagalli et al., 2001). However this association has not been consistently observed (Cook et al., 2002).

Neuroimaging studies have also provided evidence for response to antidepressant medication. Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies have reported increased baseline (rACC) activity in MDD patients who responded to treatment (R. J. Davidson et al., 2003;Mayberg et al., 1997;Saxena et al., 2003). A meta-analysis of 23 studies demonstrated that baseline activity in the rACC has been shown to reliably predict response to SSRI treatment (Pizzagalli, 2011). Interestingly, low activity in the rACC predicts remission following cognitive behavioral therapy (CBT) (Roiser et al., 2012).

1.3.4 Genetic effects

It is a well-established finding that depression clusters strongly in families. Twin/family based heritability estimates for MDD are approximately 35% and first degree-relatives of MDD patients are three times more likely to develop the condition than the general population (Geschwind & Flint, 2015). These estimate can increase to 70% if severity, relapse rate and age of onset are taken into account (Menke et al., 2012b).

For 40 years the search for candidate genes involved in the aetiology of depression has been underway. Over 100 candidate genes, based on pathophysiological notions, have been investigated to date, however studies have often been met with limited success (Shadrina et al., 2018). The most frequently investigated candidate gene is *SLC6A4*, which codes for the serotonin transporter that is responsible for the uptake of serotonin at the synaptic cleft to the presynaptic neuron, and is the drug target of SSRIs. The promoter region of *SLC6A4*, contains a polymorphism (5-HTTLPR) which has either a 44bp insertion or deletion, resulting in a long or short variation of this gene (Heils et al., 1995). The short allele is associated with decreased expression of *SLC6A4* mRNA and lower serotonin reuptake *in vitro* (Heils et al., 1996; Lesch et al., 1996). Furthermore, it has been associated with various affective disorders (Serretti et al., 2006) and with poorer antidepressant response, especially in patients of Caucasian ancestry who were treated with SSRIs (Porcelli et al., 2012). In a seminal epidemiological study, Caspi et al. investigated whether 5-HTTLPR was associated with risk of depression in a cohort of 847 participants (2003). They found that the individuals with the short allele exhibited more depressive symptoms and suicidality in response to stressful life events than individuals homozygous for the long allele. Moreover, childhood maltreatment predicted depression in adulthood only in adults carrying a short allele but not among long/long homozygotes. This study provides evidence for a gene-by-environment (G X E) interaction, in which the risks of depression conferred on an individual by genetic variants are dependent on environmental exposure to stress. These findings have been

consistently reproduced (Eley et al., 2004;Jacobs et al., 2006;Kendler et al., 2005;Wilhelm et al., 2006;Zalsman et al., 2006) with a couple of notable exceptions (Gillespie et al., 2005;Surtees et al., 2006). However recently, a large candidate gene study found no clear evidence for any gene polymorphism associations with depression or any polymorphism-by-environment moderator effects (Border et al., 2019).

Advances in microchip technology enabled researchers to conduct genome-wide association studies (GWAS) which search the entire genome for small variations, called single nucleotide polymorphisms (SNPs), which occur more frequently in people with depression, independently of any explanatory hypothesis. However GWAS involving tens of thousands of patients have failed to identify specific loci which can account for the development of depression. A mega-analysis of GWAS for MDD analysed more than 1.2 million SNPs in 18,759 European individuals (Ripke et al., 2013). No SNPs achieved genome-wide significance and further exploratory analyses failed to provide robust findings. These null findings were replicated in a GWAS combining the results of 17 population-based studies ($n=34,549$) (Hek et al., 2013).

This result is not entirely surprising as the risk of MDD is highly polygenic and MDD phenotypes are highly heterogeneous. In addition, the effects of common main genetic variants for complex human diseases are often small and therefore require far larger sample sizes than historically available with existing consortia. However, studies which have used a more homogeneous phenotypic approach have yielded significant results. A recent GWAS investigated three depression-related phenotypes: broad depression, probable MDD, and ICD-coded MDD in 322,580 UK Biobank participants (Howard et al., 2018). The authors reported 17 genetic variants associated across the three phenotypes. Gene sets were enriched in excitatory neurotransmission, mechanosensory behaviour, post synapse, neuron spine and dendrite functions. Furthermore a recent genome-wide association meta-analysis based on 135,458 cases and 344,901 controls, identified 44 independent loci associated with clinical features of MDD (Wray et al., 2018). The results

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implicated brain regions which also show anatomical differences between MDD cases and controls, confirming that depression is a brain disorder. They also showed significant associations with educational attainment and BMI, consistent with both factors being causal. An ongoing search for consistent genetic variants that contribute to depression is currently being conducted by the Psychiatric Genomics Consortium (<http://www.med.unc.edu/pgc>).

1.3.5 *Stressful life events*

Stressful life events have been shown to predict subsequent depressive episodes (Kendler et al., 1999;Kessler, 1997). Historically, studies have often focused on stressful events in adulthood, usually in the preceding 12 months before onset of depressive symptoms. These include events such as a serious illness, death of spouse, marital separation, loss of a job or major financial crisis (Brugha et al., 1985). Furthermore, in recent decades levels of stress have increased reflecting changing social and economic demands, coinciding with a rapid rise in the prevalence of depression (Kessler et al., 2003).

The literature also supports a strong relationship between events in childhood and depression (M. Li et al., 2016b). This includes physical and sexual abuse, physical and emotional neglect and exposure to domestic violence, with the more 'silent' types of abuse (neglect and emotional abuse) being the strongest risk factors (Infurna et al., 2016;Mandelli et al., 2015). Longitudinal studies have shown that adverse childhood experiences predict symptom severity, recurrence and time to remission (Fuller-Thomson et al., 2014;Gilman et al., 2013;Rhebergen et al., 2012). Furthermore, the relationship between the number and severity of adverse life events and the risk, severity and chronicity of MDD appear to be dose–response in nature (M. Li et al., 2016b).

Despite the robust literature demonstrating the link between childhood maltreatment and depression, the biological mechanisms mediating this relationship remain poorly understood and understudied (McLaughlin, 2016). Most research in this field has focused on neurobiology. As previously mentioned, hippocampal atrophy is strongly associated with depression (Schmaal et al., 2016). Reduced hippocampal volume has also been observed in people with early life adversity (McCrory et al., 2010) and there are findings to suggest that smaller volume may mediate the association between childhood abuse and adult depression (Rao et al., 2010; Vythilingam et al., 2002).

Another way that childhood maltreatment might increase the risk of depression is by increasing sensitivity to stressors later in life (Kendler et al., 2004). Neuroendocrinological processes involved in the psychophysiological response to stress may be altered by adverse childhood experience. The role of the hypothalamic-pituitary-adrenal (HPA) axis, a complex set of neuronal and endocrine interactions that comprise the stress response, has been well documented in relation to stress regulation (Gunnar & Quevedo, 2007). Chronic activation of the HPA-axis has been associated with both hippocampal atrophy, childhood maltreatment and depression (R. T. Liu, 2017), although the degree to which this is mediating the link remains unclear. The role of the HPA-axis in depression will be discussed in more detail in section 1.5.

1.4 Depression and innate immunity

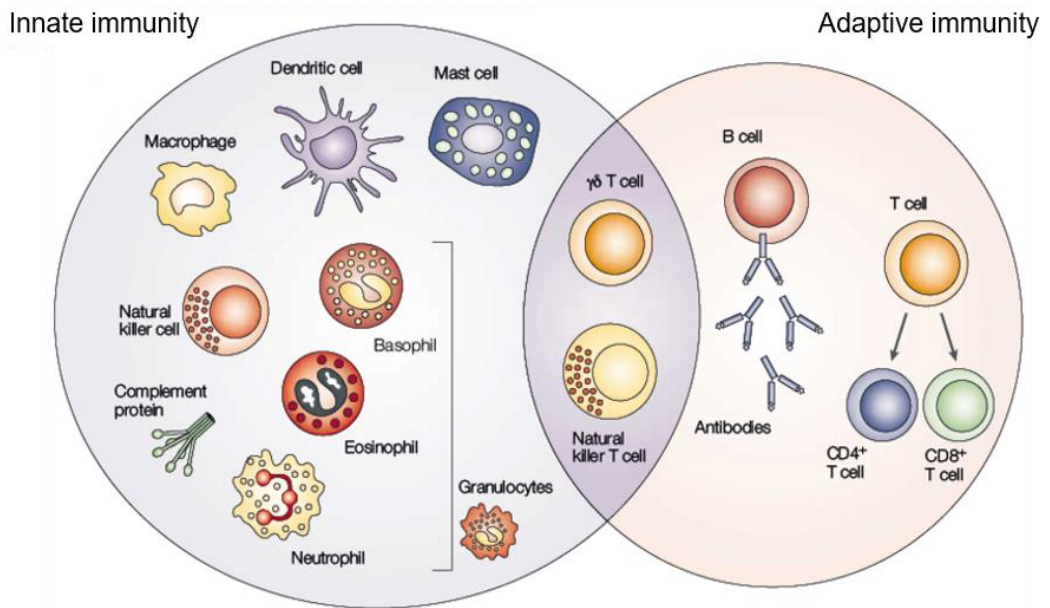
The immune system may also play an important role in the development and maintenance of depression and a vast amount of evidence supports an association between depressive symptoms and immune dysfunction (Irwin & Miller, 2007; A. H. Miller et al., 2009; A. H. Miller & Raison, 2016; Raison et al., 2006). The immune system is a complex network of organs and processes that provide protection against infection and

disease. The immune system can be broadly divided into two subsystems, innate and adaptive immunity, with extensive communication between the two (Dranoff, 2004).

The innate immune response functions as the first line of defence against infection and is rapid, broad and short lived. Mechanisms of innate immunity include phagocytes such as monocytes, neutrophils and macrophages, which ingest and destroy microbes, and natural killer cells, which respond to viral infection. Before it is overwhelmed, the innate system signals the adaptive immune response via signalling molecules known as cytokines. The adaptive immune system is a far more sophisticated line of defence and provides specific, long lasting protection. It is mediated by B-lymphocytes which produce antibodies, helping to identify and inactivate target cells, and T-lymphocytes which directly kill target pathogens and orchestrate the cell-mediated immune response (see Figure 1.1) (Toben & Baune, 2015).

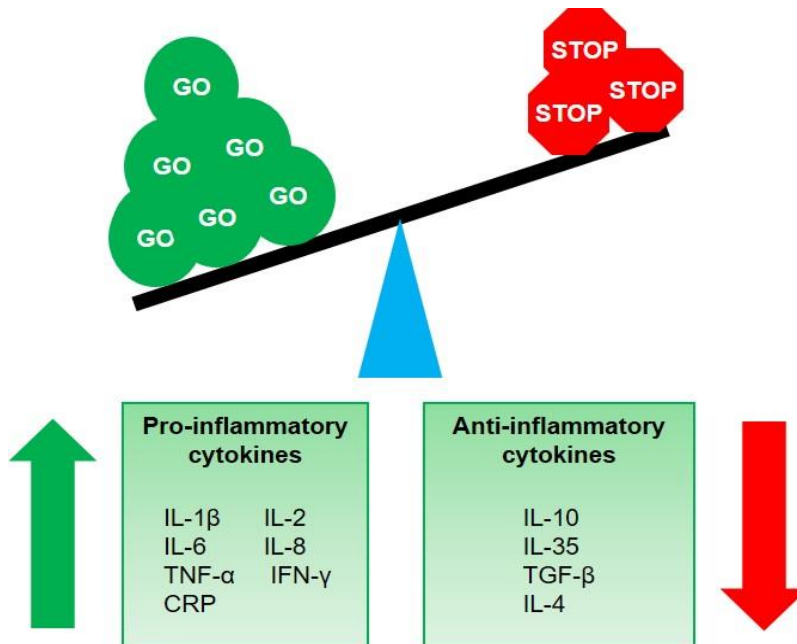
The innate and adaptive immune systems have a collaborative, bi-directional relationship, thus maintaining homeostasis (state of equilibrium). Immune cells from both the innate and adaptive systems secrete cytokines (signalling molecules), whose primary function is to regulate and mediate immunity. Pro-inflammatory cytokines such as interleukin-6 (IL-6), tumour-necrosis-factor-alpha (TNF α), and the acute phase reactant C-reactive protein (CRP), promote inflammation. Anti-inflammatory cytokines such as interleukin-10 (IL-10), interleukin-35 (IL-35) and transforming growth factor beta (TGF- β) inhibit inflammation (see Figure 1.2).

Figure 1.1 The immune system



Innate immunity is the critical line of defence against infection. Innate immunity is mediated by white blood cells including basophils, dendritic cells, eosinophils, mast cells, monocytes and macrophages, neutrophils and natural killer cells, as well as soluble factors, such as complement proteins. The adaptive immune response is slower to respond and consists of highly specialised cells which are able to recognize and remember specific pathogens. It consists of antibodies, B cells, and T cells (CD4+ and CD8+ T lymphocytes).

Figure 1.2 Balance of pro and anti-inflammatory cytokines.



Pro-inflammatory cytokines such IL-1, IL-6 and TNF- α and CRP drive the inflammatory response, whereas anti-inflammatory cytokines such as IL-10, IL-35 and TGF- β inhibit inflammation. Pro-inflammatory cytokines have been shown to be increased in people with depression.

Adapted from: Grainger, D. n.d.

However, the traditional view of the immune system as the guardian against pathogens and infection has been replaced by an appreciation of its role in the regulation of homeostatic processes throughout the body, including bi-directional interaction with other organ systems, such as the endocrine system and central nervous system (CNS) (Toben & Baune, 2015). As a result of this paradigm shift, researchers have explored the influence of immune processes within several CNS related disorders including autoimmune diseases, brain injuries, stroke, neurodegenerative diseases and depression (Capuron & Dantzer, 2003;Klegeris et al., 2007).

Much of the focus on immune activation in depression has been on the role of the innate immune response and inflammation (Dantzer et al., 2008;A. H. Miller et al., 2009), The most reliable biomarkers of increased immune response in depressed patients are elevations in the pro-inflammatory cytokines, IL-6, TNF α , and CRP in peripheral blood (Dowlati et al., 2010;Haapakoski et al., 2015;Howren et al., 2009;Zorrilla et al., 2001). In this review, we will present evidence for the role of the innate immune response before turning our attention to adaptive immunity.

1.4.1 *Pro-inflammatory cytokines and depression*

The cytokine hypothesis of depression was formulated over 20 years ago and proposes that internal stressors (e.g. infection or disease) and external stressors (e.g. diet, psychological distress) activate the immune system, via the release of pro-inflammatory cytokines, resulting in a range of behavioural, neuroendocrine and neurotransmitter alterations that are associated with depression (Maes, 1995). The behavioural changes, otherwise known as 'sickness behaviour', include reduced sociability, loss of appetite, tiredness, disrupted sleep, low mood and irritability, as well as impaired concentration and memory (Dantzer et al., 2008). These changes in motivation enable the individual to cope with infection, e.g. via energy conservation (Dantzer, 2001).

Evidence from animal studies supports the likely causal nature of this hypothesis. It has been repeatedly shown that administration of IL-1 β or TNF- α to mice or rats induces the entire range of sickness behaviors in a concentration and time dependent manner (Dantzer, 2001). These animals assume a hunched position in the corner of their cage and show little interest in their surroundings. They display decreased locomotor activity, social withdrawal, loss of appetite, increased sleep, altered cognition and increased sensitivity to pain. These data were additionally supported by pharmacological experiments using administration of lipopolysaccharide (LPS), a bacterial toxin which induces inflammation. LPS induces expression of pro-inflammatory cytokine expression in the brain (Breder et al., 1994;van Dam et al., 1992) as well as sickness behaviour in rats (Stepanichev et al., 2014). Administration of anti-inflammatory cytokines, in particular IL-10, (Bluthe et al., 1999;Dantzer et al.,1999a) and chronic treatment with antidepressants (Dunn et al., 2005) diminishes the behavioural signs of sickness attenuates much of the depressive like behaviour associated with pro-inflammatory cytokines and LPS. IL-10 deficient mice also demonstrate an exaggerated sickness behaviour in response to administration of LPS compared to wild-type mice (Dantzer et al., 2008). This exaggerated behaviour is associated with an increased expression of pro-inflammatory cytokine genes in the brain. These findings support the idea that the balance of pro and anti-inflammatory cytokines is essential to the regulation of the behavioral response to stress (see Figure 2).

Animal data also indicates that psychological stress activates pro-inflammatory cytokines and their signaling pathways both peripherally and in the CNS. Psychological stressors such as restraint or social isolation have been shown to increase concentrations of IL-1 β and TNF- α in brain regions involved in emotional regulation, and in the periphery of rats (Madrigal et al., 2002;K. A. O'Connor et al., 2003). Furthermore, depressive-like symptoms induced by social isolation can be reversed by administration of the soluble IL-1 receptor antagonist (IL-1ra) (Pugh et al., 1999). Neuronal cell loss following restraint

induced stress is also associated with increased concentrations of TNF- α (Madrigal et al., 2002).

Stress studies in humans produces similar results. Exposure to brief, natural stress such as taking an examination or experience of stressful life events such as death of a spouse is associated with increased circulating inflammatory markers (Kiecolt-Glaser et al., 2002; Marshall et al., 1998). Stressful life events are also associated with increased risk for many physical health morbidities that involve inflammatory pathophysiology (S. Cohen et al., 2007; Tosevski & Milovancevic, 2006), including CVD (Steptoe & Kivimaki, 2013). Stress experienced during academic exam periods has been associated with increased TNF- α , IL-6, IL-1Ra and interferon gamma (IFN- γ) (Maes et al., 1998). An experimental paradigm has also been employed to study the inflammatory effects of acute stress. Psychosocial stress tasks such as the Stroop colour-word interference task, public speaking, mental arithmetic, anger recall, and the Trier Social Stress Test (TSST) are typically used to induce moderate psychological stress in a laboratory setting. A meta-analysis of 49 studies that measured the effects of acute stress on circulating inflammatory markers reported significant stress-related increases in circulating IL-1 β , IL-6, IL-10 and TNF- α but not CRP (Marsland et al., 2017).

Chronic stress, such as caregiving, is also associated with inflammatory activation in healthy people. One study involving 120 mothers of children newly diagnosed with cancer, demonstrated that circulating levels of IL-6 increased over the first 6 months following the child's diagnosis and remained high at 12-month follow-up (Walsh et al., 2018). Another study including 33 adults caring for a family member with glioblastoma and 47 controls who were relatively free of major stress, showed that caregivers' monocytes showed increased expression of genes bearing response elements for nuclear-factor kappa B (NF- κ B), a key pro-inflammatory transcription factor (G. E. Miller et al., 2014). A similar study including 11 familial caregivers of brain-cancer patients and 10 matched controls, showed that caregiver's monocytes demonstrated heightened

expression of transcripts with response elements for NF κ B. Caregivers also showed increased CRP and IL-1Ra (G. E. Miller et al., 2008). A longitudinal community study followed older men and women who were caregiving for a spouse with dementia and 106 non-caregivers over 6 years (Kiecolt-Glaser et al., 2003). They found that the average rate of increase in IL-6 was four times as large in caregivers compared to non-caregivers. Furthermore, the increase in IL-6 among former caregivers was maintained even several years after the death of the spouse. This finding was independent of physical comorbidities, medications, or health behaviors that might have accounted for caregivers' increased IL-6 levels. Further analysis also revealed that this was not simply a function of depressive symptoms. In a systematic review of 41 studies, increased levels of CRP were associated with chronic psychosocial stress such as employment stress, unemployment stress, caregiver stress and discrimination (Johnson et al., 2013). However, a larger review of 151 studies which focused on stress biomarkers in informal dementia caregivers found there was mixed evidence for alterations in immune function, possibly due to multiple methodologies and variability in biomarkers (Allen et al., 2017). A more recent review of psychosocial stress of caregiving and inflammatory biomarkers found little evidence of a relationship between stress and inflammation (Potier et al., 2018). However it should be noted that few of the studies included provided data on important potential confounding factors, such as the presence of chronic disease or comorbidities known to be associated with inflammation.

Early-life stress, particularly childhood maltreatment, has been shown to be an independent risk factor for inflammation in adulthood. Danese et al. (2007) examined the effect of maltreatment on CRP levels in 866 people from the Dunedin Multidisciplinary Health and Development Study and reported that maltreated children showed a significant and graded increase in the risk for clinically relevant CRP levels, 20 years later, independently of the influence of co-occurring early life risks and adult health and health behavior. Carpenter et al. (2010) measured plasma IL-6 response to the TSST in 69 healthy adult subjects without depression, 19 of whom had experienced moderate-

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severe childhood maltreatment. Participants who had experienced childhood maltreatment demonstrated higher IL-6 release compared to those who did not. Miller and Chen explored the impact of a 'harsh family climate' on inflammatory profiles (G. E. Miller & Chen, 2010). They measured psychological stress and inflammatory biomarkers in 135 female adolescents, on four occasions, over 1.5 years and found that those participants who were reared in harsh families displayed an increasingly pro-inflammatory phenotype during the follow-up analyses. A systematic review of 20 studies also reported that a history of childhood maltreatment was associated with increased levels of CRP, fibrinogen, IL-6 and TNF- α . The results were particularly robust for CRP (Coelho et al., 2014). Following this a meta-analysis of 25 articles, including 18 CRP studies (n = 16, 870), 15 IL-6 studies (n = 3,751) and 10 TNF- α studies (n = 881), showed that individuals exposed to childhood trauma had significantly higher levels of all inflammatory cytokines (Baumeister et al., 2016a). Furthermore, sub-group analyses for specific types of trauma showed that childhood sexual abuse was significantly associated with a TNF- α and a trend for IL-6, however no association was observed for CRP. Similarly, physical abuse was significantly associated with TNF- α and IL-6 but not CRP.

Genetic studies have also provided evidence suggesting that depression and inflammation have common genetic variants and shared gene-expression pathways. A review of clinical studies by Bufalino et al. showed that functional allelic variants in the genes for interleukin -1 beta (IL-1 β), TNF- α and CRP increase the risk of depression (Bufalino et al., 2013). The Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium used GWAS data from over 60,000 participants and showed prominent links between immune gene variants and mood disorders. A more recent systematic review showed that variants in the genes for IL-1 β , IL-6, IL-10, TNF- α , CRP and phospholipase A2 are implicated in both depression and immune activation, although findings were inconsistent (J. Barnes et al., 2016). Furthermore this review also

reported that increased mRNA expression of cytokines predicted antidepressant response.

Depression is also more prevalent in patients with chronic inflammatory diseases such as cardiovascular disease and type 2 diabetes compared with the general population (Stephens, 2007). Moreover up to 50% of patients with autoimmune diseases such as rheumatoid arthritis and multiple sclerosis experience depressive symptoms (Pryce & Fontana, 2016). Pro-inflammatory cytokines also induce MDD in patients with medical illness but no history of depression. Patients receiving inflammation-based treatments develop symptoms closely resembling depression. Interferon-alpha (IFN- α) is a potent inducer of pro-inflammatory cytokines and is used to treat infectious diseases or cancer. IFN- α therapy has been shown to induce symptoms consistent with a diagnosis of MDD in patients in 30-70% of patients (Schaefer et al., 2012). It has been shown that this may be partly due to an increased biological sensitivity to IFN- α , evidenced by larger gene expression changes and specific signatures in those patients who develop IFN- α -induced depression (Hepgul et al., 2016). In addition, pre-treatment administration of the SSRI paroxetine results in significantly fewer cases of depression, minimizing the effects of IFN- α (Musselman et al., 2001). Treatment with IL-2 also induces similar effects, which are entirely reversible upon cessation of therapy (Denicoff et al., 1987).

Despite the similarity of symptoms between sickness and depression, the nature of the syndromes is different. Sickness behaviour is an adaptive response to infection and spontaneously remits upon recovery, unlike depression which persists over time. Depression could therefore be seen as a maladaptive response to stress, that occurs when the stimulus is chronic or intense, or when an individual is vulnerable to depression, for example, in individuals with a history of childhood maltreatment (Dantzer et al., 2008).

The majority of human literature is cross sectional and six meta-analyses show increases in circulating inflammatory cytokines in depressed individuals compared with non-

depressed (Dowlati et al., 2010;Goldsmith et al., 2016b;Hiles et al., 2012b;Howren et al., 2009;Kohler et al., 2017;Y. Liu et al., 2012) (see Table 1.2).

The most consistent cross-sectional findings are increased levels of circulating IL-6 and TNF- α in depressed people. A cumulative meta-analysis was also conducted in which the results from individual studies were added chronologically to determine an aggregate effect estimate (Haapakoski et al., 2015). The purpose of such an analysis is to explore whether further studies are needed to accept or reject a hypothesis. The authors examined the strength of the association between IL-6, IL-1 β , CRP, and TNF- α with MDD in 58 studies. A robust association was confirmed between both IL-6 and CRP and depression, which remained relatively stable after controlling for the effects of age, gender, body mass index (BMI) and study size or quality. However the association between TNF- α and depression was weaker and less consistent, largely due to heterogeneity in estimates and variability in sub group analyses. It is unclear whether this reflects a truly weaker relationship or is due to poor control of confounding variables or inaccuracies in measurement. It should also be noted that these studies were limited to one-time assessments of inflammatory biomarkers and depression and therefore distinctions between chronic and acute states could not be made. Furthermore cross-sectional studies are unable to establish the direction of association between inflammatory markers and depression.

Table 1.2 Meta-analyses of cross sectional studies comparing pro-inflammatory cytokine levels between depressed people and controls

Author/year	Cytokines	Population; sample size	Results
Dowlati et al. 2010	TNF- α	13 studies; $n = 788$ (438 MDD, 350 controls)	There were significantly higher concentrations of TNF-α in depressed people compared with controls WMD = 3.97 pg/ml (95% CI, 2.24 - 5.71)
	IL-1 β	9 studies; $n = 513$ (267 MDD, 246 controls)	There was no significant difference in IL-1 β levels between the depressed group and controls WMD = -1.58 pg/ml (95% CI, 3.59 - 0.43)
	IL-2	5 studies; $n = 292$ (153 MDD, 139 controls)	There was no significant difference in IL-2 levels between the depressed group and controls WMD = -5.75 pg/ml (95% CI, 100.45 - 88.96)
	IL-6	16 studies; $n = 892$ (492 MDD, 400 controls)	There were significantly higher concentrations of IL-6 in depressed people compared with controls WMD = 1.78 pg/ml (95% CI, 1.23 - 2.33)
	IL-8	4 studies; $n = 382$ (205 MDD, 177 controls)	There was no significant difference in IL-18 levels between the depressed group and controls WMD = -0.39 pg/ml (95% CI, 2.13 - 1.35)

Table 1.2 continued. Meta-analyses of cross sectional studies comparing pro-inflammatory cytokine levels between depressed people and controls

Author/year	Cytokines	Population; sample size	Results
	IFN- γ	4 studies; $n = 238$ (131 MDD, 107 controls)	There was no significant difference in IFN- γ levels between the depressed group and controls WMD = -6.63 pg/ml (95% CI, 25.91 - 12.65)
Goldsmith et al. 2016	TNF- α	7 studies; $n = 697$ (346 MDD, 351 controls)	There were significantly higher concentrations of TNF-α in depressed people compared with controls ES = 0.05 (95% CI, -0.10 - 0.19)
	IL-1 β	3 studies; $n = 262$ (105 MDD, 157 controls)	There was no significant difference in IL-1 β levels between the depressed group and controls, ES = 0.21 (95% CI, -0.04 - 0.47)
	IL-2	2 studies; $n = 100$ (82 MDD, 8 controls)	There were significantly lower concentrations of IL-2 in depressed people compared with controls ES = 0.54 (95% CI, -0.90 to -0.17)
	IL-6	7 studies; $n = 416$ (205 MDD, 211 controls)	There were significantly higher concentrations of IL-6 in depressed people compared with controls ES = 0.39 (95% CI, 0.20 - 0.59)

Table 1.2 continued. Meta-analyses of cross sectional studies comparing pro-inflammatory cytokine levels between depressed people and controls

Author/year	Cytokines	Population; sample size	Results
	IL-8	3 studies; $n = 263$ (126 MDD, 137 controls)	There was no significant difference in IL-8 levels between the depressed group and controls, ES = 0.08 (95% CI, -0.16 - 0.32)
	IL-12	2 studies; $n=195$ (82 MDD, 113 controls)	There were significantly lower concentrations of IL-12 in depressed people compared with controls ES = -0.44 (95% CI, -0.44 to -0.14)
	IFN- γ	4 studies; $n = 421$ (195 MDD, 226 controls)	There was no significant difference in IFN- γ levels between the depressed group and controls, ES = -0.03 (95% CI = -0.24 - 0.16)
Hiles et al. 2012	IL-6	96 studies (MDD/depressive symptoms and controls)	There were significantly higher concentrations of IL-6 in depressed people compared with controls, $d = 0.46$ (99% CI, 0.34 - 0.58)
Howren et al. 2009	CRP	49 studies; $n = 51,234$ (MDD/depressive symptoms)	CRP was positively associated with depression, $d = 0.15$ (95% CI, 0.10 - 0.21)
	IL-1	14 studies; $n = 756$ (MDD/depressive symptoms)	IL-1 was positively associated with depression, $d = 0.35$ (95% CI, 0.03 - 0.67)

Table 1.2 continued. Meta-analyses of cross sectional studies comparing pro-inflammatory cytokine levels between depressed people and controls

Author/year	Cytokines	Population; sample size	Results
	IL-1 α	9 studies; $n = 1,214$	IL-1α was positively associated with depression, $d = 0.25$ (95% CI, 0.04 - 0.46)
	IL-6	61 studies; $n = 24,873$ (MDD/depressive symptoms) (MDD/depressive symptoms)	IL-6 was positively associated with depression, $d = 0.25$ (95% CI, 0.18 - 0.31)
Kohler et al. 2017	TNF- α	42 studies; $n = 3077$ (1620 MDD, 1457 controls)	There were significantly higher concentrations of TNF-α in depressed people compared with controls, ES = 0.68 (95% CI, 0.43 - 0.92)
	IL-1 β	22 studies; $n = 1506$ (779 MDD, 727 controls)	There was no significant difference in IL-1 β levels between the depressed group and controls, ES = 0.03 (95% CI, -0.29 - 0.35)
	IL-1 α	4 studies; $n = 258$ (148 MDD, 110 controls)	There were significantly higher concentrations of IL-1α in depressed people compared with controls, ES = 0.45 (95% CI, 0.08 - 0.82)
	IL-6	42 studies; $n = 2770$ (1587 MDD, 1183 controls)	There were significantly higher concentrations of IL-6 in depressed people compared with controls, ES = 0.62 (95% CI, 0.49 - 0.76)

Table 1.2 continued. Meta-analyses of cross sectional studies comparing pro-inflammatory cytokine levels between depressed people and controls

Author/year	Cytokines	Population; sample size	Results
	IL-12	4 studies; $n=436$ (135 MDD, 301 controls)	There were significantly higher concentrations of IL-12 in depressed people compared with controls, ES = 1.23 (95% CI, 0.28 - 2.18)
	IL-17	3 studies; $n = 191$ (85 MDD, 106 controls)	There was no significant difference in IL-17 levels between the depressed group and controls, ES = 0.12 (95% CI, -0.54 - 0.30)
	IL-18	5 studies; $n = 278$ (135 MDD, 143 controls)	There were significantly higher concentrations of IL-18 in depressed people compared with controls, ES = 1.72 (95% CI, 0.38 - 3.06)
	IL-8	7 studies; $n = 523$ (306 MDD, 217 controls)	There was no significant difference in IL-8 levels between the depressed group and controls, ES = 0.03 ((95% CI, -0.35 - 0.41)
	IFN- γ	17 studies; $n = 1470$ (700 MDD, 770 controls)	There were significantly lower concentrations of IFN-γ in depressed people compared with controls, ES = -0.48 (95% CI, -0.94 to -0.02)
Liu et al. 2012	TNF- α	15 studies (MDD and controls)	There were significantly higher concentrations of TNF-α in depressed people compared with controls, SMD = 0.56 (95% CI, 0.13 - 0.99)

Table 1.2 continued. Meta-analyses of cross sectional studies comparing pro-inflammatory cytokine levels between depressed people and controls

Author/year	Cytokines	Population; sample size	Results
	IL-1 β	10 studies	There was no significant difference in IL-1 β levels between the depressed group and controls, SMD = -0.53 (99% CI, -1.36 - 0.32)
	IL-6	18 studies	There were significantly higher concentrations of IL-6 in depressed people compared with controls, SMD = 0.68 (95% CI, 0.44 - 0.92)

Abbreviations: IL = interleukin; TNF- tumour necrosis factor, IFN = interferon; CRP = C-reactive protein; WMD = weighted mean difference; pg/ml = picogram/millilitre; CI = confidence interval, *d* = Cohen's *d*, MDD = major depressive disorder, SMD = standard mean difference, ES = effect size

Several studies have also reported significant associations between inflammatory markers and severity of depressive symptoms (Alesci et al., 2005; G. E. Miller et al., 2002b; Motivala et al., 2005). The association remains even for mild symptoms which do not meet criteria for MDD (Suarez et al., 2004). However it should be noted that after adjustment for confounding variables, such as BMI, some studies show a significantly attenuated association (G. E. Miller et al., 2002b) and in others the association loses significance completely (Kop et al., 2002).

To address the issue of causation, several longitudinal studies have been conducted to determine whether elevated inflammation predicts symptoms of depression or whether depression predicts elevated inflammatory markers (see Table 1.3). Several studies have shown a significant association between increased levels of CRP at baseline and subsequent depressive symptoms in otherwise healthy people (Gimeno et al., 2009; Khandaker et al., 2014; Wium-Andersen et al., 2013; Zalli et al., 2016). Gender differences have also been reported, with baseline CRP predicting worsening depression for women, but not for men (Niles et al., 2018). Repeated exposure to CRP was also observed in a cohort of 2,068 older people (J. A. Bell et al., 2017a). Transient inflammation (1 episode) was not associated with developing depression, however repeated exposure (2 episodes) was associated with increased risk of future depressive symptoms among women, but not in men. However findings regarding CRP are inconsistent, with the association either not reaching significance (Copeland et al., 2012; Deverts et al., 2010; Glaus et al., 2018; Huang et al., 2019; Khandaker et al., 2014; Stewart et al., 2009) or losing significance after adjusting for confounders (Chu et al., 2018).

Studies investigating IL-6, also show that increased cytokine levels at baseline predict depressive symptoms at follow up (Chu et al., 2018; Gimeno et al., 2009; Khandaker et al., 2014; Kivimaki et al., 2014; Zalli et al., 2016). The association with IL-6 and symptom resolution has also been investigated. Using data from the Whitehall II study, Virtanen et

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al. (2015) showed that individuals with repeatedly low IL-6 levels were more likely to experience depression remission at follow-up compared with those with repeatedly high levels. The effects of chronically elevated levels of inflammation on common mental health disorders have also been investigated using the same cohort. Kivimaki et al. (2014) examined the association between IL-6 and the cumulative 10-year risk of common mental disorder among 2,757 people. Compared to participants with low IL-6 at the beginning of the study, those with high IL-6 had a greater likelihood of common mental disorder 10 years later. Furthermore, they found that more incidents of elevated IL-6 a participant experienced, the greater their 10-year risk of common mental disorder, suggesting a dose-response relationship. The association was similar for both men and women and was unaffected by acute inflammation, obesity, smoking and drug treatments. It should be noted that studies using this cohort rely on questionnaire data which is not the same as a clinical diagnosis of depression. In addition the data is from an occupational cohort which underrepresents both women and individuals from ethnic backgrounds and contains people who are likely to be healthier than the general population. However the repeated measures design is able to capture the chronicity of inflammation and the direction of association. A recent study by Huang et al. (2019) showed that baseline IL-6 predicted future depressive symptoms 7 years later, in 166 middle-aged male twins recruited from the Vietnam Era Twin Registry. In contrast, baseline IL-6 did not predict 6-year change in depressive symptoms in 263 healthy, older men and women (Stewart et al., 2009).

Only one meta-analysis of longitudinal studies has been conducted to date and showed that elevated IL-6 and CRP have a small but significant association with the subsequent development of depressive symptoms (Valkanova et al., 2013). The associations remained significant after adjusting for a variety of confounders. The effects was larger for CRP than for IL-6, however that may have been a result of the number of studies included (eight studies for CRP, $n=14,832$ and three studies for IL-6, $n=3,695$). Alternatively it may reflect a more general role for CRP in driving the inflammatory

response (Dantzer & Kelley, 2007). These findings provide support for a biological pathway from inflammation to depression. However, it remains unclear whether inflammation is a mediating risk factor or a causal factor.

Studies have also shown a significant association between increased depressive symptoms at baseline and subsequent levels of CRP (Copeland et al., 2012; Deverts et al., 2010; Huang et al., 2019; Niles et al., 2018). However some studies have reported differential findings, with one only observing an association in black people, not white people (Deverts et al., 2010) and one observing an association in men, but not women (Niles et al., 2018). Copeland et al. (2012) investigated the effect of current and cumulative episodes of depression on future CRP and reported that the association between current depression and CRP was attenuated after controlling for BMI, smoking, and medication use. In contrast the association between cumulative depression and CRP remained significant after adjusting for confounders. A significant association between increased depressive symptoms at baseline and subsequent levels of IL-6 has also been reported (Stewart et al., 2009), although null findings have also been reported (Huang et al., 2019). Furthermore, sub-types of depression may be differentially associated with future IL-6. “Atypical-melancholic” and “unspecified” MDD have been associated with decreased IL-6 and TNF- α levels, whereas other MDD subtypes were not associated with any inflammatory markers (Glaus et al., 2014). The authors speculate that this could be due to decreased levels of monocytes observed in MDD, a major source of IL-6 concentration (Whooley et al., 2008) or the anti-inflammatory effects of increased cortisol, which is also observed in MDD (Duivis et al., 2011). Taken together, these findings suggest that the association between inflammation and depression may be bidirectional.

Table 1.3 Longitudinal studies assessing associations between pro-inflammatory cytokines and depressive symptoms

Author/year	Cytokines	Population; sample size	Follow-up	Depression measure	Results
Bell et al. 2017	CRP	2,068	8 years	CES-D	Transiently CRP did not predict depressive symptoms Repeated CRP predicted depressive symptoms (OR = 1.60 (95% CI, 1.00 - 2.55))
Copeland et al. 2012	CRP	1,420	12 years	Young Adult Psychiatric Assessment	CRP did not predict depressive symptoms Cumulative baseline depressive symptoms predicted CRP ($\beta = 0.04$, $p < 0.0001$)
Chu et al. 2018	CRP, IL-6	2731	9 years	CIS-R	Baseline IL-6 predicted diurnal variation in mood (RR = 1.75 (95% CI, 1.13–2.69)) No association between CRP and depressive symptoms
Deverts et al. 2010	CRP	2,544	5 years	CES-D	Baseline depressive symptoms predicted CRP in black people only ($\beta = 0.031$, $p < 0.001$) Baseline CRP did not predict depressive symptoms
Gimeno et al. 2009	CRP, IL-6	3339-3070, (30% women)	11.8 years	GHQ	Baseline CRP ($\beta = 0.038$, $p = 0.036$) and IL-6 ($\beta = 0.041$, $p = 0.018$) predicted depressive symptoms Baseline depressive symptoms did not predict CRP or IL-6

Table 1.3 continued. Longitudinal studies assessing associations between pro-inflammatory cytokines and depressive symptoms

Author/year	Cytokines	Population; sample size	Follow-up	Depression measure	Results
Glaus et al. 2018	CRP, TNF- α , IL-6	3118 (53.7% women)	5.5 years	DIGS	<p>Baseline combined melancholic and atypical MDD predicted increased CRP ($\beta = 0.29$ (95% CI, 0.03–0.55))</p> <p>Baseline atypical MDD predicted increased CRP ($\beta = 0.32$ (95% CI, 0.10 – 0.55))</p> <p>Baseline combined melancholic and atypical MDD predicted decreased IL-6 ($\beta = -0.74$ (95% CI, -1.30 to -0.18))</p> <p>Baseline TNF-α associated with decreased risk of MDD ($\beta = 0.86$ (95% CI, 0.78 – 0.95))</p>
Huang et al. 2019	CRP, IL-6	166 (83 pairs) male twins	7 years	BDI-II	<p>Baseline CRP did not predict depressive symptoms</p> <p>Baseline depressive symptoms predicted CRP ($\beta = 0.33$, $p < 0.05$)</p> <p>Baseline IL-6 predicted depressive symptoms ($\beta = 0.22$, $p < 0.05$)</p> <p>Baseline depressive symptoms did not predict IL-6</p>
Khandaker et al. 2014	CRP, IL-6	2447	9 years	CIS-R, MFQ	<p>No association between CRP and depressive symptoms</p> <p>Baseline IL-6 predicted depressive symptoms (OR = 1.55 (95% CI, 1.13–2.14))</p>
Kivimaki et al. 2014	IL-6	2757 (24.6% women)	10 years	GHQ	Baseline IL-6 predicted depressive symptoms (OR = 1.40 (95% CI, 1.07–1.82))

Table 1.3 continued. Longitudinal studies assessing associations between pro-inflammatory cytokines and depressive symptoms

Author/year	Cytokines	Population; sample size	Follow- up	Depression measure	Results
Niles et al. 2018	CRP	13,775 (59% women)	4 years	CES-D	Depressive symptoms predicted CRP in men only ($\beta = 0.03$, $p = 0.034$) CRP predicted depressive symptoms in women only ($\beta = 0.03$, $p = 0.006$)
Stewart et al. 2009	CRP, IL-6	263 (51.7% women)	6 years	BDI-II	No association between CRP and depressive symptoms Baseline depressive symptoms predicted IL-6 ($\beta = 0.16$, $p = 0.01$) Baseline IL-6 did not predict depressive symptoms
Virtanen et al. 2015	IL-6	2,419 (33.9% women)	6.5 years	GHQ	Low baseline IL-6 predicted depressive symptom remission (RR = 1.15 (95% CI, 1.06–1.25))
Wium-Anderson et al. 2103	CRP	73,131	Average 5 years	Antidepressant use and hospitalisation with depression	Increasing CRP levels were associated with increasing risk for hospitalization with depression ($P = 4 \times 10^{-8}$ for trend)
Zalli et al. 2016	CRP, IL-6	656 (64.5% women)	5 years	CES-D	IL-6 predicted depressive symptoms (OR = 2.44, $p = 0.030$) CRP IL-6 predicted depressive symptoms (OR = 1.81, $p = 0.052$)

Abbreviations: CIS-R = Clinical Interview Schedule – Revised; MFQ = Mood and Feelings Questionnaire; GHQ = General Health Questionnaire, OR = odds ratio; RR = relative risk; BDI-II = Beck Depression Inventory-II; DIGS = Diagnostic Interview for Genetic Studies; PHQ = Patient Health Questionnaire; CRP = C-Reactive Protein; IL-6 = Interleukin-6; TNF- α = Tumour Necrosis Factor – alpha; MDD = Major Depressive Disorder; CES-D = Center for Epidemiologic Studies Depression Scale.

The relationship between early life stress, depression and inflammation in adulthood has also been investigated prospectively. Danese et al. (2008) followed a birth cohort of 1000 individuals followed up to age 32 years as part of the Dunedin Multidisciplinary Health and Development Study. Childhood maltreatment during the first decade of life was assessed retrospectively and participants were assessed for current MDD and circulating inflammatory biomarkers. The results showed that depression was associated with high levels of CRP, however this association was attenuated and lost significance after controlling for childhood maltreatment. Participants who had current depression and a history of childhood maltreatment had higher risk of CRP levels compared to controls. Moreover, even in the absence of a current depressive episode, people with a history of maltreatment were more significantly more likely to show high CRP levels than controls. However this risk was not significant for those with depression but no history of childhood maltreatment. These findings suggest that a childhood maltreatment may significantly contribute to the comorbidity of depression and inflammation. Furthermore it may explain why some depressed people exhibit elevated inflammation and others do not. The heterogeneity in the literature may reflect variation in the prevalence of childhood maltreatment. However, it should be noted that in this study, CRP was not measured before the onset of depression, therefore the direction of the effect was not tested.

Many studies have also investigated central inflammation in patients with MDD, focusing on cytokines in the cerebrospinal fluid (CSF) and immune cells in the brain, including microglia, astrocytes and oligodendrocytes (Enache et al., 2019). Microglia, the macrophages of the CNS, produce several pro-inflammatory cytokines including interleukin IL-1, IL-6, IL-12, and TNF- α (Mondelli et al., 2017). Astrocytes, the dominant glial cell in the CNS, are phagocytic and produce IL-6 and TNF- α (Rajkowska & Stockmeier, 2013). Oligodendrocytes, also glial cells, plays a key role in myelination and produce IL-1 (Mechawar & Savitz, 2016). Historically, most studies investigating central immunity have focused on post-mortem brain tissue. However an increasing number of studies are now using PET (positron emission tomography) scans to investigate

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microglia activation *in vivo* (Enache et al., 2019). A recent systematic review and meta-analysis was conducted investigating studies focusing on markers of central inflammation in patients with MDD compared with controls (Enache et al., 2019). The authors reported that depressed patients have higher CSF levels of IL-6 and TNF- α , increased microglia activation and reduced astrocytes and oligodendrocytes compared with controls. Furthermore, they observed little correlation between central and peripheral markers of inflammation. These findings suggests that central inflammation may not be a result of peripheral inflammation in MDD, although this lack of association could also be an effect of changes to the permeability of the blood brain barrier (A. H. Miller & Raison, 2016)

1.4.2 *Anti-depressant treatment and inflammation*

The effects of anti-depressant medication on cytokine levels has also been the focus of research studies, however results are inconsistent. Four meta-analyses have investigated circulating levels of inflammatory markers before and after antidepressant treatment in people with depression (see Table 1.4). The most consistent finding is a reduction in IL-6 levels following treatment (Hiles et al., 2012a; Kohler et al., 2018; Wiedlocha et al., 2018), although the effect has sometimes been small (Hannestad et al., 2011). Findings regarding other cytokines, such as, TNF- α , CRP and IL-1 β , largely due to the high levels of heterogeneity observed in the studies and the fact they tend to have small sample sizes and short duration.

To further complicate the issue, inflammation appears to be differentially related to treatment response to specific medications. In the Genome-Based Therapeutic Drugs for Depression study, a multicenter open-label randomized clinical trial, 241 men and women with MDD were randomly allocated to 12-week treatment with either escitalopram (SSRI) or nortriptyline, a tricyclic antidepressant (TCA) (Uher, 2013). Baseline CRP differentially predicted treatment outcome with the two drugs. Patients with low levels of

CRP (<1 mg/L), showed greater symptom reduction with escitalopram and patients with higher CRP levels, showed greater symptom reduction with nortriptyline. Similarly in the Combining Medications to Enhance Depression Outcomes trial, participants were randomised to a 12 week treatment of either escitalopram plus placebo (SSRI monotherapy) or bupropion plus escitalopram (Jha et al., 2017). Bupropion is a non-serotonergic antidepressant which inhibits dopamine reuptake, increases brain extracellular dopamine concentration and reduces inflammation. Patients with low CRP level (<1 mg/L) responded better to SSRI monotherapy whereas those with higher levels responded better to the combination of bupropion and SSRI.

Interaction between inflammatory proteins and medication may help explain variation in treatment outcome. A study by Yoshimura et al. (2009) compared plasma IL-6 and TNF- α levels among SSRI responsive or SNRI responsive depressed patients and healthy controls. Both IL-6 and TNF-alpha were significantly higher in depressed patients than in healthy controls and treatment with antidepressants significantly reduced levels of both. In addition, IL-6 level but not TNF-alpha, was higher in SSRI-refractory than SSRI-responsive depressed patients, and higher in SNRI-refractory than SNRI-responsive depressed patients. Strawbridge et al. (2015) examined data from 35 studies and reported that IL-6 levels decreased following antidepressant treatment, regardless of outcome. However TNF- α levels decreased only in those who responded, whereas treatment resistance was associated with persistently elevated TNF α . In addition, when the authors combined IL-6, TNF- α and CRP as a composite measure of inflammation, they found a trend between baseline inflammation and future antidepressant response. However Kohler et al. (2018) analysed data from 45 studies and reported that whilst IL-6 and TNF α were reduced following treatment, reductions were not significantly associated with treatment response.

However there is evidence from *in vitro* studies that antidepressants can also stimulate inflammation. Both imipramine (TCA) and venlafaxine (SSRI) increased the production

of IL-6 in depressed, SSRI-pretreated patients and when the drugs were used at a higher concentrations. A study by Szuster-Ciesielska et al. (2003) reported that whilst clomipramine (TCA), inhibited stimulated levels of IFN- γ , it also enhanced unstimulated levels. The finding that co-incubation with immunostimulants may modulate the immunomodulatory effect of antidepressants has implications for depressed individuals with elevated inflammatory biomarkers. It is possible that antidepressants might have differential effects, depending on pre-existing levels of inflammation. A review by Baumeister et al. (2016b) demonstrated that *in vitro* incubation with some antidepressants, such as clomipramine and fluoxetine, decrease production of IL-6, (IFN)- γ and TNF- α , whilst others, such as mirtazapine and venlafaxine, tend to increase production. In addition, the effects of anti-inflammatory effects of antidepressants appear to be dose-dependent. Diamond et al. (2006) showed that exposure to a low concentration of desipramine (TCA) resulted in an increase in IFN- γ and IL-10 production, whilst inhibiting them at higher doses.

The effects of non-pharmacological therapies on inflammation have also been explored. A study including un-medicated women suffering from first-episode depression, reductions in IL-6 were associated with a reduction in depressive symptoms following seven weeks of CBT (Gazal et al., 2013). However, a similar study reported that whilst seven weeks of CBT was associated with a reduction in depressive symptoms, IL-6 and TNF- α , there was no association between symptom remission and change in cytokines (Moreira et al., 2015). A systematic review by Lopresti (2017) reported that overall, the evidence suggests that CBT may reduce inflammatory biomarkers and that the reductions are likely to be clinically meaningful.

Taken together, these results do appear to suggest that there is some degree of normalisation of cytokine levels following both antidepressant treatment and psychotherapy. Broadly speaking, elevations in inflammatory biomarkers during depressive episodes may represent a general over-activation of the immune system,

which stabilizes upon symptom resolution (Hiles et al., 2012a). Furthermore, consistently high levels of inflammation may contribute to treatment resistance, indicating that anti-inflammatory medication may have a role to play in the treatment of those who do not respond to conventional therapies. However whilst immunomodulation appears to be evident, the effect is not always anti-inflammatory, with some cases of activated inflammation depending on dose, sample type, co-incubation and the specific protein being measured. To explore this line of enquiry further, studies have investigated whether the use of anti-inflammatory drugs could improve antidepressant response.

Table 1.4 Meta-analyses exploring the effects of antidepressants on cytokine levels

Author/year	Population	Sample size	Cytokines	Results
Hannestad et al. 2011	MDD	6 studies (<i>n</i> =115)	IL-1 β	IL-1 β was significantly decreased after antidepressant treatment SMD=-0.52 (95% CI -0.83 to -0.20)
		13 studies (<i>n</i> =274)	IL-6	There was no significant difference in IL-6 after antidepressant treatment SMD=-0.32 (95% CI -1.06 - 0.43)
		13 studies (<i>n</i> =438)	TNF- α	There was no significant difference in TNF- α after antidepressant treatment SMD=-0.13 (95% CI: -0.55 - 0.29)
Hiles et al. 2012	MDD/depressive symptoms	14 studies	IL-6	IL-6 was significantly decreased after antidepressant treatment SMD=-0.42 (95% CI -0.78 to -0.06)
		3 studies	IL-10	No significant effect of antidepressants was observed for IL-10 SMD=-0.45 (95% CI -1.03 to 0.14).
		8 studies	CRP	CRP was marginally significantly decreased after antidepressant treatment SMD=-0.57 (95% CI -1.14 to 0.01).

Table 1.4 continued. Meta-analyses exploring the effects of antidepressants on cytokine levels

Author/year	Population	Sample size	Cytokines	Results
Kohler et al. 2018	MDD	24 studies (<i>n</i> =722)	IL-6	IL-6 was significantly decreased after antidepressant treatment (Hedges <i>g</i> = -0.454, <i>P</i> < 0.001).
		10 studies (<i>n</i> = 331)	IL-10	IL-10 was significantly decreased after antidepressant treatment (Hedges <i>g</i> = -0.566, <i>P</i> = 0.012).
		23 studies (<i>n</i> = 797)	TNF- α	TNF- α was significantly decreased after antidepressant treatment (Hedges <i>g</i> = -0.202, <i>P</i> = 0.015).
Więdołcha et al. 2018	MDD	4 studies (<i>n</i> = 88)	IL-1 β	IL-1 β was significantly decreased after treatment with SSRI only (SMD = - 0.434 (95% CI - 0.903–0.348))
		7 studies (<i>n</i> = 191)	IL-2	There was no significant difference in IL-2 after antidepressant treatment (<i>p</i> > 0.05).
		5 studies (<i>n</i> = 140)	IL-4	IL-4 was significantly decreased after antidepressant treatment (SMD = - 0.726 (95% CI - 1.269 to - 0.184)).
		17 studies (<i>n</i> = 542)	IL-6	IL-6 was significantly decreased after antidepressant treatment (SMD = - 0.701 (95% C: - 1.024 to - 0.379))

Table 1.4 continued. Meta-analyses exploring the effects of antidepressants on cytokine levels

Author/year	Population	Sample size	Cytokines	Results
		6 studies (<i>n</i> = 154)	IL-10	IL-10 was significantly decreased after antidepressant treatment (SMD = - 0.763 (95% CI - 1.247 to - 0.278))
		9 studies (<i>n</i> = 325)	CRP	There was no significant difference in CRP after antidepressant treatment (<i>p</i> > 0.05).
		16 studies (<i>n</i> = 457)	TNF- α	There was no significant difference in TNF- α after antidepressant treatment (<i>p</i> > 0.05).
		6 studies (<i>n</i> = 154)	IFN- γ	There was no significant difference in IFN- γ after antidepressant treatment (<i>p</i> > 0.05).

Abbreviations: MDD = Major Depressive Disorder; IL = interleukin; TNF- α = tumour necrosis factor- alpha, IFN- γ = interferon-gamma; CRP = C-reactive protein; CI = confidence interval, MDD = major depressive disorder, SMD = standard mean difference.

1.4.3 *Nonsteroidal anti-inflammatory drugs (NSAIDs)*

Several clinical trials have been conducted to test the anti-depressant effects of anti-inflammatory drugs, in particular nonsteroidal anti-inflammatory drugs (NSAIDs) and cytokine inhibitors. NSAIDs inhibit the enzyme cyclooxygenase 2 (COX-2), which is responsible for the production of pro-inflammatory cytokines (Muller et al., 2006). Several studies have investigated the efficacy of using NSAIDs in combination with antidepressants in patients with MDD. A meta-analysis of four studies which used the selective COX-2 inhibitor, celecoxib, showed that patients receiving adjunctive celecoxib had significantly improved depression scores compared to antidepressant plus placebo (Na et al., 2014). However, potential issues such as heterogeneity and publication bias could not be sufficiently assessed due to the small number of trials included. The antidepressant effect of NSAID monotherapy was assessed in a meta-analysis of five randomised controlled trials (RCTs) in patients with active osteoarthritis (Iyengar et al., 2013). The authors reported a trend towards reduction of depression symptoms in patients taking NSAID compared to placebo. A subsequent meta-analysis by Kohler et al. investigated anti-inflammatory treatment in people with depression, regardless of concomitant disease or whether the anti-inflammatory treatment was used alone or in conjunction with antidepressants (2014). The study demonstrated a beneficial effect of NSAIDs on depressive symptoms with no evidence of increase risk of adverse effects. More recently, a meta-analysis of 6 RCTs including both MDD and bipolar disorder reported a significant moderate antidepressant effect of anti-inflammatory medication, comparable with effect sizes reported in clinical trials of antidepressants (Husain et al., 2017).

However NSAIDs are broad-spectrum anti-inflammatory drugs that also act on other biological targets, such as glucocorticoid receptors, which are also implicated in depression pathophysiology (Kappelmann et al., 2018). Therefore the exact mechanism by which they exert their antidepressant effects is still unclear. NSAIDs also increase the

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risk of CVD, a well-established depression co-morbidity (Mukherjee et al., 2001). In addition, most studies investigating NSAIDs in depression use medical populations, making it difficult to determine whether the improvement in depressive symptoms is due to improvement in physical illness. The use of cytokine inhibitors may be more useful in elucidating any anti-inflammatory effects on depression as their mechanism of action targets specific cytokine pathways and both TNF- α and IL-6 blockade have received particular attention in this regard.

1.4.4 Cytokine inhibition

TNF-alpha

The relatively recent development of drugs which inhibit the production of cytokines has provided a new way of investigating the relationship between depression and inflammation. Anti-cytokine treatment is used to treat chronic inflammatory conditions and antidepressant activity has been investigated in clinical trials where depressive symptoms were measured as a secondary outcome. The most commonly studied drugs are TNF- α inhibitors. These have been shown to reduce depressive-like symptoms in an animal model of stress (Camara et al., 2015; Krügel et al., 2013). In clinical studies, TNF- α inhibitors have been shown to reduce depressive symptoms in people with rheumatoid arthritis (RA) (Uguz et al., 2009) psoriasis (Tyring et al., 2006; C. Y. Wu et al., 2016) irritable bowel disease (Horst et al., 2014) and Crohn's disease (Lichtenstein et al., 2002; Persoons et al., 2005). A study by Arisoy et al (2013) also reported that TNF- α inhibitors significantly decreased depressive symptoms in people with ankylosing spondylitis. More importantly, this reduction was not associated with changes in markers of clinical disease activity. Similarly, a study by Uguz et al. (2009) reported that patients with RA taking anti-TNF- α drugs had a significantly lower frequency of any depressive symptoms compared to patients not receiving the drugs. As with the previous study, the reduction in symptoms was not associated with any clinical characteristics of RA. This

provides further support for the theory that inflammatory activation underpins the pathophysiology of depression. Two meta-analyses of the effect on TNF- α inhibitor therapy on depression in people with chronic physical illness have been conducted (Abbott et al., 2015; Kappelmann et al., 2018). Authors from both studies reported a small but significant beneficial effect on depression across a variety of clinical populations.

TNF- α inhibition and depression has also been explored longitudinally. Using a nationwide cohort study, 980 patients with psoriatic arthritis or psoriasis who had received anti-TNF therapy were followed over approximately 2 years (C. Y. Wu et al., 2016). The prevalence of patients taking regular antidepressants before starting biologics therapy was about 20%. The authors reported a 40% reduction in this prevalence after anti-TNF therapy at follow up. Together, these findings suggest that patients with chronic inflammatory conditions who take TNF- α inhibitors experience an improved affective state and consequently require less antidepressant medications.

To date only a few studies have been conducted to explicitly examine the effects of TNF- α inhibition on depressive symptoms in people with MDD. The first study to do this was a randomized, placebo-controlled trial by Maas et al. (2010) where older patients (>60 years) with TRD were randomized to either a single 3-mg/kg dose of the TNF- α inhibitor infliximab or placebo. The trial was unable to demonstrate a positive effect of the drug and the trial was prematurely interrupted due to difficulties with patient recruitment. Following this, Raison et al. (2013b) conducted a single-site, parallel-group, randomized, double blind trial comparing the effects of infliximab with placebo, in 60 medically stable MDD patients who were moderately resistant to treatment, as determined by a score ≥ 2 on the Massachusetts General Hospital Staging method for treatment resistance. Whilst the authors reported no significant difference in depression scores between the two groups over time, they did observe an interaction between treatment time and baseline CRP. Patients who had higher CRP (>5mg/L) at baseline demonstrated greater symptom reduction with Infliximab, whereas patients who had lower CRP (<5mg/L) demonstrated

greater symptom reduction with placebo. Exploratory analyses restricted to patients with high CRP showed that infliximab treatment resulted in treatment response in 62% of patients compared with placebo which resulted in treatment response in 33% of patients. Baseline TNF- α levels were also higher in responders compared to non-responders. The authors concluded that TNF-alpha antagonism does not have generalized efficacy in people with TRD but may reduce symptoms in those with a high inflammatory profile. This supports the notion that a subset of adults with MDD comorbid with elevated inflammation may be differentially responsive to anti-inflammatory intervention, however this result needs further replication. A subsequent study was conducted on this sample to explore the mechanisms of action involved in the response to infliximab (Mehta et al., 2013). Differential gene expression was examined in peripheral blood mononuclear cells (PBMCs) from infliximab responders compared to non-responders. Results showed that 148 transcripts were significantly associated with response to infliximab and were distinct from placebo responders. Transcripts predictive of infliximab response were enriched in pathways related to both glycolysis and gluconeogenesis and to lipid and cholesterol homeostasis. Furthermore, responders also demonstrated greater inhibition of genes related to apoptotic pathways through TNF signalling. The authors conclude that infliximab response involved regulation of metabolic genes and inhibition of genes related to innate immune activation. Therefore patients with comorbid depression, metabolic syndrome and elevated inflammatory markers may benefit from novel anti-inflammatory treatments for depression, such as anti-TNF.

Interleukin-6

To date there is a far smaller literature regarding IL-6 inhibition and depression. Preclinical evidence suggests that inhibition of IL-6 signaling may offer protection against depressive-like behavior using IL-6 knockout mice (Chourbaji et al., 2006). In response to stress (i.e., forced swim, tail suspension, foot shock), wild-type mice exhibited an increase in hippocampal IL-6 expression compared to knockout mice, whereas knockout

mice were more resilient to stress compared to wild-type. In another study the brain tissue of rats showed elevated levels of IL-6 following stress exposure. Central administration of IL-6 also produced depressive-like behaviours in mice and administration of antidepressants failed to reduce the effect. Moreover IL-6 blockade prevented the IL-6-induced behavioural changes and attenuated the IL-6 production in the brain (Sukoff Rizzo et al., 2012). Similar results were reported in a study using a social defeat stress model in mice (Hodes et al., 2014). Mice susceptible to stress had significantly higher IL-6 levels compared to controls and these effects were persistent following subsequent chronic stress (>1 month). However the injection of IL-6 monoclonal antibodies was able to successfully block the development of social avoidance. Taken together, these data indicate that IL-6 may have a pathophysiological role underlying depression and suggest that modulation of the IL-6 signaling pathway may have therapeutic potential.

Sirukumab is a human monoclonal antibody which inhibits IL-6 and is designed for the treatment of inflammatory conditions. Results from clinical trials in patients with autoimmune disease suggest that treatment with the drug may improve mental health outcomes. In a phase I, double-blind, placebo-controlled study investigating the efficacy of sirukumab in patients with systemic lupus erythematosus (SLE), mental health was measured as a secondary outcome (Szepietowski et al., 2013). The patient-reported outcome data suggests that SLE patients experienced clinically relevant improvements in mental health. Similarly, in a phase II, randomized, placebo-controlled study of the safety and efficacy of sirukumab in patients with RA, Smolen et al. (2014) measured disability-related functional outcomes, including mental health. Significant improvements were reported with sirukumab compared to placebo. These findings have led to the suggestion that sirukumab may also alleviate MDD symptoms (A. J. Zhou et al., 2017). Clinical studies exploring IL-6 blockade in MDD have yet to be published, therefore the studies described above should be interpreted with caution. They provide a rationale for

hypothesizing that IL-6 blockade may be beneficial in the treatment of depression, but proof-of-concept studies are warranted. A randomized, placebo-controlled, double-blind phase II study testing the efficacy and safety of sirukumab in participants with MDD is currently underway (NCT02473289). Until more data is available, IL-6 blockade may be considered a promising approach in the treatment of depression.

Additional clinical trials have been conducted investigating the effects of add-on anti-inflammatory medications, such as antibiotics. Minocycline is an antibiotic which can cross the blood-brain barrier and reduce inflammation. Following a pilot study, which showed that adjunctive minocycline leads to improvement in depressive symptoms of TRD (Husain et al., 2015), a larger clinical trial is currently being conducted to determine its efficacy and acceptability as a treatment option for TRD patients (NCT 03947827). Omega-3 polyunsaturated fatty acids (omega-3 PUFAs) have also been tested pharmacologically in the treatment of depression. PUFAs can reduce the production of inflammatory cytokines and can reduce inflammation through their precursor arachidonic acid. A recent meta-analysis of double-blind randomized placebo-controlled trials demonstrated that supplementation with omega-3 PUFAs with eicosapentaenoic acid are effective at reducing symptoms of depression (Liao et al., 2019). In addition, a clinical trial of a novel anti-inflammatory drug, a P2X7 receptor blocker, is currently being conducted to test its efficacy as an adjunct treatment to antidepressant medication TRD (EudraCT 2018-001884-21). These lines of enquiry are promising, although it is not yet clear whether these drugs will be effective only as augmentation for antidepressant medications, or will they be effective as a monotherapy?

1.4.5 *Mechanisms of cytokine related depression*

The pathways through which cytokines contribute to the development and maintenance of depression have also been investigated. Findings suggest that cytokine interactions are so extensive, they span every physiological domain associated with depression

pathology (A. H. Miller et al., 2009). The majority of evidence covers neurotransmitter metabolism, neural plasticity and neuroendocrine function. To date, it is unclear whether inflammatory processes during depression are initiated locally within the brain or whether they originate in the periphery and access the brain via mechanisms such as 1) passage through leaky regions in the blood-brain-barrier, 2) active uptake via transport molecules, 3) activation of endothelial cells and perivascular macrophages in the cerebral vasculature, producing local inflammation and 4) binding to receptors at peripheral vagal nerve afferents and relaying cytokine signals to relevant brain regions (Felger & Lotrich, 2013). Neurotransmitter metabolism and neural plasticity will be briefly discussed, however this PhD will focus predominantly on the interaction between cytokines and neuroendocrine function.

Neurotransmitter metabolism

As previously mentioned, the action of monoamines have dominated depression research. Once cytokines reach the brain they can affect the synthesis, release and reuptake of monoamine neurotransmitters (A. H. Miller et al., 2009). The most studied neurotransmitter in the context of depression is 5-HT and research has focused on cytokine effects on the serotonergic system. Polymorphisms in 5-HTTLPR have been shown to influence pathogenesis of cytokine-induced depression in patients receiving IFN- α treatment. The 'high transcription' serotonin transporter (5-HTT) genotype was associated with significantly fewer symptoms of depression (Bull et al., 2008). *In vitro* studies have also shown that cytokines upregulate 5-HTT, thereby reducing availability of 5-HT at the synapse (Morikawa et al., 1998; Ramamoorthy et al., 1995; Zhu et al., 2006). In addition, treatment with the SSRI, paroxetine, has been shown to reduce IFN- α induced depression (Musselman et al., 2001). Taken together these findings suggest that both drugs and gene polymorphisms that affect 5-HT metabolism influence the development of cytokine-induced depression.

Another way in which cytokines influence serotonergic pathways is via monoamine synthesis, including induction of the enzyme indoleamine 2,3-dioxygenase (IDO). IDO is a metabolic enzyme in the tryptophan–kynurenine pathway that converts tryptophan (TRP), the precursor of 5-HT, to kynurenine, resulting in reduced synthesis of 5-HT (Hestad et al., 2017). In murine studies, IDO blockade has been shown to inhibit LPS-induced depressive-like behaviour (J. C. O'Connor et al., 2009) and in humans, patients with inflammatory conditions and those treated with immunotherapy demonstrate reduced levels of plasma TRP and increased IDO and kynurenine (Bonaccorso et al., 2002; Capuron et al., 2002b; Dantzer et al., 2010).

Additional experiments have been conducted using an acute TRP depletion technique. Participants drink a TRP-free amino acid solution and within 4-12 hours plasma TRP declines to 10–50% of baseline levels. Brain serotonin has been shown to decline in association with TRP depletion in both animals and healthy volunteers (Moore et al., 2000). A mega-analysis of studies investigating TRP depletion in people with depression reported that depletion results in a clinically significant recurrence of depressive symptoms in approximately 50% of remitted depressed patients (L. Booij et al., 2002).

Neural plasticity

Whilst cytokines are usually associated with inflammation in the periphery, in the CNS their role is more pleiotropic. During homeostatic conditions, cytokines support neurogenesis and contributes to cognitive function. However during chronic inflammatory activation, cytokines can produce a host of central abnormalities including decreased neurogenesis, increased apoptosis, impaired cognitive function and increased oxidative stress (A. H. Miller et al., 2009). Peripheral administration of LPS has been shown to produce impaired hippocampal neurogenesis, increased levels of TNF- α and IL-1 β in the hippocampus, impaired spatial learning and memory and reduced expression of brain-derived neurotrophic factor (BDNF) (C. W. Wu et al., 2007).

In addition cytokine blockade has been shown to prevent the effects of stress on cognition, neurogenesis and neurotrophic factors (Barrientos et al., 2003; Ben Menachem-Zidon et al., 2008). Furthermore, cytokines are able to increase glutamate release (Ida et al., 2008) and decrease the expression of glutamate transporters (Tilleux & Hermans, 2007), resulting in decreased re-uptake. Glutamate is a major excitatory synaptic neurotransmitter and increased levels are associated with both CNS disorders and mood disorders (Mathews et al., 2012).

1.5 Depression and neuroendocrine function

One of the most consistently observed mechanistic effects of cytokines is their impact on neuroendocrine function. The association between immune activation and neuroendocrine function will be the focus for the rest of this chapter. Firstly, this next section will describe the literature regarding neuroendocrine changes in depression and then evidence for an interaction of the immune system with the neuroendocrine system within the context of depression will be presented.

1.5.1 The hypothalamic-pituitary-axis and the stress response

Selye originally described stress as a non-specific response of the body to any demand placed upon it, both physiological and psychological (Selye, 1998). It is now customary to consider a stressor as a situation or experience that threatens an individual's ability to adapt and cope (Lazarus & Folkman, 1984) and confers a real or perceived threat to homeostasis. Maintenance of homeostasis in the presence of a stressor results in a 'stress response', involving the activation of a complex range of responses involving the endocrine, nervous, and immune systems. Several structures play important roles in the regulation of the stress response, including the brain stem noradrenergic neurons, the sympatho-adrenal-medullary system, parasympathetic systems and the hypothalamic-

pituitary-adrenal (HPA) axis (S. M. Smith & Vale, 2006). Although these systems interact, this PhD will predominantly focus on the role of the HPA axis in depression.

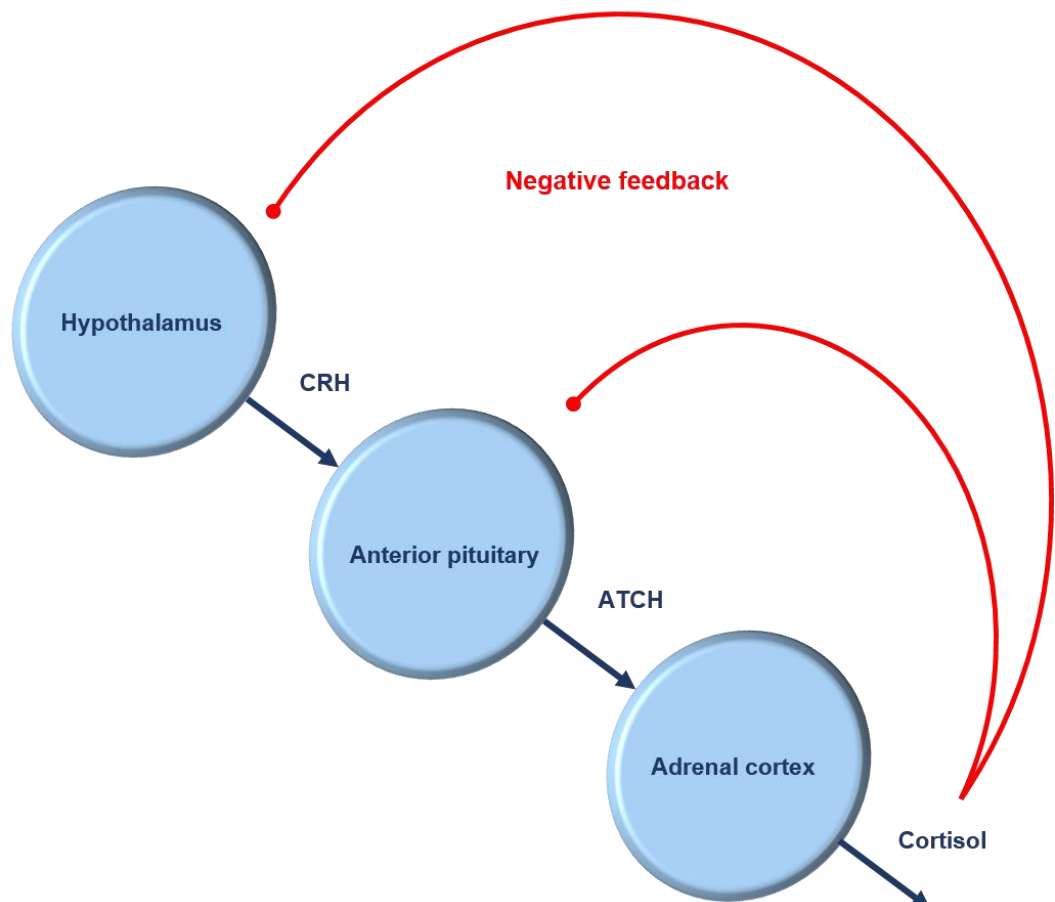
The HPA-axis (Figure 1.3) is the principle endocrine system involved in the stress response (Pariante, 2006). Upon experiencing a stressful event, the amygdala, an area of the brain that contributes to emotional processing, activates the hypothalamus. Neurons in the paraventricular nucleus (PVN) of the hypothalamus release corticotropin-releasing hormone (CRH) into the blood vessels connecting the hypothalamus and the pituitary gland. CRH stimulates the anterior pituitary gland to produce and secrete adrenocorticotrophic hormone (ACTH) into the general circulation. ACTH, in turn, induces glucocorticoid synthesis from the adrenal glands, causing the release of glucocorticoids (GC) (Raison & Miller, 2003). Glucocorticoids subsequently interact with their receptors – the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) to exert their function (see Section 1.5.5 for a full description). These hormones also inhibit further release of CRH from the hypothalamus and ACTH from the pituitary, forming a negative feedback loop (Figure 1.3).

Cortisol, the human glucocorticoid, plays an important role in regulating homeostasis under basal conditions and during stress. It regulates metabolic, autonomic and cardiovascular systems, as well as moderating behaviour. (Girod & Brotman, 2004). However, glucocorticoids are perhaps best known for their role as profound immune regulators, protecting the host against chronic inflammatory and autoimmune responses (Silverman & Sternberg, 2012). In addition, glucocorticoids have a powerful effect on the neural pathways projecting from the hippocampus, amygdala and prefrontal anterior cingulate cortex to the PVN, regulating neuronal survival and neurogenesis, as well as influencing the size of anatomical structures such as the hippocampus, which is involved in cognitive functions such as memory formation and emotional appraisal (E. R. de Kloet et al., 2007a; Nemeroff & Vale, 2005).

1.5.1 HPA axis dysregulation and depression

Stress is known to play a fundamental role in precipitating episodes of depression in predisposed individuals (Checkley, 1996). Considering the role the HPA-axis plays in mediating the stress response and brain function, it is not surprising that abnormalities in function have been observed in depressed people. One of the most robust findings in biological psychiatry is that individuals with depression exhibit hyperactivity of the HPA axis (Pariante & Lightman, 2008). This phenomenon effectively represents a prolonged stress response, regardless of an actual stressor. MDD patients demonstrate increased concentrations of cortisol in plasma, urine, and cerebrospinal fluid (CSF), an amplified cortisol response to ACTH and enlarged pituitary and adrenal glands (Pariante & Miller, 2001).

Figure 1.3 The hypothalamic-pituitary-adrenal axis.



A meta-analysis of 361 studies, spanning four decades and including 18,454 individuals, reported that approximately 60% of depressed individuals have increased levels of cortisol and ACTH compared to non-depressed (Stetler & Miller, 2011). These alterations are thought to be secondary to increased release of CRH. Depressed patients exhibit increased CRH in the CSF, increased CRH mRNA and protein in the PVN of the hypothalamus and a blunted response to ACTH challenge (Pariante & Miller, 2001). HPA axis abnormalities have also been observed post mortem in the brains of suicide victims, in particular a reduction of CRH receptors in the frontal cortex (Merali et al., 2004) and increased weight of adrenal glands (Dumser et al., 1998).

The degree of hyperactivity varies considerably across patient groups and clinical symptomology (Stetler & Miller, 2011). Evidence suggests that patients who remit have more pronounced normalization of an initially dysregulated HPA-axis compared to those who do not (Hennings et al., 2009), although findings in this area are inconsistent (Ahrens et al., 2008). HPA-axis hyperactivity is most strongly associated with melancholic depression, which accounts for about 25-30% of depressed patients (Leistner & Menke, 2018). Psychotic depression, the most severe subtype, accounting for 15-33% of depressed patients, is accompanied by the most severe HPA-axis hyperactivity (Schatzberg, 2015). A recent study using data from the English Longitudinal Study of Ageing (ELSA), showed that higher cortisol was more strongly associated with somatic rather than cognitive-affective symptoms (Iob et al., 2019).

Whilst MDD is strongly associated with HPA-axis hyperactivity, in contrast hypo-activation of the HPA axis, characterised by low levels of CRH secretion and hypocortisolism, has been associated with post-traumatic stress disorder (PTSD) and atypical, seasonal depression (Juruena et al., 2004). Hypocortisolism has been repeatedly reported in patients who have experienced a traumatic event and subsequently developed PTSD (Heim et al., 2000a). Furthermore hypocortisolism is also present in healthy people experiencing chronic stress as well as in patients with stress-

related somatic disorders, such as hypertension, chronic fatigue syndrome, fibromyalgia, chronic pain syndromes, rheumatoid arthritis and asthma (Heim et al., 2000a;Wirtz et al., 2007). Atypical depression (characterised by hypersomnia, fatigue, increased appetite and weight gain) accounts for approximately 15-30% of depression cases (Stetler & Miller, 2011). Overall, evidence suggests that people with atypical depression have lower levels of cortisol than people with melancholic depression, usually indistinguishable from that of healthy controls (Juruena et al., 2018).

Over production of cortisol is thought to damage or impede brain function, including impaired hippocampal neurogenesis, neuronal survival, neuronal excitability, learning, and memory (J. Herbert et al., 2006). In animal studies, chronic corticosterone exposure has been shown to decrease hippocampal neuronal proliferation in mice (David et al., 2009). In addition, adrenalectomy reduces levels of glucocorticoids and increases hippocampal neurogenesis in rats, which is subsequently reversed with corticosterone replacement (Gould et al., 1992). Impaired HPA-axis function, resulting in increased cortisol secretion and creating a cascade of hippocampal damage has been termed the “glucocorticoid cascade hypothesis” (Sapolsky et al., 1986). This hypothesis is now widely accepted as a pathophysiological pathway leading to brain changes associated with depression.

In humans, the effects of cortisol on brain structure and function are best characterised by patients with Cushing’s syndrome, which is a collection of symptoms which develop as a result of glucocorticoid excess. Depression occurs in about 60% of patients with Cushing's Syndrome, however these symptoms remit upon normalisation of cortisol levels (W. F. Kelly et al., 1996). Furthermore, premature cerebral atrophy and cognitive impairments also occur in these patients (N. E. Simmons et al., 2000). The effects of cortisol on the hippocampus may be particularly relevant to depression in a number of ways. Firstly, the hippocampus is thought to be an important site for negative feedback inhibition (S. M. Smith & Vale, 2006). It contains a high number of both GR and MR and

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glucocorticoid infusion into the hippocampus has been shown to reduce glucocorticoid release in response to stress. Secondly, the hippocampus is critically involved in mood processing and so damage may contribute to the debilitating symptoms of depression (Anacker et al., 2011). In addition, hippocampal damage may explain the memory defects observed in depressed individuals. Cognitive deficits are a core component of MDD and evidence suggests that alteration in cortisol levels can impair both learning and memory (Lupien et al., 2002).

Cortisol effects on the hippocampus could also help explain why people who experience childhood maltreatment are more susceptible to adult depression. Repeated exposure to stress in early life resulting in activation of the stress response and chronic cortisol release may damage the developing hippocampus. There is some evidence to suggest that brain regions have distinctly different periods of vulnerability during development. The sensitive-period hypothesis postulates that stress-sensitive brain regions have independent developmental stages when they are particularly vulnerable to the effects of early stress (Andersen et al., 2008). Preclinical data show that exposure of the immature hippocampus to CRH results in cell loss (Brunson et al., 2001) and a particular subset of cells in the immature hippocampus, but not in the adult hippocampus, have been shown to release CRH in response to stress (Y. Chen et al., 2004). Childhood maltreatment during this vulnerable period may therefore result in a 'diathesis' for depression. Severe or prolonged stress in early life triggers activation of the HPA-axis and cortisol release. This damages the developing hippocampus, resulting in a reduced ability for the HPA-axis to normalise and an increased sensitivity to stressors later in life. The experience of further stress in adulthood then results in hyper-activation of the HPA-axis, further damaging the brain and predisposing the individual to depression.

Animal studies have also shown that early-life stress produces persistent increases in HPA axis activity (Meaney, 2001). Clinical studies in healthy people (Power et al., 2012) and people suffering from PTSD (D. Bremner et al., 2007; J. D. Bremner et al., 2003)

have shown that childhood maltreatment is associated with lower morning cortisol levels and a flattening of the diurnal cycle. Adults who experienced childhood abuse also exhibit enhanced stress reactivity in response to standardized psychosocial stressors (Carpenter et al., 2011; Heim et al., 2000b; Heim et al., 2002; A. Suzuki et al., 2014).

There is also evidence to suggest that hypercortisolaemia may play a role in antidepressant resistance (Jurueña et al., 2013). Excess glucocorticoids may impair the modulation of neurotransmission. Specifically, increased production of cortisol decreases levels of serotonin and norepinephrine availability in the synaptic cleft (Y. P. Zhang et al., 2018). Following on from this finding, studies have investigated the effects of anti-glucocorticoid treatment in patients with depression, although they are still at the proof-of-concept stage. Anti-glucocorticoid drugs decrease the levels of cortisol either through inhibition of biosynthesis or through antagonism of glucocorticoid receptors (Price et al., 1996). The importance of baseline cortisol on the efficacy of these drugs has been examined in a meta-analysis of nine studies by Lombardo et al. (2019) who showed that patients treated with cortisol synthesis inhibitors, treatment responders had significantly higher baseline cortisol levels compared with non-responders, however baseline cortisol was not associated with treatment response in patients treated with a GR antagonist. This finding supports the notion that personalised treatment for patients with mood disorders may provide the greatest benefit.

1.5.2 *HPA axis and depression treatment*

Animal studies demonstrated that antidepressant medication may alter HPA-axis activity (N Barden et al., 1995; Peeters et al., 1994). Following on, clinical studies have also provided evidence of normalized HPA axis activity after successful treatment with antidepressants (Heuser et al., 1996; Linkowski et al., 1987). Plasma and saliva cortisol has been shown to be reduced following treatment with SSRIs (Dziurkowska et al., 2013; Hernandez et al., 2013), specifically: mirtazapine (Schmid et al., 2006; Schule et

al., 2003;Schüle et al., 2009); amitriptyline (Rota et al., 2005); citalopram (Nikisch et al., 2005); paroxetine (Nickel et al., 2003); sertraline (Ahmed et al., 2011;Cooney & Dinan, 2000); fluoxetine (Jazayeri et al., 2010) and escitalopram (Ahmed et al., 2011;Park et al., 2015). However findings are inconsistent with some studies reporting null effects (Deuschle et al., 2003;Kauffman et al., 2005;Muck-Seler et al., 2002;Rao et al., 2005) and some reporting time-dependent effects, with shorter administration leading to increased plasma cortisol and longer administration leading to decreased cortisol (Sagud et al., 2002).

Depressed patients with hypercortisolaemia are also less likely to achieve a significant response to antidepressants. A meta-analysis of 34 studies investigating changes in cortisol and treatment response, including 1,049 depressed patients, reported that 56% of participants had similar cortisol levels before and after treatment, regardless of symptom improvement (McKay & Zakzanis, 2010). A more recent meta-analysis of 39 studies also reported that responders and non-responders did not differ in either central HPA-axis markers (CRH and ACTH) or cortisol (S. Fischer et al., 2017a). However, further investigation revealed that when their analysis was restricted to studies which used cortisol from urine or saliva as opposed to blood, non-responders had higher pre-treatment levels than responders. This can be explained by HPA-axis interaction with the monoamine systems which are targeted by antidepressants. For example, CRH receptors are abundantly expressed in extra-hypothalamic areas, which constitute the main regions of the 5-HT and NA systems (Valentino & Commons, 2005;Valentino & Van Bockstaele, 2008). During stress CRH can modulate 5-HT and NA activity by shifting the firing pattern from phasic to tonic (Joëls & Baram, 2009). In addition, hypercortisolaemia could be a reflection of reduced negative feedback. Impaired glucocorticoid signalling not only results in hyperactivity of the HPA-axis but also in a reduced ability to inhibit inflammation (Quan et al., 2003;D. Wang et al., 2011). As already discussed, cytokines upregulate 5-HTT, reducing the bio-availability of 5-HT (Morikawa et al., 1998;Ramamoorthy et al., 1995;Zhu et al., 2006). Therefore the interplay between

alterations in HPA-axis functioning and increased inflammation could potentially render specific individuals less likely to benefit from antidepressant treatment.

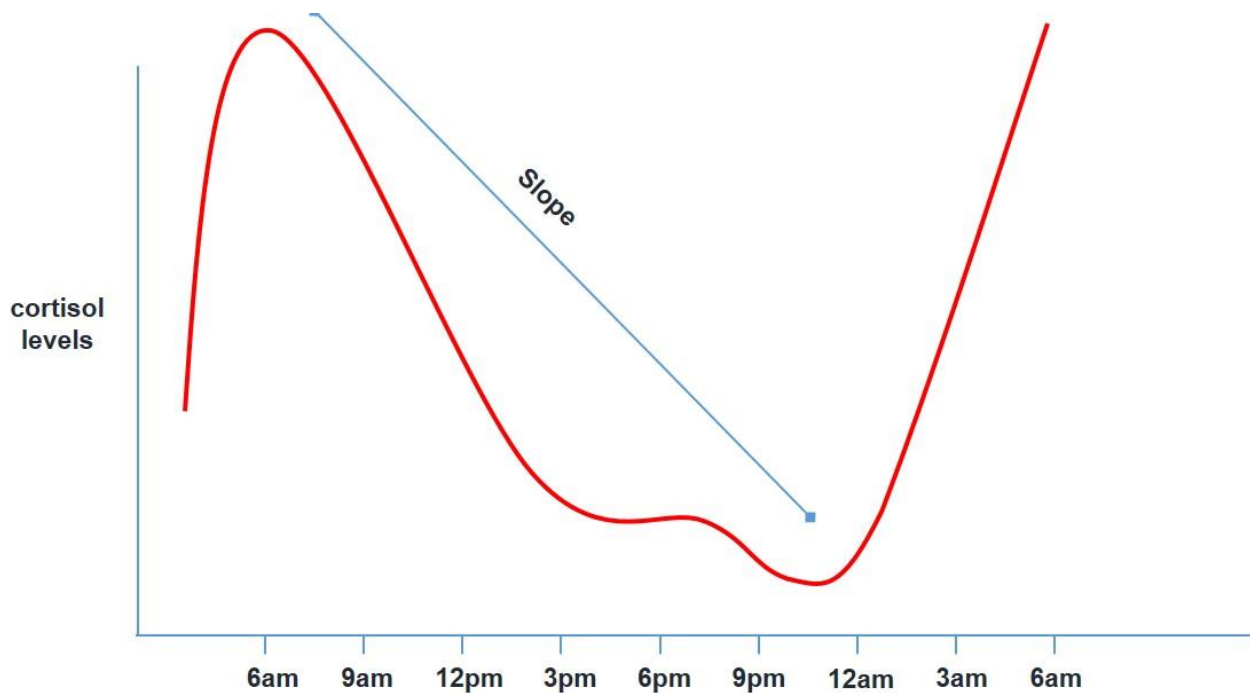
Cortisol levels following psychological therapy have also been explored. A systematic review and meta-analysis by Fischer et al. (2017b) reported that higher cortisol levels before starting psychological therapy predicts more depressive symptoms or reduced improvement at the end of treatment, although this association was by trend only. The authors speculate that psychological therapy might be ineffective for people with more pronounced HPA-axis dysregulation because high levels of cortisol may result in cognitive impairment, reducing the ability of the patient to successfully engage in therapy.

1.5.3 *Diurnal HPA axis activity*

HPA-axis researchers are also interested in the diurnal rhythm of cortisol. Cortisol secretion fluctuates according to a circadian rhythm (Figure 1.4). Under basal (i.e., unstressed) conditions, cortisol levels rise throughout the early hours, increasing sharply around 30 minutes after waking. This is referred to as the cortisol awakening response (CAR) (Fries et al., 2009). There is then a subsequent decline throughout the day with lowest levels occurring around midnight. The circadian rhythm is regulated by a central clock in the suprachiasmatic nucleus of the ventral hypothalamus, which is governed by light input received from the retina, allowing biological processes to be entrained by sunlight, and subsequently oscillate according to a 24 hour cycle (Spiga et al., 2014).

HPA-axis dysregulation and altered cortisol rhythm, can result in a change in the size of the CAR, the degree of decline in cortisol across the day (the slope) and the total or average level of cortisol across the day (area under the curve (AUC), all of which have been associated with negative health outcomes (Adam et al., 2017).

Figure 1.4 The circadian rhythm of HPA axis function



Under basal (unstressed) conditions, cortisol secretion shows a clear circadian variation over a 24 hour period. The decline of cortisol across the day is referred to as the cortisol 'slope'.

Depression and the CAR

The CAR is considered a measure of the sensitivity of the HPA-axis to naturally occurring stress (waking up) (Dedovic & Ngiam, 2015). The CAR can be calculated by either calculating change over time across multiple time points within the first 60 minutes or by calculating the CAR AUC, or the overall volume of cortisol released during this first hour. Expert consensus guidelines on the main aspects of CAR assessment has been published summarizing the evidence regarding methodological factors affecting CAR assessment and providing clear direction for future research (Stalder et al., 2016).

A number of studies have investigated the association between subclinical depression and CAR. Dedovic et al. (2010) compared young healthy adults with moderate and high levels of depressive symptoms and controls. They reported that people with sub-clinical levels of depressive symptoms failed to show a significant increase in cortisol levels after awakening. Furthermore, people with more severe symptoms also demonstrated lower

CAR AUC. A similar study in Mexican–American people with no clinical diagnosis of depression, showed that depressive symptoms were associated with flattening of the CAR. Additional analyses showed that attenuation of the initial rise in the CAR (difference between awakening and + 30 minutes) was a significant predictor of depressive symptomatology (Mangold et al., 2011). In contrast to these findings a study by Pruessner et al. (2003b) investigating mild levels of depressive symptomatology in healthy young men reported that increased symptoms were associated with a greater CAR AUC.

Alterations in CAR have also been associated with psychological traits known to increase vulnerability to depression. High levels of neuroticism (Madsen et al., 2012;Portella et al., 2005) and high-trait negative affect (Polk et al., 2005) have been associated with increased CAR in healthy volunteers. However, a negative correlation has also been observed between levels of neuroticism and CAR (Mangold et al., 2012). Furthermore some studies report no association at all (Hill et al., 2013;van Santen et al., 2011). Hopelessness has been associated with increased CAR AUC (Sjogren et al., 2006), whereas worry, rumination and loneliness have been associated with flattened CAR (Cropley et al., 2015;Leah D Doane & Adam, 2010;Kuehner et al., 2007). Associations have also been made between CAR and anticipation of future stressful events. For example, the CAR has been shown to be higher on work days compared to non-work days (Kunz-Ebrecht et al., 2004;Schlotz et al., 2004). Furthermore, this difference was associated with chronic work overload and worry (Schlotz et al., 2004).

Clinical depression has also been associated with both increased and lowered or blunted CAR. Findings from a large cohort study, including 1,588 middle-aged people, revealed that people with current and remitted MDD showed a significantly higher CAR compared with controls (Vreeburg et al., 2009a). Similar results have been reported in adult women (Dienes et al., 2013) adolescent females (Ulrike et al., 2013) and older people (Rhebergen et al., 2015). In contrast, a blunted CAR has been observed in women with

mild-to-moderate clinical depression compared with controls (Stetler & Miller, 2005). The authors reported that although both groups showed similar cortisol levels initially upon awakening, the depressed group failed to show a significant increase in the following 30 minutes and levels remained significantly lower at 60 minutes post-awakening, compared with controls. In a study of depressed patients admitted for inpatient psychotherapy CAR was also found to be blunted in depressed as compared to non-depressed patients (Huber et al., 2006).

A family history of depression may also be associated with higher CAR, suggesting a degree of heritability. A study including 49 young people without depression but who had a parent with a history of MDD, compared CAR with 55 controls without depression and no reported depression in a first-degree relative (Mannie et al., 2007). Participants with a family history of depression showed greater CAR levels compared to controls. Similar results were reported in a study which showed that non-depressed individuals with a parental history of depression had a higher CAR than those without, which was similar to that observed in people with depression (Vreeburg et al., 2010).

A number of studies have shown that childhood maltreatment disturbs the CAR. However, findings are mixed, with some studies suggesting that maltreatment leads to an increased CAR and some suggesting a blunted CAR. Childhood abuse was associated with lower morning cortisol levels at awakening, in a study of older adults, irrespective of depressive symptoms (Wieland et al., 2018). Increased CAR AUC was observed in non-depressed participants only and this was associated with childhood abuse. Childhood physical abuse and sexual abuse have been associated with greater CAR in fibromyalgia patients. Sensitivity analyses revealed that the results were not attributable to effects of depression. Another study has reported higher CAR in maltreated depressed adolescents compared to non-maltreated adolescents (Quevedo et al., 2017). In contrast, a study investigated the long-term relationship between early maltreatment and basal cortisol secretion in 623 adults adopted as children (van der Vegt

et al., 2009). The CAR AUC was lower in people who reported severe neglect or abuse compared with non-abused participants. A meta-analysis examining childhood maltreatment and diurnal cortisol regulation reported blunted waking cortisol levels but no association between childhood maltreatment and the CAR (Bernard et al., 2017).

A systematic review and meta-analysis of 147 studies was conducted by Chida and Steptoe (2009) exploring the association between the CAR and psychosocial factors including general life stress, job stress, depression, hopelessness, fatigue, burnout, exhaustion, anxiety, and negative affect, as well as positive psychological traits such as well-being and happiness. Analyses were conducted using both the change score of cortisol from the level recorded on waking (CAR) and the calculated AUC over the waking period (CAR AUC). The authors reported that general life stress were associated with an increased CAR and CAR AUC. In contrast, fatigue, burnout, or exhaustion were characterized by a reduced CAR and the CAR AUC was negatively related to posttraumatic stress syndrome, suggesting differential associations between CAR and specific psychosocial factors. No overall association was observed between depression and CAR, however rather than being a null association, this reflected the contradictory findings in the literature. These inconsistencies may be due to methodological issue such as poor controlling for confounding variables or may reflect the wide range of symptom severity, with some studies restricting the sample to patients with MDD and some including people with subclinical symptoms. Another systematic review was conducted including 151 studies examining the effects of stress on cortisol in informal dementia caregivers (Allen et al., 2017). They reported that the most consistent finding was that caregiving was generally associated with elevated cortisol levels. This increase was evident at single time points, in the CAR and across diurnal secretion. Due to the heterogeneity in the findings, a meta-analysis using p-curve analysis was conducted on 709 findings from 212 studies (Boggero et al., 2017). The authors reported inconclusive results for the CAR, however the AUC during the waking period was positively associated with depression.

The inconsistency surrounding the findings might also be related to depression severity or depressive phenotypes. A study by Veen et al. (2011) compared the association between depression and the CAR using DSM-IV categories and dimension measures of a mood questionnaire, in depressed outpatients and controls. They reported no group differences in the CAR when categorical distinctions between groups were applied. However the CAR showed statistically significant nonlinear relationships with two phenotypic dimensions: anhedonic depression and general distress. Specifically, individuals with low or mild anhedonic depression levels exhibited a similar CAR AUC as controls and those with moderate levels exhibited an increase, while those with severe levels showed a decrease in CAR AUC compared to controls. A similar inverted U shape association between CAR AUC and general distress, anhedonic depression, and anxious arousal was observed in a large cohort study of 1,029 participants with a lifetime depression and/or anxiety disorder from the Netherlands Study of Depression and Anxiety (Wardenaar et al., 2011).

Longitudinal studies have also investigated whether CAR can predict future depressive episodes. Adam et al. (2010) assessed whether CAR assessments in late adolescence could predict a clinical diagnosis of MDD one year later. Results showed that a higher CAR was associated with increased odds of having an episode of MDD over the follow-up period. Following on from these findings, Vrshek-Schallhorn et al. (2013) measured CAR in 270 older adolescents and followed them up over four years. The authors reported that baseline CAR significantly predicted major depressive episodes for 2.5 years following cortisol measurement, however beyond that time the association lost significance, suggesting increased morning cortisol may be a time-limited risk factor. Hardeveld et al (2014) also investigated whether CAR could predict time to recurrence in 549 people with a lifetime diagnosis of MDD, in remission for at least six months. Salivary cortisol samples were collected at baseline and participants were interviewed twice over a four year follow up period. Analysis revealed a higher CAR AUC was associated with time to recurrence of MDD, independently of stressful life events.

CAR has been explored in relation to symptom remission. In a study by Aubry et al. (2010) cortisol parameters were compared between patients who were in remission for at least three months and controls. Analysis revealed that CAR AUC was 51% higher in remitted patients compared to controls, suggesting a persistent exaggerated cortisol response, despite clinical recovery. A similar result was reported by Bhagwagar et al. (2003) who found that patients in clinical remission exhibited increased CAR compared to controls. Another study showed that a decrease in the CAR is predictive of antidepressant response (J. Beck et al., 2015). Patients who demonstrated a higher decrease in CAR following treatment with a serotonin–norepinephrine reuptake inhibitor (SNRI) also showed a reduction in depressive symptoms at follow up, whereas patients who did not demonstrate a reduction in CAR, failed to achieve remission. Ruhe et al. (2015) explored the longitudinal effects of SSRI treatment on cortisol in medication-free depressed patients and healthy controls. In contrast to the previous findings, CAR values were lower in the depressed group compared to controls, although this result failed to reach significance. However symptom remission was robustly associated with increased CAR in participants with depression after 12 weeks of SSRI therapy. Furthermore, the remitters had higher CARs at endpoint than controls at study-entry.

A study exploring the effects of antidepressants on cortisol including 1,526 people with either current or past depression, compared CAR between antidepressant and non-users (Manthey et al., 2011). Tricyclic antidepressant (TCA) users demonstrated a flattened CAR compared to those treated with SSRI, other antidepressants or non-users. The authors speculate that specific antidepressants may have a differential effect on the HPA-axis. Another study compared the effects of SSRI and SNRI on waking cortisol (Harmer et al., 2003). SSRI significantly enhanced the CAR, there was no effect of SNRI treatment.

To date the evidence suggests that the CAR may be heritable, however, as it is also greatly influenced by environmental factors, it may also represent a measure of

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vulnerability to depression, particularly concerning daily stress. However, longitudinal studies have demonstrated that increased CAR is associated with depression onset and recurrence, independently of stressful life events. There is also evidence to suggest differential effects on the CAR according to the type of stress experienced or the nature and severity of the depressive symptoms exhibited. The CAR may therefore be considered an index of coping which becomes dysregulated once it cannot meet the demands of stress. It is possible that beyond a certain level, persistent increased CAR may become downregulated and blunted. This could explain why some studies have reported increased CAR and others blunted CAR in relation to depression (Dedovic & Ngiam, 2015).

Depression and the cortisol slope

The decline in cortisol from morning to evening over the waking day is known as the cortisol slope. Like the CAR, the slope can be quantified in a number of ways and calculations include the number and timing of the cortisol samples across the waking day. These slopes are usually calculated by either taking a simple difference between the morning measure and the evening measure or by taking a simple difference divided by the total time between the two samples. Where multiple data points are available linear regression is used to apply a line of best fit and the slope of this line is used to estimate cortisol diurnal slope (Adam & Kumari, 2009).

The association between cortisol slope and MDD has been examined in adolescents. Doane et al. (2013) compared cortisol slope in 300 adolescents with current and past MDD with controls. The authors reported that past and current MDD were both associated with a flatter slope compared with no history of MDD. Negative emotion, including sadness and loneliness, was associated with flatter slopes and partially accounted for the associations between MDD and cortisol rhythm. Using the same sample, Adam et al.(2010) reported that higher baseline CAR was associated with a

significantly increased risk of developing MDD one year later, even when excluding individuals with baseline MDD. This suggests that a heightened CAR may play a role in the aetiology of MDD.

Self-reported depressive symptoms have been associated with a flatter slope in a random sample of 257 men and women (Sjogren et al., 2006) and a study on 126 MDD outpatients and 106 healthy controls reported that flatter diurnal cortisol patterns were associated with family history of mental illness, symptom severity and higher suffering levels (Hsiao et al., 2010). A flatter slope was also observed in women with MDD compared with controls, and with depression severity (Jarcho et al., 2013). Furthermore flatter diurnal cortisol slopes were associated with reduced cortisol response to the DST, suggesting that flatter cortisol rhythms in depression may reflect impaired GR function, particularly for those women with severe symptoms.

However, inconsistencies do exist in the literature. A study including 57 MDD patients and 40 healthy controls reported no significant difference in the diurnal cortisol variation between groups (Doolin et al., 2017). The authors speculate that this may be due to inconsistencies in saliva sampling. Another small study also reported no significant difference in cortisol slope in 13 medicated depressed patients compared with 13 healthy controls (Assies et al., 2004). In another study daily cortisol slope was steeper in the depressed compared to the non-depressed group, however the sample size was small (N=30) (S. H. Booij et al., 2015). A systematic review and meta-analysis by Adam et al. (2017) investigated diurnal cortisol slopes and physical and mental health outcomes, including depression symptoms and disorders. Flatter slopes were significantly associated with depression, however as most of the studies were cross sectional in nature, it was impossible to determine the direction of causality.

Chronic stress has been linked to flattened slope (Adam et al., 2017) and steeper slope (Ockenfels et al., 1995; Steptoe et al., 2000). A recent study explored whether HPA-axis

genetic variation explains the heterogeneity in the findings (Starr et al., 2019). The authors reported that whilst chronic stress did not directly predict diurnal slope, adolescents at low genetic risk appeared to adapt to chronic stress over six months with a high waking cortisol and steeper diurnal decline. Childhood maltreatment has also been with associated diurnal slope. In a study of adoptees, people who reported moderate maltreatment had a steeper diurnal slope and those who reported severe maltreatment had a flatter slope compared to non-maltreated adoptees (van der Vegt et al., 2009). This suggests that the nature of this relationship is influenced by the level of severity of early maltreatment. However, a meta-analysis of 27 studies reported no association between childhood maltreatment and cortisol slope (Bernard et al., 2017).

To my knowledge only one study has explicitly investigated the effects of SSRIs on cortisol slope. The study included 64 non-depressed men and women who were randomised to SSRI or placebo for six days (Ronaldson et al., 2018). Results showed that women receiving SSRIs had significantly steeper cortisol slopes compared with those receiving placebo. There was no effect of medication on cortisol slope in men. Antidepressant effects on cortisol slope have not been explored in people with depression.

Depression and the cortisol AUC

The AUC is a summary measure of the total or average level of cortisol across the day (Adam & Kumari, 2009) and changes in AUC have also been reported in people with depression. In a study assessing salivary cortisol levels in 45 females caring for a stroke survivor, Saban et al. (2012) found that cortisol levels were significantly lower across the day in women with a high level of depressive symptoms compared to women with a low level. Lower cortisol concentrations were also observed in 401 men and women in Canada with depressive symptoms (Marchand et al., 2014). In contrast, O'Brien et al. (2004) reported a 53% increase in the AUC in 61 older people with MDD, compared with

40 healthy controls. Higher mean levels of cortisol have also been reported in clinically depressed young women compared to controls (Dienes et al., 2013). In another study no difference in AUC was observed between women with MDD compared to controls (Gonul et al., 2017).

Cortisol levels have also been investigated in relation to anti-depressant response. In a study of 208 depressed patients, responders had significantly lower cortisol levels than those who did not respond (Ventura-Junca et al., 2014). Hinkelmann et al. (2012) also examined cortisol AUC in 52 MDD patients before and after 3 weeks of SSRI therapy in conjunction with an add-on treatment modulating the mineralocorticoid receptor in comparison to healthy controls. Results showed that SSRI treatment reduced cortisol in depressed patients to the level of healthy control within three weeks. Moreover, symptoms remission was strongly correlated with decreases in cortisol secretion. Ruhe et al. (2015) also showed a decrease in AUC in 70 initially drug-free MDD patients following SSRI treatment. More recently, a preliminary study including 13 adolescents with MDD, showed no differences in AUC between those with remitting symptoms compared to those with persistent symptoms (Klimes-Dougan et al., 2018).

A study including 429 international adoptees explored the influence of childhood maltreatment on anxiety and the AUC (van der Vegt et al., 2010). The results showed that in adoptees with an anxiety disorder, severe maltreatment was associated with a lower AUC than non-maltreated adoptees. In adoptees without an anxiety disorder, no difference in the AUC was observed between participants who did or did not experience severe maltreatment in childhood. This suggests that early life adversities may modify the relationship between mood disorders and basal cortisol secretion in adulthood.

Morning and evening cortisol

A systematic review and meta-analysis was conducted by Knorr et al. (2010) including 20 case-control studies, including 1,354 patients with depression and 1,052 controls. Initial findings showed that cortisol was increased in the morning and in the evening in depressed patients compared to controls, however subsequent meta-regression revealed that the difference in cortisol levels may be associated with age and the intra-assay variability of the cortisol kits and not associated with depression scores. The authors conclude that there is no firm evidence for a difference of salivary cortisol in depressed patients.

The antidepressant effects of morning cortisol have also been investigated. A study by Harmer et al. (2003) studied the effect of six-day administration of the SSRI citalopram and the SNRI reboxetine and on waking cortisol in healthy volunteers. They reported that citalopram significantly increased waking cortisol, whilst there was no effect of reboxetine.

Overall, the evidence suggests that depression is associated with dysregulation of HPA axis function, however the literature is not consistent concerning the direction of the dysregulation. For example, both heightened and blunted CARs have been reported, as have increased and decreased AUC. CAR appears to have a degree of heritability but is also associated with daily stress, specific psychological traits and severity of symptoms. Findings regarding antidepressant response are also conflicting. Clinical remission has been associated with both increased and decreased CAR and results concerning the AUC show both decreases and no change. Evidence regarding the cortisol slope is less conflicting, with most studies reporting a flatter slope in depressed people. However the effects of antidepressants on cortisol slope have yet to be determined in MDD. There appears to be a complex and important relationship between HPA-axis dysregulation

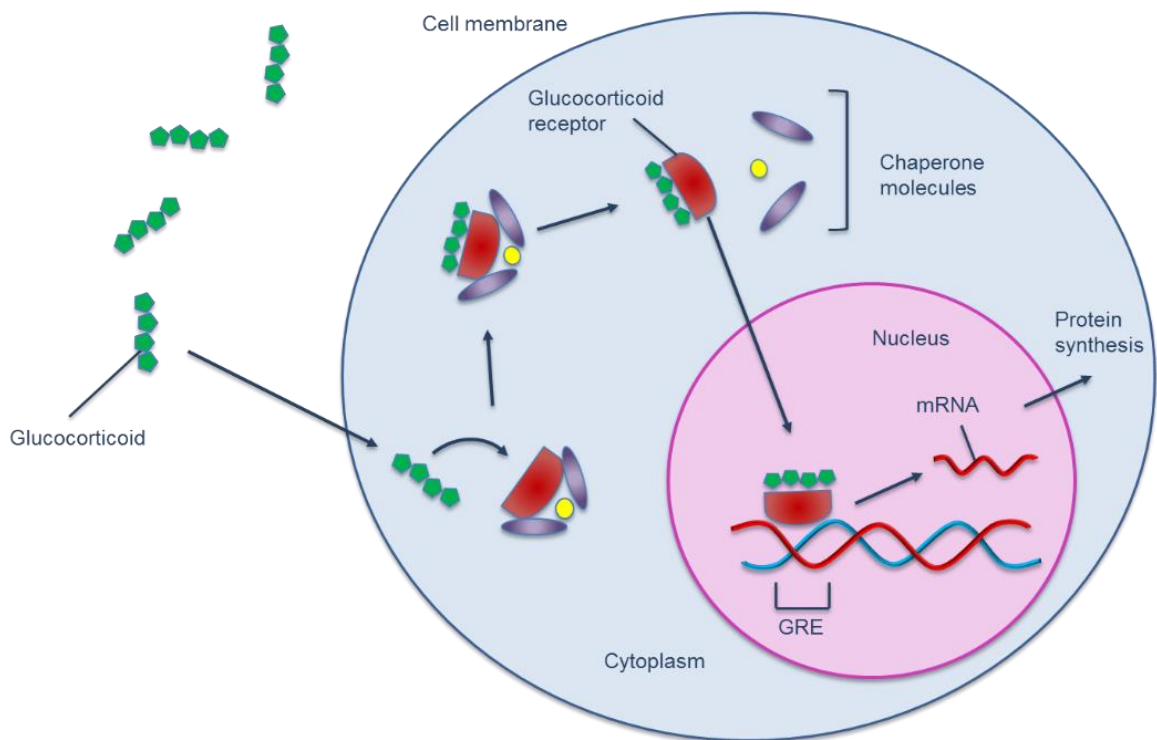
and depression, however the mechanisms underlying this dysregulation are still unclear. The prevailing model focuses on reduced corticosteroid receptor sensitivity.

1.5.4 *The role of the corticosteroid receptor*

As previously mentioned, the inhibitory effect of glucocorticoids on the HPA-axis is mediated by two intracellular receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). GRs are ubiquitously expressed and regulate function throughout the body, although numbers may differ according to cell type (Pariante, 2004). In contrast, MRs are selectively expressed in tissue such as the brain, heart, kidney and colon. MR expression is particularly high in the hippocampus and other limbic-pre-fronto-cortical regions (E. R. de Kloet et al., 2016).

The inactivated GR is located in the cytoplasm of the cell, where it resides as a complex with molecular chaperone molecules, including several heat shock proteins. Upon glucocorticoid ligand binding, such as cortisol or dexamethasone (a synthetic glucocorticoid), conformational changes occur in the GR (“activation”) and it dissociates from the chaperone proteins and translocates to the nucleus (Figure 5). There it binds to specific glucocorticoid response elements (GREs) in regulatory DNA areas (Juruena et al., 2004) or interacts with other transcription factors.

Figure 1.5 Translocation of the GR from the cytoplasm to the nucleus



The inactivated GR resides primarily in the cytoplasm, in a complex with chaperone proteins that serve to stabilize the unbound receptor. Upon binding to the glucocorticoid ("activation"), the GR sheds its chaperone proteins and translocates to the nucleus. It then binds to specific regulatory DNA areas in target genes (GREs) where it either activates or represses gene transcription. GRE: glucocorticoid response element; mRNA: messenger RNA

GREs can confer either positive or negative regulation on the genes to which they are linked. Typical target genes that are negatively regulated by GR include a large number of inflammatory proteins, including IL-6 and TNF- α (De Bosscher & Haegeman, 2009). Once activated, the GR cannot rebind with glucocorticoid, as it is dissociated from the chaperone complex, and is recycled (W. B. Pratt, 1993).

The MR is highly expressed in limbic regions of the brain and binds with high affinity to naturally occurring glucocorticoids such as cortisol and corticosterone, suggesting that it is actively occupied most of the time (Pariante & Lightman, 2008). It is believed to play a role in the regulation of circadian fluctuations in these hormones and has been shown to be both necessary and sufficient to maintain low basal corticosterone levels and low HPA-axis activity during the nadir of the circadian cycle (E. R. De Kloet et al.,

1998;Spencer et al., 1998). Conversely, the GR preferentially binds to synthetic glucocorticoids, such as dexamethasone and has reduced affinity with endogenous glucocorticoids. As such, it is thought to be more important in regulating the stress response, when levels of cortisol are high and maintaining normal levels of HPA axis activity at the peak of the circadian cycle (Juruena et al., 2006;Spencer et al., 1998). However it has also been shown that during the circadian peak or acute stress the MR also plays a role in facilitating GR regulation of the HPA-axis (Spencer et al., 1998), suggesting that rather than working independently of one another, they function co-operatively.

Evidence suggests that homeostasis depends on the coordination and balance of these two receptors (E. R. de Kloet et al., 2007b). In the early stages of the stress response, cortisol increases attention, vigilance and emotional responses, as well as facilitating a behavioural response and the MR receptors appear to mediate these actions. In the later stage of the stress response, following increases in the levels of cortisol, the GR receptors become activated and this enables the cessation of the stress response. The GR also enables memory formation of the successful behavioural response in dealing with the stressor, preparing the individual for future stressful events. It is therefore imperative that the actions of the MR and GR are appropriately coordinated. If stress signalling occurs inappropriately, impairment in cognitive function and behavioural adaptation occurs, as is observed in depression (E. R. de Kloet et al., 2005). Once the balance between MR and GR function is disturbed, the individual is less able to maintain homeostasis in the presence of an adverse experience, leading to HPA-axis dysregulation, further increasing depression risk.

1.5.5 *Mechanisms of glucocorticoid resistance*

The biological impact of glucocorticoids is not only influenced by the level of glucocorticoids released by the HPA-axis but also by how sensitive the tissue is to their

effects and many factors can affect this sensitivity (Quax et al., 2013). It is possible that hypercortisolism exhausts the ability of the GR to recycle and therefore impairs its ability to respond adequately to further stimulation (Pariante & Miller, 2001). However the literature on GR number in depression, discussed below, does not provide much support for this hypothesis. Glucocorticoid receptor affinity can also influence glucocorticoid sensitivity. Patients with Cushing syndrome demonstrate decreased glucocorticoid receptor affinity in PBMCs (Hagendorf et al., 2005). Conversely, glucocorticoid receptor affinity might be normal in patients with chronic hypocortisolism. It is thought that 30% of all patients who receive glucocorticoid treatment demonstrate glucocorticoid resistance, specifically 4–10% of asthma patients, 30% of rheumatoid arthritis patients, almost all chronic obstructive pulmonary disease and sepsis patients (P. J. Barnes & Adcock, 2009; Dendoncker & Libert, 2017) and 10–30% of untreated acute lymphoblastic leukaemia patients (Haarman et al., 2003).

Glucocorticoid bioavailability could affect GR sensitivity. The vast majority of circulating glucocorticoids are bound to plasma proteins, mostly to cortisol-binding globulin (CBG) and only free, unbound glucocorticoids are biologically active (around 10%) (Quax et al., 2013). Therefore, CBG levels determine the actual amount of cortisol available. CBG levels have been associated with cortisol and ACTH levels following social stress challenge in healthy people, suggesting a role for CBG in HPA-axis regulation (Kumsta et al., 2007). However there is no evidence of increased CBG in depressed people compared to controls (Deuschle et al., 1996; Leake et al., 1989).

In addition, the intracellular bioavailability of free cortisol is regulated 11- β -hydroxysteroid dehydrogenase (11 β HSD) (Oakley & Cidlowski, 2013). 11 β HSD is a family of enzymes which modulate the effects of glucocorticoids. 11 β HSD type 1 converts inactive cortisone into active cortisol. Conversely 11 β HSD type 2 converts cortisol into inactive cortisone. Changes in the expression of these enzymes can influence GR sensitivity (Whorwood et

al., 2001). There is some evidence to suggest that 11 β -HSD2 activity is decreased in depressed patients (Zhai et al., 2015).

Another possibility is that people with GR sensitivity may have a genetic predisposition i.e. there may be genetic variants in the GR gene (NR3C1) which influence function. In recent years a number of common SNPs have been shown to be associated with glucocorticoid sensitivity, including ER22/23EK, 9 β , N363S, TthIII, NR3C1-1, and BclI (A. T. Spijker & Van Rossum, 2012). These SNPs have also been associated with mood disorders. The ER22/23EK SNP has repeatedly been associated with a higher risk of depression (Bet et al., 2009;van Rossum et al., 2006;van West et al., 2006) and clinical response to antidepressant treatment (van Rossum et al., 2006). An association between the ER22/23EK and 9 β SNPs and depressive symptoms in combination with childhood adversity has also been observed suggesting a gene X environment interaction. The BclI polymorphism is also associated depression risk (Brouwer et al., 2006;Krishnamurthy et al., 2008;H. Y. Lee et al., 2009;van Rossum et al., 2006) and treatment response (Brouwer et al., 2006).

SNPs in the MR gene (NR3C2) have also been associated with MR function. The MR180V allele has been associated with increased cortisol response to both perceived chronic stress and acute laboratory stress (DeRijk et al., 2006;van Leeuwen et al., 2011) and with depressive symptoms (Kuningas et al., 2007). A weak association was also observed between this SNP and the CAR after a low dose of dexamethasone in elderly people (DeRijk et al., 2011). There is increasing evidence for sex differences in MR functionality. Common MR haplotypes (groups of genes inherited together from a single parent), have been associated with sex differences in suppression of the CAR following dexamethasone in healthy people (van Leeuwen et al., 2010) and with depression risk in women (Klok et al., 2011b) and with sex-specific depression susceptibility following childhood maltreatment (C. H. Vinkers et al., 2015).

Genetic variation in the FKBP5 gene may provide a genetic contribution to GR resistance. FKBP5 is a protein which forms part of the chaperone complex required for ligand-binding of the GR and subsequent compartmentalisation. Variations in the FKBP5 gene lead to increased intracellular protein expression, influencing GR function (A. T. Spijker & Van Rossum, 2012). Alleles associated with increased expression of FKBP5 following GR activation, lead to an increased GR resistance and impaired HPA-axis function in healthy controls (Binder, 2009) and are associated with antidepressant response and the recurrence of depressive episodes (Binder et al., 2004).

Another potential mechanism of GR resistance is gene expression due to alternative splicing. Whilst there is only one gene which codes for the GR, there are nine exons give rise to several splice variants (Quax et al., 2013). Most of the research has focused on GR α and GR β . GR α is the biologically active isoform, whereas GR β is inactive and incapable of glucocorticoid binding. However several genes are regulated by GR β and it is thought to negatively inhibit GR α , impairing its transcriptional activity suggesting that high levels of GR β may lead to glucocorticoid resistance. Interestingly, the expression of GR β is increased by proinflammatory cytokines and increased levels of GR β have been associated with glucocorticoid resistance in a variety of inflammatory diseases (Oakley & Cidlowski, 2013). Reduced expression of GR α mRNA has been shown in post mortem brain tissue (Perlman et al., 2004;M. Webster et al., 2002) and in PBMCs in depressed patients (Carvalho et al., 2014;Matsubara et al., 2006). However to date, the role for GR β in depression has yet to be determined.

Post mortem studies have shown decreased MR mRNA in the hippocampus (Klok et al., 2011a;Lopez et al., 1998;Medina et al., 2013) and PVN (Qi et al., 2013) of depressed patients and a difference in GR:MR ratio in depressed patients compared to controls (Medina et al., 2013;Qi et al., 2013).

1.5.6 Measurement of GR function

Over the years different approaches have been developed to test GR function, both *in vitro* and *in vivo*. Studies either measure GR number directly or assess the influence of glucocorticoids on GR function. In light of the obvious methodological issues associated with accessing GRs in the brain, most investigations rely on using peripheral cells, such as immune cells. Although differences have been observed in density between immune and neuronal GRs, similarity has been demonstrated in affinity and steroid specificity (Lowy, 1989). Furthermore Yehuda et al. (2004) showed a high correlation between the inhibitory effect of dexamethasone and GR sensitivity in mononuclear leukocytes, both *in vitro* and *in vivo*. In light of this evidence and given their obvious availability, the evaluation of peripheral cells presents a plausible model of GR investigation (Pariante & Miller, 2001).

Two different *in vitro* approaches have been used to explore GR number. Cytosolic binding assays involve lysing the cells and incubating the cytosolic fraction of the lysate with steroid (A. H. Miller et al., 1998). As previously described, once the GR has become activated it translocates from the cytoplasm to the nucleus, thereby leaving a reduced number of GRs in the cytosolic fraction. The disadvantage of this method is that the reduced number of GRs could represent either a reduction in the overall expression of GR, suggesting downregulation, or an increase in the number of GRs in the nucleus, suggesting increased activation. The second approach, whole cell binding, overcomes this issue and measures total cellular GRs. Whilst these techniques are able to provide useful data regarding GR number, they do not provide information regarding their biological effects (Quax et al., 2013).

In vitro studies investigating GR function involve isolating and cultivating immune cells (Leistner & Menke, 2018). Glucocorticoids are known to inhibit the proliferation of PBMCs in response to polyclonal mitogens and earlier studies focused on these effects (Pariante

& Miller, 2001). PBMCs were incubated with concanavalin A, phytohemagglutinin and pokeweed mitogen, followed by dexamethasone, and proliferative responses were measured. More commonly nowadays, whole blood or isolated monocytes are co-incubated with the bacterial endotoxin LPS, which is known to stimulate an immune response, and various concentrations of dexamethasone in order to determine proinflammatory cytokine production. The specific concentration of dexamethasone required for 50% inhibition of the maximum LPS-induced cytokine production observed (in the absence of dexamethasone), is referred to as the 'inhibitory concentration 50' (IC50). The IC50 is an index negatively associated with glucocorticoid sensitivity (Leistner & Menke, 2018). Reduced dexamethasone-induced inhibition of either cytokine production or mononuclear cell proliferation is interpreted as partial glucocorticoid resistance (Carvalho & Pariante, 2008). Throughout this thesis, *in vitro* assays will be referred to as 'glucocorticoid sensitivity assays'.

Genetic expression studies have also been conducted. Transcriptional profiling has been conducted in RNA from post mortem brain tissue and from peripheral blood (Leistner & Menke, 2018), mRNA expression has been measured in many cell types and GR protein has been measured using cytosolic binding and Western blot techniques (Carvalho & Pariante, 2008).

In vivo studies have predominantly used the dexamethasone suppression test (DST). The DST was first used as a diagnostic tool for screening for Cushing syndrome (Carroll et al., 1968). Dexamethasone binds to the glucocorticoid receptors and inhibits synthesis of ACTH, leading to a decrease of cortisol concentrations. Thus the DST is able to measure the negative feedback mechanism of the HPA axis. Usually an oral dose of 1.5mg dexamethasone is given to the participant at 11.00pm and plasma/saliva cortisol (and sometimes ATCH) is measured at various times the following day. Cortisol concentrations exceeding 5 µg/dl are considered an abnormal DST result and indicative of glucocorticoid resistance (reduced receptor sensitivity) (Kunugi et al., 2006). Non-

suppression of the DST has been correlated with reduced dexamethasone-induced inhibition of lymphocyte proliferation *in vitro* (Carvalho & Pariante, 2008). However, the DST has been criticised for lacking the sensitivity and specificity to reliably differentiate mood disorder patients from other psychiatric populations or healthy controls (Arana et al., 1985; Braddock, 1986).

The CRH receptors can also be investigated using the corticotrophin-releasing hormone (CRH) challenge test. Following intravenous administration of CRH, ACTH levels are measured assessing the responsivity of the pituitary to CRH (Leistner & Menke, 2018). A bolus injection with 1 µg/kg or 100 µg of CRH is administered and ACTH and cortisol levels are evaluated in the following 1–2 hours, in regular time intervals. Healthy people demonstrate an increase in ACTH between 5 and 15 minutes after CRH injection with a peak response in ACTH between 10 and 15 min and a peak response in cortisol levels between 30 and 60 min following infusion (C. de Kloet et al., 2006).

To improve the sensitivity of the DST and further explore the neuroendocrine feedback of the HPA axis, the DST was combined with the CRH test, developing the dexamethasone/corticotropin releasing hormone (Dex/CRH) challenge test (Von Werne Baes et al., 2012). A single, oral dose of 1.5mg dexamethasone is given to the participant at 11.00pm, followed by an intravenous bolus of 100µg CRH, the next day at 3pm. Patients stay in a supine position throughout the test. Blood samples are then taken at 5 time intervals between 3.00pm and 4.15pm to test for cortisol and ACTH concentrations. The 3pm blood sample measures dexamethasone suppression and the subsequent samples measure CRH response (Leistner & Menke, 2018).

Despite its popularity, the DST is not without its limitations. Firstly, not all MDD patients are non-suppressors, therefore the DST may be a marker of a specific pathophysiological subtype (W. A. Brown & Shuey, 1980). Secondly, dexamethasone only binds to the GR and therefore is unable to probe MR function and has a much longer

half-life compared with cortisol. A suppression test was devised by Pariante et al. (2002) using prednisolone, a synthetic glucocorticoid that is more similar to cortisol in its ability to bind to both the GR and MR, as well as having a similar half-life. As such, the prednisolone suppression test (PST) provides an indirect measure of GR and MR-mediated negative feedback on the HPA axis and can be used both *in vitro* and *in vivo*. It has therefore been proposed as a more naturalistic test for the HPA axis.

The PST and DST have been used in combination in depressed people to explore MR function. Results have shown evident dissociation between the cortisol responses to dexamethasone and to prednisolone, in patients compared with controls (Jurueña et al., 2006). As both glucocorticoids probe the GR and only prednisolone probes the MR, any difference in sensitivity between them can be interpreted as a selective MR sensitivity. Indeed results from research using both glucocorticoids show that depressed patients show less suppression than controls following dexamethasone administration but show normal suppression by prednisolone. Furthermore, in controls there is a correlation between prednisolone and dexamethasone suppression, indicating equal sensitivity. In contrast, no such correlation is present in depressed patients. In a similar fashion to the DST, an oral dose of 5mg prednisolone is given to the participant at 10.00pm and saliva cortisol is measured at various times the following day.

These findings are consistent with studies examining MR function in depression, using the MR antagonist, spironolactone. Spironolactone is a potent anti-mineralocorticoid which blocks the MR. An oral dose of 200-300mg spironolactone is administered to the participant and blood or salivary cortisol is measured before ingestion at baseline and every 30 minutes for up to six hours (Hinkelmann et al., 2016; Young et al., 2003). Studies exploring MR function have also used fludrocortisone which is a synthetic corticosteroid which acts largely as a powerful mineralocorticoid, with some weak glucocorticoid activity (Nicolaidis et al., 2000). Blood samples are taken at baseline and patients are

administered 0.5mg of fludrocortisone. Blood samples are then taken every hours for up to five hours and cortisol levels are measured (Lembke et al., 2013).

1.5.7 *Depression and the corticosteroid receptors*

Over the last 30 years, many studies have explored glucocorticoid function in people with depression. Due to the fact that the GR preferentially binds to synthetic GCs and most depression studies have used the DST, there is a vast bias towards studies investigating GR function compared to MR function. Furthermore, because the MR has a high affinity for endogenous corticosteroids and is therefore believed to be occupied most of the time, it was thought that the GR was more important in the stress response (Pariante & Miller, 2001). This section will first describe studies investigating corticosteroid receptor number in people with depression, before moving on to corticosteroid receptor sensitivity.

Corticosteroid receptor number and depression

Whilst the majority of early studies investigating GR number reported no difference between people with depression and healthy controls (Maguire et al., 1997; Rupperecht et al., 1991a; Rupperecht et al., 1991b; Schlechte & Sherman, 1985; A. Wassef et al., 1990; A. Wassef et al., 1992) a few studies have found a reduction in the number of GRs in depressed people (Gormley et al., 1985; Sallee et al., 1995; Whalley et al., 1986). Oral dexamethasone administration also significantly decreased glucocorticoid receptor number in DST suppressors, compared to non-suppressors (Gormley et al., 1985; Lowy et al., 1988; A. Wassef et al., 1990). More recently, a study by Calfa et al. (2003) showed a reduced number of GRs on lymphocytes from non-medicated depressed patients compared with healthy controls. Following subsequent antidepressant treatment a significant increase in GR number was observed in the depressed people. Furthermore this increase was above the level of GRs observed in the control group. Depressed patients have also been shown to have fewer GR number compared to patients with

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other psychiatric disorders (Yehuda et al., 1993). Furthermore, in a study by Sallee et al. (1995) investigating GR number in depressed adolescents, a reduction in cytosolic GR binding at baseline predicted antidepressant response, and GR number increased following successful antidepressant treatment. The finding that antidepressants upregulate GR binding is supported by a number of animal studies which demonstrate that antidepressant treatment upregulates GR protein and mRNA in the brain (Pariante & Miller, 2001).

The inconsistency in the findings may be due to patient heterogeneity, cell populations studied and methodological differences. Most of the studies which reported a reduction in GR number in depressed people compared to controls used cytosolic binding assays, however most of the studies which reported no difference used whole cell binding assays. The combination of findings (no difference in whole cell numbers and reductions in cytosolic numbers) suggests that GR alterations observed in depressed people may be due to increased translocation to the nucleus or an increase in the number of GRs which have shed their chaperone proteins following recent activation and are therefore no longer able to bind with the ligand and reactivate. As excess cortisol levels overburdening the GR could result in either of these outcomes, this would provide support for the theory that GR downregulation is secondary to hypercortisolism.

Corticosteroid receptor function and depression

In vitro measurement

In vitro studies of GR function have consistently reported significant decreased sensitivity in depressed patients (see Table 5). Wodarz et al. (1991;1992) and Rupprecht et al. (1991c) reported a significantly weaker suppressive effect of in vitro dexamethasone on mitogen-induced lymphocyte proliferation in severely depressed people. Furthermore, there was no impairment in GR function in clinically recovered patients (Wodarz et al.,

1992). Two studies by Lowry et al. (1984;1988) have also shown that oral and *in vitro* dexamethasone inhibition of lymphocyte proliferation is reduced in depressed people who are also non-suppressors on the DST. It should be noted here that the non-suppression of cortisol and lymphocyte proliferation in the same participants, using dexamethasone both *orally and in vitro*, demonstrates the utility of *in vitro* studies as direct measures of GR function (Pariante, 2004). Reduced inhibition of natural killer cell mediated cytotoxicity following *in vitro* cortisol administration has also been observed in MDD patients compared with controls (A. H. Miller et al., 1987). The relationship between depressive severity and GR function has also been explored *in vitro* (Tanke et al., 2008). An association between dexamethasone suppression of lymphocyte proliferation and MDD symptom severity was observed in 13 MDD patients.

Dexamethasone inhibition of LPS-stimulated cytokines in whole blood has also been used to explore GR function in psychiatric populations. Rohleder et al. (2004) reported that less dexamethasone was required for the suppression of both IL-6 and TNF- α in PTSD patients compared to controls, reflecting increased GR sensitivity. Miller et al. (2005b) measured dexamethasone inhibition of LPS-stimulated IL-6, in response to an acute bout of psychological stress, in 72 women, 36 with MDD and 36 healthy controls. All participants underwent a 17-minute mock-job interview before providing blood samples. Depressed participants demonstrated increased GR sensitivity compared with controls, prior to the stressor. However following the stressor sensitivity decreased among depressed individuals and increased among controls, suggesting a dysregulated stress response.

Studies have also explored GR function and antidepressant treatment. The effects of sertraline on glucocorticoid sensitivity has been investigated in PBMCs in PTSD patients (Yehuda et al., 2006). Results showed that sertraline reduced GR sensitivity compared with controls, which is line with the findings that PTSD patients exhibit enhanced sensitivity to glucocorticoids (Yehuda et al., 2004). Calfa et al. (2003) showed an

increase in cortisol inhibition of mitogen-induced lymphocyte proliferation in depressed people following 4 weeks of antidepressants, to a level even higher than that found in healthy controls. In contrast, a study by Bauer et al. (2003) demonstrated reduced inhibition of T-cell proliferation and cytokine production in TRD patients following both *oral and in vitro* administration of dexamethasone. Furthermore, Carvalho et al. (2008) showed that 24 hour incubation of whole blood with clomipramine decreased glucocorticoid inhibition of LPS induced IL-6 in controls but not in TRD patients. Therefore, TRD patients also demonstrated a lack of antidepressant effect *in vitro* suggesting that restoration of GR function is dependent on clinical remission. However it should be noted that this observed reduction in GR sensitivity following antidepressants is thought to be transient and specific to *in vitro* studies.

Chronic stress has also been associated with GR resistance. In a study measuring job strain in men, 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. In a study including 120 mothers of children with newly diagnosed cancer, those women who demonstrated higher levels of distress across the 12 months following their child's diagnosis showed increased glucocorticoid resistance. Another study examined glucocorticoid signaling in people caring for a family member with glioblastoma and healthy controls whose lives were free of major stressors (G. E. Miller et al., 2014). Results showed that caregivers demonstrated reduced expression of genes with response elements for the glucocorticoid receptor, however, there were no differences in functional glucocorticoid sensitivity between the groups; hydrocortisone was equally effective at inhibiting cytokine production following monocyte stimulation with LPS. A similar study including familial caregivers of brain-cancer patients, also showed that the caregiver's monocytes showed diminished expression of transcripts bearing response elements for glucocorticoids compared to controls (G. E. Miller et al., 2008). A longitudinal study also provided evidence for an association between

psychological distress and the development of glucocorticoid resistance in mothers of children newly diagnosed with cancer (Walsh et al., 2018).

Gene expression analyses following GR induction have also been conducted alongside neuroendocrine tests to investigate any genetic influences on GR function. Spijker et al. (2010) examined LPS-stimulated (*ex vivo*) whole blood gene expression in MDD patients. They identified a gene expression profile (*CAPRN1, CLEC4A, KRT23, MLC1, PLSCR1, PROK2, ZBTB16*) that serves as a molecular signature of MDD. Interestingly, from the seven genes identified, six are related to immune cell proliferation and one is related to regulation of circadian rhythm by the suprachiasmatic nuclei. Considerable evidence demonstrates that antidepressants enhance GR signalling *in vitro* via mRNA expression, GR protein level and GR function (Carvalho & Pariante, 2008).

Table 1.5 In vitro measurement of GR function in depression

Author/year	Sample	Study design	GR measurement protocol	Statistical analysis	Results
Bauer et al. 2003	36 TRD inpatients (15 men, 21 women), 31 healthy controls (12 men, 19 women) mean age 49yr	Difference in glucocorticoid sensitivity between pharmacologically treated MDD inpatients and healthy controls	DEX suppression of mitogen induced T-cell proliferation, LPS-induced IL-2 and TNF- α in isolated PBMCs. Comparison with DST	ANOVA, no covariates	Decreased glucocorticoid sensitivity in TRD patients compared to controls
Carvalho et al. 2008	15 TRD patients, 28 healthy controls, mean age 47	Difference in glucocorticoid sensitivity between pharmacologically treated TRD patients and healthy controls	DEX, PRED cortisol and corticosterone inhibition of LPS-induced IL-6 levels in whole blood	Mann–Whitney	Decreased glucocorticoid sensitivity in controls following incubation with antidepressants. No effect of antidepressants on glucocorticoid sensitivity in controls
Calfa et al. 2003	16 MDD patients, untreated (4 men, 12 women), 13 MDD patients treated (9 men and 4 women) and 16 healthy controls (12 men, 4 women), mean age 39yr	Difference in glucocorticoid sensitivity between pharmacologically untreated MDD patients, pharmacologically treated MDD patients and healthy controls	Cortisol suppression of mitogen-induced lymphocyte proliferation.	ANOVA Covariates: basal cortisol	Decreased glucocorticoid sensitivity in MDD patients compared to controls. Increased glucocorticoid sensitivity in MDD patients following treatment

Table 1.5 continued. In vitro measurement of GR function in depression

Author/year	Sample	Study design	GR measurement protocol	Statistical analysis	Results
Lowy et al. 1984	12 MDD inpatients (7 men, 5 women), 12 matched controls, mean age 31yr	Difference in glucocorticoid sensitivity between MDD inpatients and healthy controls	DEX suppression of mitogen induced lymphocyte proliferation	Student's t-test, ANOVA	Decreased glucocorticoid sensitivity in MDD patients compared to controls
Lowy et al. 1988	21 MDD inpatients (9 men, 12 women), 23 non-depressed psychiatric patients (15 men, 8 women) and 15 controls (9 men, 6 women), mean age 31yr	Difference in glucocorticoid sensitivity between MDD inpatients, non-depressed psychiatric patients and healthy controls	DEX suppression of mitogen induced lymphocyte proliferation. Comparison with DST	Student's t-test, ANOVA	Decreased glucocorticoid sensitivity in DST non-suppressors compared to suppressors regardless of depression status
Miller et al. 2005	36 women with MDD and 36 healthy female controls, mean age 27yr	Difference in glucocorticoid sensitivity between women with MDD and healthy controls in response to acute psychological stress	DEX suppression of LPS-induced IL-6 and TNF- α in PMBCs	ANOVA, no covariates	Decreased glucocorticoid sensitivity in women with MDD following stressor compared to controls

Table 1.5 continued. In vitro measurement of GR function in depression

Author/year	Sample	Study design	GR measurement protocol	Statistical analysis	Results
Rupprecht et al. 1991c	12 severe MDD patients (3 men, 9 women) and 13 healthy controls (3 men, 10 women), mean age 48yr	Difference in glucocorticoid sensitivity between severely depressed people and healthy controls	DEX suppression of mitogen induced lymphocyte proliferation following glucocorticoid depletion	ANOVA, no covariates	Decreased glucocorticoid sensitivity in severely depressed people compared to controls
Tanke et al. 2008	13 MDD inpatients (5 men, 8 women) mean age 48yr	Relationship between glucocorticoid sensitivity and depressive symptom severity	DEX suppression of mitogen induced lymphocyte proliferation	Logistic regression	Negative association between glucocorticoid sensitivity and depressive symptom severity
Wodarz et al. 1991	12 severe MDD inpatients (3 men, 9 women) and 13 matched healthy controls, mean age 48yr	Difference in glucocorticoid sensitivity between depressed patients and healthy controls	DEX suppression of mitogen-induced lymphocyte proliferation	ANOVA	Decreased glucocorticoid sensitivity in depressed people compared to controls

Table 1.5 continued. In vitro measurement of GR function in depression

Author/year	Sample	Study design	GR measurement protocol	Statistical analysis	Results
Wodarz et al. 1992	12 severe MDD inpatients (3 men, 9 women) and 13 matched healthy controls, mean age 48yr	Difference in glucocorticoid sensitivity between patients after clinical recovery from severe MDD and healthy controls	DEX suppression of mitogen-induced lymphocyte proliferation	ANOVA	No difference in glucocorticoid sensitivity between clinically recovered MDD patients and healthy controls

Abbreviations: GR = glucocorticoid receptor; TRD = treatment resistant depression; MDD = major depressive disorder; DEX = dexamethasone; LPS = lipopolysaccharide; IL-2 = interleukin-2, TNF- α = tumour necrosis factor – alpha; PBMCs = peripheral blood mononuclear cells; ANOVA = analysis of variance.

In vivo measurement

DST studies

In vivo measurement of glucocorticoid effects on immune function using oral dexamethasone have produced similar results as *in vitro* studies. Studies have demonstrated elevated cortisol levels in depressed patients following oral dexamethasone suppression, with decreased levels observed after recovery (Carroll et al., 1976; Carroll et al., 1968; Holsboer et al., 1982). More recently, a study by Jarcho et al. (2013) including 26 pre-menopausal depressed women and 23 never depressed controls, reported that reduced cortisol response to the DST was associated with flatter cortisol slope. This suggests that flatter diurnal cortisol patterns may be a result of impaired glucocorticoid sensitivity.

As previously mentioned, depression is not universally associated with increased cortisol levels. Melancholic depression is however reliably associated with elevations in cortisol (Leistner & Menke, 2018). It is not surprising then that melancholic individuals also exhibit non-suppression after the DST (Rush & Weissenburger, 1994). A meta-analysis of DST studies examining cortisol suppression in patients with psychotic and nonpsychotic depression and with melancholic/endogenous and non-melancholic depression was conducted by Nelson and Davis (1997). They found that the non-suppression rate was significantly higher in patients with psychotic depression (64%) compared with patients with nonpsychotic depression (41%). Non-suppression was also significantly higher in patients with melancholic depression (36%) compared to non-melancholic (12%), however the effect size was smaller than for the psychotic/nonpsychotic distinction. These findings are supported by evidence that people with psychotic depression exhibit higher basal cortisol levels compared to people with non-psychotic depression (Stetler & Miller, 2011).

DST results have also been shown to predict clinical outcome following successful antidepressant treatment. In a study of 20 patients, with initially abnormal test results, clinical remission was observed approximately three weeks after normalisation of the HPA-axis (Holsboer et al., 1982). DST normalisation in conjunction with clinical remission was also observed in 31 MDD in-patients following drug treatment (Greden et al., 1983). Ribeiro et al. (1993) conducted a meta-analysis of 144 studies exploring the DST and treatment response or clinical outcome in MDD. The authors reported that whilst baseline DST result did not predict treatment response or clinical outcome, non-suppression post treatment was associated with early relapse and poor outcome. In a more recent study of 208 depressed patients receiving antidepressant treatment, no difference was observed in DST suppression between the responders and non-responders (Ventura-Junca et al., 2014). However most recently, cortisol levels following the DST were shown to be higher in non-responders in a meta-analysis by Fischer et al. (2017a). DST results have also been shown to predict treatment response following psychological therapy (S. Fischer et al., 2017b). Taken together, these studies provide robust evidence that GR sensitivity is decreased in depression (Holsboer, 2000; Leistner & Menke, 2018; Pariante & Miller, 2001).

Increased suppression following the DST has been observed in people with a history of childhood abuse. Newport et al. (2004) reported that depressed women with a history of childhood abuse exhibited greater cortisol suppression than those with no abuse history, no MDD or both. Secondary analysis of PTSD produced similar results. Increased suppression indicates enhanced sensitivity of the HPA-axis to negative feedback and is a robust finding in PTSD (Yehuda et al., 2004). These findings may therefore reflect PTSD comorbidity rather than MDD. Chronic stress and GR function has also been investigated *in vivo*. Contrary to *in vitro* findings, increased sensitivity was reported in a study including 12 men suffering from job-related exhaustion compared to 12 matched healthy controls following the DST (Menke et al., 2014).

Dex-CRH test

The majority of studies report increased cortisol and ACTH levels in depressed people following CRH administration, despite dexamethasone pre-treatment (Bardeleben & Holsboer, 1989;Gonul et al., 2017;Heuser et al., 1994;Holsboer-Trachsler et al., 1991;Holsboer et al., 1987;Modell et al., 1997;von Bardeleben & Holsboer, 1991). Increased cortisol levels following dex-CRH challenge is also associated with age (Heuser et al., 1994;Rybakowski & Twardowska, 1999;von Bardeleben & Holsboer, 1991) and severity of symptoms (Kunugi et al., 2006;von Bardeleben & Holsboer, 1991) sleep (Friess et al., 2008) Feedback inhibition following the dex-CRH test has also been shown to be compromised in medication free MDD patients compared with controls (Paslakis et al., 2011;Sher et al., 2013). However some studies have also reported no difference in cortisol response in MDD patients compared with controls (Carpenter et al., 2009a;Spitzer et al., 2018). In clinically remitted MDD patients, higher cortisol levels on the DEX/CRH test were also associated with relapse (Appelhof et al., 2006;Aubry et al., 2007;Zobel et al., 2001) and frequency of recurrence (Hatzinger et al., 2002). Mokhtari et al. (2013) conducted a meta-analysis of the literature including eight studies of the DEX/CRH test in patients with MDD and healthy controls. The authors confirmed the finding that depressed patients release significantly more cortisol after the DEX/CRH test in comparison with healthy controls and reported a relatively large effect size of 1.34 (95% CI: 0.70–1.97).

Increased cortisol responses to the DEX/CRH test have been reported in healthy adults (without psychopathology) who were considered to have vulnerability factors such as chronic inhibited temperament (Tyrka et al., 2008b) and childhood parental loss (Tyrka et al., 2008a). However, Ising et al. (2005) investigated GR function in 74 healthy people at high genetic risk for depression compared with controls and found no difference between the groups. The authors conclude that rather than being a vulnerability factor

for depression, dysregulation of the HPA-axis is a 'neurobiological scar' which develops as a result of depression.

Several studies have investigated response to the DEX/CRH test before and after pharmacotherapy. Despite heterogeneity in the types of drugs used, overall the findings suggest normalisation of the HPA-axis following treatment (Ising et al., 2007). Differential effects of various antidepressants on DEX/CRH test results in depressed patients have also been observed. In a study of 40 MDD patients, those receiving SNRI treatment showed a gradual and significant reduction in cortisol and ACTH response, which was greatest after five weeks of treatment (Schule et al., 2006). In contrast, those receiving a noradrenergic and specific serotonergic antidepressant (NaSSA), showed significant reductions within one week. Sarubin et al. (2014) conducted a randomised, open-label 5-week trial including 60 MDD in-patients. Patients were treated with either the atypical antipsychotic drug quetiapine fumarate extended release (QXR) or the SSRI escitalopram for 5 weeks. The DEX/CRH test was performed before and after treatment. Whilst both medications showed similar clinical effects on depressive symptoms, the QXR group demonstrated immediate cortisol inhibition (week one) whereas the SSRI group showed a brief increase of cortisol release (week one), followed by a comparable decrease at follow up. For those patients who do not respond to antidepressant medication, there is evidence that successful electroconvulsive therapy can be effective in normalising cortisol response in the DEX/CRH test (Kunugi et al., 2006; Yuuki et al., 2005).

The DEX/CRH test has also been studied in people with MDD and a history of childhood maltreatment (Heim et al., 2008a). Plasma ACTH and cortisol concentrations were measured in response to DEX/CRH administration 49 healthy men, with and without MDD. Men with a history of childhood trauma demonstrated increased ACTH and cortisol compared with non-abused men. Furthermore, abused men with current MDD showed

increased responsiveness compared with both controls and men with MDD but without childhood abuse experience.

Increased response was also associated with severity and duration of symptoms. A similar study was conducted in 40 patients with severe mood disorder (Watson et al., 2007). Contrary to the previous study, there was no difference in cortisol response to the DEX/CRH test between patients reporting greater emotional neglect in childhood and controls. However, patients with lower reported levels of emotional neglect demonstrated an exaggerated response compared to controls. The relationship between emotional neglect and GR function was moderated by family history of mood disorder. Familial mood disorder was associated with a greater HPA axis response, suggesting that genetic susceptibility may interact with early adverse life events, resulting in reduced GR mediated negative feedback. Following this a systematic review was conducted by Von Werne Baes et al. (2012). They reported that overall, early life stress is associated with GR resistance in both depressed and healthy people. The authors suggest that this is partially attributable to an imbalance between GR and MR.

Dexamethasone-induced gene expression

Gene expression pattern has also been investigated following oral dexamethasone administration. Menke et al. (2012a) conducted an *in vivo* dexamethasone challenge test and compared GR-mediated changes in gene expression in PBMCs between 18 depressed patients and 18 healthy controls. They reported that the top dexamethasone regulated genes were less stimulated in depressed patients than in controls, reflecting reduced GR sensitivity. *FKBP5*, *DUSP1* and *ZBTB16* were within the top genes regulated by dexamethasone and these genes have all been associated with mood disorders. The study also showed that GR-stimulated gene expression is a better tool for discrimination between depressed patients and controls than either unstimulated gene expression or dexamethasone suppression of plasma cortisol. An investigation into

FKBP5 expression, following *in vivo* dexamethasone challenge, has also been conducted (Menke et al., 2013). FKBP5 mRNA expression was analysed in the PBMCs of 68 depressed patients and 87 healthy controls, at baseline and after oral dexamethasone administration. Patients carrying the risk allele (rs1360780) showed a reduced induction of dexamethasone-stimulated FKBP5 mRNA compared to controls. Interestingly, patients carrying the risk allele also demonstrated impaired cortisol suppression, leading the authors to propose their findings as a potential explanation as to why a disturbed HPA axis is not consistently found in patients suffering from major depression.

MR function

In light of the aforementioned DST results and evidence presented earlier that MR expression is reduced in the depressed brain, it is possible that depression is also accompanied by decreased MR activity. This hypothesis was first explored by Young et al. (2003) in a pilot study of 12 depressed patients and 12 controls. Participants were administered spironolactone, an MR antagonist, and corticotropin and cortisol secretion was measured in whole blood. In contrast to their expectations, the authors reported that MR blockade resulted in a significant increase in cortisol levels in both groups, suggesting that the MR is functional in depressed patients. In addition, depressed patients demonstrated an actual increase in cortisol secretion rather than a delay in the circadian fall, as observed in controls, suggesting that MR is functionally more active in depressed patients. Patients also demonstrated a significant increase in corticotropin levels, which was not observed in controls further indicating increased MR function.

However conflicting findings have been reported. Hinkelmann et al. (2016) conducted a randomised, double-blind, within-subject cross-over study, including 48 depressed patients and 45 controls. Participants received either spironolactone, an MR antagonist, or placebo in the afternoon and the effects on cortisol were measured at various time

points during the same day. The results showed an increase in cortisol levels across groups following spironolactone treatment. However depressed patients exhibited elevated cortisol compared to controls following placebo but not following spironolactone. These results are interesting because if the GR is impaired in depression, as a vast body of evidence suggests, then we would expect to see significant differences in cortisol release after MR blockade. In a healthy person, the GR would be still be able to inhibit the HPA-axis, despite the lack of MR binding, and so the effect of spironolactone would be modest. However in a depressed person with an impaired GR, the effect of MR blockade would eliminate HPA-axis inhibition, resulting in a substantially higher increase in cortisol. Therefore in this study, the results do not support GR resistance in depression but suggest that impaired MR signalling may be responsible for hypercortisolaemia in MDD (Hinkelmann et al., 2016).

MR dysfunction has also been observed specifically in people with psychotic MDD (Lembke et al., 2013). Cortisol levels were assessed in 14 people with psychotic MDD, 16 people with non-psychotic MDD and 19 controls before and after fludrocortisone administration. There were no differences in baseline cortisol across the groups or between people with non-psychotic MDD and controls post fludrocortisone. However cortisol levels were significantly increased in people with psychotic MDD compared to controls following fludrocortisone administration, suggesting impaired MR feedback inhibition.

MR function has been more extensively explored in patients with TRD. In a single-blind, repeated-measure design study, prednisolone (5 mg) or dexamethasone (0.5 mg) was administered to 18 severe TRD inpatients and 14 healthy volunteers (Juruena et al., 2006). Salivary cortisol was collected the following day. Depressed patients has higher baseline cortisol compared to controls after both treatments, however the depressed patients showed impaired suppression by dexamethasone and normal suppression by prednisolone, suggesting differential sensitivity. However a correlation was observed

between prednisolone suppression and dexamethasone suppression in controls, suggesting equal sensitivity. The authors interpret these findings as an indication of impaired GR function and retained MR function in depression.

In a study by Juruena et al. (2009) the PST was administered to 45 in-patients with TRD and 46 controls. Results showed that whilst in-patients with severe TRD had higher salivary cortisol levels compared with controls, both after placebo and after prednisolone administration, the suppressive effect of prednisolone was similar in both groups. Given that: 1) DST results in people with depression report impaired GR function; 2) both MRs and GRs are active in negative feedback of the HPA-axis and 3) prednisolone is active at both receptor sites, it seems plausible that MRs may play a compensatory role and increase their signalling in the context of impaired GR function. However the authors also reported that when analysis was stratified according to treatment response following intensive in-patient therapy, they observed increased post-prednisolone cortisol output in the non-responders compared with the treatment-responsive group, suggesting impaired suppression. This observation was not made in the placebo condition. The authors conclude that the compensatory role of the MR in depressed people is not functional in severely treatment-resistant patients (Juruena et al., 2009).

In light of this finding, response to the PST was investigated in relation to antidepressant treatment (Juruena et al., 2010). Saliva cortisol was measured in 28 in-patients with TRD following either placebo or prednisolone administration, before and after an inpatient treatment. The findings showed that the response to prednisolone does not change before and after inpatient treatment, even in those who respond clinically. This suggests that it is a stable aspect of HPA axis activity and is not modulated by improvement in symptoms. This is in contrast to evidence showing that the response to dexamethasone changes with symptom improvement. The authors were also able to repeat their previous findings and show that a subgroup of patients demonstrated an impaired response to prednisolone at baseline which was associated with a lack of future response to

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treatment. This response did not change between admission and follow-up. Therefore, MR dysfunction may be a stable biomarker of poor prognosis and treatment resistance (Juruena et al., 2010).

Otte et al. (2010) investigated whether GR modulation with either an MR agonist (fludrocortisone) or an MR antagonist (spironolactone) alongside SSRI therapy could improve efficacy. 64 MDD patients were randomised to fludrocortisone, spironolactone or placebo the first 3 weeks during five weeks of treatment. Whilst there was no difference in depression scores or response times overall between the groups, among the responders, patients receiving fludrocortisone and SSRI improved treatment response by six days. This suggests that, in patients who respond to antidepressant treatment, stimulation of MR improves the effects of SSRIs.

MR blockade was also used in a study by Juruena et al. (2013) who explored the effect of a) stimulation with prednisolone; b) prednisolone with spironolactone and c) spironolactone alone on GR/MR function in 24 TRD patients and 24 controls. TRD patients had increased cortisol levels compared to controls in all three conditions. As expected, in controls, spironolactone increased cortisol compared to placebo. Additionally, administration of spironolactone plus prednisolone decreased the suppressive effect of prednisolone. However in TRD patients, cortisol levels did not increase in response to spironolactone and the combination of spironolactone with prednisolone had no effect on the suppressive effects of prednisolone. This suggests an inability of MR to exhibit negative feedback on the HPA-axis in TRD patients (Juruena et al., 2013).

Overall, taken together with the existing data on GR function, the findings suggest that an imbalance between the GR and MR may be important in the dysregulation of the HPA-axis. The evidence for GR resistance in depression is consistent, however the role of the MR in depression is still largely unclear, with many questions still unanswered. MR

expression appears to be decreased in depression and yet MR function appears to be increased, although not for all subgroups. It has been shown that GR function is necessary for HPA-axis feedback inhibition when cortisol is high but also that MR can both facilitate GR signalling (Spencer et al., 1998) and compensate for its impairment (Jurueña et al., 2009). Therefore the balance between these two receptors is thought to be important to HPA-axis regulation and imbalance may be a risk factor for mental health disorders (E. R. de Kloet, 2014). To date only one study has investigated both GR and MR function in MDD (Jurueña et al., 2006). Further studies are required examining both GR and MR function simultaneously in this population. To date MR function in depression has only been investigated *in vivo*. Whilst these studies provide an indirect measure of MR-mediated negative feedback on the HPA axis, they do not measure MR function directly. *In vitro* studies are therefore required to directly probe the MR in depressed people.

1.6 Inflammatory neuroendocrine interaction in depression

So far this thesis has presented evidence for both immune activation with increased levels of cytokines, and HPA-axis hyperactivity with GR resistance in depressed people. However the presence of both inflammation and hypercortisolism is rather puzzling. Glucocorticoids are critical in suppressing inflammation and autoimmune responses (McEwen et al., 1997; Silverman & Sternberg, 2012) and are believed to play a major role in protecting the brain against inflammation (Nadeau & Rivest, 2003). This suppressive effect appears to not always be present in depressed people. Indeed growing data suggests that inflammation itself may contribute to glucocorticoid resistance (Pace et al., 2007b). In the following section I will first discuss how neuroendocrine abnormalities can exacerbate inflammation and then I will examine the effects of inflammation on neuroendocrine function.

1.6.1 *Glucocorticoids effects on cytokines*

Reduced glucocorticoid inhibition on inflammation

Neuroendocrine systems can influence inflammatory systems in two ways. Firstly, dysfunctional glucocorticoid signalling could be less capable of inhibiting a pro-inflammatory response. A study exploring the effects of chronic stress in caregivers showed that whilst their cortisol levels were the same as that of the controls, their gene expression profiles in PBMCs shifted from glucocorticoid signalling to inflammatory signalling through diminished expression of transcripts bearing response elements for each pathway (G. E. Miller et al., 2008). This suggests that even without increased cortisol, chronic stress can induce glucocorticoid resistance and increased inflammation.

Animal studies provide some evidence for a shift from glucocorticoid signalling to inflammatory signalling. In a mouse model of social stress, mice demonstrated impaired nuclear translocation of the GR and increased activation of NF-kappa β in macrophages (Quan et al., 2003). The impact of reduced glucocorticoid signalling on inflammation has been demonstrated in a study by Wang et al. (2011) where they observed the effects of GR blockade on depressive-like behaviour in rats. Administration of GR antagonist 3 days before LPS injection resulted in blockade of GR, significantly increased depressive-like behaviour and increased levels of TNF α and IFN- γ . However a similar study showed GR blockade showed a decrease or no effect on cytokine release (B. Yang et al., 2008). There is therefore some evidence to support the hypothesis that impaired GR function leads to both inflammatory activation and depressive symptoms but this is by no means conclusive.

Glucocorticoids may have pro-inflammatory effects

Secondly, there is a possibility that GCs could be pleiotropic and have context specific, pro-inflammatory functions. This hypothesis is in direct contrast to the one above in that it is not a dysfunctional GR that causes inflammation but that inflammation is a result of normal glucocorticoid signalling (Horowitz et al., 2013). There is some evidence to suggest that glucocorticoids can promote an adaptive inflammatory response and that they are anti-inflammatory at very low and high doses but pro-inflammatory at intermediate doses.

Mouse macrophages show increased phagocytosis following IFN- γ stimulation when incubated with an intermediate dose of glucocorticoids, than when incubated with IFN- γ alone (Warren & Vogel, 1985). Similarly, rat macrophages pre-treated with glucocorticoids prior to LPS and IFN- γ stimulation have demonstrated increased levels of IL-6 and TNF- α (Smyth et al., 2004). Furthermore this increase in inflammatory activation occurs in a dose-response fashion, but only at intermediate concentrations (T. Y. Zhang & Daynes, 2007). Taken together these studies have implications for patients with depression, as cortisol levels in depressed people are only modestly elevated, similar to the studies above (Fang et al., 1981; Halbreich et al., 1985).

The timing of glucocorticoid administration may also have an impact on the nature of its inflammatory effects. For example, prior exposure to corticosterone in rats before LPS stimulation, resulted in increased IL-1 β , TNF- α and IL-6 levels. However exposure to corticosterone after LPS stimulation suppressed this pro-inflammatory response (Frank et al., 2010). These findings are further supported by studies involving 'stress induced priming'. Frank et al. (2012) exposed rats to inescapable tailshock (a paradigm used to induce acute stress and depressive-like behaviour) and then challenged the hippocampal microglia with LPS ex-vivo, resulting in increased IL-6, IL-1 β and NK-k β . Prior treatment with either GR antagonist or adrenalectomy blocked this response.

The importance of timing has also been shown in clinical studies. In a study by Barber et al. (Barber et al., 1993) hydrocortisone treatment 12 or 144 hours before LPS administration resulted in increased levels of IL-6 and TNF- α , however hydrocortisone treatment either immediately before or 6 hours before LPS resulted in a reduction in inflammatory cytokines. A study in healthy people showed that baseline cortisol concentrations did not exert an anti-inflammatory effect and that an intermediate concentration of cortisol treatment before LPS injection resulted in maximal inflammatory responses, whilst a high dose had no effect (Yeager et al., 2011). Finally, glucocorticoids may also increase cytokine production via metabolic pathways. Increased levels of cortisol results in an increase in central obesity, insulin resistance and dyslipidaemia. This increase of adipose tissue can then increase levels of IL-6 (Yudkin et al., 2000).

There is also some evidence for MR signalling effects on cytokine production in murine models (J. Chen et al., 2016). MR agonist has been shown to enhance production of L-1 β and IL-6 mRNA induced by LPS in both cerebrospinal fluid and prefrontal cortex of rats (Bay-Richter et al., 2012). Furthermore, a positive correlation was observed between IL-1 β in CSF and depressive-like behaviour.

1.6.2 *Cytokine effects on glucocorticoids*

HPA-axis function

Cytokines can affect neuroendocrine function via two main mechanisms: activation of the HPA-axis and direct effects on the GR (Horowitz et al., 2013). Firstly, cytokines have been shown to promote the release of CRH, ACTH and cortisol (A. H. Miller et al., 2009). Chronic administration of cytokines has been shown to increase CRH, ACTH and cortisol in animal studies (Besedovsky & del Rey, 1996; Felger et al., 2007). This has also been demonstrated in patients undergoing acute IFN- α treatment in the context of medical illness. IFN- α is used clinically in the treatment of both cancer and infectious diseases

due to its antiviral and anti-proliferative effects (Pace & Miller, 2009a). It has been shown to increase HPA-axis activity within a few hours, *in vitro* and *in vivo*, resulting in marked increases in ACTH and cortisol (Gisslinger et al., 1993) and depressive symptoms (Capuron et al., 2002a). ACTH and cortisol was measured in 14 patients with malignant melanoma, before and after IFN- α therapy (Capuron et al., 2003). ACTH and cortisol responses were significantly higher in the patients who subsequently developed depressive symptoms compared to those who did not. Furthermore, the degree of hyperactivity after the first dose of IFN- α predicts depressive symptoms, suggesting some patients may have a pre-existing sensitivity of the HPA axis and predisposition to depression. In contrast, chronic exposure to IFN- α therapy has been associated with a flattening of the diurnal cortisol slope and increased evening plasma cortisol concentrations in 33 patients receiving treatment for hepatitis C (Raison et al., 2010). Furthermore, these effects were correlated with increases in depression. Release of TNF- α has also been shown to elevate levels of ACTH, CRH, and cortisol (Black, 1994; Dantzer et al., 1999b) and upregulate the HPA-axis (Ma et al., 2016). These studies are of particular importance because they suggest a direction of causality, namely that increased inflammation causes HPA-axis dysfunction. However it is still unclear whether this is a result of the effects of cytokines on HPA-axis activity or an effect on the GR (Horowitz et al., 2013). Furthermore, it should also be noted that these findings were made in people with very serious advanced diseases that are known to affect immune function, so they may not generalise to people with depression and no physical illness.

A few cross-sectional studies have reported concurrent indices of plasma cortisol levels and circulating inflammatory markers in people with MDD compared with healthy controls, with the majority focusing on IL-6 and TNF- α . Several studies have reported increased plasma cortisol in conjunction with increased IL-6 in depressed people (Carvalho et al., 2008; Carvalho et al., 2013; Karlović et al., 2012; Martinac et al., 2017; Trzonkowski et al., 2004). However, other studies have reported similar plasma cortisol levels between depressed people and controls alongside increased IL-6 in the

depressed groups (Alesci et al., 2005;Humphreys et al., 2006;Maes et al., 1995;Maes et al., 1993b) and one study reported no difference in either plasma cortisol or IL-6 in depressed patients compared with controls (Marques-Deak et al., 2007). Higher plasma cortisol has also been reported along with both increased TNF- α (Y. Chen et al., 2017;Kahl et al., 2017;Kahl et al., 2015) and decreased TNF- α (Rudzki et al., 2017) in depressed patients compared with controls. However, lower plasma cortisol and lower TNF- α levels have also been reported in MDD patients (Lopes et al., 2012). In general, studies reporting plasma cortisol levels provide inconsistent support for the notion that increased inflammation activates the HPA-axis in MDD.

Studies investigating saliva cortisol and circulating inflammatory cytokines are fewer and have often focused on sub-group comparisons. Simmons et al. (2018) reported higher saliva cortisol in depressed participants with reduced appetite compared with healthy controls but there was no difference in either IL-6 or CRP. Conversely, in depressed participants with increased appetite both IL-6 and CRP were increased but there was no significant difference in cortisol, suggesting that the relationship between HPA-axis function and inflammation may differ according to distinct depression subtypes. Another study by Bauer et al. (2003) showed that basal morning saliva cortisol levels and TNF- α levels did not differ between patients and controls

To date only two studies reported cortisol awakening response as an outcome measure. Lamers et al. (2013) analysed data from 776 people from the Netherlands Study of Depression and Anxiety and reported that CRP, IL-6 and TNF- α levels were significantly elevated in atypical depression compared to controls. Regarding saliva cortisol, the CAR AUC and diurnal slope was significantly higher in melancholic depression compared with controls. This finding lends further support to the notion that depressive subtypes differ in their biological correlates and suggests that HPA-axis hyperactivity is a distinct feature of melancholic depression, whereas increased inflammation is a feature of atypical

depression. Verduijn et al. (2015) used data from the same cohort study to investigate inflammatory and HPA-axis dysregulation across 8 consecutive stages of MDD progression, from familial risk to chronic MDD. A linear increase of CRP, IL-6 and saliva was observed across the entire sample. Significant trends of dysregulations in IL-6 and cortisol AUC across stages were present for at-risk individuals, however no trends were found in dysregulations for any of the mechanisms across more progressive stages of full-threshold MDD. This suggests that the mechanisms involved in depression aetiology may be less important in the progression and maintenance of MDD.

In order to address the conflicting nature of these findings a very recent systematic review and meta-analysis was conducted. Perrin et al. (2019) included 32 studies comparing cortisol and inflammatory cytokine levels in MDD patients compared with controls and reported a trend for an association between increased cortisol and increased IL-6 and TNF- α . The cross-sectional nature of study does not allow for causal inference but this study provides support for an association between cytokine production and HPA-axis activity. However, due to the lack of studies reporting cortisol within the first hour of waking, it is still unclear whether there is a relationship between inflammation and diurnal variations in cortisol. Further research is need to identify specific associations between inflammatory biomarkers and diurnal cortisol parameters.

GR function

Secondly, cytokines are able to directly impair GR function. The effects of cytokines on GR function are extensive and include GR translocation to the nucleus, GR protein-protein interactions, GR binding to response elements on DNA and increased expression of the GR isoforms GR α and GR β (Pace & Miller, 2009b). Furthermore, cytokines can affect the bioavailability of glucocorticoids by inhibiting 11 β HSD (Kossintseva et al., 2006) and CBG (Emptoz-Bonneton et al., 1997). Evidence also suggests that cytokines

can decrease GR translocation via a number of GR signalling pathways (Pace et al., 2007a). For example, IFN- α activates the signal transducer and activator of transcription (STAT) pathway, which binds to the activated GR in the nucleus preventing the GR binding to its DNA response element (Hu et al., 2009). Nuclear factor-kB (NF-kB) also disrupts GR function through a similar protein-protein interaction (Smoak & Cidlowski, 2004). Translocation of the GR from the cytoplasm to the nucleus is inhibited by activation of the mitogen-activated protein kinase (MAPK) pathway (X. Wang et al., 2004). Together these studies show that there are a plethora of ways in which cytokines can impair GR function and increase HPA-axis activity.

A large body of evidence in cell models suggests that pro-inflammatory cytokine administration (IL-1 β , IL-2, IL-4, IL-15, TNF- α and IFN- α) can disrupt some aspect of GR function (Goleva et al., 2002; Onda et al., 2006; Pace et al., 2011; Xu et al., 2004) however the exact mechanism associated with each cytokine has yet to be identified (Horowitz et al., 2013). TNF- α has also been shown to increase GR β expression in cell models and this increase was correlated with the development of GC resistance (J. C. Webster et al., 2001). Cytokine induced GR resistance has also been demonstrated in animal studies. GR resistance, as represented by DEX non-suppression, has been observed in rats injected with herpes simplex-1 (HSV-1) and endotoxin (Bener et al., 2007; Weidenfeld & Yirmiya, 1996). In another study IL-1 receptor knockout mice failed to induce GR resistance in response to stress (Engler et al., 2008).

In humans, several studies have assessed the results of the DST in conjunction with inflammatory biomarkers. An early study investigated whether IL- β production is related to HPA-axis activity in depressed in-patients (Maes et al., 1993a). Cortisol levels before and after the DST were measured, as well as mitogen-stimulated supernatant IL- β production in PBMCs, in 28 depressed in-patients and 10 healthy controls. Results showed a significant correlation between IL- β production and post-DST cortisol levels in both the depressed and control groups. Up to 25% of the variance in the DST results

could be explained by IL- β production, supporting the hypothesis that HPA-axis hyperactivity is enhanced by pro-inflammatory cytokines.

Another study by Landmann et al. (1997) compared TNF- α levels and plasma cortisol levels, before and after oral administration of DEX, in depressed people and controls. TNF- α and cortisol response to DEX did not differ between the groups. Circulating TNF- α has also been investigated alongside peripheral GR function, as demonstrated by reduced cutaneous glucocorticoid receptor sensitivity in people with TRD. Fitzgerald et al. (2006) reported that TRD patients demonstrated a reduced vasoconstrictor response to topical application of steroids and significantly higher levels of both IL-6 and TNF- α compared to controls. Furthermore, there was a significant inverse correlation between TNF- α concentration and vasoconstriction response in the MDD group. The results failed to show a significant difference in plasma cortisol between the TRD patients and controls, nor any correlation between plasma cortisol and inflammatory cytokines or depressive symptoms. The authors speculate that these findings may reflect a direct influence of inflammatory cytokines on the GR, as opposed to via increased HPA-axis activity and subsequent hypercortisolaemia.

Lisi et al. (2013) assessed salivary cortisol circadian rhythm after low dose DST and gene expression of IL-6 in MDD patients before and after 8 months of antidepressant drug treatment. At baseline, IL-6 expression was markedly increased in the MDD patients compared to healthy controls, although due to high variability this did not reach significance. A strong trend to reduction at follow-up was observed although this also failed to reach significance. DST results showed normal suppression in healthy controls, whereas 30% of the depressed group demonstrated non-suppression and remained non-suppressors at follow-up. However, they found no differences among the profiles of daily cortisol secretions between the groups at either time point and inflammatory changes were not associated with levels of circulating glucocorticoids. The authors conclude that immune dysregulation in MDD is independent from HPA axis function.

However there is also conflicting evidence that elevated cytokines in depressed patients are associated with GR resistance. Schuld et al. (2003) investigated the interaction of inflammatory and HPA-axis changes MDD by studying TNF- α and IL-6 levels and ACTH response to the combined DEX/CRH test on 14 MDD patients. TNF- α levels were significantly and negatively correlated with the amount of ACTH released following the DEX/CRH test. This suggests that HPA system over-activity is accompanied by a reduced production of TNF- α . In contrast, IL-6 production was not correlated with HPA-axis activity. The authors speculate that this discrepancy may be a reflection of a difference in sensitivity of glucocorticoids to IL-6 and TNF-a. Higher IL-6 levels are known to be needed to significantly stimulate the HPA-axis during experimental immune stimulation in vivo (Schuld et al., 2000;Spath-Schwalbe et al., 1998). The findings from this study stand in direct contrast to the plethora of studies which demonstrate both increased HPA-axis activity and increased cytokine levels suggesting that cytokines activate the HPA-axis. The negative correlation between TNF- α and the DEX/CRH test results presented here suggest that HPA activity suppresses the cytokine system.

A similar result was replicated in a study by Himmerich et al. (2006), who examined alterations in plasma levels of TNF- α , levels of its soluble receptors p55 (sTNF-R p55) and p75 (sTNF-R p75) as well as response to the DEX/CRH test on admission and at discharge in 70 depressed inpatients without inflammation. The authors reported a negative correlation between TNF- α and the response to the CRH/DEX test at admission and a trend towards a negative correlation with the cortisol response. This suggests that HPA-axis activity in acute depression suppresses the release of TNF- α . Furthermore, a positive correlation between TNF- α and CRH/DEX test outcome was observed in remitted patients following successful antidepressant treatment and normalisation of the HPA-axis. This implies that upon resolution of depressive symptoms, the TNF- α system increases its control over the HPA-axis. Together the results of this study suggest that during a depressive episode, the HPA-axis is activated, which in turn suppresses the TNF- α system. Upon remission, the HPA-axis normalizes. However the degree to which the

HPA-axis normalizes appears to be dependent on TNF-activity. Patients in remission who demonstrate no increase or even a reduction in TNF- α between admission and discharge showed greater HPA-axis normalisation, whilst patients with increased TNF- α at discharge showed less normalisation. It should be noted that neither the Schuld (2003) study nor the Himmerich et al. (2006) study used a control group, making inferences about MDD rather tenuous.

A systematic review and meta-analysis of nine studies which reported DST results and cytokine levels between depressed patients and controls, observed a trend for higher GR resistance in depressed individuals which was associated with increased cytokine levels Perrin et al. (2019). However it should be noted that DST studies measure peripheral GR function only and do not directly measure GR sensitivity. Additionally, findings cannot be translated into inferences regarding the CNS. Due to the small number of studies included in this analysis, the authors adopted a broad inclusion criteria including studies involving patients with co-morbidities such as cancer, resulting in potential confounding.

Studies have also investigated *in vitro* measures of GR function and inflammatory cytokines in MDD. An early cross-sectional study in 24 depressed inpatients and 8 healthy controls measured IL-6 production in culture supernatants of mitogen-stimulated PBMCs and post-dexamethasone cortisol (Maes et al., 1993b). The authors reported that IL-6 activity was significantly correlated with post-dexamethasone cortisol values across the sample, suggesting that IL-6 may stimulate the HPA-axis. Carvalho et al. (2008) measured GR function in people with TRD by glucocorticoid inhibition of LPS-stimulated IL-6 levels following incubation with the tricyclic antidepressant, clomipramine. Patients had higher plasma IL-6 and higher plasma cortisol compared with controls. The authors observed no difference in GR sensitivity between the groups, however fact that GR function appeared normal in the presence of hypercortisolaemia suggests a functional GR difference between patients and controls.

Gene expression studies have also investigated mRNA levels of genes belonging to GR function and inflammation in depressed people. Cattaneo et al. (2012) reported a dissociation between biomarkers that predicted future response and those that were targeted by antidepressants, before and after 8 weeks of antidepressant treatment. Increased baseline levels of the inflammation-related genes, IL-1 β , macrophage inhibiting factor and TNF- α , predicted lack of response to antidepressants, but successful antidepressant response was not associated with a reduction in the levels of these genes. In contrast, successful antidepressant response was associated with a reduction in the levels of the gene for IL-6 and GR associated gene expression. Another study by Carvalho et al. (2014) compared gene expression between 47 medication-free melancholic MDD inpatients and 42 healthy controls reported that immune cells in MDD patients overexpress inflammatory genes and under express the active GR variant, GR α , suggesting reduced sensitivity of monocytes to glucocorticoids. Furthermore, the reduced GR α expression correlated strongly to the increased expression of inflammatory genes.

A review of four studies that assessed GR function either by *in vitro* assays or gene expression was unable to report a positive association between GR resistance and inflammation (Perrin et al., 2019). However the effect size observed was larger than for studies focused on DST results or plasma/saliva cortisol levels. However, it should be noted that this analysis included a gene expression study, not reported in this review, involving CHD patients with comorbid depression, which creates an issue of confounding. *In vitro* studies provide the most direct and controlled measure of GR sensitivity, therefore may be more likely to detect any significant effects. Further *in vitro* studies required to test this hypothesis.

The conflicting findings in the overall literature likely reflect the variability of measures used. Perrin et al. (2019) suggest that combining multiple independent measures of HPA-axis function is likely to provide the best opportunity for detecting any trends and

allow for better comparison across studies. A more comprehensive characterisation of GR function, in tandem with analysis of the major cytokines implicated in depression such as IL-6 and TNF- α , would help to disentangle the complex relationship between HPA-axis dysfunction and inflammation in MDD. Furthermore, studies should adopt more stringent inclusion criteria, restricting study populations to MDD patients without medical or psychiatric comorbidities. The inclusion of medication-free patients is also of importance as antidepressants are known to modulate cytokines (Hiles et al., 2012a; Kohler et al., 2018; Wiedlocha et al., 2018) and HPA-axis activity (Heuser et al., 1996; Linkowski et al., 1987).

1.6.3 *Summary of evidence*

In summary, there is a vast amount of information implicating inflammation in the pathogenesis of depression. Patients with depression show increased biomarkers for inflammation, particularly IL-6, TNF- α and CRP, administration of cytokines induces depressive symptoms and both acute and chronic stress activates an inflammatory response. In addition, depressed people demonstrate a dysregulated HPA-axis, as demonstrated by increased levels of glucocorticoids and disturbed diurnal rhythm, although the direction of dysregulation regarding specific diurnal cortisol parameters is not entirely clear. The presence of both inflammation and hypercortisolism in MDD presents something of a paradox. Glucocorticoids should theoretically dampen down an inflammatory response and yet it appears that they do not always do that.

This thesis has presented evidence for a complex interaction between inflammatory activation and neuroendocrine function and suggested possible explanatory mechanisms, including cytokine activation of the HPA-axis, cytokine induced GR dysfunction and reduced inflammatory inhibition as a result of dysfunctional glucocorticoid signalling. To date most studies have focused on *either* inflammation *or* HPA-axis function, although a few have reported associations between the two, with

conflicting results. There seems to be a reciprocal relationship between inflammation and HPA-axis function however the nature of this relationship has yet to be fully elucidated.

In vitro assessment of GR sensitivity has rarely been explored in conjunction with inflammation, despite that fact that it may offer the most effective measure. There is also a very little known about MR function in depression and a complete lack of research investigating any relationship with inflammation. Most studies report average cortisol measures, only two studies have reported cortisol awakening responses and indices of inflammation. To date there have been no studies reporting *in vitro* GR and MR function results, diurnal cortisol outcomes and inflammatory cytokine levels in people with MDD compared with controls. In order to address this gap in the literature, this PhD will simultaneously investigate multiple biological differences between people with MDD and healthy controls, including inflammatory cytokine levels (IL-6 and TNF- α), *in vitro* assessments of GR and MR sensitivity and diurnal cortisol rhythm.

1.6.4 Research aims

The aim of Study 1 of this PhD is to investigate the relationship between the immune system and HPA-axis function in people with depression.

Study 1a will focus on the following research questions:

1. How do inflammatory profiles differ between people with MDD and healthy controls?
2. How does glucocorticoid and mineralocorticoid sensitivity differ between people with MDD and healthy controls?

3. How does diurnal cortisol rhythm differ between people with MDD and healthy controls?
4. Is there an association between increased inflammation and HPA-axis function in MDD?

In addition, it is unclear how these factors, and the relationships between them, change in relation to symptom remission. There is some evidence to suggest that inflammation is attenuated following successful antidepressant treatment and particularly high levels of inflammation may contribute to treatment resistance. However there are mixed findings with some findings reporting a pro-inflammatory effect of antidepressants. Results regarding clinical recovery and diurnal cortisol are also mixed with no clear agreement concerning the direction of association. GR function studies suggest increased sensitivity following successful antidepressant treatment, however it is still unclear whether MR sensitivity is associated with symptom remission. The longitudinal association between inflammation and HPA-axis function has yet to be fully explored. To date there have been no studies reporting *in vitro* GR and MR function results, diurnal cortisol outcomes and inflammatory cytokine levels longitudinally in people with MDD.

Therefore, study 1b of this PhD will also investigate the following research questions:

1. Are changes in inflammatory markers associated with change in depressive symptoms over time?
2. Are changes in glucocorticoid and mineralocorticoid sensitivity associated with change in depressive symptoms over time?
3. Are changes in diurnal cortisol rhythm associated with change in depressive symptoms over time?

4. Are changes in inflammation associated with changes in HPA-axis function over time?

1.7 Chapter summary

Depression is a heterogeneous disorder, with high co-morbidity, a variable trajectory, an unpredictable response to treatment, and as yet, no established mechanism. The monoamine hypothesis, which remains the dominant model of depression, fails to account for why some people develop depressive symptoms and others do not, or why some people respond to treatment whilst others appear resistant. The aetiology of depression is likely to be far more complex than simply a disorder of the brain, and will almost certainly include alterations in multiple biological systems, including the immune system and the HPA-axis.

A large body of evidence demonstrates alterations in immune function in depression, yet this relationship is also far from simple. Depression is associated with increased inflammation, however this is not a universal phenomenon. There is considerable variability in biomarker levels between individuals and the effect sizes are small, likely reflecting the heterogeneity of the condition. Inflammation may be causally related to some cases of depression but not all, and the degree to which inflammation plays a role is still unclear. Further complications arise from fact that inflammation is associated with both physical and additional psychiatric disorders and is moderated by a multitude of factors such as age, body mass index, gender, medication use and childhood maltreatment. There appears to be a degree of normalisation of the inflammatory response following antidepressant treatment, however findings are inconsistent.

The relationship between inflammation and depression may also be moderated by glucocorticoids. Indeed one of the most reliable findings in depression research is hyperactivity of the HPA-axis. However dysfunction is not evident in all depressed people

and the degree of hyperactivity varies considerably across patients groups. Antidepressants may impact HPA-axis function, however the findings in this area are inconclusive. Mounting evidence points to the role of the glucocorticoid receptor and GR resistance is reliably demonstrated in depressed people, whilst the role of the MR is still unknown. To further complicate things, the relationship between inflammatory cytokines and glucocorticoid function is likely to be bidirectional, with alterations in either system having consequences for the functionality of the other. However the exact nature of this relationship has yet to be characterised. Taken together the evidence suggests that immune dysregulation and HPA-axis dysfunction interact to influence the development and maintenance of depression.

2. Study 1 - The Resist Study: Introduction and methods

2.1 The Resist Study

As outlined in Chapter 1, despite a robust literature demonstrating increased levels of inflammation and a disturbed HPA-axis in MDD few clinical studies have investigated associations between these aspects. In addition, an emerging body of research indicates that there may be abnormalities in adaptive immune function in people with depression and that this may also be associated with both inflammation and HPA-axis function. However findings in this field are sparse and inconsistent. By taking a multiple biological systems approach and exploring associations between these systems, the complex underpinnings of MDD may be further elucidated. This may help us understand the pathophysiology of depression and guide treatment.

The Resist Study was set up to: 1) explore the association between increased inflammation and HPA-axis function in MDD and 2) explore any association between Tregs and HPA-axis function in MDD. This study was an observational study which assessed circulating inflammatory biomarkers, HPA-axis function, Tregs and psychosocial measures. In addition, people with depression were also assessed at six week follow-up to determine whether any changes in these parameters were associated with changes in depression.

The inflammatory markers measured in the Resist Study included IL-6 and TNF- α . Neuroendocrine parameters included diurnal cortisol profiles and corticosteroid receptor sensitivity. Circulating Treg frequencies were also measured. Major depressive disorder was measured by structured clinical interview. A number of psychosocial factors were also measured via self-report. These included depressive symptoms, perceived stress, life events and childhood maltreatment and health behaviours including smoking and BMI. The focus of Chapters 3, 4 and 6 will be on the differences in these variables and

associations between them. Chapter 3 will focus on cross-sectional analyses of inflammation and neuroendocrine function, chapter 4 will focus on the longitudinal follow-up analyses and chapter 6 will focus on Treg frequencies.

2.2 Methods

2.2.1 Study design

The Resist Study was an observational, case-control study, including people with symptoms of depression and healthy controls. Depressed people attended two assessments: a baseline assessment and a follow-up assessment six weeks later. This follow up assessment was selected in order to ascertain whether any changes in psychological symptoms were associated with any biological changes over time. Healthy controls attended baseline assessment only. Full details of the study protocol are provided in section 2.2.5.

2.2.2 Sample size

A statistical power analysis was performed for sample size estimation, based on data from several studies. Haapakoski et al. (2015) published a cumulative meta-analysis investigating studies comparing inflammatory biomarkers in MDD patients with healthy controls. The authors reported a medium-sized effect for IL-6 ($n = 31$, combined $d = 0.54$, total n (MDD) = 1045, total n (non-MDD) = 977) and a small effect for TNF- α ($n = 31$, combined $d = 0.40$, total n (MDD) = 1214, total n (non-MDD) = 1262). Another study by Huber et al. (2006) compared the CAR between depressed and non-depressed people and reported a small effect size ($d = 0.55$). Grosse et al. (2015) published a study comparing Tregs frequency between MDD patients and controls prior to antidepressant treatment ($n = 40$ in each group). The study showed a medium affect size ($d = 0.5$). As no previous studies have reported effect sizes for differences in GR or MR sensitivity

using corticosteroid inhibition of LPS-stimulated cytokines in whole blood, power calculations directly relevant to these neuroendocrine parameters, could not be carried out. Due to time and laboratory constraints, the Resist Study aimed to have a sample of 90 participants (MDD group $n = 60$, control group $n = 30$). We performed statistical power analyses using G*Power software which revealed that with a sample of 60 depressed participant and 30 controls we would have >65% power to detect any differences in IL-6, TNF- α , Tregs and the CAR between depressed people and healthy controls.

2.2.3 *Recruitment*

We planned to recruit 90 participants, 60 people with symptoms of depression and 30 healthy controls. We decided upon this sample size to allow for attrition. Attrition is a particularly large problem in mood disorder studies, where participants may feel less motivated to attend assessments or provide samples, or may feel ashamed of their symptoms.

We initially planned to recruit depressed participants via a randomised control trial, PANDA, (UCL, 2015) in collaboration with Professor Glyn Lewis (GL) from the Division of Psychiatry at University College London (UCL). The PANDA trial was investigating the severity and duration of depressive symptoms that are associated with a clinically important response to SSRIs in people with depression. The PANDA researchers recruited depressed patients from primary care, who were not currently being treated with antidepressants. They then randomised them to either sertraline or placebo and followed them up at two, six and 12-week time points. We selected this trial because it would have given us participants who were free from antidepressants at baseline. We would then have been able to observe any effect of antidepressants on inflammatory biomarkers. Our aim was to use the psychological data collected by the PANDA researchers and to collect additional biological data at baseline and six-week follow-up.

Patients in the PANDA trial who had indicated that they agreed to be contacted with information of any future studies relating to depression were considered for the Resist study. At the baseline interview, the PANDA researcher assessed depressive symptoms using the Clinical Interview Schedule-Revised (CIS-R) and the Patient Health Questionnaire-9 (PHQ-9). If the participant was categorised as meeting depression criteria for the Resist study they were given the Resist PIS and asked if they would also like to be contacted by the Resist researcher to discuss the study in more detail. Details of potential participants were then passed to the Resist researcher by telephone. Recruitment began in August 2016, however uptake for the PANDA trial was very slow and although seven participants were referred, we only successfully recruited one. Six exclusions were due to physical co-morbidities and one was due to participant family bereavement. In order to reach our target it was necessary for us to expand our recruitment via additional methods.

In October 2016, we began recruiting directly from GP practices. In order to keep our recruitment as parallel to the PANDA trial as possible, we worked alongside the same Clinical Research Networks as the PANDA study. In total, we successfully recruited six GP practices. GPs were asked to identify potential participants with depression during consultation, provide them with a patient information sheet (Appendix 11.1) and refer them via email to the researcher if they were interested in participating. Potential participants were then contacted by phone to introduce the study and conduct screening, if appropriate. 52 patients were referred via GP practices and we successfully recruited 18 people with symptoms of depression. 36 participants were excluded at screening, five due to bi-polar disorder and the remaining 31 due to physical co-morbidities and /or medications. Five patients did not attend their baseline assessment.

In January 2017, we approached iCope Psychological Therapies Service in Camden and Islington, in collaboration with Professor Steve Pilling from the Division of Psychology and Language Sciences at UCL, to discuss recruiting via their service. iCope is part of

the Camden and Islington NHS Foundation Trust and offers treatment for a range of psychological problems, including anxiety and depression. Clients can self-refer or be referred via their GP. The recruitment process consisted of an honorary assistant psychological practitioner conducting monthly database searches of clients with symptoms of depression, who had previously consented to being contacted regarding research projects. A list of potential participants was then sent to a clinician for eligibility screening. Potential participants were then referred to the study via email and were then contacted by phone or email. In total, 97 potential participants were referred from iCope, of which we successfully recruited 11 people with depression. 41 clients did not respond to invitation, five declined, two were excluded (one for symptoms of anxiety only and one for physical co-morbidity), one had incorrect contact details and two did not attend.

We also recruited people with depression online. We created a study page on the website 'Call For Participants' (Call For Participants, 2018) (Appendix 11.2). This acted as an advert for our study, which was published in a public, on-line community. Potential participants were then able to contact us directly if they wished to partake. In order to provide additional support for participants recruited online who may not yet be receiving clinical care for depression, a signposting sheet was given to them at the baseline assessment, containing information on how to seek help via their GP or local IAPT service and contact information for mental health helplines (Appendix 11.3). We successfully recruited two people with symptoms of depression via 'Call For Participants'.

We also recruited people with depression through UCL. Posters were created and displayed across the campus (Appendix 11.4) and the study was featured in the student newsletter. Students or staff were then able to contact us directly if they wished to participate. We recruited 13 people with symptoms of depression from UCL.

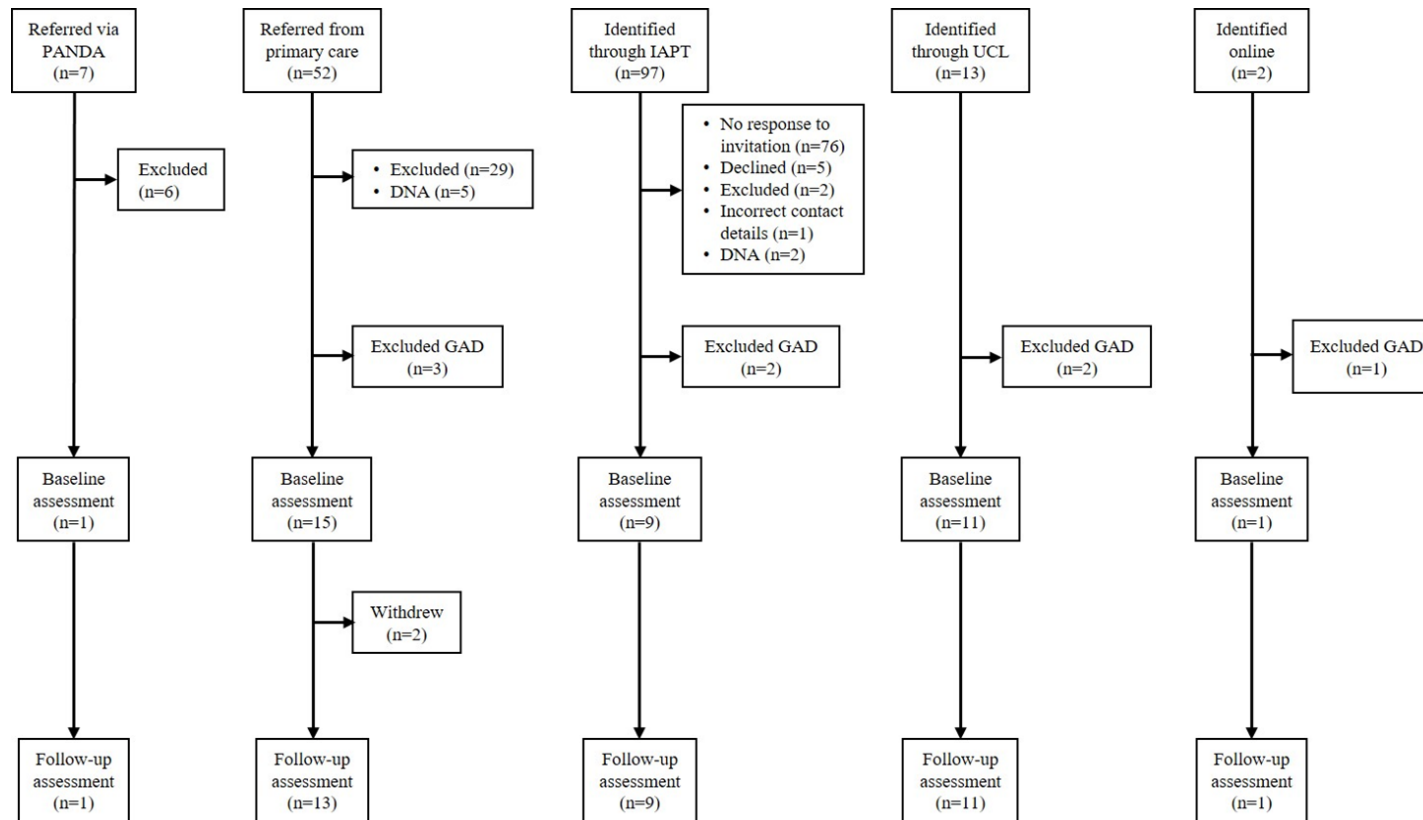
We initially planned to recruit healthy controls via the same GP practices as the PANDA study. This method was chosen in order to demographically match our healthy controls

with our depressed participants as well as possible. Practice staff were provided with eligibility criteria and asked to conduct a database search. A list of potential participants was then passed to the GP for eligibility screening. Letters of invitation, containing the researcher's contact details, were sent out to patients, along with patient information sheets (Appendix 11.5) via Docmail® (CFH Docmail Ltd, 2018). Upon receiving the letter of invitation, people who were interested in joining the study contacted the researcher either by phone or email. Mailings were sent out from three practices between October 2016 and March 2017, to 1,432 patients. This resulted in only two patients contacting the researcher to discuss the study. Due to the low response rate and the associated costs of conducting mail outs, we decided to recruit healthy controls from within UCL and QMUL. We recruited four healthy controls from QMUL and 22 healthy controls from UCL.

Furthermore, we recruited a number of healthy controls from the NIHR Clinical Research Network North West London (National Institute for Health Research, 2018). Staff who were associated with supporting recruitment for our study in primary care volunteered to be healthy controls. We recruited six healthy controls from NIHR. In total, we recruited 34 healthy controls and 45 people with MDD. Recruitment pathways are illustrated in Figure 2.1.

All data were collected with the written informed consent of the participants. The study obtained ethical approval in April 2016 (West Midlands - South Birmingham National Health Service (NHS) Research Ethics Committee, 16/WM/0143). Additional approval was obtained from the Health Research Authority in August 2016. The study was published on ClinicalTrials.gov on January 2016 (ClinicalTrials.gov Identifier: NCT02657798) (U.S. National Library of Medicine, 2018)

Figure 2.1 Recruitment pathways for people with depression.



Abbreviations: DNA = did not attend; GAD = generalised anxiety disorder.

2.2.4 *Participants*

A priori exclusion criteria included people with a diagnosis of other psychiatric disorders such as psychosis, schizophrenia, bipolar disorder, mania, hypomania, dementia, and eating disorder. Based on the baseline assessment we also excluded people who had a primary diagnosis of generalised anxiety disorder (GAD), agoraphobia, panic disorder or specific (isolated phobia). Full details of the measures used during assessments are provided in section 2.2.6.

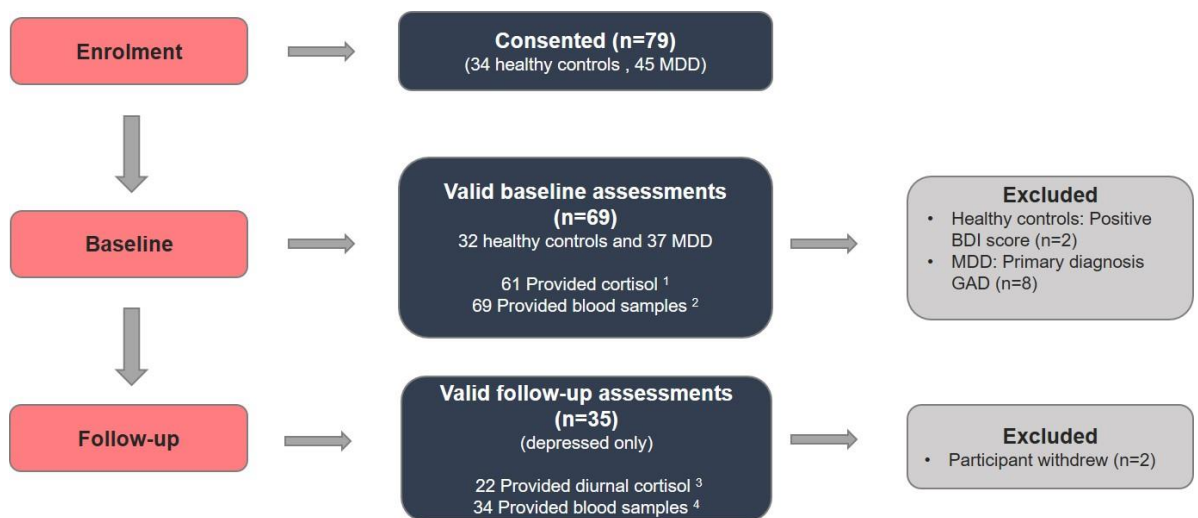
Inclusion criteria were that people had to be able to complete the psychological interview and questionnaires in English and be between 18 and 74 years of age. In order to exclude people who may have underlying systemic inflammation due to medical comorbidity, we excluded with significant physical illness (e.g. severe allergies, hypertension, cancer and hematological, autoimmune, cardiovascular, endocrine, pulmonary, renal, hepatic, gastrointestinal or neurological disease) as well as those who had viral illnesses during the preceding two weeks. In order to control for the effects of medications on inflammatory pathways we excluded people taking corticosteroid medications, people with a history of hypersensitivity to corticosteroids or steroid use, people who had been prescribed antibiotics in the last six months, anti-inflammatory medication in the last three months or prescribed any medications for the treatment of insomnia including hypnotic drug therapy, sedatives and melatonin in the last six weeks. Furthermore, we excluded people who smoked more than 25 cigarettes per day, people who were drug or alcohol dependent, pregnant or lactating women. The inclusion and exclusion criteria for healthy controls was identical with one notable exception: healthy controls needed to have no history or current symptoms of depression.

79 people in total consented to join the study, 45 people with symptoms of depression and 34 healthy controls. Unfortunately, this was below our target; however, after 16 months of recruitment we decided that due to the time constraints of the project,

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further recruitment would not be possible. Out of these 79 participants, two healthy controls were excluded at baseline due a BDI score of ‘mild mood disturbance’ (BDI scores 14 and 16). This score was not substantiated by the CIS-R and so they were unable to be recruited into the ‘depressed’ arm of the study. Eight depressed participants were excluded at baseline due to a CIS-R primary diagnosis of GAD. In addition, two depressed participants dropped out of the study after baseline assessment. One stated that they no longer had time and the other gave no reason. Of the 69 participants assessed at baseline, 61 provided baseline saliva samples. Three participants stated that they had forgotten to post their samples, one was unable to take a sample due an accident and four stated that they were unable to take the samples due to inconvenience such as interfering with their work schedule. A flow diagram of participant data collection and attrition is provided in Figure 2.2.

Figure 2.2. Flow diagram of participant data collection and attrition from the Resist Study.



1 Eight participants failed to post diurnal cortisol samples after baseline assessments. Three forgot to post the samples. One was unable to take the samples due to an accident. Four were unable to take the samples due to inconvenience.

2 All participants provided blood samples at baseline.

3 13 participants failed to provide diurnal cortisol samples after follow-up assessment. Five were unable to take the samples due to inconvenience. Eight stated that they had forgotten to post them.

4 Problems with blood collection.

2.2.5 *Study protocol*

Baseline: All participants attended baseline assessments at either their GP practice (if they were recruited via the practice) or at UCL. Assessments were always conducted between 8.00am and 9.00am to ensure homogeneity. Participants provided written consent (Appendix 11.6). Following this the researcher conducted a brief interview to collect demographic information. Participants were then asked to complete questionnaires containing measures of depressive symptoms including depression, perceived stress and life-time events. Depressed patients only were asked to complete the CIS-R to provide a probable diagnosis of clinical depression. Following this, a baseline blood sample was taken (approximately 50ml). Participants were then presented with a saliva sampling kit and instructed on how to use them. The kit included five pre-labelled 'salivette' collection tubes (Sarstedt, Leicester, UK) and a cortisol sampling diary (Appendix 11.7). The cortisol diary contained instructions on how and when to collect 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 five saliva samples over the course of a day, on waking, 30 minutes after waking (30+), 60 minutes after waking (60+), 4pm and bedtime. Participants stored their samples in the refrigerator before returning them to the researcher via Freepost.

Follow-up (six weeks after baseline): Depressed participants only attended follow-up assessments at either their GP practice or at UCL. Participants were asked to complete questionnaires containing measures of psychiatric symptoms including depression, affect, changes in sleep and appetite, perceived stress and childhood maltreatment. Following this, a blood sample was taken. Participants were then presented with another saliva sampling kit and asked to complete it exactly as before and post back to the researcher.

2.2.6 *Psychosocial Measures*

Questionnaire measures were selected for several reasons. 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. Two depression measures were chosen and used simultaneously at baseline. This approach was adopted to ensure that our identification of depressed people was robust and generalisable across scales, as recommended by Fried and Nesse (2015b). Details of the individual measures used are provided below and full versions of the study questionnaires are provided in Appendices.

Sociodemographic information

Sociodemographic information was gathered from all participants to include age, body mass index (BMI), ethnicity, marital status, employment status, smoking and alcohol intake. All sociodemographic information was self-reported. BMI was calculated as (kg/m²). Participants were characterised as normal (18.5 to 24.9) or overweight/obese (25 and above). We did not have any participants who had a BMI under 18.5. Marital status was defined as 'married' or 'unmarried' and ethnicity was defined as 'white' or 'non-white'. Employment status was defined as 'employed' or 'unemployed'. Smoking was defined as 'current smoker' or 'non-smoker'. Non-smokers included people who had never smoked and those who had given up smoking. Alcohol consumption was defined as '>one drink per month' or '<one drink per month'.

Clinical information

Clinical information was collected from all participants and included physical health comorbidities and medication history. Mental health history was also collected and included number of previous depressive episodes, length of current depressive episode, additional mental health comorbidities and treatment history, including both pharmacotherapy and psychotherapy.

Depression

Depression was measured in two ways for depressed participants, interview (CIS-R) and questionnaire (BDI-II), in order to allow internal replication. It was not possible to conduct validity tests between the two measures, as their constructs are not identical (the CISR includes mixed depression and anxiety, whereas the BDI-II only measures depression). However, there was a positive correlation between the total scores of the two measures, $r=0.550$, $p<0.001$. Healthy controls were only asked to complete the BDI-II.

CISR

MDD was assessed during baseline interview using the computerised version of the Clinical Interview Schedule-Revised (CIS-R) (Lewis et al., 1992). The CIS-R is a widely used standardised measure of depression and anxiety disorders in community and primary care research. It is a fully structured assessment, suitable for lay interviewers, and as such can be self-administered using a personal computer. It is considered to be one of the most reliable and valid measures of minor psychiatric disorders in the community and is based on International Classification of Diseases, 10th Revision (ICD-10) criteria (Head et al., 2013; World Health Organization, 1992).

The CIS-R produces a 10-item report, which is scored on a 0-4 scale (except the depressive ideas item 0-5), depending on symptom severity and frequency (Lewis et al., 1992). It yields diagnostic categories including, depressive disorder, generalised anxiety disorder, mixed anxiety and depressive disorder as well as a total depression score of 0 to 21. The total depression score reflects the severity of depressive symptoms experienced in the preceding week (Khandaker et al., 2014). A total score of 12 or more indicates a clinically significant level of distress.

The CISR also includes a question about duration of current depressive episode. After the depressive symptoms section the question asks about the following categories: less than 2 weeks, between 2 weeks and 6 months, between 6 months and 1 year, between 1 and 2 years, between 2 and 5 years, between 5 and 10 years and more than 10 years. In addition symptom scores for fatigue, concentration, and sleep problems are calculated.

Beck Depression Inventory (BDI-II)

Depressive symptoms were also measured using the Beck depression Inventory II (BDI-II) (Appendix 11.8). The BDI-II is also widely used as an outcome measure in studies of depression in both clinical and non-clinical populations (A. T. Beck et al., 1996a). The BDI-II has been comprehensively reviewed and found to demonstrate high reliability, content and structural validity and be able to discriminate between depressed and non-depressed participants (Y.-P. Wang & Gorenstein, 2013). It has also been shown to be sensitive to change across time and therefore suitable for use in studies with repeated measures (Richter et al., 1998). It is also endorsed by the National Institute for Health and Clinical Excellence (NICE) for use in primary care in measuring baseline depression severity and responsiveness to treatment (Smarr & Keefer, 2011).

It is a 21-item self-report questionnaire which provides a group of four statements per question. Participants are asked to choose one statement in each group which best describes the way they have been feeling over the past two weeks. The questions refer to this timeframe in order to correspond with the DSM-IV criteria for major depressive disorder. The BDI-II was scored by summing the scores of each item. A four-point scale indicates degree of severity; items are rated from 0 (not at all) to 3 (extreme form of each symptom) with a highest possible score of 63 and a lowest possible score of 0. A score of 0-13 indicates normal mood, 14-19 indicates mild depression, 20-28 indicates moderate depression and 29-63 indicates severe depression (A. T. Beck et al., 1996a). The Cronbach's alpha for the BDI-II in this sample at baseline (n=69) was 0.95. The Cronbach's alpha at follow-up (n=35) was 0.87.

Life Events

Stress is widely acknowledged as an important factor in the aetiology of depression. There is a wealth of evidence demonstrating a causal relationship between stressful life events, such as a divorce or the death of a spouse, and the development of depression (Kendler & Gardner, 2016). Life events were assessed using the List of Threatening Experiences (LTE) questionnaire (Brugha et al., 1985) (Appendix 11.9). This is a widely used measure and has been found to be valid and reliable in both psychiatric patients (Brugha et al., 1985) and a population based sample (Rosmalen et al., 2012).

The LTE is a 12-item self-report questionnaire, which asks about unpleasant events over the last 12 months. The LTS includes items such as 'You yourself suffered a serious illness, injury or an assault' and 'You had a separation due to marital difficulties'. In each case, participants are asked to indicate whether each of the 12 different life events occurred (yes/no). The LTE total score is the sum of the item scores (maximum score=12).

Perceived Stress

The degree to which participants appraised the situations in their lives as stressful was measured using the Perceived Stress Scale (PSS) (S. Cohen et al., 1983) (Appendix 11.10). The PSS was chosen because it provides additional data regarding life situations that is not solely based on the qualities of the events themselves, but rather on personal and contextual factors, such as an individual's ability to cope. It has also been shown to be reliable and valid in non-clinical populations (Roberti et al., 2006) and in a psychiatric sample (Hewitt et al., 1992).

The PSS is a 10-item self-report questionnaire, which asks about feelings and thoughts over the last month. The PSS includes items such as 'In the last month, how often have you felt that you were unable to control the important things in your life?' and 'In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?' In each case, respondents are asked how often they felt a certain way and are scored on a five-point Likert scale, ranging from 0 'Never' to 4 'Very often'. Items 4, 5, 7, and 8 are reverse scored. The Cronbach's alpha for the PSS in this sample at baseline (n=69) was 0.91. The Cronbach's alpha at follow up (n=35) was 0.82.

Childhood Maltreatment

There is an extensive body of research which shows that childhood maltreatment increases the risk of adult psychopathology (Thabrew et al., 2012). Exposure to physical, sexual and emotional abuse and neglect have all been linked to depression in adulthood. Childhood maltreatment was measured using the Childhood Experience of Care and Abuse Questionnaire (CECA-Q) (Bifulco et al., 2005) (Appendix 11.11). The CECA-Q is a self-report instrument that has been shown to be reliable and valid in a clinical population (N. Smith et al., 2002) and a community sample (Bifulco et al., 2005). This

instrument is considered a first choice tool for broad clinical research (Thabrew et al., 2012).

The CECA-Q is a self-report questionnaire used to assess neglect, antipathy, physical abuse, and sexual abuse. There are 16 parental care items; 8 neglect items defined in terms of parental disinterest in material care, health, schoolwork, and friendships and 8 antipathy items defined as parental hostility, coldness, or rejection. In each case, respondents are asked to answer questions in terms of 'How you remember your mother/father in your first 17 years', including items such as 'She made me feel unwanted' and 'He would leave me unsupervised before I was 10 years old'. All items were presented separately for each parent and were scored on a five-point Likert scale ranging from 1 'Yes definitely' to 5 'No, not at all'. The mid-point, 3, was labelled as 'Unsure'. Items 2, 3, 5, 8, 11, 12, 13 and 14 are reverse scored. A cut off score of 25, 22 or 24 or more indicates severity for maternal and paternal antipathy, maternal neglect and paternal neglect, respectively. Cronbach's alpha scores were determined for the two CECA-Q dimensions of antipathy and neglect (n=66) and found to be 0.83 and 0.88, respectively.

Physical abuse is defined in terms of hitting by parents or other older household members. There is one screen item which asked 'When you were a child or teenager were you ever hit repeatedly with an implement (such as a belt or stick) or punched, kicked or burnt by someone in the household?' rated 0 'No' or 1 'Yes'. In additions, there are four supplementary items for assessing the severity of physical abuse which included items such as 'Did the hitting happen on more than one occasion?' and 'Were you ever injured e.g. bruises, black eyes, broken limbs?'. Items are rated 0 'No' or 1 'Yes' and summed to give a total score of 0-4. A cut off score of one or more indicates severity.

There are also three screen items for sexual abuse which is defined by physical contact or approach of a sexual nature by any adult, excluding consensual sexual contact with

peers. This section includes items such as 'Did anyone force you or persuade you have sexual intercourse against your wishes before age 17?'. Items are rated 0 'No', 1 'Unsure' or 1 'Yes'. Severe sexual abuse is assessed with seven additional items including items such as 'Was the other person a relative?' and 'Did this person do it to you on more than one occasion?' . Items are rated 0 'No' or 1 'Yes' and summed to give a total score of 0-7. Scoring was based on initially summing the screening items and then summing the severity items separately A cut off score of one or more indicates severity.

2.2.7 *Biological Measures*

Plasma IL-6 and TNF- α determination

Plasma IL-6 and TNF- α levels were measured at both baseline and follow-up assessment. IL-6 and TNF- α production was measured using a commercially available enzyme-linked immunosorbent assays (ELISA), from R&D Systems®, Inc. A more detailed description of the cytokine determination assay protocols will be provided in Section 3.3.1 of this thesis.

Cortisol sampling

Diurnal salivary cortisol secretion was measured at both baseline and follow up. Salivary cortisol is routinely used as a measure of HPA-axis function. It has been shown to be a reliable measure of 'free' unbound plasma cortisol, which is considered to be the biologically active fraction (Kirschbaum & Hellhammer, 1989, 2000). High correlations between salivary cortisol and unbound free cortisol level in plasma and serum have been demonstrated, which are consistent throughout circadian rhythm (D. H. Hellhammer et al., 2009). Saliva cortisol was chosen as a specimen over blood analysis for methodological reasons. Saliva sampling is a comparatively inexpensive and non-invasive method, avoiding issues associated with repeated venipuncture, making it an

ideal choice for ambulatory measurement of cortisol in naturalistic settings (Kirschbaum & Hellhammer, 1989). Furthermore, salivary cortisol is stable at room temperature for two weeks after sampling, allowing for participants to post their samples back to the laboratory, without the need for centrifugation. A description of the calculation of diurnal cortisol parameters will be provided in Section 4.3.1 of this thesis.

Corticosteroid receptor sensitivity

GR and MR sensitivity was measured by dexamethasone and prednisolone inhibition of lipopolysaccharide (LPS) induced IL-6 levels in whole blood. IL-6 production was measured using a commercially available Luminex technology kit for IL-6 from Bio-RAD®. A more detailed description of the corticosteroid sensitivity assay protocol will be provided in Section 3.3.3 of this thesis.

Measurement of Treg percentages

Peripheral blood mononuclear cell (PBMC) suspensions were prepared from sodium-heparinized blood via Ficoll gradient centrifugation according to standard methods and on the same day as the blood was collected. Samples were frozen in 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until further analysis.

Tregs were identified as CD4⁺CD25⁺Foxp3⁺ cells. Membrane staining was performed to identify CD4 and CD25 and intracellular staining was performed to identify FoxP3. Stained cells were analysed using flow cytometry. A more detailed description of the Treg measurement protocol will be provided in Section 6.3.1 of this thesis.

2.3 Data Storage

All Resist data were handled according to the Data Protection Act 1998 as well as UCL Information Security Policy. The project was registered with the UCL Data Protection Office and the Health Research Authority. All data were anonymized using unique study IDs. UCL's Data Safe Haven (DSH), a technical solution for transferring and storing information that is highly confidential, was used to store and handle data. DSH has been certified to the ISO27001 information security standard and conforms to the NHS Information Governance Toolkit. All paper questionnaires were anonymised and stored in a locked filing cabinet in locked offices at UCL. Biological samples were assigned a study identification number and did not display any personal data. They were held at UCL and QMUL laboratories for relevant analysis, with access limited to members of the study team. 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. Plasma and samples from each participant are currently being securely stored in -20°C freezers in a code-protected laboratory. Leukocyte samples are currently being securely stored in liquid nitrogen in a code-protected laboratory. Anonymised raw data were entered onto a computer database for statistical analysis. All data from the Resist Study may be kept in the secure manner described above for up to 20 years prior to being destroyed.

2.4 Statistical analyses

All statistical analyses were performed using SPSS version 22.0 software (SPSS Inc., Chicago, Illinois, USA). The significance level was set to $p < 0.05$ (two-tailed) for all analyses. Specific details of statistical analyses carried out are included in chapters 4

and 5 which deal with IL-6 and TNF- α levels, corticosteroid receptor function, diurnal cortisol, and measurement of regulatory T cell percentages.

2.5 My involvement and contribution

The research question regarding depressive symptoms and inflammatory biomarkers was established between myself and Dr Livia Carvalho who has worked extensively in this field. Dr Carvalho established the collaboration with the PANDA Study and introduced me to Professor Glyn Lewis. Together we designed the Resist study. I was responsible for the development and creation of all the study materials. I applied for all regulatory approvals and was responsible for all recruitment and data collection. I conducted all psychological interviews and was solely responsible for all blood and saliva sampling. I also carried out all corticosteroid receptor sensitivity assays, plasma IL-6 and TNF- α determination assays and Treg flow cytometry assays. I was responsible for the recoding of all the saliva samples collected 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

3. Study 1a - The Resist Study results:

The association between TNF- α , IL-6 and HPA-axis function in people with MDD compared with healthy controls

3.1 Introduction

As described in the literature review, evidence suggests an important role for immune activation and neuroendocrine dysfunction in the pathology of depression. Increases in levels of pro-inflammatory cytokines such as IL-6 and TNF- α , have been robustly reported in depressed patients. Dysregulation of the HPA-axis is one of the most consistent neurobiological findings in this population, although the exact nature of disturbances in diurnal rhythm and the role of the MR are yet to be clarified. Inflammatory cytokines activate the HPA-axis, resulting in an increase in cortisol, and glucocorticoids are known to suppress the production of inflammatory cytokines during immune challenge. It is therefore reasonable to hypothesize that inflammatory and neuroendocrine alterations in depression might interact, yet, to date, these systems have largely been investigated separately. The few that have investigated relationships between the two have reported conflicting results. Furthermore, studies have often relied on single but variable measures of HPA-axis function, adding to the inconsistency. By examining differences in multiple biomarkers across both systems and exploring any association between them, we hope to advance our understanding of the complex mechanisms involved. In this section I will present results from the Resist Study concerning differences in and associations between inflammation and HPA-axis function in depressed people compared with healthy controls. These results may tell us more about the ways in which these different biological systems interact in the pathophysiology of depression.

3.2 Hypotheses

Many cross-sectional studies have demonstrated increased levels of inflammatory biomarkers in depressed people. Several meta-analyses have reported increased levels of IL-6 and TNF- α (Dowlati et al., 2010;Goldsmith et al., 2016b;Hiles et al., 2012b;Howren et al., 2009;Kohler et al., 2017;Y. Liu et al., 2012). A cumulative meta-analysis also confirmed the findings for IL-6 ((Haapakoski et al., 2015). We sought to replicate this finding in the current sample. Therefore **I hypothesise that people with MDD will have significantly increased levels of inflammation compared with healthy controls, specifically increased levels of plasma IL-6 and TNF-a (hypothesis 1).**

Regarding neuroendocrine function, *in vitro* studies of the GR have consistently reported decreased sensitivity in depressed patients (Bauer et al., 2003;Calfa et al., 2003;Lowy et al., 1984;Lowy et al., 1988;G. E. Miller et al., 2005b;Rupprecht et al., 1991c;Tanke et al., 2008;Wodarz et al., 1991;Wodarz et al., 1992). *In vivo*, DST studies (Gormley et al., 1985;Lowy et al., 1988;A. Wassef et al., 1990) and Dex-CRH studies (Bardeleben & Holsboer, 1989;Gonul et al., 2017;Heuser et al., 1994;Holsboer-Trachsler et al., 1991;Holsboer et al., 1987;Modell et al., 1997;von Bardeleben & Holsboer, 1991) also provide robust evidence that GR sensitivity is decreased in depression. Overall there is a lack of studies investigating the MR in depression and what evidence there is, is mixed, with some studies demonstrating retained sensitivity (Juruena et al., 2006;Juruena et al., 2009), some demonstrating increased sensitivity (Young et al., 2003) and some demonstrating impairment (Hinkelmann et al., 2016;Juruena et al., 2009;Juruena et al., 2013;Lembke et al., 2013). Therefore, in order to address this gap in the literature, this study will explore both GR and MR sensitivity between people with MDD. Based on the findings above, **I hypothesise that people with MDD will exhibit significantly decreased glucocorticoid sensitivity (hypothesis 2) and significantly different mineralocorticoid sensitivity compared with controls (hypothesis 3).**

The evidence for hyperactivity of the HPA-axis in depression is robust, however findings regarding depression and diurnal cortisol profiles are mixed. The majority of cross-sectional and longitudinal studies provide evidence for associations between increased CAR and clinical depression (Dienes et al., 2013;Hardeveld et al., 2014;Rhebergen et al., 2015;Ulrike et al., 2013;Vreeburg et al., 2009a). However, other studies have reported lowered or blunted CAR in depressed people (Huber et al., 2006;Stetler & Miller, 2005). Similarly, both increases and decreases in the CAR have been associated with symptom remission (J. Beck et al., 2015;Ruhe et al., 2015). Increased AUC has also been reported in clinically depressed people (Dienes et al., 2013;O'Brien et al., 2004), however one study found no difference between depressed people and controls (Gonul et al., 2017). Most studies have reported a flatter slope in depressed people (Adam et al., 2017;Hsiao et al., 2010;Jarcho et al., 2013), although there is also some inconsistency with some a few studies reporting either a steeper slope (S. H. Booij et al., 2015) or no difference between depressed people and healthy controls (Assies et al., 2004;Doolin et al., 2017). In order to address these inconsistencies in the literature we compared diurnal cortisol rhythms between people with MDD and healthy controls. Based on the balance of findings, **I hypothesise that people with MDD will exhibit significantly altered diurnal cortisol secretion compared to healthy controls, including increased CAR, increased AUC and flatter cortisol slope (hypothesis 4).**

In terms of the relationship between these pathways, very few MDD studies have explored associations between these parameters. One study demonstrated that the degree of DEX non-suppression following the DST was associated with IL-6 levels in PBMCs in both depressed people and healthy controls (Maes et al., 1993b). Another study showed that plasma TNF- α was correlated with peripheral GR resistance following topically applied glucocorticoids in depressed people only (Fitzgerald et al., 2006). Conversely, TNF- α has been negatively associated with HPA-axis function following the DEX/CRH test in depressed people in two studies (Himmerich et al., 2006;Schuld et al., 2003). Whilst the findings presented are conflicting, there is a robust literature

demonstrating that depression is accompanied by both increases in inflammatory cytokines and decreases in GR sensitivity. It is therefore reasonable to suppose that these factors may be associated. An association between inflammation and MR sensitivity has yet to be explored.

Baseline serum/saliva cortisol has been positively associated with inflammatory biomarkers in both depressed people and healthy controls (Y. Chen et al., 2017; Cubala & Landowski, 2014). IL-1 β mRNA has been negatively associated with the CAR (Doolin et al., 2017) and IL-6 has been positively associated with the AUC in depressed people but not controls (Maes et al., 1995). To date, the relationship between TNF- α and diurnal cortisol rhythm in MDD patients have not been explored. In order to address these gaps in the literature we investigated whether there was any association between inflammatory biomarkers and HPA-axis parameters. Therefore, **I hypothesise that inflammatory biomarkers will be significantly, positively associated with increased CAR, AUC and flatter slope and negatively associated with GR sensitivity in people with MDD (hypothesis 5).**

As well as being associated with depression, inflammation is also associated with perceived stress (Johnson et al., 2013; G. E. Miller et al., 2008; G. E. Miller et al., 2014; Walsh et al., 2018) and stressful life events (Kiecolt-Glaser et al., 2002; Maes et al., 1998; Marshall et al., 1998) in healthy people. It has also been associated with childhood maltreatment (Carpenter et al., 2010; Coelho et al., 2014; G. E. Miller & Chen, 2010) in both healthy and people with MDD (Danese et al., 2008; Zeugmann et al., 2013). GR resistance has also been associated with perceived stress in healthy people (Menke et al., 2014; G. E. Miller et al., 2008; G. E. Miller et al., 2014; Walsh et al., 2018) and with childhood maltreatment in both depressed and healthy people (Von Werne Baes et al., 2012). Little is known about the relationship between psychosocial factors and MR function. The findings for the effects of perceived stress on diurnal cortisol are conflicting. Evidence exists for both positive associations (Allen et al., 2017; Boggero et al., 166

2017;Chida & Steptoe, 2009) and negative associations (Boggero et al., 2017) between stress and the CAR in healthy people. Similarly perceived stress has been positively associated (Ockenfels et al., 1995;Steptoe et al., 2000) and negatively associated (Adam et al., 2017) with the slope. Findings regarding diurnal cortisol and childhood maltreatment are also mixed, with some studies suggesting that maltreatment leads to an increased CAR (Quevedo et al., 2017;Wielaard et al., 2018) in both depressed and non-depressed people and some suggesting a blunted CAR (van der Vegt et al., 2009). Moderate maltreatment has been associated with a steeper diurnal slope and severe maltreatment with a flatter slope (van der Vegt et al., 2009). However, a meta-analysis reported no association between either the CAR or the slope and childhood adversity (Bernard et al., 2017). In order to investigate any effects of these psychosocial factors, exploratory analyses will be conducted including any of the variables which differ between the groups.

3.3 Biological measures

3.3.1 Plasma IL-6 and TNF- α determination

Protocol

Sodium-heparinised blood was centrifuged (500g, room temp, 10mins). The plasma was then removed, avoiding any red cells, transferred to 1.5ml polypropylene tubes, and stored at -20° until analysis. Plasma IL-6 and TNF- α levels were determined using commercially available, solid-phase enzyme-linked immunosorbent assays (ELISA) from R&D Systems®, Inc. The inter- and intra-assay coefficient of variation (CV) for IL-6 analysis was 7.8% and 7.4% respectively, and the mean minimum detectable dose was 0.039pg/ml. The inter- and intra-assay coefficient of variation (CV) for TNF- α analysis was 6.5% and 2% respectively, and the mean minimum detectable dose was 0.049pg/ml. The assays were performed in duplicate.

3.3.2 Corticosteroid receptor sensitivity

Reagents

RPMI 1640 medium (Sigma, 500ml, sterile, R8758); foetal calf serum (FCS) (Gibco 10270); penicillin/streptomycin (Sigma, 500ml, sterile, P4458); HEPES buffer (Fisher BioReagents, 1M solution, pH 7.3, BP299-100); penicillin/streptomycin (Sigma, sterile, P4458-100ml); LPS (Sigma), 10mg, L2630; DEX (Sigma), D4902; prednisolone (Sigma), P-6004.

Protocol

GR and MR sensitivity was measured by dexamethasone and prednisolone inhibition of LPS-stimulated IL-6 levels using *in vitro* glucocorticoid and mineralocorticoid sensitivity assays, retrospectively (See Figure 1). The protocol for both assays was identical apart from the choice of corticosteroid. The protocol was based on a previously published method with slight modifications (Carvalho et al., 2008). This method was chosen over an *in vivo* Dex/CRH test as it is a more direct measure of corticosteroid receptor function. Sodium-heparinised blood was diluted ten-fold using RPMI 1640 medium supplemented with 10% foetal calf serum, 2.5% hepes buffer and 1% PEN-STREP (Penicillin-Streptomycin). The following concentrations of dexamethasone and prednisolone were used: 10^{-6} , 10^{-7} , 10^{-8} , and 3×10^{-9} . 540µl of diluted blood was then 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 and stored at -20° until analysis. The assays were performed in duplicate.

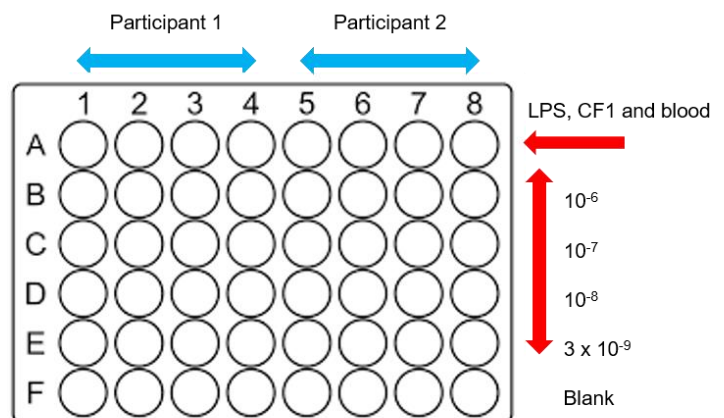
IL-6 level measurement was carried out using a commercially available Luminex technology kit for IL-6 from Bio-RAD®. The inter- and intra-assay coefficient of variation

(CV) for IL-6 analysis was 6.8% and 4.5% respectively, and the mean minimum detectable dose was 0.36 pg/ml.

Of the 67 participants who were included in the analysis, seven healthy control samples were excluded due to researcher error, leaving a study sample of 60 (23 HC and 37 MDD).

Figure 3.1 The glucocorticoid sensitivity assay.

1. Whole blood was diluted with RPMI, foetal calf serum and penicillin-streptomycin.
2. LPS was added to 36 wells of a 48 well plate.
3. Culture medium was added to four or the remaining wells.
4. Either dexamethasone (GR) or prednisolone (MR) were added to 32 wells in serial concentrations.
5. Whole blood from two participants was then added to each well in duplicate.
6. The plate was incubated for 24 hours.
7. The supernatant was removed and analysed for IL-6.



Glucocorticoid suppression was calculated by normalising all data to LPS-stimulated IL-6 levels in the absence of either DEX or prednisolone expressed as 100%. Specifically, the calculation of the percentage inhibition of IL-6 by the glucocorticoids was as follows:

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

Percentage inhibition for each concentration of DEX and prednisolone was then calculated using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA) in order to calculate the log inhibitory concentration 50% (IC₅₀) values of the dose-

response curve of DEX and prednisolone suppression of IL-6 production. The IC_{50} represents the concentration of substance or drug required to bring about 50% functional inhibition. Log IC_{50} values are inversely proportional to glucocorticoid sensitivity. Higher log IC_{50} values indicate that more DEX or prednisolone was required to suppress IL-6 production by 50%, and thus decreased GR and/or MR sensitivity.

Due to difficulty in converging dose-response curves for IC_{50} values in Graphpad, we were unable to compute DEX log IC_{50} values for 12 MDD participants and PRED log IC_{50} values for 21 MDD participants and 10 healthy controls. Therefore DEX log IC_{50} values were successfully computed for 48 participants (25 MDD and 23 HC) and PRED log IC_{50} values were successfully computed for 29 participants (16 MDD and 13 HC).

3.3.3 *Calculation of diurnal cortisol parameters*

All saliva samples were collected using 'salivettes' (Sarstedt, Leicester, UK) and were stored at $-20^{\circ}C$ for analysis at a later date. Cortisol levels were measured using a time-resolved immunoassay with fluorescence detection at the University of Dresden. The inter- and intra-assay coefficient of variation (CV) for cortisol analysis was $<9\%$ and $<6\%$ respectively.

Four different indices of HPA axis function were computed: cortisol awakening response (CAR), cortisol output during first hour of awakening (CAR AUC), total cortisol output (AUC) and cortisol slope across the day. Of the 61 participants who provided saliva samples, two had extreme values and were excluded from the analysis, leaving a sample of 59 (28 HC and 31 MDD). Of the 59 participants included in the analysis, there were some missing cortisol samples as follows: one at waking + 60 minutes, three at 4pm and one at bedtime.

The CAR was calculated by subtracting the waking from the waking + 30 min values. When calculating the CAR, five participants were excluded who declared a waking sample collection time before the waking time, one participant who failed to post their sampling diary and one participant who failed to note their waking time. 13 individuals were also excluded who reported a delay of >15 min between waking and taking the 'waking' sample, leaving a sample of 39 (20 HC and 19 MDD). 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 slope of decline in cortisol across the day was calculated by computing the difference between waking and bedtime values and dividing by the time elapsed between the two samples, expressed in nmol/L/min. Higher values indicate a steeper decrease in cortisol over the day. Cortisol slope was calculated if the participant had waking cortisol and bedtime cortisol. 58 participants had sufficient data for the calculation of cortisol slope (one participant did not provide a bedtime sample) (27 HC and 31 MDD).

The CAR AUC, or overall volume of cortisol released during the first hour, was calculated using the cortisol AUC with respect to ground method, as described by Pruessner and colleagues (J. C. Pruessner et al., 2003a). The CAR AUC calculation included waking, waking + 30 minutes and waking + 60 minutes samples. Therefore, CAR AUC was calculated for the 58 participants who provided these saliva samples successfully. Total cortisol output over the day was assessed by calculating the cortisol AUC with respect to ground (with 30+ excluded). Cortisol AUC was calculated only for those who provided saliva samples for waking, waking + 60, 4pm and bedtime cortisol. Therefore, cortisol AUC was calculated for the 55 participants who provided all these saliva samples successfully (25 HC and 30 MDD). There was no significant difference mean waking times between people with MDD and healthy controls ($t(55) = -1.179, p = 0.243$). The mean waking time for people with MDD was 7.26am ($SD = 1.39$) and the mean waking time for healthy controls was 6.57am ($SD = 1.17$).

3.4 Statistical analysis

Kolmogorov-Smirnov tests were used to test for the normality of the distribution and a natural log transformation was conducted when the test revealed deviation from normality. Log transformations did not normalise the distribution for age, BDI scores at baseline, PSS scores at baseline and LTE scores, so non-parametric tests were performed to compare the groups on these variables. Regarding the CECA.Q, a composite variable was computed to summarise how many adversities had been experienced by each participant, following the guidelines published by Bifulco et al. (2005). This strategy was based on a previously published method with slight modifications (Trotta et al., 2016). Non-parametric tests were also used for this variable.

LPS values, all percentage inhibition values and plasma IL-6 and TNF- α values were not normally distributed in both groups. Log transformation normalised the distributions for baseline LPS, all percentage inhibition values and plasma IL-6. Non-parametric tests were applied for the analysis of TNF- α . In relation to salivary cortisol, normality tests revealed that baseline cortisol levels at waking + 60 minutes, 4pm and bedtime were not normally distributed. Log transformations normalised the distributions for these variables. All other baseline cortisol variables were normally distributed. Levene's test was used to test for homogeneity of variance and corrected T-tests were used where appropriate.

Independent-Samples Mann-Whitney U tests and chi-square tests were used to compare the MDD group with the control group on all socio-demographic and clinical characteristics. Differences in biological parameters between the groups were conducted using Independent Samples T-tests and Independent-Samples Mann-Whitney U tests. For comparison of more than two groups, One-way ANOVA analysis or Kruskal–Wallis test were performed, as appropriate. Where there were significant differences between the groups on any of the sociodemographic variables, multivariable linear regression analyses were conducted, where the variables of interest were included as predictor

variables. Pearson's R correlations and Spearman's correlations were used to ascertain whether there was any association between inflammatory and HPA-axis pathways and between these biological pathways and baseline mood. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA).

3.5 Results

3.5.1 Participants

At baseline, blood was successfully drawn from 37 people with MDD and from 32 healthy controls. Saliva samples for cortisol measurement were collected from 31 people with MDD and 30 healthy controls. Participants with at least one of these biological measures (cortisol, cytokine measurement or receptor sensitivity) were included in the main sample of this study ($n = 69$). Two healthy controls had both cortisol and cytokine values outside 3 SD from the mean and were removed from the analysis, leaving an analytic sample of 37 people with MDD and 30 controls.

Table 3.1 summarises the sociodemographic characteristics of the participants. Of the 67 participants included in the analysis, 37 had MDD and 30 were healthy controls. The overall sample had an age range of 21-61 years ($M = 32.72$, $SD = 10.24$) and were almost two-thirds women (62.7%). Two-thirds were normal weight (67.2% BMI<25) and white (67.2%). Most were unmarried (82.1%) and employed (74.6%). 16.4% of the sample were smokers and two-thirds of people consumed an alcoholic drink more than once a month (67.2%).

There was no significant difference in age ($U = 666.5$, $p = 0.159$), gender ($\chi^2 = 0.010$, $df = 1$, $p = 0.921$), BMI ($\chi^2 = 2.224$, $df = 1$, $p = 0.136$) marital status ($\chi^2 = 0.57$, $df = 1$, $p = 0.811$), ethnicity ($\chi^2 = 0.937$, $df = 1$, $p = 0.333$) or employment status ($\chi^2 = 2.175$, $df = 1$, $p = 0.140$) between the MDD group and controls. There was a significant difference in

smoking ($\chi^2 = 6.778$, $df = 1$, $p = 0.009$) and alcohol consumption ($\chi^2 = 4.058$, $df = 1$, $p = 0.044$). People with MDD were more likely to smoke (MDD: $n = 10$, 27%; controls: $n = 1$, 4.9%) and less likely to drink alcohol (MDD: $n = 21$, 56.8%; controls: $n = 24$, 80%) compared with controls.

Table 3.2 summarises the clinical characteristics of the participants. Two thirds of the people in the depressed group had experienced depression previously (67.6%). One third of the depressed group were taking antidepressants (30%) and one fifth were receiving psychotherapy at baseline (22%). Regarding severity of depressive symptoms, almost half of the depressed group were experiencing severe depression (43.2%), 5.4% had moderate depression, 10.8% had mild depression and 8.1% had mixed anxiety and depressive disorder (mild).

As expected, BDI scores were found to be very low among the controls ($BDI < 3$) and there was a significant difference between the groups ($U = 1,109$, $p < 0.001$). There was also a significant difference in PSS scores between the two groups ($U = 1,064$, $p < 0.001$). People with MDD had significantly higher levels of perceived stress ($Mdn = 23.00$, $IQR = 6$) compared with controls ($Mdn = 10.50$, $IQR = 7$) There was no significant difference in LTE scores ($U = 611.5$, $p = 0.460$). In terms of childhood maltreatment, there was a borderline significant difference between the groups in total adversity score ($U = 552.5$, $p = 0.082$). People with MDD were more likely to have experienced childhood adversity ($Mdn = 1.00$, $IQR = 2$) compared with controls ($Mdn = 0.00$, $IQR = 1$). The groups did not differ in their experience of physical abuse ($\chi^2 = 0.472$, $df = 1$, $p = 0.492$). There was a significant difference in their experience of sexual abuse ($\chi^2 = 4.433$, $df = 1$, $p = 0.035$). People with MDD were more likely to have experienced childhood sexual abuse ($n = 13$, 37.1%) compared with controls ($n = 4$, 13.8%).

Table 3.1. Sociodemographic characteristics of the study sample at baseline (n=67)

	People with MDD (n=37)	Healthy controls (n=30)	Group difference (p value)
	<i>Mean ± SD or Median ± IQR or N (%)</i>	<i>Mean ± SD or Median ± IQR or N (%)</i>	
Age (years)	*32 ± 23	*28 ± 7	0.159
Female	23 (62)	19 (63)	0.921
BMI			0.136
	<i>Normal</i>	22 (60)	23 (77)
	<i>Overweight/obese</i>	15 (41)	7 (23)
Marital status (Married)	7 (22)	5 (17)	0.811
Ethnicity (White)	24 (75)	22 (73)	0.333
Employed	25 (68)	25 (83)	0.140
Smoker	10 (27)	1 (3)	0.009
Alcohol (> 1 drink per month)	21 (57)	24 (80)	0.044

Abbreviations: MDD = major depressive disorder, SD = standard deviation; BMI = body mass index. *Median/interquartile range.

Table 3.2. Clinical characteristics of the study sample at baseline (n=67)

	People with MDD (n=37)	Healthy controls (n=30)	Group difference (p value)
	<i>Mean ± SD or Median ± IQR or N (%)</i>	<i>Mean ± SD or Median ± IQR or N (%)</i>	
Previous depression	25 (68)	-	-
Antidepressant use at baseline	11 (30)	-	-
Psychotherapy at baseline	8 (22)	-	-
BDI II score	*25 ± 13	*2 ± 3	<0.001
CISR score	22.51 ± 8.25	-	-
CISR primary diagnosis			
<i>Mild depressive episode</i>	19 (51)	-	-
<i>Moderate depressive episode</i>	2 (5)	-	-
<i>Severe depressive episode</i>	16 (43)	-	-

Abbreviations: MDD = major depressive disorder, SD = standard deviation; BDI – Beck Depression Inventory II, CISR = Clinical Interview Schedule-Revised. *Median/interquartile range.

Table 3.2 continued. Clinical characteristics of the study sample at baseline (n = 67)

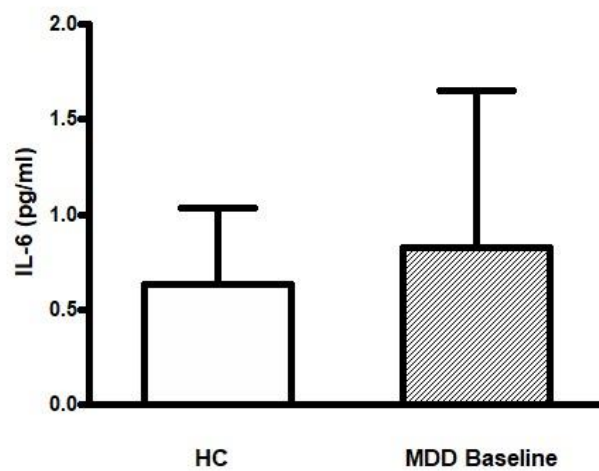
	People with MDD (n=37)	Healthy controls (n=30)	Group difference (p value)
	<i>Mean ± SD or Median ± IQR or N (%)</i>	<i>Mean ± SD or Median ± IQR or N (%)</i>	
PSS score	*23.00 ± 6	*10.50 ± 7	<0.001
LTE score	*1 ± 3	*1 ± 2	0.460
Childhood maltreatment			
<i>Total adversity score</i>	*1.00 ± 2	*0.00 ± 1	0.082
<i>Physical abuse</i>	3 (9)	4 (14)	0.492
<i>Sexual abuse</i>	13 (37)	4 (14)	0.035

Abbreviations: MDD = major depressive disorder; SD = standard deviation; PSS = Perceived Stress Scale; LTE – List of Threatening Events.
*Median/interquartile range.

3.5.2 Inflammatory biomarkers

To analyse differences in inflammation, plasma IL-6 and TNF- α levels were compared between the groups. There was no significant difference in IL-6 levels between people with MDD and healthy controls ($t(65) = -0.522, p = 0.604$) (striped bar represents people with MDD) (Table 3.3, Figure 3.2).

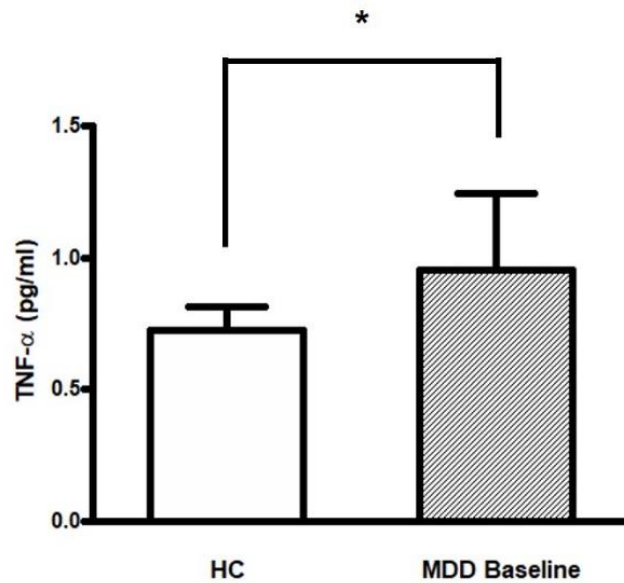
Figure 3.2. Plasma IL-6 levels in healthy controls and people with MDD at baseline.



Data shown are raw values presented as mean \pm SD in pg per ml. Healthy controls (n=30), people with MDD (n=37).

In contrast, there was a significant difference in TNF- α levels between the groups, ($U = 843.50, p < 0.001$). TNF- α levels were higher in people with MDD compared to in healthy controls (Table 3.3, Figure 3.3.)

Figure 3.3. Plasma TNF- α levels in healthy controls and people with MDD at baseline.



Data shown are raw values presented as median \pm interquartile range in pg per ml. * Statistically significant at $P < 0.05$. Healthy controls ($n=30$), people with MDD ($n=37$).

Because the groups differed in terms of smoking and alcohol consumption, analyses were carried out to ascertain whether these health behaviours impacted the findings. Linear regression revealed that TNF- α levels were not attenuated by the inclusion of these individual health factors as covariates. The results showed that the overall model was statistically significant ($F(3, 63) = 3.245, p = 0.028$) and that the independent variables explained 13.4% of the variance in TNF- α . Study group was significantly associated with TNF- α levels ($\beta = 0.388, t(66) = 3.063, p = 0.003$). However smoking status and alcohol consumption were not significantly associated with TNF- α ($\beta = -0.094, t(66) = -0.758, p = 0.451$; and $\beta = 0.151, t(66) = 1.248, p = 0.217$, respectively).

Table 3.3. Mean inflammatory and corticosteroid receptor sensitivity parameter values and p values from analyses comparing differences between people with MDD at baseline and healthy controls

	People with MDD (n=37)	Healthy controls (n=30)	Group difference (p value)
	<i>Mean ± SD or median ± interquartile range</i>	<i>Mean ± SD or median ± interquartile range</i>	
Plasma IL-6 (pg/ml) (n=67)	0.83 ± 0.82	0.63 ± 0.40	0.604
Plasma TNF-α (pg/ml) (n=67)	*0.96 ± 0.45	*0.72 ± 0.22	<0.001
IL-6 concentration with LPS only (pg/ml) (n=60)	151.15 ± 103.28	150.87 ± 80.54	0.527
% LPS induced IL-6 levels with DEX 10 ⁻⁶ (pg/ml) (n=55)	19.74 ± 26.73	6.02 ± 2.87	0.011
% LPS induced IL-6 levels with DEX 10 ⁻⁷ (pg/ml) (n=58)	27.61 ± 34.58	9.05 ± 6.43	0.004
% LPS induced IL-6 levels with DEX 10 ⁻⁸ (pg/ml) (n=60)	79.85 ± 80.28	47.15 ± 17.26	0.220
% LPS induced IL-6 levels with DEX 3 x 10 ⁻⁹ (pg/ml) (n=59)	123.56 ± 84.58	99.09 ± 36.12	0.776
% LPS induced IL-6 levels with PRED 10 ⁻⁶ (pg/ml) (n=57)	46.90 ± 68.22	12.43 ± 4.56	0.001
% LPS induced IL-6 levels with PRED 10 ⁻⁷ (pg/ml) (n=60)	83.30 ± 87.69	49.05 ± 16.15	0.634

Abbreviations: MDD = major depressive disorder; DS = standard deviation; IL = interleukin; pc = picogram; ml = millilitre; TNF = tumour necrosis factor; LPS – lipopolysaccharide; DEX = dexamethasone; PRED = prednisolone. Mean and median scores represent raw values. *median/interquartile range.

Table 3.3. continued. Mean inflammatory and corticosteroid receptor sensitivity parameter values and *p* values from analyses comparing differences between people with MDD at baseline and healthy controls

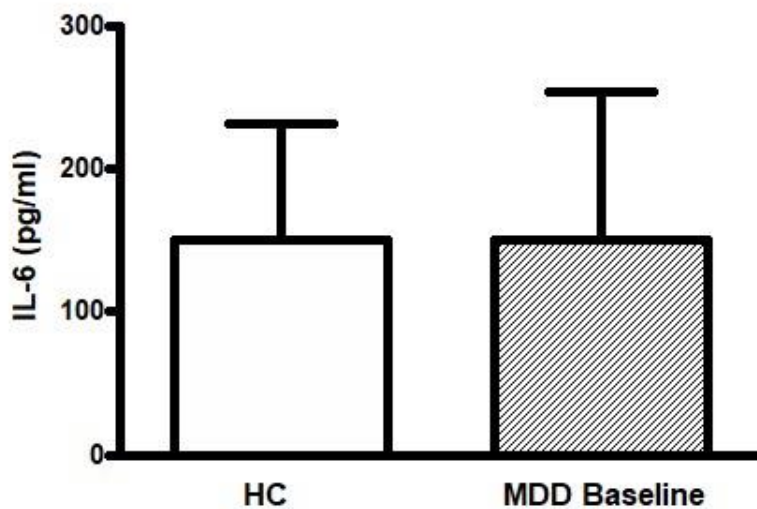
	People with MDD (n=37)	Healthy controls (n=30)	Group difference p-value
	<i>Mean ± SD</i>	<i>Mean ± SD</i>	
% LPS induced IL-6 levels with PRED 10 ⁻⁸ (pg/ml) (n=60)	148.70 ± 95.58	97.25 ± 28.12	0.494
% LPS induced IL-6 levels with PRED 3 x 10 ⁻⁹ (pg/ml) (n=53)	164.13 ± 118.01	109.61 ± 46.37	0.750
DEX IC ₅₀ (n=48)	9.50E-8 ± 1.67E-7	1.91E-8 ± 2.20E-8	0.034
PRED IC ₅₀ (n=29)	1.68E-7 ± 2.63E-7	1.29E-7 ± 1.06E-7	0.597

Abbreviations: MDD = major depressive disorder; SD = standard deviation; IL = interleukin; pc = picogram; ml = millilitre; DEX = dexamethasone; PRED = prednisolone. Mean and median scores represent raw values.

3.5.3 Corticosteroid receptor sensitivity

Firstly, LPS induced IL-6 levels were measured and compared between the groups, in the absence of corticosteroids. Stimulation with LPS increased IL-6 levels in MDD patients and in healthy controls to similar levels, ($t(58) = 0.637, p = 0.527$) (Table 3.3, Figure 3.4.).

Figure 3.4. LPS induced IL-6 in healthy controls and people with MDD at baseline.



Data shown are raw values presented as mean \pm S.E.M. in pg per ml. in Healthy controls ($n=23$), people with MDD ($n=37$).

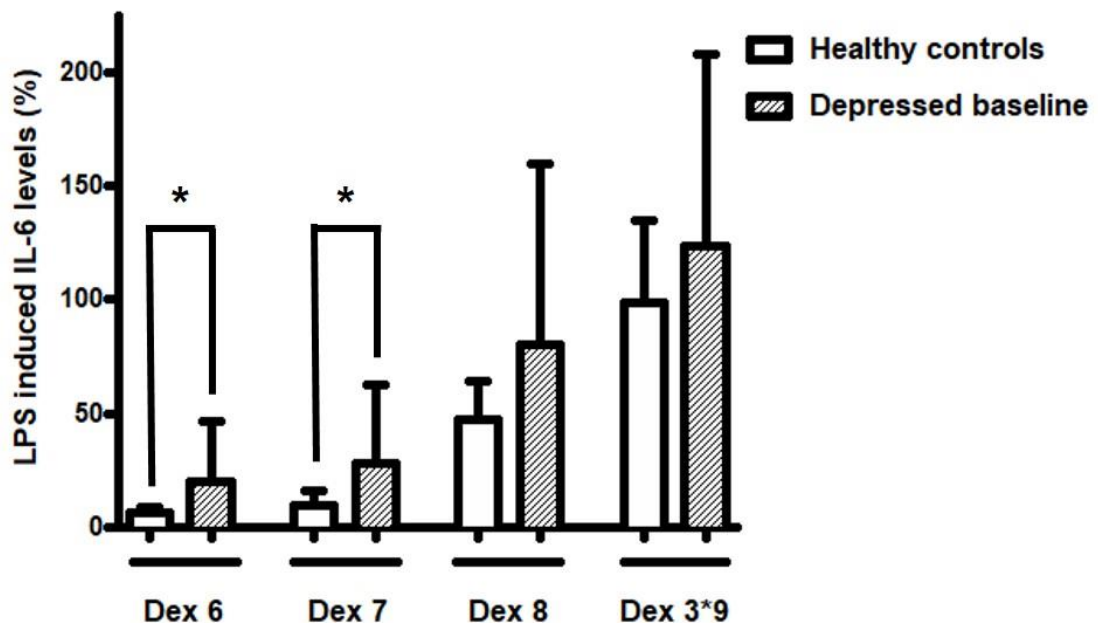
Next, corticosteroid sensitivity in people with MDD and healthy controls was investigated. Data are presented as glucocorticoid inhibition of LPS-stimulated IL-6 levels as a percentage. Both DEX and PRED induced a concentration-dependent inhibition of LPS-stimulated IL-6 levels in both groups.

There was a difference in the effects of in vitro incubation with DEX between the groups. In people with MDD, IL-6 levels were higher after incubation with DEX compared with controls at the highest concentration, 10^{-6} ($F(1, 43) = 7.07, p=0.011$). Including smoking status and alcohol consumption in the analysis showed that the overall model was not statistically significant ($F(3, 51) = 2.188, p = 0.101$). Study group was significantly

associated with IL-6 levels ($\beta = 0.362$, $t(54) = 2.486$, $p = 0.016$). However smoking status and alcohol consumption were not significantly associated with IL-6 levels ($\beta = -0.063$, $t(54) = -0.443$, $p = 0.659$; and $\beta = 0.151$, $t(54) = 1.971$, $p = 0.276$, respectively).

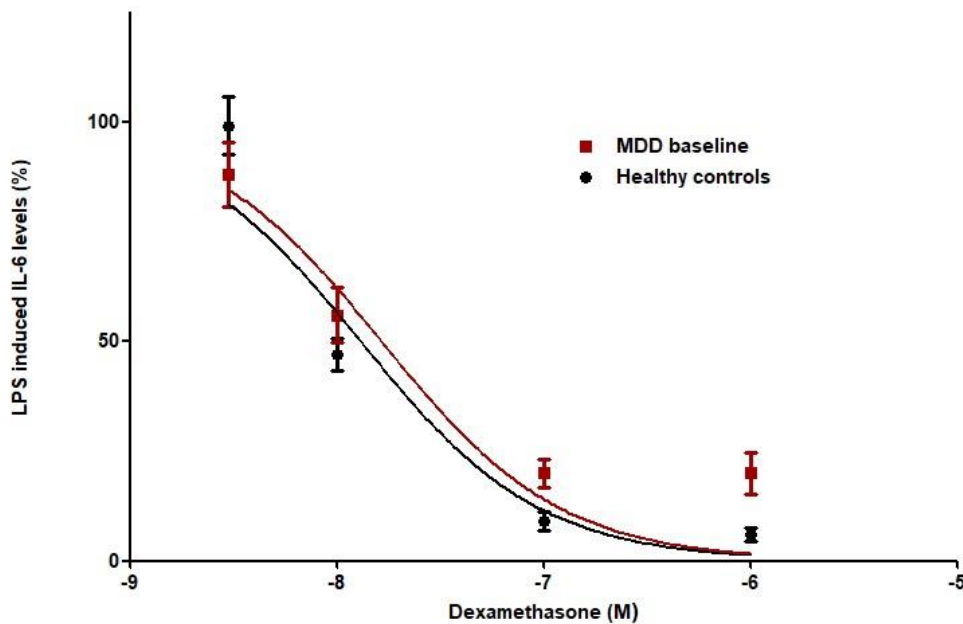
LPS-stimulated IL-6 levels were also higher in people with MDD after incubation with DEX at the second highest concentration, 10^{-7} ($F(1, 54.52) = 8.82$, $p = 0.004$). Including smoking status and alcohol consumption in the analysis showed that the overall model was statistically significant ($F(3, 54) = 3.598$, $p = 0.019$). Study group was significantly associated with IL-6 levels ($\beta = 0.410$, $t(57) = 2.986$, $p = 0.004$). However smoking status and alcohol consumption were not significantly associated with IL-6 levels ($\beta = -0.033$, $t(57) = -0.249$, $p = 0.805$; and $\beta = 0.239$, $t(57) = 1.850$, $p = 0.070$, respectively). However this difference was not present at the two lowest concentrations 10^{-8} , and 3×10^{-9} ($p > 0.05$). (Table 3.3, Figure 3.5). The dose-response curve is shown in Figure 3.6.

Figure 3.5. Dexamethasone suppression of LPS induced IL-6 in healthy controls and people with MDD at baseline.



Data shown are raw values presented as mean \pm SD in pg per ml. * Statistically significant at $P < 0.05$. Healthy controls ($n = 23$), people with MDD ($n = 37$).

Figure 3.6. Dexamethasone inhibition of LPS stimulated IL-6 levels in healthy controls and people with MDD at baseline.

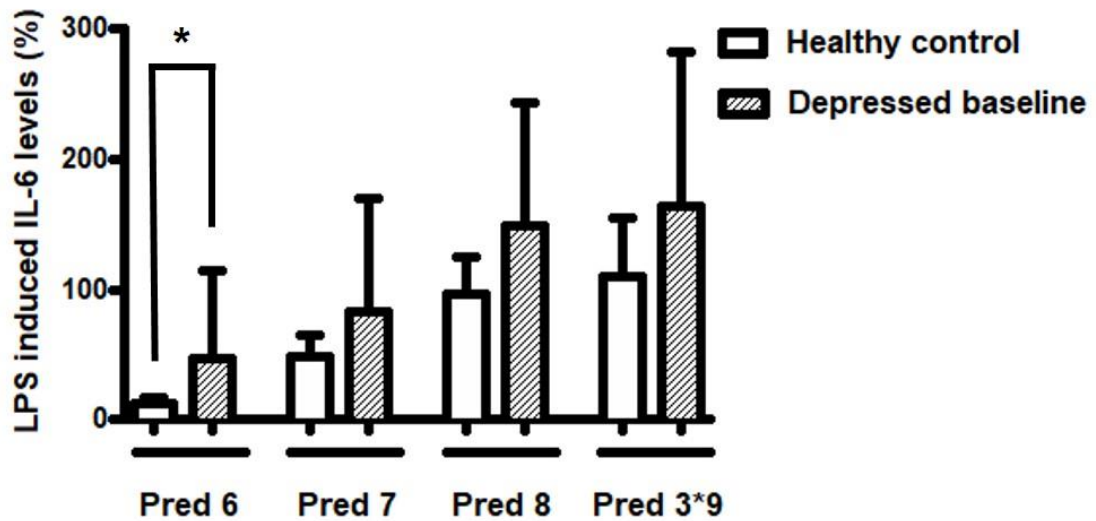


Data are shown as mean \pm S.E.M of the % DEX inhibition. Healthy controls ($n = 23$), people with MDD ($n = 37$).

There was also a significant difference in DEX IC_{50} values between the groups ($t(24.9) = -2.248, p = 0.034$). Compared to healthy controls, people with MDD required a significantly larger concentration of DEX to inhibit LPS-induced IL-6 by 50% (Table 3.3). These findings suggests that GR sensitivity was reduced in people with MDD compared to controls.

There was a difference in the effects of in vitro incubation with PRED between the groups (Figure 3.7). In people with MDD, LPS-stimulated IL-6 levels were higher after incubation compared with controls at the highest concentration of PRED, 10^{-6} ($F(1, 51.68) = 12.94, p = 0.001$). However this difference was not present at the lower concentrations ($p > 0.05$). A slope for baseline PRED IC_{50} values was unable to converge, therefore a dose response curve for PRED has not been presented here.

Figure 3.7. Prednisolone suppression of LPS induced IL-6 in healthy controls and people with MDD at baseline.



Data shown are mean \pm SD in pg per ml. * Statistically significant at $P < 0.05$. Healthy controls ($n=23$), people with MDD ($n=37$).

Including smoking status and alcohol consumption in the analysis showed that the overall model was statistically significant ($F(3, 44) = 4.171, p = 0.011$) and that the independent variables explained 22.1% of the variance in DEX IC_{50} values. Study group was significantly associated with DEX IC_{50} values ($\beta = 0.458, t(47) = 3.115, p = 0.003$), however smoking status and alcohol consumption were not significantly associated with DEX IC_{50} ($\beta = -0.218, t(47) = -1.480, p = 0.146$; and $\beta = 0.275, t(47) = 1.971, p = 0.055$, respectively). In contrast, there was no significant difference in prednisolone IC_{50} values between the groups ($t(20.6) = -0.537, p = 0.597$) (Table 3.3). Overall these findings suggest that there was less functional ability of the MR to respond to corticosteroids in depressed people.

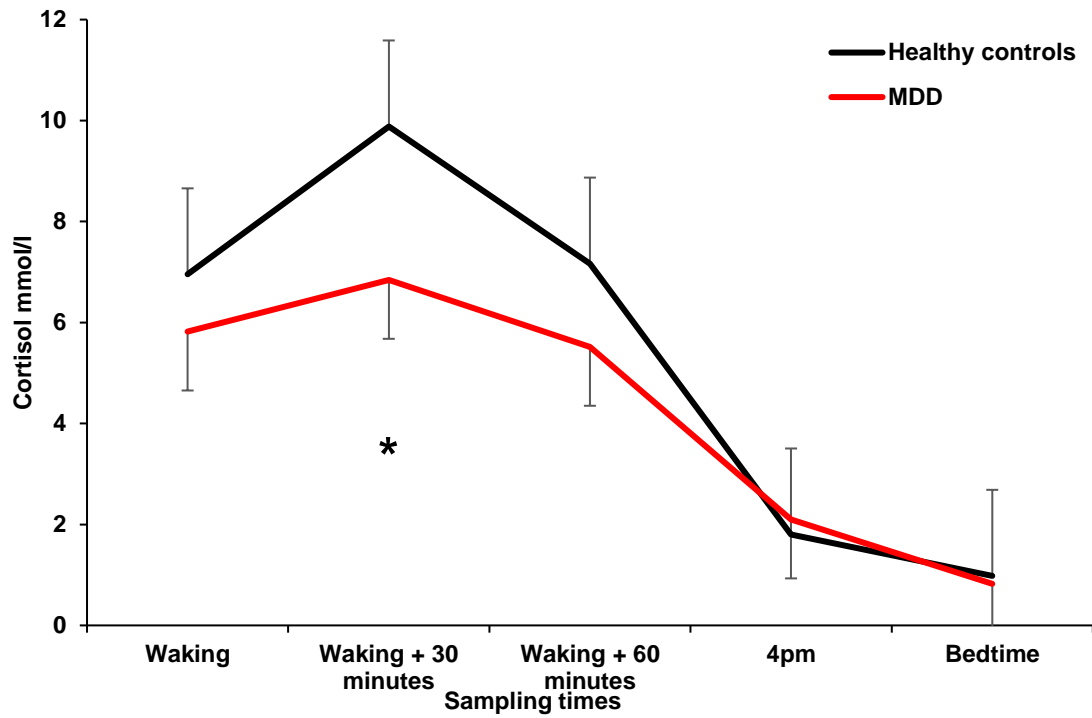
3.5.4 Diurnal cortisol

The analyses of diurnal cortisol in relation to study groups are summarised in Table 3.4. A graphical representation of mean cortisol values across the day in both the MDD and controls groups is provided in Figure 3.8. There was a significant difference in waking cortisol + 30 minutes between people with MDD and healthy controls ($t(57) = 2.419, p = 0.019$). Waking cortisol + 30 minutes was lower ($M = 6.84, SD = 4.19$) compared to controls ($M = 9.88, SD = 5.43$). After adjustment for smoking and alcohol the overall model was not statistically significant ($F(3, 55) = 2.323, p = 0.085$). Study group was no longer significantly associated with waking cortisol + 30 ($\beta = -0.259, t(58) = -1.873, p = 0.066$). Smoking status and alcohol consumption were also not significantly associated with waking cortisol + 30 ($\beta = -0.090, t(58) = -0.146, p = 0.279$; and $\beta = 0.002, t(58) = 0.015, p = 0.988$, respectively).

There was also a significant difference between the groups regarding the CAR AUC ($t(56) = 2.670, p = 0.010$). The CAR AUC was smaller in the MDD groups ($M = 6.57, SD = 3.36$) compared to controls ($M = 9.15, SD = 4.00$). After adjustment for smoking and alcohol the overall model was not statistically significant ($F(3, 54) = 2.473, p = 0.071$). Study group was significantly associated with CAR AUC ($\beta = -0.301, t(57) = -2.174, p = 0.034$), with smaller values in the participants with MDD. However smoking status and alcohol consumption were not significantly associated with CAR AUC ($\beta = -0.090, t(57) = -0.672, p = 0.504$; and $\beta = 0.028, t(57) = 0.214, p = 0.832$, respectively).

There were no significant differences between the groups in terms of waking cortisol ($t(57) = 1.200, p = 0.235$), waking + 60 minutes ($t(56) = 1.658, p = 0.103$), cortisol at 4pm ($t(54) = -1.439, p = 0.156$), bedtime cortisol ($t(56) = 0.895, p = 0.375$), CAR ($t(37) = 1.628, p = 0.112$), AUC ($t(53) = 0.953, p = 0.345$) or cortisol slope ($t(56) = 1.152, p = 0.254$).

Figure 3.8. MDD versus healthy controls: Mean salivary cortisol values across the day averaged across all participants.



Saliva samples were taken on waking (n=59), waking+30mins (n=59), waking+60mins (n=58), 4pm (n=56) and at bedtime (n=58) in people with MDD (red line) and healthy controls (black line). Error bars represent SEM. Data shown are raw values.* Statistically significant at $P < 0.05$.

Table 3.4. Mean cortisol parameter values and p values from analyses comparing differences between people with MDD at baseline and healthy controls.

	People with MDD (n=37)	Healthy controls (n=30)	Group difference (p value)
<i>Diurnal cortisol parameters</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	
Waking cortisol (nmol/L) (n=59)	5.82 ± 3.24	6.95 ± 4.01	0.235
Waking cortisol +30 minutes (nmol/L) (n=59)	6.84 ± 4.19	9.88 ± 5.43	0.019
Waking cortisol +60 minutes (nmol/L) (n=58)	5.52 ± 3.79	7.17 ± 3.93	0.103
4pm cortisol (nmol/L) (n=56)	2.10 ± 1.52	1.80 ± 1.88	0.156
Bedtime cortisol (nmol/L) (n=58)	0.82 ± 1.61	0.98 ± 1.42	0.375
CAR (nmol/L) (n=39)	0.98 ± 4.53	3.28 ± 4.31	0.112
CAR AUC (nmol/L) (n=58)	6.57 ± 3.37	9.15 ± 4.00	0.010
AUC (nmol/L) (n=55)	45.71 ± 23.50	51.80 ± 23.69	0.345
Cortisol slope (nmol/L) (n=58)	0.01 ± 0.00	0.01 ± 0.00	0.254

Abbreviations: MDD = major depressive disorder; SSD = standard deviation; nmol = nanomoles; L = litre; CAR = cortisol awakening response; AUC = area under the curve. Mean and median scores represent raw values.

3.5.5 *Inter-correlations between the systems*

To explore the relationship between HPA-axis function and inflammatory activation, differences in correlations between variables were examined. All biological correlations are presented in Table 3.5. There was a significant negative correlation between IL-6 and the CAR AUC in healthy controls ($r = -0.532$, $p = 0.004$). A similar pattern of association was observed in the MDD group, however this failed to reach Bonferroni significance ($r = -0.423$, $p = 0.018$). There were negative patterns of association between IL-6 and the AUC in both groups (HC: ($r = -0.474$, $p = 0.017$), MDD: ($r = -0.451$, $p = 0.012$)), which again failed to reach adjusted significance. There also appeared to be a negative pattern of association between TNF- α and the CAR AUC and the AUC in the MDD group, but not in the control group (MDD: CAR AUC ($r = -0.357$, $p = 0.049$), AUC ($r = -0.378$, $p = 0.039$), HC: CAR AUC ($r = -0.097$, $p = 0.63$), AUC ($r = -0.165$, $p = 0.43$)). However this was not significant following Bonferroni correction.

To explore the relationships between biological factors and perceived stress, differences in correlations between the groups were examined. Correlations were only conducted on biological variables which were shown to be significantly different between groups. The results are presented in Table 3.6. No significant correlations were observed.

Table 3.5. Correlations between inflammatory biomarkers and HPA-axis parameters in people with MDD and healthy controls

	IL-6	TNF- α
MDD		
DEX IC ⁵⁰	-0.088	0.313
PRED IC ⁵⁰	0.092	0.156
CAR	-0.066	-0.018
CAR AUC	-0.423*	-0.357*
AUC	-0.451*	-0.378*
Cortisol slope	-0.348	-0.190
Healthy Controls		
DEX IC ⁵⁰	-0.154	0.039
PRED IC ⁵⁰	0.120	0.231
CAR	-0.202	0.011
CAR AUC	-0.532#	-0.097
AUC	-0.474*	-0.165
Cortisol slope	-0.314	-0.016

Abbreviations: MDD = major depressive disorder; DEX = dexamethasone; PRED = prednisolone; IL = interleukin, TNF- α = tumour necrosis factor alpha, CAR = cortisol awakening response; AUC = area under the curve. **Note:** * $p < 0.05$; ** $p < 0.01$; # $p < 0.008$ (critical p-value after Bonferroni correction).

Table 3.6. Correlations between biological and perceived stress in people with MDD and healthy controls

	Perceived stress score	
	Correlation coefficient	<i>p</i> -value
MDD		
TNF- α	-0.261	0.119
% LPS induced IL-6 levels with DEX 10 ⁻⁶	0.244	0.171
% LPS induced IL-6 levels with DEX 10 ⁻⁷	0.186	0.284
DEX IC ₅₀	-0.051	0.810
% LPS induced IL-6 levels with PRED 10 ⁻⁶	0.209	0.236
Waking cortisol +30 minutes (nmol/L)	0.030	0.871
CAR AUC	0.019	0.921
Healthy Controls		
TNF- α	0.217	0.249
% LPS induced IL-6 levels with DEX 10 ⁻⁶	0.001	0.996
% LPS induced IL-6 levels with DEX 10 ⁻⁷	-0.067	0.761
DEX IC ₅₀	-0.017	0.939
% LPS induced IL-6 levels with PRED 10 ⁻⁶	-0.204	0.352
Waking cortisol +30 minutes (nmol/L)	-0.308	0.110
CAR AUC	-0.342	0.081

Abbreviations: MDD = major depressive disorder; DEX = dexamethasone; PRED = prednisolone; IL = interleukin, TNF- α = tumour necrosis factor alpha, CAR = cortisol awakening response; AUC = area under the curve; BDI = Beck Depression Inventory; PSS = Perceived Stress Scale; LTE = List of Threatening Experiences. **Note:** $p < 0.007$ = critical p -value after Bonferroni correction.

3.5.6 Sensitivity analyses

Depressive symptom severity

Given that depressive symptom severity has been associated with increased inflammation and HPA-axis dysfunction, I ran exploratory analyses in order to identify whether any differences in the findings emerged when the analysis was restricted to people with moderate/severe depressive symptoms at baseline. The main analyses were repeated following the removal of those participants who scored <20 on the BDI, indicating a score of mild depression. This decision was based on the guidelines from Beck et al. which suggest that a score of 20 denotes the cut-off for moderate depression (1996a). The BDI was chosen instead of the CISR as it is a more conservative and more widely used scale, and has been endorsed by NICE for use in measuring baseline depression severity (Smarr & Keefer, 2011). According to the BDI, 11 people had mild baseline depressive symptoms (score <20) and were removed from the analysis, resulting in a reduced MDD sample of 26.

The results confirmed the findings from the main analysis for both inflammatory biomarkers. There was no significant difference between the groups for IL-6 ($p = >0.05$) and TNF- α was significantly higher in the MDD group compared with controls ($p = 0.002$). Regarding corticosteroid receptor function, the results for the effects of in vitro incubation with both DEX and PRED remained unchanged. In the MDD group, LPS-stimulated IL-6 levels were significantly higher after incubation with DEX at 10^{-6} ($p = 0.019$) and 10^{-7} ($p = 0.014$) and with PRED at 10^{-6} ($p = 0.011$), compared with controls. However the difference in DEX IC₅₀ values between the groups lost significance ($p = 0.151$), likely reflecting a loss of power.

The result for waking cortisol +30 minutes became borderline significant, with lower levels in the MDD group ($p = 0.058$). However a significant difference emerged for waking

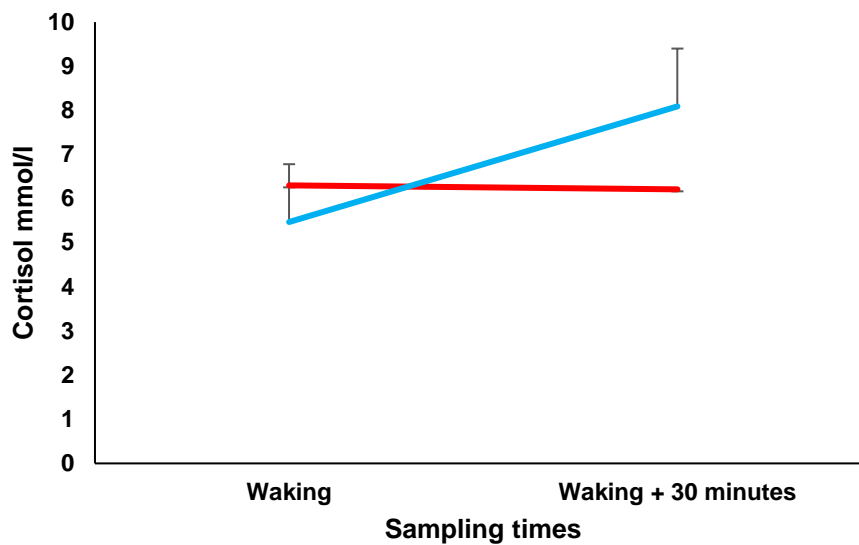
cortisol +60 minutes ($t(46) = 2.183, p = 0.034$), with cortisol levels being lower in the MDD group ($M = 4.66, SD = 3.04$) compared with controls ($M = 7.17, SD = 3.93$). The difference in the CAR AUC remained significant, with lower levels in the MDD group ($p = 0.021$). In addition, the difference in the AUC became borderline significant ($t(44) = 1.982, p = 0.054$). The AUC was lower in the MDD group ($M = 39.17, SD = 18.58$) compared with controls ($M = 51.80, SD = 23.69$). There were no significant differences in the CAR or cortisol slope using the restricted analysis ($p = >0.05$).

Childhood sexual abuse

As previously shown, the MDD and control group differed significantly in terms of experiencing childhood sexual abuse, with the MDD group experiencing significantly higher levels than controls. Therefore, I also ran an exploratory analysis to determine whether there were any biological differences between depressed people with and without a history of abuse. The main analyses were repeated including two groups; depressed individuals with a history of childhood sexual abuse and depressed individuals without a history of childhood sexual abuse. Due to the fact that there was a borderline significant difference in terms of total adversity score, I also conducted a sensitivity analysis for this composite variable. I observed a similar pattern of results for both analyses, therefore only the sexual abuse findings are presented.

The results showed that there was no significant difference in inflammatory activation or in corticosteroid sensitivity parameters between the groups (all $p = >0.05$). Regarding diurnal cortisol, there was a difference between the groups regarding the CAR ($t(17) = -2.354, p = 0.031$). Depressed individuals with no history of childhood sexual abuse had a significantly flatter CAR than those with a history of abuse (MDD with no history of abuse: $M = -1.33, SD = 4.6$; MDD with a history of abuse: $M = 3.05, SD = 3.48$), (see Figure 3.9). There were no other significant differences in diurnal cortisol parameters (all $p = >0.05$).

Figure 3.9. Depressed individuals with and without a history of childhood sexual abuse: difference in the CAR between groups.



Blue line = depressed with history of abuse; red line = depressed with no history of abuse. Error bars represent SEM.

3.6 Discussion

3.6.1 Aims and hypotheses

The aim of this study was to compare differences in inflammatory cytokines, corticosteroid receptor sensitivity and diurnal cortisol rhythm in depressed people compared with healthy controls. I also sought to explore whether there are any associations between a) any of these biological parameters and b) between biological and psychological parameters. I hypothesised that people with MDD would have significantly increased levels of inflammation compared to controls, specifically increase in plasma IL-6 and TNF- α (hypothesis 1). I hypothesised that people with MDD would exhibit significantly decreased GR sensitivity compared with controls (hypothesis 2) and significantly different MR sensitivity (hypothesis 3). I hypothesised that people with MDD will exhibit significantly altered diurnal cortisol secretion compared to controls,

specifically increased CAR, increased AUC and flatter cortisol slopes (hypothesis 4). We hypothesised that in people with MDD, inflammation will be significantly correlated with HPA-axis function, specifically a negative correlation between inflammation and GR sensitivity and a positive correlation between IL-6 and the AUC (hypothesis 5). I also conducted an exploratory analysis to investigate possible associations between any altered biological pathways and psychosocial stress.

3.6.2 *Summary of results*

The results showed that hypothesis 1 was partially confirmed: Inflammatory activation was increased in depressed people compared to controls, as indicated by significantly higher TNF- α levels in the MDD group than in the control group. In contrast, there were no significant differences in IL-6. Hypothesis 2 was confirmed: I observed decreased GR sensitivity in the MDD group compared with healthy controls. This was indicated by the fact that depressed people required a significantly larger concentration of DEX to inhibit LPS-induced IL-6 at the two highest concentrations. Furthermore DEX IC₅₀ values were higher in the MDD group. Hypothesis 3 was confirmed: I observed decreased MR sensitivity in the MDD group, indicated by the fact that depressed people required a significantly larger concentration of PRED to inhibit LPS-induced IL-6 at the highest concentration. Hypothesis 4 was not confirmed: contrary to my prediction, the MDD group exhibited a smaller CAR AUC compared with controls. There was no significant difference in the AUC or slope between the groups. Hypothesis 5 was not confirmed: a significant relationship between IL-6/TNF- α and the CAR AUC/AUC was observed in the MDD group, however counter to expectations, these associations were negative. Neither inflammatory biomarker was associated with GR sensitivity. No associations between biological variables and perceived stress were observed. I will discuss possible explanations for these findings later in the Discussion section.

Exploratory analyses revealed that restricting the depressed sample to only those with moderate/severe symptoms had no effect on the findings regarding inflammation. Whilst LPS-induced IL-6 levels were higher than controls after incubation with DEX at the two highest concentrations in the restricted sample, the IC⁵⁰ findings lost significance. MR sensitivity results remained unchanged. There was no effect on the findings for CAR AUC. Additional exploratory analyses to examine the influence of childhood sexual abuse, showed that depressed individuals with no history of childhood sexual abuse had a significantly flatter CAR than those with a history of abuse sexual abuse. There was no significant differences in inflammatory activation or in corticosteroid sensitivity parameters between depressed people with or without a history of abuse. I will discuss possible explanations for this pattern of results later in the Discussion section.

3.6.3 *Hypothesis 1: Differences in inflammatory biomarkers*

In the current study TNF- α levels were significantly higher in people with depression but there was no difference in levels of IL-6. These findings are in contrast to the literature which robustly demonstrates increases in both TNF- α and IL-6 in depressed people (Dowlati et al., 2010;Goldsmith et al., 2016b;Kohler et al., 2017;Y. Liu et al., 2012). In fact increased IL-6 levels is considered among the most reliable of findings in this field, and is supported by data from cross-sectional meta-analyses (Haapakoski et al., 2015;Hiles et al., 2012b;Howren et al., 2009), predictive associations of IL-6 with the future development of depression (Chu et al., 2018;Gimeno et al., 2009;Glaus et al., 2014;Khandaker et al., 2014;Kivimaki et al., 2014;Valkanova et al., 2013;Virtanen et al., 2015), evidence of a dose–response relationship (Kivimaki et al., 2014) and associations with therapeutic response to antidepressants (Hiles et al., 2012a;Kohler et al., 2018;Wiedlocha et al., 2018). To my knowledge only one other study has reported similar findings. Zou (2018) compared 117 MDD patients with 102 healthy controls and reported that compared to healthy controls, the patients with MDD had significantly higher levels TNF- α , however there was no significant differences in the levels of IL-6.

There are number of possible explanations for why we observed different results. Firstly, several of the studies mentioned above did not restrict their sample to individuals who met criteria for MDD as defined by the DSM-V or ICD-10, but relied on the presence of depressive symptoms using brief questionnaires (e.g., General Health Questionnaire (GHQ); Hamilton Rating Scale for Depression (HAM-D); Beck Depression Inventory (BDI)) (Gimeno et al., 2009;Goldsmith et al., 2016b;Hiles et al., 2012a, 2012b;Howren et al., 2009;Kivimaki et al., 2014;Valkanova et al., 2013;Virtanen et al., 2015). As discussed in Chapter 1, Section 1.2, these instruments do not include any essential core symptoms or require symptoms to disrupt normal daily functioning, and do not provide a diagnosis of a clinical disorder (Fried & Nesse, 2015b). In addition, questionnaires such as the GHQ focus on cognitive symptoms, ignoring the somatic aspects of depression. This is of particular relevance as somatic symptoms such as insomnia, increased appetite, and weight gain appear to be differentially related to inflammation, suggesting that depressive symptoms have different biological correlates (Duivis et al., 2013;F. Lamers et al., 2013). It is therefore possible that the findings from these studies reflect a broader sample than in this study which only included people who met ICD-10 criteria for MDD and therefore reflected a more homogenous phenotype. Whilst meta-analyses indicate an overall increase in inflammation in MDD, they also demonstrate a considerable amount of heterogeneity. Methodological and sampling differences further exacerbate this variability.

In addition, many of the studies above did not exclude or account for physical (Chu et al., 2018;Glaus et al., 2014;Goldsmith et al., 2016a;Hiles et al., 2012b;Khandaker et al., 2014) or psychiatric co-morbidities (Chu et al., 2018;Dowlati et al., 2010;Goldsmith et al., 2016a;Haapakoski et al., 2015;Hiles et al., 2012a, 2012b;Howren et al., 2009;Kivimaki et al., 2014;Valkanova et al., 2013;Virtanen et al., 2015). The results from these studies may well be confounded by inflammation which has an alternative aetiology. Interestingly, the only study which reported similar findings to the present study, used both a clinical diagnosis of MDD based on DSM-V criteria and a depressive symptom

questionnaire (Zou et al., 2018). This was also the only study to incorporate comprehensive exclusions similar to the current study, including people with other psychiatric comorbidities, physical health conditions such as autoimmune diseases, cancer, systemic diseases, chronic infection and those taking immunomodulatory treatment or antibiotic therapy. In light of these observations, it may be that our findings reflect a more pure MDD sample than is often included in depression research. It is crucial that samples constituting more homogeneous phenotypes are taken into account in future studies.

Another possible explanation may be related to depressive subtypes. Whilst the association between depression and inflammation may seem conclusive, it may not apply to all cases. MDD is a psychiatrically heterogeneous disorder and it is reasonable to assume that this heterogeneity may also be reflected in levels of inflammation. In recent years, evidence has emerged for differential associations between depression subtypes and inflammation (B. W. J. H. Penninx et al., 2013). Melancholic and non-melancholic patients have been shown to exhibit differential inflammatory profiles and there is some evidence that cytokine levels normalize with clinical improvement only in melancholic depression (Rothermundt et al., 2001). Data from 776 people from the Netherlands Study of Depression and Anxiety demonstrated that people with atypical depression had significantly higher levels of inflammatory markers compared with people with melancholic depression (F. Lamers et al., 2013). Another study also reported that IL-6 levels were higher in atypical cases versus melancholic cases of depression (Rudolf et al., 2014). However it should be noted that some studies have reported no difference (Dunjic-Kostic et al., 2013; Karlović et al., 2012). A more recent systematic review reported that IL-6 is increased in melancholic vs. non-melancholic MDD but that there are no differences in TNF- α between subgroups, suggesting that only IL-6 has the potential to discriminate between the MDD subtypes (C. Yang et al., 2018). It therefore remains to be shown whether inflammation is of pathogenic significance for all types of depression. In the present study, subtypes such as melancholic and atypical depression

were not identifiable using our chosen measures, therefore it is unclear which subgroups our sample captured. This may explain why this study was unable to provide support for the hypothesis that MDD is associated with comprehensive inflammatory activation.

Another possible explanation may be related to the effect of antidepressants in our study. There is some evidence that cytokine plasma concentrations change during treatment with antidepressants, although the results are conflicting. The most consistent finding is a reduction in IL-6 levels following treatment with several different antidepressants (Hiles et al., 2012a; Kohler et al., 2018; Wiedlocha et al., 2018). Furthermore, a meta-analysis investigating inflammation in MDD and clinical response showed that IL-6 levels decreased with antidepressant treatment, regardless of symptom remission (Strawbridge et al., 2015). Due to the fact that one third of our depressed participants were taking various antidepressants, analysis comparing differences in IL-6 between depressed people taking antidepressants, depressed people not taking antidepressants and healthy controls were conducted. There were no significant differences between the groups ($p = >0.05$), suggesting that there was no effect of antidepressant use.

It is possible that the elevated TNF- α observed in the present study could be explained by TRD. Persistently elevated TNF- α has been associated with treatment resistance, as shown in a study by Strawbridge et al. (2015) where significant decreases in TNF- α were observed only in treatment responders. Further support for the role of TNF- α in TRD comes from studies investigating the TNF α antagonist, Infliximab, which has been shown to reduce depressive symptoms in patients with TRD, particularly in those people with high inflammatory profiles (Raison et al., 2013a). This may explain why another meta-analysis, which did not include treatment response in their analysis, reported that antidepressant treatment did not reduce TNF- α levels (Hannestad et al., 2011). Given that in our sample two thirds of the people in the depressed group had experienced depression previously, analyses comparing differences in TNF- α between those who had experienced previous depression and those who had not, were conducted. The results

of this analysis showed that depressed individuals with a history of depression had significantly higher levels of TNF- α compared with controls ($p = 0.002$), however whilst depressed individuals without a history of depression also had higher levels of TNF- α , this did not reach significance ($p = 0.060$). This should be interpreted with caution as the depressed group with no history was very small ($n=12$) and the result was borderline significant, suggesting a similar trend.

3.6.4 Hypothesis 2: Differences in glucocorticoid sensitivity

In line with my hypothesis, GR sensitivity was reduced in depressed people compared with healthy controls. To reiterate, in the present study, I used an *in vitro* glucocorticoid sensitivity assay which measured glucocorticoid inhibition of LPS-stimulated IL-6 levels in peripheral, whole blood. This technique has not been used previously to compare basal GR sensitivity in MDD patients and healthy controls. Reduced sensitivity was demonstrated by increased LPS-stimulated IL-6 levels in the presence of the two highest concentrations of DEX and by increased IC⁵⁰ values. The finding reported here supports the literature which has consistently reported significant decreased sensitivity in depressed patients. *In vitro* studies on immune cells from peripheral blood have shown that the ability of glucocorticoids to inhibit mitogen-induced lymphocyte proliferation is impaired in depressed patients (Lowy et al., 1984;Lowy et al., 1988;Rupprecht et al., 1991c;Wodarz et al., 1991;Wodarz et al., 1992). *In vivo* studies using the DST have produced similar results. People with depression consistently demonstrate increased cortisol following oral DEX administration (Holsboer, 2000;Leistner & Menke, 2018;Pariante & Miller, 2001). DEX administration also significantly decreases mitogen-induced lymphocyte proliferation in DST suppressors, compared to non-suppressors (Gormley et al., 1985;Lowy et al., 1988;A. Wassef et al., 1990).

3.6.5 Hypothesis 3: Differences in mineralocorticoid sensitivity

In line with my prediction, depressed people demonstrated impaired MR function compared with controls, demonstrated by decreased inhibition of LPS-stimulated IL-6 in the presence of the highest concentration of PRED. This is also in line with a study by Juruena et al. (2013) who also reported that MR blockade increased cortisol and decreased the suppressive effect of prednisolone in controls but not TRD patients. Findings from a study by Hinkelmann et al. (2016) reported that depressed patients exhibited higher baseline cortisol values compared to healthy individuals, however they observed no difference in cortisol between groups following MR antagonism. The authors suggest that these results indicate impaired MR function based on the following reasoning: if the GR was impaired in MDD, MR blockade would result in significantly increased cortisol as the HPA-axis would be entirely disinhibited. If however, the MR was impaired, the GR would be able to inhibit the HPA-axis once cortisol levels had risen. Therefore the lack of cortisol increase following MR blockade suggests intact GR function, which means that the difference in basal cortisol must be a reflection of impaired MR function. In the present study we also observed impaired DEX suppression, therefore we can only conclude that both the GR and MR are dysregulated in people with MDD.

MR dysfunction has also been observed in people with psychotic MDD (Lembke et al., 2013). Cortisol levels were significantly increased in people with psychotic MDD compared to controls following treatment with MR agonist. Psychosis was an exclusion criteria in the present study, so we can only infer that MR dysfunction was observed in non-psychotic depression. These findings are in contrast with the findings of Young et al. (2003) and Juruena et al. (2006) who both reported that the MR is functional in depressed patients. Two subsequent studies by Juruena et al. (2010;2009) also reported that MR function was similar in TRD patients and controls, however they did observe impaired MR sensitivity in those patients who failed to respond to intensive in-patient therapy compared with those who did. Furthermore, this response did not change

between admission and follow-up, suggesting that the MR may not be functional in severely treatment-resistant patients. In the current study we did not recruit any severely TRD in-patients and so it is unlikely that our lack of findings reflect severe TRD. However to explore this further secondary analysis was conducted with a subset of depressed individuals who had mild baseline depressive symptoms in order to explore whether the effect remained (score >20) (n=11). Results showed that there was no change in the findings, with decreased MR sensitivity still present at the highest concentration of PRED ($p = 0.014$). Therefore this study does not support the hypothesis that depression severity is the only factor for MR deficiency.

The null finding in relation to the PRED IC^{50} value may be a reflection of the fact that prednisolone is less sensitive at lower concentrations compared with dexamethasone, therefore higher concentrations may be required to reach the IC^{50} . Furthermore, due to financial constraints we were only able to use four concentrations of each corticosteroid, making it more difficult to create the dose-response curve necessary to compute the IC^{50} values.

3.6.6 Hypothesis 4: Differences in diurnal cortisol rhythm

In contrast to my hypothesis this study found that waking cortisol + 30 minutes and the CAR AUC was reduced in depressed people, compared with controls. The findings in the current study are in line with a study by Dedovic et al. (2010) who observed a blunted CAR in people with sub-clinical depression. The findings also support previous studies which found a blunted CAR in patients with MDD (Doolin et al., 2017; Huber et al., 2006; Stetler & Miller, 2005) as well as those which have reported an association between psychological traits known to increase vulnerability to depression and attenuation of the CAR, such as neuroticism, loneliness and worry (Cropley et al., 2015; Leah D Doane & Adam, 2010; Kuehner et al., 2007; Mangold et al., 2012). Furthermore, increases in CAR have been associated with clinical remission following SSRI therapy (Ruhe et al., 2015)

However there is a great deal of heterogeneity in the literature and our findings are also at odds with other studies. A large cohort study showed that both currently depressed and remitted MDD patients had an increased CAR compared with controls (Vreeburg et al., 2009a). Increased CAR has also been associated with clinical depression in women (Dienes et al., 2013) adolescent females (Ulrike et al., 2013) and older people (Rhebergen et al., 2015). Increased CAR has been reported in remitted MDD patients compared with controls (Aubry et al., 2010; Bhagwagar et al., 2003) and decreases in CAR have been shown to be predictive of antidepressant response (J. Beck et al., 2015). Longitudinal studies have also shown that increases in CAR AUC are associated with time to recurrence in remitted patients (Hardeveld et al., 2014). In healthy populations increased CAR has been associated with depressive symptoms (M. Pruessner et al., 2003b), neuroticism (Madsen et al., 2012) and high-trait negative affect (Polk et al., 2005). Furthermore longitudinal studies have shown that higher CAR in adolescence predicts future diagnosis of MDD (Adam et al., 2010; Vrshek-Schallhorn et al., 2013). The largest meta-analysis to date reported that the AUC during the waking period was positively associated with depression (Boggero et al., 2017).

The heterogeneity in the findings may reflect methodological inconsistencies. The majority of the studies mentioned above, including the present study, only collected saliva samples on a single day. It has been suggested that samples collected on a single day are more susceptible to state factors and that CAR determination over two to six days is required for reliable trait measures (J. Hellhammer et al., 2007). The conflicting findings in the literature may therefore be a reflection of situational factors, rather than a reliable measure of HPA-axis activity.

Possible mechanisms underlying the attenuated CAR AUC observed in the present study can only be speculated on. One potential explanation is that in people with chronic or recurrent depression, the regulatory mechanisms underlying the CAR become exhausted, as is seen in patients who exhibit hypocortisolaemia (Chida & Steptoe,

2009). Hypocortisolism has been reported in PTSD patients, in healthy people experiencing chronic stress and in patients with stress-related somatic disorders (Heim et al., 2000a;Wirtz et al., 2007). Hypocortisolism may be a consequence of hippocampal atrophy. Increased cortisol secretion creates a cascade of hippocampal damage (Sapolsky et al., 1986). The hippocampus is part of a mood regulatory network (Anacker et al., 2011), involved in HPA-axis negative feedback inhibition (S. M. Smith & Vale, 2006) and is involved in memory formation and emotional appraisal (Nemeroff & Vale, 2005). It is possible that in the early stages of depression, HPA-axis hyperactivity and subsequent hypercortisolaemia damages the hippocampus, resulting in decreased ability to meet these regulatory demands, resulting in HPA-axis exhaustion and hypocortisolism. This hypothesis is supported by studies showing that hippocampal volume is associated with a reduced CAR in depressed people (Dedovic et al., 2010) and healthy people (J. C. Pruessner et al., 2005;M. Pruessner et al., 2007). In the present study only 25% of the depressed group had been experiencing the current depressive episode for more than 2 years, however given that 68% of depressed people had a history of previous depressive episodes, the potential explanation of an exhausted HPA-axis is plausible.

Another possibility is that increases in the CAR may be affected by the disrupted sleep often observed in people with depression. Depression is associated with sleep disturbances such as altered sleep continuity, sleep depth and REM sleep (Baglioni et al., 2016;M. J. Murphy & Peterson, 2015). In the majority of people, cortisol is secreted in two-four distinct bursts over the waking period (Chida & Steptoe, 2009). If depressed people experience disrupted sleep or wake up during an earlier phase of the pre-awakening cortisol rise, these bursts may be secreted over a longer period of time, reducing the intensity of the CAR. It is also possible that the CAR was affected by antidepressant use. Animal studies have shown that antidepressant medication may alter HPA-axis activity (N Barden et al., 1995;Peeters et al., 1994) and cortisol is reduced in depressed people following treatment with SSRIs (Dziurkowska et al., 2004

2013;Hernandez et al., 2013). In order to explore whether sleep influenced the results for the CAR AUC in the present study, analyses were conducted to investigate whether sleep problems predicted CAR AUC values. Comparisons with healthy controls could not be conducted as CIS-R data was only collected for depressed patients. Results showed that sleep did not predict CAR AUC in people with depression ($p = 0.05$).

Childhood abuse and the CAR

There was also an effect of childhood sexual abuse on the CAR in depressed people. Depressed people who had not experienced sexual abuse had a significantly flatter CAR compared with those who had. Our findings are in line with studies which have shown that childhood trauma leads to increased HPA-axis activity (Heim et al., 2008b;Heim et al., 2000b;Kumari et al., 2013). Heim et al. (2000b) found that women with current depressive symptoms and a history of childhood abuse exhibited increased cortisol stress responses compared with non-abused depressed women. In a similar study with men, Heim et al. (2008b) showed that men with current MDD and history of childhood trauma exhibited increases in cortisol responses to Dex/CRH test compared with depressed men without a history of childhood abuse. Increased neuroendocrine function has also been observed in depressed abused children compared with depressed non-abused (Kaufman et al., 1997). Our findings also support studies examining the CAR specifically. A study by Quevedo et al. (2017) reported higher CAR in maltreated depressed adolescents compared to non-maltreated depressed adolescents. Another study by Lu et al. (2016) reported that people with a history of childhood trauma exhibited an enhanced CAR and CAR AUC, regardless of depression score. Most notably, there was no association between MDD in participants with no trauma history and with elevated cortisol levels. Childhood abuse has also been significantly correlated with increased CAR AUC, in a study of older adults, although this was only observed in non-depressed participants (Wielgaard et al., 2018).

However the literature regarding relations between HPA-axis markers and history of childhood maltreatment are conflicting and findings from the current study are at odds with other studies which indicate that hypo-activity of the HPA axis in adults is associated with a history of childhood abuse in healthy people (D. Bremner et al., 2007; J. D. Bremner et al., 2003; Carpenter et al., 2011; Carpenter et al., 2009b; Hinkelmann et al., 2013; Power et al., 2012; Voellmin et al., 2015) and people with depression (Hinkelmann et al., 2013). Morning cortisol levels have explicitly been shown to be lower in people who report severe neglect or abuse compared with non-abused participants (van der Vegt et al., 2009). Regarding the CAR specifically, a meta-analysis examining childhood maltreatment and diurnal cortisol regulation reported blunted waking cortisol levels but no association between childhood maltreatment and the CAR (Bernard et al., 2017).

Our findings are in accordance with the hypothesis of a long-lasting impact of stressful experiences during childhood on HPA-axis activity. The *'Risky Families Model'* (Repetti et al., 2002) posits that childhood adversity creates a cascade of risk, beginning early in life. This includes psychological vulnerabilities, such as deficits in social competence and emotion regulation, and an inclination to compensate for them with poor health behaviours. Constant or recurrent exposure to stress, alongside deficits in emotion processing, social competence, and behavioural self-regulation may lead to alterations in HPA axis function. The effects of a biologically dysregulated response to stress may be particularly harmful during early childhood when the normal development of biological regulatory systems occurs. Furthermore they may be cumulative over the lifespan, result in chronically increased cortisol, which in time leads to wear and tear known as allostatic load (McEwen, 1998). This can be interpreted as an inability to adapt to stress and effectively "turn off" the biological stress response when appropriate (McEwen, 2007). As previously described chronic activation of the HPA-axis may result in the mechanisms underlying the CAR becoming exhausted. This notion is supported by evidence that stress response dysregulation associated with childhood maltreatment may be mediated by epigenetic modifications such as increased methylation of the GR (McGowan et al., 2006).

2009;Perroud et al., 2011;Tyrka et al., 2012) and demethylation of FKBP5 (Klengel et al., 2013).

Childhood maltreatment may therefore result in a distinct form of depression with different neuroendocrine function compared with depression without maltreatment. Maltreated depressed patients exhibit unique clinical features, such as prolonged and more severe depression and a lower responsiveness to treatment compared with non-maltreated depressed patients (Quevedo et al., 2017). This hypothesis is supported by evidence that depressed patients with history of abuse have a lower responsiveness to treatment compared to those with no history (Harkness et al., 2012;Nanni et al., 2012;Shamseddeen et al., 2011) and an increased risk of developing recurrent and persistent depressive episodes (Nanni et al., 2012). This had led clinicians to suggest that depressed patients with history of childhood maltreatment, may require specialized clinical approaches (Shamseddeen et al., 2011). This thesis has already called into question the assumption that depression is a homogeneous disorder. Given that the clinical and biological variations in MDD are extensive, with different subtypes reflecting different pathophysiologies, it is not unreasonable to suggest that the additional experience of childhood maltreatment may result in a unique phenotype. In the present study, a history of childhood adversity may have increased the CAR in depressed people to a level above that of those with no history, but not to the level of healthy controls.

Another influencing factor may be the effect of disrupted sleep. This section has already discussed how sleep disturbances may interact with diurnal cortisol rhythm, resulting in a blunting of the CAR. It is also possible that the hyper-vigilance and arousal which accompanies irregular sleep patterns may result in the increased CAR observed in our depressed sample. Support for this hypothesis comes from findings which show that childhood maltreatment is associated with sleep disorders in adulthood (Anda et al., 2006;Bader et al., 2007a;Bader et al., 2007b;Daniel P Chapman et al., 2013;D. P. Chapman et al., 2011;Schafer & Bader, 2013). Findings using data from the World

Mental Health Survey has shown that childhood sexual abuse in particular is associated with both sleep disturbances and mood disorders in adulthood (Gal et al., 2011). In a study of women participants with a history of childhood sexual abuse reported significantly greater rates of sleep disturbances than controls above and beyond depression (Noll et al., 2006). Sleep disturbances were also associated with depression. Childhood sexual abuse is also associated with more awakenings and arousal (Bader et al., 2007a;Bader et al., 2007b), more nightmares (Agargun et al., 2003) and insufficient sleep in adulthood (Daniel P Chapman et al., 2013). In the study by Quevedo et al. (2017) where higher CAR was reported in maltreated depressed adolescents compared to non-maltreated depressed, a trend was also observed for higher insomnia in depressed participants who has experienced maltreatment. However this interpretation ought to be taken with caution and further research is necessary to provide more robust support for this explanation.

3.6.7 *Hypothesis 5: Immuno-neuroendocrine relationships*

The results provided evidence of a relationship between inflammatory activation and diurnal cortisol rhythm. IL-6 was negatively associated with the CAR and CAR AUC in both groups. TNF- α was associated with reduced CAR AUC and AUC in the depressed group only, suggesting a link between dysregulation of the HPA axis and immune system. (Huber et al., 2006;Stetler & Miller, 2005). To date there is only one study to assess diurnal cortisol rhythm and plasma TNF- α in people with MDD. Lamers et al. (2013) used data from 776 people from the Netherlands Study of Depression and Anxiety to compare inflammatory markers, including TNF- α , and saliva cortisol awakening curves in people with melancholic depression, atypical depression and healthy controls. The observed that people with melancholic depression had significantly higher CAR AUC compare with controls, however there was no significant difference in TNF- α . Conversely, people with atypical depression had significantly higher levels of TNF- α compared with controls but no significant difference in CAR AUC. This finding suggests

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that HPA-axis hyperactivity is a distinct feature of melancholic depression, whereas increased inflammation is a feature of atypical depression.

Studies reporting both TNF- α and cortisol levels have reported conflicting results. Higher plasma cortisol has been reported along with both increased (Y. Chen et al., 2017; Kahl et al., 2017; Kahl et al., 2015) and decreased TNF- α (Rudzki et al., 2017) in depressed patients compared with controls. Lower plasma cortisol and lower TNF- α levels have also been reported in MDD patients (Lopes et al., 2012). This is the first study to assess diurnal cortisol rhythm and TNF- α in people with MDD, regardless of subtypes. A negative association between the CAR and TNF- α was reported in patients with ADHD (Corominas-Roso et al., 2017) and similarly, an inverse association between waking cortisol and TNF- α was observed in a population-based sample (DeSantis et al., 2012). This result supports the notion that dysregulation of the HPA-axis, as seen in depression, may be associated with higher levels of inflammation, however the direction of the association remains unclear

Together, these results suggest possible underlying neurobiological differences between depressed people and controls and that impaired HPA-axis activity could be less capable of inhibiting a pro-inflammatory response. Examining the directionality of the causal relationships is beyond the scope of this thesis, however the reasons for these associations may be speculated on. In the initial stages of stress, activation of the HPA-axis occurs, resulting in secretion of cortisol and subsequent down-regulation of pro-inflammatory cytokines. However, a prolonged stress response and chronic stimulation of the HPA-axis may lead to HPA-axis fatigue, resulting in hypocortisolism. This is accompanied by shift in the cytokine milieu with increased production of pro-inflammatory cytokines and decreased production of anti-inflammatory cytokines (Varghese et al., 2016). Persistent HPA-axis hyperactivity may also result in GR downregulation. Hypercortisolism could theoretically exhaust the capacity of the GR to recycle, resulting in a reduced ability to provide negative feedback.

Animal models of stress provide some evidence for a shift from glucocorticoid signalling to inflammatory signalling (Quan et al., 2003;D. Wang et al., 2011), although this finding has not always been replicated (B. Yang et al., 2008). A study exploring the effects of chronic stress in caregivers also showed that the gene expression profiles shifted from glucocorticoid signalling to inflammatory signalling (G. E. Miller et al., 2008). There is therefore some evidence to support the hypothesis that impaired GR function leads to both inflammatory activation and depressive symptoms but this is by no means conclusive.

3.6.8 *Perceived stress*

The results provided no evidence of a correlation between biological dysregulation in depressed people and perceived stress. This is surprising given that psychosocial stress has been associated with increased inflammatory cytokines in many studies (Hansel et al., 2010;Rohleder, 2014). TNF- α specifically has previously been associated with work stress in non-depressed people (Grossi et al., 2003;von Känel et al., 2008) and people who report high stress perception have a significantly higher production of TNF-alpha (Maes et al., 1998). There is also evidence that life stress is associated with dysregulated CAR and CAR AUC (Boggero et al., 2017;Chida & Steptoe, 2009) and with GR resistance (Menke et al., 2014;G. E. Miller et al., 2008;G. E. Miller et al., 2014;Walsh et al., 2018). Furthermore, a robust and causal association exists between stress and MDD (Hammen, 2005). Given that in the present study depressed people reported increased levels of stress and demonstrated increased TNF- α levels and a dysregulation of the HPA-axis compared with controls we would expect to see some associations.

There is evidence to suggest that the type and timing of stress may exert differential effects on inflammation and depression. A longitudinal study with cancer patients showed that whilst childhood trauma and recent life events were risk factors for higher depressive symptoms, only childhood trauma and not recent events explained the

association between TNF- α and depressive symptoms (Archer et al., 2012). A cross-sectional study including 214 MDD patients 180 healthy controls reported that whilst there was a relationship between childhood sexual abuse and both TNF- α and IL-6 in MDD patients, levels of TNF- α and IL-6 were not associated with recent stressful experiences (Grosse et al., 2016a). In this study we did not observe any association between childhood adversity and inflammation, however the lack of association of more recent stressors with biological dysregulation may be due to their timing. Recent life stress may occur in a less vulnerable stage compared to early life, when the immune function is more plastic. Following stress exposure, an immature immune system can develop a hyper-sensitive response pattern, leading to chronic inflammation (G. E. Miller et al., 2011). By adulthood people may have developed better resilience to stress and adopted psychosocial resources which moderate its effects. Indeed this speculative explanation is supported by the sociodemographic nature of this sample, which contained a high proportion of university staff/students from high socioeconomic backgrounds. It is therefore possible that the current sample may be biased towards individuals who are possess more protective factors (social support and coping skills) than would be observed in the larger population. This may have acted as a confounding variable, buffering the effects of stress and mitigating any biological sequelae (Potier et al., 2018). Thus, the present study could be under-estimating the true magnitude of associations between biological disparities and psychosocial stress.

Another possible explanation for this could be that psychosocial factors are differentially associated with HPA-axis function. A systematic review and meta-analysis by Chida and Steptoe (2009) reported that work stress and general life stress were associated with an increased CAR, whereas fatigue, burnout, or exhaustion were associated with a reduced CAR. It is therefore possible the lack of association reported here is a reflection of heterogeneity in psychosocial factors. In the present study the measures used did not enable the discrimination of 'stress types' therefore it is not possible to test this hypothesis.

3.6.9 *Strengths and limitations*

This study has both strengths and weaknesses. The diagnoses of depression was always carried out using the CIS-R, which is a fully structured assessment and considered to be one of the most reliable and valid measures of minor psychiatric disorders in the community (Head et al., 2013; Lewis et al., 1992). Furthermore depression status was confirmed by a specific scale, BDI-II, a widely used outcome measure in studies of depression in both clinical and non-clinical populations (A. T. Beck et al., 1996a). The groups did not differ significantly on socio-demographic factors, with the exception of smoking and alcohol consumption and these were controlled for in sensitivity analyses. The study only included participants without other comorbid psychiatric or medical disorders. The prevalence of depression is higher in patients than in the general public. Wang et al. (2017) estimated that the prevalence of depression in different specialties varied from 17.0% to 53.0%. Depression is particularly prevalent in patients with chronic inflammatory and autoimmune diseases (Pryce & Fontana, 2016; Steptoe, 2007). Furthermore, medications used to treat medical disorders can also affect the biological pathways investigated. Therefore, in order to determine that any differences observed could reasonably be attributed to organic depression, a highly selected sample was included. Consequently, the inclusion criteria used in this study increases the internal validity but decreases the external validity and the generalisability of the results.

However, our sample size was small and we did not reach our recruitment target, which limited statistical power. It is therefore possible that this study was underpowered to detect certain effects. This factor also reduced the number of subgroup analyses that could be included, such as comparisons between treatment groups. As has already been discussed in this section, it is possible that there were different inflammatory/neuroendocrine profiles between depressed participants undergoing

specific therapies or between participants with different subtypes of depression. Larger studies of people with MDD are needed to establish the robustness of the findings.

We also included individuals who had mixed depression and anxiety. The CISR has a hierarchy so that if someone meets criteria for both depression and anxiety then the *primary* diagnosis is depression and the *secondary* diagnosis is anxiety. The high comorbidity rate of depression with anxiety disorders meant that excluding these participants would have devastated our recruitment. Regarding *secondary* diagnoses, we recruited 13 people with GAD, nine with mixed depression and anxiety disorder (mild), three with panic disorder, three with mixed depression and anxiety, one with GAD (mild), one with agoraphobia and one with specific (isolated) phobia. In total we also recruited three individuals with a *primary* diagnosis of 'mixed depression and anxiety disorder (mild)' and these participants were coded as 'mild depression'. We recruited six participants with no *secondary* diagnoses, all of whom had a *primary* diagnosis of mild depression. Anxiety is highly prevalent in MDD populations and is associated with worse outcomes. Due to our small sample size we did not have enough power to stratify our results according to *secondary* diagnoses. However it should be borne in mind that our sample was psychiatrically heterogeneous.

The study was cross-sectional, so no causal conclusions can be drawn. It is possible that depressive symptoms activate the immune system, resulting in neuroendocrine changes. As discussed in Chapter 1, stressful life events (Kiecolt-Glaser et al., 2002) and chronic stress (Walsh et al., 2018) are associated with increased inflammatory biomarkers. However, it is also possible that heightened inflammation precedes the development of depression as demonstrated by those patients receiving inflammation-based pharmacotherapy (Capuron et al., 2009). Furthermore, we observed a relationship between HPA-axis dysregulation and cytokine production, however it is not possible to determine the direction of this relationship.

Regarding childhood maltreatment, our use of retrospective measures may have influenced our findings. A systematic review and meta-analysis by Baldwin et al. (2019) including 16 studies and 25,471 individuals, found that prospective and retrospective measures of childhood maltreatment showed poor agreement. Moreover, more than half of individuals with prospective observations of childhood maltreatment did not report it retrospectively. It is therefore possible that in this study participants may have under reported experiences of childhood adversity.

It is unknown how closely the participants adhered to the cortisol saliva sampling protocol in this study. When measuring hormones known to have a profound circadian rhythm, the exact timing of sample collection is of paramount importance (Kudielka et al., 2003). Furthermore, cortisol levels are influenced by several other factors, such as caffeine, food intake and nicotine consumption, affecting what would otherwise be a typical cortisol profile. Indeed research has shown that a significant number of individuals do not comply with saliva sampling regimen unless they are aware of being monitored (Kudielka et al., 2003). Lack of fidelity may have therefore reduced the reliability of our cortisol data. Cortisol samples were also collected on a single day and as already discussed, this makes them more susceptible to situational factors, possibly further reducing reliability. Additionally, night-time cortisol was not measured so it was not possible to assess total 24 hour cortisol output. Cortisol levels reach its lowest level at about midnight and then rise throughout the early hours, increasing sharply around 30 minutes after waking (Fries et al., 2009). In order to explore whether sleep disruption played a role in the reduced CAR observed in the MDD group, examining the gradual night-time increase in these participants would have been of interest.

Regarding corticosteroid receptor sensitivity, performing assays in whole blood rather than in isolated PBMCs may have influenced our findings. This analysis does not account for differences in immune cell populations, which could vary between participants (Burnsides et al., 2012). However this technique is fast and given the time limitations of

this study, it was considered the most appropriate. Future studies could carry out these tests in isolated PBMCs to validate these findings. Moreover, these assays only provide a proxy measure of GR and MR sensitivity via corticosteroid suppression of LPS-induced IL-6 release. The addition of assays measuring gene expression of GR up and downregulated genes would help to establish GR responsiveness. Measurement of receptor levels may also add value to these findings, by determining receptor mRNA levels, measuring the rate of translocation of the receptors into the cell nuclei or DNA binding. Future studies could include measures of receptor levels and sensitivity to provide a more comprehensive investigation of the mechanisms involved.

3.7 Conclusion

In conclusion, these results indicate that inflammatory activation is increased, whilst GR and MR function is reduced in people with depression. We also observed a flattening of the CAR in depressed people. Furthermore, there appears to be a relationship between inflammatory cytokines and diurnal cortisol rhythm. However it is not clear from these findings whether these factors change with improvements in depressive symptoms. In the following chapter I will present the findings from the follow-up analysis, which will explore whether any observed psychological changes are accompanied by biological changes. The results of this may tell us more about how these systems interact.

4. Study 1b - The Resist Study follow-up results:

Changes in depression and associations with inflammatory biomarkers and HPA-axis function at 6 week follow-up

4.1 Introduction

As outlined in Chapter 3, findings from the Resist Study demonstrated inflammatory activation and neuroendocrine dysfunction in depressed people compared with controls. Recent evidence suggests that the dysfunction observed in biological pathways in people with depression may normalise following symptom resolution and that persistently high levels of inflammation may contribute to treatment resistance. It is therefore of interest to explore whether any changes in physiological parameters in depressed people during the six week follow-up period are associated with any changes in depressive symptoms. If the inflammatory and neuroendocrine disturbances previously observed are indeed a physical manifestation of MDD, we would expect that any degree of normalisation would be, at least in part, associated with symptom remission. To date no study has investigated changes in circulating inflammatory cytokines, GR and MR function and diurnal cortisol rhythm simultaneously in MDD patients, over time. In this section I will present results from the follow-up part of the Resist Study. Baseline measurements of depressive symptoms, inflammatory cytokines, diurnal cortisol parameters and corticosteroid receptor function in depressed people at baseline were compared with measurements at six weeks. These results may tell us more about the underlying pathways involved in clinical recovery and help identify those who may be less likely to respond to conventional treatment.

4.2 Hypotheses

Meta-analyses have consistently demonstrated that IL-6 levels are reduced following antidepressant treatment, although the effect may be small (Hannestad et al., 2011; Hiles 216

et al., 2012a;Kohler et al., 2018;Wiedlocha et al., 2018). However findings regarding TNF- α are inconclusive. IL-6 levels have been shown to decrease following antidepressant treatment, regardless of outcome (Strawbridge et al., 2015). However TNF- α levels appear to decrease only in those who respond. Furthermore treatment resistance has been associated with persistently elevated TNF- α . Other findings suggest that reductions in both IL-6 and TNF- α are not associated with treatment response (Kohler et al., 2018). Psychological therapy has also been shown to reduce inflammatory biomarkers, including IL-6 and TNF- α (Lopresti, 2017). In general, there appears to be some degree of normalisation of cytokine levels following antidepressant treatment. Furthermore, persistently high levels of inflammation may contribute to treatment resistance. Therefore **I hypothesise that any change in depressive symptoms will be influenced by baseline levels of inflammation and predicted by change in inflammation over time (hypothesis 1).**

Regarding corticosteroid receptor function, there is some evidence that increased GR number in depressed people is associated with antidepressant treatment and that antidepressants upregulate GR binding (Calfa et al., 2003;Sallee et al., 1995). Cortisol inhibition of mitogen-induced lymphocyte proliferation improves in MDD patients following antidepressant therapy (Calfa et al., 2003), whilst pharmacologically treated TRD in-patients show reduced inhibition of T-cell proliferation and cytokine production following *in vitro* administration of DEX (Bauer et al., 2003;Carvalho et al., 2008). These findings suggest that restoration of GR function is dependent on clinical remission. *In vivo* studies have also shown that increased GR sensitivity is associated with symptom improvement (S. Fischer et al., 2017a;Greden et al., 1983;Holsboer et al., 1982), however this has not always been reported (Ribeiro et al., 1993;Ventura-Junca et al., 2014). Overall the findings suggest normalisation of the HPA-axis following treatment, although differential effects of various antidepressants have also been observed. Regarding MR function, there is evidence to suggest that MR sensitivity does not change before and after antidepressant treatment, even in those who respond clinically (Jurueña

et al., 2010). However individuals who do not respond to antidepressant treatment demonstrate impaired MR sensitivity compared to those who do respond (Juruena et al., 2010;Juruena et al., 2009). MR function may therefore be a predictor of treatment response. Therefore **I hypothesise that any change in depressive symptoms will be influenced by corticosteroid sensitivity at baseline and predicted by change in corticosteroid sensitivity over time (hypothesis 2).**

In terms of diurnal cortisol rhythm, symptom remission has been robustly associated with increased CAR following SSRI treatment in depressed people who had previously exhibited a blunted CAR compared with controls (Harmer et al., 2003;Ruhe et al., 2015). This is supported by findings from a large cohort study which showed that significantly higher CARs were found in remitted patients compared to controls (Vreeburg et al., 2009a). Higher AUC has also been observed in remitted patients compared with controls (Aubry et al., 2010). Therefore **I hypothesise that any change in depressive symptoms will be influenced by diurnal cortisol rhythm at baseline and predicted by change in cortisol rhythm over time (hypothesis 3).**

In terms of the relationship between these pathways, only two MDD studies have explored associations between changes in these parameters over time. Himmerich et al. (2006) examined alterations in TNF- α and response to the DEX/CRH test on admission and at discharge in MDD inpatients and reported a positive correlation between TNF- α and CRH/DEX test outcome in remitted patients. In contrast, Lisi et al. (2013) assessed cortisol circadian rhythm after low dose DST and gene expression of IL-6 in MDD patients before and after 8 months of antidepressant drug treatment. IL-6 was reduced at follow-up although was not significant. DST results showed normal suppression in healthy controls, whereas 30% of the depressed group demonstrated non-suppression and remained non-suppressors at follow-up. However, they found no differences among the profiles of daily cortisol secretions between the groups at either time point and inflammatory changes were not associated with levels of circulating glucocorticoids. In

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light of the fact that we observed a negative association between IL-6/TNF- α and the CAR AUC/AUC in depressed people in Study 1a, we would expect to see a relationship between any changes in these pathways at follow-up. Therefore **I hypothesise that any change in inflammatory cytokines will be associated with change in CAR AUC/AUC (hypothesis 4).**

4.3 Biological measures

All assays were repeated as per the protocols in Chapter 3, Section 3.3.

4.3.1 *Plasma IL-6 and TNF- α determination*

35 participants with MDD attended follow-up assessments and 34 participants provided blood samples. Plasma IL-6 and TNF- α data was available for all 34 participants at follow-up.

4.3.2 *Corticosteroid receptor sensitivity*

Two participants were excluded from the corticosteroid receptor sensitivity analysis at follow-up due to difficulties accessing the samples following incubation, resulting in a sample of 32. DEX log IC⁵⁰ values were successfully computed for 29 participants, 20 of which also had DEX log IC⁵⁰ values a baseline. PRED log IC⁵⁰ values were successfully computed for 20 participants, 12 of which also had PRED log IC⁵⁰ values at baseline. Therefore comparisons between log IC⁵⁰ values at baseline and follow-up were conducted in 20 people for DEX and 12 for PRED.

4.3.3 *Calculation of diurnal cortisol parameters*

Of the 35 depressed participants assessed at follow-up, 22 provided saliva samples. As with the previous analysis, the cortisol data were cleaned and the same indices of HPA axis function were computed. Of the 22 participants included in the follow-up analysis, there were some missing cortisol samples: one at waking + 30 minutes and two at 4pm. When calculating the CAR, we omitted 6 individuals who reported a delay of >15 min between waking and taking the 'waking' sample and one individual who failed to provide a sample at waking + 30 minutes, leaving a sample of 15. 11 participants had CAR data at both time points and were included in the analytic sample. CAR AUC was calculated for 21 participants and 20 had data at both time points and AUC was calculated for 20 participants, 18 of whom had data at both time points. All 22 participants had sufficient data for the calculation of cortisol slope and 21 participants had data at both time points.

4.4 **Statistical analysis**

Kolmogorov-Smirnov tests were used to test for the normality of the distribution for all variables at follow-up and a natural log transformation was conducted when the test revealed deviation from normality. BDI and PSS scores at both time points were normally distributed. LPS, percentage inhibition values, plasma IL-6 and plasma TNF- α values were not normally distributed. Log transformation normalised the distributions for baseline LPS, all percentage inhibition values and plasma IL-6. Non-parametric tests were applied for the analysis of TNF- α . In relation to salivary cortisol, normality tests revealed that follow-up cortisol levels at waking, waking + 30 minutes and bedtime were not normally distributed. Log transformations normalised the distributions for these variables.

Repeated Measures T-Tests were used to compare all parametric variables between baseline and follow-up. Related-Samples Wilcoxon Signed Rank tests were used to

compare non-parametric variables. Where there were significant differences between the time points on any of the biological variables, secondary analyses were conducted. To determine whether severity of baseline depression affected the change in IL-6 repeated measures ANCOVAs were conducted, including baseline depression score as a covariate. Separate analysis were conducted using CISR score and baseline BDI. There was no difference between the results and so the BDI analyses have been reported. In addition, to explore whether change in BDI score was a predictor of change in IL-6, linear regression analyses were also conducted, with baseline BDI included as a covariate. In order to determine whether any biological changes were influenced by age, sex, smoking or alcohol consumption, repeated measures ANCOVAs were conducted including these variables. Pearson's R correlations and Spearman's correlations were used to ascertain whether there was any association between change in inflammatory and HPA-axis pathways and between changes in these biological pathways and changes in mood. Change scores were calculated as value at follow-up – value at baseline. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA).

4.5 Results: People with MDD at baseline and six week follow-up

4.5.1 Participants

Data is presented for participants who provided at least one biological sample at follow up (n = 34). Table 4.1 summarises the clinical characteristics of the participants at baseline and follow-up. One third of the depressed group were taking antidepressants (32%) and one fifth were receiving psychotherapy (21%) at baseline. There were no differences in either of these measures at follow-up. There was a significant difference in BDI scores between baseline and follow-up ($t(33) = 3.952, p < 0.001$). Participants had significantly lower depressive symptoms at follow-up ($M = 19.03, SD = 8.77$) compared with baseline ($M = 24.59, SD = 7.74$). However, the change in BDI score varied from -27

to 12 suggesting that some participants exhibited a decrease in depressive symptoms, whilst others exhibited an increase. There was also a trend towards significance for PSS score ($t(33) = 1.859, p = 0.072$). Participants reported lower levels of perceived stress at follow-up ($M = 22.09, SD = 5.88$) compared with baseline ($M = 24.12, SD = 5.48$).

Table 4.1. Clinical characteristics of people with MDD at baseline and follow-up (n=34)

	Baseline	Follow-up	Group difference (p value)
	<i>Mean ± SD or N (%)</i>	<i>Mean ± SD or N (%)</i>	
Antidepressant use	11 (32)	11 (32)	-
Psychotherapy	7 (21)	7 (21)	-
BDI II score	24.59 ± 7.17	19.03 ± 8.77	<0.001
PSS score	24.12 ± 5.48	22.09 ± 5.88	0.072

Abbreviations: SD = standard deviation; BDI – Beck Depression Inventory; PSS = Perceived Stress Scale.

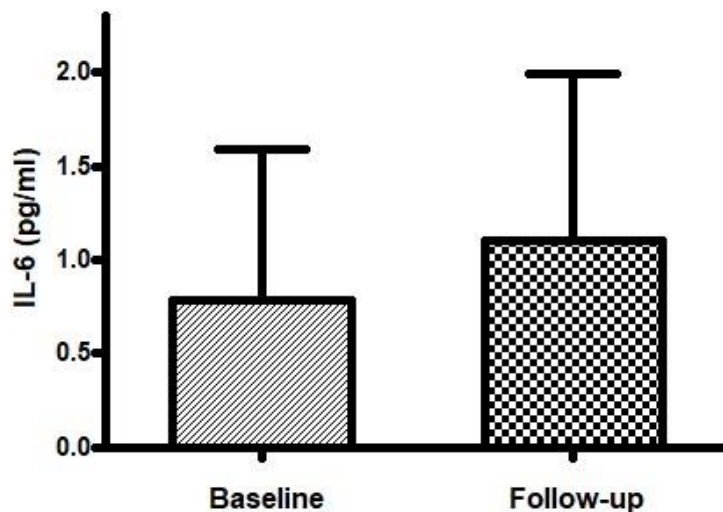
4.5.2 Inflammatory biomarkers

To explore differences in inflammatory activation, plasma IL-6 and TNF- α levels were compared between the time points. There was a trend towards significance for IL-6 ($t(34) = -1.785, p = 0.084$). IL-6 levels were higher on average at follow-up compared to baseline (Table 4.2, Figure 4.1, hashed bars represent baseline, checked bars represent follow-up). This is in contrast to our expectations, as we would anticipate IL-6 to be reduced in conjunction with decreased depressive symptoms.

We then explored whether the observed reduction in depressive symptoms remained significant after controlling for baseline IL-6. Results from ANCOVA showed that there was still a significant reduction in BDI score over time, independently of IL-6 ($F(1,32) =$

5.964, $p = 0.020$) and there was no significant interaction effect between the variables ($F(1,32) = 1.492, p = 0.231$). Linear regression was also conducted to ascertain whether change in IL-6 was associated with change in depression score over time, adjusted for age, sex, smoking or alcohol consumption. Results showed that change in IL-6 was not a significant predictor of change in BDI score ($\beta = 0.422, p = 0.075$). Reversed analyses showed no significant difference in IL-6 over time after adjustment for baseline BDI score and showed that change in BDI was not a significant predictor of change in IL-6 (both $p = >0.05$).

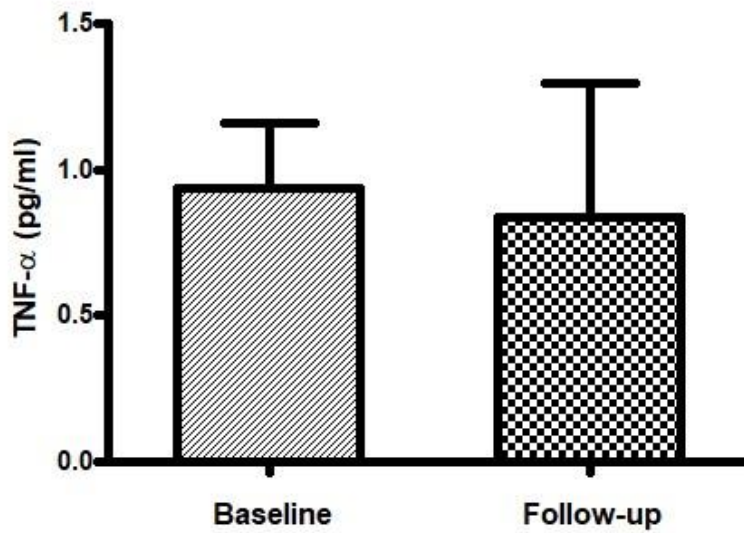
Figure 4.1. Plasma IL-6 levels in people with MDD at baseline and follow-up (n=34).



Data shown are mean \pm SD in pg per ml. * statistically significant at $P < 0.05$

TNF- α levels were reduced in depressed people at follow-up, although this did not reach significance ($Z = -0.542, p = 0.588$) (Table 4.2, Figure 4.2).

Figure 4.2. Plasma TNF- α levels in people with MDD at baseline and follow-up (n=34).



Data shown are median \pm interquartile range in pg per ml.

Table 4.2. Mean inflammatory and corticosteroid receptor sensitivity parameter values and p values from analyses comparing differences in people with MDD at baseline and follow-up (n=34).

	Baseline	Follow-up	Group difference (p value)
	<i>Mean ± SD or median ± interquartile range</i>	<i>Mean ± SD or median ± interquartile range</i>	
Plasma IL-6 (pg/ml) (n=34)	0.77 ± 0.80	1.07 ± 0.89	0.084
Plasma TNF-α (pg/ml) (n=34)	*0.94 ± 0.37	*0.84 ± 0.53	0.588
IL-6 concentration with LPS only (pg/ml) (n=32)	154.87 ± 107.98	148.57 ± 85.71	0.500
% LPS induced IL-6 levels with DEX 10 ⁻⁶ (pg/ml) (n=29)	20.99 ± 28.33	9.43 ± 7.88	0.061
% LPS induced IL-6 levels with DEX 10 ⁻⁷ (pg/ml) (n=31)	29.18 ± 36.42	13.07 ± 9.20	0.074
% LPS induced IL-6 levels with DEX 10 ⁻⁸ (pg/ml) (n=32)	82.94 ± 85.50	48.61 ± 21.78	0.185
% LPS induced IL-6 levels with DEX 3 x 10 ⁻⁹ (pg/ml) (n=32)	121.41 ± 84.68	93.96 ± 37.58	0.567
% LPS induced IL-6 levels with PRED 10 ⁻⁶ (pg/ml) (n=30)	49.95 ± 72.08	17.78 ± 11.43	0.012
% LPS induced IL-6 levels with PRED 10 ⁻⁷ (pg/ml) (n=32)	85.76 ± 93.69	46.33 ± 20.05	0.301

Abbreviations: MDD = major depressive disorder; DS = standard deviation; IL = interleukin; pc = picogram; ml = millilitre, TNF = tumour necrosis factor, LPS – lipopolysaccharide; DEX = dexamethasone; PRED = prednisolone. Mean and median scores represent raw values. *median/interquartile range.

Table 4.2 continued. Mean inflammatory and corticosteroid receptor sensitivity parameter values and p values from analyses comparing differences in people with MDD at baseline and follow-up (n = 34)

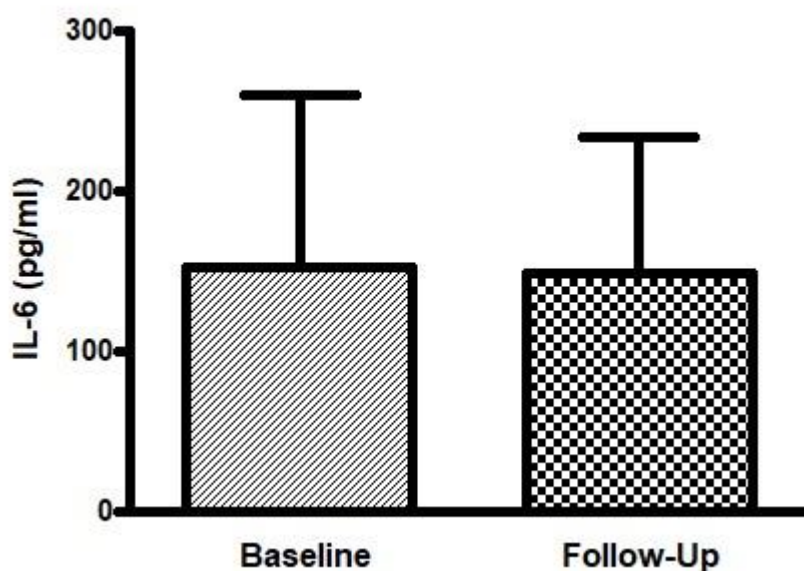
	Baseline	Follow-up	Group difference (p value)
	<i>Mean ± SD</i>	<i>Mean ± SD</i>	
% LPS induced IL-6 levels with PRED 10 ⁻⁸ (pg/ml) (n=32)	144.22 ± 96.43	97.52 ± 51.74	0.051
% LPS induced IL-6 levels with PRED 3 x 10 ⁻⁹ (pg/ml) (n=30)	166.07 ± 121.77	127.56 ± 76.81	0.639
DEX IC ₅₀ (% inhibition of LPS stimulated IL-6 levels) (n=20)	8.28E-8 ± 1.63E-7	1.94E-8 ± 1.83E-8	0.085
PRED IC ₅₀ (% inhibition of LPS stimulated IL-6 levels (n=12)	1.49E-7 ± 2.51E-7	2.25E-7 ± 2.27E-7	0.408

Abbreviations: MDD = major depressive disorder; SD = standard deviation; IL = interleukin; pc = picogram; ml = millilitre; DEX = dexamethasone; PRED = prednisolone. Mean and median scores represent raw values.

4.5.3 Corticosteroid receptor sensitivity

LPS induced IL-6 levels were measured and compared in depressed people between baseline and follow-up, in the absence of corticosteroids. As expected, there was no difference in IL-6 levels in MDD patients between the time points, ($t(31) = -0.682, p = 0.5$) (Table 4.2, Figure 4.3).

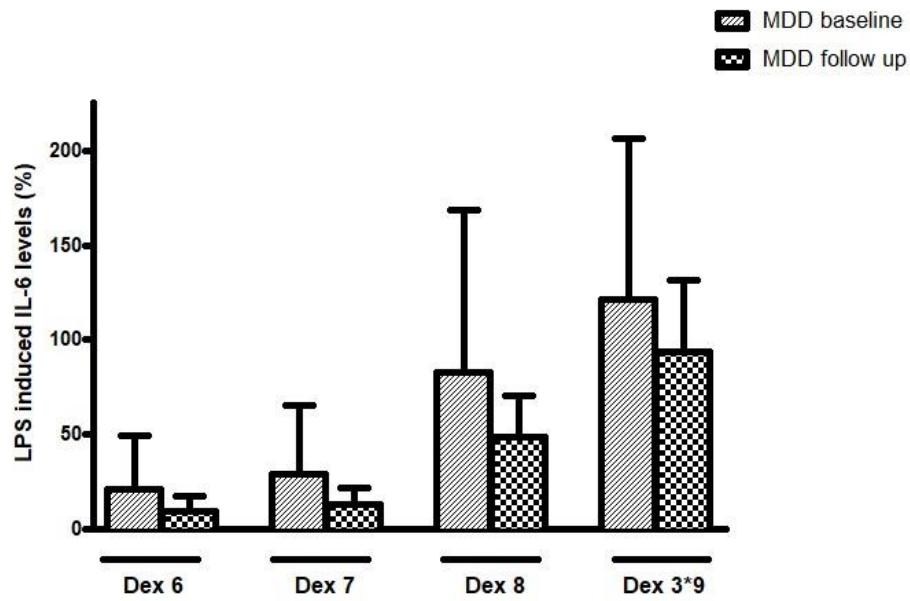
Figure 4.3. LPS induced IL-6 in people with MDD at baseline and follow-up (n=32).



Data shown are mean \pm SD in pg per ml.

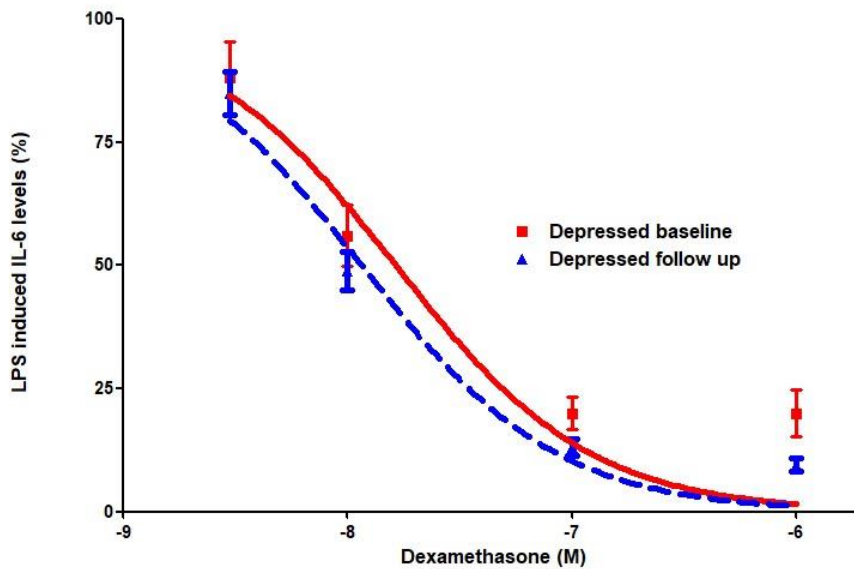
Next, corticosteroid sensitivity was compared at baseline and follow-up. Data are presented as glucocorticoid inhibition of LPS-stimulated IL-6 levels as a percentage. There was a difference in the effects of in vitro incubation with DEX between the time points. However, whilst IL-6 levels were lower after incubation with DEX for all concentrations at follow-up, none of them reached significance ($p > 0.05$) (Figure 4.4). The dose-response curve is shown in Figure 4.5.

Figure 4.4. DEX suppression of LPS induced IL-6 in people with MDD at baseline and follow up (n=32).



Data shown are mean \pm SD in pg per ml.

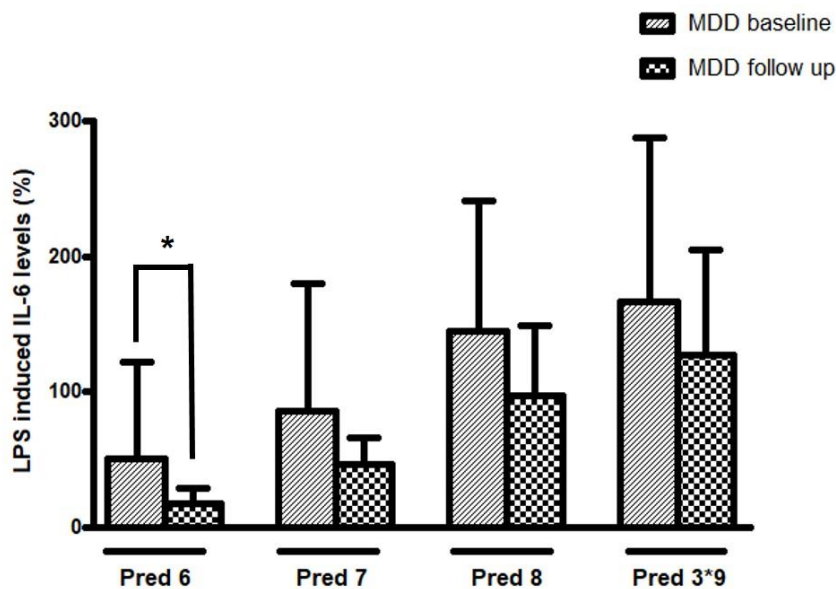
Figure 4.5. DEX inhibition of LPS stimulated IL-6 levels in people with MDD at baseline and follow-up (n = 20).



Data are shown as mean \pm S.E.M of the % DEX inhibition.

LPS-stimulated IL-6 levels were also lower after incubation with PRED for all concentrations at follow-up. There was a significant difference in the effects of in vitro incubation with the highest concentration of PRED between the time points ($t(29) = 2.690$, $p = 0.012$). IL-6 levels decreased from baseline to follow-up, suggesting some improvement in MR function. However this difference was not significant at the lower concentrations ($p > 0.05$) (Table 4.2, Figure 4.6).

Figure 4.6. PRED suppression of LPS induced IL-6 in people with MDD at baseline and follow up (n=32).



Data shown are mean \pm SD in pg per ml. * statistically significant at $P < 0.05$.

The reduction in depressive symptoms lost significance after adjusting for baseline PRED 10^{-6} . Results from ANCOVA showed that there was no longer a significant reduction in BDI score over time ($F(1,30) = 0.55$, $p = 0.816$) and there was no significant interaction effect ($F(1,30) = 0.980$, $p = 0.330$). Linear regression was also conducted to ascertain whether change in IL-6 levels was associated with change in depression score over time, adjusted for age, sex, smoking or alcohol consumption. Results showed that change in IL-6 was not a significant predictor of change in BDI score ($\beta = 0.639$, $p = 0.158$). Reversed analyses showed no significant difference in LPS-stimulated IL-6

levels over time after adjustment for baseline BDI score and showed that change in BDI was not a significant predictor of change in IL-6 levels (both $p = >0.05$).

There was a trend towards significance for DEX IC₅₀ values ($t(19) = 1.816, p = 0.085$). DEX IC₅₀ values were lower on average at follow-up compared with baseline. This suggests that depressed people required a lower concentration of DEX to inhibit LPS-induced IL-6 by 50% at follow-up. Neither baseline DEX IC₅₀ ($\beta = 2.571, p = 0.374$) or change in DEX IC₅₀ were significant predictors of change in BDI over time ($\beta = 2.733, p = 0.337$) after adjusting for age, sex, smoking or alcohol consumption (all $p = >0.05$). In contrast, there was no significant difference in PRED IC₅₀ values between the groups ($t(11) = -0.861, p = 0.408$). A slope for the baseline PRED IC₅₀ values was unable to converge, therefore a dose response curve for PRED has not been presented here.

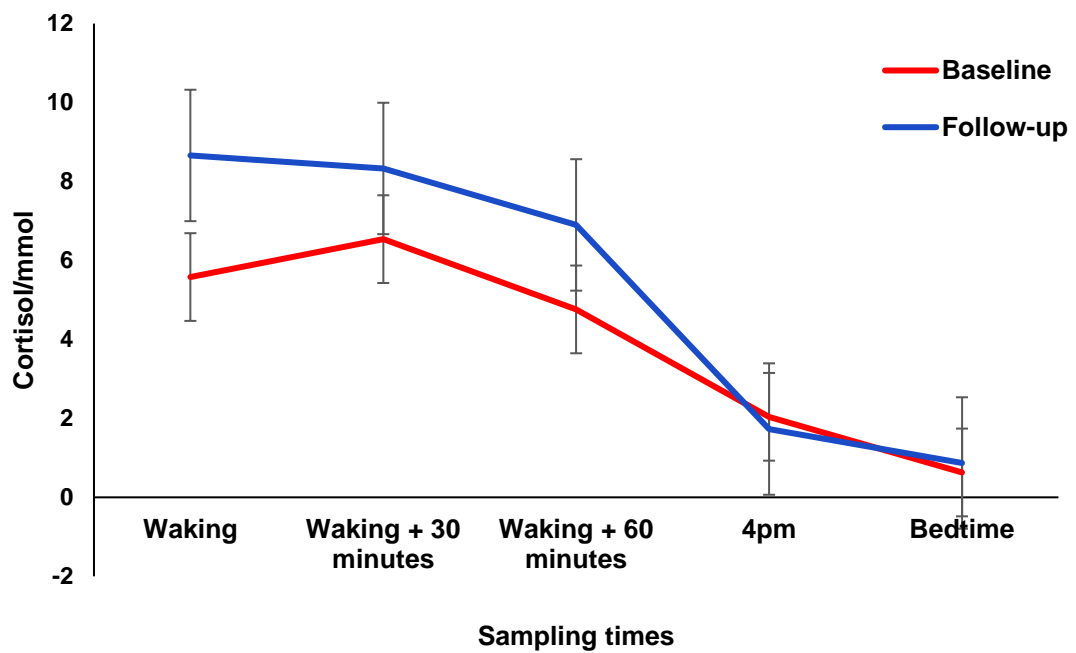
4.5.4 Diurnal Cortisol

The analyses of diurnal cortisol in relation to time points are summarised in Table 4.3. A graphical representation of mean cortisol values across the day at baseline and follow-up is provided in Figure 4.7. There was a borderline significant difference between the time points in terms of waking cortisol + 60 minutes ($t(20) = -1.944, p = 0.066$). Saliva cortisol levels were higher on average at follow-up compared to baseline. Results showed that the observed reduction in BDI score was independent of cortisol at waking + 60 minutes ($F(1,27) = 5.426, p = 0.028$) and there was no significant interaction effect ($F(1,30) = 0.021, p = 0.886$). Cortisol at waking + 60 minutes was not a significant predictor of change in BDI score ($\beta = -0.268, p = 0.312$) adjusted for age, sex, smoking and alcohol consumption. Reversed analyses showed no significant difference in cortisol levels over time after adjustment for baseline BDI score and showed that change in BDI was not a significant predictor of change in cortisol levels (both $p = >0.05$).

There was also a borderline significant difference between CAR AUC values ($t(19) = -1.957$, $p = 0.065$). CAR AUC values increased on average at follow-up compared to baseline, almost to the level observed in healthy controls, suggesting a normalisation of the HPA-axis. The reduction in depressive symptoms lost significance after adjusting for baseline CAR AUC. Results from ANCOVA showed that there was no longer a significant reduction in BDI score over time ($F(1,27) = 0.764$, $p = 0.390$) and there was no significant interaction effect ($F(1,27) = 1.035$, $p = 0.318$). Change in CAR AUC was not a significant predictor of change in BDI score ($\beta = 0.345$, $p = 0.112$) adjusted for age, sex, smoking and alcohol consumption. Reversed analyses showed no significant difference in CAR AUC over time after adjustment for baseline BDI score and showed that change in BDI was not a significant predictor of change in CAR AUC (both $p = >0.05$).

Cortisol slope also showed a trend towards significance ($t(20) = -1.827$, $p = 0.083$). Cortisol slope values were higher on average at follow-up compared to baseline. The reduction in BDI was not robust to adjustment for baseline cortisol slope ($F(1,27) = 1.443$, $p = 0.240$) and there was no significant interaction effect ($F(1,27) = 1.337$, $p = 0.258$). Change in cortisol slope was not a significant predictor of change in BDI score ($\beta = 0.349$, $p = 0.207$) adjusted for age, sex, smoking and alcohol consumption. Reversed analyses showed no significant difference in cortisol slope over time after adjustment for baseline BDI score and showed that change in BDI was not a significant predictor of change in cortisol slope (both $p = >0.05$).

Figure 4.7. MDD baseline versus follow-up: Mean salivary cortisol values across the day averaged across all participants



Saliva samples were taken on waking, waking+30mins, waking+60mins, 4pm, 8pm, and at bedtime in people at baseline (red line) and at 6 week follow-up (blue line). Error bars represent S.E.M. Data shown are raw values.

Table 4.3. Mean cortisol parameter values and p values from analyses comparing differences between people with MDD at baseline and follow-up

	Baseline	Follow-up	Group difference (p value)
<i>Diurnal cortisol parameters</i>	<i>Mean ± SD</i>	<i>Mean ± SD</i>	
Waking cortisol (nmol/L) (n=21)	5.68 ± 3.02	8.66 ± 7.76	0.327
Waking cortisol +30 minutes (nmol/L) (n=20)	6.54 ± 3.90	8.33 ± 1.14	0.342
Waking cortisol +60 minutes (nmol/L) (n=21)	4.76 ± 3.30	6.98 ± 4.04	0.066
4pm cortisol (nmol/L) (n=18)	2.04 ± 1.69	1.73 ± 1.46	0.182
Bedtime cortisol (nmol/L) (n=21)	0.63 ± 0.61	0.87 ± 0.19	0.160
CAR (nmol/L) (n=11)	0.33 ± 3.63	-0.77 ± 6.88	0.563
CAR AUC (nmol/L) (n=20)	6.2 ± 3.25	8.85 ± 5.22	0.065
AUC (nmol/L) (n=18)	39.73 ± 16.52	49.47 ± 28.88	0.204
Cortisol slope (nmol/L) (n=21)	0.01 ± 0.00	0.01 ± 0.01	0.083

MDD = major depressive disorder; SSD = standard deviation; nmol = nanomoles; L = litre; CAR = cortisol awakening response; AUC = area under the curve. Mean scores represent raw values.

4.5.5 Inter-correlations between the systems

To explore the relationship between change in HPA-axis function and change in inflammatory activation, correlations between change scores were conducted. The results are presented in Table 4.4. There was a negative pattern of association between change in TNF- α and change in the CAR, suggesting that as TNF- α decreases the CAR increases ($r = -0.624$, $p = 0.040$). However this was not significant following Bonferroni correction. There were no other significant associations.

Table 4.4. Correlations between change in inflammatory biomarkers and change in HPA-axis parameters in people with MDD

	IL-6	TNF- α
MDD		
DEX IC ⁵⁰	-0.152	0.245
PRED IC ⁵⁰	-0.028	0.406
CAR	-0.464	-0.624*
CAR AUC	-0.017	0.041
AUC	-0.203	-0.025
Cortisol slope	0.118	0.248

Abbreviations: MDD = major depressive disorder; DEX = dexamethasone; PRED = prednisolone; IL = interleukin, TNF- α = tumour necrosis factor alpha, CAR = cortisol awakening response; AUC = area under the curve. **Note:** * $p < 0.05$; ** $p < 0.01$; # $p < 0.008$ (critical p-value after Bonferroni correction).

Correlations were also conducted to explore the relationships between changes in biological factors and changes in mood. The results are presented in Table 4.5. There was a negative pattern of association between change in perceived stress and the AUC ($r = -0.530$, $p = 0.024$). However this did not reach Bonferroni significance. There was also a trend towards a positive association between change in perceived stress and TNF- α ($r = 0.334$, $p = 0.053$). Taken together these findings imply that as stress decreases, there is a reduction in inflammation and a normalisation of HPA-axis function. No other significant correlations were observed.

Table 4.5. Correlations between changes in biological and psychological variables in people with MDD

	BDI	PSS
MDD		
IL-6	0.237	0.180
TNF- α	0.249	0.334
DEX IC ₅₀	-0.116	-0.114
PRED IC ₅₀	-0.309	0.092
CAR	-0.036	0.243
CAR AUC	-0.018	-0.299
AUC	-0.216	-0.530*
Slope	0.062	-0.336

Abbreviations: MDD = major depressive disorder; DEX = dexamethasone; PRED = prednisolone; IL = interleukin, TNF- α = tumour necrosis factor alpha, CAR = cortisol awakening response; AUC = area under the curve; BDI = Beck Depression Inventory; PSS = Perceived Stress Scale; LTE = List of Threatening Experiences. **Note:** * $p < 0.05$; ** $p < 0.01$; # $p < 0.006$ (critical p-value after Bonferroni correction).

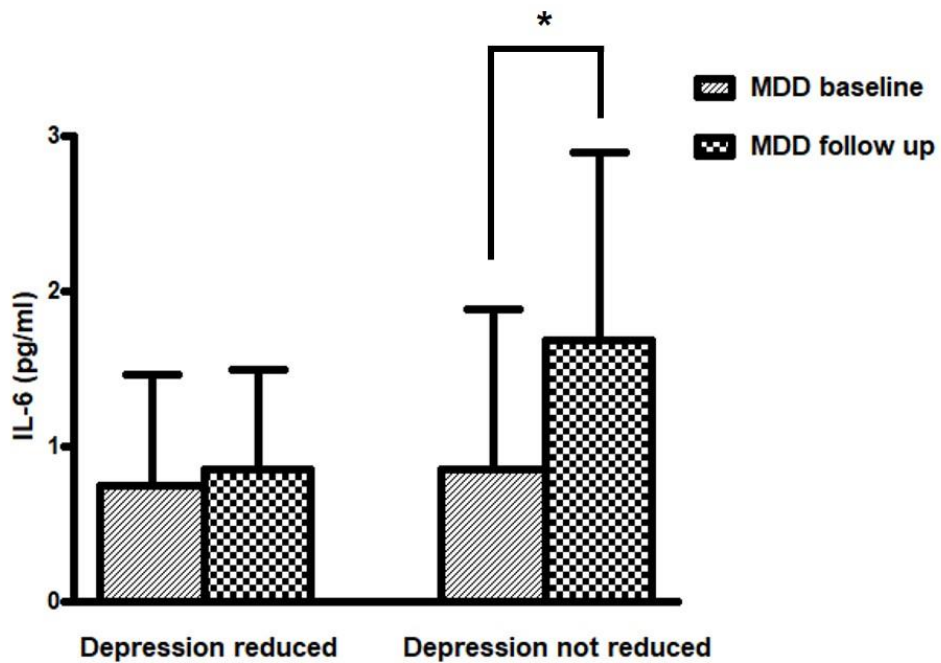
4.5.6 Sensitivity analyses

As described in Section 4.5.1, overall participants had significantly lower depressive symptoms, at follow-up compared with baseline. However this decrease was not a universal phenomenon across the sample; 25 participants experienced a reduction in BDI-II score, 7 experienced an increase and 2 did not experience any change. Therefore, I also ran an exploratory analysis to investigate whether these clinical differences impacted on the findings. The main analyses were repeated with the group split into those who demonstrated a reduction in BDI-II score ($n=25$) and those who did not ($n=9$). The results for inflammatory biomarker and corticosteroid receptor function are presented in Table 4.6.

The stratified analysis showed that there was a significant increase in IL-6 at follow-up for individuals who did not show any improvement in depressive symptoms ($t(8) = -2.858$, $p = 0.021$). IL-6 levels were 0.85 ± 1.03 pg/ml at baseline and 1.68 ± 1.21 pg/ml at follow-up. However for individuals who did show an improvement in depressive symptoms, there was no significant change in IL-6 ($t(24) = -0.409$, $p = 0.686$). IL-6 levels were 0.75 ± 0.72 pg/ml at baseline and 0.86 ± 0.64 pg/ml at follow-up. This finding suggests that the increase in IL-6 observed in the main analysis is largely being driven by people with persistent depressive symptoms (Table 4.6, Figure 4.8, hashed bars represent baseline, checked bars represent follow-up). There was no significant change in TNF- α for individuals who showed an improvement in depressive symptoms ($Z = -1.257$, $p = 0.209$) or for individuals who did not show an improvement in depressive symptoms ($Z = -0.840$, $p = 0.401$).

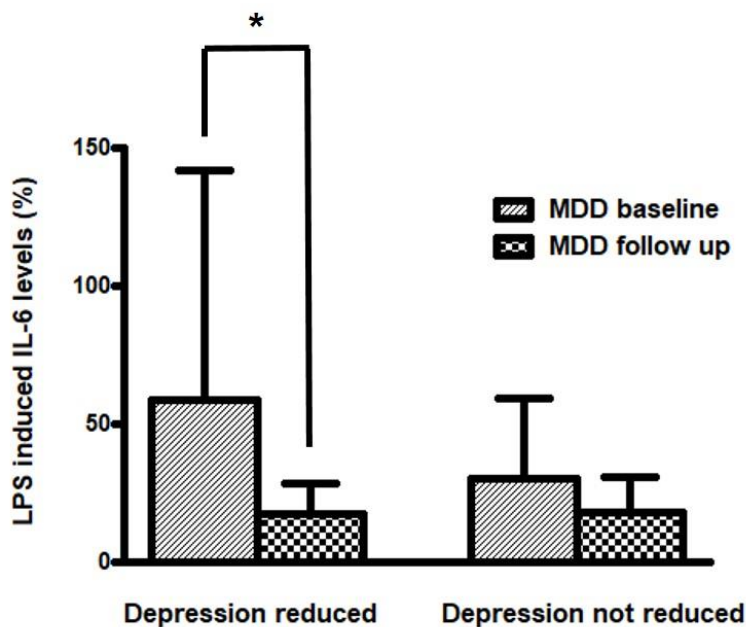
Regarding corticosteroid receptor sensitivity, there was a significant increase in MR sensitivity at follow-up for individuals who showed an improvement in depressive symptoms. LPS-stimulated IL-6 levels were lower at follow-up compared with baseline following incubation with the highest concentration of prednisolone, 10^{-6} ($t(20) = 2.491$, $p = 0.022$) (Table 4.6, Figure 4.9, hashed bars represent baseline, checked bars represent follow-up). However for individuals who did not show an improvement in depressive symptoms, whilst there was a reduction in IL-6 levels at follow-up, this did not reach significance ($t(8) = 1.027$, $p = 0.355$). There was no significant correlation between change in plasma IL-6 and change in MR sensitivity in either group (depression decreased: ($r = -0.029$, $p = 0.902$); depression not decreased ($r = -0.067$, $p = 0.865$)). Stratification by symptom recovery had no effect on GR sensitivity or saliva cortisol findings (all $p = >0.05$).

Figure 4.8. Plasma IL-6 levels at baseline and follow-up according to symptom reduction.



People with MDD who experienced a reduction of depressive symptoms ($n=25$); no reduction ($n=9$). Data shown are mean \pm SD in pg per ml. * statistically significant at $P<0.05$.

Figure 4.9. Prednisolone suppression of LPS induced IL-6 at the highest concentration, 10^{-6} , at baseline and follow-up, according to symptom resolution.



People with MDD who experienced a reduction of depressive symptoms ($n=21$); no reduction ($n=9$). Data shown are mean \pm SD in pg per ml. * statistically significant at $P<0.05$.

Table 4.6. Mean inflammatory and corticosteroid receptor sensitivity parameter values and p values from analyses comparing differences in people with MDD at baseline and follow-up according to depressive symptom resolution (n=34)

	Depressive symptoms reduced (n=25)			Depressive symptoms not reduced (n=9)		
	Baseline	Follow-up	P-value	Baseline	Follow-up	P-value
	<i>Mean ± SD or median ± interquartile range</i>	<i>Mean ± SD or median ± interquartile range</i>		<i>Mean ± SD or median ± interquartile range</i>	<i>Mean ± SD or median ± interquartile range</i>	
Plasma IL-6 (pg/ml) (n=34)	0.75 ± 0.72	0.86 ± 0.64	0.686	0.85 ± 1.03	1.68 ± 1.21	0.021
Plasma TNF-α (pg/ml) (n=34)	*0.92 ± 0.38	*0.84 ± 0.45	0.209	*0.99 ± 0.56	*1.10 ± 0.56	0.401
IL-6 concentration with LPS only (pg/ml) (n=32)	171.14 ± 116.88	154.31 ± 95.18	0.933	113.30 ± 70.08	113 ± 89	0.106
% LPS induced IL-6 levels with DEX 10 ⁻⁶ (pg/ml) (n=29)	24.50 ± 32.18	9.18 ± 7.37	0.055	13.20 ± 15.21	9.98 ± 9.38	0.798
% LPS induced IL-6 levels with DEX 10 ⁻⁷ (pg/ml) (n=31)	31.71 ± 40.46	13.26 ± 8.93	0.130	22.99 ± 24.84	12.61 ± 10.38	0.336
% LPS induced IL-6 levels with DEX 10 ⁻⁸ (pg/ml) (n=32)	91.21 ± 97.21	46.83 ± 17.28	0.251	61.81 ± 40.54	53.14 ± 31.39	0.513
% LPS induced IL-6 levels with DEX 3 x 10 ⁻⁹ (pg/ml) (n=32)	136.90 ± 93.97	98.17 ± 39.31	0.452	81.81 ± 39.57	83.21 ± 32.28	0.793
% LPS induced IL-6 levels with PRED 10 ⁻⁶ (pg/ml) (n=30)	58.54 ± 83.23	17.23 ± 11.04	0.022	29.89 ± 29.49	17.98 ± 12.61	0.335
% LPS induced IL-6 levels with PRED 10 ⁻⁷ (pg/ml) (n=32)	98.28 ± 104.46	48.10 ± 20.13	0.346	53.78 ± 49.04	41.82 ± 20.29	0.700
% LPS induced IL-6 levels with PRED 10 ⁻⁸ (pg/ml) (n=32)	156.78 ± 99.50	103.76 ± 53.87	0.126	112.14 ± 84.81	81.57 ± 44.67	0.189
% LPS induced IL-6 levels with PRED 3 x 10 ⁻⁹ (pg/ml) (n=30)	188.62 ± 128.74	132.41 ± 83.96	0.410	113.46 ± 88.71	116.26 ± 59.67	0.662
DEX IC ₅₀ (n=20)	8.44E-8 ± 1.72E-8	1.71E-8 ± 1.62E-8	0.173	7.97E-8 ± 1.59E-7	2.38E-8 ± 2.23E-8	0.345
PRED IC ₅₀ (n=12)	1.21E-7 ± 2.07E-7	2.13E-7 ± 2.24E-7	0.542	1.78E-7 ± 3.06E-7	2.36E-7 ± 2.52E-7	0.638

Abbreviations: MDD = major depressive disorder; SD = standard deviation; IL = interleukin; TNF = tumour necrosis factor; pc = picogram; ml = millilitre; LPS – lipopolysaccharide; DEX = dexamethasone; PRED = prednisolone. Mean and median scores represent raw values. *median/interquartile range.

4.6 Discussion

4.6.1 *Aims and hypotheses*

The aim of this study was to observe any changes in inflammatory and neuroendocrine biomarkers in depressed people at baseline and six week follow-up, and explore whether these changes predicted any change in depressive symptoms. I hypothesised that any change in depressive symptoms will be influenced by baseline levels of inflammation and predicted by change in inflammation over time (hypothesis 1). I hypothesised that any change in depressive symptoms will be influenced by corticosteroid sensitivity at baseline and predicted by change in corticosteroid sensitivity over time (hypothesis 2). I hypothesised that any change in depressive symptoms will be influenced by diurnal cortisol rhythm at baseline and predicted by change in cortisol rhythm over time (hypothesis 3). Finally I hypothesised that any change in inflammatory cytokines will be associated with change in CAR AUC/AUC (hypothesis 4).

4.6.2 *Summary of results*

The results showed that hypothesis 1 was not confirmed: there were no significant changes in inflammatory biomarkers at follow-up. Hypothesis 2 was partially confirmed: there was a significant increase in MR sensitivity over time and the reduction in BDI score at follow-up was not robust to adjustment for MR sensitivity at baseline, however there was no interaction between the two variables and the change in sensitivity was not a significant predictor of change in BDI score. Hypothesis 3 was not confirmed: there were no significant differences in diurnal cortisol at follow-up. Finally hypothesis 4 was not confirmed: there were no significant association between change in inflammatory cytokines and change in CAR AUC/AUC.

4.6.3 Hypothesis 1: Differences in inflammatory biomarkers

We did not observe any significant change in inflammation, despite observing a significant reduction in depressive symptoms. Furthermore IL-6 appeared to be increased, albeit not significantly, at follow-up. However, this may be explained by the high variability in our IL-6 data, which had high standard deviations relative to the mean values. This suggests that the mean score is a poor fit of the observed data and is unlikely to represent the sample (Field, 2017). Furthermore, the change in IL-6 varied from -2.46 to 3.67pg/ml, suggesting that some participants exhibited an increase in IL-6, whilst others exhibited a decrease. Sensitivity analyses showed that the observed increase in IL-6 was primarily driven by individuals who did not show any clinical recovery, suggesting that increased inflammation may be associated with persistent depressive symptoms.

This interpretation is also in line with a study which showed that cytokine levels are associated with treatment response following antidepressant therapy. Plasma IL-6 levels have been shown to be reduced in treatment responders compared with non-responders, suggesting that higher IL-6 activity is associated with the refractoriness of depression, and might be a predictor of treatment response (Yoshimura et al., 2009). A meta-analysis by Hiles et al. (2012a) provided evidence which demonstrated that with a reduction in depression, there is a co-occurring reduction in inflammation. Strawbridge et al. (2015) examined data from 35 studies and reported that IL-6 levels decreased following antidepressant treatment, regardless of outcome. Similar findings have been reported regarding of non-pharmacological therapies. A reduction in depressive symptoms following CBT therapy has been associated with reductions in IL-6 in un-medicated women (Gazal et al., 2013). A systematic review by Lopresti (2017) also reported that CBT may reduce inflammatory biomarkers.

Our results showed that TNF- α levels were decreased at follow-up, however this finding did not reach significance. The literature regarding TNF- α and clinical remission are inconclusive. Some studies have shown that levels of TNF- α are lower following antidepressant treatment, although the effect size was small (Kohler et al., 2018). Other studies have not found any significant effect of antidepressant treatment on TNF- α levels (Hannestad et al., 2011; Wiedlocha et al., 2018). This inconsistency in the literature may be explained by the finding that decreases in TNF- α may be restricted to treatment responders (Strawbridge et al., 2015). The meta-analyses by Hannestad et al (2011) and Wiedlocha et al. (2018), which reported null findings, did not consider treatment response.

Treatment responders are usually classified as those who demonstrate a 50% reduction in depressive symptoms (Hiles et al., 2012a; Masson & Tejani, 2013). The *minimum clinically important difference*, defined as the minimum change in an outcome in which the patient perceives a difference (usually because of a therapy/intervention), has been identified as a 30% reduction by one study (Wilson, 2008) and as a change of at least 5 points on the BDI after treatment according to two studies (Dworkin et al., 2008; Hiroe et al., 2005). In the present study, the mean reduction in depressive symptoms was modest, 22.61% or 5.56 points on the BDI-II. This is not surprising given that this was an observational study and we did not intervene in treatment. Therefore it is possible that the small reductions in depressive symptoms we observed reflect small reductions in inflammation, which we were underpowered to detect.

4.6.4 Hypothesis 2: Differences in corticosteroid receptor function

We did not observe any significant change in GR sensitivity. Whilst LPS-induced IL-6 levels were lower for all DEX concentrations at follow-up, none of them reached significance. There was however, a trend for lower DEX IC₅₀ values at follow-up compared to baseline, suggesting that depressed people required a lower concentration

of DEX to inhibit LPS-induced IL-6 by 50% at follow-up and therefore may have experienced an improvement in GR function. However, neither baseline DEX IC₅₀ values nor change in DEX IC₅₀ values were significant predictors of change in BDI over time.

Studies investigating the effects on antidepressants have provided evidence of normalized HPA-axis feedback after successful treatment (Heuser et al., 1996; Linkowski et al., 1987). An *in vitro* study of GR function has shown that following treatment, an increase in GR number is concurrent with a tendency to improve depressive symptoms (Calfa et al., 2003). *In vivo* studies have reported similar results. Depressed in-patients who were also non-suppressors to the DST, demonstrated progressive normalisation of DST responses in conjunction with clinical improvement (Greden et al., 1983). Other studies have reported findings which support an opposite direction of effect. Patients with initially abnormal DST results have demonstrated clinical remission three weeks after normalisation of the HPA-axis (Holsboer et al., 1982). However, other studies report no difference in DST suppression between the responders and non-responders (Ventura-Junca et al., 2014). A recent meta-analysis showed that cortisol levels following the DST were higher in non-responders (S. Fischer et al., 2017a). Similar results have been reported following the DEX/CRH test. Reductions in cortisol and ACTH response have been associated with SNRI and NaSSA treatment (Schule et al., 2006) and SSRI and QXR treatment (Sarubin et al., 2014). Successful electroconvulsive therapy may also be effective in normalising cortisol response in the DEX/CRH test (Kunugi et al., 2006; Yuuki et al., 2005).

These findings concur with studies investigating severely treatment-resistant patients. Bauer et al. (2003) showed that TRD patients demonstrated reduced inhibition of T-cell proliferation and cytokine production following both *oral and in vitro* administration of DEX. Similarly, Carvalho et al. (2008) showed that clomipramine increased glucocorticoid inhibition of LPS induced IL-6 levels in the whole blood in healthy controls

but not in TRD patients. Together, these results suggest that recovery of GR function is dependent on clinical remission.

We did observe a trend for improved GR function and so may speculate on possible mechanisms of action. In the present study, one third of the depressed participants were taking antidepressants, therefore it is possible that the trend for improved GR function was a neurobiological effect of the medications. Four days of treatment with citalopram has been shown to increase both glucocorticoid and mineralocorticoid receptor sensitivity in healthy humans (Pariante et al., 2004). Antidepressants may target the GR directly at various functional points, including GR mRNA expression, nuclear translocation of the GR and GR-mediated gene transcription (Anacker et al., 2011). The exact mechanism of action is still unidentified but one possibility is that antidepressants enhance GR translocation and function via signal transduction pathways involving cyclic adenosine monophosphate (cAMP) (Rangarajan et al., 1992). Miller et al. (2002a) showed that the phosphodiesterase type-4 inhibitor, rolipram, which antagonizes cAMP breakdown and therefore enhances cAMP-dependent protein kinase A activation, increases GR-mediated gene transcription *in vitro* and significantly enhances GR function. Antidepressants also influence other intracellular protein kinases (Nalepa & Vetulani, 1991) which may be involved in *decreases* in GR-mediated gene transcription (Budziszewska et al., 2000). Upon immediate glance this may appear to suggest a reduction in GR function, however decreased GR expression can also reflect increased GR-mediated gene transcription and subsequent GR downregulation, and therefore reflect an increase in GR sensitivity. Finally, antidepressants may indirectly enhance GR function by inhibiting membrane transporters, which eject glucocorticoids from the cytoplasm, thus increasing the intracellular concentration of glucocorticoids (Pariante et al., 2003).

There was a significant improvement in MR sensitivity, as demonstrated by increased LPS-stimulated IL-6 levels in the presence of the highest concentration of PRED, 10^{-6} .

The observed reduction in BDI score at follow-up was not robust to adjustment for baseline MR sensitivity, and there was no significant interaction between the two. Additionally, the change in MR sensitivity was not a significant predictor of change in BDI score. However, exploratory analyses showed that the improvement in MR sensitivity was only significant in individuals who demonstrated a reduction in BDI-II score (n=21), suggesting that clinical recovery may be moderated by MR function.

To my knowledge only one study has investigated MR function and treatment response. Juruena et al. (2010) reported that the cortisol response to the PST did not change in depressed people following antidepressant treatment, even in those who responded, suggesting that MR function is not influenced by antidepressants or symptom remission. The same authors also found that depressed patients tend to have a normal response to prednisolone even in the presence of an impaired response to dexamethasone, suggesting that there is no impairment of MR function associated with depression (Juruena et al., 2006; Juruena et al., 2009). In contrast, we have shown that depressed people do exhibit impairment in MR function and do demonstrate improvement alongside clinical recovery.

One possible explanation for the divergent findings may be related to treatment resistance. Although, participants in both studies were all classified as having unipolar MDD according to either DSM-IV or ICD-10 criteria, patients in the Juruena studies described above (2006, 2010; 2009) were all considered to be treatment resistant on the basis of previous non-response to at least two different antidepressants. In the present study, we did not collect data regarding previous antidepressant use, however 65.7% of the depressed participants had experienced previous depression. Therefore we were able to conduct secondary analysis to explore whether recurrent depression may play a role in MR function. We repeated the prednisolone suppression analysis on a subset of individuals who had a history of at least one previous episode (n=19). Results showed that there was no longer a significant difference in the effects of in vitro incubation with

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the highest concentration of PRED between the time points ($t(18) = 1.483, p = 0.155$). This finding suggests that MR function may be moderated by clinical improvement but not in those with recurrent symptoms.

4.6.5 Hypothesis 3: Differences in diurnal cortisol

Whilst we did not observe any significant difference in diurnal cortisol rhythm over the time-points, the findings for the CAR AUC did show a trend towards an increase. This trend lends some support to a study by Ruhe et al. (2015) who reported increased CAR in severely depressed patients who achieved remission following antidepressant therapy. However in the present study, we did not find that the trend in increased CAR was associated with change in symptoms. This may reflect the relatively small decrease in depressive symptoms we observed. In the Ruhe et al. study, remission was defined as ≤ 7 on the Hamilton Depression Rating Scale (HDRS) which denotes normal mood (Zimmerman et al., 2013). A score of ≤ 13 indicates normal mood on the BDI-II (A. T. Beck et al., 1996a). Only 4 of the participants in our study had a BDI score of ≤ 13 , therefore we were unable to determine whether clinical remission was associated with CAR AUC.

The trend observed in our study also lends some support to findings from a large cohort study which reported significantly higher CARs in remitted patients compared to controls (Vreeburg et al., 2009a) and another study which also found higher CARs in drug-free remitted patients (Aubry et al., 2010). In contrast, decreased CAR has also been associated with antidepressant response in depressed people (J. Beck et al., 2015). Evidence from healthy populations is also inconclusive. SSRI administration has been found to increase (Harmer et al., 2003; Ronaldson et al., 2018) and decrease (U. Knorr et al., 2012) waking cortisol.

We also observed a trend towards a steeper cortisol slope at follow-up, although this was not associated with improvement in clinical symptoms. A steeper cortisol slope can be due to increased waking or decreased evening cortisol values. In the present study whilst there was no significant change in evening cortisol ($p = >0.05$), suggesting that the alteration in cortisol slope was driven by the increase in CAR AUC. In chapter 3 we reported that the CAR AUC was reduced in the MDD group, compared with controls, a finding which is supported by several other studies (Dedovic et al., 2010; Doolin et al., 2017; Huber et al., 2006; Stetler & Miller, 2005). Increased waking cortisol is an adaptive phenomenon, preparing the body upcoming challenges in the day ahead and has been associated with increased cognition (Kramer et al., 2019; Law et al., 2013). Therefore if this mechanism fails, as demonstrated by a blunted CAR/CAR AUC, this may result in depressive symptoms. In contrast increased CAR has also been associated with depression (Dienes et al., 2013; Rhebergen et al., 2015; Ulrike et al., 2013; Vreeburg et al., 2009a). Furthermore, both hyperactivity and hypo-activity of the HPA-axis have been associated with depression relapse (Bockting et al., 2012; Hardeveld et al., 2014). It may therefore be that symptom remission is associated with normalisation of the HPA-axis, regardless of the direction of the initial dysfunction.

It is difficult to compare our results with previous work because the relationship between depression remission and cortisol slope has not yet been explored. One study has investigated the effects of antidepressants and cortisol slope in healthy people. Ronaldson et al. (2018) reported that women receiving SSRIs had significantly steeper cortisol slopes compared with those receiving placebo but did not observe any effect of medication on cortisol slope in men. In order to test whether there was any effect of sex on our findings, secondary analysis was conducted. There was no main effect or interactive effect of sex on cortisol slope (both p values > 0.05).

Antidepressants have been shown to have differential effects on cortisol. TCA use has been associated with flattened CAR compared to other antidepressants in depressed

people (Manthey et al., 2011). SSRI has been shown to enhance the CAR in healthy people compared to SNRI which had no effect (Harmer et al., 2003). However a recent review of the effects of psychotropic medication on cortisol reported that antipsychotics and antidepressants are associated with a reduction in cortisol secretion (Subramaniam et al., 2019). In the present study all depressed individuals who were taking antidepressants were receiving SSRI medications. One individual was receiving benzodiazepine medication and one was receiving antipsychotic medication. Therefore if there was any effect of medication on cortisol in this study, it is likely to be a mechanism of SSRI therapy.

In terms of mechanisms, there are several ways in which SSRIs could alter HPA axis function. The serotonin system and HPA axis are intricately related (Porter et al., 2004). Serotonin receptor agonists have been shown to induce serum cortisol in healthy people (Pitchot et al., 2002) and plasma cortisol in rats (Mikkelsen et al., 2004). SSRI medication may therefore modulate cortisol levels via the serotonin receptors. Immunohistochemical studies have demonstrated that serotonin receptors are present on neurons in the PVN of the hypothalamus – a structure that initiates the activation of the HPA-axis (Lanfumeij et al., 2008).

SSRIs may also influence the HPA-axis via their effect on melatonin levels. Several studies report lower plasma melatonin levels in depressed patients compared with controls (Beck-Friis et al., 1985a; Beck-Friis et al., 1985b; Paparrigopoulos, 2002). There is evidence that melatonin modulates the HPA-axis and restores function, particularly in the context of stress from animal studies. Rats demonstrate hypertrophy of the adrenal and pituitary glands following pinealectomy (Konakchieva et al., 1997; Wurtman et al., 1959), decreased adrenocortical response when stressed and treated with melatonin and increased HPA-axis sensitivity to the DST (Konakchieva et al., 1998). Melatonin has also been found to increase GR mRNA expression (N. Barden et al., 2005) and inhibit GR function in mice (Presman et al., 2006). Studies in depressed patients suggest that

antidepressants increase circulating melatonin levels (Bearn et al., 1989; Kennedy & Brown, 1992; Palazidou et al., 1992; Schmid et al., 2006; Thompson et al., 1985). A study by Carvalho et al. (2009) showed that melatonin synthesis was increased following antidepressant treatment compared with placebo, independently of clinical remission.

As previously discussed, SSRIs could influence the HPA-axis via modulation of the GR and in the present study we observed a trend for improved GR function. Flatter cortisol slopes have previously been associated with impaired GR sensitivity (Jarcho et al., 2013). In light of this, we assessed the correlations between GR sensitivity and cortisol slope in depressed people at baseline and follow-up and found no significant associations ($p = >0.05$).

4.6.6 Hypothesis 4: Inter-correlations between the systems

In line with our hypothesis, we observed a negative pattern of association between change in TNF- α and change in the CAR, suggesting that as TNF- α decreases the CAR increases, however this did not reach significance. We interpret this finding as follows: TNF- α and the HPA axis have a reciprocal relationship, mutually influencing each other. Increases in TNF- α stimulate the HPA axis, while the HPA axis suppresses TNF- α production. It is possible that in people with chronic or recurrent MDD, HPA-axis hyperactivity might eventually exhaust the regulatory mechanisms underlying the CAR, resulting in an impaired cortisol response to stress. This reduced cortisol response might then be less capable of inhibiting TNF- α production. Upon clinical improvement the HPA-axis normalises and the CAR increases, gaining back control over the TNF- α system. This may explain why we might observe an increase in CAR in association with a decrease in TNF- α in a group of people with MDD who demonstrate a reduction in depressive symptoms.

This finding is in contrast to those of Himmerich et al. (2006) who examined alterations in TNF- α and response to the DEX/CRH test on admission and at discharge in MDD inpatients. They reported a positive correlation between TNF- α and CRH/DEX test outcome in remitted patients following successful antidepressant treatment and normalisation of the HPA-axis. Their interpretation of this finding is that during a depressive episode, HPA-axis activity is increased, overriding the suppressive effects of TNF- α . However, the inhibitory effects of the HPA axis on TNF- α are still active, leading to a suppression of TNF- α production in depressed patients with a hyperactive HPA-axis. Upon symptom remission, the HPA axis normalizes although, potentially increasing TNF- α levels. This suggests that clinical remission is associated with increased TNF- α control over the HPA-axis. An inverse association between TNF- α and the neuroendocrine response to the DEX/CRH test in MDD has also been reported by Schuld et al.(2003) cross-sectionally.

In light of these conflicting findings it may be that symptom remission is associated with normalisation of the HPA-axis, and that the direction of the initial dysfunction determines the associated effect on TNF- α . Therefore in the case of hyperactivity as demonstrated by Himmerich et al. (2006), as the HPA-axis system normalises, TNF- α levels increase. However in the case of HPA-axis exhaustion as implied by the findings of our study, as the HPA-axis system normalises, TNF- α levels decrease. Larger longitudinal studies exploring TNF- α and HPA-axis function in MDD are required to disentangle this relationship.

4.6.7 *Strengths and limitations*

Many of the limitations of the present study were discussed in detail in Chapter 3 which described the cross-sectional analysis. The main strength of this study is that it is the first to investigate changes in inflammation, GR and MR function and diurnal cortisol

rhythm simultaneously in MDD patients, over time. Another strength of this study is the low attrition rate, with ascertainment of clinical outcomes at follow-up in 95% of depressed participants, however as previously mentioned sample size was relatively small (n=35) which likely limited statistical power. It's important to note that this was an observational study and we did not interfere with any pharmacological or psychological treatment. Furthermore, patients were taking several different SSRI medications and some were receiving psychotherapy. Sample size limited the number of sub-group analyses we could conduct, therefore we cannot be certain of any specific treatment effects.

However, despite the lack of study intervention, we still observed a decrease in depressive symptoms over time. It is not clear why we observed this improvement in symptoms. Whilst the baseline assessment was not framed as a therapeutic encounter, it is possible that participants found the interview helpful and provided them with an opportunity to "get things off their chest". For individuals who have few opportunities to talk about mental health problems, such interviews have potential benefits (Latkin et al., 2017). It is therefore possible that the improvement in clinical symptoms we observed may only have been transient. Furthermore, this finding may possibly reflect demand characteristics, where participants who had a positive experience during the baseline interview may have unconsciously responded more optimistically to the questionnaires at follow-up, in line with a misinterpretation of the aim of a study.

Our study was also limited by a relatively short follow-up period. Antidepressant treatment duration ranged from 1-36 months, therefore it is possible that some people were waiting for any therapeutic effect to occur. Larger studies of patients with MDD, with longer follow-up are needed to establish the robustness of the findings.

4.6.8 *Conclusion*

In conclusion, these results indicate that improvements in depressive symptoms are accompanied by increases in MR sensitivity. In addition they may be accompanied by improved GR sensitivity and CAR AUC. These findings also tentatively imply that changes in TNF- α might be associated with changes in the CAR. However no firm conclusions can be made regarding these findings. Further longitudinal studies are required to fully explore these relationships.

5. Literature review: Regulatory T cells and depression

5.1 T-cells and depression

In addition to evidence for increased innate immune responses, a body of evidence is now beginning to emerge suggesting a role for the adaptive immune system in both the pathophysiology and resolution of depression (A. H. Miller, 2010;Toben & Baune, 2015). As previously mentioned, the adaptive arm of the immune system largely consists of lymphocytes that exert a delayed response to inflammatory challenge. Lymphocytes are broadly categorised into B cells and T cells. Activated B cells orchestrate the humoral response via antibody secretion and activated T cells differentiate into either CD8⁺T cells or CD4⁺T cells (Herkenham & Kigar, 2017). CD8⁺ or 'cytotoxic T cells' act as killer cells, destroying infected cells. CD4⁺T cells are divided into subtypes: 'T helper (Th) cells', which initiate immune responses and regulatory T cells (Tregs) which are immunosuppressive. Most studies into adaptive immunity and depression have focused on CD4⁺ and CD8⁺ proliferation.

Alterations in T cell function were first observed in patients with severe MDD. These patients demonstrate significantly decreased PBMC proliferation in response to T cell mitogens (Kronfol et al., 1983;Schleifer et al., 1984). Whilst subsequent studies have reported conflicting results, meta-analyses have reported robust decreases in T cell responses in both stressed and depressed individuals (T. B. Herbert & Cohen, 1993;Irwin & Miller, 2007;Zorrilla et al., 2001). Decreased T cell function in depressed patients has also been demonstrated *in vivo* using skin response to antigens (Hickie et al., 1993;Sephton et al., 2009). Meta-analyses have also demonstrated that T cell number and *in vitro* proliferation is significantly reduced in patients with depression (Irwin & Miller, 2007;Zorrilla et al., 2001). Increases in the CD4⁺/CD8⁺ ratio (Zorrilla et al., 2001) as well as decreases (Pavon et al., 2006) have also been observed. Currently it is unclear what the clinical implications of this imbalance might be on depression.

The exact mechanisms involved in T cell responses is not yet understood, however a number of hypotheses have been suggested. Firstly, there is evidence of accelerated apoptosis of CD4⁺T cells in patients with depression (Eilat et al., 1999;Ivanova et al., 2007;Agnieszka Szuster-Ciesielska et al., 2008). Chronic stress also induces T-cell apoptosis in humans and animals (Sakami et al., 2002;Shi et al., 2003). This increase in apoptosis could be explained by tryptophan depletion (A. H. Miller, 2010). As previously mentioned, cytokines induce IDO, which converts tryptophan to kyurenine, resulting in reduced serotonin synthesis and increased depression (Dantzer et al., 2008;Hestad et al., 2017). Tryptophan is also an essential proliferative stimulus for effector T cells, and in situations of tryptophan depletion T cells undergo apoptosis (Beissert et al., 2006;Mellor et al., 2003).

Secondly, T-cells could potentially be suppressed by glucocorticoids. As previously discussed, activation of the HPA-axis and subsequent cortisol release is a characteristic of depression. Furthermore, glucocorticoids are known to inhibit inflammation, mediate cell redistribution and initiate T-cell apoptosis (McEwen et al., 1997). Despite the intuitive appeal of this explanation, no association has been found between cortisol levels and *in vitro* decreased T-cell proliferation in response to mitogen stimulation in patients with depression (Kronfol et al., 1986). Further evidence against this hypothesis comes from more recent studies which demonstrate that the inhibitory effect of cortisol on *in vitro* T-cell proliferation is actually reduced in depressed patients (Bauer et al., 2003;Pariante & Miller, 2001;Raison & Miller, 2003). Evidence also shows that dexamethasone induced cell redistribution is greater in healthy controls compared to TRD patients (Bauer et al., 2002). This may be the result of insufficient glucocorticoid signalling. Several studies have reported a reduction in cytosolic GR binding in PBMCs (Pariante & Miller, 2001). As previously discussed, this may be a result of exposure to pro-inflammatory cytokines.

Furthermore, inflammatory cytokines, such as TNF- α , may also directly impair T-cell function in patients with depression. As this thesis has already shown, TNF- α is robustly associated with depression (Dowlati et al., 2010;Haapakoski et al., 2015;Howren et al., 2009). Chronic exposure of TNF- α to T cells has been shown to decrease both proliferation and cytokine production in murine models and in patients with rheumatoid arthritis, both *in vitro* and *in vivo* (Cope et al., 1997;Cope et al., 1994;L. F. Lee et al., 2008). Moreover, T cell function can be restored by treatment with anti TNF- α monoclonal antibodies such as infliximab and adalimumab (Bayry et al., 2007;Cope et al., 1994;L. F. Lee et al., 2008).

T cell function related polymorphisms have also been identified. A study by Wong et al. (2008) found that two SNPs critical for antigen processing and T cell differentiation, *PSMB4* (proteasome beta4 subunit) and *TBX21* (T bet) were associated with susceptibility to MDD. Furthermore, analyses revealed a dose-response effect, with participants who had three risk alleles being 10 times more likely to have the diagnosis of MDD. They also identified four other SNPs important to T cell function which were associated with antidepressant response (*CD3E*, *PRKCH*, *PSMD9* and *STAT3*). Another SNP in a proteasome gene (*PSMD13*), known to contribute to T cell function, has been shown to confer a two-fold greater risk for treatment resistance depression (Minelli et al., 2015). These findings suggest that T cell abnormalities may be involved in the aetiology of depression.

T cell dysfunction could also explain the physical comorbidities associated with depression (A. H. Miller, 2010). As discussed earlier this disorder is associated with a wide variety of physical health conditions, including infectious disease and cancer. Leserman et al. (2008) conducted a systematic review of studies investigating depression and HIV progression and reported that chronic depression is associated with decreases in CD4⁺T cells and greater risk for clinical decline and mortality in HIV patients. Similarly data from the HIV Epidemiology Research Study showed that HIV+

women with chronic depressive symptoms had a significantly reduced CD4⁺T cell count and were 2 times more likely to die than women with no depressive symptoms (Ickovics et al., 2001). In a study involving 72 women with metastatic breast cancer, women reporting more depressive symptoms showed suppressed T cell immunity indicated by a decreased antigen response (Sephton et al., 2009). Interestingly suppressed T cell immunity was also associated with higher mean diurnal cortisol concentrations, highlighting the interaction between inflammatory and neuroendocrine function.

5.2 Regulatory T cells

Whilst immune activation is an obvious requirement for defence against pathogens, like all biological process it needs to be controlled or risks becoming pathological. An overactive or inappropriate immune response can lead to excess inflammation, resulting in tissue damage and loss of organ function (Plitas & Rudensky, 2016). A subset of CD4⁺T cells with immunosuppressive properties, known as regulatory T cells, or Tregs, are known to suppress the immune response, thereby maintaining immune homeostasis. These cells act as messengers in the negative feedback loop of the immune response and are the primary component in the maintenance of self-tolerance and prevention of autoimmunity (Corthay, 2009). They are synthesized during inflammation to regulate the production of pro-inflammatory cytokines and control unwanted immune responses by various mechanisms, such as inhibition of antigen presenting cells, killing effector cells, metabolic disruption via degradation of adenosine triphosphate and the secretion of anti-inflammatory cytokines such as IL-10, IL-35 and TGF- β (Grant et al., 2015). Indeed, animal studies demonstrate that depletion of Tregs in healthy individuals provokes an acute inflammatory response that leads to lethal autoimmunity (Fontenot et al., 2003). However, whilst Tregs control inflammation, inflammation also controls Tregs (Klatzmann & Abbas, 2015). In the context of a highly inflammatory environment, Tregs become unstable and lose their suppressive function (DuPage & Bluestone, 2016).

Tregs are characterised by expressing the CD4 T cell co-receptor, the interleukin (IL2) receptor α -chain (CD25) and the lineage-specific transcription factor Foxp3 and are identified as CD4⁺CD25⁺FoxP3⁺ Tregs (Plitas & Rudensky, 2016). Additional highly expressed markers on Tregs include, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), glucocorticoid-induced tumour necrosis factor receptor family-related gene (GITR), lymphocyte activation gene-3 and CD127 (Corthay, 2009). However growing evidence suggests that these markers may not be strictly Treg specific, and appear to be markers of general T cell activation. A truly specific molecular marker for Treg cells has yet to be elucidated.

Multiple studies have assessed the frequency of Tregs in PBMCs of patients with autoimmune diseases, often with conflicting results. Studies investigating Tregs in rheumatoid arthritis have reported both increases (Han et al., 2008;van Amelsfort et al., 2004) and decreases in Treg percentages in patients compared with controls (Lawson et al., 2006), whereas others have found no evidence of any difference (Cao et al., 2003;Mottonen et al., 2005). Similarly in multiple sclerosis increases (Kumar et al., 2006), decreases (Venken et al., 2008) and no differences (Feger et al., 2007;Haas et al., 2005) in Tregs have been reported. Increased Tregs have also been observed in type 1 diabetes (Marwaha et al., 2010), whilst others have reported null findings (Brusko et al., 2005;Lindley et al., 2005). Treg deficiency has been reported in patients with IPEX syndrome (immunodysregulation, polyendocrinopathy and enteropathy, X-linked) which is associated with autoimmune disease in multiple endocrine organs (Wildin et al., 2002). The only research to demonstrate consistent findings has been regarding SLE. Patients with SLE reliably show decreases in Tregs and this is associated with disease severity (Gerli et al., 2009). A defect in the suppressive ability of Tregs has also been observed in several autoimmune diseases (Costantino et al., 2008).

5.3 Regulatory T cells in depression

Given the role of Tregs in modulating cytokine production and the increased cytokine levels observed in MDD, it seems plausible that Tregs may be implicated in the aetiology of depression. Tregs produce the anti-inflammatory cytokines IL-10 and transforming growth factor- β (TGF- β) (Haroon et al., 2012) and decreased levels of IL-10 and TGF- β , as well as an increased IL-6/IL-10 ratio, have been observed in depressed people (Dhabhar et al., 2009; L. Sutcgil et al., 2007). Nevertheless, there is limited data investigating Tregs in depression. Studies using animal models of depression have reported a decreased frequency of Tregs. Reduced Tregs have been significantly associated with the onset of depressive-like behaviour in mice, in the context of a chronic unpredictable mild stress paradigm (Hong et al., 2013). Treg cell depleted mice also exhibited anxious and depressive behaviors in an elevated plus maze, tail suspension task, and forced swim test when compared with the non-stressed wild-type group (S.-J. Kim et al., 2012). In a rat model of postnatal depression, Tregs were decreased and negatively associated with serum IL-1 β and IL-6 (J. Li et al., 2016a). In addition, treatment with the antidepressant fluoxetine resulted in a subsequent increase in Tregs.

In humans, the effect of acute psychological stress on Tregs has also been explored. In a study by Freier et al. (2010), 31 healthy young males underwent a brief laboratory stressor and effects on Tregs and other T cell subpopulations were measured. A significant stress-induced decrease in Tregs and an increase in T effector cells was observed, suggesting a shift towards an immune-enhancing environment. In contrast, an increase in the proportion of Tregs was also observed in 41 undergraduate students in response to examination stress, which was accompanied by an increase in inflammatory cytokines (Hoglund et al., 2006). The association between inflammatory and neuroendocrine responses to acute stress and Treg frequency have also been investigated. In a study including 121 healthy older men and women from the Whitehall II cohort, a blunted cortisol and elevated IL-6 response to acute psychophysiological

stress testing was associated with a greater Treg percentage three years later (Ronaldson et al., 2015). Furthermore, the percentage of Tregs was independently associated cross-sectionally with higher levels of depressive symptoms.

The course of Treg abnormalities have been explored in individuals at risk for mood disorders. T cell subsets from 140 children of a parent with bipolar disorder were assessed at three time-points: adolescence, young adulthood and adulthood. Tregs exhibited a dynamic course over time with reduced levels of Tregs in adolescence, which were correlated with a high expression of pro-inflammatory genes in monocytes, and a reduced relative number of Th1, Th17 cells in young adulthood (Snijders et al., 2016). Th1 cells produce IFN- γ , IL-2, and TFN- β (Romagnani, 1999) and Th17 cells produce the highly inflammatory cytokine IL-17 (Tesmer et al., 2008). Both Th1 and Th17 cells are thought to play an important role in the induction of autoimmune diseases. This study demonstrated a high inflammatory state at adolescence and an anti-inflammatory state in young adulthood. In adulthood these states normalized. No significant associations between Tregs and mood disorders were found. Tregs have also been shown to be increased in women with postnatal depressive symptoms (Krause et al., 2014). In a study of 100 pregnant women, Tregs were shown to be increased both prenatally and postnatally in women with postnatal depression. Furthermore, Tregs also strongly predicted postnatal depressive symptoms

Few studies have investigated Treg frequencies in psychiatric populations. Sommershof et al. (2009) reported a 48% reduction in the proportion of Tregs in 19 PTSD patients compared to 27 controls. Functionally, this reduction was associated with a significantly increased *ex vivo* proliferation of anti-CD3 stimulated T cells, suggesting a potential explanation for the increased susceptibility to infections, and inflammatory and autoimmune diseases observed in PTSD. However findings are conflicting with a similar study finding no differences in the proportion of Tregs between 23 PTSD patients and matched controls, although they did observe a less suppressive phenotype (Jergović et al., 2018).

al., 2014). An increase in the proportion of Tregs in 26 medicated participants with stable schizophrenia compared with and 17 healthy controls has also been reported (D. L. Kelly et al., 2018). Elevated Tregs also correlated with fewer negative symptoms, suggesting that Tregs may contribute to improved negative symptoms in successful treatment in schizophrenia.

Decreases in Treg cell populations have been noted in patients with depression (Table 5.1). A study by Li et al. (2010) reported a decrease in CD4⁺CD25⁺ Tregs, IL-10 and TGF- β and increased IL-2 in 27 patients with MDD compared to 27 healthy controls. They also reported that in the overall sample, Tregs were correlated with inflammatory cytokines, indicating a role for Tregs in the immune imbalance observed in MDD. Furthermore they found lower levels of serotonin in the plasma and 5-HT1a receptor in Tregs. This suggests a potential interaction between serotonin, its receptor, and Tregs in major depression. These findings are in line with the monoamine theory of depression postulates that the underlying pathology of depression is a depletion in the levels of neurotransmitters (Delgado, 2000). Interestingly, serotonin also plays a role in immune signalling in both innate and adaptive immune cells (Ahern, 2011). Further research is needed to investigate the mechanism of action in the interaction between serotonin and Tregs in depression. Another study reported a significant decrease in circulating Tregs and a significant increase in Th17 cells in 40 MDD patients compared to 30 healthy controls (Y. Chen et al., 2011). The study also showed that MDD patients had increased serum IL-17 and a higher mRNA expression of the Th17 cell transcription factor, ROR γ t. Further research is also needed regarding the relationship between Th17 cells and Tregs in the pathogenesis of autoimmunity in MDD.

Grosse et al. (2016b) observed decreased percentages of Tregs, in association with monocyte gene expression 50 MDD patients compared with 58 controls. In addition they also observed increased serum epidermal growth factor (EGF) and increased inflammatory monocyte gene expression (IL-6, IL-1 β and TNF- α), further confirming

inflammatory monocyte activation. EGF has been identified as an inflammatory mediator (Schipper et al., 2012). The authors also found a significant negative correlation of Treg percentages with the inflammatory monocyte gene expression in MDD patients. These findings demonstrate interrelated disruption of both the innate and adaptive immune system in MDD patients.

However there is inconsistency in the literature. A study by Suzuki et al. (2017) compared differences in the percentages of lymphocytes in 54 un-medicated MDD patients and 56 healthy controls. MDD patients demonstrated a significantly increased percentage of CD127^{low}CCR4⁺ Treg cells compared with controls. CCR4 is a chemokine receptor and CCR4⁺ Tregs produce more IL-10 and are thought to have increased suppressive function (Molinaro et al., 2015). Similarly, a study by Patas et al. (2018) reported a significantly higher frequency of CD4⁺CD25⁺CD127^{low/-} Tregs in 20 medication-free MDD patients compared to 20 matched controls. Increases in Tregs were not associated with serum levels of ACTH or cortisol.

Glucocorticoid signaling of adaptive immune cells and associations with pro-inflammatory cytokines was investigated in 35 MDD patients and 35 healthy controls by Hasselmann et al. (2018). The authors measured monocyte and T-cell phenotypes, including Tregs, gene expression of *GR* and *MR* in both monocytes and T cells, diurnal HPA axis activity and serum levels of IL-6, IL-1 β and TNF- α . The results showed a significantly higher number of monocytes but no significant differences in frequencies of and T-cell subset in MDD patients compared with controls. They also found lower expression of *GR* in MDD patients compared with controls, but this was restricted to monocytes only. There was no significant difference in *MR* expression in either cell type, in HPA-axis activity or in any of the cytokines between the groups. These findings suggest that impaired GR signaling may not be mediated by the adaptive immune system.

The effects of anti-depressants on Treg populations have also been investigated. Himmerich et al. (2010) measured Treg frequencies and plasma cytokine levels in 16 patients at the point of referral for a depressive episode. Patients were treated with different kinds of antidepressant drugs according to their doctor's choice and, following six weeks of antidepressant therapy, Treg and cytokine levels were re-assessed, along with improvements in mood. Results showed that levels of Tregs increased significantly after antidepressant therapy, whilst depressive symptoms, IL-1 β serum levels and LPS-stimulated IL-1 β and IL-6 production decreased significantly. The authors also reported that whilst change in depressive symptoms was not correlated with change in Tregs, the more severely depressed the patients were at baseline, the more their Tregs increased.

Following this, Grosse et al. (2015) investigated whether antidepressant therapy was predictive of clinical response. Leukocyte subsets were examined in 40 medication-free patients with non-psychotic MDD and 40 matched controls who were recruited to a randomized double-blind antidepressant trial. Significantly lower levels of Tregs were found in MDD patients compared with controls, prior to treatment. Following seven weeks of treatment, a significant and substantial increase in Tregs was observed in MDD patients and Treg percentages normalised, comparable with controls. However, the increase in Tregs was not associated with clinical response. A more recent study including 47 MDD patients who were being treated with antidepressants and 47 controls, showed that the percentage and absolute count of Tregs were significantly higher in the medicated, depressed group (Mohd Ashari et al., 2019). However, there was no association between Treg frequency and severity of depressive symptoms.

Table 5.1. Studies examining regulatory T cell levels in people with depression

Author/year	Sample	Study design	Treg markers	Statistical analysis, covariates	Results
Chen et al. 2011	40 MDD patients (25 men, 15 women), mean age 35yr, 30 matched controls	Difference in Tregs and Th17/Treg ratios between MDD patients and controls	CD4+ CD25+ FoxP3+	Student's <i>t</i> -test	MDD patients showed reduced Tregs and increased Th17 cells compared with controls
Grosse et al. 2015	40 medication-free inpatients with melancholic, non-psychotic MDD (16 men, 24 women) mean age 52yr, 40 matched healthy controls	Differences in percentages of circulating Tregs between MDD patients and controls before and after treatment with either venlafaxine or imipramine.	CD4+ CD25 ^{high} FOXP3+	ANCOVA; age and sex	MDD patients showed reduced percentages of Tregs compared with controls before treatment. Increases in Tregs in MDD patients after treatment. Treg levels were not predictors of clinical outcome of treatment.
Grosse et al. 2016	50 MDD patients, mean age 33yr, 58 matched healthy controls	Differences in levels of circulating Tregs between MDD patients and controls and association with monocyte activation	CD4+ CD25 ^{high} FoxP3+	ANCOVA; age, gender, BMI, smoking	Tregs decreased in MDD patients compared with controls. No difference in MDD patients. Tregs negatively correlated with inflammatory monocyte activation in MDD patients.
Himmerich et al. 2010	16 depressed patients (5 men, 11 women), mean age 42yr	Differences in Treg percentages in MDD patients before and after antidepressant treatment	CD4+ CD25 ^{high}	ANOVA	Tregs increased and depression scores decreased after antidepressant therapy.
Li et al. 2010	27 MDD patients (13 men, 14 women), mean age 30yrs, 27 matched healthy controls	Differences in levels of circulating Tregs between MDD patients and controls	CD4+ CD25+	Student's <i>t</i> -test	MDD patients showed significantly decreased numbers of Treg compared with controls.

Table 5.1 continued. Studies examining regulatory T cell levels in people with depression

Author/year	Sample	Study design	Treg markers	Statistical analysis, covariates	Results
Patas et al. 2018	20 un-medicated MDD patients (9 men, 11 women), mean age 37yr, 20 matched controls	Difference in T cell phenotype and FoxP3 mRNA expression in purified CD4+ T cells between MDD patients and controls and association with ACTH and cortisol	CD4+ CD25 ^{high} CD127 ^{low/-}	Paired Wilcoxon signed-rank test, Spearman's rank correlation test	MDD patients showed a significantly higher frequency of Tregs and increased FoxP3 mRNA expression compared with controls
Suzuki et al. 2017	54 un-medicated MDD patients (13 men, 41 women), mean age 34yr, 56 matched controls	Difference in Treg percentage between MDD patients and controls and association with sleep disturbance	CD25+ CD127 ^{Low} CCR4+	Student's <i>t</i> -test ANCOVA; BMI, age, sex, batch effects	MDD patients showed a significantly increased percentage of Tregs compared with controls. Sleep disturbance not related to Treg counts
Hasselmann et al. 2018	34 un-medicated MDD patients (25 women, 9 men), mean age 32, 34 matched controls	Difference in monocyte and T cell phenotype, cell-specific GR and MR RNA expression, saliva cortisol rhythm, IL-6, TNF- α and IL-1 β .	CD4+ CD25+ CD127-	Paired samples T-test, repeated measures ANOVA, Spearman's correlation	MDD patients had higher frequency of monocytes and lower GR expression in monocytes compared to controls. No difference in Tregs, cortisol, cytokines or MR expression.

Abbreviations: MDD = major depressive disorder, Tregs = regulatory T cells, ANCOVA = analysis of covariance, BMI = body mass index, mRNA = messenger ribonucleic acid, ACTH = adrenocorticotrophic hormone.

The exact mechanism by which antidepressants affect Tregs is not yet understood and further studies investigating Treg function are required. The increase in Tregs during treatment may explain the observed decrease in cytokines and may be particularly important for those patients with severe MDD. It is plausible that Treg stimulation could thus be used as an adjunct treatment of MDD with the aim of reversing inflammatory activation and low mood.

Summary of evidence

Taken together these findings tentatively suggest that the adaptive arm of the immune system may play an important role in the aetiology and treatment of MDD. However the evidence is sparse and findings are inconsistent with some studies suggesting increased Tregs and some suggesting decreased Tregs in people with depression. There appear to be links between Tregs and cytokine levels, potentially highlighting a new focus of research into how immunological pathways interact in the pathophysiology of depression. Research investigating associations between Tregs and neuroendocrine function in MDD has only just begun, however so far there is little evidence supporting a relationship. To date no-one has investigated the association between Tregs and circulating TNF- α levels, GR and MR sensitivity or diurnal cortisol patterns in depressed patients.

Therefore, study 1 of this PhD will also investigate the following research questions:

1. Are Treg frequencies different between people with MDD and healthy controls?
2. Are Tregs associated with inflammatory activation or HPA-axis function in MDD?

6. Study 1c - The Resist Study results:

Alterations in regulatory T-cells and their association with inflammation and HPA-axis function in people with depression

6.1 Introduction

In Chapter 6 I will present results from the Resist Study concerning differences in Treg frequency between people with MDD and healthy controls and associations with both inflammation and HPA-axis function. Exploring differences in Tregs between depressed people and healthy controls, and associations with more established depression related pathways, may teach us about the role of adaptive immunity in the pathophysiology of depression.

6.2 Hypotheses

Only a small number of studies have explored Treg frequencies in MDD and the findings are inconclusive. Five studies have reported decreased Tregs in depressed people compared with controls (Y. Chen et al., 2011;Grosse et al., 2015;Grosse et al., 2016b;Y. Li et al., 2010) whilst two have reported increased levels of Tregs (Patas et al., 2018;H. Suzuki et al., 2017) and one has reported no difference (Hasselmann et al., 2018). In order to address this inconsistency in the literature, we sought to explore differences in Tregs between depressed people and healthy controls. On the balance of the evidence to date, **we hypothesise that people with MDD will have a significantly decreased frequency of circulating Tregs compared with healthy controls (hypothesis 1).**

To date only three studies have investigated the association between Tregs and inflammatory activation in MDD, all showing a negative association with increases in pro-inflammatory cytokines (Y. Chen et al., 2011;Grosse et al., 2016b;Y. Li et al., 2010). Increases in Tregs following antidepressant therapy have also been associated with

decreases in pro-inflammatory cytokines (Himmerich et al., 2010). Only one study has specifically explored the relationship between Tregs and IL-6, reporting an inverse association (Grosse et al., 2016b). The relationship between Tregs and TNF- α has not yet been clarified. Therefore we sought to explore the relationship between Tregs and both IL-6 and TNF- α . In line with the previous findings regarding IL-6 and on the basis of our findings from Study 1a regarding TNF- α , **we hypothesise that the frequency of circulating Tregs in people with MDD will be significantly, negatively associated with inflammatory cytokines (hypothesis 2).**

To date only two studies have explored the relationship between Tregs and neuroendocrine dysregulation in MDD patients. Patas et al. (2018) found no associations between serum levels of ACTH or cortisol and Tregs and Hasselmann (2018) reported no difference in *GR* or *MR* expression in Tregs between MDD patients and healthy controls. To date no one has investigated associations between Tregs and either GR and MR function or diurnal cortisol rhythm in MDD. To address this gap in the literature, we investigated whether there is any association between Tregs and HPA-axis function in people with MDD. Based on the robust evidence for disturbed HPA-axis function in MDD and tentative evidence for reduced Tregs **we hypothesise that the frequency of circulating Tregs in people with MDD will be significantly, negatively associated with any disturbances in HPA-axis function (hypothesis 3).**

6.3 Biological measures

6.3.1 Regulatory T cell determination

Reagents

Phosphate-buffered saline (PBS) (Gibco, pH 7.2, 1 X, 20012027); Ficoll® Paque Plus (GE Healthcare, 17-1440-02), foetal calf serum (FCS) (Gibco 10270); RPMI 1640

medium (Sigma, 500ml, sterile, R8758); penicillin/streptomycin (Sigma, 500ml, sterile, P4458); HEPES buffer (Fisher BioReagents, 1M solution, pH 7.3, BP299-100); Dimethyl sulfoxide (Sigma, sterile-filtered, D2650-100ML), LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Brilliant Violet 525 (L34965); anti-CD4 Alexa Fluor 700 (Biolegend, 877-246-5343), anti-CD25 Brilliant Violet 650 (Biolegend, 877-146-5343); FoxP3 PE-Cyanine 7 (eBioscience, 25-4777-41); Foxp3/Transcription Factor Staining Buffer Set (eBioscience, 00-5523-00).

Panel

As mentioned in Chapter 5, Section 5.2, Tregs are characterised by high amounts of extracellular CD4 and CD25 and intracellular expression of Foxp3, therefore these markers were chosen to identify Tregs in this study. A fluorochrome-conjugated antibody panel was created using FluoroFinder, LLC, and is presented in Table 6.1.

Table 6.1. Flow cytometry fluorochrome-conjugated antibody panel.

Laser	Filter	Marker	Fluorochrome
641	730/45	CD4	AF700
405	660/20	CD25	BV650
561	780/60	FoxP3	PE-Cy7
405	525/50	Live/dead	BV525

Controls

To ensure the accuracy of our FACS analysis we employed several methodologies recommended by Tung and colleagues (2007); compensation controls, live/dead discrimination and fluorescence-minus-one (FMO) controls.

Compensation controls: When conducting multi-fluorochrome flow cytometry, the emission spectra of the various fluorochromes can overlap, resulting in detection in a different channel, known as 'spill-over'. In order to control for this, we corrected the analysis of our FACS data using automatic compensation within FACSDiva software (BD Biosciences, California, USA). Prior to analysing the samples, automatic compensation was computed using single stained cells for each antibody used. A negative, unstained control was also used. This compensation was then applied to the dataset and any spectral overlap was automatically subtracted from the detected signal. Single cell staining was only conducted once, prior to full sample analysis.

Live/dead discrimination: Dead cells have greater auto-fluorescence and non-specifically bind to fluorochrome-conjugated antibodies, potentially leading to false positives. Therefore it is essential that they are excluded from the analysis. Whilst identification of dead cells can be partly achieved using a gate, based on forward and side scatter, this method will not exclude all dead cells. Elimination of dead cells is further improved by including a 'live/dead' stain, which stains dead cells much more brightly than live cells, making them easier to identify. In this study, a live/dead stain was added to the samples prior to fixation/permeabilization.

Fluorescence-minus-one (FMO) controls: FMO control is a strategy used to distinguish between positive and negative cells when using multiple fluorochromes. An FMO control consists of all the fluorochromes in a panel, minus the one being measured. By including FMO controls in the analysis, the maximum fluorescence expected for a given subset, in a given channel, when the fluorochrome-conjugated antibody used in that channel is excluded, can be calculated. This allows for correct identification of the true upper boundary for negative cells. Cells from the study sample were used for FMO analysis. The FMOs used in the study are shown in Table 6.2.

Table 6.2. Fluorescence-minus-one (FMO) controls.

Antibody	AF700	BV650	PE-Cy7	BV525
CD4 FMO	---	CD25	FoxP3	Live/dead
CD25 FMO	CD4	---	FoxP3	Live/dead
FoxP3 FMO	CD4	CD25	---	Live/dead
Live/dead	CD4	CD25	FoxP3	---

Protocol

In vitro isolation of lymphocytes from human peripheral blood

Following previous plasma separation of the samples, PBS was added to the four heparin plasma tubes to the 10ml level and inverted several times to mix. 10mls Ficoll-Paque media was added to two 50ml polypropylene tubes, using a syringe. The diluted blood sample was carefully layered (20mls) onto the Ficoll-Paque media solution (10mls), to avoid mixing. The polypropylene tubes were centrifuged (1000 x g, room temperature, 15 mins) and the upper plasma layer was drawn off using a sterile Pasteur pipette, leaving the mononuclear cell layer undisturbed at the interface. The cells were then transferred to a clean 50ml polypropylene tube, using a sterile Pasteur pipette and PBS was added to a total volume of 50ml. The cells were suspended and re-centrifuged (500 x g, room temperature, 10 mins). The supernatant was discarded and the cells were gently re-suspended in 1ml of cold culture fluid (RPMI 1640 medium; penicillin/streptomycin, HEPES buffer; foetal calf serum) and placed on ice. Viable cells were then counted in a 1:20 Trypan Blue dilution (10 μ l cells; 90 μ l PBS; 100 μ l 0.4% Trypan Blue) under a microscope using a hemocytometer. Cells which had excluded the dye were counted. The cell suspension was then aliquoted into cryovials in volumes to achieve 10 x 10⁶ cells per vial and culture fluid was added were necessary to achieve a total volume of 500 μ l per vial. 1ml of dimethyl sulfoxide was then added to a

polypropylene tube with 4mls of culture fluid and the solution was filtered. 500µl of the solution was slowly added to each cryovial to achieve a final volume of 1ml. Cryovials were then added to a 1°C/min freezing container (Nalgene® Mr. Frosty) and stored at -80° for 24 hours, after which they were transferred to liquid nitrogen awaiting analysis.

Cell preparation

FACS analysis was conducted in batches of between 5 and 10 samples. PBMCs were removed from liquid nitrogen and defrosted in 1ml of 37°C FCS. The cells were then added to a 15ml polypropylene tube containing 10mls 37°C culture fluid and washed (500 x g, room temperature, 5 mins). The supernatant was discarded, the cells were re-suspended in 5ml of culture fluid and the wash was repeated. A final wash in 5mls PBS was then performed, the supernatant was discarded and the cells were re-suspended. Viable cells were then counted in a 1:4 dilution with 0.4% Trypan Blue, under a microscope using a hemocytometer. Cells which had excluded the dye were counted. The mean number of cells contained per cryovial were 8×10^6 .

Cell surface staining for single stain controls

5×10^6 cells per antibody were added to four individual wells in a round bottomed 96 well Corning® microplate and one labelled for each of the antibodies (CD4, CD25, FoxP3, live/dead). The plate was then spun (500 x g, room temperature, 5 mins) and the supernatant was discarded. 3µl of each conjugated antibody was added to corresponding well with 100µl PBS and mixed. The plate was then wrapped in aluminium foil and refrigerated for 30 minutes. The plate was then washed twice in 200µl of FACS buffer (1 X PBS and 2% FCS; 500 x g, 5 mins). 200µl FACS buffer was added to each control well and the cells were re-suspended, added to labelled 5ml polystyrene FACS tubes, containing 100µl FACS buffer, covered with aluminium foil and refrigerated, awaiting analysis.

Cell surface staining for viability dye, CD4 and CD25

5 x 10⁶ cells per sample were added to individual wells in a round bottomed 96 well Corning® microplate for full panel staining. In order to include FMO controls, 5 x 10⁶ cells per FMO were also added to individual wells. The plate was then spun (500 x g, room temperature, 5 minutes) and the supernatant was discarded. 3µl of CD4, CD25 and viability dye conjugated antibodies per sample was added to an Eppendorf 1.5ml microcentrifuge tube, with 50µl PBS per sample, plus an additional 50µl PBS, and vortexed. 50µl of the antibody mix was added to each sample well. 3µl of each individual antibody was added to an Eppendorf 1.5ml microcentrifuge tube for each FMO, minus the antibody being measured, with 100µl PBS, and vortexed. 50µl of each FMO antibody mix was added to each FMO well. The plate was then wrapped in aluminium foil and refrigerated for 30 minutes. The plate was then washed twice in 200µl of FACS buffer (1 X PBS and 2% FCS; 500 x g, 5 minutes). 200µl FACS buffer was added to the FMO control for FoxP3 well and these cells were re-suspended, added to a 5ml polystyrene FACS tube, containing 100µl FACS buffer, covered with aluminium foil and refrigerated.

Intracellular staining

Fixation and Permeabilization was conducted according to manufacturer instructions (eBioscience, California, USA). Foxp3 Fixation/Permeabilization working solution was prepared by mixing 1 part of Foxp3 Fixation/Permeabilization Concentrate with 3 parts of Foxp3 Fixation/Permeabilization Diluent. 100µl of the working solution was added to the sample/FMO wells and the cells were re-suspended, covered with foil and refrigerated for 30 minutes. A 1X working solution of Permeabilization Buffer was prepared by mixing 1 part of 10X Permeabilization Buffer with 9 parts of distilled water. 100 µl of Permeabilization Buffer was added to each sample/FMO well and the plate was then washed (500 x g, room temperature, 5 minutes). The supernatant was discarded and the wash was repeated. 6µl per sample of FoxP3 antibody was added to an

Eppendorf 1.5ml microcentrifuge tube with 200µl Permeabilization Buffer per sample and vortexed. 100µl of the FoxP3 solution was added to each sample/FMO well, covered with foil and incubated at room temperature for 30 minutes. 100 µl of Permeabilization Buffer was added to each sample/FMO well and the plate was then washed (500 x g, 5 minutes). The wash was repeated. The cells were re-suspended in 200µl FACS buffer, added to 5ml polystyrene FACS tubes, containing 100µl FACS buffer, covered with aluminium foil and refrigerated, awaiting analysis.

Flow cytometry analysis for identification of Tregs

Stained cells were analysed by four-colour flow cytometry (LSR I, BD Biosciences, California, USA) and analysed using Flowjo (Tree Star Inc. Ashland, Oregon, USA) software. We employed a gating strategy to quantify Treg populations as follows:

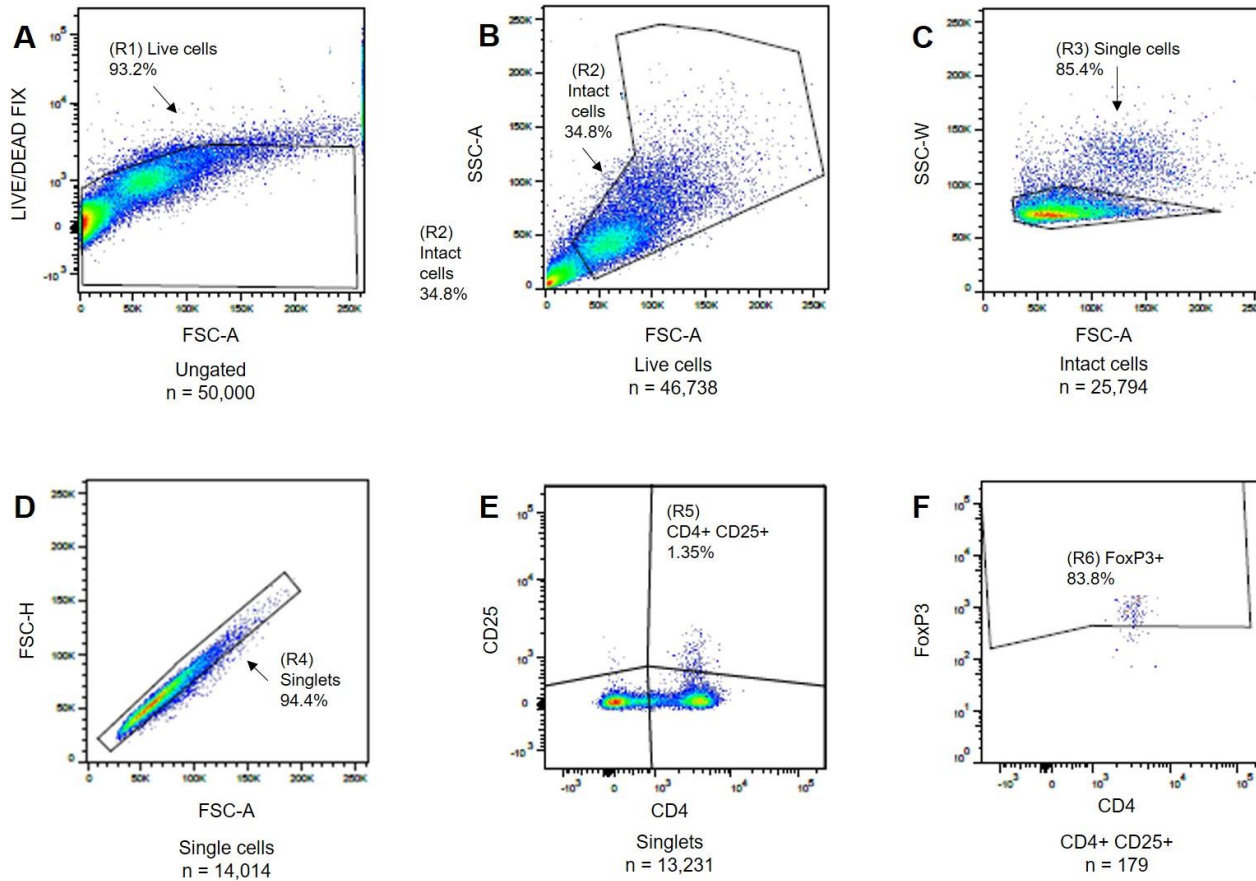
1. In order to discern between living and dead cells, we first created a Forward Scatter Area (FSC-A) versus Live/dead stain plot and a region was inserted around the viable cell population (Figure 6.1(A), R1 - 'Live cells').
2. A FSC versus Side Scatter Area (SSC-A) gate was then applied to further identify viable cells. FSC-A and SSC-A give an estimation of the size and granularity of the cells respectively, allowing for the additional exclusion of debris and dead cells (Figure 6.1(B), R2 - 'Intact cells').
3. A Side Scatter Width (SCC-W) versus FSC-A gate was then applied to eliminate doublets (single events that consist of 2 independent cells). Flow cytometry is based on single cell analysis and doublet discrimination allows for increased accuracy in determining population frequency. Doublet exclusion can be performed by plotting the width against the area for FSC and SSC. Doublets will have double width of single

cells, whilst the height is approximately the same. Therefore disproportions between height and width can be used to identify doublets (Figure 6.1(C), R3 - 'single cells').

4. A Forward Scatter Height (FSC-H) versus FSC-A was also applied to eliminate doublets. Doublets have increased area whilst similar height to single cells. Therefore disproportions between height and area can be used to identify doublets (Figure 6.1(D), R4 - 'Singlets').
5. The cells were then gated by their expression of CD4 and CD25 to identify CD4+CD25+ cells (Figure 6.1(E), R5 - 'CD4+CD25+').
6. Finally, the expression of FoxP3 was gated on CD4+CD25+cells to identify CD+CD25+FoxP3 Tregs (Figure 6.1(F), R6 - 'FoxP3').

The final measure determines the percentage of CD4+CD25 T cells which co-express FoxP3.

Figure 6.1. Flow cytometry gating strategy for Tregs.



A) live cells were identified based on live/dead stain; (B) intact cells were selected based on forward and side scatter; (C) single cells were selected based on forward scatter area and side scatter width; (D) singlets were identified based on forward scatter area and forward scatter height; (E) T cells co-expressing CD4 and CD25 are shown; (F) expression of FoxP3 for a healthy control (n=179).

6.4 Statistical analysis

The Frequency of Parent statistic was calculated in FlowJo (Tree Star Inc. Ashland, Oregon, USA) to determine the percentage of CD4+CD25+ T cells represented by FoxP3 Tregs. Frequency of Parent is calculated by dividing the number of cells in the subpopulation by the number of cells in its direct ancestor population.

Kolmogorov-Smirnov tests revealed that Treg frequency was normally distributed and Levene's test revealed that homogeneity of variance was equal for the groups. Differences in Treg frequency between the groups were conducted using Independent Samples T-tests. Pearson's R correlations and Spearman's correlations were used to ascertain whether there was any association between Treg frequency and inflammatory and HPA-axis pathways. One-way ANOVAs with Gabriel comparisons were used in sensitivity analyses to explore the impact of childhood sexual abuse. All inferential statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, Illinois, USA).

6.5 Results

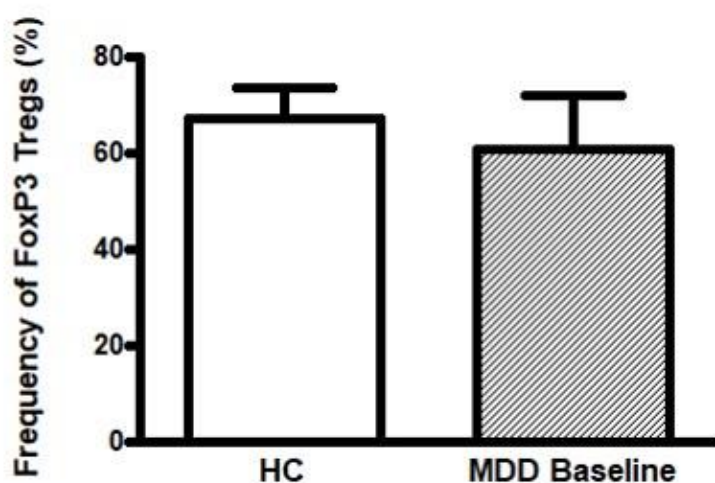
6.5.1 Participants

Tregs were successfully isolated from 36 people with MDD and from 31 healthy controls. The sociodemographic and clinical characteristics of the participants did not differ from those reported in Chapter 3, Section 3.5.1, so are not presented again here.

6.5.2 CD4+CD25+FoxP3+ Treg frequency

The percentage of CD4+CD25+ cells expressing FoxP3 was compared between the groups. There was no significant difference in Tregs between people with MDD and healthy controls ($t(65) = -0.364, p = 0.717$) (Figure 6.2, striped bar represents people with MDD).

Figure 6.2. The frequency of peripheral CD4+CD25+FoxP3 Treg cells in healthy controls and people with MDD.



People with MDD (n=36) healthy controls (n=31). Data shown are percentage of CD4+CD25+T cells, presented as mean \pm SD.

6.5.3 Correlations with inflammatory biomarkers

To explore the relationship between Tregs and inflammatory activation, correlations between variables were examined. Correlations are presented in Table 6.3. There were no significant associations between Treg frequency and any of the other biological variables. The only association to approach significance was with the CAR in healthy controls ($p = 0.078$), suggesting that as Tregs increase, CAR values may also increase.

Table 6.3. Correlations between Tregs, and inflammatory biomarkers/HPA-axis parameters in people with MDD and healthy controls

	Frequency of CD4+CD25+FoxP3 Tregs			
	MDD		Healthy controls	
	Correlation coefficient	<i>p</i> -value	Correlation coefficient	<i>p</i> -value
Inflammation				
IL-6	0.071	0.680	0.098	0.601
TNF- α	0.138	0.421	0.139	0.455
Corticosteroid receptor function				
DEX IC ⁵⁰	-0.043	0.842	0.101	0.604
PRED IC ⁵⁰	0.026	0.925	-0.254	0.401
Diurnal saliva cortisol				
CAR	0.072	0.777	0.386	0.084
CAR AUC	-0.123	0.518	0.245	0.208
AUC	-0.133	0.492	-0.232	0.254
Cortisol slope	-0.117	0.538	0.202	0.302

Abbreviations: MDD = major depressive disorder; IL-6 = interleukin 6; TNF- α = tumour necrosis factor alpha; DEX = dexamethasone, PRED = prednisolone, CAR = cortisol awakening response, AUC = area under the curve.

6.5.4 Sensitivity analyses

Depressive symptom severity

Exploratory analyses were conducted in order to identify whether any differences between the groups emerged when the analysis was restricted to people with moderate/severe depressive symptoms only. The main analysis was repeated following the removal of those participants who were classified as experiencing mild depressive symptom according to the BD-II. 11 people had mild baseline depressive symptoms (score <20) and were removed from the analysis, resulting in a reduced MDD sample of 25 people with moderate to severe symptoms. Results showed that symptom severity did not have an impact on the results. There was no significant difference in Tregs

between people with moderate/severe MDD and healthy controls ($t(54) = -0.458, p = 0.649$).

Antidepressant use

Little is known about the effects of anti-depressants on Treg populations. Therefore, I ran exploratory analyses to determine whether there was any difference in Treg frequency between depressed people taking antidepressants and depressed people who were antidepressant free. Results showed that there was no difference in Treg frequency between the groups ($t(34) = -1.232, p = 0.226$).

6.6 Discussion

6.6.1 Aims and hypotheses

The aim of this study was to compare differences in Tregs in depressed people compared with healthy controls. I also sought to explore whether there are any associations between Tregs and both inflammatory and neuroendocrine function. I hypothesised that people with MDD would have significantly decreased frequency of circulating Tregs compared with healthy controls (hypothesis 1). I also hypothesised that the frequency of circulating Tregs in people with MDD would be significantly, negatively associated with inflammatory cytokines (hypothesis 2) significantly associated with HPA-axis function (hypothesis 3).

6.6.2 Summary of results

The results of this study do not provide support for these hypotheses. There was no significant difference in circulating Tregs in depressed people compared to controls. Sensitivity analysis, restricting the depressed sample to only those with moderate/severe

symptoms had no effect on the findings. Additional analyses examining the use of antidepressants also showed no effect on the results. There were no significant associations between circulating Tregs and inflammatory biomarkers and no significant associations between circulating Tregs and HPA-axis function. I will discuss possible explanations for these findings in the following sections.

6.6.3 Hypothesis 1: Differences in Tregs

According to our results the number of CD4+CD25+FoxP3+ cells in the PBMCs from MDD patients was comparable to that from healthy controls, suggesting that there is no significant decrease in the frequency of circulating Tregs in MDD. Our findings support those of Hasselmann et al. (2018) who also reported no differences in Tregs between antidepressant-free MDD patients and controls. However, this is in contrast to several studies which have shown reduced Treg frequency in depressed people (Y. Chen et al., 2011;Grosse et al., 2015;Grosse et al., 2016b;Y. Li et al., 2010). However there are also inconsistencies in the literature with increased Treg frequency also being reported (Patas et al., 2018;H. Suzuki et al., 2017).

The inconsistency in the literature may be attributed to variation in Treg markers. Some studies defined Treg cells solely on the expression of the cell surface proteins and did not include the classic intracellular marker of Treg cells, FoxP3 (Himmerich et al., 2010;H. Suzuki et al., 2017). Furthermore there are inconsistencies in the choice of cellular markers used, with some studies using additional activation markers, such as CD127 (Hasselmann et al., 2018;Patas et al., 2018;H. Suzuki et al., 2017) and CCR4 (H. Suzuki et al., 2017). In the current study we focused on CD4, CD25 and FoxP3, which are largely acknowledged to identify the major population of Treg cells (Plitas & Rudensky, 2016).

However, CD4+CD25+FoxP3 Tregs are both phenotypically and functionally heterogeneous and inclusion of co-stimulatory/co-inhibitory molecules may help further identify functionally suppressive Tregs (X. Chen & Oppenheim, 2011). As such our null finding may reflect insufficient Treg characterisation. However, several studies have reported Treg reductions in MDD using the same classification as in the current study (Y. Chen et al., 2011;Grosse et al., 2015;Grosse et al., 2016b). Tregs are a very active area of research, and additional targets are still emerging (X. Chen & Oppenheim, 2011). As a definitive molecular marker for Treg cells has yet to be elucidated, future studies should incorporate functional analyses of the suppressive capacity of Tregs in order to better determine their role in MDD.

To date, depressive symptom severity in relation to circulating Tregs has only been explored in two studies. Himmerich et al. (2010) reported that baseline levels of Tregs did not differ between those with mild symptoms and those with moderate to severe symptoms. Mohd Ashari et al. (2019) also reported that depressive symptoms scores were not correlated with Treg percentage or number in antidepressant treated MDD patients. To explore any influence of symptom severity in the current study, secondary analysis was conducted with a subset of depressed individuals who had moderate to severe baseline depressive symptoms (score >20) (n=25). Results showed that there was no change in the findings, with no significant difference in Tregs between the groups ($p = 0.649$).

More conclusive findings have come from studies investigating the effects of antidepressant therapy on Treg levels. Himmerich et al. (2010) found that Tregs increased significantly during antidepressant treatment. Furthermore, the more severely depressed the patients were at baseline, the more their Tregs increased. This finding was replicated by Grosse et al. (2015) and Mohd Ashari et al. (2019) who also reported that the percentage and absolute count of Tregs were significantly higher in depressed people following antidepressant therapy. These findings are in line with those from

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studies investigating autoimmune diseases, in which Tregs have been shown to increase in patients responding to anti-inflammatory therapy (Boissier et al., 2009). However the mechanism of action of antidepressants remains incompletely understood. Antidepressants may activate Tregs in patients with MDD, restoring immune balance, halting the inflammatory process and thereby reducing depressive symptoms. Alternatively, antidepressants may influence Tregs indirectly, via their effects on cytokine production. As discussed in Chapter 1, Section 1.4.3, antidepressants have been shown to reduce pro-inflammatory cytokines such as IL-6 (Hiles et al., 2012a; Kohler et al., 2018; Wiedlocha et al., 2018) and TNF- α (Kohler et al., 2018) and the activity of Tregs can depend highly on the influence that these cytokines have on their differentiation, maintenance and function (La Cava, 2008). IL-6 completely inhibits the generation of Tregs (Bettelli et al., 2006) and reduces their suppressive effect (Wan et al., 2007) and TNF- α has been shown to have both inhibitory effects (Valencia et al., 2006) and stimulatory effects (X. Chen et al., 2007) depending on the expression of the TNF- α receptors TNFR1 and TNFR2. In order to explore whether Treg frequency in people with MDD varied according to antidepressant treatment in the current study, we conducted a sensitivity analysis. The results showed no difference between those who were taking antidepressants and those who were not ($p = 0.226$).

One possible explanation for the inconsistency in the findings regarding Treg frequencies in MDD is the variability in markers used to characterise Treg populations. Some studies rely only on the cell surface markers CD4 and CD25 (Himmerich et al., 2010; Li et al., 2010; Patas et al., 2018; Suzuki et al., 2017) others include FoxP3 (Y. Chen et al., 2011; Grosse et al., 2015; Grosse et al., 2016) and others include additional markers such as CD127 (Hasselmann et al., 2018; Patas et al., 2018; H. Suzuki et al., 2017) and CCR4 (H. Suzuki et al., 2017). In the present study we characterised Tregs as CD4+CD25+FoxP3 T cells. Ideally a common measure of Treg frequency would be used, however in the absence of a consensus regarding Treg characterisation this is difficult. This creates a challenge in this field of research.

6.6.4 Hypothesis 2: Relationships with inflammatory biomarkers

We did not observe a correlation between Tregs and pro-inflammatory cytokines. This is contrast to findings from several studies which have reported a link between Tregs and inflammatory activation (Y. Chen et al., 2011;Grosse et al., 2016b;Himmerich et al., 2010;Y. Li et al., 2010). Li et al. (2010) reported decreases in Tregs and the anti-inflammatory cytokines, IL-10 and TGF- β and increases in the pro-inflammatory cytokine, IL-2, in MDD patients compared with controls. They also reported that in the overall sample, Tregs were correlated with inflammatory cytokines. Chen et al. (2011) found significantly higher Th17 cells, which produce the pro-inflammatory cytokines IL-17, IL-21 and IL-22, and significantly lower Tregs, in MDD patients compared with controls. This suggests that the balance between inflammation and regulation was tilted towards an inflammatory state. Studies have shown that Th17 cell differentiation requires both TGF- β and IL-6 (McGeachy et al., 2007), however the effect of TGF- β appears to be dose-dependent, with very low concentrations resulting in Th17 cells and high concentrations promoting production of Tregs (L. Zhou et al., 2008). Increased levels of plasma/serum IL-6 have been robustly reported in people with MDD (Hiles et al., 2012b), whilst the anti-inflammatory cytokine TGF- β 1 is significantly lower in depressed patients compared with controls (Musil et al., 2011;Levent Sutcigil et al., 2008). Therefore it has been hypothesised that the cytokine milieu in MDD is favoured towards the development of Th17 cells and the inhibition of Tregs (Y. Chen et al., 2011). Grosse et al. (2016b) found that MDD patients aged \geq 28 years demonstrated decreased Tregs and this was correlated with increased inflammatory monocyte gene expression (IL-6, IL-1 β and TNF- α). Tregs have also have also been shown to be increased following antidepressant treatment in conjunction with decreases in IL-6 and IL-1 β (Himmerich et al., 2010). Taken together these findings show that impaired immune suppression and inflammatory activation co-occur in the same depressed patients and suggest that Treg deficiency may contribute to the inflammatory dysregulation observed in MDD.

6.6.5 *Hypothesis 3: Relationships with neuroendocrine function*

We also did not observe a correlation between Tregs and neuro-endocrine function. To date, only two studies have investigated Tregs and markers of HPA-axis activity in MDD. A study by Patas et al. (2018) reported increased frequency of Tregs in MDD patients but no association with ACTH or cortisol levels. Another study by Hasselmann et al. (2018) reported that whilst *GR* expression was lower in monocytes in MDD patients compared with controls, there was no difference in *GR* expression in Tregs. Studies investigating *GR* sensitivity have often used glucocorticoid suppression of mitogen induced T-cell proliferation (Bauer et al., 2003; Calfa et al., 2003; Lowy et al., 1984; Lowy et al., 1988; Rupprecht et al., 1991a; Tanke et al., 2008; Wodarz et al., 1991; Wodarz et al., 1992). This finding suggests that the *GR* resistance reported by proliferation assays is unlikely to be explained by reduced *GR* expression in T cells, but may be mediated by other components of the innate immune system. Taken together these findings suggest that any observed alterations in Treg frequency are unlikely to be explained by the HPA-axis dysfunction observed in MDD, however further studies are required in order to determine whether any relationship exists.

6.6.6 *Strengths and limitations*

In addition to the strengths and limitations mentioned in Chapter 3, there are some other considerations. A strength of our study is the inclusion of FoxP3 as an intracellular marker. Some studies have relied on CD4+CD25+ cells which may not provide a reliable assessment of Treg frequency (Himmerich et al., 2010; Y. Li et al., 2010). CD25 is expressed by various immune cells and is not specific to Tregs (Brusko et al., 2005). The lineage-specific transcription factor Foxp3 is required for the differentiation and function of Tregs (Plitas & Rudensky, 2016). Furthermore, continued Foxp3 expression in mature Tregs is needed to maintain transcriptional and functional capacity (Williams &

Rudensky, 2007). It is therefore essential to include CD4+CD25+FoxP3 Tregs in order to ensure reliable measurement of functional Tregs.

There are also limitations to this study. First, we did not collect data on physical activity, a lack of which can have negative consequences on immune function (Irwin & Miller, 2007). In addition we did not collect data on diet, which has also been shown to influence Tregs, particularly vitamins A and D, gluten, fatty acids and probiotics (Issazadeh-Navikas et al., 2012). Finally, we did not collect longitudinal data on Tregs due to financial and time constraints and so were unable to investigate any changes in Tregs over time.

We explored whether our null finding might be explained by a lack of power. Previous studies have used sample sizes ranging from 20-50 participants in each group, which is line with our sample size. However as discussed above comparisons between findings from these studies is difficult as it is unclear whether in fact they are measuring the same cell populations. We therefore conducted a post-hoc power analysis to calculate the sample size we would need to show a significant difference considering the effect size we observed. The result suggested we would need a sample size of 3,086 to observe a statistical difference, given an alpha of 0.05 and 80% power. This very large number suggests that even though our sample size was fairly small, a reasonable increase would not have impacted on the findings. This provides support for the argument that this finding was a genuine null effect, and that the difference in Tregs between the groups was negligible. Furthermore, the correlations between Tregs and inflammatory cytokines were in the opposite direction to our hypothesis, making it very unlikely that a larger sample would have shown the predicted negative association.

It is also worth noting that future studies should incorporate measures of cytokine signalling in Tregs, such as the TNF receptors, TNFR1 and TNFR2. All human Tregs express TNFR2 and at a much higher density than TNFR1 (Faustman & Davis, 2013). CD4+CD25^{hi} Tregs express the highest level of TNFR2, thus TNF is likely to activate

more functionally suppressive Tregs (Chen & Oppenheim, 2010). TNFR2 + Tregs are highly suppressive in vitro, whereas TNFR2- Tregs have minimal suppressive function. There is evidence from animal models that TNFR2 signalling promotes Treg activity. TNFR2 activates and induces proliferation of Tregs (Chen et al., 2007) and TNFR2 expression indicated maximally suppressive Tregs in a mouse tumour model (Chen et al., 2008). In a clinical trial of Type 1 diabetes, TNF- α agonists increased Treg production (Faustman et al., 2012), however to date, TNF- α receptor expression on Tregs in patients with depression has not been investigated.

6.6.7 *Conclusion*

In conclusion, these results indicate that there is no difference in Treg frequency between people with MDD and healthy controls. Furthermore we found no evidence of any relationship between Treg frequency and either inflammatory cytokines or markers of HPA-axis function. Further studies using functional analyses of the suppressive capacity of Tregs are required to explore their role in MDD.

7. Literature review: Understanding depression and inflammation in cardiovascular disease mortality

7.1 Introduction

In this chapter I will define and describe the pathology and prevalence of CVD. I will then provide evidence for depression as a risk factor for both CVD morbidity and mortality. Following this I will describe the literature regarding current understandings of the biological mechanisms linking depression and CVD, with a focus on the role of inflammation. The aim of this chapter is to highlight the importance of depression in CVD progression and prognosis and to try to shed some light on the role of inflammation as a contributory factor. This chapter will also highlight some of the limitations of the work to date.

7.2 Cardiovascular disease: pathogenesis and prevalence

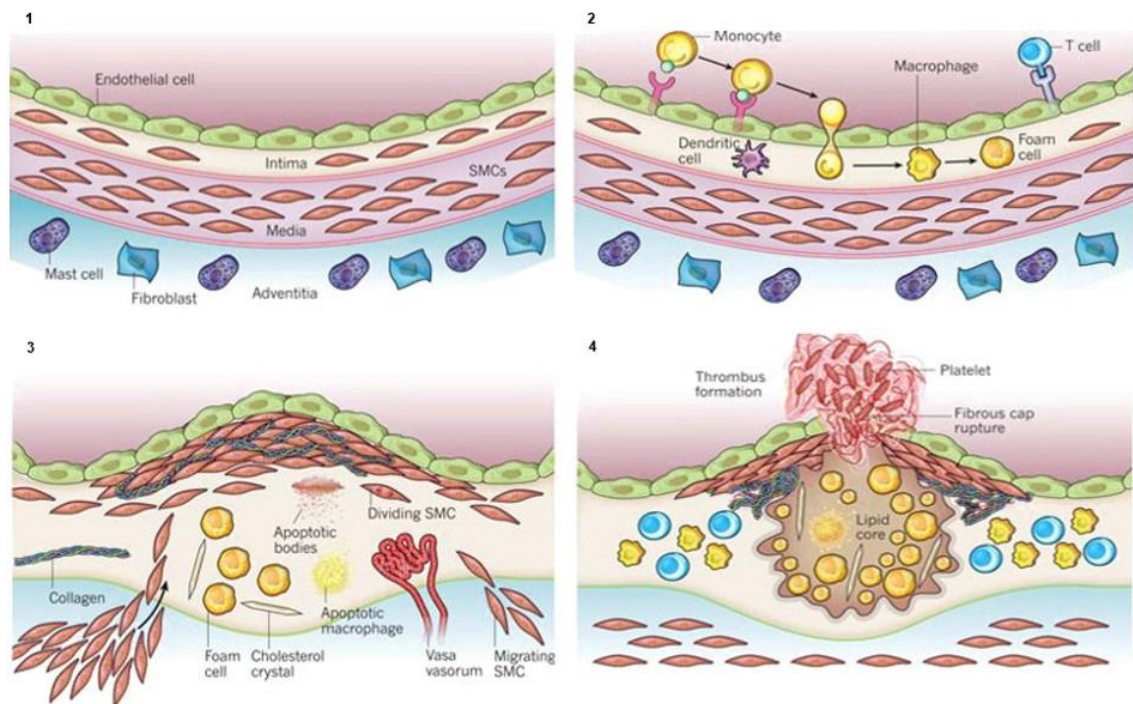
7.2.1 Pathogenesis

Cardiovascular diseases (CVDs) are a group of disorders of the circulatory system and include coronary heart disease (CHD; a disease of the blood vessels supplying the heart muscle) and cerebrovascular disease (a disease of the blood vessels supplying the brain). The main underlying pathological process in CVD is atherosclerosis, the development of atheromatous plaques in the inner lining of the arteries (Hansson & Hermansson, 2011). Historically, atherosclerosis was considered to be solely due to the accumulation of fatty deposits in the arterial wall. However it is now acknowledged that it is a complex disease characterized by intense immunological activity both at the beginning of plaque formation through to the occurrence of an acute event resulting from plaque erosion or rupture (Hansson & Libby, 2006; Pant et al., 2014). Whilst atherosclerosis accumulates over the lifespan, global comparative risk assessment

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studies have estimated that approximately 80% of deaths from CHD and nearly 70% of deaths from stroke in the world were attributable to a small number of physiological and behavioural risk factors, including hypertension, dyslipidaemia, smoking, high BMI, alcohol use, low intake of fruits and vegetables and lack of physical inactivity (Ezzati et al., 2003).

Figure 7.1. Stages in the development of atherosclerosis



Box 1 shows the normal artery and boxes 2-4 show the progression of atherosclerosis. Adapted from Libby, Ridker & Hansson (2011)

The changes that occur in the artery during disease progression are illustrated in Figure 1. The artery is made up of three layers (See Figure 7.1, Box 1). The inner layer is the intima and consists of a layer of endothelial cells. The middle layer is the media and consists of vascular smooth muscle cells (VSMCs). The outer layer is the adventitia which contains mast cells, nerve endings and microvessels. Atherogenesis is thought to begin with alterations in the layer of endothelial cells in the intima (See Figure 7.1, Box 2). Hypercholesterolemia, hypertension and pro-inflammatory mediators can cause activation of the endothelium and an inflammatory response in the artery wall. Activated

endothelial cells, which usually resist attachment of passing white blood cells, express leucocyte adhesion molecules. These adhesion molecules cause the passing white blood cells to adhere to their surface. The captured cells, predominantly monocytes, then migrate into the intima, where they mature into macrophages. At the same time, the permeability of the endothelium increases, allowing low-density lipoprotein (LDL) particles to cross into the artery wall. The macrophages then ingest the LDL particles and become foam cells. Plaque formation also involves proliferation of SMCs in the intima and additional recruitment of SMCs from the media (See Figure 7.1, Box 3). The SMCs then synthesise collagen, elastin and proteoglycans which form the extracellular matrix and form a fibrous cap that covers the plaque. Within the plaque, some of the foam cells die and some release lipids. The accumulation of dead cells and lipids form a lipid-rich necrotic core. The clinical consequences of plaque formation occur due to flow-limiting stenosis (narrowing of the lumen), that in turn results in tissue ischaemia. If a plaque ruptures it can trigger a thrombosis at the surface, which can interrupt blood flow locally or detach to become an embolus and lodge in distal arteries (See Figure 7.1, Box 4). Furthermore, plaque rupture can result in the release of the pro-coagulant material in the plaque's core, causing an increase in platelet aggregation, humoral coagulation and formation of a thrombus. Ischemia can have life threatening consequences. If it occurs in the coronary artery it can result in myocardial infarction (MI) and in the brain it can result in ischemic stroke (Libby et al., 2011) (Hansson, 2005).

7.2.2 *Prevalence*

According to the World Health Organisation, CVD is the leading cause of death globally (2017a). There were an estimated 17.9 million CVD related deaths in 2016, representing 31% of all deaths worldwide. Of those deaths, 85% were due to MI and stroke. In the UK approximately 7 million people are living with CVD and CVD related deaths cause 25% of all deaths each year (The British Heart Foundation, 2018). Healthcare costs relating to CVD are estimated at £9 billion each year and costs to the UK economy is estimated

to be £19 billion annually (*ibid*). CHD is the one of the UK's leading causes of death and recent estimates reveal that it is responsible for over 66,000 deaths in the UK each year (*ibid*). In the UK, one in seven men and one in twelve women die from CHD and it kills more than twice as many women in the UK as breast cancer (*ibid*). Since the 1960s the UK death rate from CVD has declined by more than 75% and the annual number of CHD deaths in the UK has fallen by more than 50% (*ibid*). These reductions are largely attributable to improvements in medical treatment and a reduction in risk factors, particularly improvements in the management of hypertension (O'Flaherty et al., 2013). Decreases in other CVD risk factors such as cholesterol, smoking and physical activity are far more modest and are counteracted by increases in obesity and type 2 diabetes.

7.3 Depression and cardiovascular disease: Introduction

As mentioned in the previous section, there are a number of well-established risk factors for CVD. More recently, a multitude of studies have confirmed an independent association between depression (defined here as either MDD or significant depressive symptoms with substantial functional impairment) and CVD. Depression is robustly shown to increase cardiovascular risk (Correll et al., 2017;Cuijpers & Smit, 2002;Gan et al., 2014;Nicholson et al., 2006;Rugulies, 2002;Van der Kooy et al., 2007;Q. Wu & Kling, 2016;Wulsin & Singal, 2003) and people with established CVD are more likely to experience depressive episodes (Carney & Freedland, 2008;Thombs et al., 2006). In this section, I will review the epidemiological evidence linking these two disorders before moving on to discuss potential underlying mechanisms.

7.4 Depression in patients with existing cardiovascular disease

Depression is also common and persistent in people with established CVD. Depressive symptoms occur in approximately 1 out of every 5 patients, a prevalence that is at least 3 times greater than in the general population (B. E. Cohen et al., 2015). Lane et al.

(2002) assessed the prevalence and persistence of symptoms of depression in 288 patients during the first 12 months following acute MI. Elevated depressive symptoms were reported in 31% of patients during hospitalisation, in 38% four months later and in 37% 12 months later. A cross-sectional study of 1,024 patients with stable CHD showed that 20% had elevated depressive symptoms (Ruo et al., 2003). A systematic review of studies examining depression following acute MI, including data on more than 14,000 patients, confirmed this finding (Thombs et al., 2006).

A meta-analysis of 16 studies, involving 10,175 post-MI patients, investigated to what extent depression independently predicts prognosis (Meijer et al., 2013). The association between depression and cardiac events was partly attenuated after adjustment for disease severity and health variables. However, after full adjustment for age, gender, smoking, diabetes, BMI, history of MI, left ventricular ejection fraction (LVEF), and Killip class, depressive symptoms were still associated with a 13% increased risk of future cardiac events.

CVD severity is also associated with depression. LVEF, a measure of how much blood is expelled from the left ventricle on each contraction (commonly expressed as a percentage), was assessed alongside depressive symptoms in 1,989 MI patients (van Melle et al., 2005). During hospitalisation, depressive symptoms was higher in patients with LVEF dysfunction and lower LVEF was independently associated with a higher rate of depression from 3-12 months following MI. Depressive symptoms are also strongly associated with patient-reported health status, including symptom burden, physical limitation, quality of life, and overall health, even after accounting for objective measures of cardiac function in patients with CHD (Ruo et al., 2003).

Patients with comorbid CVD and depressive symptoms are at increased risk for recurrent cardiovascular events and mortality. Meta-analyses have reported that depressed patients are twice as likely to die or suffer future major cardiovascular events compared

with non-depressed (Barth et al., 2004;Meijer et al., 2011;Nicholson et al., 2006;van Melle et al., 2004). Furthermore, a dose-response relationship has been observed between depression symptoms during post-MI hospitalization and cardiac mortality (Lesperance et al., 2002). Patients with higher baseline depression scores had worse long-term cardiac prognosis regardless of symptom changes. Post-stroke depression has also been associated with increased mortality (Bartoli et al., 2018;Cai et al., 2019).

Depression has been shown to increase incident heart failure (HF) in CVD patients as well as increased hospitalisation in patients with established HF (Freedland et al., 2011;Rutledge et al., 2006). A meta-analysis of 36 publications reported the presence of clinically significant depression in 21.5% of HF patients (Rutledge et al., 2006). Moreover, depressed HF patients demonstrate higher rates of death and secondary events, increased health care use, and higher rates of hospitalization (Fan et al., 2014;Jiang et al., 2001;Rutledge et al., 2006). Functional severity of heart failure is also associated with depression. In a study including 682 hospitalised patients with chronic HF, prevalence ranged from as low as 8% among patients with no limitation of physical activity to as high as 40% among patients unable to carry on any physical activity without discomfort (Freedland et al., 2003). Among patients undergoing coronary artery bypass graft (CABG) surgery, depression is associated with prolonged hospitalisation (Beresnevaitė et al., 2010), progression of CVD (Wellenius et al., 2008) and mortality (Blumenthal et al., 2003). Depression also independently predicts mortality in patients with an implantable cardioverter-defibrillator (van den Broek et al., 2013).

Depressive symptoms are even more prevalent in stroke survivors. A systematic review and meta-analysis of 61 studies reported that 31% of stroke survivors experience depression (Hackett & Pickles, 2014). The authors also report that depressive symptoms resolve spontaneously within a few months of onset for most patients, with few receiving any antidepressant treatment. A more recent meta-analysis and meta-regression

including 128 studies showed that depression was present in 33.5% of people after stroke (Mitchell et al., 2017).

Despite improvements in medical treatment, intervention and care over the last 25 years, the association between post-MI depression and mortality is stable (Meijer et al., 2011). Faced with a wealth of studies exploring this relationship, the American Heart Association conducted a systematic review on depression and adverse medical outcomes after acute coronary syndrome (ACS), including 53 studies and 4 meta-analyses (Lichtman et al., 2014). On the strength and consistency of the evidence examined, they issued a scientific statement elevating depression to the status of a risk factor for adverse medical outcomes in patients with ACS.

The association between depression and CVD is both complex and bidirectional (Lippi et al., 2009). When depression and CVD present together, the prognosis for both worsens (Dhar & Barton, 2016). Polsky et al. (2005) examined the risk of developing significant depressive symptoms after a new medical diagnosis of various long term conditions, including cancer, diabetes, hypertension, CHD, arthritis, chronic lung disease and stroke. After eight years of follow-up, patients with CHD had consistently higher risk of developing depression than those with other diagnoses. Kendler et al. (2009) conducted a time-dependent analysis to clarify the causal relationship between MDD and CHD in a large population-based sample of older, Swedish twins. They reported that the risk of depression following CHD onset was greater than the risk of CHD following MDD. The future risk for CHD was also strongly related to the severity and recurrence of MDD. In contrast it has been shown that although depression may be triggered by CVD, the adverse outcomes associated with depression are not explained by CVD severity (Diez Roux et al., 2006; Lett et al., 2008).

7.5 Depression as a cardiovascular risk factor

Over 100 studies and eight meta-analyses have demonstrated that people with depression are at increased cardiovascular risk compared to the general population (Correll et al., 2017;Cuijpers & Smit, 2002;Gan et al., 2014;Nicholson et al., 2006;Rugulies, 2002;Van der Kooy et al., 2007;Q. Wu & Kling, 2016;Wulsin & Singal, 2003). Nicholson et al. (2006) conducted a meta-analysis of 21 aetiological studies, measuring depression with follow-up for fatal CHD/incident MI. The analysis included 124,509 participants and 416 cardiac events over a mean follow-up period of 10.8 years. The results showed that people with depression had an 80% increased risk of developing CHD. However lack of adjustment for confounding variables such as lifestyle and socio-demographic factors, means that this result is likely to be an overestimation.

Findings from more recent meta-analyses of prospective cohort studies support an association between depression and CVD risk. Gan et al. (2014) conducted an analysis of 30 studies, including 893,850 participants and reported that individuals with depression had a 30% increased risk of for CHD compared with non-depressed people. This association remained significant after adjustment for confounders. Similarly, another meta-analysis of 19 studies including 323,709 participants, reported that depression was associated with a 31% increase in the risk of MI and a 36% increase in the risk of coronary death (Q. Wu & Kling, 2016).

The most comprehensive large-scale meta-analysis to date assessed the worldwide prevalence and incidence of CVD in people with specific severe mental illness (Correll et al., 2017). The analysis included 3,211,768 patients and 113,383,368 controls. CVD prevalence was 11.7% in MDD patients and MDD was significantly associated with CVD and CHD after adjusting for potential confounders. People with MDD also had a 72% increased risk of CVD and a 63% increased risk of CHD and CVD related mortality.

Many studies have specifically reported an association between depressive symptoms and CVD mortality in older people. Vinkers et al. (2004) followed 500 people, 85 years and over, from the population-based Leiden 85-plus Study, for three years. Depression was independently associated with a two-fold increase of all-cause mortality. Both CVD mortality and non-CVD mortality contributed equally to the increased risk. Depressed mood was also associated with an increased risk of CVD mortality in 3,701 men aged > 70 years in the US (B. W. Penninx et al., 1998), chronic depression was associated with all-cause mortality in 1,784 older men and women in Taiwan (Teng et al., 2013) and both moderate and severe depression predicted mortality in 3,746 non-demented older community-living persons in Amsterdam (Schoevers et al., 2009). A meta-analysis of 52 studies, including 47,625 older people found that people with depressive symptoms had a higher risk of stroke and all-cause mortality but not of MI (Eurelings et al., 2018). However, these findings are not entirely consistent, with some studies reporting no association (Callahan et al., 1998; Cuijpers, 2001; Hybels et al., 2002; McCusker et al., 2006).

Evidence also extends to subclinical CVD processes such as peripheral atherosclerosis (Seldenrijk et al., 2010), impaired endothelial function (van Dooren et al., 2016) and increased arterial stiffness (M. Hamer et al., 2010; Seldenrijk et al., 2011). There is also an association between lower, sub clinical symptoms of psychological distress and CVD mortality in healthy people. A meta-analysis of 10 large prospective cohort studies from the Health Survey for England, measured psychological distress in 68,222 participants and followed them over 8 years (Russ et al., 2012). The authors observed a dose-response relationship between psychological distress and mortality across the full range of severity. Sub-clinically symptomatic patients had a 29% increased risk of CVD related death and a 20% increased risk of all-cause mortality. This association remained after adjusting for age, sex, SES, BMI, systolic blood pressure, physical activity, smoking status, alcohol consumption and diabetes at baseline.

Finally, there is evidence that this relationship is bi-directional. A history of CVD can increase the risk of developing depressive symptoms and clinical disorders, either directly through physiological outcomes or indirectly via biological and psychosocial modifications (B. W. Penninx, 2016). Comparatively few studies have investigated the role of CVD in increasing the risk of depression. A Swedish study including 30,374 twins reported that onset of CAD predicted concurrent and ongoing risk for depression (Kendler et al., 2009). Furthermore, the effect of CAD onset on MDD risk was much stronger than the reverse. There is also a high prevalence of depressive symptoms following acute MI, which persist over time (Lauzon et al., 2003). Therefore, the association between depression and CVD can be best considered a metaphorical 'downward spiral' in which depression and cardiovascular disease exist in a mutually reinforcing relationship (B. W. Penninx, 2016).

7.6 Mechanisms linking depression to CVD

In light of the strength of evidence described in the previous section, it is clear that CVD and depression are linked. However the definitive, underlying mechanisms associating the two disorders have yet to be fully elucidated, making targeted pharmacological, psychological and preventative interventions impossible (Halaris, 2013a). The difficulty in identifying these mechanisms is due to the complex interactions between psychological factors such as health behaviours and social isolation, and several biological factors such as abnormalities in the autonomic nervous system, the vascular and hematologic systems, the neuroendocrine system and the immune system,. Based on the current literature, inflammation emerges as the dominant protagonist, involved in all stages of CVD progression and a robust biomarker of clinical depression.

This section will briefly describe the main behavioural and biological underpinnings of the co-morbidity between depression and CVD. However it is beyond the scope of this thesis to provide a comprehensive discussion of available clinical and research data for

each of the mechanisms presented. In the following section, the specific role of inflammation will be described in more detail.

7.6.1 *Behavioural and psychosocial factors*

Behavioural factors, including physical inactivity, medication non-adherence, smoking, alcohol consumption and poor diet, and psychosocial factors, such as social isolation often cluster together in people with depression (Bonnet et al., 2005). Furthermore, post-MI patients are less likely to adhere to recommended behaviour and lifestyle changes intended to reduce the risk of further cardiac events (Ziegelstein et al., 2000). Several studies have shown that behavioural processes explain a substantial amount of the association between depression and CVD (Gale et al., 2014;M. Hamer, 2012;M. Hamer et al., 2008b;Whooley et al., 2008;Win et al., 2011). However due to the bidirectional nature of these associations, it is difficult to determine causality (Whooley & Wong, 2013). Regardless of this, psychological interventions that aim to reduce CVD risk in mental health populations are now encouraged to focus on health behaviours (M. Hamer et al., 2008a;Whooley et al., 2008;Win et al., 2011).

Physical inactivity

The beneficial effects of physical activity on somatic disorders are well established (Aune et al., 2015;Haskell et al., 2007;Warburton & Bredin, 2017). Furthermore, there is evidence that physical activity also confers beneficial effects on mental health (Fox, 1999;Harvey et al., 2010;Wiles et al., 2007). A prospective study using data from the Whitehall II cohort examined physical activity and symptoms of depression at three time points, over 8 years, in 9,309 participants (Azevedo Da Silva et al., 2012). Results showed that the recommended levels of physical activity were associated with reduced risk of depressive symptoms and that experiencing depressive episodes was associated

with increased risk of not meeting the recommended levels of physical activity. These findings were also observed in a study of 2,230 adolescents (Stavrakakis et al., 2012).

There is also evidence that physical inactivity may partially mediate the relationship between depressive symptoms and mortality (Brummett et al., 2003;M. Hamer et al., 2008a;M. Hamer et al., 2008b;Whooley et al., 2008;Win et al., 2011). In the Heart and Soul Study, a prospective cohort study of 1,017 outpatients with stable CHD, depressive symptoms were associated with a 50% greater rate of cardiovascular events (Whooley et al., 2008). After adjustment for physical activity the association was reduced by 31.7%. A similar study of 5,888 older adults reported that depressive symptoms and physical inactivity independently increased CVD mortality risk and were strongly associated with each other (Win et al., 2011). They concluded that physical inactivity accounted for a significant proportion of the risk of cardiovascular mortality due to depressive symptoms.

Medication non-adherence

Depression may also lead to increased CVD mortality through its negative effects on medication adherence. Depression has been associated with non-adherence to cardiac medication (Gehi et al., 2005;Goldstein et al., 2017;Rieckmann et al., 2006) and unsurprisingly non-adherence is associated with mortality in patients who have experienced an MI (Rasmussen et al., 2007).

HF patients who have depressive symptoms and are non-adherent to their medication are 5 times more likely to experience a cardiac event compared with those who are free of depressive symptoms and medication adherent (J. R. Wu et al., 2013). In the Heart and Soul study, medication non-adherence attenuated the association between depressive symptoms and cardiovascular events by 5.3% (Whooley et al., 2008).

Smoking

According to the British Heart Foundation, smokers are almost twice as likely to experience an MI compared with people who have never smoked (2019). Even passive smoking (defined as non-smokers living with someone who smokes) is associated with a 25-30% increase in risk of CHD (Whincup et al., 2004). Smoking cessation significantly reduces the risk of cardiac events and mortality (Athysos et al., 2013). Research shows the smoking rate for people with depression is about twice the rate in the general population (Mathew et al., 2017) and the odds of successful abstinence are lower for those with depression compared with non-depressed individuals (J. Cooper et al., 2016).

The association between depression and smoking is bidirectional, with smokers being more likely to experience depression and those with a history of depression being more likely to smoke (Breslau et al., 1998). Smoking has been proposed as a mediator in the relationship between depression and CVD. In the Heart and Soul study, smoking attenuated the association between depressive symptoms and cardiovascular events by 10.9% (Whooley et al., 2008).

Alcohol

The relationship between alcohol and CVD is a hotly debated topic, particularly regarding the controversial U/J-shaped curve which refers to the potentially cardio protective effect of moderate drinking. Findings from several studies indicate that low intake of alcohol may have a beneficial effect on the reduction of CDV risk (Camargo et al., 1997;Gaziano et al., 1993;Rimm et al., 1991;Suliga et al., 2019), although this claim has subsequently been challenged (Toma et al., 2017). There is some evidence that alcohol affects biological pathways implicated in CHD risk, such as hypertension (L. Chen et al., 2008;Roerecke et al., 2017), BMI (Cho et al., 2015) and lipids (Holmes et al., 2015;Tabara et al., 2016). A combined analysis of individual-participant data for 599,912 current drinkers in 83 prospective studies showed that there is no threshold which lower

alcohol consumption stopped being associated with lower CVD risk (except MI) (Wood et al., 2018).

Much of the contention surrounding the 'abstainer risk' hypothesis comes from study design limitations. Individuals who abstain from alcohol often do so due to increased illness, disability, frailty, medication use or historical alcohol abuse (Fillmore et al., 2006) and this could bias risk estimates for those who are lifelong abstainers (Rehm et al., 2008). However several studies, where former drinkers are removed, continue to show an increased risk of CVD in those who abstain compared with moderate drinkers (S. Bell et al., 2017b; Bergmann et al., 2013; Mukamal et al., 2003). In order to assess whether stability in alcohol intake levels are important in risk estimates O'Neill et al. (2018) conducted a meta-analysis of six cohort studies, including 35,132 individuals, and calculated alcohol intake trajectories over approximately 10 years. They reported that those individuals who abstain from drinking (long term or more recently) and those who inconsistently consume moderate amounts of alcohol intake have a higher risk of experiencing CHD. These findings support the notion of a cardio protective effect of moderate alcohol consumption, providing it is stable. Further research suggests that this protective effect may be explained by inflammatory activation. A 12 year follow-up study including 8,209 older people from the Whitehall II study showed that stable moderate drinkers had lower concentrations of CRP, IL-6 and IL-1 RA compared to non-drinkers during the following 12 years (Bell et al., 2017b).

The relationship between alcohol consumption and depression has also been investigated, with conflicting results. Some studies have shown that heavy, binge drinking is associated with an increased risk of depression (Choi & DiNitto, 2011; Paljärvi et al., 2009; JianLi Wang & Patten, 2002), however this has not always been reported (Haynes et al., 2005). Other studies have reported that people who drink in moderation have a lower risk of depression compared to those who abstain (Gea et al., 2013; Gea et al., 2012), however the reverse has also been reported (Haynes et al., 2005). Using data

from the Whitehall II Study, Bell & Britton (2015) followed 7,478 middle aged men and women, who were free from depression at baseline, for 25-28 years. They found no significant association between drinking patterns and subsequent depression. Another study using the English Longitudinal Study of Ageing (ELSA) cohort also reported no association between drinking patterns and depression in older adults (Garcia-Esquinas et al., 2018). In the Heart and Soul study, regular alcohol consumption only attenuated the association between depressive symptoms and cardiovascular events by 0.5%, suggesting it is not an important biological mediator (Whooley et al., 2008).

Poor diet

Dietary patterns are also associated with both depression and CVD. In particular adherence to a Mediterranean diet (a higher consumption of vegetables, fruit, whole grains, fish and low-fat dairy) is associated with reduced cardiovascular risk (Dobrosielski et al., 2017; Ros et al., 2014; Sofi et al., 2014) and lower depression incidence (Akbaraly et al., 2009; Lassale et al., 2018; Molendijk et al., 2018).

A prospective study of 22,786 Spanish young adults reported that Mediterranean diet was associated with both reduced CVD and depression (Carlos et al., 2018). In a study of 2,171 patients with a history of MI, a Mediterranean style dietary pattern was associated with less depressive symptoms (Rius-Ottenheim et al., 2017). The association between depression and CVD risk was mediated by adherence to a Mediterranean diet in the ATTICA study (Antonogeorgos et al., 2012). Additionally, positive feelings had a protective effect on CVD risk and this was also mediated by Mediterranean eating pattern. A Mediterranean has also been shown to mediate the relationship between depression and ACS prognosis (Chrysohoou et al., 2011; Notara et al., 2016).

Social isolation

Social isolation has been associated with increased mortality risk in post-MI patients (Berkman et al., 1992;Case et al., 1992;Ruberman et al., 1984). In a study of 430 patients with significant CAD, those with three or fewer people in their social support network had more than twice the risk for cardiac mortality (Brummett et al., 2001). The authors also reported that social isolation was also associated with lower income, higher hostility ratings and higher smoking rates.

Social isolation has been associated with depression in a multitude of studies (Cacioppo et al., 2006;Ge et al., 2017;Heikkinen & Kauppinen, 2004;Kawachi & Berkman, 2001). A prospective cohort study of 34,653 American adults found that the absence of close friends was associated with an increased risk of MDD (Chou et al., 2011).

As part of the Enhancing Recovery in Coronary Heart Disease (ENRICHD) pilot study, social support and depressive symptoms were measured in 196 MI patients (Barefoot et al., 2003). The prevalence of depression symptoms across the group was high. Furthermore, high social support was associated with lower depressive symptoms both in hospital and at two week follow up. Another study reported that depressive symptoms and lack of social integration independently predicted recurrent cardiac events in 292 women with CHD (Horsten et al., 2000). When the these two risk factors occurred together, the risk was almost four times higher than without them, regardless of other clinical prognostic factors.

7.6.2 *Biological factors*

Biological dysregulation has also been implicated in the association between depression and CVD. The next section describes evidence for biological factors, including autonomic nervous system (ANS) dysfunction, endothelial dysfunction, platelet activation, common

genetic factors, mental stress–induced ischemia, activation of the HPA-axis and inflammation. It should be noted that many of these factors are also associated with poor health behaviours and it is yet unclear to what degree they are behavioural consequences or parallel mechanisms linking depression and CVD (Whooley & Wong, 2013).

Autonomic dysfunction

The autonomic nervous system (ANS) is divided into the sympathetic nervous system (SNS) and the parasympathetic nervous system (PSNS) that act to increase and decrease heart rate, cardiac contractility and vasodilation, respectively (Whooley & Wong, 2013). Sympathetic activation can be caused by physical activity, coronary ischemia, heart failure, and mental stress and has been shown to increase the risk of adverse cardiovascular outcomes, including mortality (Curtis & O'Keefe, 2002).

Acute stress activates sympathetic and inhibits parasympathetic nerves as part of the fight or flight response (B. W. Penninx, 2017). People with depression are thought to have an ANS that is in a relative state of increased sympathetic and decreased parasympathetic activation. Depressed CHD patients tend to have higher levels of circulating catecholamines, such as norepinephrine, epinephrine, and cortisol, which are markers of sympathetic activation (Carney et al., 2005b).

Another measure of ANS activity is heart-rate variability (HRV). HRV is the variation in the time interval between heartbeats and reflects the balance between the SNS and PSNS, with low HRV suggesting increased SNS activity or decreased PSNS activity. Low HRV is a predictor for CVD and mortality (J. M. Dekker et al., 2000) and for somatic symptoms associated with CVD such as, tachycardia, hypertension, serum triglycerides, serum glucose, and low high-density lipoprotein (HDL) cholesterol (Licht et al., 2013).

A meta-analysis of 18 studies investigating the impact of depression on HRV, including 673 depressed participants without CVD and 407 controls, reported that depression is associated with reduced HRV, which decreases with increasing depression severity (Kemp et al., 2010). However findings are inconsistent with several studies reporting no association (Kemp et al., 2013;Licht et al., 2008;Licht et al., 2015). These conflicting results may be explained by antidepressant use, which has been consistently associated with low HRV. In the meta-analysis by Kemp et al. (2010) TCAs were found to decrease HRV, which was also confirmed in the studies by Licht et al. (2008;2015). Evidence also exists for the reducing effects of SNRIs and SSRIs, but this effect appears to be much smaller and findings are inconsistent (B. W. Penninx, 2017).

Decreased HRV has also been associated with depression in patients after acute MI (Carney et al., 2005a;Carney et al., 2001) and in patients with depressive symptoms and stable CAD (de Jonge et al., 2007). In 907 patients from the Cardiovascular Health Study, Kop et al. (2010) reported that adjusting for HRV attenuated the association between depression and increased cardiovascular mortality risk. However, the Heart and Soul Study found that adjustment for HRV did not change the effect size of the association between depressive symptoms and cardiovascular events (Whooley et al., 2008).

Endothelial dysfunction

Endothelial dysfunction is also associated with most cardiac risk factors and is involved in all stages of atherosclerosis (Hadi et al., 2005). The endothelium, or lining of the blood vessel wall, can become inflamed in the presence of CVD risk factors such as smoking and hypercholesterolemia. This results in an increase in vasoconstrictive and prothrombotic factors, increasing the risk of a cardiac event. Depression has also been associated with endothelial dysfunction, even in people free of other CVD risk factors (Broadley et al., 2002;D. C. Cooper et al., 2011;Rybakowski et al., 2006). A meta-

analysis including 12 studies and 1,491 people, including healthy adults and cardiovascular patients, found an association similar in magnitude to the association between obesity and systolic blood pressure (D. C. Cooper et al., 2011). In patients with traditional cardiac risk factors the association was even stronger.

Platelet activation

Factors that promote platelet activation and coagulation play a key role in thrombus formation and the progression of CVD (Serebruany et al., 2003). Increased platelet activity and elevated coagulation markers, particularly platelet factor 4 and beta-thromboglobulin, have been observed in patients with depression and CVD (Kuijpers et al., 2002;Serebruany et al., 2003;Shimbo et al., 2002), However data on platelet activity in depression in general is inconclusive and demands further exploration (von Kanel, 2004). Measuring platelet function is complex and no single test currently exists. Furthermore, several methodological issues mean that correlating measures of platelet function with clinical outcomes is problematic (Gurbel et al., 2007).

Common genetic factors

For several decades, it has been known that CHD tends to cluster families (Dai et al., 2016;Musunuru & Kathiresan, 2019;Rissanen, 1979;Rose, 1964). As previously discussed, depression is also more common among first degree-relatives of individuals who have been depressed (Geschwind & Flint, 2015). Scherrer et al. conducted a study examining the genetic and environmental contributions to the covariation of depressive symptoms and CVD in a sample of 6,903 male-male twins from the Vietnam Era Twin Registry (2003). They found that CVD was more common among individuals reporting five or more symptoms of depression and among those with more severe symptoms. The genetic correlation between CVD and depression was significant and showed that that nearly 20% of genetic influences were common across both phenotypes. A
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systematic review of genome wide and candidate gene studies investigating genetic variants associated with CVD and mood disorders reported 24 potential pleiotropic genes that are likely to be shared between the two (Amare et al., 2017). Taken together, these findings suggest that lifetime co-occurrence of these CVD and depression is partly explained by common genetic risk factors.

As previously mentioned in Chapter 1, the short allele of a polymorphism in the promoter region of the serotonin transporter gene (5-HTTLPR) is associated with increased risk of depression in medically healthy individuals (Caspi et al., 2003; Serretti et al., 2007) and poor antidepressant response (Porcelli et al., 2012). In the Heart and Soul study, the association of 5-HTTLPR with depression and 24-hour urinary norepinephrine excretion in 557 outpatients with CHD was examined (Otte et al., 2007). The authors reported that the short allele was associated with depression and greater 24-hour urinary norepinephrine excretion. These findings suggest that the 5-HTTLPR genotype may be an important vulnerability factor for both depression and adverse outcomes in CHD patients.

Activation of the HPA-axis

As previously discussed in Chapter 1, Section 1.5.2, hyperactivity of the HPA-axis and elevated cortisol in depressed patients is an enduring and well-replicated finding (Pariante & Lightman, 2008). It has also been posited that HPA-axis dysfunction may contribute to the pathogenesis of depression and comorbid CVD. From a physiological perspective, cortisol affects the metabolism of almost every tissue in the body and plays an important role in lipid and glucose metabolism (Baxter & Forsham, 1972). HPA axis dysregulation and the resulting prolonged exposure to elevated levels of glucocorticoids is also related to many CVD risk factors such as hypertension, dyslipidemia, insulin resistance, glucose intolerance, and central adiposity (B. R. Walker, 2007). In a comprehensive review by Girod and Brotman (2004), three main physiological roles of

glucocorticoids are identified. Firstly, they prime the metabolic, autonomic, psychological, haemostatic and cardiovascular aspects of the stress response in preparation for everyday stress. Secondly, they suppress the inflammatory response and cellular proliferation, preventing autoimmunity and circulatory collapse. Thirdly, they play a role in body composition, affecting insulin resistance and lipogenesis. Whilst some of these functions, such as the suppression of inflammation, may be beneficial to the cardiovascular system, others such as hypertension and insulin resistance may be harmful. On balance it is likely that increased glucocorticoid production is deleterious to cardiovascular health (Girod & Brotman, 2004).

Indeed, alterations in diurnal cortisol have been associated with CVD risk in several observational studies. Salivary cortisol reactivity over the first 20 and 60 min after awakening has been positively associated with progression of intima media thickness in women (Eller et al., 2005). In the Multi-Ethnic Study of Atherosclerosis Stress Study, coronary artery calcification was associated with a flatter decline in morning cortisol in 464 older men and women (Hajat et al., 2013). In the CARDIA Study, coronary calcification was associated with a flatter cortisol slope in 718 healthy middle-aged adults (Matthews et al., 2006). Cortisol AUC has also been associated with atherosclerosis of the carotid arteries in 1,866 healthy, elderly people in the Rotterdam Study (M. J. Dekker et al., 2008).

Cortisol patterning has also been explored in people with established CVD. CAD patients have been shown to demonstrate higher 24 hour cortisol secretion and a flattened diurnal slope, compared to clinically healthy controls (Nijm et al., 2007). The CAR has been shown to be flattened in men with CVD (Vreeburg et al., 2009b). Flatter cortisol rhythms have also been associated with depression in CAD patients (Bhattacharyya et al., 2008) and CHD patients with a history of MI have been shown to have lower AUC values compared to CHD patients who had no previous MI, suggesting that cortisol output is associated with CHD severity (Merswolken et al., 2013). Alterations in diurnal cortisol

profile have also been associated with depressed mood in CABG surgery. In a study of 171 patients undergoing first-time, elective CABG surgery, a steeper cortisol slope measured 60 days after surgery predicted reduced odds of depression 12 months after surgery (Poole et al., 2016). However, the Heart and Soul Study found that adjustment for 24-hour excretion of cortisol levels did not change the strength of association between depressive symptoms and adverse cardiovascular events (Whooley et al., 2008).

Dysregulation of the HPA axis has also been associated with cardiovascular mortality. In a study using data from the Whitehall II cohort, diurnal cortisol profiles were examined in 4,047 civil servants who were followed up over a six year period (Kumari et al., 2011). Results showed that flatter cortisol slopes were associated with increased risk of all-cause mortality and that this was mainly driven by an increased risk of cardiovascular deaths.

This contribution of HPA-axis dysfunction to depression and comorbid CVD may be mediated, at least in part, by the loss of glucocorticoid receptor sensitivity. This was investigated in a study by Nikkheslat et al. (2015) who measured plasma and salivary cortisol, gene expression of GR and in vitro GR sensitivity in 83 CHD patients with and without comorbid depression. Results showed that depressed CHD patients had lower plasma and saliva cortisol levels and a reduction in GR expression and sensitivity. In a study including 382 in-patients with mood disorder, non-suppression to the DST and higher baseline serum cortisol predicted CVD death, suggesting that HPA axis dysregulation may be a mediating factor between depression increased CVD mortality risk (Jokinen & Nordstrom, 2009).

A potential mechanism whereby impaired GR sensitivity increases CVD risk is via reduced negative feedback on inflammatory signalling, resulting in immune activation. Importantly, disruptions of the HPA-axis may be enhanced by pro-inflammatory cytokines, which further impair GR receptor functioning, creating a feed-forward cascade

and reflecting a complex bidirectional biological dialogue between the immune system and the HPA-axis (Sasayama et al., 2011).

7.7 Inflammation, depression and CVD

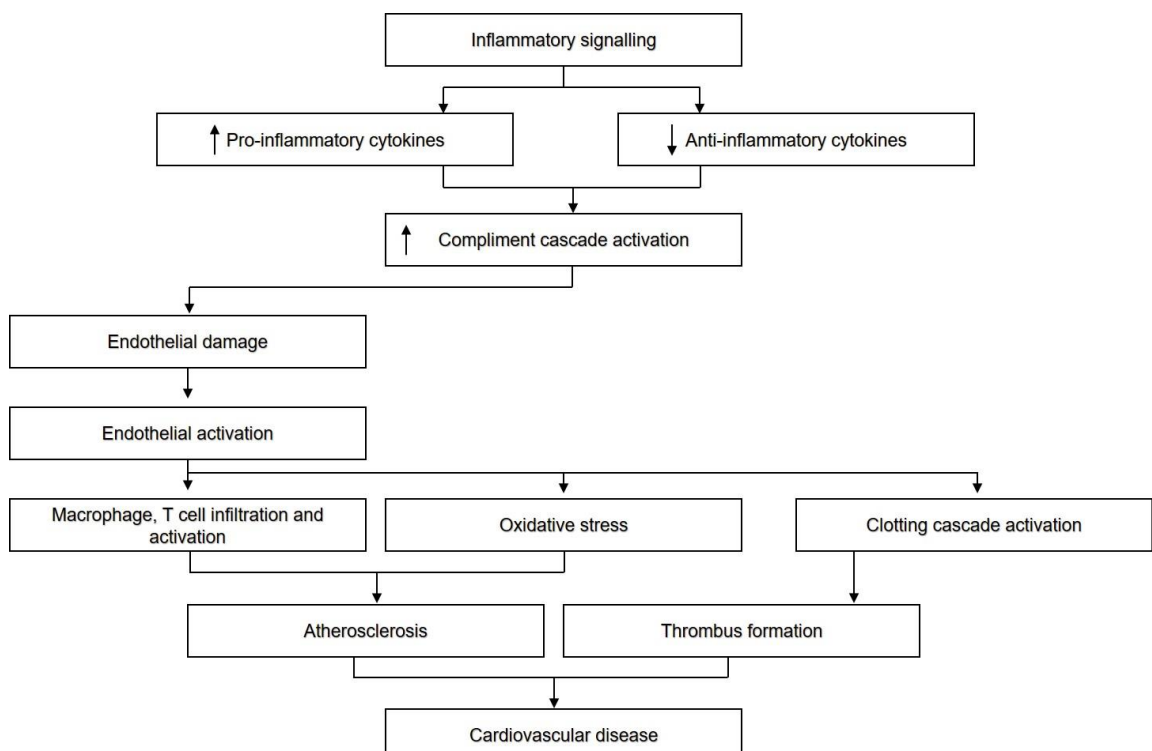
As previously discussed in Chapter 1, Section 1.4.2, meta-analyses have robustly demonstrated that inflammatory biomarkers (IL-6, TNF- α , CRP) are associated with depression (Haapakoski et al., 2015). Chronic, systemic increases in pro-inflammatory cytokines also predict CVD events and mortality in both healthy people (Cesari et al., 2003; Danesh et al., 2008; Kaptoge et al., 2012; Kop et al., 2010; Woodward et al., 2007) and patients with established disease (Heeschen et al., 2000; Kop et al., 2011). Results from a meta-analysis of 54 prospective cohort studies, including 160,309 people with no history of CVD, showed that CRP is consistently associated with CHD, ischaemic stroke and mortality (Kaptoge et al., 2010). Indeed, atherosclerosis is now considered a fundamentally inflammatory disease (Ross 1999). There is now a growing consensus that inflammation may be a key biological link in the comorbidity of depression and CVD (Golia et al., 2014; Halaris, 2013c; Joynt et al., 2003; Kop & Gottdiener, 2005; Lippi et al., 2009).

7.7.1 Biological pathways

Over the past two decades a number of pro-inflammatory mediators have been shown to play a central role in the initiation, progression, and complications of atherosclerosis. These including acute-phase proteins, CRP, fibrinogen, immunoglobulins, adhesion molecules, and cytokines (Halaris, 2013b). This role may be characterised by interactions between inflammatory signalling cascades and several CVD related pathways, as illustrated in Figure?. Inflammatory factors, such as CRP, activate the classical complement cascade. The complement system is a complex protein network of the innate immune system that influences many processes involved in the development

and progression of atherosclerosis, (Carter, 2012; Hertle et al., 2014). The complement system is thought to contribute to several aspects of endothelial dysfunction, including promotion of endothelial cell activation, leukocyte adhesion, increased endothelial permeability, monocyte infiltration into the extracellular matrix, and stimulation of cytokine release from VSMCs (Steyers & Miller, 2014). Local inflammatory cytokines in atherosclerotic plaques also initiate the adaptive immune response, resulting in the infiltration of T-cells. CD4+ T helper cells and CD8+ cytotoxic T cells promote endothelial dysfunction, lipid accumulation in macrophages with subsequent foam cell formation and cell death (Ammirati et al., 2015). In addition, innate immune activation promotes oxidative stress. Oxidative stress broadly describes an imbalance between reactive oxygen species (ROS) and antioxidants (Sies, 2015). Macrophages release ROS, which promote platelet activation, endothelial dysfunction, apoptosis and thrombosis of atherosclerotic plaques (Cervantes Gracia et al., 2017; Santilli et al., 2015). Innate immune cells also express tissue factor, which initiates the clotting cascade, resulting in thrombus formation (Iba & Levy, 2018).

Figure 7.2. Interactions between inflammatory signalling and CVD related pathways.



Adapted from Baune et al. (2012).

7.7.2 Association between depression and inflammation in CVD

Studies examining both depression and inflammation cross-sectionally in CVD populations have reported mixed results. Bankier et al. (2009) examined 72 stable CHD outpatients with MDD and found a significant association between MDD and CRP. Depressive symptoms have been associated with increased CRP in 65 patients who were recovering from ACS (G. E. Miller et al., 2005a). Shimbo et al. (2006) measured depression remission status three months after an ACS event in 103 patients and found that persistent depression was significantly associated with raised CRP. A more recent study including 164 post-ACS patients, depressive symptoms were independently associated with elevated markers of IL-17 (Celano et al., 2017). Mixed findings were reported by Lespérance et al. (2004) who studied 481 patients 2 months after hospitalization for ACS. Depressed patients showed significantly higher levels of soluble intercellular adhesion molecule 1 but not IL-6. Similarly Frasure-Smith et al. (2009) found that CRP but not IL-6 was elevated in depressed cardiac patients compared with non-depressed. In contrast, a study by Kim et al. (2018) including 969 ACS patients reported that higher IL-6 and IL-18 levels were independently associated with depression. IL-6 was also shown to be associated with pre-operative depression in 236 patients two weeks before cardiac surgery (Ai et al., 2005). Increased levels of TNF- α have also been observed in depressed MI-patients compared with non-depressed (2014). Increased TNF- α , but not IL-6 or IL-1beta, has also been associated with depression in heart failure patients (Moorman et al., 2007).

However, some studies have only reported null findings. In a case-control study of 57 depressed patients and 46 non-depressed patients, who had all been hospitalized for MI, Schins et al. (2005) reported no differences in IL-6 or TNF- α between groups. Similarly, a study of 247 women who had survived acute MI found no evidence of a relationship between depressive symptoms and CRP, IL-6 or IL-1ra (Janszky et al., 2005). IL-1beta was also shown to be not associated with depression in older cardiac

patients (Lyness et al., 2001). However, a meta-analysis by Howren et al. (2009) confirmed that CRP, IL-6, and IL-1 are associated with depression in CVD populations.

It should be noted that findings from studies including CVD patients need to be interpreted with caution. Medications such as statins and aspirin are known to have anti-inflammatory effects (Greenwood & Mason, 2007; Vane & Botting, 2003) and the predictive effects of inflammatory cytokines on depression in ACS patients is attenuated in patients taking statins (S. W. Kim et al., 2018). Despite these findings, medications have not been consistently controlled for in the literature. Furthermore, the quality of published evidence on CRP and CVD prognosis has been criticised by some, particularly regarding publication bias, confounding and methodology, undermining the reliability of this relationship (Hemingway et al., 2010).

7.7.3 *Depression, inflammation and CVD risk*

Given the fact that both depression and inflammation appear to exist simultaneously in cardiac patients, it is possible that inflammation may moderate the relationship between depression and CVD (Shimbo et al., 2005). Depression and inflammation may both be causal to CVD but via independent pathways. Conversely, there may be a shared aetiology that is itself associated with CVD. Inflammation may cause both depression and CVD, with no direct relationship existing between depression and CVD. Finally, depression may precede inflammation. In this scenario, inflammation may mediate the relationship between depression and CVD, explaining its existence. Or depression may moderate the relationship between inflammation and CVD, influencing its strength. Prospective studies have attempted to explore this issue in both CVD and healthy populations.

Several studies have investigated the role of depression status as a prognostic factor in patients with existing CVD. A study by Frasure-Smith et al. (2007) explored the impact

of both depression and inflammation on cardiac prognosis in 741 men with stable CAD. The authors reported that depression and CRP interacted in predicting future cardiac events: only those men with low levels of depression and low levels of CRP demonstrated low risk of major adverse cardiac events. In addition, the effect of CRP was largely restricted to the non-depressed men and the effect of elevated depression symptoms was largely restricted to those with lower levels of CRP. This finding does not support the notion that inflammation is acting as a mediator or that inflammation and depression are operating via separate pathways, but that both factors mutually influence each other.

However, in another study, Frazier et al. (2009) measured CRP and depressive symptoms in 490 patients following elective coronary stent insertion and followed them up over two years. The results showed that both depression and CRP were predictive of a future major adverse cardiac event. Furthermore, depression was a stronger predictor when inflammatory proteins were removed from the analysis, suggesting that inflammation may be a partial mediator of depression and cardiac outcomes. Vaccarino et al. (2007) followed 559 women with suspected coronary ischemia over 5.9 years. They reported that depression predicted CVD and that CRP explained 13% and IL-6 explained 4% of the association, suggesting a minor role of inflammation in the relationship between depression and CVD. Similarly in the Heart and Soul study, which followed 1,017 stable CHD patients over 4.8 years, CRP only explained a small part of the association between depressive symptoms and cardiovascular events (Whooley et al., 2008).

Population-based studies of community samples have also explored these relationships, mainly in older people. The results of these studies largely support the idea that depressive symptoms and inflammatory biomarkers increase CVD risk independently. Nabi et al. (2008) used data from 6,396 CHD free civil servants from the Whitehall II Study, (4,453 men and 1,943 women) who were followed up over 11 years. Negative

affect, psychological distress, and inflammatory markers predicted incident CHD, however there was no association between psychological factors and increased inflammation. In a Canadian cohort study, including 1,794 participants, depression and inflammation were associated at baseline and each significantly predicted CHD separately (K. W. Davidson et al., 2009). Furthermore, when they were analysed in the same model, each remained significant. In a nested case-referent study within the Prospective Epidemiological Study of Myocardial Infarction (PRIME) study, Empana et al. (2005) followed 9,758 healthy, middle-aged, CHD free men over 5 years and reported that baseline depressed mood was associated with future CHD and that this association remained stable after adjustment for inflammatory markers. Similar findings were observed by Arbelaez et al. (2007), who showed that CRP did not mediate the relationship between depressive symptoms and risk of ischemic stroke in 5,525 elderly people. CRP was also shown not to explain any association between depression and mortality in the National Diet and Nutrition Survey in older adults (2011).

However some studies do report minimal effects of mediation. A study by Surtees et al. (2008b), including 19,649 people (41-80 years), found that baseline depression was associated with incident ischemic heart disease (IHD) one year later and that CRP explained 15% of the association. Hughes et al. (2016) followed 2,389 older men over 18 years and reported that CRP explained 7.3% of the association between depressive symptoms and all-cause mortality. Findings from the Cardiovascular Health Study showed that IL-6 attenuated the depression-CVD mortality association by 7.9% in older people (Kop et al., 2010). In another study of 6,576 healthy people, CRP accounted for 5.5% of the association between psychological distress and CVD events (M. Hamer et al., 2008a). Finally, Hiles et al. (2015) found that CRP explained 8.1 % and IL-6 10.9 % of the effect of depression on cardiovascular events in a study including 1,692 participants. Taken together these studies suggest that whilst inflammation may confer nominal effects of mediation, it is unlikely to explain the associations observed between depression and CVD.

Given the fact that depression and inflammation appear to have largely independent roles in the prediction of cardiovascular disease, it is possible that the presence of both may further increase cardiovascular disease risk. To date only one study has investigated the combined effect of depressive symptoms and elevated inflammation on CVD development. Using data from the MONICA–KORA Augsburg Cohort Study, Ladwig et al. (2005) analysed CRP and depressive symptoms in 3,021 healthy, older men who were followed up over 7.7 years. They reported that compared with men who had low CRP and no depressive symptoms, men with high CRP and depressed mood had an increased risk of a future CHD event, however men with high CRP and no depressed mood had no increased risk, indicating that there is a synergy between inflammation and depression in CHD prediction. There was a significant interaction between depressive symptoms and inflammation after adjustment for age, although this lost significance in the fully adjusted model. The authors speculate that both conditions may share a common underlying mechanism. However this study was restricted to older men and so it is unclear whether this effect is present in women, therefore further studies including both men and women are required.

7.7.4 *Gender differences in depression and mortality*

Largely due to the fact that more men die from CHD than women, and at a younger age (40–60 years), there is a bias in the literature towards studies which include a majority of men (Fairweather, 2014; Shaw et al., 2009). Furthermore, data are not typically analysed separately according to sex. This means that our understanding of the factors important to CVD risk are skewed in favour of men.

Some studies have reported sex differences in the association between late-life depression and mortality, with several reporting a greater mortality risk in men. For example, Ryan et al. (2008) explored the association between depression and four year survival in 7,363 elderly people. They reported that increasing depression severity was

associated with incrementally higher mortality risk in men only. In women the association between depression and mortality was less marked and only significant for severe depression in the absence of treatment. Anstey & Luszcz (2002) measured depressive symptoms in 1,947 elderly people and showed that in men, incident depression was associated with mortality but not in women. MDD was shown to be an independent risk factor for mortality in men only in a study of 1,000 elderly people by Jeong et al. (2013). The relationship between depression and mortality risk was also shown to be considerably stronger in men compared to women in the Bambuí Cohort Study of Aging (Diniz et al., 2014). Differential effects have also been reported by a study showing that chronic depression predicts increased mortality in older women and incident depression predicts increased mortality in older men (Teng et al., 2013).

Regarding CVD specifically, a prospective cohort study of 3,701 elderly people found that newly depressed mood was independently associated with an increased risk of CVD mortality, new CVD events and new coronary heart disease events in men, but not women (B. W. Penninx et al., 1998). Furthermore, newly depressed older men were twice as likely to have a CVD event as those who were never depressed, whilst this finding was not present in women. A systematic review reported that depression was associated with significantly increased risk of cardiovascular death for men, but not women (Wulsin et al., 1999). In contrast, a prospective longitudinal study of 860 women from the Geelong Osteoporosis Study showed that baseline depression predicted 18-year CHD incidence, adjusting for a wide variety of risk factors (O'Neil et al., 2016).

Sex differences in inflammation have been reported, including higher CRP levels in women (by potentially up to 60%) compared to men, with and without CVD risk factors (Sobhani et al., 2018). Furthermore, CRP is a strong independent risk factor for CVD in women (Ridker et al., 1998). Increased inflammatory biomarkers are also associated with several cardiovascular risk factors, such as high body mass index, blood pressure, and smoking status in healthy, CVD-free women (Bermudez et al., 2002). This suggests

that increases in inflammation may be of particular importance for cardiovascular risk in women. Whether the inflammation and depression link is particularly significant for women is still unknown.

7.8 Chapter summary

Overall the evidence suggests that cardiovascular disease is epidemiologically linked to both depression and inflammation. A robust literature has demonstrated that people with depression or elevated inflammation are at increased mortality risk, particularly from CVD. Furthermore depressive symptoms and inflammatory biomarkers co-vary in those with established CVD. Despite the magnitude of literature in this field, the exact nature of the relationship between these two conditions remains elusive. The majority of the evidence suggests that depression and inflammation influences CVD risk via independent pathways, with minimal effects of mediation or moderation. The effect of combined depression and inflammation has only been investigated in one study and that was restricted to older men only. It is still therefore unclear whether this combination confers a significantly greater CVD risk than either depression or inflammation alone, and whether this risk is increased in both men and women. Therefore, study 2 of this PhD will investigate the following research questions:

- a) Do people with both depressive symptoms and increased inflammation have significantly greater CVD mortality risk than people with depressive symptoms or inflammation alone?

- b) Do depressive symptoms moderate the mortality risk associated with increased inflammation or does inflammation mediate the association between depressive symptoms and CVD mortality?

8. Study 2 - Combined influence of depressive symptoms and systemic inflammation on all-cause and cardiovascular mortality in ELSA

8.1 Overview

This chapter concerns the findings from our analysis of the ELSA dataset. As discussed in Chapter 7 the combined effect of depression and inflammation on mortality has not been investigated fully. The aim of this study was to prospectively investigate the combined association of these factors with the prediction of CVD and all-cause mortality in 5,328 older men and women from the ELSA cohort. A brief overview of the ELSA cohort and study background is presented followed by the methods and results of this study, and a discussion of the findings. The results of this study have been published in *Psychological Medicine* (Lawes et al., 2018).

8.2 The English Longitudinal Study of Ageing (ELSA) Cohort

ELSA is a prospective study of community-dwelling people aged 50 and over, that collects health, social and economic data. The present sample contains data from up to seven waves of data collection, spanning fourteen years and includes objective and subjective data relating to health, biological markers of disease, economic circumstance, social participation and well-being. The study was designed to investigate the complex relationships relating to the process of ageing, including health trajectories, life expectancy and the relationship between physical and mental health. For more information on ELSA see <http://www.elsa-project.ac.uk/>.

ELSA commenced in 2002, and the sample has been followed up every 2 years. Sample members are drawn from respondents to the Health Survey for England (HSE). Data are collected during face-to-face interviews, using computer-assisted interviewing and self-completion questionnaires, with additional nurse visits for the assessment of biomarkers

every 4 years. Wave 1 took place in 2002-2003 and included a baseline interview. It consisted of 11,391 study members and was deemed to be nationally representative. Wave 2 took place in 2004-2005 and consisted of an interview and a health examination that study participants were added from HSE to maintain the size and representativeness of the panel.

8.3 My contribution to the ELSA Study

As part of a team of researchers involved in this study, I contributed to the study design under guidance from Dr Livia Carvalho, Professor Andrew Steptoe and Professor Glyn Lewis. I was responsible for the interpretation of the results from the statistical analyses, under guidance from Dr Panayotes Demakkakos. Furthermore I reported the results of this study in a first-author publication (Lawes et al., 2018)

8.4 Introduction

As previously mentioned in Sections 1.2 and 2.5, a number of population-based studies have reported that people with depressive symptoms are at greater risk of mortality (Cuijpers & Smit, 2002;Lasserre et al., 2016), particularly from CVD (Correll et al., 2017). Many studies have specifically reported an association between depressive symptoms and mortality in older people (Eurelings et al., 2018), however these findings are not consistent (Callahan et al., 1998;Cuijpers, 2001;Hybels et al., 2002;McCusker et al., 2006). Systemic increases in pro-inflammatory cytokines also predict CVD events and mortality in people with (Heeschen et al., 2000;Kop et al., 2011) and without CVD (Cesari et al., 2003;Danesh et al., 2008;Kaptoge et al., 2012;Kop et al., 2010;Woodward et al., 2007). Extensive evidence also exists for an association between depressive symptoms and inflammation, with increased inflammatory biomarkers in depressed people compared with controls (Dowlati et al., 2010;Goldsmith et al., 2016b;Hiles et al.,

2012b;Howren et al., 2009;Kohler et al., 2017;Y. Liu et al., 2012). However, it is unclear whether the increased mortality risk in depressed people is due to inflammation.

As previously discussed, it is possible that inflammation may be mediating the association between depression and mortality, however evidence is inconsistent. Some studies demonstrate that depressive symptoms increase CVD risk independently of inflammation (Arbelaez et al., 2007;K. W. Davidson et al., 2009;Empana et al., 2005;Nabi et al., 2008), whilst others have reported minimal effects of mediation (M. Hamer et al., 2008a;Hiles et al., 2015;Hughes et al., 2016;Kop et al., 2010;Surtees et al., 2008b). To our knowledge only one study has investigated the combined effect of depressive symptoms and elevated inflammation on CVD development. Ladwig et al. (2005) showed that combined depressed mood and high inflammation conferred a significantly greater risk of future cardiac events than either depressed mood or high inflammation alone in older men. It is still unclear whether this combination confers a significantly greater CVD risk than either depression or inflammation alone, and whether this risk is increased in both men and women.

Gender differences have also been observed in the association between late-life depression and mortality. Several studies report a greater mortality risk in men (Anstey & Luszcz, 2002;Diniz et al., 2014;Jeong et al., 2013;Ryan et al., 2008;Teng et al., 2013), particularly for CVD (B. W. Penninx et al., 1998;Wulsin et al., 1999). Sex differences in inflammation have also been reported, including higher inflammation in women compared to men (Sobhani et al., 2018). Furthermore, inflammation is a strong independent risk factor for CVD in women (Bermudez et al., 2002;Ridker et al., 1998). Whether the inflammation and depression link is particularly significant for women is still unknown.

In this study, we used data from ELSA to investigate the combined effects of depressive symptoms and inflammation on CVD and all-cause mortality risk in older men and

women. In particular, we sought to investigate whether depressive symptoms moderate the mortality risk associated with increased inflammation or whether inflammation could be mediating the association between depressive symptoms and mortality.

8.5 Hypotheses

Given that both depression and inflammation predict CVD and all-cause mortality, and the combination of both factors has been shown to increase the risk of future cardiac events in men (Ladwig et al., 2005), **I hypothesise that people with both depressive symptoms and inflammation will have significantly greater mortality risk than people with depressive symptoms or inflammation alone (hypothesis 1).**

The evidence for inflammation as a mediator suggests that any effect is likely to be minimal, **therefore I hypothesise that depressive symptoms and inflammation will have largely independent effects on mortality (hypothesis 2).**

8.6 Materials and methods

8.6.1 *Study Population*

The study included 5,328 people, aged 52 to 89 years, from an initial cohort of 8,670 who participated in the interview at wave 2. We excluded 1,084 individuals who did not participate in the health examination survey, 134 who did not consent for their vital status data to be included, 1,411 who were unable to provide a blood sample, 330 individuals whose CRP values were unavailable or not reliable. The latter included samples which were lost in the post, received later than five days after collection, considered unusable by the laboratory or of insufficient amount to be analysed. Detailed information about how the ELSA data was collected and processed into its current format and about how each variable was coded is available at:

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<https://discover.ukdataservice.ac.uk/series/?sn=200011>. We also excluded 155 participants from the analysis because their CRP levels were ≥ 20 mg/L, allowing for the elimination of individuals with acute inflammation, while another 229 participants were excluded because of missing values in the covariates

8.6.2 *Assessment of inflammation*

Blood samples were taken by the study nurse at wave 2 and serum CRP was analysed by Royal Victoria Infirmary, Newcastle. High sensitivity plasma CRP level was dichotomised into two categories: < 3 mg/L was defined as normal and 3-20mg/L was defined as high. This cut off point is based on guidelines from the Center for Disease Control and Prevention and the American Heart Association suggesting that plasma CRP values of > 3.0 mg/L might be predictive of cardiovascular disease (Pearson et al., 2003).

8.6.3 *Assessment of depressive symptoms*

Depressive symptoms were measured at wave 2 (2004-2005) using the eight-item Centre for Epidemiological Studies Depression Scale 8 (CES-D8) which is a self-report questionnaire designed to measure depressive symptomatology in the general population (Radloff, 1977). Respondents were asked how often they felt depressed, felt that everything was an effort, slept restlessly, were happy, felt lonely, enjoyed life, felt sad, and could not get going. The two positive items ('was happy' and 'enjoyed life') are reverse coded, so a higher score here also indicates a more depressed mood. We subsequently derived a summary CES-D score by adding responses to all eight dichotomous questions (possible range: 0–8). Exact wording of the different items can be found in Appendix 11.12. The eight-item abbreviated version of the CES-D has been widely used, is internally consistent, has been validated in both the general population (Van de Velde et al., 2009) and older adults, and shows a comparable construct of depression across eleven different countries (Missinne et al., 2014). The presence of

depressive symptoms was defined as CES-D ≥ 4 as per previous publications (Demakakos et al., 2013; Mark Hamer et al., 2012; Malgaroli et al., 2017; Mhaoláin et al., 2012; Steffick, 2000). This conservative threshold has been found to produce comparable results to the >16 cut off on the well-validated 20-item CES-D scale (Radloff, 1977; Steffick, 2000).

8.6.4 *Depressive symptoms and inflammation as a combined variable*

In order to ascertain whether the combination of depressive symptoms and inflammation predicts mortality, the variables based on wave 2 assessments were combined and four new variables were computed: 'no depressive symptoms/low inflammation', 'no depressive symptoms/high inflammation', 'depressive symptoms/low inflammation' and 'depressive symptoms/high inflammation'.

8.6.5 *Mortality*

Mortality was ascertained for a mean 7.7 year period for consenting study members (5,328) by linking to the UK National Health Service mortality register up until 12th November, 2015. In England, all deaths need to be registered within five days, therefore participants not registered as dead were assumed to be alive. Deaths were classified according to International Classification of Diseases (ICD) 10th Edition. Deaths with ICD10 codes I00 to I99 were classified as cardiovascular deaths.

8.6.6 *Covariates*

All covariates were collected at wave two (2004-2005) with the exception of education and sex which were collected at wave one. All covariates were determined by self-report, with the exception of body mass index (BMI). Age was treated as a continuous variable. Socioeconomic status (SES) was operationalized by using marital status

(married/cohabiting vs. not married/single/divorced), level of education (degree/higher/A-level, GCSE/O-level/other, no qualifications) and total wealth in tertiles. Total wealth was defined from the sum of financial, physical (e.g. businesses, land) and housing wealth, minus debts and pension payments. Health behaviours included: smoking (never smoked, ex-smoker, current smoker) and BMI (<25kg/m², 25-29.99kg/m², >30+kg/m²) (Banks et al., 2006). The presence of chronic diseases were added as separate variables and defined as yes/no. They were calculated as lifetime self-reported physician diagnoses of chronic conditions (i.e. cardiovascular disease (myocardial infarction and stroke), chronic lung disease, cancers (of any site) and emotional, nervous and psychiatric problems).

The covariates were included because they have all been shown to be associated with mortality and are therefore potential confounders (Marmot et al., 2012; Pletcher & Moran, 2017; N. D. Wong, 2014). Furthermore, health behaviours such as smoking and BMI were included as they have been shown to mediate the relationship between depression and mortality (Joynt et al., 2003).

8.7 Statistical analysis

Baseline characteristics were analysed by depressive symptoms and inflammation levels. Statistical tests examined the associations of demographic variables with depressive symptoms/inflammation levels. ANOVA tests were used for continuous variables and Chi square tests were used for categorical variables.

Differences between the characteristics of the participants included and excluded from the analysis were analysed. Statistical tests examined the difference in demographic variables between included and excluded samples. ANOVA tests were used for continuous variables and Chi square tests were used for categorical variables.

The association of depressive symptoms and inflammation levels (separately and in combination) with CVD and all-cause mortality were assessed by Cox proportional hazards regression models. The proportionality assumption was tested using Nelson-Aalen cumulative hazard curves and Schoenfeld residuals. We inspected the plots and the global test ($p = 0.8052$) confirming that we do not have a violation of the proportional assumption. Survival time was measured in months, from the date of interview in wave 2 (2004-2005) to the date of death or 12 November 2015, whichever was first. Kaplan Meier survival curves are available in Appendix 11.12.

We investigated whether there were significant interactions between age or sex and depression/inflammation categories in order to determine whether the association between depression/inflammation levels and mortality varied according to age or between men and women. We used the likelihood ratio test to compare the goodness of fit of a stratified model. We found no interaction between sex and depressive symptoms, however we did find that the association between inflammation and mortality varied significantly by sex (P value = 0.013). There was also an age interaction between inflammation and mortality, however this disappeared once we stratified our analysis by sex, therefore we only present the sex-stratified analyses. We first fitted a basic unadjusted model, which was followed by an age-adjusted model. We then additionally adjusted for socioeconomic variables, health behaviours and chronic diseases.

To investigate moderation we tested whether a multiplicative interaction between depressive symptoms and inflammation was significantly associated with mortality. To investigate mediation we first examined the association between depressive symptoms and mortality and then added inflammation to see how much of the association was explained. All analyses were performed using STATA 13.0 (StataCorp LP, College Station Texas).

8.8 Results

8.8.1 Participants

Table 8.1 represents social-demographic characteristics in depression/inflammation categories in men and women separately. Men and women with concurrent depressive symptoms and high inflammation were more likely to be poorer, less educated, more likely to smoke, have a higher BMI and were more likely to have chronic lung disease and emotional, nervous and psychiatric problems. There were no significant overall differences in the frequency of cardiovascular disease or cancer between depression/inflammation levels in either men or women.

There were 420 all-cause male deaths (including 112 CVD related) over mean of 7.6 years follow-up and 334 all-cause female deaths (including 109 CVD related) over a mean of 7.8 years follow-up. Out of a total of 5,328 people, we identified 420 all-cause deaths in men and 334 in women during 18,594 and 22,519 person-years, respectively.

An analysis was conducted to determine any differences between the analytic sample and the excluded sample. Table 8.2 summarises the results. Participants included and excluded from the analysis did not differ significantly in terms of sex. The group included in the analysis was younger, less likely to be depressed, more likely to be married, more educated and wealthier than the group who was excluded.

Table 8.1. Baseline characteristics of men and women aged 52-89 years by depressive symptoms and inflammation level

	Men					Women				
	Depressive symptoms/inflammation level					Depressive symptoms/inflammation level				
	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value
Number	1,551	668	132	98		1,542	864	275	198	
Mean age (SD) (years)	64.9 (8.8)	67 (9.1)	64.5 (9.1)	66.9 (9.9)	<0.001	65 (9.0)	66.5 (9.2)	67.3 (10.3)	67.4 (9.7)	<0.001
Marital status (%)										
Married	1,252 (80.7)	501 (75)	79 (59.9)	60 (61)	<0.001	1,023 (66.3)	530 (61.3)	128 (46.6)	83 (41.9)	<0.001
Not married	299 (19.3)	167 (25)	53 (40)	38 (39)		519 (33.7)	334 (38.7)	147 (53.5)	115 (58.1)	

Table 8.1. continued. Baseline characteristics of men and women aged 52-89 years by depressive symptoms and inflammation level

	Men					Women				
	Depressive symptoms/inflammation level									
	No depressive symptoms/normal CRP	No depressive symptoms/high CRP	Depressive symptoms/normal CRP	Depressive symptoms/high CRP	P value	No depressive symptoms/normal CRP	No depressive symptoms/high CRP	Depressive symptoms/normal CRP	Depressive symptoms/high CRP	P value
Education (%)										
Degree/Higher/A-level	700 (45.1)	228 (34.1)	43 (32.6)	27 (27.6)	<0.001	458 (29.7)	191 (22.1)	51 (18.6)	39 (19.7)	<0.001
GCSE/O-level/Other qualification	444 (28.6)	203 (30.4)	37 (28)	27 (27.6)		555 (36)	281 (32.5)	98 (35.6)	53 (26.8)	
No qualification	407 (26.2)	237 (35.5)	52 (39.4)	44 (44.9)		529 (34.3)	392 (45.4)	126 (45.8)	106 (53.54)	

Table 8.1. continued. Baseline characteristics of men and women aged 52-89 years by depressive symptoms and inflammation level

	Men					Women				
	Depressive symptoms/inflammation level					Depressive symptoms/inflammation level				
	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value
Total Wealth (%)										
Richest tertile	688 (44.4)	203 (30.4)	40 (30.3)	17 (17.4)	<0.001	681 (44.2)	228 (26.4)	73 (26.6)	28 (14.1)	<0.001
Intermediate tertile	531 (34.2)	252 (37.7)	35 (26.5)	35 (35.7)		486 (31.5)	323 (37.4)	89 (32.4)	82 (41.4)	
Poorest tertile	332 (21.4)	213 (31.9)	57 (43.2)	46 (46.9)		375 (24.3)	313 (36.2)	113 (41.1)	88 (44.4)	

Table 8.1. continued. Baseline characteristics of men and women aged 52-89 years by depressive symptoms and inflammation level

	Men					Women				
	Depressive symptoms/inflammation level									
	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value
Smoking status (%)										
Never a smoker	496 (32)	149 (22.3)	34 (25.8)	15 (15.3)	<0.001	743 (48.2)	379 (43.9)	107 (38.9)	65 (32.8)	<0.001
Ex-smoker	894 (57.6)	383 (57.3)	73 (55.3)	57 (58.2)		634 (41.1)	355 (41.1)	126 (45.8)	79 (39.9)	
Current smoker	161 (10.4)	136 (20.4)	25 (18.9)	26 (26.5)		165 (10.7)	130 (15.1)	42 (15.3)	54 (27.3)	

Table 8.1. continued. Baseline characteristics of men and women aged 52-89 years by depressive symptoms and inflammation level

	Men					Women				
	Depressive symptoms/inflammation level									
	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value
Body Mass Index (%)										
<25kg/m ²	403 (26.8)	123 (19.3)	35 (27.8)	20 (23.5)	<0.001	606 (40.5)	121 (14.9)	98 (37)	32 (17.5)	<0.001
25-29.99kg/m ²	777 (51.7)	306 (48.1)	59 (46.8)	32 (37.7)		628 (42)	285 (35)	113 (42.6)	65 (35.5)	
>30+kg/m ²	323 (21.5)	207 (32.6)	32 (25.4)	33 (38.8)		262 (17.5)	408 (50.1)	54 (20.4)	86 (47)	

Table 8.1. continued. Baseline characteristics of men and women aged 52-89 years by depressive symptoms and inflammation level

	Men					Women				
	Depressive symptoms/inflammation level									
	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	P value
Chronic disease										
CVD (% yes)	115 (7.41)	61 (9.13)	14 (10.61)	14 (14.29)	0.052	48 (3.11)	31 (3.59)	14 (5.09)	14 (7.07)	0.026
Cancer (% yes)	85 (5.48)	46 (6.89)	7 (5.30)	11 (11.22)	0.093	139 (9.01)	58 (6.73)	26 (9.49)	14 (7.07)	0.191
Chronic lung disease (% yes)	78 (5.03)	70 (10.48)	10 (7.58)	18 (18.37)	<0.001	55 (3.57)	69 (8.00)	31 (11.31)	33 (16.67)	<0.001
Emotional, nervous, psychiatric problems (% yes)	81 (5.22)	32 (4.79)	25 (18.94)	22 (22.45)	<0.001	159 (10.31)	81 (9.40)	58 (21.17)	51 (25.76)	<0.001

Abbreviations: CRP = C-reactive protein; SD = standard deviation; kg = kilogram; m2 = meters squared.

Table 8.2. Characteristics of participants included and excluded from the analyses

	Participants included in the survival analysis (n=5,328)	Participants excluded due to no mortality data (n=281)	All other participants excluded (n=3,061)	P value
Men (%)	2,449 (45.95)	123 (43.77)	1,340 (43.78)	0.137
Mean age (SD) (years)	65.67 (9.16)	69.46 (10.29)	67.73 (9.75)	>0.001
Depression status (% yes)	703 (13.19)	49 (19.07)	565 (19.60)	>0.001
Marital status (% yes)	3,656 (68.62)	163 (58.01)	1,893 (61.84)	>0.001
Education (%)				>0.001
Degree/Higher/A-level	1,737 (32.60)	78 (28.36)	838 (27.41)	
GCSE/O-level/Other	1,698 (31.87)	75 (27.27)	840 (27.48)	
No qualification	1,893 (35.53)	122 (44.36)	1,379 (45.11)	

Table 8.2. continued. Characteristics of participants included and excluded from the analyses

	Participants included in the survival analysis (n=5,328)	Participants excluded due to no mortality data (n=281)	All other participants excluded (n=3,061)	P value
Wealth (%)				>0.001
Wealthiest tertile	1,958 (36.75)	89 (32.01)	820 (27.84)	
Intermediate tertile	1,833 (34.40)	83 (29.86)	938 (31.85)	
Poorest tertile	1,537 (28.85)	106 (38.13)	1,187 (40.31)	

Abbreviations: SD = standard deviation.

8.8.2 *Depressive symptoms and inflammation as a combined predictor of CVD mortality*

In men, depressive symptoms alone were not associated with any significant increase in risk of death, whilst high inflammation was associated with a 238% (HR: 3.38; 95% CI 2.23-5.10) increased risk. Men with both depressive symptoms and high inflammation had a 584% (HR: 6.84; 95% CI 3.71-12.6) increased CVD mortality risk. This association remained significant after adjustment for age, SES and health behaviours, with men who had both depressive symptoms and high inflammation demonstrating a 289% (HR: 3.89; 95% CI 2.04-7.44) increased risk of death (P value <0.001) (Table 8.3 and Figure 8.1). In women the associations were more modest and failed to reach significance (Table 8.3).

8.8.3 *Depressive symptoms and inflammation as a combined predictor of all-cause mortality*

In men, the direction of results was similar for all-cause mortality. Depressive symptoms alone were not associated with a significant increase in risk of death, whilst high inflammation was associated with a 91% (HR: 1.91; 95% CI 1.55-2.36) increased risk, compared to men with neither. Men with both depressive symptoms and high inflammation had a 241% (HR: 3.41; 95% CI 2.39-4.86) increased mortality risk. This association was attenuated after adjustment for age, SES and smoking. However the association remained significant, with men who had both depressive symptoms and high inflammation demonstrating a 140% (HR: 2.40; 95% CI 1.65-3.48) increased risk of death (Figure 8.1).

The results for women were different. In the unadjusted model, both depressive symptoms and high inflammation separately increased risk of death by 85% (HR: 1.85; 95% CI 1.32-2.59) and 48% (HR: 1.48; 95% CI 1.16-1.90), respectively. The combination

of both depressive symptoms and high inflammation increased risk to 89% (HR: 1.89; 95% CI 1.29-2.76), only marginally more than depressive symptoms alone. The increased risk of depressive symptoms and high inflammation to all-cause mortality in women was explained by age as after adjustments the risk was no longer significant (Table 8.3).

8.8.4 Effects of moderation and mediation

Moderation analysis showed that an interaction term of depressive symptoms by inflammation was not significantly associated with mortality in all categories: all-cause mortality in men ($p=.426$); all-cause mortality in women ($p=.155$); CVD mortality in men ($p=.868$) and CVD mortality in women ($p=.481$). This suggests that the mortality risk conferred by increased levels of inflammation is not further augmented by depressive symptoms. Mediation analysis showed that the strength of the association between depressive symptoms and mortality was not reduced by including inflammation, suggesting a direct effect of depressive symptoms on mortality risk (Table 8.4).

Table 8.3 Association between depressive symptoms/inflammation and all-cause and cardiovascular mortality by sex

	Men				Women			
	Depressive symptoms/inflammation level							
	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms / high CRP
Model								
All-cause mortality								
Model 1 HR	1.00	1.91	1.42	3.41	1.00	1.48	1.85	1.89
(95% CI)	(reference)	(1.55-2.36)	(0.93-2.17)	(2.39-4.86)	(reference)	(1.16-1.90)	(1.32-2.59)	(1.29-2.76)
p-values ^a		<0.001	0.104	<0.001		0.002	<0.001	0.001
Model 2 HR	1.00	1.63	1.47	3.03	1.00	1.23	1.34	1.44
(95% CI)	(reference)	(1.32-2.00)	(0.97-2.25)	(2.12-4.32)	(reference)	(0.96-1.57)	(0.95-1.87)	(0.98-2.10)
p-values ^b		<0.001	0.072	<0.001		0.104	0.093	0.060
Model 3 HR	1.00	1.54	1.29	2.59	1.00	1.18	1.27	1.30
(95% CI)	(reference)	(1.25-1.90)	(0.84-1.98)	(1.80-3.74)	(reference)	(0.92-1.51)	(0.90-1.78)	(0.89-1.92)
p-values ^c		<0.001	0.242	<0.001		0.199	0.168	0.176

Table 8.3 continued. Association between depressive symptoms/inflammation and all-cause and cardiovascular mortality by sex

Model	Men				Women			
	Depressive symptoms/inflammation level							
	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms / high CRP
All-cause mortality								
Model 4 HR	1.00	1.49	1.27	2.40	1.00	1.09	1.18	1.15
(95% CI)	(reference)	(1.20-1.84)	(0.83-1.96)	(1.65-3.48)	(reference)	(0.84-1.41)	(0.84-1.66)	(0.77-1.70)
p-values ^d		<0.001	0.273	<0.001		0.536	0.336	0.501
Model 5 HR	1.00	1.46	1.24	2.09	1.00	1.11	1.12	1.10
(95% CI)	(reference)	(1.18-1.81)	(0.80-1.90)	(1.43-3.07)	(reference)	(0.85-1.44)	(0.79-1.58)	(0.74-1.65)
p-values ^e		<0.001	0.337	<0.001		0.436	0.336	0.629

Table 8.3 continued. Association between depressive symptoms/inflammation and all-cause and cardiovascular mortality by sex

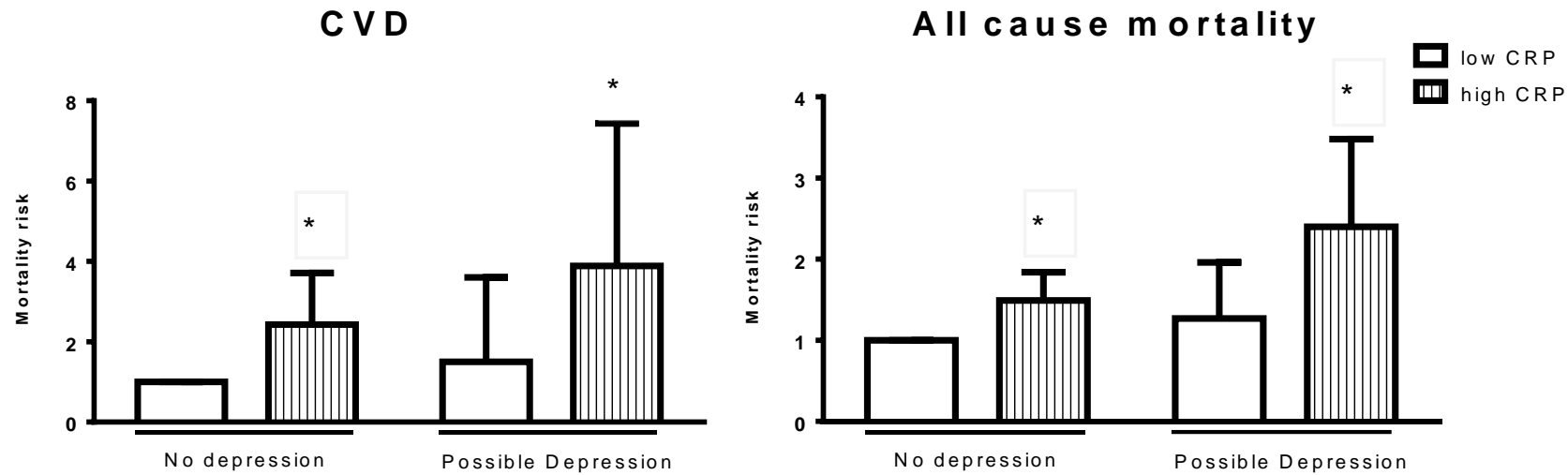
Model	Men				Women			
	Depressive symptoms/inflammation level							
	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms / high CRP
Cardiovascular disease mortality								
Model 1 HR (95% CI) p-values ^a	1.00 (reference)	3.38 (2.23-5.10) <0.001	1.86 (0.79-4.38) 0.159	6.84 (3.71-12.6) <0.001	1.00 (reference)	1.45 (0.96-2.2) 0.079	1.27 (0.66-2.44) 0.417	1.29 (0.61-2.71) 0.508
Model 2 HR (95% CI) p-values ^b	1.00 (reference)	2.81 (1.86-4.26) <0.001	1.91 (0.81-4.51) 0.140	5.78 (3.13-10.68) <0.001	1.00 (reference)	1.14 (0.75-1.73) 0.540	0.81 (0.42-1.56) 0.531	0.93 (0.44-1.97) 0.857
Model 3 HR (95% CI) p-values ^c	1.00 (reference)	2.56 (1.68-3.89) <0.001	1.51 (0.63-3.62) 0.354	4.50 (2.39-8.47) <0.001	1.00 (reference)	1.09 (0.71-1.67) 0.685	0.76 (0.39-1.46) 0.407	0.84 (0.40-1.80) 0.658
Model 4 HR (95% CI) p-values ^d	1.00 (reference)	2.43 (1.59-3.71) <0.001	1.50 (0.63-3.60) 0.361	3.89 (2.04-7.44) <0.001	1.00 (reference)	0.92 (0.59-1.44) 0.715	0.69 (0.35-1.33) 0.267	0.69 (0.32-1.50) 0.353

Table 8.3 continued. Association between depressive symptoms/inflammation and all-cause and cardiovascular mortality by sex

Model	Men				Women			
	Depressive symptoms/inflammation level							
	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms/ high CRP	No depressive symptoms/ normal CRP	No depressive symptoms/ high CRP	Depressive symptoms/ normal CRP	Depressive symptoms / high CRP
Cardiovascular disease mortality								
Model 5 HR	1.00	2.42	1.44	3.25	1.00	0.96	0.69	0.68
(95% CI)	(reference)	(1.58-3.69)	(0.60-3.46)	(1.66-6.40)	(reference)	(0.62-1.50)	(0.35-1.35)	(0.32-1.49)
p-values ^e		<0.001	0.413	0.001		0.866	0.275	0.338

Abbreviations: HR = Hazard ratio; CI = Confidence interval; CRP= C-reactive protein. Cox regression survival analysis models, stratified by sex, are adjusted as follows: ^aInflammation and depressive symptoms as main effects; ^bas model 1, plus adjustment for age; ^cas model 2, plus adjustment for socioeconomic variables (marital status, level of education, total wealth), ^das model 3, plus adjustment for health behaviours (smoking, body mass index) and ^ea model 4, plus individually adjustment for chronic diseases (cardiovascular disease, cancers, chronic lung disease and emotional, nervous and psychiatric problems)

Figure 8.1. Adjusted hazard ratios for CVD and all-cause mortality according to levels of depressive symptoms and inflammation in men.



* Statistically significant at $P < 0.001$.

Abbreviations: CVD = Cardiovascular disease; CRP = C-reactive protein. Hazard ratios are adjusted for age, socioeconomic variables (marital status, level of education, total wealth), health behaviours (smoking, body mass index) and chronic diseases (cardiovascular disease, cancers, chronic lung disease and emotional, nervous and psychiatric problems). $n=5,328$

Table 8.4. Mediation analyses (n=5,328)

	Model 1^a	Model 1 + dichotomous CRP^b	Model 1 + continuous CRP^c
Men			
All-cause mortality (420 deaths)			
HR (95% CI)	1.61 (1.06-2.44)	1.56 (1.03-2.37)	1.65 (1.08-2.50)
Cardiovascular mortality (112 deaths)			
HR (95% CI)	1.73 (0.83-3.61)	1.59 (0.76-3.29)	1.82 (0.73-3.79)
Women			
All-cause mortality (334 deaths)			
HR (95% CI)	1.01 (0.70-1.45)	1.02 (0.71-1.46)	1.00 (0.69-1.44)
Cardiovascular mortality (109 deaths)			
HR (95% CI)	0.55 (0.27-1.13)	0.55 (0.27-1.14)	0.55 (0.27-1.13)

Abbreviations: HR = Hazard ratio; CI = Confidence interval; CRP= C-reactive protein

Mediation analysis models, stratified by sex, are adjusted as follows: ^a Chronic symptoms of depression (wave 1 and wave 2) as main effects, plus adjustment for age, socioeconomic variables (marital status, level of education, household wealth) and chronic disease (cardiovascular disease, cancers, chronic lung disease); ^b as model, 1 plus adjustment for CRP dichotomised into two categories: <3mg/L defined as normal and 3-20mg/L defined as high; and ^c as model 1, plus adjustment for continuous CRP.

8.9 Discussion

8.9.1 *Aims and hypotheses*

The aim of this study was to examine the combined effect of depressive symptoms and inflammation on CVD and all-cause mortality in a large cohort of older adults. We also sought to investigate whether depressive symptoms moderate the mortality risk associated with increased inflammation or whether inflammation could be mediating the association between depressive symptoms and mortality. I hypothesised that people with both depressive symptoms and inflammation will have significantly greater mortality risk than people with depressive symptoms or inflammation alone (hypothesis 1). I also hypothesised that depressive symptoms and inflammation will have largely independent effects on mortality (hypothesis 2).

8.9.2 *Summary of results*

The results from this study provide partial support for both these hypotheses. We found that older men, with both depressive symptoms and high levels of inflammation, have a significantly increased risk of CVD and all-cause mortality compared to men with depressive symptoms or inflammation alone. However we found no significant increased risk in women. In addition, our findings demonstrate independent effects of depressive symptoms and inflammation on mortality, finding no evidence of either moderation or mediation.

8.9.3 *Depression, inflammation and mortality*

We demonstrated an increased risk of all-cause mortality in the comparison between men with high and low baseline levels of inflammation. The addition of depression to the model increased the risk substantially suggestive of a particularly high-risk phenotype in men. This supports findings in healthy, older men showing that high inflammation

predicted cardiovascular events only in people with depressed mood (Ladwig *et al.*, 2005). These findings suggest that depression and inflammation might cause CVD through separate physiological pathways, such as elevated interleukin-6 upstream of CRP (Ridker, 2016), triglycerides (Parekh *et al.*, 2017), cortisol as a result of stress-induced hyperactivity of the hypothalamic-pituitary-adrenal axis (Jokinen and Nordstrom, 2009), endothelial dysfunction (Chen *et al.*, 2011) and platelet activation (Williams *et al.*, 2014).

Our study found no significant interaction between depressive symptoms and inflammation on mortality and no effect of mediation, a finding which is supported by other studies, but not by all. To our knowledge, there is only one other study which looked at the potential synergistic effect of depressive symptoms and inflammation in the prediction of cardiovascular events. Ladwig showed a significant interaction between depressive symptoms and inflammation in the prediction of cardiovascular events, suggesting a shared underlying mechanism (Ladwig *et al.*, 2005). These authors, however, have only investigated men.

Most previous studies have investigated the effects of inflammation mediating the association between depression and all-cause or CVD mortality showing either no or small effect. Similar to our study, Empana *et al.*, (2005) showed that men with depressive symptoms had a 53% increase in the odds of CHD and the association remained unchanged when inflammatory markers were added to the model. Davidson *et al.*, 2009 found that depressive symptoms increased the risk of incident CHD ; this risk was not explained by increased inflammation in either men or women. Nabi *et al.*, 2008 also found an association between psychological distress and incident CHD which remained after adjustment for inflammatory markers. Contrary to our study and the studies cited above, there has also been reports of mediation. Hughes *et al.*, (2016) reported an association between depressive symptoms and all-cause mortality which was partly explained by inflammation (CRP 7.3%). Inflammation partly mediated the predictive

value of depressive symptoms by 6.5% in cardiovascular mortality risk (Kop *et al.*, 2010), and by 8.1% in cardiovascular hospitalization (Hiles *et al.*, 2015). This supports the knowledge that other pathways are also involved on the depression and mortality link.

8.9.4 *Effect of gender on the link between depressive symptoms, inflammation and mortality*

Clear sex-specific differences were observed in the inter-relationships of depressive symptoms, inflammation and mortality in this study. The combination of depressive symptoms and inflammation only conferred an increased mortality risk in men. A previous population cohort study investigating inflammation and mortality also showed sex differences. Ahmadi-Abhari *et al.* reported that high levels of CRP increased mortality risk in men at lower clinical threshold categories than in women, most notably for CVD mortality (2013). A similar trend has also been observed in the development of CHD. In a study of older men and women, Cushman *et al.* showed that the presence of high inflammation was predictive of CHD in men with intermediate Framingham Risk Scores, whereas it only became predictive of CHD in women with a high Framingham risk (2005). Furthermore a large meta-analysis showed that inflammation only discriminated 10-year risk of cardiovascular events in men, but not in women (Kaptoge *et al.*, 2012). Some reports on the association between depression and inflammation also support our findings, although not all. Two large population cohort studies have shown that major depression/depressive symptoms and inflammation are more strongly associated in men than in women (Ford and Erlinger, 2004, Elovainio *et al.*, 2009). However in contrast, in a study of 508 healthy adults, depressive symptoms were only associated with inflammation in women, not in men (Ma *et al.*, 2010). Furthermore, a study of women with suspected coronary ischemia demonstrated a robust association between depressive symptoms and inflammation which was not explained by CVD risk factors (Vaccarino *et al.*, 2007).

Further speculation on gender differences is inspired by a recent review from Raison and Miller (2017). The authors propose that in evolutionary terms, depression may have provided an adaptive advantage to women. Inflammation was detrimental to fertility in ancestral environments (Van Bodegom, 2007; Schaller, 2011; Koybayashi, 2013). Depressive symptoms promoted sickness behaviours (e.g. lethargy, psychomotor slowing and social withdrawal) which provided increased protection against pathogens (e.g. by conserving energy for an immune response), thereby reducing the need for a high inflammatory response. This is supported by findings which show that women demonstrate increased levels of depression in response to inflammatory challenge compared to men (Moieni *et al.*, 2015b; Udina *et al.*, 2012). If women are more likely to develop depression in response to immune activation then it is possible that the presence of depressive symptoms is likely to reflect less severe underlying biological pathology compared to men and consequently lower mortality risk. Whilst this explanation is intuitively appealing, further research is required to confirm gender specific immune mechanisms in depression.

Another possible explanation for our observed sex differences is the influence of sex hormones, particularly the protective effect of oestrogen on the heart. In people under 75, the incident of cardiovascular related death is lower in women than in men (British Heart Foundation, 2014), with the development of atherosclerosis occurring post menopause in 95% of women (Fairweather, 2014). Even at 65-74 years of age, almost two decades after the average occurrence of menopause, women have substantially lower incidence of CVD compared to men. This suggests that former exposure to endogenous estrogen may be atheroprotective long after menopause. Women have also been shown to have longer telomeres than men (Gardner *et al.*, 2014). Shorter telomeres have been associated with early death in the general population (Weischer *et al.*, 2012) although findings are inconsistent (Bojesen, 2013). Telomere length has been more robustly associated with CHD, independently of traditional vascular risk factors (Haycock *et al.*, 2014). It is not yet understood exactly why women differ from men in the

development of CHD, however to date women have been underrepresented in cardiovascular trials, resulting in a bias towards factors which are relevant to disease aetiology in men (Fairweather, 2014).

Current National Institute for Health and Clinical Excellence (NICE) guidelines recommend the use of the QRISK2 risk assessment tool to assess risk for the primary prevention of CVD in both men and women up to 84 years (National Institute for Health and Care Excellence, 2014). There has been some debate as to whether or not the addition of circulating inflammatory markers, such as CRP, should now be included in screening measures for cardiovascular risk (Pearson *et al.*, 2003, Peters *et al.*, 2013). The main uncertainty seems to be whether the modest increases in risk associated with higher inflammation can significantly affect health. This is understandable when considering that population studies have shown that CRP is a relatively moderate predictor of CVD risk, yielding an odds ratio of 1.5 when comparing the top baseline tertile with the bottom (Danesh *et al.*, 2004). In addition, when compared to traditional risk factors, such as smoking and total cholesterol, CRP only slightly improved their predictive value. Our study demonstrates that men with comorbid depressive symptoms and high inflammation constitute a clinically meaningful risk category. In light of these findings, it might be worth considering inflammation as a cardiovascular risk factor in depressed men. This could help identify patients who may benefit from targeted prophylactic intervention, improving screening efficacy and cardiovascular outcomes.

8.9.5 *Study strengths and limitations*

The strengths of our study include the prospective design, the presence of both women and men and the nationally representative nature of the ELSA cohort. Despite this, our study also has limitations. The first limitation of this study is that we measured depressive symptoms and inflammation at only one time point at wave 2 (2004-2005) and did not investigate any change in levels at a later points. This design was chosen in order to

maximize the number of participants with inflammation and depressive symptoms at baseline and to allow a longer follow-up period to capture mortality.

Nevertheless, we did not find an association of depressive symptoms and mortality in the absence of inflammation. In a recent study using the same cohort, which considered depressive symptoms across several years, a dose-response association was observed between persistency of depressive symptoms across time and mortality risk (White *et al.*, 2016). Similar to these findings, data from the Longitudinal Aging Study Amsterdam (LASA) also found that transient depressive episodes did not predict mortality although chronic depression did (Geerlings *et al.*, 2002). Secondly, we also did not control for medication use in our analysis, as this data was not available. Statins, for example, present anti-inflammatory effects (Antonopoulos *et al.*, 2012) and therefore medication use may have interfered with current findings. Thirdly, our study had a smaller proportion of female deaths compared to male deaths and therefore it is unclear whether our lack of association in women reflects an absence of an effect or is a result of insufficient power. The only closest previous study to date investigated combined depressive symptoms and inflammation in the development of CVD and was restricted to men. In order to explore this we conducted a post hoc power calculation in STATA. The calculation showed that the study was sufficiently powered to detect an effect in men for both CVD and all-cause mortality (CVD & all-cause: power=1.00), however in women there was insufficient power (CVD: power=0.52, all-cause: power=0.14). We cannot speculate as to whether this is due to the fact that our effect size is very small or whether there is in fact no effect in women. The only other study to investigate combined depressive symptoms and inflammation in CVD development restricted their sample to men only. Therefore, we had no prior effect on which to base a power calculation. Finally, in our study depressive symptoms were measured by an 8 item self-reported questionnaire, rather than a diagnostic interview. Short scales such as this have previously been criticised for a lack of specificity. To address this, we defined depressive

symptoms using a conservative cut-off of 4, which increases the measure's ability to discriminate between true and false positives.

8.9.6 *Conclusion*

In conclusion, we demonstrated that men with concurrent depressive symptoms and increased inflammation constitute a high-mortality risk phenotype. This risk is particularly high for cardiovascular related death. These findings have clinical implications for the treatment and prevention of depression and inflammation in men. Subgroups of depressed individuals with comorbid inflammation may benefit from additional anti-inflammatory pharmacotherapy. Further research is needed to investigate whether combined interventions improve outcomes.

9. Discussion

9.1 Overview

There is a robust literature associating immune dysregulation with depression, however the exact nature of this relationship is not completely understood. An interaction between immune and neuroendocrine function provides a potential explanatory pathway. This PhD consisted of two studies that aimed to explore the role of inflammation in depression, using two different methods of investigation. Firstly, an observational case-control study was used to assess differences in immune and HPA-axis function, and associations between them, in people with MDD compared to healthy controls. In addition, this study investigated the longitudinal association between inflammation and HPA-axis function in relation to depressive symptom remission. Secondly, a prospective cohort study was used to investigate the combined effects of depressive symptoms and inflammation on mortality risk.

In Study 1a (presented in Chapter 3), differences in inflammatory cytokines, diurnal cortisol rhythm and *in vitro* corticosteroid function were investigated cross-sectionally in 37 people with MDD and 30 healthy controls. Following on from this, in Study 1b (presented in Chapter 4) changes in depressive symptoms, inflammatory cytokines, diurnal cortisol parameters and corticosteroid receptor function were investigated longitudinally in people with MDD. Study 1c (presented in Chapter 6) investigated differences in Treg frequency and associations with inflammatory cytokines and neuroendocrine function in people with MDD compared with healthy controls. Study 2 (presented in Chapter 8) assessed the combined effects of depressive symptoms and inflammation on CVD and all-cause mortality risk in older people. In this chapter the hypotheses and findings of these two studies will be briefly summarised and the contribution of these studies to the literature will be highlighted. Limitations of this thesis, implications of this work and ideas for future research will also be discussed.

9.2 Main findings and their implications

9.2.1 Study 1a: *The Resist Study results: The association between inflammation and HPA-axis function in people with MDD compared with healthy controls*

There is an extensive literature (Chapter 1, Section 1.4.2) implicating inflammation in the pathogenesis of depression. Several meta-analyses show increases in pro-inflammatory cytokines in depressed people compared with healthy controls (Dowlati et al., 2010; Goldsmith et al., 2016b; Hiles et al., 2012b; Howren et al., 2009; Kohler et al., 2017; Y. Liu et al., 2012). Longitudinally, inflammation has been associated with the subsequent development of depressive symptoms (Valkanova et al., 2013) and depressive symptoms have been associated with subsequent levels of inflammation (Copeland et al., 2012; Deverts et al., 2010; Huang et al., 2019; Niles et al., 2018; Stewart et al., 2009). However the pathways through which cytokines contribute to the development and maintenance of depression are yet to be completely characterised.

A large body of evidence (Chapter 1, Section 1.5) indicates that dysregulation of the HPA-axis also exists in depressed people (Stetler & Miller, 2011). However the direction of dysregulation regarding specific diurnal cortisol parameters is unclear. Furthermore, whilst there is robust evidence of reduced GR sensitivity *in vitro* (Bauer et al., 2003; Calfa et al., 2003; Lowy et al., 1984; Lowy et al., 1988; G. E. Miller et al., 2005b; Rupprecht et al., 1991c; Tanke et al., 2008; Wodarz et al., 1991; Wodarz et al., 1992) and *in vivo* (Bardeleben & Holsboer, 1989; Gonul et al., 2017; Gormley et al., 1985; Heuser et al., 1994; Holsboer-Trachsler et al., 1991; Holsboer et al., 1987; Lowy et al., 1988; Modell et al., 1997; von Bardeleben & Holsboer, 1991; A. Wassef et al., 1990), the role of the MR in MDD is not fully understood. There appears to be an association between inflammation and HPA-axis function, however the nature of this relationship has yet to

be fully elucidated. The few studies that have explored this relationship have largely reported single measures of HPA-axis function and produced conflicting results.

In Study 1a we hypothesised that people with MDD would exhibit increased levels of inflammation (IL-6 and TNF- α), disturbed corticosteroid sensitivity (decreased GR sensitivity, altered MR sensitivity) and altered diurnal cortisol secretion (increased CAR, increased AUC and flatter cortisol slope), compared with healthy controls. It was also hypothesised that increased inflammation would be associated with disturbances in HPA-axis function in the depressed group. Finally, exploratory analyses were conducted to investigate possible associations between any altered biological pathways and psychosocial stress.

We observed evidence of dysregulation in both immune and neuroendocrine function in people with MDD and some of the hypotheses were supported. Consistent with the literature, TNF- α levels were increased in depressed people compared to controls, however there were no significant differences in IL-6. In line with our prediction, both GR and MR sensitivity was reduced in the MDD group. Contrary to expectations, the CAR AUC was reduced in the MDD group, however there were no significant differences in the AUC or slope between the groups. We also observed a relationship between inflammatory activation and diurnal cortisol rhythm. IL-6 was negatively associated with the CAR and CAR AUC in both groups, whereas TNF- α was associated with reduced CAR AUC and AUC in the depressed group only. Neither inflammatory biomarker was associated with corticosteroid sensitivity. No associations between biological and psychosocial stress variables in depressed people were observed. Sensitivity analyses restricting the sample to those with moderate/severe depressive symptoms did not alter the pattern of results. Additional exploratory analyses to examine the influence of childhood sexual abuse, showed that depressed people who had not experienced sexual abuse had a significantly lower CAR compared with depressed people who had.

Study 1a has contributed to the field by showing dysregulation of multiple biological pathways in people with MDD. It confirmed previous findings of increased inflammatory activation in MDD, specifically plasma level of TNF- α (Dowlati et al., 2010; Goldsmith et al., 2016b; Kohler et al., 2017; Y. Liu et al., 2012). However, contrary to the literature we found no evidence of elevations in IL-6, despite this being the most reliable finding in this field (Haapakoski et al., 2015). It is unclear why we observed this null finding, however there are a number of possible explanations. The reliance on using brief questionnaires to measure depressive symptoms by many published studies may reflect a broader sample than in this study, which only included individuals who met criteria for MDD. There is also the issue of confounding; much of the literature includes studies which fail to exclude or account for physical or psychiatric co-morbidities. This inevitably allows for the inclusion of inflammation which is not specific to MDD. Another consideration is that inflammation may not be universally related to all depressive subgroups. MDD is a psychiatrically heterogeneous disorder and inflammatory cytokines may be differentially associated with specific clusters of symptoms (C. Yang et al., 2018). There is also tentative evidence to suggest that the elevated TNF- α we observed may reflect individuals with more persistent depression, however this should be interpreted with caution as this study was underpowered to perform subgroup analyses.

As hypothesised, GR sensitivity was reduced in depressed people compared with healthy controls. This is in line with findings from several other *in vitro* studies (Lowy et al., 1984; Lowy et al., 1988; Rupprecht et al., 1991; Wodarz et al., 1991; Wodarz et al., 1992) which have used synthetic glucocorticoids to inhibit mitogen-induced lymphocyte proliferation. Study 1a measured glucocorticoid inhibition of LPS-stimulated IL-6 levels in peripheral whole blood, *in vitro*. To the best of my knowledge this has only been previously used in a study investigating patients with TRD (Carvalho et al., 2008), PTSD (Rohleder et al., 2004), and in depressed women undergoing acute stress testing (G. E. Miller et al., 2005b). Many studies have used the DEX/CRH test *in vivo* (Bardeleben & Holsboer, 1989; Gonul et al., 2017; Heuser et al., 1994; Holsboer-Trachsler et al., 352

1991;Holsboer et al., 1987;Modell et al., 1997;von Bardeleben & Holsboer, 1991), however this provides a measure of HPA-axis activity rather than a direct measure of GR function (Pariante, 2004).

This study also reported impaired MR function in depressed people. The literature regarding MR function in MDD is inconclusive, with studies reporting conflicting results. There is some evidence to suggest that MR signalling is increased in MDD, compensating for impaired GR function, but that this compensatory increase is absent in a subgroup of severely depressed individuals who are exceptionally treatment resistant (Juruena et al., 2010;Juruena et al., 2009). The balance between GR and MR may therefore be crucial in identifying patients who may be the least responsive to treatment. To date only one study has investigated both receptors in depression and this study only included people with TRD, reporting impaired GR function and retained MR function. (Juruena et al., 2006). In contrast to the findings reported by Juruena et al. (2006) we observed impaired function in both receptors. Furthermore, secondary analyses provided evidence that MR resistance was present even in those with mild symptoms. To date, MR function in MDD has only been investigated *in vivo*, using oral administration of either prednisolone or spironolactone.

Regarding diurnal cortisol rhythm, we found that waking cortisol + 30 minutes and the CAR AUC was reduced in depressed people compared with controls, suggesting reduced sensitivity of the HPA-axis to naturally occurring stress. Contrary to my hypotheses, we observed no difference in either the AUC or the slope between depressed people and controls. The literature in this field is enormously inconsistent. Cross-sectional studies, longitudinal studies and those investigating antidepressant response have all reported conflicting findings. However the largest and most recent meta-analysis reported that the AUC during the waking period was positively associated with depressive symptoms, which is in direct contrast to our finding (Boggero et al., 2017). Our findings tentatively imply that the regulatory mechanisms underlying the CAR

may be exhausted in depressed people, possibly due to previous HPA-axis hyperactivity. We also observed an effect of childhood sexual abuse on the CAR. Depressed people who had not experienced sexual abuse had a significantly flatter CAR compared with those who had. The literature is inconsistent regarding the HPA-axis and childhood maltreatment. In line with our finding, studies specifically investigating the CAR and childhood maltreatment in depressed adults have reported increased CAR in depressed people with a history of childhood abuse compared to those without (Lu et al., 2016;Quevedo et al., 2017). It is possible that this finding reflects a distinct subtype of depression with different neuroendocrine function. However this interpretation should be treated with caution and further research is needed.

The review of the published literature conducted for Chapter 1 found several studies exploring both inflammation and neuroendocrine function in people with MDD and associations between them, however results are conflicting. This could be explained by the variability of measures used in this field and the reliance of single measures of HPA-axis function. A recent review (Perrin et al., 2019) called for future research to combine multiple independent measures of HPA-axis activity and GR function in order to provide the best opportunity for detecting any trends in biological dysregulation. Therefore this study has addressed this gap in the literature by including assessments of inflammatory cytokine levels (IL-6 and TNF- α), *in vitro* measures of GR and MR sensitivity and diurnal cortisol rhythm simultaneously.

We observed that higher levels of plasma TNF- α were associated with a less pronounced CAR, in depressed people, however this association was not observed for healthy controls. A flattening of the CAR has previously been associated with depressive symptoms (Dedovic et al., 2010;Mangold et al., 2011) and MDD (Huber et al., 2006;Stetler & Miller, 2005), however studies reporting both TNF- α and cortisol levels have reported conflicting results. Higher plasma cortisol has been reported along with both increased (Y. Chen et al., 2017;Kahl et al., 2017;Kahl et al., 2015) and decreased

TNF- α (Rudzki et al., 2017) in depressed patients compared with controls. Lower plasma cortisol and lower TNF- α levels have also been reported in MDD patients (Lopes et al., 2012). This is the first study to assess diurnal cortisol rhythm and TNF- α in MDD. A negative association between the CAR and TNF- α has been reported in patients with ADHD (Corominas-Roso et al., 2017) and similarly, an inverse association between waking cortisol and TNF- α was observed in a population-based sample (DeSantis et al., 2012).

This result supports the notion that dysregulation of the HPA-axis, as seen in depression, may be associated with higher levels of inflammation, however the direction of the association remains unclear. Due to the cross sectional nature of this study, we cannot differentiate whether the reduced CAR we observed caused higher TNF- α levels or was itself caused by higher TNF- α levels. It is possible that a chronically activated and subsequently impaired HPA-axis is less capable of inhibiting a pro-inflammatory response, resulting in a shift towards an inflammatory state (Varghese et al., 2016). It is also possible that increased inflammatory cytokines activate the HPA-axis, leading to subsequent dysregulation. This finding needs to be corroborated by further research. In addition, whilst blood samples were taken between 8.00am and 10.00am, allowing us to capture any dynamic relations between inflammation and the CAR and control for circadian variabilities between the two, we cannot exclude the possible effects of additional “third” variable that influences both cortisol rhythm and cytokine levels. Studies experimentally altering cortisol or cytokine levels will provide more data regarding causality.

Finally, we did not observe any associations between any of the biological variables which differed between the groups and psychosocial stress. This finding was unexpected given that we observed both inflammatory and neuroendocrine dysregulation alongside reports of increased perceived stress in the MDD group. This could be explained by the nature of our sample which included a high proportion of people from high socioeconomic

backgrounds, who may have better psychosocial resources. The lack of association may also be a reflection of heterogeneity in psychosocial factors, as qualitatively different types of stress were not explored in this study.

Strengths and limitations

The limitations of this study were addressed in Chapter 3. The main limitations were that our sample size was small, limiting statistical power and the cross-sectional design, which prevented us from drawing any causal conclusions. The study was further limited by potential under-reporting of childhood adversity by the use of retrospective accounts. It is also possible that participants did not adhere to the cortisol saliva sampling protocol resulting in reduced reliability of our cortisol data. Additionally, performing the corticosteroid receptor sensitivity assays in whole blood rather than in isolated PBMCs prevented us from observing any differences in immune cell populations. Finally the inclusion of gene expression assays would help to establish GR responsiveness.

Despite these considerations (and others discussed in Chapter 3) I believe this study offers a novel contribution to the literature for several reasons. Firstly, this study investigated associations between multiple biological pathways and suggests that there is a relationship between TNF- α and diurnal cortisol rhythm in MDD. Secondly, this study is the first to explore both GR and MR in a study of people with MDD compared to healthy controls and has provided evidence for impaired function in both receptors. Thirdly, this study has used an *in vitro* approach to determine receptor sensitivity.

9.2.2 *Study 1b: The Resist Study follow-up results: Changes in depression and associations with inflammatory biomarkers and HPA-axis function at 6 week follow-up*

Following on from Study 1a, Study 1b (presented in Chapter 4) compared baseline measurements of depressive symptoms, inflammatory cytokines, diurnal cortisol parameters and corticosteroid receptor function in depressed people at baseline and at six weeks follow-up. There is robust evidence (Chapter 1, Section 1.4.3) that circulating IL-6 levels are reduced following antidepressant treatment (Hannestad et al., 2011; Hiles et al., 2012a; Kohler et al., 2018; Wiedlocha et al., 2018). However the findings regarding TNF- α are less convincing (Hannestad et al., 2011; Hiles et al., 2012a; Wiedlocha et al., 2018). There is conflicting evidence regarding corticosteroid receptor function and antidepressant treatment (Chapter 1, Section 1.5.8). Increased GR function following antidepressant therapy has been demonstrated *in vitro* in people with MDD (Calfa et al., 2003) but not in those who are treatment resistant (Bauer et al., 2003; Carvalho et al., 2008). *In vivo* findings suggest increased GR sensitivity is associated with symptom improvement (S. Fischer et al., 2017a; Greden et al., 1983; Holsboer et al., 1982), however null findings have also been reported (Ventura-Junca et al., 2014). There is evidence suggesting that MR sensitivity does not change with antidepressant treatment (Juruena et al., 2010), however treatment resistant individuals demonstrate impaired MR sensitivity compared to those who respond (Juruena et al., 2010; Juruena et al., 2009). Symptom remission has been robustly associated with increased CAR following SSRI therapy (Harmer, Bhagwagar, Shelley, & Cowen, 2003; Ruhe et al., 2015). Higher AUC has also been observed in remitted patients compared with controls (Aubry et al., 2010). In Study 1b the associations between any changes in biological parameters and changes in depressive symptoms were assessed in 34 people with MDD, over a six week follow-up period. It was hypothesised that any changes in depressive symptoms would be influenced by baseline levels of inflammation, corticosteroid sensitivity and diurnal cortisol rhythm, and predicted by change in these biomarkers over time.

A significant reduction in depressive symptoms was observed at follow-up, however contrary to our hypothesis we did not observe any significant changes in either IL-6 or TNF- α . IL-6 appeared to be increased at follow-up, however the data exhibited high variability. Additional analyses showed that in people who did not show any clinical recovery, IL-6 levels were increased, suggesting that increased inflammation may be associated with persistent depressive symptoms. TNF- α levels were decreased at follow-up, however this did not reach significance. These null findings could be explained by the modest reduction in depressive symptoms we observed, which may have reflected small reductions in inflammation, which we were underpowered to detect.

We did observe a significant improvement in MR sensitivity, however there was no significant association with improvement in depressive symptoms. Further analyses showed that the improvement in MR sensitivity was only significant in individuals who experienced a degree of clinical recovery and was not significant in those who had a history of previous depressive episodes. This suggests that MR function may be associated with clinical improvement but not in those with recurrent symptoms. We also observed a trend for an increase in GR sensitivity over time and speculate that this may be an effect of antidepressants. Previous studies have reported normalisation of HPA-axis feedback after antidepressant treatment (Heuser et al., 1996; Linkowski et al., 1987). One third of the depressed participants in the study were taking antidepressants, therefore any improvement in GR function could be a neurobiological effect of medication. To date MR function in depression has only been investigated longitudinally *in vivo*.

We also observed a trend towards an increase for the CAR AUC at follow-up although this was not associated with improvement in clinical symptoms. This is in agreement with some but not all of the previous studies. In light of the blunted CAR AUC in our depressed participants at baseline, reported in Chapter 3, these results may be interpreted as a

normalisation of the HPA-axis. We also observed a trend towards a steeper cortisol slope independent of symptom remission. In the absence of a significant change in evening cortisol this was likely driven by the increase in CAR AUC. Changes to cortisol slope have not previously been explored longitudinally in depressed people.

In addition, we observed an apparent trend towards a negative pattern of association between change in TNF- α levels and change in the CAR, suggesting that as TNF- α decreases the CAR increases. This finding builds on the results from Study 1a which demonstrated an association between increased TNF- α and a blunted CAR AUC in depressed people at baseline. The implication of this finding is that in those people who experience chronic or recurrent depression, an exhausted HPA-axis becomes incapable of restraining a stimulated TNF- α system. Once clinical symptoms improve, the HPA-axis normalises gaining back control over TNF- α production.

Strengths and limitations

The limitations of this study were addressed in Chapters 3 and 4. The main limitation of the follow-up study was the relatively short follow-up period, which may not have been able to capture any therapeutic effects in people who had recently started antidepressant treatment. In addition it is not clear why we still observed a decrease in depressive symptoms over time. Participants were not given any treatment or advice over this period and none of the participants reported any change in their treatment at follow-up. It is possible that the interviews provided some transient therapeutic benefit or that participants may have unconsciously responded to the questionnaires more positively at follow-up, having misinterpreted the aim of a study.

Despite these considerations (and others) these findings are novel and contribute to the literature in this field by being the first to investigate changes in inflammation, diurnal cortisol rhythm and *in vitro* corticosteroid function over time in the same MDD sample. It

has demonstrated that improvements in depressive symptoms co-occur with increased MR sensitivity. Finally it has indicated a possible longitudinal relationship between TNF- α and diurnal cortisol rhythm in MDD patients, although this interpretation should be treated with caution and needs further corroboration.

9.2.3 *Study 1c: The Resist Study results: Alterations in regulatory T-cells and their association with inflammation and HPA-axis function in people with depression*

In Study 1c (presented in Chapter 6) I examined the differences in Treg frequency between people with MDD and healthy controls, using data from the Resist Study. This study built upon the findings of Study 1a by assessing the role of adaptive immune function in MDD and any relationship with established depression related pathways. There is only a small literature examining Tregs in MDD and the results to date are inconclusive. Treg frequency has been shown to be both decreased (Y. Chen et al., 2011;Grosse et al., 2015;Grosse et al., 2016b;Y. Li et al., 2010) and increased (Patas et al., 2018;H. Suzuki et al., 2017) in depressed people compared with controls. Another study has reported no difference between the groups (Hasselmann et al., 2018). There is huge variability between these studies regarding markers used to characterise Treg populations and the subsets with which they compare them. Study 1c has contributed to the field by investigating the difference in the percentage of CD4+CD25+ T cells expressing FoxP3 in people with MDD compared with controls. On the balance of the evidence to date, it was hypothesised that depressed people would have a significantly decreased frequency of circulating Tregs compared with healthy controls.

We also investigated the relationship between circulating Tregs and circulating inflammatory biomarkers (IL-6 and TNF- α), *in vitro* GR and MR function and diurnal cortisol rhythm. Findings from several studies have suggested an association between Tregs and inflammatory activation (Y. Chen et al., 2011;Grosse et al., 2016b;Himmerich

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et al., 2010;Y. Li et al., 2010). One study has reported an inverse association with IL-6 and TNF- α gene expression, but not circulating IL-6 levels (Grosse et al., 2016b). Associations between Tregs and circulating TNF- α have not yet been investigated. Taking the findings of Study 1a into consideration, we hypothesised that the frequency of circulating Tregs in people with MDD would be significantly, negatively associated with both IL-6 and TNF- α . To date only two studies have investigated Tregs and HPA-axis activity simultaneously in MDD. A study by Patas et al. (2018) found no association with serum ATCH or cortisol levels and another study by Hasselmann et al. (2018) reported no difference in *GR* expression in Tregs in MDD patients compared with controls. To date no one has investigated associations between Tregs and either GR/MR function or diurnal cortisol rhythm in MDD. Based on our findings in Study 1a regarding disturbed HPA-axis function and the tentative evidence for reduced Tregs in MDD we hypothesised that the frequency of circulating Tregs in people with MDD would be significantly associated with HPA-axis function.

The main finding of the study was that, in contrast to expectation, there was no significant difference in circulating Tregs between depressed people and healthy controls. This finding corroborates that of Hasselmann et al. (2018) who observed no differences in Tregs in un-medicated MDD patients. Given the variability in methodology in this field, the inconsistency in the findings regarding Treg frequencies in MDD is unsurprising. Post-hoc power analysis supported the conclusion that the null effect we observed was genuine and that the findings would not have reached significance with any reasonable increase in sample size.

Contrary to hypothesis, we did not observe a correlation between Tregs and pro-inflammatory cytokines in MDD. Grosse et al. (2016b) have previously reported a negative association with gene expression of both IL-6 and TNF- α , however no association was observed with serum levels of IL-6. Circulating TNF- α levels have not previously been investigated in conjunction with Tregs in MDD. Theoretically we would

expect to see impaired immune suppression and inflammatory activation in the same people, however given out null findings regarding Tregs this result is not surprising. Similarly, we did not observe a correlation between Tregs and neuro-endocrine function in depressed people. The literature regarding this relationship has only just begun, however the evidence so far has provided little support for an association between the two. Further studies are required in order to determine whether any relationship exists.

Strengths and limitations

The limitations of this study were addressed in Chapter 6. The main limitations were a lack of data on physical activity, which is known to have negative consequences on immune function and a lack of data on diet, which has also been shown to influence Tregs. We also did not collect longitudinal data on Tregs and so were unable to investigate any changes in Tregs over time.

Despite these limitations these findings are novel and contribute to the literature in this field by being the first to investigate the difference in the percentage of CD4+CD25+ T cells expressing FoxP3 in people with MDD compared with controls. Furthermore, the relationship between Tregs and circulating IL-6 and TNF- α , or GR and MR sensitivity has not yet been investigated. The null findings from this study suggest that there is no difference in Tregs between people with MDD and healthy controls.

9.2.4 Study 2: Combined influence of depressive symptoms and systemic inflammation on all-cause and cardiovascular mortality in the English Longitudinal Study of Ageing (ELSA)

Moving on from the laboratory analyses in Study 1, Study 2 (presented in Chapter 8) took an epidemiological approach to examining depressive symptoms and inflammation.

This study investigated the combined effects of depressive symptoms and inflammation

on CVD and all-cause mortality risk in 5,328 older men and women from the English Longitudinal Study of Ageing (ELSA). The background to this study was that people with depression or elevated inflammation are at increased mortality risk (Lasserre et al., 2016;Schulz et al., 2002;Wulsin et al., 2005;Wulsin et al., 1999), particularly from CVD (Correll et al., 2017;Laursen et al., 2007;Q. Wu & Kling, 2016). However the relationship between these factors remains poorly understood. It has been proposed that inflammation may mediate the association between depression and mortality, however, evidence is inconsistent with majority of the evidence suggesting independent associations (K. W. Davidson et al., 2009;Empana et al., 2005;M. Hamer et al., 2011;Nabi et al., 2008;Surtees et al., 2008a) or minimal effects of mediation (Hiles et al., 2015;Hughes et al., 2016;Kop et al., 2010;Vacarino et al., 2007). One study has examined the combined effects of depressive symptoms and inflammation on the prediction of coronary events. Ladwig et al. (2005) showed that depressive symptoms and CRP interact in the prediction of cardiovascular events, however this study was restricted to men only. It remains unclear whether this combination confers a greater risk than either depression or inflammation alone, in both men and women.

In addition, this study investigated whether depressive symptoms moderate the mortality risk associated with CRP or whether CRP mediates the association between depressive symptoms and mortality. Based on previous findings we hypothesised that people with both depressive symptoms and elevated CRP would have significantly greater mortality risk than people with depressive symptoms or increased CRP alone.

As predicted, we found that older men (≥ 50 years) with both depressive symptoms and high levels of CRP had an increased risk of CVD and all-cause mortality, independent of age, socioeconomic variables and health behaviours. This risk was considerably higher than that conferred by high CRP alone. There was no significant increase in mortality risk associated with depressive symptoms. However contrary to our hypothesis, neither depressive symptoms or inflammation or the combination of both significantly predicted CVD or all-cause mortality in older women. In addition, the results demonstrated

independent effects of depressive symptoms and inflammation on mortality, finding no evidence of either moderation or mediation.

The sex-specific differences we observed in this study are of particular interest. Sex differences regarding inflammation and CVD risk have been reported previously, demonstrating increased vulnerability in men compared with women (Ahmadi-Abhari et al., 2013; Cushman et al., 2005; Kaptoge et al., 2012). Possible explanations include the protective influence of oestrogen which is thought to have cardio-protective effects until approximately 70 years of age (Fairweather, 2014). Furthermore, men exhibit shorter telomeres than women. Shorter telomeres have been associated with mortality and CVD risk (Gardner et al., 2014; Haycock et al., 2014; Weischer et al., 2012). From an evolutionary perspective, it is also possible that depression confers an adaptive advantage for women. Depressive symptoms promote sickness behaviours, reducing the need for an inflammatory response, which is harmful to fertility. Their presence is therefore less likely to reflect severe biological pathology (A. H. Miller & Raison, 2016).

Strengths and limitations

The strengths of this study include the prospective design, the inclusion of both women and men and the nationally representative nature of the cohort. The main limitations of this study is that we measured depressive symptoms and inflammation at only one time point only and that we did not control for medication use. Our study had a smaller proportion of female deaths compared to male deaths possibly reflecting insufficient power and depressive symptoms were measured by an 8 item self-reported questionnaire, rather than a diagnostic interview.

9.3 Overall summary of findings in this thesis

Taken together, some but not all of our hypotheses were supported by the data in this PhD. These studies have provided support for dysregulation in both immune and neuroendocrine function in people with MDD, highlighting increased TNF- α production, a blunted CAR AUC and reduced sensitivity of both the GR and MR. The results of this PhD also indicate that there is a relationship between TNF- α and diurnal cortisol rhythm in MDD. We have also demonstrated that men with concurrent depressive symptoms and increased inflammation constitute a high-mortality risk phenotype. This work has shed light on some previously unexplored associations and in combination these studies contribute to the literature investigating depression and inflammation.

9.4 Methodological issues and limitations

The findings presented in this thesis have to be interpreted in the context of their limitations. The shortcomings of each study were discussed at the end of every chapter. Therefore, in this section only the most important issues and broader, overarching limitations will be discussed.

9.4.1 The study samples

This PhD used two different samples. The samples for Study 1 were taken from the Resist Study. Study 1 was an observational, case-control study of 37 people with MDD and 30 healthy controls. In Study 2 the participants (n=5,328) were taken from the English Longitudinal Study of Ageing epidemiological cohort at wave 2 (2004-2005). In Study 1, one advantage of the sample used was that they all had a diagnosis of MDD. This was obtained using the CIS-R, which is a fully structured diagnostic interview, generating diagnoses which meet ICD-10 criteria for MDD. Therefore we did not solely rely on the presence of depressive symptoms which may have resulted in a lack of

specificity. Another advantage was that each participant had to meet strict exclusion criteria (see Section 2.2.4) in order to take part in the study. This reduced confounding due to comorbid psychiatric or medical disorders and medication use which may have affected the biological pathways under investigation. In Study 2, an advantage of the sample was the inclusion of both women and men and the nationally representative nature of the ELSA cohort.

However, these participant samples were not without limitations. In the Resist Study the strict exclusion criteria vastly reduced the number of eligible participants due to the high number of co-morbidities in this population. We planned to recruit 60 people with MDD via a randomised control trial, PANDA. Due to low uptake we changed the recruitment strategy to include participants from primary care practices across London, iCope Psychological Therapies Service in Camden and Islington, UCL staff and students and via an online website. In total, recruitment was conducted over a 16-month period but despite these endeavours, we only achieved 62% of our target. Approximately 65% of depressed patients have at least one concurrent psychiatric disorder and 60% have at least one concurrent general medical condition (Otte, 2008). Comorbidity therefore appears to be the rule rather than the exception. Feedback from GPs was that the exclusion criteria was too restrictive. Furthermore, 56% of the patients that were eventually referred from GP practices, were subsequently excluded during screening. The consequence of this was that we had a smaller than anticipated sample size, potentially resulting in a loss of power.

Another shortcoming of the recruitment strategy that applies to Study 1 is that the recruitment method differed for the depressed participants and healthy controls. Due to the low response rate and the associated costs of conducting mail outs via GP practices, we decided to recruit healthy controls from the staff and student population at UCL and QMUL and from staff within the NIHR Clinical Research Network. Consequently, this

sample came from a higher socioeconomic background than the depressed group and are not representative of non-depressed people living in London.

Another consideration regarding the exclusion criteria is that whilst it increased the internal validity of the study, enabling us to more confidently assess the biological correlates of MDD, it also provided us with a sample which does not accurately represent the MDD community. This artificially rendered our sample more medically healthy and psychiatrically singular than in the general MDD population and decreased the generalisability of the results.

Regarding the ELSA sample, there are very few ethnic minority participants, largely due to costs associated with establishing representative oversamples (Steptoe et al., 2013). When ELSA started in 2002, the national census indicated that 3.2% of men and women aged 50 and over were from ethnic minorities. This is the same proportion as in ELSA, therefore the sample is representative of the older population in England, but that leads to a rather small number of black, Asian and minority ethnic people. In 2011 the national census indicated that 19.5% of people were from ethnic minorities, therefore the ELSA cohort may not adequately reflect the current general population in England. The 2014 Adult Psychiatric Morbidity Survey found the prevalence of common mental health problems to vary significantly by ethnic group for women (McManus et al., 2016). Black British women (29.3%) were more found to be more likely to have a common mental health problem than white British women (20.9%). The under-representation of these groups may impair the ability of ELSA to fully capture the burden of depressive symptoms in women.

Additionally data regarding medication use is not collected in the early waves of ELSA. For example, statins confer anti-inflammatory effects (Antonopoulos et al., 2012). Antidepressants are also known to reduce circulating inflammatory biomarkers, including CRP (Hiles et al., 2012a) and antidepressant use is prevalent in 10% of elderly people

in the UK (Tamblyn et al., 2019). Therefore medication use may have interfered with our findings.

Finally, the use of a structured depression interview is not possible in ELSA given the large, multipurpose design of the study. Thus, we relied on self-reported depressive symptoms, measured by an eight-item questionnaire. As previously mentioned, short scales such as this can result in a lack of specificity. Whilst we attempted to address this issue by using a conservative cut-off score to define the presence of depressive symptoms, it is possible that we still included false positives.

9.4.2 *Biological measurement issues*

In Study 1, diurnal cortisol secretion was measured using five saliva samples taken over the course of one day. It has been suggested that samples collected on a single day are more susceptible to state effects and that sampling over two to six days is required for reliable trait measures (J. Hellhammer et al., 2007). This means that in our sample diurnal rhythm may have been affected by transient, situational factors rather than long-standing factors. Furthermore individuals suffering with depression often experience disrupted routines, often as a result of sleep disturbance (Baglioni et al., 2016; M. J. Murphy & Peterson, 2015). Therefore reliability may have been improved by taking measures over the course of several days. The healthy control group would likely have had less variability in their routine and so measurements over a single day may have been more representative than in the depressed group. In addition, depressed people often experience problems with memory (Roca et al., 2015) and a deficit in motivation (Callaghan et al., 2018) making it less likely that they will adhere to the sampling protocol. In fact on average, twice the number of depressed people compared with controls, failed to provide at least one saliva sample at baseline (MDD = 6, HC = 3). At follow-up an average of 16 depressed people failed to provide at least one sample. It therefore seems that there that there may have been an issue of fidelity in the depressed group.

However it was decided to measure diurnal cortisol secretion over the course of one day only in order to minimise participant burden. This was particularly relevant for the depressed group as if the process had been overly cumbersome, it may also have deterred them from attending follow-up. Recruitment and retention of depressed participants is notoriously difficult in clinical research (J. S. L. Brown et al., 2019) and so limiting the inconvenience and effort required is of paramount importance. In fact at follow-up we observed a very low attrition rate (5%), which given our already small sample size, was critical.

Cortisol can be measured in several biological specimens including saliva, blood, urine, and hair. We chose to measure cortisol in saliva for a number of reasons. Firstly saliva sampling is a comparatively inexpensive method and in order to investigate multiple biological parameters we had to impose every reasonable financial constraint. Secondly, saliva sampling is a non-invasive method, avoiding issues associated with repeated venipuncture and allows for ambulatory assessment in a naturalistic setting, where participants are collecting their own samples (Kirschbaum & Hellhammer, 1989). Repeated blood sampling across the day would be impractical and would likely impact on recruitment and retention. Furthermore, salivary cortisol is stable at room temperature for two weeks after sampling, allowing for participants to post their samples back to the laboratory, without any degradation. Finally, saliva cortisol provides a measure of 'free', biologically active cortisol (Kirschbaum & Hellhammer, 1989, 2000). There are high correlations between salivary cortisol and unbound free plasma cortisol consistent throughout circadian rhythm (D. H. Hellhammer et al., 2009).

In Study 1 corticosteroid sensitivity was measured using glucocorticoid and mineralocorticoid sensitivity assays. Specifically, GR and MR sensitivity was measured by dexamethasone and prednisolone suppression of LPS induced IL-6 levels in whole blood. We chose an *in vitro* method as this approach provides a more controlled and direct measure of corticosteroid sensitivity compared with the DST. However, these

assays still only provide a proxy measure of receptor sensitivity. The addition of assays measuring gene expression of GR up and downregulated genes would help to establish GR responsiveness. Measurement of receptor mRNA levels, the rate of translocation of the receptors into the cell nuclei or DNA binding would also be of value. In addition we chose whole blood to allow for rapid measurement, however measurement in isolated monocytes or lymphocytes would have allowed for a more focused measure of IL-6 suppression. The production of inflammatory cytokines differs between specific leukocyte subsets. For example, LPS-induced IL-6 is mainly produced by monocytes (Berczi, 1998). There are also differences in frequency of immune cell populations, which could vary between participants (Burnsides et al., 2012). Finally, the inclusion of additional outcomes known to be affected by glucocorticoids would allow us to examine broader effects. The results of Study 1 should be interpreted with these issues borne in mind.

In Study 1c we determined frequency of FoxP3 expressing cells within a CD4+CD25+ subset. There is a great deal of inconsistency in the literature regarding the markers used to characterise Treg populations and the subsets with which they compare them, which may account for the conflicting findings. For example, some studies measure the percentage of Tregs in peripheral blood lymphocytes (2015;2016b;2010), another determined the ratio of CD4+CD25+FoxP3 cells to CD4+ cells (2011), another quantified the frequency of CD25^{high}CD127^{low}- T cells in a subset of CD4+ T cells (2018), another measured the frequency of CD127^{low}CCR4+ and memory Tregs in PBMCs and another identified the percentage of CD4+ CD25+CD127- cells in lymphocytes (2018). Some studies rely only on the cell surface markers CD4 and CD25 (Himmerich et al., 2010;Y. Li et al., 2010;Patas et al., 2018;H. Suzuki et al., 2017), whilst others include the classic intracellular marker of Treg cells, FoxP3 (Y. Chen et al., 2011;Grosse et al., 2015;Grosse et al., 2016b). We based our choice of Treg identification on the following rationale. In humans, up to 30% of peripheral blood CD4+ T cells express CD25 (Ng et al., 2001). However only 1~2% of CD4+ cells with the highest level of CD25 exhibit the

suppressive function of Tregs (Baecher-Allan et al., 2001; Baecher-Allan et al., 2002) and high expression of FoxP3 (Miyara et al., 2009). In contrast, at least 50% of total FoxP3+ human Tregs are CD25^{low} and some do not express CD25 at all, therefore identification of Tregs based on CD25^{high} will fail to capture the majority of human CD4+FoxP3+ Tregs (X. Chen et al., 2010). Furthermore, activated CD4+ effector cells also express high levels of CD25 (Seddiki et al., 2006), potentially leading to the identification of false positives. Foxp3 is generally considered a master regulator of Treg function. The essential role of FoxP3 is illustrated by the fact that mutations of the human FoxP3 gene have been shown to cause IPEX syndrome (Bennett et al., 2001). FoxP3 expression is critical for the survival of Tregs, their ability to produce IL-2 and proliferate following T cell receptor engagement (Gavin et al., 2007). Furthermore, loss of FoxP3 expression by fully differentiated Tregs causes a loss of suppressor function (Williams & Rudensky, 2007). Therefore the identification of FoxP3 expression in CD4+CD25+ cells is essential for accurate Treg identification. By determining the percentage of CD4+CD25+ T cells which express FoxP3 we can be more confident that we are measuring suppressive Tregs and not effector cells. However in the absence of a consensus regarding Treg characterisation and a common measure of Treg frequency, comparisons with other studies is difficult. The results of Study 1c should therefore be treated with a great deal of caution.

9.4.3 *Antidepressant use*

Study 1 was originally designed to explore the effect of antidepressants on the biological pathways implicated in depression. We intended to recruit via a randomised control trial, which was recruiting depressed patients from primary care, who were not currently being treated with antidepressants. Participants were then to be randomised to either sertraline or placebo and followed up over 12 weeks. We selected this trial because it would have given us participants who were free from antidepressants at baseline and enabled us to observe any antidepressant effects. However, due to the recruitment issues already

discussed, we decided to recruit from primary care directly. This inevitably meant that we lost our 'clean' baseline and ended up recruiting people receiving a variety of treatments. In fact 30% of the depressed group were taking antidepressants at baseline with treatment duration ranging from 1-36 months. The consequence of this is that we were no longer able to examine any causal effects of antidepressants and any interpretations regarding changes in biology are merely speculative. Nonetheless, we did include sensitivity analyses where appropriate, to explore whether any observed biological changes may have been influenced by antidepressant use and found no evidence of an effect.

Depressive symptoms significantly improved at follow-up, but it is unclear why. For those individuals receiving antidepressant medication there could have been an effect of the drug. However given that the therapeutic action of antidepressant treatments usually takes several weeks to appear (Ferrari & Villa, 2017) and in our sample only 22% of participants had been taking them for a minimum of six weeks, we cannot confidently infer any treatment effect. Further issues come from our relatively short follow-up period, which hinders our ability to capture any therapeutic effects in the 8% of people who had only recently started antidepressant treatment. Furthermore, given the fact that 57% of the depressed group were not receiving either antidepressant medication or psychotherapy and we provided no therapeutic intervention, it is unclear why, overall, their depressive symptoms improved.

9.5 Suggestions for future research

The studies presented in this thesis have highlighted gaps in the literature and demonstrated the need for further research in this field. Suggestions for future research were provided in the Discussion section of each chapter. In this section I will outline more general ideas for future studies investigating the biological correlates of depression.

9.5.1 *The heterogeneity of depression*

Despite decades of research, very basic questions regarding depression remain unanswered. Specifically, the aetiology of depression remains unclear and the ineffectiveness of antidepressants beyond placebo for the majority of patients remains unresolved. Research in depression typically involves three stages, firstly the researcher selects a rating scale or clinical interview from a vast array of choices and a series of mood, cognitive and somatic symptoms are measured. Secondly, these symptoms are summed, creating a severity score which is compared to a pre-defined threshold, in order to distinguish individuals with MDD from healthy controls. Thirdly, the researcher investigates whether patients and controls differ in terms of biological markers (Studies 1a and 1) or physical health outcomes (Study 2), or whether biological markers are associated with treatment response or symptom remission (Study 1b).

This poses several problems. As discussed in Chapter 1, Section 1.2, patients with depression exhibit a wide range of different symptoms and may even present with the opposite symptoms. For example, a patient who gains weight, sleeps too much and moves around slowly would score the same as a patient who loses weight, can't sleep and moves around quickly. Fried et al. (2015a) identified 1,030 unique symptom profiles in 3,073 MDD patients, calling into question the notion of MDD as a homogenous syndrome. It seems therefore to make little sense to investigate biological markers in a population who demonstrate such variability in symptoms. In addition, the definition of MDD is indisputably vague. Symptom questionnaires such as the BDI, GHQ and HAM-D differ greatly from the DSM-V criteria for MDD (Fried & Nesse, 2015b). A content analysis of seven common depression scales produced 52 disparate symptoms with low overlap with DSM-5 MDD (Fried, 2017). This inconsistency is reflected in the fact that over the last century more than 280 measures of depression have been developed and published, and dozens of MDD subtypes have been suggested and subsequently discounted (Santor & Gregus, 2006). One would struggle to envision similar peculiarities

in establishing a diagnosis of a medical illness such as cancer or CVD. Thus the assumption that these symptoms represent interchangeable indicators of the same distinct disease category is questionable.

It has been suggested that MDD should not be understood as a homogenous condition, but as a heterogeneous cluster of symptoms, which have a reciprocal relationship with each other (Fried, 2015). For example, insomnia can cause fatigue, resulting in concentration and psychomotor problems. Studies have also shown that specific MDD symptoms differ in their relations with inflammatory biomarkers. Jokela et al. (2016) demonstrated that independent associations with CRP were only evident with sleep problems, tiredness or lack of energy and changes in appetite, in line with the notion of sickness behaviour. Associations between inflammation and cognitive and emotional symptoms of depression were not independent of other depression symptoms. A more recent birth cohort study including 2,731 participants showed that IL-6 was specifically associated with somatic/neurovegetative symptoms such as fatigue, sleep disturbances and diurnal variation in mood (Chu et al., 2019). A study using the ELSA cohort reported that CRP exhibited a stronger relationship with somatic than with cognitive-affective symptoms (Job et al., 2019). Another recent study also reported a specific association between IL-6 and sleep disturbance in people with MDD (M. Wang et al., 2019). Identifying specific symptom associations may help identify treatment targets and markers of treatment response in clinical trials of anti-inflammatory drugs for the treatment of depression. Clusters of symptoms also differ in their response to antidepressant treatment. Chekroud et al. (2017) showed that antidepressants were more effective at treating core emotional symptoms than they were at treating sleep or atypical symptoms. Furthermore, MDD symptoms differentially predict relapse (Chekroud et al., 2016)

In light of these considerations, several practical improvements to MDD measurement can be suggested. Firstly, more thorough assessments of compound symptoms could

be made. Insomnia and hypersomnia are opposites and combining them into 'sleep' is unhelpful. Similarly, psychomotor retardation has four times the effect on impairment of psychosocial functioning than psychomotor agitation (Fried & Nesse, 2014). Secondly, as in Study 1a different depression instruments should be used simultaneously and conclusions should be considered robust only if results generalise across scales. Finally, by focusing on specific symptoms which provide more reliable units of measurement, researchers may be able to make progress in identifying distinct underlying biological processes. This could lead the way to a more personalised treatment of depression that accounts for the heterogeneity of MDD.

9.5.2 *HPA-axis measurement*

Studies 1a and 1b provided evidence for the utility of incorporating multiple measures of GR resistance and HPA-axis dysfunction. Dysregulation was observed in terms of GR and MR sensitivity, as well as diurnal cortisol rhythm represented by a flattening of the CAR AUC. This comprehensive approach allowed for the observation of a relationship between inflammatory activation and HPA-axis dysfunction which may otherwise have been overlooked.

Studies investigating inflammation and GR resistance have reported conflicting results. A recent meta-analysis concluded that the variability of measures assessing GR resistance makes interpretation of the literature difficult (Perrin et al., 2019). Studies often rely on single measures of HPA-axis function, each of which has its own limitations. Studies including plasma or salivary cortisol appear to provide the largest effect but often fail to include assessments of diurnal cortisol rhythm. Diurnal variations in cortisol levels are a well-established phenomenon of the HPA-axis and yet this aspect of dysregulation is often overlooked. Most studies investigating inflammation and GR resistance measure morning cortisol but fail to target the following hour, preventing the assessment of diurnal rhythm (Perrin et al., 2019). The reliability of the cortisol data is also hindered by the fact

that the window of measurement spans 9.00am to 11.00am, creating widespread variability. Furthermore, while cortisol levels indicate the degree of HPA-axis activation, they do not directly probe the corticosteroid receptors.

Many studies rely on the DST as a measure of GR resistance, however this only provides a measure of peripheral GR function and findings do not translate to the CNS. Whilst *in vitro* assays also suffer from translational limitations, they do provide a more controlled, direct measure of receptor sensitivity. The greatest effect size appears to come from studies using an *in vitro* approach and/or GR expression (Perrin et al., 2019). Furthermore, the inclusion of a measure of MR function is wholly absent among these studies.

In light of these limitations, employing a combination of techniques to explore associations between inflammation and HPA-axis function will maximise the likelihood of observing any relationship between the two. There is a need for larger studies, measuring various cytokines and using various measures of GR and MR resistance, including *in vitro* assays and assessments of diurnal cortisol rhythm, in MDD. This will provide a broad characterisation of the relationship between inflammation and HPA-axis function.

9.6 Final conclusions

This thesis has presented the findings from two studies which have used a mixture of laboratory testing and epidemiological methods. I believe each of the studies presented have added something new to the field. Study 1a was the first to measure inflammation, diurnal cortisol rhythm and GR and MR function simultaneously in a cohort of people with MDD. The findings of this study suggest that depressed people experience dysregulation regarding inflammatory cytokine production, diurnal cortisol rhythm and both GR and MR sensitivity. In addition, the findings suggest a relationship between TNF- α and diurnal

cortisol rhythm in depression. Study 1b was the first to investigate changes in inflammation, diurnal cortisol rhythm and *in vitro* corticosteroid function over time in the same MDD sample. The results demonstrated that improvements in depressive symptoms co-occur with increased MR sensitivity. Study 1c was the first to investigate the difference in the percentage of CD4+CD25+ T cells expressing FoxP3 in people with MDD compared with controls. Furthermore, it was the first to explore the relationship between Tregs and circulating IL-6 and TNF- α , or GR and MR sensitivity in MDD. The findings suggest that there is no difference in Tregs between people with MDD and healthy controls. Finally Study 2 is the first to investigate the combination of inflammation and depressive symptoms on mortality risk, in both men and women and demonstrated that men who exhibit both risk factors constitute a high-mortality risk phenotype, particularly in relation to CVD.

10. References

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10.1 Patient information sheet for participants with MDD

RESIST: Understanding the role of depression in heart disease

Invitation to participate

You are being invited to take part in a research study, the RESIST study, which is being conducted as part of a PhD by Miss Samantha Lawes at University College London. Please take time to read through this information carefully and discuss it with others if you wish.

The purpose of the study

Depression and heart disease are common health problems. People who suffer with depression are at greater risk of heart problems. It is not yet understood why this happens. A growing number of scientists believe that depression and heart disease may be influenced by the immune system. One of the body's natural responses to infection or injury is called inflammation but if this is chronic it can lead to health problems. Differences in levels of inflammation between people may lead to differences in molecules in our blood. In this study, we will compare the blood of people with depression to people who do not have depression.

Why have I been chosen to be invited?

We are interested in patients who have been diagnosed with depression to help us with the study. You have been given this information sheet because your GP thinks you may have depression. However, no information will be passed to the RESIST study team from by your GP.

Do I have to take part?

No, taking part in this study is voluntary. It is up to you to decide whether or not to take part. Whether or not you decide to take part will not affect the standard of care you receive from your general practice. If you are interested in taking part you can contact the researcher by phone or email to discuss the study in more detail. If you decide to participate the researcher will arrange to meet with you. This interview can take place either at your GP surgery, UCL or your home. At this point you will also have the opportunity to raise any questions about the study. If you agree to take part, the researcher will ask you to complete a consent form and you will be given a copy. You are free to withdraw at any time without giving a reason.

What will taking part involve?

Once you provide consent you will undertake study assessments, the most convenient time will be at the same interview. The assessments can take place either at your GP surgery, UCL or your home and should take around 45 minutes. The researcher will ask you to complete some short questionnaires on paper. These questions will be about events in your life, current symptoms of depression and general well-being. We will collect from you a 30ml sample of blood. We will also ask you to provide five saliva samples over a 24-hour period, complete a sampling diary and post them to us. We will provide you with the necessary equipment, including a saliva sampling kit, sampling diary

and a pre-paid envelope. You will need to complete a further assessment 6 weeks later. This follow-up assessment can take place either at your GP surgery, UCL or your home and will last about 45 minutes. The researcher will ask you to complete the same questionnaires as at your first visit. On this occasion we will also ask you to complete an additional short questionnaire about your childhood. We will also collect from you another 30mls of blood.

As the researcher may be visiting you in your home, risks to the researcher will be assessed and UCL's Lone Worker Policy (2012) will be followed.

What is being tested?

We will use your blood and saliva to look at molecules that increase the risk of developing cardiovascular disease.

What will happen to my sample and medical information?

Your samples will be taken to a UCL laboratory by the researcher where they will be stored and later analysed, with access limited to members of the study team. They will be stored for 5 years by the research group, pending ethical approval for use in future research. Samples will be processed, stored and disposed of in accordance with all applicable legal and regulatory requirements, including the Human Tissue Act 2004 and any amendments thereafter.

Your sample and medical information will be assigned a number. This number, without your name or any other identifiable details, will be how researchers keep track of samples and information. All data will be kept separate from personally identifiable information in a secure system, ensuring that all information remains anonymous.

Will personal information about me be kept confidential?

We will follow ethical and legal practice and all information about you will be handled in confidence. Your research data will be kept in a secure system at UCL designed for handling identifiable data which has been certified to the ISO27001 information security standard and conforms to the NHS Information Governance Toolkit. Your medical records will not hold any of your results from this research. Your samples can not be linked back to you as they will be coded with your participant number (not your name). This data is only accessible to the research team. It is likely that the results of this study will be published; however your name will not appear on any publications or reports about this research. At the end of the study, all personal identifiable data will be destroyed.

What will happen to the results of the research study?

When the study is completed, the results will be published in a health care journal so health care professionals can see the results. If published, your identity and personal details will be kept confidential. No named information about you will be published in any reports about the study.

Are there any disadvantages in taking part?

Blood will be collected by venepuncture which involves a minimal risk procedure. You will be asked to provide five saliva samples and complete a diary which will involve a small amount of time. Some of the questions in the questionnaire about your childhood, which is given at the follow-up interview, ask about physical and sexual abuse. This may cause feelings of distress for some people and we will direct you to counselling if needed. Support is available via www.samaritans.org and www.nhs.uk/conditions/stress-anxiety-depression. It is important that we ask these questions in order to identify potential risk factors for depression. Since your care is not being affected in any way, there are no other disadvantages associated with taking part in the study.

What are the possible benefits of taking part?

Although we cannot promise that this study will be of immediate benefit to you, by taking part you will help to increase the knowledge of what causes people to be more susceptible to depression and heart disease. We hope that in the future this knowledge may lead to the development of better treatments and improved prevention of these illnesses. You may benefit from this.

Who is funding and organising the research?

The research is being conducted by University College London. It is funded by the British Heart Foundation.

Who has reviewed the study?

The study has been reviewed by the Health Research Authority and the NHS Research Ethics Committee.

What if there is a problem?

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated by members of staff you may have experienced due to your participation in the research, National Health Service or UCL complaints mechanisms are available to you. Please ask your research doctor if you would like more information on this.

In the unlikely event that you are harmed by taking part in this study, compensation may be available.

If you suspect that the harm is the result of the Sponsor's (University College London) or the hospital's negligence then you may be able to claim compensation. After discussing with your research doctor, please make the claim in writing to Professor Glyn Lewis who is the Chief Investigator for the research and is based at University College London. The Chief Investigator will then pass the claim to the Sponsor's Insurers, via the Sponsor's office. You may have to bear the costs of the legal action initially, and you should consult a solicitor about this.

What do I do now?

Thank you for considering taking part in the research. If you are happy to take part, please sign the Permission to Contact form and a RESIST researcher will contact you within the next few days.

If you would like independent advice about participating in this study you can contact the Patient Advice and Liaison Service (PALS) via your GP surgery, NHS 111 or [www.nhs.uk/Service-Search/Patient-advice-and-liaison-services-\(PALS\)/LocationSearch/363](http://www.nhs.uk/Service-Search/Patient-advice-and-liaison-services-(PALS)/LocationSearch/363).

If you would prefer not to take part, you need not do anything.

If you have any questions about the study please contact:

Student researcher:

Samantha Lawes: [REDACTED]

Chief Investigator:

Professor Glyn Lewis

Tel: 020 7679 1682

Fax 020 7916 8542

Email: samantha.lawes.13@ucl.ac.uk

UCL Research Department of Epidemiology and Public Health

Psychobiology Group

1 - 19 Torrington Place, London WC1E 6BT

Tel: 0207 679 9253

Email: glyn.lewis@ucl.ac.uk

THANK YOU FOR YOUR TIME

10.2 On-line advert recruiting participants with MDD

Invitation to participate in a depression study at UCL

05 May 2017



Over the past 2 weeks, have you been bothered by:

- Little interest or pleasure in doing things?
- Feeling down, depressed, or hopeless?

If you answered 'yes' to either question you could be eligible to take part in a UCL study investigating the relationship between depression and heart disease.

The study would involve TWO sessions where we would ask you to complete some questionnaires and provide a blood and saliva sample (note: these sessions take place on Monday –Thursday mornings). You will be reimbursed £10 for each session attended.

Requirements

- **Age 18-74**
- **Men and women**
- **Currently experiencing low mood**
- **Otherwise healthy**
- **NOT to be pregnant or breastfeeding**
- **Able to attend at 9.00am Monday-Thursday**
- **Able to attend 2 x assessment sessions**
- **Able to read, understand and complete questionnaires**

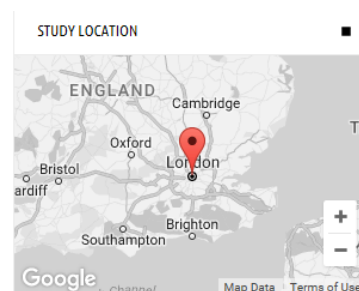
Ethical approval

This study has been approved by the Health Research Authority Ethics Committee (16/WM/0143)

About the researcher

Psychobiology Group, Department of Behavioural Science and Health, 1-19 Torrington Place, University College London, WC1E 7HB

Contact researcher <https://www.ucl.ac.uk/iehc/research/behavioural-science-health/research/psychobiology/resist/index>





If you experience symptoms of depression for most of the day, every day for more than two weeks, you should seek help from your GP.

Many people wait a long time before seeking help for depression, but it's best not to delay. The sooner you see a doctor, the sooner you can be on the way to recovery.

It's particularly important to speak to your GP if you:

- have symptoms of depression that aren't improving
- find your mood affects your work, other interests, and relationships with your family and friends
- have thoughts of suicide or self-harm

Adult Improving Access to Psychological Therapies programme (IAPT)

IAPT is a free NHS talking therapy service for adults, who are worried or have low mood. They provide a range of treatment programmes including one to one therapy, counselling and group work.

There are two ways to get help from IAPT.

1. Talk to your GP or another health professional about being referred to your local IAPT service.
2. Refer yourself by calling IAPT directly. Details of local IAPT services are available on the NHS Choices website.

Mental health helplines

There are also a number of helplines which offer support for people suffering from depression or anxiety:

- ***Mind***

Promotes the views and needs of people with mental health problems.

Phone: 0300 123 3393 (Mon-Fri, 9am-6pm) Website: www.mind.org.uk

- ***Anxiety UK***

Charity providing support if you've been diagnosed with an anxiety condition.

Phone: 08444 775 774 (Mon-Fri, 9.30am-5.30pm) Website: www.anxietyuk.org.uk

- ***Depression Alliance***

Charity for sufferers of depression. Has a network of self-help groups.

Website: www.depressionalliance.org

10.4 UCL posters for recruiting participants with MDD



Over the past 2 weeks, have you been bothered by:

- **little interest or pleasure in doing things?**
- **feeling down, depressed, or hopeless?**



If you answered 'yes' to either question you could be eligible to join the RESIST study

- We are investigating the role of inflammation in the relationship between depression and heart disease
- Participation would involve two sessions, ~ 60 mins
- Participation will involve some questionnaires and a blood and saliva sample
- You will be reimbursed £10 for your time

For more information or to volunteer for this study, please contact:

Samantha Lawes

Tel: 020 7679 1682 Mobile: 07891 432226

Email: samantha.lawes.13@ucl.ac.uk

10.5 Letters of invitation for healthy controls via GP practices

<< Patient name>>

<< Address >>

<Date>

Dear <<Patient name>>

RESIST: Understanding the role of depression in heart disease

I am writing to tell you about a research study taking place at University College London, which you may be able to help with. This study is investigating why people who suffer with depression are at greater risk of heart problems.

We are interested in patients who have been diagnosed with depression as well as healthy volunteers to help us with the study. You have been identified as a potential healthy volunteer by your GP practice.

The RESIST study is aiming to recruit volunteers for a single interview, including providing a blood and saliva sample. We hope that you would be interested in taking part in this worthwhile study but before you decide, it is important for you to understand why the research is being done and what it will involve.

I have enclosed an information sheet giving more details about the RESIST study and how it is run. Please take time to read it through and consider whether you are willing to be contacted by the RESIST research team about this study.

You do not have to take part in the study, but we hope that as many people as possible will take part. Please let the research team know if you are interested in participating by contacting them on 020 7679 1682 or at samantha.lawes.13@ucl.ac.uk.

Thank you for your help.

Yours sincerely,

<GP>

10.6 Consent form



RESIST: Understanding the role of depression in heart disease

A copy of this consent form will be given to you.

Participant ID number:

Please initial box

1. I confirm that I have read and understand the patient information sheet v2.0 05/05/2017 for the RESIST study. I have had the opportunity to consider the information, ask questions and have these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw, without giving a reason, and without my medical care or legal rights being affected.

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from UCL from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

4. I agree to take part in the above study

5. I agree to provide blood sample and saliva samples.

6. I agree to my GP being informed about my participation in this study.

10.7 Example page from cortisol sampling diary

TUBE 1 : AS SOON AS YOU WAKE UP

1. **What is the time now?** _____ a.m. / p.m.
2. **What was the exact time you collected the sample?** _____ a.m. / p.m.
- 2a. Was there a delay between waking up and collecting your first sample? **Yes** **No**
- 2b. ↪ If yes, how long? _____ hrs & _____ minutes

In the last 30 minutes how much did you feel.....

	Not at all					Very much
	1	2	3	4	5	
3. In control	1	2	3	4	5	
4. Tired	1	2	3	4	5	
5. Happy	1	2	3	4	5	
6. Frustrated or angry	1	2	3	4	5	
7. Sad	1	2	3	4	5	
8. Stressed	1	2	3	4	5	
9. Pain	1	2	3	4	5	
10. 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
Eat a meal?	No	Yes
Do any exercise?	No	Yes
Smoke any cigarettes?	No	Yes

10.8 Beck Depression Inventory II

YOUR FEELINGS

Please read each group of statement carefully, then pick out **one statement** in each group which best describes the way you have been feeling during the **past two weeks, including today**. Tick the statement you have picked.

If several statements in the group seem to apply equally well, simply tick the statement which has the largest number. Be sure that you do **not** tick more than one statement for item 16 (change in sleeping pattern) and item 18 (change in appetite).

1. Sadness	I do not feel sad	0	<input type="checkbox"/>
	I feel sad most of the time	1	<input type="checkbox"/>
	I am sad all the time	2	<input type="checkbox"/>
	I am so sad or unhappy that I can't stand it	3	<input type="checkbox"/>
2. Pessimism	I am not discouraged about my future	0	<input type="checkbox"/>
	I feel more discouraged about my future than I used to be	1	<input type="checkbox"/>
	I do not expect things to work out for me	2	<input type="checkbox"/>
	I feel my future is hopeless and will only get worse	3	<input type="checkbox"/>
3. Past Failure	I do not feel like a failure	0	<input type="checkbox"/>
	I have failed more than I should have	1	<input type="checkbox"/>
	As I look back, I see a lot of failures	2	<input type="checkbox"/>
	I feel I am a total failure as a person	3	<input type="checkbox"/>
4. Loss of Pleasure	I get as much pleasure as I ever did from the things I enjoy	0	<input type="checkbox"/>
	I don't enjoy things as much as I used to	1	<input type="checkbox"/>
	I get very little pleasure from the things I used to enjoy	2	<input type="checkbox"/>
	I can't get any pleasure from the things I used to enjoy	3	<input type="checkbox"/>
5. Guilty Feelings	I don't feel particularly guilty	0	<input type="checkbox"/>
	I feel guilty over many things I have done or should have done	1	<input type="checkbox"/>
	I feel guilty most of the time	2	<input type="checkbox"/>
	I feel guilty all of the time	3	<input type="checkbox"/>
6. Punishment Feelings	I don't feel I am being punished	0	<input type="checkbox"/>
	I feel I may be punished	1	<input type="checkbox"/>
	I expect to be punished	2	<input type="checkbox"/>
	I feel I am being punished	3	<input type="checkbox"/>
7. Self-Dislike	I feel the same about myself as ever	0	<input type="checkbox"/>
	I have lost confidence in myself	1	<input type="checkbox"/>
	I am disappointed in myself	2	<input type="checkbox"/>
	I dislike myself	3	<input type="checkbox"/>

8. Self-Criticism	I don't criticise or blame myself more than usual	0	<input type="checkbox"/>
	I am more critical of myself than I used to be	1	<input type="checkbox"/>
	I criticise myself for all of my faults	2	<input type="checkbox"/>
	I blame myself for everything bad that happens	3	<input type="checkbox"/>
9. Suicidal Thoughts or Wishes	I don't have any thoughts of killing myself	0	<input type="checkbox"/>
	I have thoughts of killing myself, but I would not carry them out	1	<input type="checkbox"/>
	I would like to kill myself	2	<input type="checkbox"/>
	I would kill myself if I had the chance	3	<input type="checkbox"/>
10. Crying	I don't cry any more than I used to	0	<input type="checkbox"/>
	I cry more than I used to	1	<input type="checkbox"/>
	I cry over every little thing	2	<input type="checkbox"/>
	I feel like crying, but I can't	3	<input type="checkbox"/>
11. Agitation	I am no more restless or wound up than usual	0	<input type="checkbox"/>
	I feel more restless or wound up than usual	1	<input type="checkbox"/>
	I am so restless or agitated that it's hard to stay still	2	<input type="checkbox"/>
	I am so restless or agitated that I have to keep moving or doing something	3	<input type="checkbox"/>
12. Loss of Interest	I have not lost interest in other people or activities	0	<input type="checkbox"/>
	I am less interested in other people or things than before	1	<input type="checkbox"/>
	I have lost most of my interest in other people or things	2	<input type="checkbox"/>
	It's hard to get interested in anything	3	<input type="checkbox"/>
13. Indecisiveness	I make decisions about as well as ever	0	<input type="checkbox"/>
	I find it more difficult to make decisions than usual	1	<input type="checkbox"/>
	I have much greater difficulty in making decisions than I used to	2	<input type="checkbox"/>
	I have trouble making any decisions	3	<input type="checkbox"/>
14. Worthlessness	I do not feel I am worthless	0	<input type="checkbox"/>
	I don't consider myself as worthwhile or useful as I used to	1	<input type="checkbox"/>
	I feel more worthless as compared to other people	2	<input type="checkbox"/>
	I feel utterly worthless	3	<input type="checkbox"/>
15. Loss of Energy	I have as much energy as ever	0	<input type="checkbox"/>
	I have less energy than I used to have	1	<input type="checkbox"/>
	I don't have enough energy to do very much	2	<input type="checkbox"/>
	I don't have enough energy to do anything	3	<input type="checkbox"/>

16. Change in Sleeping Pattern	
I have not experienced any change in my sleeping pattern	0 <input type="checkbox"/>
I sleep somewhat more than usual	1a <input type="checkbox"/>
I sleep somewhat less than usual	1b <input type="checkbox"/>
I sleep a lot more than usual	2a <input type="checkbox"/>
I sleep a lot less than usual	2b <input type="checkbox"/>
I sleep most of the day	3a <input type="checkbox"/>
I wake up 1-2 hours early and I can't get back to sleep	3b <input type="checkbox"/>
17. Irritability	
I am no more irritable than usual	0 <input type="checkbox"/>
I am more irritable than usual	1 <input type="checkbox"/>
I am much more irritable than usual	2 <input type="checkbox"/>
I am irritable all the time	3 <input type="checkbox"/>
18. Change in Appetite	
I have not experienced any change in my appetite	0 <input type="checkbox"/>
My appetite is somewhat less than usual	1a <input type="checkbox"/>
My appetite is somewhat greater than usual	1b <input type="checkbox"/>
My appetite is much less than before	2a <input type="checkbox"/>
My appetite is greater than usual	2b <input type="checkbox"/>
I have no appetite at all	3a <input type="checkbox"/>
I crave food all the time	3b <input type="checkbox"/>
19. Concentration Difficulty	
I can concentrate as well as ever	0 <input type="checkbox"/>
I can't concentrate as well as usual	1 <input type="checkbox"/>
It's hard to keep my mind on anything for very long	2 <input type="checkbox"/>
I find I can't concentrate on anything	3 <input type="checkbox"/>
20. Tiredness or Fatigue	
I am no more tired or fatigued than usual	0 <input type="checkbox"/>
I get more tired or fatigued more easily than usual	1 <input type="checkbox"/>
I am too tired or fatigued to do a lot of things I used to do	2 <input type="checkbox"/>
I am too tired or fatigued to do most of the things I used to do	3 <input type="checkbox"/>
21. Loss of Interest in Sex	
I have not noticed any recent change in my interest in sex	0 <input type="checkbox"/>
I am less interested in sex than I used to be	1 <input type="checkbox"/>
I am much less interested in sex now	2 <input type="checkbox"/>
I have lost interest in sex completely	3 <input type="checkbox"/>

10.9 List of Threatening Events

EVENTS IN YOUR LIFE

In the next section 12 unpleasant events are listed. Please indicate if you have experienced these events in the past **12 months**.

1. You yourself suffered a serious illness, injury or an assault	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
2. A serious illness, injury or assault happened to a close relative	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
3. Your parent, child or spouse died	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
4. A close family friend or another relative (aunt, cousin, grandparent) died	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
5. You had a separation due to marital difficulties	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
6. You broke off a steady relationship	Yes <input type="checkbox"/>
	No <input type="checkbox"/>

7. You had a serious problem with a close friend, neighbour or relative	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
8. You became unemployed or you were seeking work unsuccessfully for more than 1 month	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
9. You were sacked from your job	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
10. You had a major financial crisis	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
11. You had problems with the police and a court appearance	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
12. Something you valued was lost or stolen	Yes <input type="checkbox"/>
	No <input type="checkbox"/>

1.1 Perceived Stress Scale

YOUR FEELINGS AND THOUGHTS

Please read each group of statements carefully, then pick out **one statement** in each group which best describes how often you felt or thought a certain way. Tick the statement you have picked.

1. In the last month, how often have you been upset because of something that happened unexpectedly?	never	0	<input type="checkbox"/>
	almost never	1	<input type="checkbox"/>
	sometimes	2	<input type="checkbox"/>
	fairly often	3	<input type="checkbox"/>
	very often	4	<input type="checkbox"/>
2. In the last month, how often have you felt that you were unable to control the important things in your life?	never	0	<input type="checkbox"/>
	almost never	1	<input type="checkbox"/>
	sometimes	2	<input type="checkbox"/>
	fairly often	3	<input type="checkbox"/>
	very often	4	<input type="checkbox"/>
3. In the last month, how often have you felt nervous and "stressed"?	never	0	<input type="checkbox"/>
	almost never	1	<input type="checkbox"/>
	sometimes	2	<input type="checkbox"/>
	fairly often	3	<input type="checkbox"/>
	very often	4	<input type="checkbox"/>
4. In the last month, how often have you felt confident about your ability to handle your personal problems?	never	0	<input type="checkbox"/>
	almost never	1	<input type="checkbox"/>
	sometimes	2	<input type="checkbox"/>
	fairly often	3	<input type="checkbox"/>
	very often	4	<input type="checkbox"/>
5. In the last month, how often have you felt that things were going your way?	never	0	<input type="checkbox"/>
	almost never	1	<input type="checkbox"/>
	sometimes	2	<input type="checkbox"/>
	fairly often	3	<input type="checkbox"/>
	very often	4	<input type="checkbox"/>

6. In the last month, how often have you found that you could not cope with all the things that you had to do?	never	0	<input type="checkbox"/>
	almost	1	<input type="checkbox"/>
	never	2	<input type="checkbox"/>
	sometimes	3	<input type="checkbox"/>
	fairly often	4	<input type="checkbox"/>
	very often		
7. In the last month, how often have you been able to control irritations in your life?	never	0	<input type="checkbox"/>
	almost never	1	<input type="checkbox"/>
	sometimes	2	<input type="checkbox"/>
	fairly often	3	<input type="checkbox"/>
	very often	4	<input type="checkbox"/>
8. In the last month, how often have you felt that you were on top of things?	never	0	<input type="checkbox"/>
	almost never	1	<input type="checkbox"/>
	sometimes	2	<input type="checkbox"/>
	fairly often	3	<input type="checkbox"/>
	very often	4	<input type="checkbox"/>
9. In the last month, how often have you been angered because of things that were outside of your control?	never	0	<input type="checkbox"/>
	almost never	1	<input type="checkbox"/>
	sometimes	2	<input type="checkbox"/>
	fairly often	3	<input type="checkbox"/>
	very often	4	<input type="checkbox"/>
10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	never	0	<input type="checkbox"/>
	almost never	1	<input type="checkbox"/>
	sometimes	2	<input type="checkbox"/>
	fairly often	3	<input type="checkbox"/>
	very often	4	<input type="checkbox"/>

1.2 Childhood Experience of Care and Abuse Questionnaire

YOUR CHILDHOOD EXPERIENCE

The following section is about how you remember your mother and father in your first 17 years.

Please read each statement carefully. Each statement is rated on a 5-point scale from 'yes definitely' to 'no, not at all'. Please pick out the number which best describes how you felt and circle it.

As you remember your mother figure in your first 17 years:

	Yes definitely		Unsure	No, not at all	
1. She was very difficult to please....	5	4	3	2	1
2. She was concerned about my worries....	5	4	3	2	1
3. She was interested in how I did at school....	5	4	3	2	1
4. She made me feel unwanted....	5	4	3	2	1
5. She tried to make me feel better when I was upset....	5	4	3	2	1

	Yes definitely		Unsure	No, not at all	
6. She was very critical of me....	5	4	3	2	1
7. She would leave me unsupervised before I was 10 years old....	5	4	3	2	1
8. She would usually have time to talk to me....	5	4	3	2	1
9. At times she made me feel like I was a nuisance....	5	4	3	2	1
10. She often picked on me unfairly....	5	4	3	2	1
11. She was there if I needed her....	5	4	3	2	1
12. She was interested in who my friends were....	5	4	3	2	1
13. She was concerned about my whereabouts....	5	4	3	2	1
14. She cared for me when I was ill....	5	4	3	2	1

	Yes definitely		Unsure	No, not at all	
15. She neglected my basic needs (e.g. food and clothes)....	5	4	3	2	1
16. She did not like me as much as my brothers and sisters.... (leave blank if no siblings)	5	4	3	2	1

As you remember your father figure in your first 17 years:

	Yes definitely		Unsure	No, not at all	
1. He was very difficult to please....	5	4	3	2	1
2. He was concerned about my worries....	5	4	3	2	1
3. He was interested in how I did at school....	5	4	3	2	1
4. He made me feel unwanted....	5	4	3	2	1
5. He tried to make me feel better when I was upset....	5	4	3	2	1

	Yes definitely		Unsure	No, not at all	
6. He was very critical of me....	5	4	3	2	1
7. He would leave me unsupervised before I was 10 years old....	5	4	3	2	1
8. He would usually have time to talk to me....	5	4	3	2	1
9. At times he made me feel like I was a nuisance....	5	4	3	2	1
10. He often picked on me unfairly....	5	4	3	2	1
11. He was there if I needed him....	5	4	3	2	1
12. He was interested in who my friends were....	5	4	3	2	1
13. He was concerned about my whereabouts....	5	4	3	2	1

	Yes definitely		Unsure	No, not at all	
14. He cared for me when I was ill....	5	4	3	2	1
15. He neglected my basic needs (e.g. food and clothes)....	5	4	3	2	1
16. He did not like me as much as my brothers and sisters.... (leave blank if no siblings)	5	4	3	2	1

The following section is about physical punishment by a parent figure or other household member.

17a. When you were a child or a teenager were you ever hit repeatedly with an implement (such as belt or stick), or punched, kicked or burnt by someone in the household?	Yes	<input type="checkbox"/>
	No	<input type="checkbox"/>

If you answered 'yes' to question 17a, please continue to question 17b. If you answered 'no' to question 17a please continue to question 18.

	Mother figure	Father figure
17b. How old were you when it began?	Age.....	Age.....
17c. Did the hitting happen on more than one occasion?	Yes <input type="checkbox"/> No <input type="checkbox"/>	Yes <input type="checkbox"/> No <input type="checkbox"/>
17d. How were you hit? Please circle as appropriate	Belt or stick Punched/kicked Hit with hand Other	Belt or stick Punched/kicked Hit with hand Other
17e. Were you ever injured e.g. bruises, black eyes, broken limbs?	Yes <input type="checkbox"/> No <input type="checkbox"/>	Yes <input type="checkbox"/> No <input type="checkbox"/>
17f. Was this person so angry they seemed out of control?	Yes <input type="checkbox"/> No <input type="checkbox"/>	Yes <input type="checkbox"/> No <input type="checkbox"/>

The following section is about unwanted sexual experiences before age 17.

18. When you were a child or teenager did you ever have any unwanted sexual experiences?	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
	Unsure <input type="checkbox"/>
19. Did anyone force you or persuade you to have sexual intercourse against your wishes before age 17?	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
	Unsure <input type="checkbox"/>
20. Can you think of any upsetting sexual experiences before age 17 with a related adult or someone in authority (e.g. teacher)?	Yes <input type="checkbox"/>
	No <input type="checkbox"/>
	Unsure <input type="checkbox"/>

If you answered 'yes' or 'unsure' to any of the three questions above, please continue to question 63. If you answered 'no' to all the three questions above, you have finished the questionnaire.

	First experience	Other experience
21a. How old were you when it began?		
	Age.....	Age.....
21b. Was the other person someone you knew?	Yes <input type="checkbox"/>	Yes <input type="checkbox"/>
	No <input type="checkbox"/>	No <input type="checkbox"/>

	First experience	Other experience
21c. Was the other person a relative?	Yes <input type="checkbox"/>	Yes <input type="checkbox"/>
	No <input type="checkbox"/>	No <input type="checkbox"/>
21d. Did the other person live in your household?	Yes <input type="checkbox"/>	Yes <input type="checkbox"/>
	No <input type="checkbox"/>	No <input type="checkbox"/>

21e. Did this person do it to you on more than one occasion?	Yes	<input type="checkbox"/>	Yes	<input type="checkbox"/>
	No	<input type="checkbox"/>	No	<input type="checkbox"/>
21f. Did it involve touching private parts of your body?	Yes	<input type="checkbox"/>	Yes	<input type="checkbox"/>
	No	<input type="checkbox"/>	No	<input type="checkbox"/>
21g. Did it involve touching private parts of the other person's body?	Yes	<input type="checkbox"/>	Yes	<input type="checkbox"/>
	No	<input type="checkbox"/>	No	<input type="checkbox"/>
21h. Did it involve sexual intercourse?	Yes	<input type="checkbox"/>	Yes	<input type="checkbox"/>
	No	<input type="checkbox"/>	No	<input type="checkbox"/>

THANK YOU FOR YOUR TIME

1.3 Center for Epidemiologic Studies Depression Scale

Study participants were asked about the occurrence (yes/no) of eight depressive symptoms:

“Now think about the past week and the feelings you have experienced. Please tell me if each of the following was true for you much of the time during the past week”:

- a) Much of the time during the past week, you felt depressed?
1 Yes
2 No

- b) Much of the time during the past week, you felt that everything you did was an effort?
1 Yes
2 No

- c) Much of the time during the past week), your sleep was restless?
1 Yes
2 No

- d) Much of the time during the past week, you were happy?
1 Yes
2 No

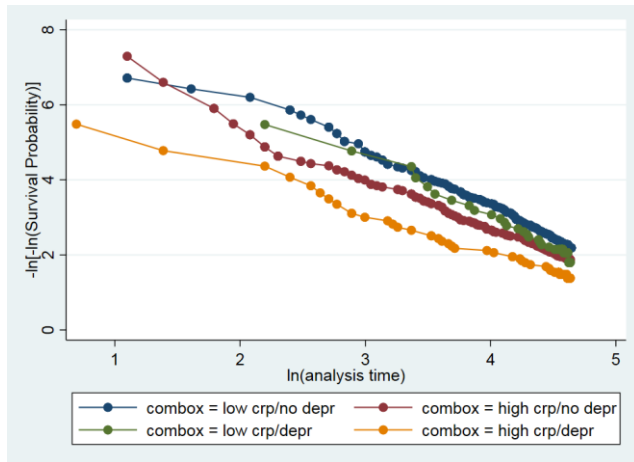
- e) Much of the time during the past week, you felt lonely?
1 Yes
2 No

- f) Much of the time during the past week, you enjoyed life?
1 Yes
2 No

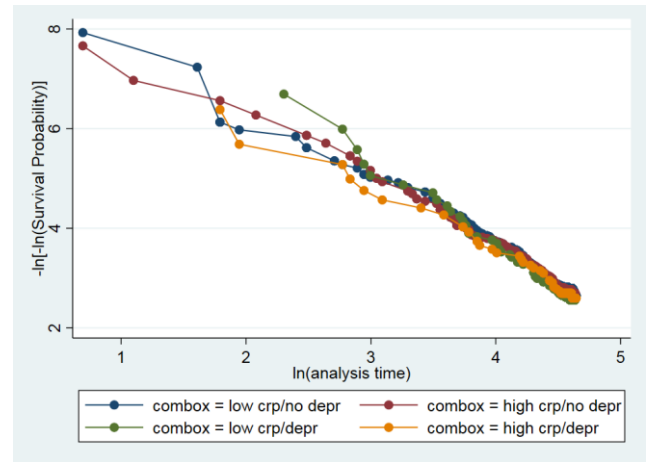
- g) Much of the time during the past week, you felt sad?
1 Yes
2 No

1.4 Kaplan-Meier Survival curves

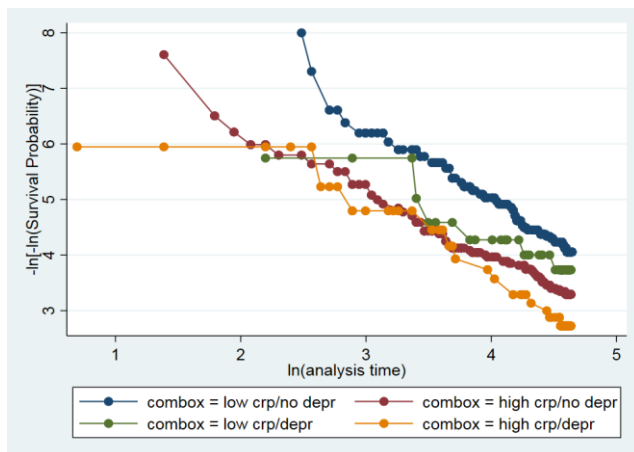
(A)



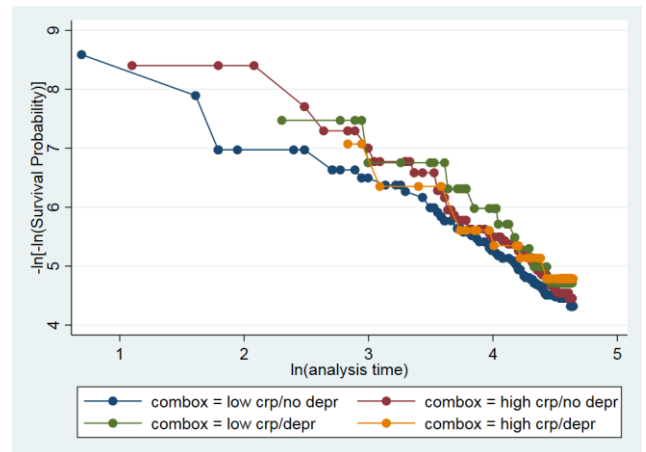
(B)



(C)



(D)



Curves provided for all-cause and CVD mortality, stratified by sex.

Abbreviations: CRP = C-reactive protein; depr = depressive symptoms; CVD = cardiovascular disease. (A) = all-cause mortality in men; (B) = all-cause mortality in women; (C) = CVD mortality in men; (D) = CVD mortality in women.