

NEUROINFLAMMATION IN ALCOHOL DEPENDENCE

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Submitted for the degree of Doctor of Philosophy

September 2014

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ABSTRACT

Alcohol dependence damages the brain through a multiplicity of factors including thiamine deficiency, liver disease, head injuries, and repeated episodes of alcohol withdrawal. Alcohol withdrawal is a potential opportunity for reducing damage as it is an intensive time of contact between doctors and patients. Pre-clinical models of alcohol dependence have demonstrated activation of microglia, resident tissue macrophages, and expression of cytokines and other inflammatory mediators both in the brain and peripheral blood during alcohol withdrawal. These changes were associated with neuronal death, and learning deficits. Similar processes may occur in man as increased microglial numbers, increased chemokine expression and raised circulating pro-inflammatory cytokines have been reported in alcohol dependence.

The aim of the thesis was to characterise the peripheral cytokine profile during alcohol detoxification, to investigate whether there are relationships between peripheral cytokines and clinical features of alcohol withdrawal, to investigate neuroinflammation in alcohol dependence by using [¹¹C]PBR28 Positron Emission Tomography to assess microglial activation in recently abstinent alcohol dependent patients in vivo and to investigate how these processes relate to elevated cortisol and elevated cerebral glutamate reported in alcohol withdrawal respectively.

The longitudinal study undertaken in 51 alcohol dependent patients during detoxification demonstrated that both pro- and anti-inflammatory cytokines, and chemokines, decreased significantly during detoxification while T cell cytokines increase. IL-6 was positively associated with withdrawal severity and depressive symptoms during withdrawal. The chemokine CCL-2 was positively associated with performance on cognitive tasks. Serum cortisol was in the high normal range and decreased during detoxification. The salivary Cortisol Awakening Response (CAR) was also in the normal range at

all time points. Both serum cortisol and the CAR were positively correlated with IL-6 concentrations suggesting hyperfunction of the HPA axis during alcohol detoxification may relate in part to inflammatory stimulation.

The PET study comparing alcohol dependent men in early abstinence and healthy controls was undertaken using the ligand, [¹¹C]PBR28 that binds to the Translocator Protein 18 kDa (TSPO) richly expressed in microglia. Alcohol dependent patients had lower TSPO binding in the hippocampi than healthy controls. TSPO binding in the hippocampus was also positively correlated with performance on tests of verbal memory. This suggests that hippocampal microglial loss or dysfunction may be related to memory problems in alcohol dependence. Given that benzodiazepines are used to treat alcohol withdrawal, an *in vitro* binding study was conducted to investigate whether benzodiazepines would significantly block [¹¹C]PBR28 binding and found that benzodiazepines do not block a significant proportion of TSPO at all but the highest clinical doses. The relationship between brain glutamate, as measured with Magnetic Resonance Spectroscopy, and microglial activation was investigated. Alcohol dependent patients had significantly lower glutamate + glutamine (Glx) concentrations in the occipital cortex with no difference in glutamate concentrations in the anterior cingulate cortex (ACC).

In summary, there are changes in both peripheral and brain inflammatory processes in early abstinence from alcohol dependence that are related to clinical symptoms. Peripheral pro-inflammatory cytokines are raised early in detoxification relative to late detoxification and are related to withdrawal and affective symptoms. Surprisingly, evidence of decreased microglial function in the hippocampus was found and this related to poorer cognitive function, suggesting a positive role for immune cells in the brain in alcohol dependence. Inflammatory processes were related to HPA axis function during detoxification but not to changes in brain glutamate concentrations. In conclusion, characterisation of inflammation through multiple approaches in

this series of studies demonstrates the likely importance of such processes and provides novel approaches for treatment to reduce brain damage due to alcoholism.

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Declaration of Originality

This thesis represents my own ideas, study design and analysis. In the course of my research, I have relied on others to perform analysis where this has necessarily taken place remotely, or has required knowledge and skills beyond my competency. Where this has been the case, I have indicated so in the text. The published works of others that have contributed to my ideas are indicated via citations.

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ACKNOWLEDGEMENTS

I would like to thank the following people, without whom this thesis would not have been completed:

Professor Anne Lingford-Hughes for her support and mentoring over the past seven years during my time as an Academic Clinical Fellow in University of Bristol and subsequently as a Clinical Research Fellow at Imperial College London, for her help in shaping the ideas that underpin the thesis, for her willingness to allow me to run with my ideas and tangents and for her valuable feedback on my hypotheses, conclusions and writing style;

Dr Ilan Rabiner, for his support and advice throughout the PhD, but especially during the planning and execution of the scanning study, his Socratic questioning which enabled me to clarify my ideas, his unflappability in the face of mishaps and his incisive comments on papers and chapters;

Professor Martin Wilkins and Professor Paul Matthews, for offering me the opportunity provided by the Fellowship and for their mentorship throughout;

The Wellcome Trust and GlaxoSmithKline for providing me with financial support and generous research resources during the PhD;

Qi Guo who performed the pre-processing, and parametric analysis of the PET data;

Ashley Gilmour, who performed the Luminex analysis of the cytokine data;

Les Huson, who provided invaluable advice regarding the statistical approach in Chapter 2;

Christine Parker who supervised the binding study and David Owen who taught me to bind;

Mike Newson who helped me with, and taught me, salivary cortisol ELISA;

Teams at the Hammersmith Hospital, Ealing Hospital and Charing Cross who analysed the TSPO genotype and clinical blood tests

The clinicians and NIHR recruiting staff who trusted me with their patients and spent time recruiting in busy clinics: Dr Karim Dar, Dr Jeff Fehler, Dr Raquin Cherian, Brenda Clarke, Hamjit McGeever and Antoinette McNulty;

The brave volunteers for taking part in studies that required considerable commitment of time and some discomfort;

The donors and scientists from the Multiple Sclerosis Brain Bank, who provided the samples for the binding study;

The Neuropsychopharmacology Unit, for their scientific input, and moral and practical support, especially Darren, Robin, Louise, David and Liese;

Margaret Collett, who ordered everything and made sure all the volunteers were paid;

The team at Imanova for their flexibility, good humour and technical expertise in running the scanning days;

The staff at the Wellcome Trust Clinical Research Facility, who booked and supported my screening appointments;

My family and friends, for tolerating four years of neglect;

And Nicholas for proof reading and cheerleading, keeping the domestic ship afloat, and everything else.

GLOSSARY

ACC	Anterior cingulate cortex
ACTH	Adreno-corticotropic hormone
ADPs	Alcohol-dependent patients
ALT	Alanine transaminase
ARBD	Alcohol-related brain damage
ARSAC	Administration of Radioactive Substances Advisory Panel
AUQ	Alcohol Urge Questionnaire
BBB	Blood brain barrier
BDI	Beck Depression Inventory
CAR	Cortisol Awakening Response
CBG	Cortisol binding globulin
CCL	Chemokine with adjacent cysteine residues near amino terminal
CCL-2	Monocyte Chemoattractant Protein 1
CCL-3	Macrophage Inflammatory Protein 1-Alpha (MIP-1 α)
CCL-4	Macrophage Inflammatory Protein 1-Beta (MIP-1 β)
CCL-5	Regulated On Activation, Normal T cell Secreted (RANTES)
CCL-11	Eosinophil chemotactic protein (Eotaxin)
[Cho]	Choline concentration
CIWA-Ar	Clinical Institute Assessment of Withdrawal from Alcohol –revised
CNS	Central nervous system
[Cr]	Creatine concentration
CRP	C-reactive protein
CRH	Corticotrophin
CSF	Cerebrospinal fluid
CXCL	Chemokine in which cysteine residues are separated by a single amino acid
CXCL-8	Interleukin 8
CXCL-9	Monokine induced by gamma interferon
CXCL-10	Interferon gamma-induced Protein 10
DLPFC	Dorsolateral prefrontal cortex
DSM-IV	Diagnostic and Statistical Manual, Fourth Edition
ELISA	Enzyme-Linked Immunosorbent Assay
FAS	Phonemic verbal fluency test, using the letters 'F', 'A' and 'S'
FSS	Fatigue Severity Scale
GGT	Gamma-glutamyl transferase
GFP	Green fluorescent protein
[Glu]	Glutamate concentration
[Gln]	Glutamine concentration
[Glx]	Glutamate + glutamine concentration
GM-CSF	Granulocyte macrophage colony stimulating factor
HABs	High affinity binders
HCS	Healthy controls
HDs	Heavy drinkers
HPA axis	Hypothalamo-pituitary-adrenal axis
HPLC	High performance liquid chromatography
ICD-10	International Classification of Diseases, 10 th Edition
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IL-1β	Interleukin 1 Beta

IL-1RA	Interleukin 1 receptor antagonist
IL-2	Interleukin 2
IL-2R	Interleukin 2 receptor
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17	Interleukin 17
LABs	Low affinity binders
LOD	Limit of detection
LPS	Lipopolysaccharide
MABs	Mixed affinity binders
MAR	Missing at random
MCAR	Missing completely at random
MCI	Mild cognitive impairment
MCV	Mean corpuscular volume
MEGAPRESS	Mescher-Garwood point resolved spectroscopy
[mI]	Myoinositol concentration
MINI-6	Mini-International Neuropsychiatric Interview, Version 6
MLE	Maximum likelihood estimation
MMSE	Mini-mental state examination
MPRAGE	Magnetisation Prepared Rapid Gradient
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple Sclerosis
[NAA]	N-acetyl aspartate concentrations
NHS	National Health Service
OCC	Occipital cortex
OCDS	Obsessive Compulsive Drinking Scale
PBR	Peripheral benzodiazepine receptor
PET	Positron emission tomography
POC	Parieto-occipital cortex
PRESS	Point resolved spectroscopy
PVE	Partial volume effect
ROCF	Rey-Osterrieth Complex Figure
ROI	Region of interest
SADQ	Severity of Alcohol Dependence Questionnaire
SSAI	Spielberger State Anxiety Inventory
STAI	Spielberger Trait Anxiety Inventory
TAC	Time activity curve
TE	Echo time
TNF-α	Tumour Necrosis Factor alpha
TR	Repetition time
TSPO	Translocator Protein 18 kDa
WMS	Weschler Memory Scale – paragraph version

CHAPTER 1

INTRODUCTION

1.1 Overview

The aim of my thesis is to characterise peripheral inflammation and neuroinflammation during alcohol withdrawal and early abstinence, and to understand how these responses relate to clinical measures and objective neurochemical and neuroendocrine derangements during this period. My hypothesis in broad terms is that both peripheral inflammation and neuroinflammation occur in man during alcohol withdrawal and early abstinence and that these are related to both withdrawal severity and cognitive impairment during this period. I also hypothesise that these inflammatory processes correspond to hypothalamo-pituitary-adrenal axis derangements and increased brain glutamate during withdrawal.

This is motivated by pre-clinical evidence that suggests a cerebral immune response – so-called ‘neuroinflammation’ – occurs in the setting of chronic alcohol intake and withdrawal, and that this may relate to brain damage. Clinical observations in alcohol-dependent patients regarding systemic cytokines, which are known to reach the brain and influence cerebral immunity, provides further support for the idea that both peripheral inflammation and neuroinflammation may contribute to brain damage occurring during withdrawal and early abstinence. This has considerable clinical implications.

In recently abstinent alcohol-dependent patients, I have (1) characterised peripheral inflammation by measuring circulating cytokine activity and (2) neuroinflammation, captured through microglial activation with [¹¹C]PBR28 (Translocator Protein 18kDa (TSPO)) Positron Emission Tomography (PET). I have explored the association between (3) cytokines and hypothalamo-pituitary-adrenal axis dysfunction by measuring the cortisol awakening response and serum cortisol. I also explored the relationship between (4) [¹¹C]PBR28 and brain glutamate as measured by Magnetic Resonance Spectroscopy (MRS).

The remainder of this introduction sets out the background to each of these approaches and the preclinical and clinical observations that support the hypothesis that inflammatory processes are important in alcohol withdrawal and early abstinence. Key concepts in peripheral inflammation such as the function of circulating cytokines and their influence on brain function will be explored. Neuroinflammation will be defined and critiqued and microglial activation and its role as a marker of neuroinflammation in clinical research explained. Abnormalities in hypothalamo-pituitary-adrenal axis function and in brain glutamate transmission during withdrawal and early abstinence, means of investigating these, and the ways in which inflammatory processes may be hypothesised to relate to them will be described. Finally, the aims and hypotheses will be laid out in detail.

1.1.1 Alcohol-related brain damage is an increasing problem

Alcohol damages the brain. Neurological syndromes representing extreme forms of alcohol-related neurodegeneration are well-recognised, occurring in the context of co-morbid nutritional deficiencies, electrolyte shifts, or other end-organ damage: Wernicke-Korsakoff syndrome (WKS), Marchiafava-Bignami disease, central pontine myelinolysis, cerebellar degeneration, and hepatic encephalopathy. Alcohol-related brain damage (ARBD) is a diffuse presentation of cognitive impairment in multiple domains that has been recognised more recently. Although synonymous with alcohol-associated dementia (DSM-IV) ARBD is the preferred term (Jauhar and Smith, 2009) because the disorder is not progressive with abstinence. Rather, in the context of abstinence, 25% remain severely affected, 25% improve minimally, 25% improve moderately and 25% recover completely (Royal College of Psychiatrists, 2014). In this respect, ARBD is more similar to traumatic brain injury than it is to other forms of dementia (Thomson et al., 2012).

1.1.2 ARBD involves multiple domains of function but is non-progressive

ARBD is defined as impairment in multiple cognitive domains occurring within three years of prolonged heavy drinking (Oslin et al., 1998). The diagnosis is supported by improvement with abstinence, ataxia, other alcohol-related end organ damage and cerebellar atrophy on Magnetic Resonance Imaging and is cast in doubt by anomia, or clinical or neuroimaging evidence of cerebrovascular disease (Oslin et al., 1998). ARBD predominantly affects people between the ages of 40 and 60 years old (Royal College of Psychiatrists, 2014). A high proportion of patients are likely to go

unrecognised because of a lack of knowledge of the condition and the stigma against patients with addictions (Wilson, 2011). Unlike patients with other dementia syndromes, patients with ARBD are more likely to show agitation, disinhibition, delusional experiences and aggression (Ferran, 1996). This presents challenges for patient management that are exacerbated by the paucity of expertise in the area in mainstream adult or older adult psychiatry services (Wilson, 2011) (Royal College of Psychiatrists, 2014) (Thomson et al., 2012). Alcohol is also a factor in the development of other forms of dementia: heavy drinking is associated with a 4.6 times increased risk of developing dementia (Saunders et al., 1991).

1.1.3 There are cognitive deficits in alcohol dependence even in those without frank ARBD

ARBD represents a severe degree of a continuum of cognitive impairment. Even apparently healthy alcohol-dependent patients (ADPs) exhibit impairment across multiple domains of cognitive function (Davies et al., 2005)(reviewed in (Stavro et al., 2013)). Deficits in episodic memory (Fama et al., 2004) (Davies et al., 2005) (Stavro et al., 2013), spatial and verbal memory and executive function have been described consistently (Stavro et al., 2013). Episodic memory deficits have been reported to exceed those explained by poor executive function alone (Pitel et al., 2007). Longitudinal studies (Bartels et al., 2007), and cross-sectional studies of patients who are more than one year abstinent (reviewed in (Stavro et al., 2013) suggest that recovery may occur but that deficits persist for some months of abstinence. Recovery of cognitive function is dependent on continued abstinence (Pitel et

al., 2009) (Johnson-Greene et al., 1997). However, long-term remission from alcohol dependence occurs in less than 50% of patients (Drugs, 2013) and relapse occurs early in abstinence for a similar proportion of patients (Hayashida et al., 1989).

1.1.4 Subclinical cognitive impairment may impact motivation and relapse

There is no consensus in the literature regarding the relationship between cognitive impairment and prognosis in healthy ADPs. Although cognitive deficits may predispose to the development of addiction, evidence from a longitudinal study in adolescence, supports the worsening of cognition with continued substance use (Tapert and Brown, 1999). Early reports suggested that poor cognitive function had an adverse effect on prognosis: patients with poorer performance on cognitive tasks were more likely to leave residential rehabilitation and had more problems in general functioning (Bowden et al., 2001). A regression analysis of a cohort with severe dependence found an association between poor cognitive test performance and relapse at 6 months (Allsop et al., 2000). Later reports, however, have failed to show significant relationships between verbal and spatial memory and relapse (Bartels et al., 2007) (Pitel et al., 2009). Associations between aspects of executive function and relapse are found in some studies (Blume et al., 2005) (Bartels et al., 2007) but not others (Pitel et al., 2009). The link between cognitive function and outcome may be mediated by the impact of poor cognition on subjective factors such as motivation to change (Le Berre et al., 2012), self-efficacy (Bates et al., 2006) and compulsion to use (Rupp et al., 2012), and behavioural measures such as treatment compliance.

1.2 Pathogenesis of alcohol-related brain damage is complex

1.2.1 Thiamine deficiency has been emphasised

The pathogenesis of alcohol-related brain damage is multi-factorial, including direct effects of alcohol, alcohol withdrawal, nutritional deficiencies, head injury, hepatic encephalopathy, and electrolyte shifts (reviewed in (Zahr et al., 2011)). Clinically, thiamine deficiency has been emphasised, as Wernicke-Korsakoff syndrome is a well-recognised consequence of alcohol dependence (Pilling et al., 2011) (Royal College of Psychiatrists, 2014). There is also evidence that both cognitive deficits and regional brain volume deficits seen in ADPs with clinical Wernicke-Korsakoff syndrome occur to a lesser extent in those without clinical Wernicke-Korsakoff syndrome (Pitel et al., 2011)(reviewed in (Zahr et al., 2011)). National guidelines recommend using parenteral thiamine on admission for detoxification, to reverse thiamine deficiency (Pilling et al., 2011). Despite this, alcohol-related brain damage continues to be a problem (Wilson, 2011). Animal studies where the relative contributions of poor nutrition and alcohol dose can be controlled suggest that alcohol itself is neurotoxic (Zahr et al., 2009) and alcohol promotes thiamine deficiency-associated neurotoxicity (Ke et al., 2009).

1.2.2 Episodes of detoxification contribute to alcohol-related brain damage

This thesis will focus on alcohol withdrawal and early abstinence, as episodes of detoxification are also relevant to the pathogenesis of alcohol-related brain damage. They are of special interest to the clinical researcher, as they coincide with clinical contact, offering an opportunity for preventative

intervention. Alcohol withdrawal is the product of adaptive changes in receptor expression in response to chronic alcohol intake. These include down-regulation and changes in receptor subtype expression in γ -aminobutyric acid A (GABA-A) receptors such that the brain is less sensitive to GABA-ergic inhibition, and upregulation and changes in receptor subtype expression in glutamatergic N-methyl-D-aspartate receptors such that the brain is more sensitive to excitatory glutamatergic transmission. (reviewed in (Clapp et al., 2008)).

Repeated episodes of detoxification are associated both with increased severity of withdrawal symptoms and an increased risk of seizures (reviewed in (Duka et al., 2004)), and an equivalent process is described in animal models (Stephens, 2001). The phenomenon is analogous to 'kindling' in the generation of epilepsy from repeated electric stimulation. Pre-clinical studies of alcohol withdrawal have proposed a possible mechanism, as repeated episodes of withdrawal are associated with progressive increases in extracellular glutamate and increased neuronal death. Animal behavioural studies have described deficits in forms of learning such as aversive conditioning following multiple episodes of withdrawal (Stephens, 2001). Similarly, a relationship has also been demonstrated between multiple detoxifications and progressive impairment in frontal lobe test performance, though this is mediated partly by other factors e.g. length of drinking history (Duka, 2003). There is also impairment in emotional processing, particularly a sensitivity to fear, in clinical populations (Townshend, 2003).

1.3 Neuroinflammation may contribute to alcohol-related brain damage

1.3.1 Inflammation in the central nervous system is tightly regulated

The role of the immune system in the central nervous system is of increasing interest in the pathogenesis of both acute brain damage from stroke or blunt trauma, and chronic neurodegenerative conditions. Previously, the brain was thought to be 'immune privileged', or lacking a specific immune response (Galea, 2007). Changes in the morphology of astrocytes, the support cells of the central nervous system (CNS), and in microglia, the resident CNS macrophages, around sites of tissue injury, were seen as a passive 'bystander effect'. It is now recognised, however, that the CNS immune response is highly regulated rather than absent. Adjustments include down-regulation of microglia by neurons, an antibody-predominant response, and inhibition of T cell invasion by CNS expression of molecules that trigger T cell death (Galea, 2007). Therefore the predominant immune response – so-called 'neuroinflammation' (O'Callaghan et al., 2008) – involves astrocytes and microglia, and local expression of inflammatory mediators such as cytokines and prostaglandins, while invasion of peripheral immune cells is absent or late.

The immune response is associated with changes in the appearance of microglia and astrocytes, a process called 'activation'. In the case of microglia, there is a progressive change from the highly ramified morphology, where ramifications are retracted, and in full activation an amoeboid shape is

taken and migration and proliferation may occur. Activation can be detected via histology, although immunohistochemistry has revealed that morphologically activated microglia are associated with different functions (Perry et al., 2010). It is the 'M1' microglial activation – the type of activation induced *in vitro* by interferon- γ , or *in vivo* by neuronal damage – that is associated with production of pro-inflammatory cytokines, prostaglandins and reactive oxygen species. Levesque, among others, have proposed that this leads to a vicious circle where microglial products cause further neuronal damage, which in turn further activates microglia (Levesque et al., 2010).

1.3.2 Pre-clinical models of alcohol dependence demonstrate neuroinflammation

Pre-clinical models of alcohol dependence, especially those with repeated cycles of withdrawal, show widespread microglial activation. Activation has been demonstrated to peak within two days of alcohol withdrawal but persist for up to three weeks (Obernier et al., 2002). Cerebral expression of pro-inflammatory cytokines and chemokines such as IL-1 β , TNF- α and CCL-2 persist for ten days following alcohol withdrawal, outlasting detectable expression in the liver and blood (Qin et al., 2008). Other inflammatory mediators such as iNOS are reported and are associated with neuronal death. Most importantly from a translational perspective, both inflammatory mediators and cell death can be suppressed by pre-treatment with simple non-steroidal anti-inflammatory drugs (Pascual et al., 2007)

1.3.3 Alcohol contributes to neuroinflammation via direct and indirect mechanisms

There are several mechanisms – direct and indirect – whereby alcohol dependence is thought to induce neuroinflammation. *In vitro* studies from a single laboratory have demonstrated astroglial and microglial activation in response to alcohol via toll-like receptor 4 (TLR4), a receptor that responds to bacterial proteins and damage-associated proteins. Knock-out of the TLR4 gene has also been reported to diminish both alcohol-related microglial activation and neuronal death (Alfonso-Loeches et al., 2010). Subsequent to the discovery of TLR4 stimulation by alcohol, induction of down-stream intracellular inflammatory signaling proteins and transcription factors such as HMBG1 and NFkB by alcohol have been described (Crews et al., 2013).

Alcohol withdrawal may also precipitate neuroinflammation. As outlined above, microglial activation has been observed to peak on day 2 of withdrawal, the timing making it unlikely direct TLR4 stimulation is responsible (Nixon et al., 2008). Elevated extra-cellular glutamate measured by microdialysis has been recorded in brain areas such as the hippocampus where microglial activation during withdrawal is also prominent (Ward et al., 2009), raising the possibility that elevated glutamate may precipitate microglial activation. Microglia have also been shown to be sensitive to glutamate elevations in other contexts (Vinet et al., 2012) (Palazuelos et al., 2009) (Lambertsen et al., 2009).

Systemic inflammatory effects also contribute to neuroinflammation in alcohol dependence. Small bowel bacterial overgrowth has been described in alcohol dependence with increased bacterial translocation, with increased portal and circulating concentrations of lipopolysaccharide and peptidoglycan. Although it had been thought that bacterial translocation stopped very soon after cessation of alcohol, recent evidence (Leclercq et al., 2014) suggests that circulating peptidoglycan concentrations remain increased throughout withdrawal, and into early abstinence. These bacterial proteins are important because they stimulate Kupffer cells (hepatic macrophages) to produce pro-inflammatory cytokines and chemokines, with systemic and cerebral consequences. Thus the study described in Chapter 2 concerns systemic cytokines and their relationship to clinical symptoms and cognitive function during alcohol detoxification.

1.4 Cytokines and alcohol dependence

1.4.1 Cytokines are chemical messengers of the immune system

Cytokines are soluble regulatory peptides (<30kDa) (Dinarello, 2007) that coordinate the innate and adaptive immune response. They are similar to hormones, in that they may be produced by one cell to act on another via cell surface receptors, sometimes at a distance. They differ, however, in that most cells of the body can produce cytokines, that they are expressed in response to specific stimuli, rather than in regular circadian cycles, and that they have a physiological effect at much smaller – picomolar – concentrations (Dinarello, 2007).

Cytokines are classified according to action (see Table 1.1). Innate immune cytokines are involved with the initial, non-specific response to infection. Historically, they have been divided into pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines such as IL-1, TNF- α and IL-6 augment the immune response by inducing expression of acute phase proteins such as C reactive protein (CRP), producing fever and activating T and B lymphocytes (Kronfol and Remick, 2000). Anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist, IL-1RA, a soluble IL-1 receptor, suppress inflammation. Cytokines associated with adaptive, specific immunity, relate to lymphocyte function. CD4⁺ or T helper (Th) lymphocyte polarisation leading to a pathogen-specific immune response is stimulated by specific cytokines with IL-12 inducing Th1 polarisation and IL-4, Th2 polarisation. Polarisation in turn is associated with specific cytokines with Th1 cells IL-2, TNF- α and IFN- γ and Th2 cells producing IL-4, IL-6, and IL-13. Soluble IL-2 receptors (IL-2R) are a general marker of the adaptive response and concentrations are correlated with the degree of T cell activation (Maes, 2011). Finally, chemokines are small cytokines that induce the chemotaxis of white blood cells, arrest their movement and co-ordinate their extravasation from blood to inflamed tissue (Ransohoff, 2009).

1.4.2 Cytokines access the central nervous system and affect brain function

Circulating pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 can affect the brain as they are able to penetrate it directly via vagal afferents (Dantzer et al., 2008) and several humoral routes: specific blood brain barrier transporters (Banks et al., 1989) (Banks et al., 1994); diffusion in BBB-deficient areas

(Capuron and Miller, 2011) and induction of endothelial prostaglandin expression stimulating activation of perivascular macrophages and parenchymal microglia to produce cytokines. Cerebral pro-inflammatory cytokines stimulate neurons in the hypothalamus and limbic regions, giving rise to fever, HPA axis activation and the constellation of behaviours known as 'sickness behaviour': decreased motor activity, social interaction, and response to reward (Dantzer and Kelley, 2007). Conversely, anti-inflammatory cytokines inhibit this response (Bluthe et al., 1999).

Table 1.1: Classification of cytokines

Cytokine class	
Cytokines associated with innate immunity	
Pro-inflammatory cytokines	IL-1 β , TNF- α , IL-6, GM-CSF
Anti-inflammatory cytokines	IL-10, IL-1-RA
Type 1 interferon	IFN- α
Cytokines related to T cell activation	
Non-specific T cell cytokines	IL-2, IL-7, IL-15, IL-2R
Th-1 associated cytokines	IL-12, IFN- γ
Th-2 associated cytokines	IL-4, IL-5, IL-13
Th-17 associated cytokines	IL-17
Chemokines	
Chemokines with a CXC motif*	CXCL-8 (IL-8), CXCL-9 (MIG), CXCL-10 (IP-10)
Chemokines with a CC motif	CCL-2 (MCP-1), CCL-3 (MIP-1 α), CCL-4 (MIP-1 β), CCL-5 (RANTES), CCL-11 (Eotaxin)

Legend: Chemokines are additionally classified according to molecular structure. In CXC chemokines, the two amino-terminal cysteine residues are separated by a single amino acid, whereas in CC chemokines, these are adjacent.

It is not known how or whether chemokines access the central nervous system, but strong positive correlations have been described between serum

CCL-2 and cerebrospinal fluid CCL-2 (Westin et al., 2012). In clinical populations, increased peripheral pro-inflammatory cytokines and chemokines are associated with neuropsychiatric disorders such as delirium (Rudolph et al., 2008) (Terrando et al., 2010) (Cibelli et al., 2010), dementia (Holmes et al., 2009) (Zuliani et al., 2007) and depression (Dowlati et al., 2010) (Liu et al., 2012). Increased pro-inflammatory cytokines are associated with poorer prognosis in dementia (Holmes et al., 2009) and treatment response in depression (Yoshimura et al., 2009).

1.4.3 There are increased circulating cytokine levels in alcohol-dependent patients

Increased circulating cytokine and chemokine concentrations in association with heavy alcohol use, dependence and withdrawal have been described by several studies (see Table 1.2). Epidemiological studies show that there is a J-shaped relationship between alcohol intake, and both IL-6 and C-reactive protein (CRP) (Volpato et al., 2004) (Pai et al., 2006) (Marques-Vidal et al., 2012). There is a direct, dose-dependent relationship between serum CXCL-8 (IL-8) concentration and alcohol consumption (Gonzalez-Quintela et al., 2007). Studies of peripheral cytokine concentrations in alcohol-dependent patient populations predominantly focus on recently abstinent populations (see Table 1.2). An increase in IL-6 has been reported in the first few days of abstinence (Latvala et al., 2005) (Gonzalez-Quintela et al., 1999) (Gonzalez-Quintela et al., 2000) (Nicolaou et al., 2004) (Garcia-Valdecasas-Campelo et al., 2007) (Vidali et al., 2008) (Leclercq et al., 2012) but returns to control levels within the first week (Khoruts et al., 1991) (Gonzalez-Quintela et al.,

2000) (Chatzipanagiotou et al., 2010) (Gonzalez-Reimers et al., 2012) (Leclercq et al., 2012). TNF- α is raised in early abstinence in most, but not all studies (Khoruts et al., 1991) (Kiefer et al., 2002) (Nicolaou et al., 2004) (Latvala et al., 2005) (Garcia-Valdecasas-Campelo et al., 2007) (Gonzalez-Quintela et al., 2008) (Vidali et al., 2008) (Leclercq et al., 2012). This may reflect specific pathology in alcohol dependence, relating to an autoimmune reaction to peroxidised lipids present in a sub-set of patients (Vidali et al., 2008), or to stage of liver disease (Vidali et al., 2008) (Gonzalez-Quintela et al., 2008).

The anti-inflammatory cytokine IL-10 is increased early in withdrawal according to some, but not all reports (Gonzalez-Quintela et al., 1999) (Gonzalez-Quintela et al., 2000) (Nicolaou et al., 2004) (Garcia-Valdecasas-Campelo et al., 2007) (Leclercq et al., 2012) and like IL-6, falls to control levels by the end of one week (Gonzalez-Quintela et al., 2000) (Leclercq et al., 2012). This is unsurprising given that pro- and anti-inflammatory cytokines are often co-expressed in order to modulate the immune response. Cytokines relating to T cell activation and chemokines have been measured in relatively few studies during early abstinence and there have been inconsistent findings for IL-2, IL-12, IFN- γ , IL-13, and CXCL-8 (IL-8) (Gonzalez-Quintela et al., 1999) (Nicolaou et al., 2004) (Garcia-Valdecasas-Campelo et al., 2007) (Vidali et al., 2008) (Laso et al., 1998) (Gonzalez-Reimers et al., 2012). No increases in IL-4 and IL-5 have been reported (Gonzalez-Reimers et al., 2012).

In summary, a wide range of cytokines associated with both innate and adaptive immunity are elevated in the peripheral blood of alcohol-dependent patients during the first few days of abstinence, although not invariably. Although the elevations reported are two to three times the mean of the control population (Gonzalez-Quintela et al., 2000) (Kiefer et al., 2002) (Latvala et al., 2005), similar to patient populations with depression (Krishnadas and Cavanagh, 2012), it is important to note that these are far smaller differences than would be seen in acute sepsis, where ten- to one hundred- fold increases are common.

1.4.4 Multiple cytokines can be measured simultaneously using Multiplex platforms

Circulating cytokines can be measured in the serum using immunoassays. Until recently, enzyme-linked immunosorbent assays (ELISAs) have been used to measure individual cytokines. This method, however, uses a relatively large amount of serum to measure a single cytokine (Vignali, 2000). Multiplex systems have been developed in order to provide information regarding co-expressed cytokines and therefore, cytokine networks, and enable simultaneous measurement of multiple cytokines using the same volume of body fluid as would be used for a single ELISA experiment, and have a similar sensitivity to ELISA (de Jager et al., 2003).

Table 1.2: studies of peripheral blood cytokine concentrations in alcohol-dependent patient (ADP) populations

Study	Population	Cytokines	Tissue/body fluid	Assay	Results (relative to controls)
Khoruts 1991	Healthy ADPs 7-14 days abstinent (n=21)	TNF- α	Serum	ELISA	Healthy ADPs: all \leftrightarrow
	Controls (n = 20)	IL-1		Bioassay	Cirrhosis: all \uparrow
	Abstinent ADPs with cirrhosis(n=20)	IFN- γ			Alcoholic hepatitis: all \uparrow
	Alcoholic hepatitis patients (n=8)	IL-6			
Laso 1998	Healthy alcoholics <24 hrs abstinent (n = 14)	IL-4	Serum	ELISA	Healthy ADPs
	Controls (n = 10)	IL-12			All \leftrightarrow
	ADPs with cirrhosis <24 hrs abstinent (n = 6)	IFN- γ			ADPs with cirrhosis:
	ADPs with cirrhosis >1 yr abstinent (n = 6)				IL-12 \uparrow in recently abstinent group
Gonzalez- Quintela 1999	Healthy ADPs <72 hours abstinent (n=65)	IL-4	Serum	ELISA	IL-4 below limit of detection
Controls (n=40)	IL-5	IL-5 below limit of detection			
	IL-6	IFN- γ detectable in 6% of alcoholics (0% of controls)			
	IL-8	IL-6 \uparrow			
	IL-10	IL-8 \uparrow			
	IL-12	IL-10 \uparrow			
	IL-13	IL-12 \uparrow			
IFN- γ	IL-13 \uparrow				
Laso 1999	Healthy ADPs <24 hrs abstinent (n = 10)	IL-2	Whole blood	Flow cytometry	Healthy ADPs:
	Controls (n = 10)	IL-4		Chemiluminescent	IL-2 \uparrow
	ADPs with cirrhosis <24 hrs abstinent (n = 10)	IFN- γ		antibody	IFN- γ \uparrow
	ADPs with cirrhosis >1 yr abstinent (n = 10)				IL-4 \leftrightarrow
					ADPs with cirrhosis <24hrs abstinent:
					IL-2 \uparrow but < healthy alcoholics
					IFN- γ \uparrow but < healthy alcoholics
					IL-4 \leftrightarrow
					ADPs with cirrhosis >1yr abstinent:
					IL-2 \leftrightarrow
					IFN- γ \leftrightarrow
					IL-4 \uparrow
Gonzalez-	Healthy ADPs (n=29) measured on day 1 (24-48	IL-6	Serum	EIA	IL-6 \uparrow

Study	Population	Cytokines	Tissue/body fluid	Assay	Results (relative to controls)
	Controls (n=30)				
Nicolaou 2004	Healthy ADPs 24 ± 12 hours abstinent (n= 43)	TNF-α	Serum	ELISA	TNF-α ↔
	Controls (n= 20)	IL-6			IL-6 ↑
		IL-8			IL-8 ↔
		IL-10			IL-10 ↔
		IL-12			IL-12 ↔
Latvala 2005	Healthy ADPs 2 ± 2 days abstinent (n=54)	TNF-α	Serum	ELISA	<u>Healthy ADPs:</u>
	ADPs with alcoholic liver disease (fatty liver, hepatitis, fibrosis or cirrhosis) (n=32)	IL-6			TNF-α ↑
		IL-8			IL-6 ↑
		IL-10			IL-8 ↑
		IL-2			IL-10 ↔
					IL-2 ↔
					<u>ADPs with cirrhosis:</u>
					TNF-α ↑
					IL-6 ↑
					IL-8 ↑
					IL-10 ↑
					IL-2 ↑
Garcia-Valdecasas-Campelo 2007	Healthy ADPs admitted for alcohol withdrawal ie v early abstinence (n=12)	TNF-α	Serum	Chemiluminescent assay	Healthy ADPs:
	Controls (n=12)	IL-1β			IL-8 ↑; others ↔
	ADPs with cirrhosis in v early abstinence (n=10)	IL-6		ELISA	Alcoholics with cirrhosis:
		IL-8			IL-8 ↑; others ↔
		IL-10			
Gonzalez-Quintela 2008	Healthy ADPs admitted to hospital for treatment of withdrawal ie recently abstinent (n=35)	TNF-α	Serum	Chemiluminescent immunoassay	TNF-α ↑
	Alcoholics with acute liver damage admitted to hospital (n = 44)				TNF-α ↑ associated with AST >2x ULN and advanced liver disease
	Alcoholics with advanced liver disease (n = 30)				
	Healthy controls (n = 460)				
Vidali 2008	Healthy ADPs < 4 days abstinent (n=29)	TNF-α	Serum	ELISA	TNF-α and IL-2 ↑ in those with antibodies to peroxidised lipids
	Controls (n =34)	IL-2			Elevation of TNF-α + antibodies predictive of advanced ALD
	Alcoholics with liver disease < 4 days abstinent (n = 30)	IL-6			
		IL-8			
Gonzalez-Riemers 2012	ADPs without cirrhosis admitted to hospital 10-15 days abstinent (n=31)	TNF-α	Serum	Chemiluminescent immunoassay	TNF-α ↑
		IL-6			IL-6 ↔

Study	Population	Cytokines	Tissue/body fluid	Assay	Results (relative to controls)
	ADPs with cirrhosis 10-15 days abstinent (n=16)	IFN- γ		ELISA	IFN- γ \uparrow
	Controls (n = 18)	IL-10			IL-10 \uparrow
		IL-13			IL-13 \uparrow
		IL-4			IL-4 \downarrow
Le Clerq 2012	Healthy ADPs (n = 52) measured on day 1-2 (T1) and day 18-19(T2) of detoxification	TNF- α	Plasma	Multiplex	TNF- α \uparrow
		IL-6			IL-6 \uparrow
	Controls (n = 16)	IL-10			IL-10 \uparrow
					All decreased from T1 to T2

1.5 Circulating cytokines and Hypothalamo-Pituitary-Adrenal (HPA) axis abnormalities during alcohol withdrawal

1.5.1 A brief introduction to the hypothalamo-pituitary-adrenal (HPA) axis

Derangement of hypothalamo-pituitary-adrenal (HPA) axis function is consistently reported in alcohol withdrawal. Chapter 3 investigates the relationship between cytokines and cortisol during alcohol detoxification. The HPA axis is an endocrine system that contributes to the body's response to physiological or psychological stress via effects on glucose availability and the immune system. Corticotrophin Releasing Hormone (CRH) is released from paraventricular nucleus of the hypothalamus into the hypophyseal portal system in response to stressors. CRH stimulates release of adrenocortico-stimulating hormone (ACTH) into the systemic circulation, which in turn stimulates adrenal production of cortisol. Cortisol binds to glucocorticoid receptors with relatively weak affinity, resulting in stimulation at high doses only. This inhibits further HPA axis activation.

1.5.2 Alcohol dependence is related to abnormally high morning cortisol levels

Both actively drinking alcohol-dependent patients (Mendelson et al., 1971) (Stokes, 1973) (Adinoff et al., 2003) and patients in alcohol withdrawal have been reported to have higher morning cortisol levels than control samples (Errico et al., 2002) (Keedwell et al., 2001) (Adinoff et al., 1991) (Leggio et al., 2008) (Kruger et al., 2006). Morning cortisol levels (a single blood sample around 9am) are used clinically to detect gross HPA derangements, as in Cushing's syndrome or Addison's disease. ADPs in withdrawal have higher

cortisol than while they are still drinking, suggesting that withdrawal represents an acute-on-chronic stressor (Adinoff et al., 2003).

A morning cortisol increase of between 2 to 5 fold in ADPs relative to those in a matched control sample has been reported (Adinoff et al., 1991) (Keedwell et al., 2001). Whilst such levels are higher than those in a control population, or in the same patients after a longer abstinence duration, they still fall within clinical laboratory normal ranges for cortisol, which are set for diagnosing primary adrenal or pituitary pathology (Kruger et al., 2006) (Leggio et al., 2008). This implies that alcohol dependence is associated with a more subtle derangement of HPA axis function than primary endocrine disease in the majority of cases, though in rare cases, alcohol-related pseudoCushing's syndrome has been reported (Besemer et al., 2011).

A loss of circadian variation in those patients with moderate to severe withdrawal symptoms has been reported (Adinoff et al., 1991) (Mukai et al., 1998), though the circadian rhythm appears preserved in those with mild symptoms or those who are receiving medication for detoxification (Keedwell et al., 2001) (Adinoff et al., 1991) (Mukai et al., 1998). ACTH and cortisol levels have been found to decrease after two to six weeks of abstinence (Errico et al., 2002). Counterintuitively, once withdrawal symptoms have resolved, lower levels of cortisol are associated with an elevated risk of relapse (Kiefer et al., 2006).

1.5.3 Blunted cortisol responses to challenge are found in alcohol dependence

The observation that cortisol levels are higher in ADPs prompted further investigation of the function of the HPA axis employing challenge studies, using synthetic hormones such as dexamethasone and corticotrophin-releasing hormone. Suppression tests have revealed no consistent abnormality although a recent review found that studies in early abstinence reported a greater proportion of patients with non-suppression than in later abstinence (Besemer et al., 2011). Stimulation tests using CRH have consistently described a lower increase in cortisol – that is a hyporesponsive HPA axis (Merry and Marks, 1972) (Coiro and Vescovi, 1999) (Adinoff et al., 2005) (Bailly et al., 1989) – in ADP men. In contrast, a single study in women found no abnormality (Adinoff et al., 2010).

HPA axis hyporesponsiveness has also been found in ADPs using psychological stress as a probe (Lovallo et al., 2000) (Errico et al., 2002) (Junghanns et al., 2005) (Fox et al., 2012). The Cortisol Awakening Response – the physiological rise in cortisol of between 35% – 70% (Wust et al., 2000b) (Clow et al., 2010) occurring within the first thirty minutes of waking – has been used as a more easily administered challenge which is feasible in large studies; individuals collect saliva samples themselves during the first half hour to hour after waking using specially prepared dental swabs. It is unaffected by smoking, gender or length of sleep (Wust et al., 2000b). The single study of the Cortisol Awakening Response in ADPs found blunted responses in recently abstinent ADPs relative to those who had been

abstinent for some months (Junghanns et al., 2007) suggesting hyporesponsiveness to physiological, as well as psychological stressors in early abstinence.

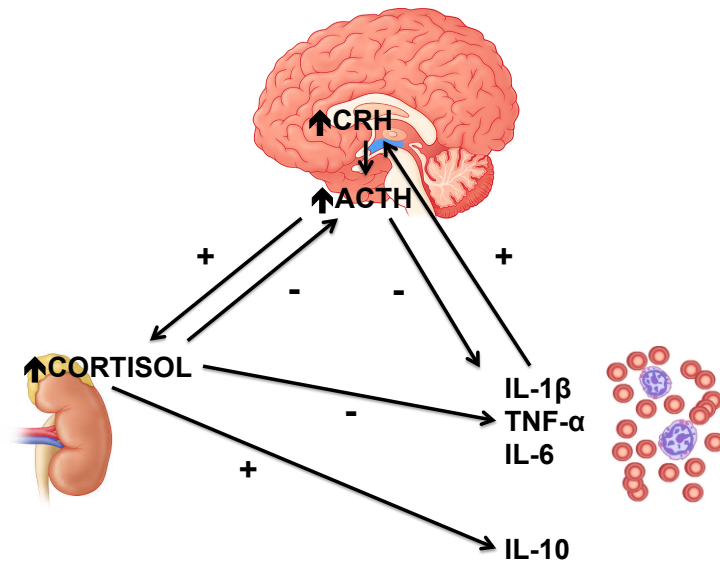
1.5.4 There are specific relationships between HPA axis function and cytokine expression

It is important to characterise the relationship between the HPA axis and cytokines in alcohol withdrawal, as under physiological conditions the HPA axis is activated by peripheral inflammatory stimuli, and provides a major negative feedback loop (Rhen and Cidlowski, 2005). The main anti-inflammatory action of cortisol is inhibition of inflammatory transcription pathways such as Nuclear Factor K β (NFK β). Increase in cortisol is thus associated with suppression of pro-inflammatory cytokines such as IL-6, Th-1 cytokines such as IL-12, and an increase in anti-inflammatory cytokines such as IL-10 (van der Poll et al., 1996), as illustrated in Figure 1.1. Stimulated ACTH production may also suppress inflammation: ACTH was inversely correlated with TNF- α after CRH challenge (Schuld et al., 2003).

Under homeostatic conditions, pro-inflammatory and Th-1 cytokines are suppressed by ACTH and cortisol. Under chronic inflammatory states, glucocorticoid receptor (GR) function is impaired, and the inflammatory stimulation of the HPA axis is not limited by negative feedback via cortisol (Pace and Miller, 2009) (Zunszain et al., 2011). Thus, suppression of certain cytokines by ACTH and cortisol does not occur. Additionally, cytokines may contribute to poor GR function by inhibiting glucocorticoid receptor

translocation, affecting interactions with other proteins, and reducing binding of the glucocorticoid receptor (Pace et al., 2007).

Figure 1.1: Relationship between HPA axis function and cytokines



Legend: Peripheral pro-inflammatory cytokines, IL-1 β , TNF- α and IL-6, stimulate the HPA axis resulting in cortisol production. Both cortisol and ACTH are associated with suppression of pro-inflammatory cytokines and cortisol is associated with increased anti-inflammatory IL-10.

Dexamethasone non-suppression in alcohol-dependent patients in early abstinence (Besemer et al., 2011) suggests that GR function is impaired in this population. Studies measuring cortisol and immune measures concurrently in surgical patients with alcohol abuse or dependence, however, found raised anti-inflammatory cytokines (Sander et al., 2005) and decreased Th-1 associated lymphocytes (Spies et al., 2004), which is inconsistent with glucocorticoid resistance. The latter abnormality was reversed by HPA axis inhibition (Spies et al., 2006), providing further support for GR sensitivity rather than resistance. These findings may have been affected by the acute inflammatory milieu of surgery and a more heterogenous patient population

(abuse and dependence). Against this background, Chapter 3 investigates the relationship in a naturalistic clinical sample of alcohol-dependent patients undergoing planned detoxification.

1.6 Microglial activation in alcohol-dependent patients during early abstinence

Chapter 5 describes an imaging experiment using [¹¹C]PBR28 Positron Emission Tomography to characterise activated microglia in alcohol-dependent patients. Microglia are the resident tissue macrophages of the central nervous system (CNS), comprising 10% of CNS cells. There are four distinct populations of microglia classified according to their anatomical location – parenchymal, perivascular, meningeal and choroid plexus microglia – and these have differing immune functions (Ransohoff and Cardona, 2010). They have small cell bodies with multiple thin branching ramifications. This morphology enables microglia to detect and respond dynamically to minute changes in their environment, even while in a morphological state previously known as ‘resting’, but now more accurately called ‘surveillant’ (Nimmerjahn et al., 2005) (Ransohoff and Cardona, 2010). In response to injury their ramifications extend to the site of injury within seconds and fuse with the ramifications of other microglia to form a wall between healthy and injured tissue (Davalos et al., 2005).

With more significant injury, microglia proliferate and change from a highly branched to a more amoeboid, macrophage-like structure. This change is termed ‘activation’. As described above, several functional states of activation

are recognized, and two specifically named: an 'M1' neurotoxic phenotype that produces pro-inflammatory cytokines, reactive oxygen species (Chao et al., 1992), and excitotoxins (Piani et al., 1992); and a neurotrophic 'M2' phenotype which secretes anti-inflammatory cytokines, nerve growth factors and clears debris via phagocytosis (Morgan et al., 2004). It is hypothesised that further functional types of activated microglia exist (Perry et al., 2010) and that the type of activation may be contingent both upon the microenvironment (Morgan et al., 2004), neuronal regulation (Nakajima et al., 2007), and systemic inflammation (Wright et al., 2014). Either overactivity of the M1 phenotype or suppression of the M2 phenotype could be associated with exacerbation of an acute or chronic neural insult.

1.6.1 Microglial activation relates to the extent of damage in a variety of conditions

Clinical imaging studies have found relationships between microglial activation and structural measures of damage or clinical measures of cognitive decline in a variety of neurodegenerative diseases. An inverse relationship between Mini-Mental State Examination (MMSE) score and binding of [¹¹C]PK11195, a radiotracer with affinity for a protein highly expressed in activated microglia (see below), has been found in mild cognitive impairment (Edison et al., 2008). In Multiple Sclerosis (MS), studies using [¹¹C]PK11195 have found a positive correlation with atrophy in areas unaffected by acute lesions (Versijpt et al., 2005), and that high [¹¹C]PK11195 binding was associated with greater disability in progressive MS (Giannetti et al., 2014). Similarly, in an acute insult such as stroke, microglial activation around the lesion was associated with a worse clinical outcome (Thiel and

Heiss, 2011). However, microglial activation in projection areas was associated with better outcome, perhaps representing disparate functional activation in different brain areas (Thiel and Heiss, 2011).

1.6.2 Microglial activation is associated with alcohol withdrawal pre-clinically

Pre-clinical studies have demonstrated microglial activation or proliferation during alcohol withdrawal (Nixon et al., 2008), (Marshall et al. 2013). Particularly, microglial activation has been reported in the hippocampus in areas where glutamate was increased (Ward et al., 2009). Microglial proliferation is detectable for up to three weeks after withdrawal (Obernier et al., 2002), (Marshall et al. 2013). There is debate over the functional phenotype of activated microglia during alcohol withdrawal. Pascual and colleagues described increased inducible nitric oxide synthetase and cyclooxygenase in pre-clinical models, which would be consistent with an inflammatory milieu involving M1 microglial activation (Pascual et al., 2007). Further, another pre-clinical study found initial inflammatory microglial activation in the hippocampus and parietal cortex, with expression of TNF- α and IL-1 β during the first four days of withdrawal, associated with cognitive decline, followed by proliferation of trophic microglia around fourteen days post withdrawal, associated with cognitive recovery (Zhao et al., 2013). In contrast, a more recently published paper found that microglial activation during alcohol withdrawal was not full activation, occurred after neuronal death, and was associated with increased expression of IL-10 (anti-inflammatory), rather than TNF- α (inflammatory) (Marshall et al. 2013). Hence there is uncertainty about whether microglia may exacerbate damage or be protective. Although the most recent findings were published

subsequent to the design of my study, the pleiotropic nature of microglia and the uncertainty regarding their role in other conditions led me to investigate the relationship between microglial activation and cognitive function as a proxy of acute damage following detoxification.

Human tissue post-mortem studies in alcohol-dependent patients have shown increased microglial numbers, increased inflammatory chemokine CCL-2 expression, and increased expression of inflammatory receptors and transcription factors. It has been suggested that inflammatory processes may be related to alcohol-related brain damage (Zahr et al., 2011). It has also been suggested that inflammatory pathways are involved in plasticity of striatal-frontal circuits related to compulsive behaviour in addiction (Crews et al., 2011).

1.6.3 Microglial activation can be detected in humans in vivo using Positron Emission Tomography (PET)

Activated microglia can be imaged *in vivo* in humans using PET. This brain imaging technique involves the intravenous injection of a 'radiotracer' or 'radioligand', which is a chemical labelled with a radioisotope, in such small amounts ('tracer' amounts) that it is able to bind to the protein of interest without perturbing the biological system concerned. The isotope decays, emitting a positron, the antimatter equivalent of an electron. The positron travels – usually a distance <1mm – until it encounters an electron, which it annihilates. When this occurs, two gamma photons are emitted, travelling in opposite directions. A ring of detectors containing scintillation material, in the

scanner itself, detect gamma photons. Those that reach them simultaneously are detected as representative of a single annihilation and the scanner uses information from simultaneous pairs of gamma photons incident on detectors opposite one another to locate and quantify the amount of radioactivity in a given area of brain.

This information is gathered for separate periods of time during the scan in a 'time-activity curve' plotting radioactivity over time for each brain voxel. Arterial blood is obtained from the radial artery continuously at first and then at intervals later in the scan, to measure both radioactivity and metabolite concentrations in the plasma. This information is used to generate a metabolite-corrected input function, the time activity curve of the radioactivity curve in the blood. These measurements are used to estimate a model of tracer pharmacokinetics, which captures the movement of the tracer from plasma, to tissue, to bound and back. Information about the tracer pharmacokinetics and binding in the brain is used in model selection, as are mathematical best fit criteria such as the Akaike Information Criteria (Akaike, 1974).

1.6.4 The PET target representing microglial activation is the Translocator Protein (18kDa)

PET radioligands that bind the Translocator Protein (18kDa) (TSPO) are used to detect microglial activation. The TSPO is a mitochondrial membrane protein found in a heteromer with a voltage-dependent anion channel and an

adenine nucleotide transporter (McEnery et al., 1992). It has multiple functions, including translocation of cholesterol during steroid synthesis, calcium homeostasis, and cell death regulation (Papadopoulos et al., 2006). It is highly expressed in steroid-producing tissues (Papadopoulos et al., 2006) and is conserved across species. TSPO is expressed in cerebral connective tissue: arteriolar smooth muscle, perivascular macrophages, the choroid plexus, ependyma, meninges, and the olfactory bulb and pituitary (Turkheimer et al., 2007). Expression in brain parenchyma under normal conditions has long been thought to be low. Recently however, expression has been reported in healthy human endothelium, choroid, human glia and microglia (Cosenza-Nashat et al., 2009), with higher binding in the hippocampus than in other brain regions (Gulyas et al., 2009), in neural progenitors *in vitro* (Varga et al., 2009), and in mature granule cell neurons in the hippocampus and Purkinje cells in the cerebellum of transgenic Translocator Protein 18kDa-Green Fluorescent Protein (TSPO-GFP) mice (Wang et al., 2012).

Microglia in a ramified state, express TSPO, but at relatively low levels (Banati et al., 2004). When microglia become activated, their expression of TSPO is increased at least twofold (Banati et al., 2000). This increase appears to relate to activation in addition to proliferation (Ito et al., 2010). On a sub-cellular level the increase in signal is associated with an increase in the number of mitochondria, mitochondrial elongation, and arrangement in ring aggregates (Banati et al., 2004). Although microglia and astrocytes increase expression of TSPO when activated, the distribution of the TSPO-PET signal (most commonly of [¹¹C]PK11195) in *in vivo* studies of a variety of brain

pathologies corresponds to microglia rather than astrocytes (for a review, see (Venneti et al., 2006)). Although very early increase in [^{11}C]PK11195 binding following a neurotoxic lesion reflects a mixed glial/microglial response, the profile of the signal in terms of time course, peak and persistence of the signal is consistent with ongoing microglial activation rather than a mixed signal (Venneti et al., 2006).

PET imaging with ligands for the TSPO has already been used to characterise microglial activation in neurodegenerative conditions, hepatic encephalopathy, stroke and traumatic brain injury (Edison et al., 2008), (Thiel and Heiss, 2011), (Ramlackhansingh et al., 2011). [^{11}C]PK11195 is the tracer most frequently used to study differences in TSPO binding (Cagnin et al., 2007). There are, however, some methodological issues with [^{11}C]PK11195 PET. Despite high affinity to the TSPO *in vitro*, it has a low specific-to-nonspecific binding ratio *in vivo* (Banati et al., 2000) and is difficult to quantify in plasma (Cleij, 2003), (Kropholler et al., 2005). Newer tracers have a superior signal-to-noise ratio (Van Camp et al., 2010), but suffer from other limitations. A single nucleotide alanine to threonine polymorphism affects the affinity of new generation ligands for the TSPO (Owen et al. 2011). Those who are homozygous for the alanine nucleotide are 'high affinity binders' (HABs) in that TSPO radioligands bind to their binding site on the TSPO with a high affinity. Those who are homozygous for the threonine nucleotide are 'low affinity binders' (LABs) in that TSPO radioligands bind to their binding site with low affinity. No specific [^{11}C]PBR28 PET signal is detectable in the brain in LABs. For this reason they were previously known as 'non-binders' (Imaizumi

et al., 2007), (Imaizumi et al., 2008). Heterozygotes, or 'mixed affinity binders' (MABs) have mixed TSPO, comprising 50% TSPO with the high affinity sites and 50% with the low affinity sites. It is therefore necessary to screen for the TSPO polymorphism prior to scanning, to exclude LABs. Knowledge of genotype is also important in interpreting scanning data, as specific binding in MABs measured by PET *in vivo*, is around half that of HABs (Kreisl et al. 2012).

Alcohol-dependent patients are usually treated with benzodiazepines during alcohol withdrawal. This complicates TSPO PET as certain benzodiazepines, including diazepam, bind to the TSPO. In fact, the TSPO was originally characterised as a ligand for benzodiazepines in the peripheral organs, which explains its the name it was first given, the Peripheral Benzodiazepine Receptor (Papadopoulos et al., 2006).

Previous *in vitro* radioligand binding studies in human brain had been published only for certain benzodiazepines, and under conditions that did not approximate the *in vivo* environment in terms of pH or temperature (Rao and Butterworth, 1997). Further, the effect on benzodiazepine affinity of the genetic polymorphism that affects radioligand binding is unknown. For this reason, I performed radioligand competition assays with commonly prescribed benzodiazepines including diazepam, chlordiazepoxide, and their metabolites, which are described in Chapter 4.

1.6.5 The affinity of benzodiazepines for the TSPO can be established using radioligand binding assays

Competition assays use a fixed concentration of a radioactive tracer or 'hot' ligand, for example, [³H]PK11195 or [³H]PBR28, of known affinity for a receptor. This is incubated with homogenised tissue containing the receptor of interest, in this case, brain, and varying concentrations of the 'cold' ligand, the benzodiazepine of interest. Each concentration of benzodiazepine is incubated at body temperature with the tissue and radioligand in physiological buffer until equilibrium is reached. The process is stopped by harvesting of the tissue, with the 'cold' ligand and radioligand labelling the receptors of interest, onto discs of filter paper. These are then placed in a vial of scintillation fluid and the amount of radioactivity is measured by a beta-counter. The affinity, or inhibition constant, of the cold ligand can then be calculated using the Cheung-Prossoff equation, as follows:

$$K_i = IC_{50} / \left(1 + \frac{[L]}{K_D} \right)$$

where K_i is the affinity of the cold ligand, IC_{50} is the concentration at which the cold ligand displaces 50% of the hot ligand, L is the concentration of the hot ligand, and K_D is the affinity of the hot ligand. This competition study was therefore necessary to determine whether the TSPO-PET study could be undertaken in patients taking, or having recently taken, benzodiazepines.

1.7 The relationship between brain glutamate and microglial activation

The increase in extracellular glutamate is a key neurobiological derangement during alcohol withdrawal and has been related to neuronal death. Chapter 6

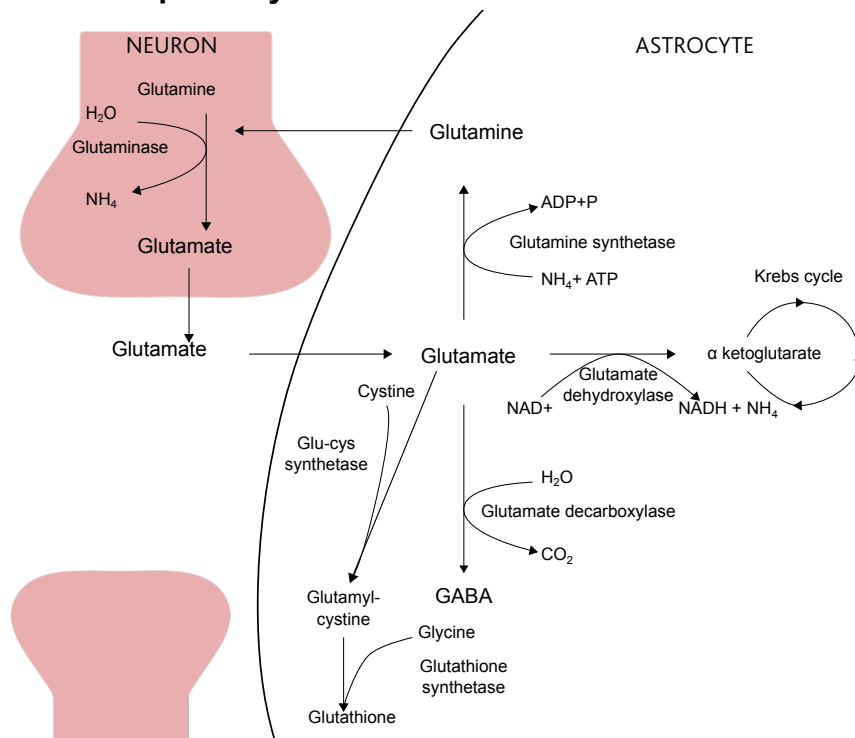
describes Magnetic Resonance Spectroscopy undertaken to establish the relationship between TSPO expression, as a proxy of microglial activation, and MRS brain glutamate concentrations. Microglial activation in response to increased glutamate provides a plausible mechanism for the increase in microglial activation during alcohol withdrawal, as set out below, in contrast to the multiple mechanisms of microglial activation identified during chronic alcohol drinking.

Glutamate is the primary excitatory neurotransmitter in the central nervous system. It participates in cellular metabolism via conversion to α -ketoglutarate, a substrate of the Krebs cycle, and is also the precursor of γ -hydroxy-amino-butyrate (GABA) the predominant inhibitory neurotransmitter and glutathione, a compound involved in conjugating toxic metabolites (Erecinska and Silver, 1990) (Bak et al., 2006). Glutamate does not cross the blood brain barrier, so it is synthesised *de novo* from glucose in the brain (Hertz et al., 1999).

Glutamate acts at an array of ionotropic and metabotropic receptors (these and its regulation are extensively reviewed in (Clapp et al., 2008)). There are three types of ionotropic receptors: N-methyl-D-aspartate (NMDA), AMPA and kainate, which are situated post-synaptically. Binding of glutamate to these receptors causes influx of positively charged ions the post-synaptic neuron and evokes a rapid neuronal response. Metabotropic glutamate receptors are situated pre-synaptically and on glial and microglial cells, and modulate glutamate release. Glutamate transmission via NMDA receptors is crucial in

memory function as it is involved in the process of long-term potentiation that underpins synaptic plasticity. It is highly regulated as excess calcium influx via NMDA receptors can result in NMDA-mediated excitotoxicity. Synaptic glutamate is rapidly taken up by astrocytic transporters and converted into glutamine, which is not a neurotransmitter, so can be safely released and taken up by neurons, for conversion into glutamate (Hertz et al., 1999) (see Figure 1.2).

Figure 1.2: The relationship between glutamate and glutamine, and other metabolic pathways in the brain



Acute alcohol intake inhibits ionotropic glutamate receptors, particularly NMDA receptors (Lovinger et al., 1989) (Hoffman, 2003). Chronic alcohol intake is associated with an up-regulation of NMDA receptors in both pre-clinical models (Floyd et al., 2003) (Snell et al., 1996) and post mortem alcoholic brain samples (Freund and Anderson, 1996). *In vitro* studies have

reported that neurons exposed to alcohol have more NMDA receptors clustered around synapses. NMDA receptors also become more sensitive to glutamate, so more calcium ions enter the cell for a given glutamate concentration, which is attributed to a change in subunit composition (for a review see (Nagy, 2008)).

Not only is the alcohol-dependent brain therefore more primed to respond to glutamate, but it is also exposed to more extracellular glutamate during alcohol withdrawal. Concentrations of extracellular glutamate as measured by microdialysis in animals (De Witte et al., 2003) and in cerebrospinal fluid (CSF) and plasma in man, are reported to be increased during alcohol withdrawal (Tsai et al., 1998) (Brousse et al., 2012) while GABA concentrations are lower in man (Tsai et al., 1998) (Brousse et al., 2012). Subsequent cycles of withdrawal in animal models are associated with progressive increases in glutamate and other excitatory amino acids (Dahchour and De Witte, 2003). Pre-clinical studies have demonstrated an association between increased NMDA transmission and cell death in the hippocampus during alcohol withdrawal (Mayer et al., 2002). In humans, CSF concentrations of excitatory neurotransmitters correlate with oxidative stress markers, which implies glutamate has a role in withdrawal-related damage (Tsai et al., 1998). Withdrawal seizures can be prevented in pre-clinical models by NMDA antagonists, which suggests they may also relate to excess excitatory neurotransmission (Stepanyan et al., 2008). Traditionally, benzodiazepines which potentiate GABA-A transmission have been used to treat symptoms of alcohol withdrawal (Pilling et al., 2011) (Lingford-Hughes et

al., 2012b), though NMDA antagonists such as MK801 and memantine have been shown to prevent alcohol withdrawal-related seizures in rodents (Stepanyan et al., 2008). In humans, medications that inhibit glutamatergic transmission, such as memantine, have had comparable effects to diazepam in the treatment of alcohol withdrawal (Krupitsky et al., 2007).

Magnetic resonance spectroscopy (MRS) has been used to measure brain glutamate in alcohol-dependent patients. MRS is a form of Magnetic Resonance Imaging (MRI) used to detect concentrations of brain metabolites. Human brain MRS most commonly detects hydrogen atoms, which are abundant, using a standard head coil.

MRS is based on the principle that when the brain is placed within a magnetic field, atomic nuclei within the tissue will predominantly spin around an axis aligned with the direction of the magnetic field. Pulsed radiofrequency waves knock the nuclei off their axes, causing them to flip to a perpendicular axis and then relax towards the aligned axis. This oscillation occurs at a particular frequency, or 'resonance'. The magnetic field produced causes an electric current in the head coil, which is acquired by the scanner. The specific frequency is influenced by the surrounding electron clouds, which vary according to molecular make-up. These cause a local magnetic field that 'shields' the nuclei from the external magnetic field to a small extent. Thus hydrogen nuclei within different compounds resonate at different frequencies.

The difference in resonance confers information about molecular structure and is measured in parts per million (ppm). Some metabolites, such as glutamate, yield multiple peaks as hydrogen nuclei exist in three different environments in the molecule. The x axis scale of the MRS spectrum generated by the scan is of ppm. The y axis represents the amplitude of the oscillation, and is proportional to the total concentration of the metabolite.

Pre-processing is necessary to deal with some of the challenges inherent in MRS. First, 'shimming' is performed by altering the electrical currents in the head coil, in order to render the magnetic field as homogenous as possible, thus maximising the signal-to-noise ratio. Second, water suppression pulses are performed, as the concentration of water in the brain is several thousand fold the concentration of the metabolites of interest.

MRS detects total glutamate ([Glu]) and glutamine ([Gln]) concentrations in the voxel of interest, and so cannot distinguish between metabolic and synaptic pools. It has been proposed that glutamine could represent a marker of glutamatergic neurotransmission, as it is produced from synaptic glutamate taken into astrocytes (Yuksel and Ongur, 2010) (Theberge et al., 2002). [Glx] is a composite measurement of glutamate and glutamine concentration that is calculated when their separate spectra cannot be resolved. It has been proposed that [Glx] represents the total glutamate store available for metabolic and synaptic purposes (Yuksel and Ongur, 2010).

In cross-sectional studies comparing controls with ADPs (summarised in Table 1.3), most published after the beginning of my study, there have been no consistent findings, which may reflect differences in the region selected, magnet strength and duration of abstinence. For instance, both increased and decreased [Glu] has been reported in the anterior cingulate cortex (ACC) (Lee et al., 2007) (Thoma et al., 2011) with no differences in [Glu] in frontal white matter (Ende et al., 2013), dorsolateral prefrontal cortex, and occipital cortex (Mon et al., 2012) in ADPs compared with healthy controls. Acamprosate, a derivative of taurine, an amino acid that had been demonstrated to reduce extracellular glutamate levels during repeated withdrawal cycles pre-clinically, was associated with a decrease in [Glu] during early abstinence (Umhau et al., 2010).

[Glu] and [Glx] relate to important clinical variables in ADPs. An inverse correlation reported between [Glu] and cognitive function in depressed patients provides additional weight to the argument that [Glu] is related to damage (Ende et al., 2013). However in binge drinking patients, lower [Glu] was related to a history of blackouts suggesting that in the acute setting higher [Glu] compensates for the amnestic effect of alcohol (Silveri et al., 2014). Increased [Glx] has been reported in the occipital cortex of those ADPs who smoked in one study, relative to ADPs who did not smoke, and controls (Mason et al., 2006), but no different to healthy controls in the nucleus accumbens and ACC in another (Bauer et al., 2013). A positive correlation has also been found between craving and [Glx] (Bauer et al., 2013). Given the integral role of glutamate in the neurobiology of alcohol

withdrawal, and its relationship with clinical variables, it was important to investigate the relationship between microglial activation and glutamate in man. The pre-clinical association between the two (Ward et al., 2009) strengthened the rationale for this study.

Table 1.3: MRS studies of glutamate, glutamine, [Glx] in alcohol dependence

Study	Population	Voxels	Findings
Bauer 2013	29 ADPs 10 days abstinent 31 HCs	NAcc ACC	↑[Glu] in NAcc ↔[Glu] in ACC ↔ [Glx], [NAA], [Cho], [Cr] in both Glx in ACC related to craving
Ende 2013	23 actively drinking HDs 9 HCs	Frontal white matter	↑[Cho] ↔[Glu] ↔[NAA] ↔[Cr] Inverse correlation between [Glu] and cognitive function
Yeo 2013	213 HDs abstinent for 24 hrs 66 HCs	ACC	↑[Glx], [NAA], [Cr], [Cho]
Hermann 2012	47 ADPs 2 scans: day 1 and day 14 of abstinence 57 HCs	ACC	↑[Glu] on day 1 [Glu] decreased to control levels by day 14 ↓[NAA] on day 1
Mon 2012	20 ADPs abstinent for 1 week 36 ADPs abstinent for 5 weeks 16 HCs 1 week 5 weeks	ACC POC DLPFC	At 1 week: ACC: ↓[Glu], [NAA], [Cho], [Cr] ↔[GABA+], [ml] POC, DLPFC: no differences Normalisation by 5 weeks abstinence Inverse correlation between [ml] and cognitive function
Thoma 2011	7 ADPs actively drinking	ACC	↑[Gln] ↓[Glu]

	6 ADPs 1 year abstinent 17 HCs		↔ [NAA], [mI], [Cho], [Cr] No difference between actively drinking and abstinent groups
Umhau 2010	2 scans during first month of abstinence 15 ADPs on acamprosate 18 ADPs on placebo	ACC	[Glu]↓ in acamprosate group [Glu]↑ in placebo group (after treatment)
Lee 2007	13 ADPs abstinent 15 d (14 – 28) 18 HCs	ACC Insula	↓[Cho], [Cr] in ACC ↑[Glu]; [Cr] in ACC Glu:Cr directly correlated with short term memory
Mason 2006	2 scans during first month of abstinence 16 ADPs 8 HCs	OCC	<i>Smoking v.s. non-smoking ADPs:</i> ↑[Glx] in smoking ADPs ↓[GABA+] in smoking ADPs <i>ADPs vs HCs:</i> ↔[Glx], [NAA], [Cho], [Cr]

Legend: ACC = anterior cingulate cortex; ADPs = alcohol-dependent patients; [Cho] = choline concentration; [Cr] = creatine concentrations; DLPFC = dorsolateral prefrontal cortex; [GABA+] = Gamma amino butyric acid concentration; [Gln] = glutamine concentration; [Glu] = glutamate concentration; [mI] = myoinositol concentration; [NAA] = N-acetyl aspartate; NAcc = nucleus accumbens; POC = parieto-occipital cortex; HDs = heavy drinkers; HCs = healthy controls; OCC = occipital cortex

1.8 Aims and hypotheses

The aim of my thesis was, first, to ascertain whether there was evidence of peripheral inflammation and neuroinflammation during alcohol withdrawal and early abstinence. Second, I aimed to investigate the relationship between these immune processes and the neurochemical and neuroendocrine derangements that characterise the withdrawal-early abstinence period. Third, I aimed to explore whether there was a relationship between inflammatory processes and clinical features of withdrawal and early abstinence, in particular impaired cognitive function. Thus I aimed not only to establish evidence for the presence of peripheral inflammation and

neuroinflammation, but also the possible clinical relevance of these processes and the way in which they related to the neurobiology of alcohol withdrawal and early abstinence.

Concerning peripheral inflammation, I hypothesised that circulating pro-inflammatory cytokines and chemokines would be raised in early detoxification relative to late detoxification, that there would be a positive relationship between cytokine concentrations and withdrawal severity and an inverse relationship between cytokines and cognitive function. I hypothesised that hypothalamo-pituitary-adrenal axis function would be deranged, as described previously, and that this derangement would correlate with pro-inflammatory cytokine concentrations.

I hypothesised that there would be a widespread increase in [^{11}C]PBR28 V_T , signalling microglial activation in the brain during early abstinence from alcohol, particularly in the hippocampus where it had been described pre-clinically. Further, I hypothesised that there would be an inverse relationship between [^{11}C]PBR28 V_T and performance on cognitive tests. Based on pre-clinical evidence describing co-occurring microglial activation and increased extracellular glutamate during withdrawal, I hypothesised that brain glutamate as detected would be increased and that there would be a positive relationship between glutamate and [^{11}C]PBR28 V_T .

CHAPTER 2

PERIPHERAL CYTOKINE PROFILE IN ALCOHOL DETOXIFICATION:

RELATIONSHIP TO CLINICAL SYMPTOMS

2.1 Introduction

2.1.1 Circulating cytokines and chemokines are increased during alcohol detoxification.

The peripheral blood cytokines TNF- α , IL-1 β and IL-6 are raised in early abstinence in alcohol dependence, as described in Chapter 1 Section 4 (Gonzalez-Quintela et al., 1999), (Gonzalez-Quintela et al., 2000), (Kiefer et al., 2002), (Nicolaou et al., 2004), (Latvala et al., 2005), (Garcia-Valdecasas-Campelo et al., 2007), (Gonzalez-Quintela et al., 2008), (Gonzalez-Reimers et al., 2012), (Leclercq et al., 2012). In addition, IL-6 and the chemokine CXCL-8 (IL-8) are positively correlated with alcohol consumption in population studies (Volpato et al., 2004) (Gonzalez-Quintela et al., 2007). Pro-inflammatory cytokine levels of two to three times greater than control values have been reported, elevations of similar magnitude to those found in other chronic inflammatory conditions such as rheumatoid arthritis (Gottenberg et al., 2012) and depression (Krishnadas and Cavanagh, 2012). Circulating cytokines are well-recognised to be of clinical relevance to alcoholic liver disease (Thurman, 1998); and raised cytokines related to innate and T cell function, TNF- α and IL-2, are associated with more advanced stages (Vidali et al., 2008), (Gonzalez-Quintela et al., 2008), (Laso et al., 1998). This chapter sets out the evidence that circulating cytokines may also be of

relevance to the brain in alcohol dependence and describes an experiment that aims to characterise the relationship between circulating cytokines, alcohol withdrawal and cognitive function in early abstinence.

2.1.2 Cytokines communicate with the brain and may influence neurotransmission

Circulating cytokines may be of relevance to pathological processes in the brain because pro-inflammatory cytokines, TNF- α , IL-1 β and IL-6 communicate with the brain via the vagus nerve, passive diffusion in blood-brain barrier (BBB) deficient regions, specific BBB transporters and stimulation of perivascular macrophages, leading to cerebral cytokine expression and microglial activation (Dunn, 2006). Cytokines bind to specific receptors present on neurons and glia and influence neurotransmission and neuroendocrine function in numerous ways (Dunn et al., 2005) (Dunn, 2006). Of interest in alcohol dependence is the relationship between NMDA receptor function and IL-6, which is protective in some *in vitro* models of excitotoxicity when applied acutely (Yamada and Hatanaka, 1994) (Ali et al., 2000) but in others, increases damage when applied chronically (Qiu et al., 1998).

Furthermore, the hypothalamo-pituitary-adrenal (HPA) axis, the function of which is altered during alcohol withdrawal, is potently stimulated by peripheral pro-inflammatory cytokines, particularly IL-1 β and TNF- α (reviewed in (Haddad et al., 2002)). There are also behavioural, emotional and cognitive changes associated with increased expression of circulating pro-inflammatory cytokines (reviewed in (Dantzer and Kelley, 2007)). For example, 'sickness

behaviour' characterised by disrupted sleep, fatigue, irritability and low mood in response to an infection, is thought to occur via cytokine-related modulation of the HPA axis, and altered processing in midbrain and limbic structures (Harrison et al., 2009a), (Harrison et al., 2009b). Of relevance to this study is that a recent pre-clinical study found similar behavioural alterations during alcohol withdrawal to those observed following injection of lipopolysaccharide (LPS), a bacterial protein used to induce sickness behaviour (Richey et al., 2012).

As well as clinical evidence of peripheral cytokine elevation in alcohol withdrawal, there is pre-clinical evidence of elevated cytokines in the brain during withdrawal. Cerebral expression of cytokines TNF- α , IL-1 β and CCL-2 (Qin et al., 2008) (Zhao et al., 2013), or their messenger RNA, an indirect measure of protein expression, has been reported during alcohol withdrawal (Whitman et al., 2013). Microglial activation associated with increased extracellular glutamate concentrations during withdrawal has also been described (Ward et al., 2009). Pre-clinical evidence suggests that peripheral inflammation may exacerbate withdrawal severity. Repeated peripheral injection of lipopolysaccharide, or injection of pro-inflammatory cytokines TNF- α or IL-1 β , or the chemokine CCL-2, sensitised animals to withdrawal-related anxiety in a similar fashion to psychological stress (Breese et al., 2008), perhaps in part via modulation of the HPA axis (Knapp et al. 2008 2008). Longitudinal studies found elevated IL-6 (Chatzipanagiotou et al., 2010), TNF- α , and IL-10 (Gonzalez-Quintela et al., 2000) (Leclercq et al., 2012) early in withdrawal, when withdrawal symptoms were most intense, but reduced one

week later. IL-12 and CXCL-8 have been measured in a single longitudinal study, which showed no changes (Gonzalez-Quintela et al., 2000).

2.1.3 Circulating cytokines: a link with depression and cognitive impairment in alcohol dependence?

In addition to their potential relationship to alcohol withdrawal severity, cytokines may be related to common complications of alcohol dependence, that is, depression and cognitive impairment. Increased circulating cytokines have been described in depression. In particular, raised peripheral blood TNF- α , IL-1 β , IL-6, and IL-2R have been found consistently (for recent meta-analyses, see (Dowlati et al., 2010), and (Liu et al., 2012)) and have clinical relevance, as higher IL-6 may predict poor treatment response (Yoshimura et al., 2009). A single study investigated the relationship between depression and cytokines in an alcohol-dependent population found an inverse association between depressive symptoms and the anti-inflammatory cytokine IL-10 after one week of abstinence, but no associations with pro-inflammatory cytokines TNF- α and IL-6 (Leclercq et al., 2012). This analysis, however, did not take into account alcohol withdrawal severity, which could influence depressive symptoms.

Inflammatory signals including cytokines, prostaglandins and inducible nitric oxide, may also be involved in cognitive impairment in alcohol dependence. Pre-clinical binge alcohol models have shed light on three aspects. First, microglial activation has been associated with perseverative behaviour following alcohol administration (Obernier et al., 2002). Second,

prostaglandin expression is associated with neuronal death following alcohol administration (Pascual et al., 2007). Third, indomethacin pre-treatment prevents neuronal death caused by alcohol (Pascual et al., 2007). Peripheral cytokines are associated with cognitive impairment in a variety of other disorders. In Alzheimer's disease, higher serum TNF- α concentrations were associated with cognitive deterioration (Holmes et al., 2009). Higher IL-6 concentrations are associated with worse cognitive function in healthy older adults (Mooijaart et al., 2013), in heart failure (Athilingam et al., 2013) and in depression (Elderkin-Thompson et al., 2012). Finally, increased serum and CSF concentrations of CCL-2, a pro-inflammatory chemokine, have been associated with Alzheimer's Disease (Sun et al., 2003) (Westin et al., 2012).

In summary, pre-clinical evidence suggests that alterations in peripheral cytokine levels may be involved in the pathogenesis of alcohol withdrawal, and observational clinical studies suggest that peripheral cytokines are raised during alcohol withdrawal. There is also pre-clinical and clinical evidence to suggest that peripheral pro-inflammatory cytokines may be important in depression and cognitive impairment, two common complications of alcohol dependence. In particular, pro-inflammatory cytokines TNF- α , IL-1 β and IL-6, T cell cytokine IL-2R, and chemokines CCL-2 and CXCL-8, are of interest. Although increased concentrations of a number of serum cytokines in alcohol dependence have been described, relatively few have been measured, so a broader knowledge of the cytokine milieu is lacking.

2.2 Aims and Hypotheses

Against this backdrop, the aims of this study were:

1. To identify changes in circulating cytokines and chemokines occurring in a naturalistic patient sample during alcohol detoxification,
2. To elucidate the relationship between cytokines and clinical symptoms in the sample.

Based on previous evidence, I hypothesised that both pro-inflammatory (TNF- α , IL-1 β , IL6) and anti-inflammatory cytokines (IL-10, IL-1RA) would decrease during detoxification, and that pro-inflammatory cytokines would be related to severity of withdrawal. Further, I hypothesised that cytokines and chemokines previously identified in association with depression – TNF- α , IL-6, IL-10 and IL-2R – and cognitive impairment – TNF- α , IL-6, and CCL-2 – in non-alcohol-dependent populations, would be associated with a simple measure of depression and performance on brief cognitive tests in an alcohol-dependent population. In the absence of existing evidence, exploratory analyses were undertaken to characterise the time course of remaining cytokine and chemokine levels during detoxification, and their relationships with one another.

2.3 Methods

2.3.1 Participants and recruitment

59 participants were recruited on admission to a dedicated addiction psychiatric in-patient unit for elective detoxification from alcohol between July 2011 and February 2012. Assessment of capacity to consent to research was performed prior to taking consent. Informed consent was taken during the

admission clerking or later on the day of admission, when participants were judged no longer incapacitated by intoxication. All patients met criteria for alcohol dependence according to ICD-10 criteria, as judged by the admitting psychiatrist. Exclusion criteria included inability to follow the procedures of the study, recent participation in a clinical trial and recent significant blood loss. Patients who developed pneumonia were excluded, as the associated increase in circulating cytokines would be likely to overwhelm any change relating to alcohol withdrawal (McInnes, personal communication), but minor soft tissue infections were allowed. Five participants withdrew consent before the first data collection point. Three further patients were diagnosed with pneumonia during their stay and their data are excluded from analysis.

Clinical information regarding participants' physical and psychiatric co-morbidity was obtained from patient interview, information from GP records, and blood tests. Current co-morbidity was diagnosed clinically and with information from blood tests and medical notes. For example, alcoholic hepatitis was considered to be present if the patient had right upper quadrant pain, elevated liver function tests, and elevated bilirubin, but no previous diagnosis of cirrhosis. Previous co-morbidity was obtained from GP records, as acute hospital or specialist out-patient records were not routinely available in mental health trusts. Caine criteria for risk of Wernicke-Korsakoff syndrome (Caine et al., 1997) were derived from patient interview and medical notes.

Patients were visited three times: in early detoxification (day 2-3 of admission), mid-detoxification (day 4-6) and late (day 7-10) detoxification. All

visits occurred between 8am and 10am in the morning. Visits were necessarily brief so as not to interfere with administration of medications and the programme of psychosocial interventions on the ward. The revised Clinical Assessment of Withdrawal from Alcohol (CIWA-Ar) (Sullivan et al., 1989) was administered. Visual analogue scales for depression, anxiety and craving, anchored by statements such as 'not at all depressed', 'moderately depressed' and 'the most depressed ever', were completed. Patients completed paper and pencil cognitive tests during the final visit. Trail Making Tests A and B (Reitan, 1958), and phonemic verbal fluency were chosen as tests of frontal lobe function, and the Weschler Memory Scale paragraph test (Weschler, 1987) was chosen as a test of verbal memory. These were chosen as they were easy and quick to administer, and are known to be sensitive to cognitive impairment secondary to alcohol dependence in 'healthy' alcohol-dependent patients (Davies et al., 2005).

2.3.2 Blood measures and analysis

Blood was taken for cytokine concentration, high sensitivity C reactive protein (CRP) as a general measure of systemic inflammation and gamma glutamyl transferase as a marker of hepatic oxidative stress between 8am and 10am and transported on ice. Blood samples were centrifuged (1300G, 10 min) and serum stored in sterile microcentrifuge tubes at -80°C within 90 minutes of collection.

Samples for cytokine analysis were analysed by collaborators at the University of Glasgow using Luminex Multiplex Human 25-plex plates

(Luminex, Austin, USA) performed in duplicate. Each well in the Luminex plate contained a specified number of populations of micropores, small spheres stained with red and orange fluorescence in different proportions, such that different populations have different spectroscopic signatures (Vignali, 2000). In each population of micropores a separate assay for a single cytokine occurs, similar to an ELISA. Each micropore carries capture antibodies for a single cytokine. A green fluorescence-labelled detection antibody is incubated with the body fluid of interest in the well containing the micropores. The Luminex machine reads the red and orange fluorescence from the micropores in order to distinguish different populations of micropores and the amount of green fluorescence attached to each. By measuring the green fluorescence against the amount detected using internal standards, it is possible for the machine to quantify the amount of each cytokine present in the fluid. High sensitivity CRP (0.3 – 500mg/dl) and gamma glutamyl transferase were analysed by clinical biochemistry services at the Hammersmith Hospital.

Patients were asked to collect salivary cortisol samples on waking, and at 15 minute intervals for the following 45 minutes, at the same three stages of detoxification. These samples were analysed using a standard ELISA assay (DRG Salivary Cortisol ELISA, DRG Instruments GmbH, Germany), performed in duplicate. In addition, serum cortisol and cortisol binding globulin was analysed by Hammersmith Hospital biochemistry services. This will be discussed in Chapter 3.

2.3.3 Statistical analysis

51 participants were included in the analysis. Data were inspected graphically via Q-Q plots to identify distribution and outliers and D'Agostino-Pearson omnibus normality tests implemented in GraphPad Prism Version 6 were performed, with information from both used to make decisions about normality. Those data with a log-normal distribution were log-transformed to aid comparison of means and standard deviations, and for statistical procedures assuming a normal distribution.

There were a number of cytokines where several data points were below the limit of detection (LOD) of the assay, as shown by Table 2.1. Where this affected more than 25% of the sample (TNF- α , IL-1 β , IFN- γ , IL-2, IL-4, IL-7, IL-15, IL-17, CXCL-9, GM-CSF), the cytokines were excluded from further analysis. TNF- α and IL-1 β are known to be difficult to detect. For this reason, IL-1RA, which antagonises the action of IL-1 β and as such is regulated as a counter-balance, was used as a surrogate marker for IL-1 β as it has been previously (Maes, 2011). For those cytokines with less than 25% values below the LOD, values were estimated using maximum likelihood estimation (MLE), implemented in Matlab (Mathworks, 2008). MLE is an accepted method that produces less biased estimates in data with a known distribution than simpler methods such as LOD/2 (Helsel, 2010). Given that cytokines are known to follow a normal or log-normal distribution, this was the most appropriate choice.

The other form of missing data in the study resulted from missed visits or unrecorded information in the medical notes, withdrawal of consent, or self-discharge (summarised in Table 2.2). Two patients missed their first visit, four their second, and four their final visit. I assumed that this missing data could be considered Missing At Random (MAR) and Missing Completely at Random (MCAR), as 'missingness' was likely to be explained either by clinical factors known to the research team, such as poor liver function necessitating admission to the acute hospital, or by completely random factors, such as the morning group meeting starting early (Donders et al., 2006). This form of missing data was handled where possible using statistical models that accommodated missing values, such as mixed models implemented in SPSS 20.0, or in the case of the multivariate analysis of variance, via a complete case analysis.

Mixed statistical models were implemented in SPSS 20.0 to examine changes in cytokine concentrations over time, and explore how cytokines relate to withdrawal symptoms, depression, and craving. Mixed statistical models are able to handle incomplete datasets, adjust for differences in variance across time points using different covariance structures, and can be used to examine relationships between two sets of repeated measures variables. The covariance structure for the models was chosen to optimise model fit according to the Akaike Information Criteria (Akaike, 1974) and Likelihood Ratio statistic and model fit examined graphically. In the case of mixed models used to examine the effects of cytokine titres on clinical symptoms, the number of independent variables examined in each model was restricted

to a maximum of five, using the rule of thumb that ten participants are required per variable examined (Field, 2012). A Bonferroni correction for multiple comparisons was performed to set a significance level for the main comparisons relating to cytokine changes over time; significance for exploratory hypotheses was set at $p < 0.05$. Exploratory analysis of the relationships between different circulating cytokines was undertaken using bivariate parametric correlations at each time point. These are displayed below via graphical correlation 'heatmaps' using Excel (Microsoft).

Multivariate analysis of variance (MANOVA) was used to ascertain the relationship between cytokines and performance on cognitive tests. Since performance on different cognitive tests is consistently reported to be highly correlated, a multivariate analysis was used to ascertain whether there was a relationship between cytokine levels and 'cognitive function', a linear composite of performance on the following tests: Trails A score, Trails B score, FAS score, and both immediate and delayed Weschler Memory Scale paragraph version. A median split of the cytokine concentrations was used to generate 'high' and 'low' concentration groups for comparison, as this was necessary in order to perform a multivariate analysis. A complete case analysis was performed as MANOVA excludes missing values and SPSS does not support a MANOVA using multiply imputed data. Levene's equality of variances and Box's test were checked to ensure model assumptions were met. Post-hoc univariate ANOVAs were employed to investigate particular relationships between cytokines and verbal memory and frontal lobe function,

where a significant relationship between a cytokine and ‘cognitive function’ had been found, using a Sidak correction for multiple comparisons.

Table 2.1: Proportion of cytokines above the assay limit of detection

Cytokine	% of samples with detectable values
Pro- and anti-inflammatory cytokines	
IL-1 β	48
TNF- α	32
IL-6	96
GM-CSF	25
IL-10	80
IL1-RA	93
Type 1 interferon	
IFN- α	100
Cytokines related to T cell activation	
IL-2	14
IL-7	14
IL-15	43
IL-2R	100
Th1 cytokines	
IL-12	100
IFN- γ	8
Th2 cytokines	
IL-4	41
IL-5	75
IL-13	100
Th17 cytokines	
IL-17	10

Cytokine	% of samples with detectable values
Chemokines	
CXCL-8 (IL-8)	92
CXCL-9 (MIG)	62
CXCL-10 (IP-10)	100
CCL-2 (MCP-1)	100
CCL-3 (MIP-1 α)	100
CCL-4 (MIP-1 β)	100
CCL-5 (RANTES)	100
CCL-11 (Eotaxin)	100

Table 2.2: Summary of data missing completely at random/missing at random

Variables	Number missing	% missing
Caine criteria	7	13.7%
Trails A&B	6	11.8%
Weschler Memory Scale, FAS	5	9.8%
Bloods and psychological measures (time point 3)	4	7.8%
Bloods (time point 1 and time point 2)	3	5.9%
Recent alcohol use	1	2.0%

2.4 Results

2.4.1 Clinical characteristics

Patient demographics are presented in Table 2.3. The mean alcohol intake per day over the previous month was 33 UK standard alcohol units (259 grams) per day. Patients had undergone a median of two detoxifications previously with the majority (54%) having a history of previous alcohol

withdrawal seizures. The median number of Caine criteria was 1 (range 0 – 4). Co-morbid nicotine dependence was present in a large proportion (68%) of patients and cannabis, cocaine and heroin dependence in a sizable minority (11%).

Table 2.3: Clinical characterisation: Demographic features and substance misuse

Demographic details	
Age	47 ± 12
Gender	33 M : 18 F (65% M)
Years of drinking	33 ± 13
Mean alcohol intake/day in month prior to admission (Standard Alcohol Units)	33 ± 15
Mean alcohol intake/day in month prior to admission (g)	259 ± 105
Number of previous detoxifications	2 (range 0 – 13)
History of alcohol withdrawal seizures	28 (54%)
Caine criteria	1 (range 0 – 4)
Co-morbid drug use	
Nicotine	35 (68%)
Cannabis	11 (20%)
Cocaine	6 (11%)
Opiates	6 (11%)

Physical co-morbidity is described in Table 2.4. The most common physical co-morbidity was concurrent gastrointestinal inflammation (25%). A significant minority suffered from symptomatic alcoholic liver disease: alcoholic hepatitis (18%) or decompensated cirrhosis (10%). Clinical blood tests on admission (Table 2.5) showed that with the exception of liver enzymes, blood parameters were in the normal range on admission.

Table 2.4: Clinical characterisation: physical co-morbidity

Current physical co-morbidity	
Liver	
Cirrhosis	7 (14%)
Decompensated cirrhosis	5 (10%)
Chronic viral hepatitis	4 (8%)
Alcoholic hepatitis	9(18%)
Gastrointestinal tract	
GI inflammation	13 (25%)
Chronic pancreatitis	2 (4%)
Other systemic sources of inflammation	
Soft tissue infections	4 (8%)
COPD	4 (8%)
Connective tissue disease	2 (4%)
Diabetes mellitus	2 (4%)
Atherosclerosis	3 (6%)
Psoriasis	1 (2%)

Table 2.5: Routine clinical blood results on admission

Blood marker	Mean ± SD	Geometric mean	Normal range
White cell count	6.5 ± 2.2	6.2	3 – 10
Platelets	162 ± 81	140	150 – 400
Albumin	43 ± 5	43	34 – 50
Bilirubin	15 ± 15	11	0 – 20
Alanine transferase	64 ± 47	50	10 – 35
Alkaline phosphatase	118 ± 57	107	35 – 105
Gamma glutamyl transferase	625 ± 846	258	6 – 42

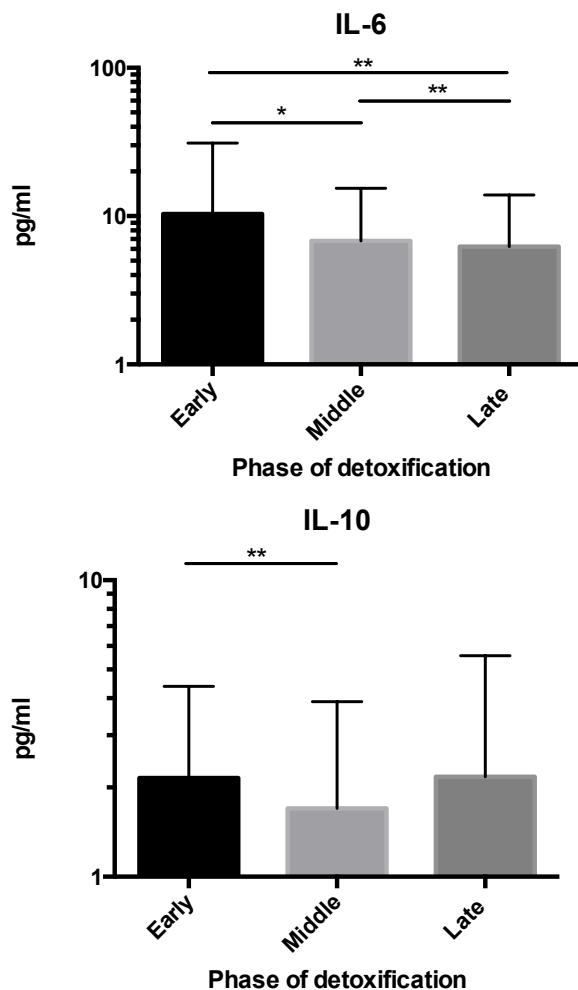
This table shows those blood values that were related to inflammation or to liver function, taken from routine clinical blood tests on admission. CRP, MCV and INR were not routinely recorded so are not included.

2.4.1 Changes in pro-inflammatory and anti-inflammatory cytokines during detoxification

Cytokine and chemokine values and statistical test results are presented in Tables 2.6 and 2.7. There was a significant association with time point for IL-

6 ($p = 0.003$) and IL-10 concentrations ($p = 0.000$) (see Figure 2.1). Post-hoc Bonferroni corrected pair-wise comparisons found that IL-6 decreased in a progressive way with significant differences between each time point (early>middle: $p = 0.015$; middle>late: $p = 0.003$), while IL-10 significantly decreased early in withdrawal only (early>middle: $p = 0.000$). IL-1RA did not change significantly. Neither TNF- α nor IL-1 β were detectable in the majority of samples.

Figure 2.1: IL-6 and IL-10 decrease significantly during detoxification

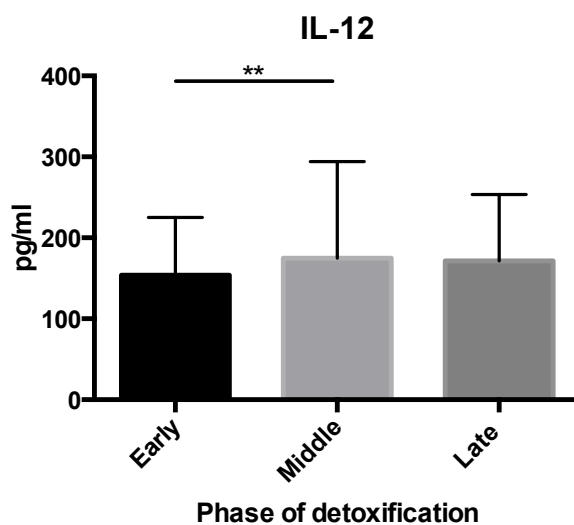


Legend: Stars denote Bonferroni-corrected significant post-hoc pairwise comparisons: * $p < 0.05$; ** $p < 0.01$

2.4.2 Changes in T cell cytokines during detoxification.

The T cell regulatory cytokine IL-12(p35/p40) changed significantly during detoxification ($p = 0.000$). Post-hoc pair-wise comparisons indicated that there was a significant increase between early and mid-detoxification ($p = 0.000$). IL-2R, IL-5 and IL-13 did not change significantly. IL-2 and IL-4 were not detectable in a majority of samples.

Figure 2.2: IL-12 increases significantly during detoxification

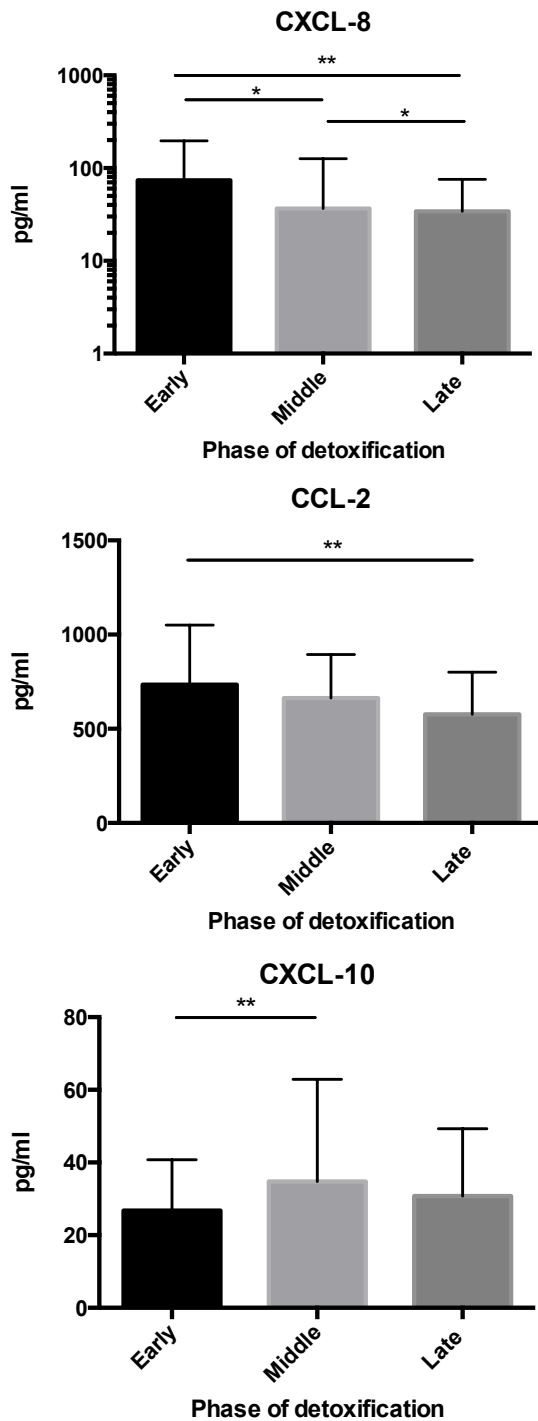


Legend: Stars denote Bonferroni-corrected significant post-hoc pairwise comparisons: * $p < 0.05$; ** $p < 0.01$

2.4.3 Changes in chemokines during detoxification

Both CXCL-8 and CCL-2 changed significantly during detoxification (CXCL-8: $p = 0.001$; CCL-2: $p = 0.003$). Post-hoc pair-wise comparisons indicated that CCL-2 was lower at the later time point relative to the early time point ($p = 0.001$), CXCL-8 decreased progressively (early>middle: $p = 0.032$; middle>late: $p = 0.011$; early>late: $p = 0.000$). CXCL-10 changed significantly during detoxification ($p = 0.003$). The change characterized by post-hoc testing was a significant increase occurring between early and mid detoxification (early<mid: $p = 0.003$). CCL-3, CCL-4, CCL-5 and CCL-11 did not change significantly during detoxification.

Figure 2.3: CXCL-8 and CCL-2, while CXCL-10 increases during detoxification



Legend: Stars denote Bonferroni-corrected significant post-hoc pairwise comparisons: * $p < 0.05$; ** $p < 0.01$

2.4.4 Changes in CRP and GGT during detoxification

High sensitivity C-reactive protein (hsCRP) changed significantly during detoxification ($p = 0.000$). Post-hoc analysis revealed that hsCRP increased between the early and middle time point ($p = 0.000$) but then decreased between the middle and late time point ($p = 0.009$) though at the late time point it was still higher than the early time point ($p = 0.011$). Gamma glutamyl transferase decreased significantly ($p = 0.000$) and progressively (early>middle: $p = 0.001$; middle>late: $p = 0.003$).

Table 2.6: Descriptive statistics (mean, SD, and geometric mean for log-transformed cytokines)

Cytokine	Early detoxification	Mid detoxification	Late detoxification
Pro- and anti-inflammatory cytokines			
Log IL-6	1.57 ± 1.267	1.40 ± 1.057	1.17 ± 1.252
IL-6	4.81	4.06	3.98
Log IL-10	0.33 ± 0.932	0.06 ± 0.898	0.20 ± 0.965
IL-10	1.39	1.06	1.21
Log IL-1RA	3.64 ± 1.409	3.73 ± 1.534	3.86 ± 1.399
IL-1RA	38.22	42.05	42.36
Type 1 interferon			
Log(IFN- α)	3.43 ± 0.429	3.39 ± 0.498	3.34 ± 0.673
IFN- α	31.0	29.6	29.6
Cytokines related to T cell activation			
IL-2R	137 ± 63.37	155 ± 127.1	159 ± 144.0
Log IL-5	0.97 ± 0.608	0.98 ± 0.617	1.00 ± 0.588
IL-5	2.62	2.65	2.66
Log IL-12	4.97 ± 0.343	5.05 ± 0.382	5.07 ± 0.352
IL-12	144	157	153
Log IL-13	1.78 ± 0.423	1.78 ± 0.447	1.83 ± 0.430
IL-13	5.92	5.95	6.04

Cytokine	Early detoxification	Mid detoxification	Late detoxification
Chemokines			
Log CXCL-8 (IL-8)	3.30 ± 1.161	3.09 ± 1.511	2.75 ± 1.488
CXCL-8	27.00	21.95	21.12
Log CXCL-10 (IP-10)	3.30 ± 0.689	3.48 ± 0.747	3.40 ± 0.674
CXCL-10	27.0	32.4	29.7
CCL-2 (MCP-1)	733 ± 315.9	646 ± 240.6	570 ± 222.0
Log CCL-3 (MIP-1 α)	3.34 ± 0.557	3.34 ± 0.627	3.29 ± 0.640
CCL-3	28.2	28.5	27.8
Log CCL-4 (MIP-1 β)	4.19 ± 0.560	4.12 ± 0.649	4.21 ± 0.604
CCL-5	66.0	61.4	64.94
Log CCL-5 (RANTES)	8.36 ± 0.446	8.36 ± 0.274	8.40 ± 0.307
CCL-5	4274	4283	4336
CCL-11 (Eotaxin)	61.7 ± 30.32	56.3 ± 25.40	56.0 ± 27.18
Acute phase proteins			
Log(CRP)	1.05 ± 1.189	1.77 ± 1.051	1.45 ± 1.078
CRP	2.85	5.88	4.14
Oxidative stress markers			
Log(GGT)	5.44 ± 1.466	5.38 ± 1.365	5.31 ± 1.191
GGT	230	216	216

Legend: Mean and standard deviations (cytokines in ng/ml and log-transformed cytokines) and geometric mean (ng/ml) provided for cytokines that were log-transformed for analysis.

Table 2.7: Variation in cytokines and CRP during detoxification

	Df	F statistic	p
Pro- and anti-inflammatory cytokines			
Log IL-6	2, 90.114	6.106	0.003**
Log IL-10	2, 90.714	8.679	0.000***
Log IL-1RA	2, 92.656	0.908	0.407
Type 1 interferon			
Log(IFN- α)	2, 86.998	1.028	0.362
Cytokines related to T cell activation			
IL2R	2, 89.339	0.675	0.512
Log IL-5	2, 92.016	0.269	0.765
Log IL-12	2, 93.031	12.703	0.000***
Log IL-13	2, 90.603	2.297	0.106
Chemokines			
Log CXCL-8 (IL-8)	2, 90.776	7.209	0.001**
Log CXCL-10 (IP-10)	2, 91.089	6.204	0.003**
CCL-2 (MCP-1)	2, 87.078	6.388	0.003**
Log CCL-3 (MIP-1 α)	2, 89.87	3.346	0.040*
Log CCL-4 (MIP-1 β)	2, 91.223	1.721	0.185
Log CCL-5 (RANTES)	2, 86.973	0.872	0.422
CCL-11 (Eotaxin)	2, 89.083	1.869	0.160
Acute phase proteins			
Log(CRP)	2, 88.177	17.916	0.000***

Legend: **p <0.01; *** p <0.001.

2.4.5 Relationship between cytokines at each time point during detoxification

Correlations between cytokines at each time point are graphically displayed in Figures 2.4a – c as ‘heatmaps’. Of interest is that the cytokines and chemokines that changed during detoxification are not correlated with many other cytokines or chemokines, that is, they did not appear to function as a network at all time points. IL-6 is strongly inversely associated with CCL-5 at all time points but is correlated with Th-2 related cytokines at the mid and late detoxification time points only. IL-10 is correlated with the T cell regulatory

cytokine IL-2R throughout detoxification, but is associated with the chemokines CCL-2, 3, and 4 only at the mid and late time points. IL-12 is associated with the anti-inflammatory IL-1RA and the chemokines CCL-5 and CXCL-10 at all time points. CCL-2 is associated with CCL-11 at the early and late time points, but not the mid time point, and with IL-10 during mid and late detoxification. CXCL-8 is inversely related to CCL-5 throughout detoxification. In contrast, the cytokines and chemokines that did not change significantly during detoxification are highly correlated with many other cytokines and chemokines, and the pattern of correlations is similar at different time points. For example, IL-2R, is positively correlated with six to eight cytokines during detoxification.

2.4.6 Changes in clinical symptoms during detoxification

Mean CIWA-Ar score during early detoxification was 10 (with a standard deviation of 7), corresponding to severity meriting pharmacological treatment. At mid-detoxification it reduced significantly in severity (for $\log(\text{CIWA})$: $p = 0.001$), to 6 (± 4), and remained 6 (± 5) during late detoxification (post-hoc: early>middle: $p = 0.032$; early>late: $p = 0.001$). Depression also reduced significantly during detoxification ($\log(\text{Depression})$: $p = 0.04$) from 42 (± 31), a measurement approaching 'quite depressed', to 34 (± 31) then to 30 (± 30) corresponding to 'a little depressed'. Craving decreased significantly from a mean of 37 (± 34) early in detoxification, to 25 (± 29) mid detoxification, then 17 (± 25) in late detoxification (for $\log(\text{Craving})$ $p = 0.002$).

Figure 2.4a: Heatmap of bivariate correlations between cytokines in early detoxification

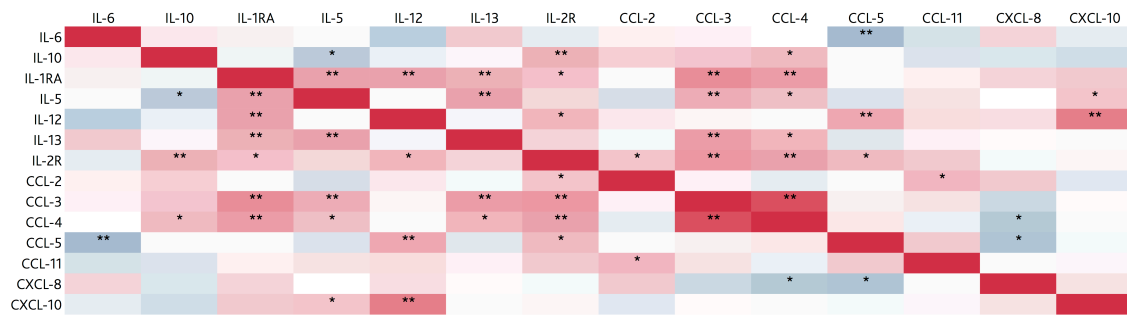


Figure 2.4b: Heatmap of bivariate correlations between cytokines in mid detoxification

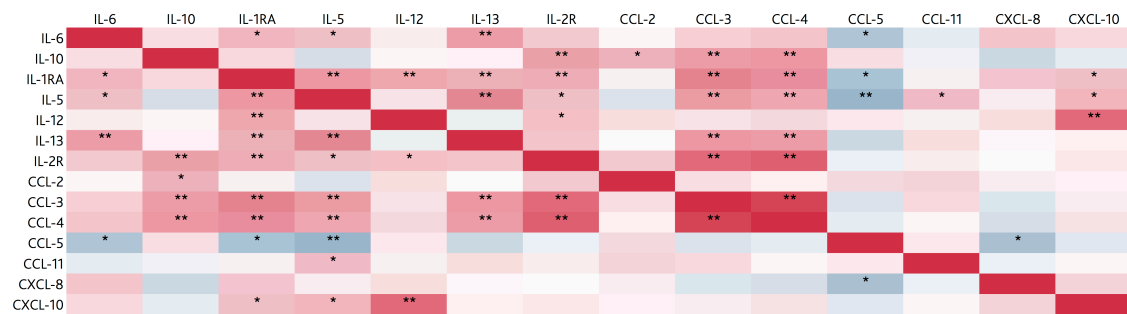
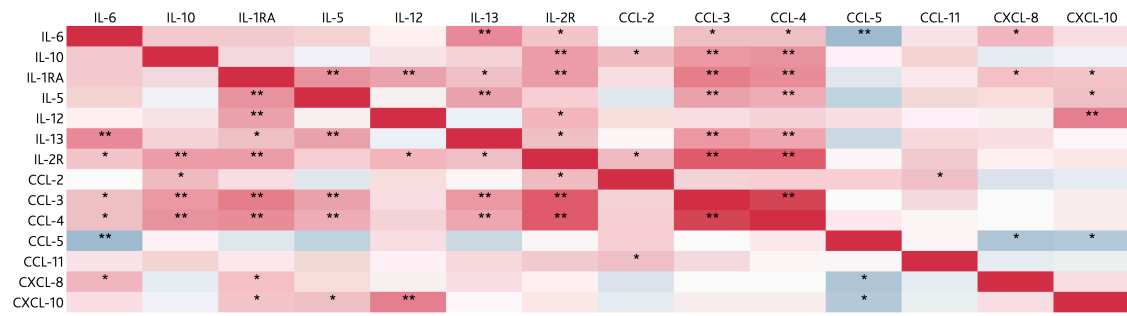


Figure 2.4c: Heatmap of bivariate correlations between cytokines in late detoxification



Legend: The heatmaps show positive (red) and negative correlations (blue) where the intensity of the colour is related to the value of Pearson's r. A key is displayed next to Figure 4a. * denotes $p < 0.05$. ** denotes $p < 0.01$.

Colour key:

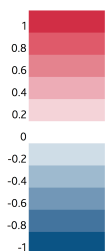
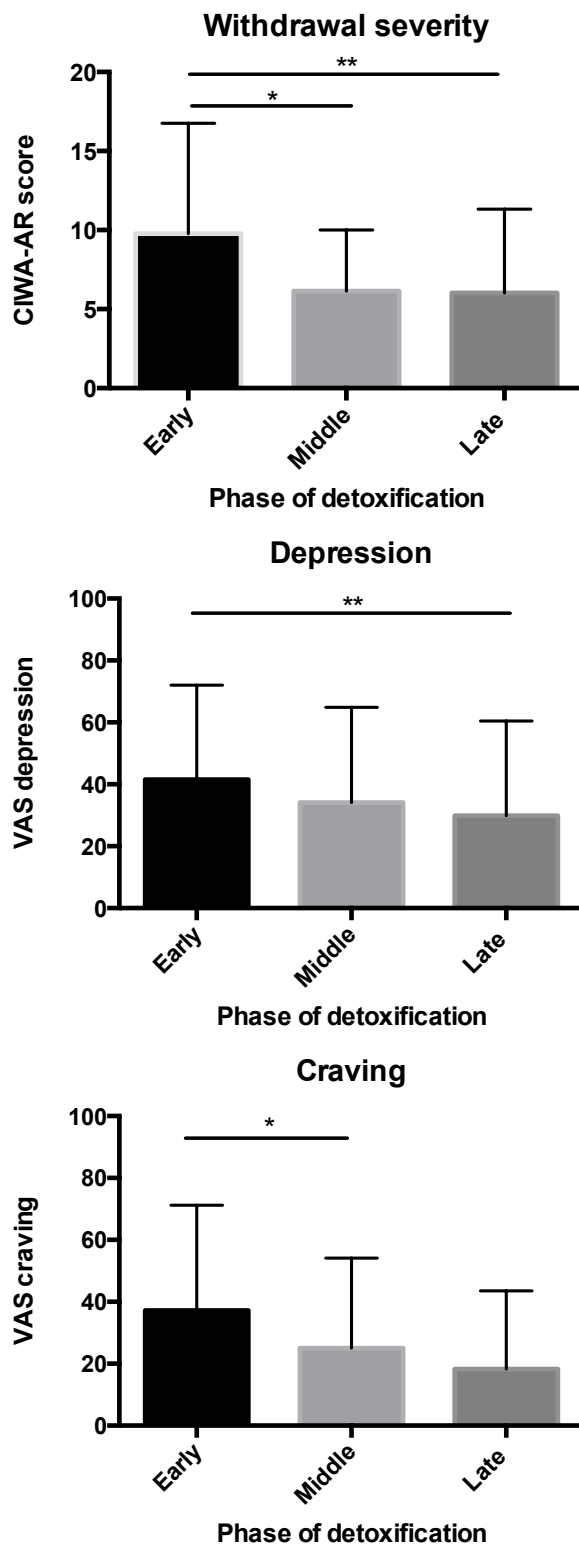


Figure 2.5: Withdrawal symptoms, depression and craving decrease during detoxification



Legend: Stars refer to Bonferroni-corrected significant post-hoc pair-wise comparisons: * $p < 0.05$; ** $p < 0.01$

2.4.5 Relationship between cytokines and clinical symptoms during alcohol detoxification

There was a significant positive association of IL-6 concentrations with withdrawal severity across all time points (log(IL-6) $F(1, 92.323) 7.060, p = 0.009$). When recent alcohol use, previous detoxifications and time point were controlled for, the positive association remained significant ($F(1, 91.626) = 4.851, p = 0.030$). The effect size was 0.125 (95% confidence intervals 0.012 – 0.237) implying a 12.5% change in withdrawal severity for each percentage change in IL-6.

There was also a significant positive association between IL-6 concentrations and depression (effect size 0.356, 95% C.I. 0.125 - 0.587, $F(1, 98.318) 3.063, p = 0.003$). This association persisted when antidepressant treatment and withdrawal severity were taken into account, with an adjusted effect size of 0.245 (95% C.I. 0.014 – 0.476, $F(1, 104.581) 4.435, p = 0.038$). Withdrawal severity was also significantly **positively** associated with depression (effect size 0.582, 95% C.I. 0.271 – 0.893, $F(1, 110,75) 13.756, p = 0.000$), but IL-2R and antidepressant treatment were not ($p > 0.05$).

2.4.6 Relationship between cytokines and chemokines, and cognition

Performance on cognitive tasks in this population fell below reported population norms, though a statistical comparison was not possible given that we did not have a control group. The mean score for phonemic verbal fluency was 8.33 ± 3.45 , below the age-related norm of 40 ± 10.7 – adjusted for 0-8 years of education (Tombaugh et al., 1999). Similarly, the mean number of

minutes to complete Trail Making Task A was 59 ± 37 seconds and Task B was 155 ± 90 seconds while reported means and ranges for a similar normal age group are 31 seconds (range 18-56 seconds) and 64 seconds (range 32 – 92 seconds) respectively (Tombaugh, 2004). Mean performance on immediate recall in the Weschler Memory Scale paragraph test was 6 ± 2.3 and on delayed recall was 4 ± 2.7 . Published age-matched norms are 13 ± 3.3 and 9 ± 3.9 respectively (Abikoff et al., 1987).

CCL-2 was found to be associated positively with 'cognitive function', a linear composite of performance on the following tests: Trails A score, Trails B score, FAS score, Weschler Memory Scale paragraph score (immediate) and Weschler Memory Scale paragraph (delayed) (Pillai's trace; $V = 0.331$ $F(5, 37) = 3.655$; $p = 0.009$). The association persisted when age and recent alcohol dose (alcohol units/day) had been taken into account (Pillai's trace; $V = 0.388$; $F(4, 35) = 5.536$; $p = 0.001$). Post-hoc univariate ANOVAs showed that CCL-2 concentration was positively associated with performance on Trails A ($F(1, 38) = 14.839$; $p = 0.000$), Trails B ($F(1, 38) = 9.522$; $p = 0.004$) and the delayed Weschler Memory Scale ($F(1, 38) = 6.935$; $p = 0.012$). Post-hoc contrasts showed that high CCL-2 was associated with better cognitive function in the following tasks ($p < 0.05$): completing the Trail Making Tasks (log(Trails A time)) was 0.479 (0.177 – 0.852) longer in the low CCL-2 group than the high CCL-2 group; for log(Trails B time) the difference was 0.515(0.177 – 0.852). Similarly, patients remembered 1.725 (95% C.I. 0.399 - 3.05) fewer phrases in the delayed Weschler Memory Task in the low CCL-2 group relative to the high CCL-2 group. IL-6 and CXCL-8 concentrations were

not associated with cognitive function. Recent alcohol use was associated with poorer cognitive function in the multivariate analysis ($V = 0.282$; $F(4, 38) = 3.434$; $p = 0.018$). Post-hoc univariate analyses indicated that recent alcohol use had a significant effect on delayed verbal memory ($F(1, 38) = 10.134$; $p = 0.003$). There was no overall effect of age on cognitive function in the multivariate analysis.

2.5 Discussion

This study is the first to measure twenty-five circulating cytokines and chemokines at three time points during alcohol detoxification in a large naturalistic cohort of alcohol-dependent in-patients, generating a highly comprehensive profile of cytokine expression. The findings from this cohort replicated the findings from studies measuring only a handful of cytokines: that the concentrations of IL-6 and IL-10 decrease significantly during detoxification. I have demonstrated an association between IL-6 and IL-10 and hepatic oxidative stress, suggesting a hepatic origin for these cytokines. I have demonstrated for the first time that the Th-1 cytokine IL-12 and the chemokine CXCL-10 increase during detoxification, while the inflammatory chemokines CXCL-8 and CCL-2 decrease. While cytokines and chemokines which did not change were highly positively correlated with one another, those which did change were correlated with few other cytokines and chemokines. Of note, IL-6 was related to both withdrawal severity and depression during detoxification and this association persisted even when other factors that would influence these symptoms, for example withdrawal severity in the case of depression, were accounted for. I found an association between the

inflammatory chemokine, CCL-2, and better cognitive function, though the difference in cognitive task performance between those participants with high CCL-2 and those with low CCL-2 was small.

The characteristics of the sample were broadly representative of patients in UK alcohol inpatient treatment services. Their mean age was 47, reflecting a typical age when individuals come into contact with health services for treatment of alcohol dependence (Pilling et al., 2011). There were some differences with the sample having a higher proportion of women than epidemiological studies of alcohol dependence (Drummond, 2005) (Proudfoot et al., 2002), a higher median number of detoxifications than in previous studies of cognition in alcohol dependence (Duka et al., 2003) and the majority (54%) had a history of withdrawal seizures. This likely reflects the clinical context of the study since in-patient detoxification is indicated for those with complications of withdrawal and end-organ damage. This explains the high prevalence of seizures, greater number of detoxifications – which make complications more likely – and proportion of women, who are more vulnerable to physical complications of alcohol dependence (Nolen-Hoeksema, 2004). The median score for Caine criteria of 1 suggested that most patients had missed meals but did not have clinical evidence of Wernicke-Korsakoff syndrome or other neurological complications of malnutrition. Co-morbid nicotine dependence and use of other illicit drugs were present in a greater proportion of participants than in the general population, but reflects rates of co-morbidity in substance misuse services. The physical co-morbidity reflected physical complications of chronic alcohol,

cigarette or drug intake. Although liver enzymes were increased, albumin and bilirubin were within normal limits, suggesting preserved synthetic and metabolic function. White cell counts were within normal limits suggesting no sepsis.

The decrease in IL-6 and IL-10 during detoxification is consistent with previous reports, which found differences in these cytokines between the day of admission for detoxification and a week later (Gonzalez-Quintela et al., 2000) (Leclercq et al., 2012). This study refines and extends earlier reports by establishing that IL-10 decreases early, and IL-6 continues to decrease progressively. Other cytokines TNF- α and IL-1 β were not detectable in our sample; such difficulty in detection in peripheral serum has previously been described (Maes, 2011). In our sample, IL-1RA, used as a surrogate marker for IL-1 β , did not change during detoxification. In addition to replicating previous findings that many cytokines decrease during detoxification, I have demonstrated for the first time that both the T cell regulatory cytokine IL-12(p40/p35), and chemokines change during detoxification. This is in contrast with a previous report, which found no change in CXCL-8 and IL-12 during detoxification (Gonzalez-Quintela et al., 2000). In contrast, CXCL-8 decreased and IL-12 increased significantly in our sample. The discrepancy may relate to the fact that in the previous study the second measurement took place over a wider range – from day 2 to day 15 – that may have obscured changes.

Chemokines other than CXCL-8 have not been studied previously in detoxification. While CCL-2 decreased, suggesting that its function in this context related to that of IL-6 and CXCL-8, CXCL-10 increased. CXCL-10 was first characterised as an IFN- γ induced chemokine, and has chemotactic effect on Th-1 T cells (Luster et al., 1985) (Eriksson et al., 2013). Taken together with the parallel increase in IL-12, this change indicates an increase in Th-1 function. In total, decrease in macrophage-associated cytokines, early suppression of Th-1 cytokines and no change in Th-2 cytokines are broadly consistent with the changes that would be expected in the context of increased sympathetic activity at the early, but not later time points. A possible implication is that this signature relates to alcohol withdrawal itself, as sympathetic hyperactivity is a feature of early withdrawal (reviewed in (Kahkonen, 2004)).

Of interest is that the cytokines that decreased during detoxification – IL-6, IL-10, CXCL-8 and CCL-2 – were not consistently strongly correlated with one another during detoxification. This suggests that they do not operate as part of a consistent cytokine network under these circumstances. It is probable that mediators that I did not measure, for example noradrenaline, and those that I was unable to detect, such as TNF- α , influenced their expression. This requires further investigation. IL-12 and CXCL-10 were positively correlated throughout detoxification suggesting that they form a single cytokine network that would be consistent with the Th-1 related function for both mediators.

This is the first study to report a relationship between IL-6 and withdrawal symptoms during alcohol detoxification. The finding remained significant when variance related to alcohol dose before detoxification, previous detoxifications and time point in detoxification was taken into account. The relationship between alcohol withdrawal and circulating cytokines is likely to be complex, because alcohol affects all components of the so-called 'gut-liver-brain axis' (Wang et al., 2010) (see Figure 2.6). Chronic alcohol consumption is associated with small bowel bacterial overgrowth and increased bacterial translocation with increased lipopolysaccharide (LPS) in the portal vein (Leclercq et al., 2012). LPS stimulates activation of Kupffer cells, hepatic macrophages, which produce cytokines such as IL-1 β , TNF- α and IL-6, and chemokines such as CCL-2 and CXCL-10 (Wang et al., 2010). IL-1 β , TNF- α and IL-6 access the brain via vagal afferents and via the stimulation of inflammatory signalling in cerebral endothelial cells, which in turn stimulate perivascular macrophages (Dantzer et al., 2008).

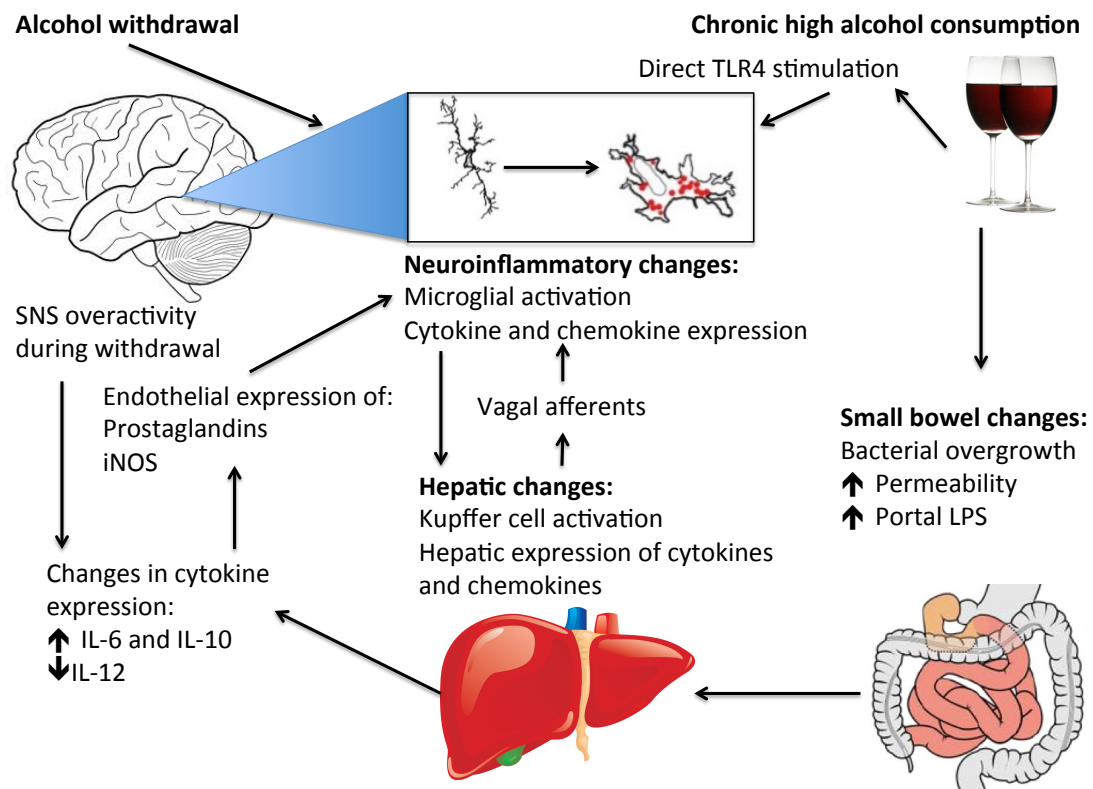
Even though circulating cytokines may not have come from the brain directly, the brain influences circulating cytokine production by the liver and spleen. Alcohol withdrawal is associated with increased cerebral expression of IL-1 β , TNF- α and CCL-2 (Zhao et al., 2013) (Qin et al., 2008) with similar, though shorter-lived, cytokine and chemokine expression in the liver (Qin et al., 2008). Central nervous system pro-inflammatory cytokine expression is reported to give rise to Kupffer cell activation, macrophage recruitment and expression of CCL-2 and CXCL-10 (Campbell et al., 2005), (Campbell et al., 2008).

Sympathetic hyperactivity is a hallmark of early alcohol withdrawal (reviewed in (Glue and Nutt, 1990) and (Kahkonen, 2004)). The effect of sympathetic activity on cytokine expression is complex, as adrenergic receptors on immune cells differ according to subset; but suppression of Th-1 associated cytokine expression such as IL-12 and increase in expression of IL-6, IL-10 and CXCL-8 has been described (reviewed in (Elenkov et al., 2000)). Although hepatic production of cytokines may also relate to increased bacterial translocation early in detoxification (Leclercq et al., 2012), the relationship between IL-6 and withdrawal symptoms across all time points suggest that the brain may be the predominating driver of hepatic cytokine and chemokine expression in this instance. In particular, increased bowel permeability and increased systemic LPS is not present at later time points in detoxification (Leclercq et al., 2012).

This study demonstrates for the first time that IL-6 concentrations are associated with depression in an alcohol-dependent population throughout detoxification. The association remained when withdrawal symptom severity was accounted for. This is consistent with studies in depressed populations (Dowlati et al., 2010) suggesting that IL-6 plays a key role in depression. No association between IL-10 and depression was found in our sample, although an inverse association between IL-10 in late detoxification, but not in early detoxification, has been reported previously (Leclercq et al., 2012). However, Leclercq and colleagues performed bivariate correlations at each separate time point and did not take withdrawal severity into account. Unlike studies in

depression, the current study found no association between IL-2R and depression suggesting that changes in adaptive immunity in depressed populations (Maes, 2011) are different from those with co-morbid alcohol dependence, at least during detoxification.

Figure 2.6: Relationship between alcohol dependence and circulating cytokines



Legend: This figure illustrates the relationships between peripheral inflammation and cerebral microglial activation and cytokine expression. Both peripheral inflammatory drive from portal LPS and cerebral stimulation of hepatic Kupffer cell activation and cytokine expression are likely to contribute to increased circulating cytokines in the setting of alcohol detoxification. iNOS = inducible nitric oxide synthetase; LPS = lipopolysaccharide; SNS = sympathetic nervous system; TLR4 = Toll-like receptor 4.

Finally, I found an association between only one of the cytokines and chemokines, CCL-2, hypothesised to be associated with cognitive function. Performance on tasks of verbal memory and frontal lobe function was not as proficient at the end of detoxification compared with published population

norms (Abikoff, et al. 1987) (Tombaugh, 1999) (Tombaugh, 2004). It was not possible to compare their performance with healthy controls directly as this study lacked a control group. Further, the differences were small and of probable limited clinical relevance. There are previous reports of increased peripheral CCL-2 in mild cognitive impairment (MCI) compared to healthy control participants (Galimberti et al., 2006), (Westin et al., 2012). In the context of MCI, higher CSF CCL-2 levels at baseline did not relate to cognitive performance based on the Mini-Mental State Examination at baseline, but were associated with a greater cognitive deterioration over the next five years (Westin et al., 2012). The disparity between these findings and mine may relate to differences in cognitive tests used – for example the Mini-Mental State Examination does not emphasise frontal lobe function – or differences in disease processes.

Previously, increased CCL-2 expression has been described in post-mortem brain samples from alcohol-dependent patients (He and Crews, 2008). CCL-2 is a chemokine, which is produced by microglia and perivascular macrophages in the brain under conditions of chronic cerebral inflammation, such as experimental autoimmune encephalitis, attracting circulating monocytes to the brain (reviewed in (Ransohoff, 2009)). Increased CCL-2 expression, both in liver and brain, has also been noted in conditions of chronic hepatic inflammation such as primary bile duct ligation, and has been associated with microglial activation in this context (D'Mello et al., 2013). Although not an expected association, my finding is supported by pre-clinical literature suggesting that increase in CCL-2 may be adaptive: CCL-2 or CCR-

2 knock-out mice exhibit lower alcohol intake in a free-choice alcohol administration paradigm and longer loss of righting reflex during alcohol dosing at a given concentration relative to C57B/6 wild-type mice, which could be consistent with lower tolerance to the intoxicating effects of alcohol (Blednov et al., 2005).

Factors related to cognitive impairment in alcohol dependence, such as history of head injury, previous detoxifications, and thiamine deficiency, were not accounted for in the multivariate analysis. Very few patients had a history of clinically significant head injury, so this was excluded from analysis. Variation in Caine criteria and previous detoxifications were omitted because the sample size prohibited inclusion of many independent variables, and cytokines were prioritised as the focus of the study. Further work in a larger sample should be done to explore the relationship between CCL-2 and other clinical variables related to cognitive function in this population.

2.5.1 Limitations

Many cytokines were not detectable in a majority of sample, which could reflect insufficient sensitivity of the Luminex technique compared to ELISA. Previous studies (Gonzalez-Quintela et al., 2000), (Leclercq et al., 2012) that have reported increased TNF- α in early abstinence, and TNF- α and IL-1 β stimulate IL-6 production, so a concomitant increase would be expected. Further investigation of the dynamics of cytokines related to innate and adaptive immunity elucidated by this study is warranted, using more sensitive methods.

This study lacked a control group, which means that it is unclear whether cytokines were in the normal range in this sample. After consideration, a control group was not recruited because the focus of the study was the longitudinal course of cytokines during alcohol detoxification, and whether cytokine concentrations were predictive of clinical symptoms within this patient population. Cytokines in healthy populations are also subject to fluctuations, which may render a single cross-sectional measure uninformative. In addition, given the aim of determining the impact of alcohol detoxification on cytokine levels, a number of 'control' groups might have been required e.g. those with hepatic disease, other physical diseases, or depression. Recruiting such a range of populations was outside the remit of the study, but should be collated in order to characterise causes and consequences of cytokines and chemokines in alcohol detoxification.

2.6 Conclusion

In summary, I have demonstrated that IL-6, IL-10, CXCL-8, and CCL-2 decreased significantly during alcohol detoxification, while IL-12, CXCL-10 and CRP increased. IL-6 was related to withdrawal severity and depression. CCL-2 was related to improved cognitive function, though the differences were small and of uncertain clinical significance. Circulating cytokines are likely to be of largely hepatic origin. The time course of the cytokines and chemokines, and relationship to clinical symptoms of withdrawal across time points, suggest that they represent a peripheral signature of the neurobiology of alcohol withdrawal. Sympathetic hyperactivity during early withdrawal is likely to contribute to the increase in cytokines associated with innate

immunity – IL-6, IL-10 – relative to later time points together with the suppression of Th-1 related cytokines. Cerebral cytokine expression may influence peripheral expression of chemokines such as CCL-2. This further supports a view that the peripheral cytokines during alcohol detoxification reflect cerebral processes during alcohol withdrawal and associated with depression and cognitive impairment.

CHAPTER 3

THE HYPOTHALAMO-PITUITARY ADRENAL AXIS DURING DETOXIFICATION AND ITS RELATIONSHIP TO CYTOKINES

3.1 Introduction

The previous chapter described changes in cytokines during detoxification and their relationship to clinical features. As pro-inflammatory cytokines stimulate the hypothalamo-pituitary-adrenal (HPA) axis, and the HPA axis provides a major negative feedback mechanism for the immune system (Rhen and Cidlowski, 2005), this chapter describes investigations performed to characterise the relationship between cytokines and the function of the HPA axis. In the Introduction, I outlined evidence that morning cortisol was elevated in alcohol-dependent patients (ADPs) during intoxication, and even more during withdrawal.

Stimulation tests using pharmacological challenges, have also provided evidence for HPA axis dysfunction in ADPs. Most commonly, a diminished response to stimulation has been reported. Physiological challenges, such as the Cortisol Awakening Response (CAR), the rise in cortisol occurring in the first 30 minutes of waking, have shown a similarly blunted response in early abstinence. The advantages of the CAR is that it represents a response to a physiological stimulus rather than pharmacological stimuli at supra-physiological levels, and can be collected by patients in their home or in their room, if in-patients, so interferes minimally with their ward routine.

Pro-inflammatory cytokines such as IL-1, TNF- α and IL-6 stimulate the HPA axis, resulting in an acute elevation in cortisol. When cortisol is elevated acutely, pro-inflammatory cytokines such as IL-6, and Th-1 cytokines such as IL-12 are suppressed, while anti-inflammatory cytokines such as IL-10 are not. Under chronic conditions of elevated cortisol, glucocorticoid resistance may develop and this relationship may be altered. In Chapter 2, I reported elevations in both the pro-inflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 in early detoxification relative to late detoxification. The aim of this investigation was to characterise the relationship between morning cortisol, and Cortisol Awakening Response – as a measure of HPA axis responsiveness to challenge, with cytokine levels and changes during alcohol detoxification.

3.2 Hypotheses

Based on previous evidence and my data regarding cytokines during detoxification, I hypothesised that:

1. Serum cortisol would be elevated during early detoxification relative to late detoxification.
2. There would be a relationship between serum cortisol and the cytokines IL-6, IL-12, and IL-10.
3. Cortisol Awakening Response would show a relationship to IL-6 and IL-10.

The relationship between HPA axis and cytokines depends on whether the inflammatory stimulation of the HPA axis predominates, or whether the immunosuppressive drive of the HPA axis is over-riding. For this reason, I was unable to be more specific about the expected nature of the relationship.

If the balance in the HPA-cytokine relationship was in favour of cytokines I expected there to be a positive correlation between IL-6 and cortisol with no relationship with IL-10 and IL-12. If cortisol predominated, I expected an inverse correlation with IL-6 and IL-12, and a positive correlation with IL-10.

3.3 Methods

3.3.1 Sample collection

Participants were asked to collect Cortisol Awakening Response (CAR) samples three times during the study – early, middle and late detoxification. They were given four sample collection kits with written instructions to obtain samples using specially prepared cotton wool swabs (Sarstedt, North Carolina) at four time points: immediately upon waking, and 15, 30 and 45 minutes later. They were instructed not to smoke or brush their teeth during collection and not to eat or drink 10 minutes before sample collection. Samples were stored in the ward sample fridge until the arrival of the research team, then centrifuged and the saliva stored at -20°C until analysis. The research team confirmed verbally with the patient that they had collected the samples as directed.

Serum for morning cortisol and cortisol binding globulin was collected as part of the sample for cytokine analysis, between 8am and 10am. Storage and processing were as described in Chapter 2.

3.3.2 Sample analysis

Saliva samples were analysed by Michael Newson and myself, using commercially available ELISA kits (DRG, Germany). The detectable range was 0.537ng/ml – 80ng/ml. The intra-assay and inter-assay reproducibility were between 1.47% – 4.52% and 5.82% – 7.47% respectively. Increase in cortisol was calculated by subtracting the initial salivary cortisol value from the highest value among the other four values. I chose this approach, rather than calculating an area under the curve, or using the 30 minute value specifically, which are the two most commonly reported methods for analysing the CAR, for two reasons. First, it allowed for slight inaccuracies in timing of sample collection (Griefahn and Robens, 2011). Second, in some patients, sample volumes from specific time points were too small to be analysed so the complete set of four was not available. Serum samples were analysed for cortisol using commercial chemiluminescent microparticle immunoassay kits in the local hospital laboratory. The lower limit of detection was 1ug/dl and the assay had a coefficient of variation of <10%. The normal reference range for cortisol used by the hospital laboratory was 160-550nM.

3.3.3 Statistical analysis

The Free Cortisol Index, a proxy measure of unbound – and therefore biologically available – cortisol, was calculated by dividing cortisol concentration by Cortisol Binding Globulin concentration (le Roux et al., 2002). It is particularly of use in conditions where CBG changes, such as the decrease seen in acute sepsis. The distribution of cortisol, Cortisol Binding Globulin and Free Cortisol Index was inspected graphically to ascertain

whether they derived from a parametric distribution. Data points (in this case, one value) that exceeded three standard deviations were excluded from the analysis. Mixed models were applied in SPSS 20.0 (IBM) to ascertain the effect of time point on cortisol and Free Cortisol Index. Parametric correlations for each time point were undertaken to examine the relationship between cytokines (log-transformed) and cortisol, as the HPA axis is stimulated by pro-inflammatory cytokines and in turn, provides negative feedback to the innate immune system, suppressing their production (see Figure 1.1 on page 37 for a schematic diagram of these relationships). These were not corrected for multiple comparisons owing the risk of Type II error, but confidence intervals (bootstrapped using SPSS 20.0, IBM) have been supplied as suggested by Field (Field, 2012).

3.4 Results

The demographic characteristics of the sample were as reported in Chapter 2. Those samples that had been collected incorrectly, for example during the night, over a twenty-four hour period, or using a different body fluid, were not processed further. Those samples where inadequate saliva had been produced for the first collection point were excluded from analysis. Few patients were able to complete Cortisol Awakening Response sample collection, despite written instructions and prompting from ward staff. Eleven completed all three collections, while four more completed early and late collections, four early and middle collections, and four middle and late collections. Those who completed samples did not differ significantly from the main group in terms of mean CIWA-Ar score, mean depression or craving

rating, serum cortisol or Free Cortisol Index, or IL-6, IL-10, IL-12, CCL-2, CCL-11 or CXCL-8 concentrations at any time point, or on performance on any cognitive test ($p > 0.05$). Serum specimens were available from 51 patients.

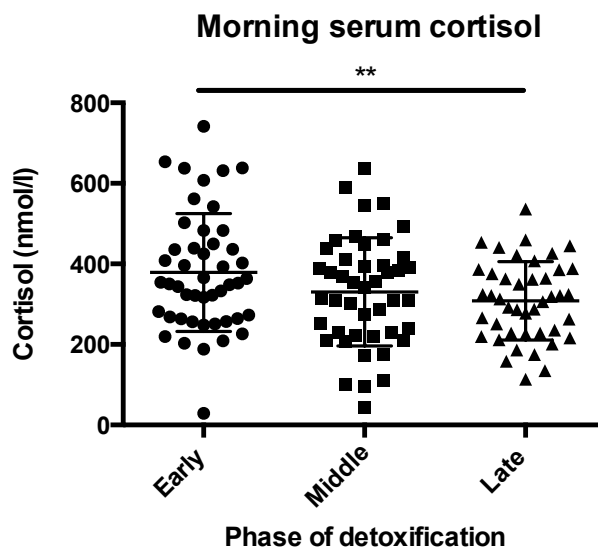
3.4.1 Profile of serum cortisol

There was a significant effect of time point on total serum cortisol ($F(2, 46) = 5.249$; $p = 0.009$), shown in Figure 3.1, though no significant effect of time point on the Free Cortisol Index ($F(2, 40) = 1.393$; $p = 0.260$). The mean cortisol concentration for each time point fell *within the clinical laboratory normal range of between 160nM and 550nM* (early: $374.9 \pm 147.21\text{nM}$; mid-detoxification: $328.3 \pm 133.93\text{nM}$; late: $309.3 \pm 96.48\text{nM}$). However there was a minority of patients whose cortisol exceeded the normal range. This proportion was 15% in early detoxification, 4% in mid detoxification and 0% in late detoxification (see Figure 3.2). Of note, a smaller proportion of patients had morning cortisol levels below normal range: 2% of participants had a lower than normal morning cortisol during early detoxification, 9% during mid detoxification and 5% during late detoxification. The relative proportions of normal, abnormally high and abnormally low serum cortisol, were significantly different between time points (chi-square (24.52, 4) $p < 0.0001$).

There was no statistically significant difference between cortisol concentrations in those patients who had withdrawal scores above the treatment threshold (>10) and those with withdrawal scores below the treatment threshold (early, middle and late: $p > 0.20$); neither was there a

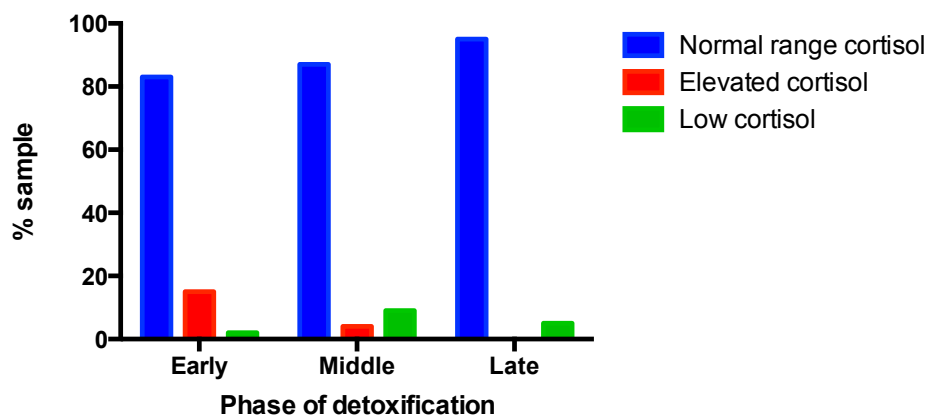
correlation between withdrawal severity and cortisol (early, middle and late: $p > 0.40$). Cortisol Binding Globulin concentrations did not change during detoxification ($p > 0.05$): early: 54.6 ± 9.32 ; middle: 53.2 ± 9.37 ; late: 53.2 ± 9.23 mg/l. Free Cortisol Index also did not change significantly during detoxification ($p > 0.05$): early: 6.8 ± 2.52 nmol/mg; middle: 6.2 ± 2.68 nmol/mg; late: 6.3 ± 2.88 nmol/mg.

Figure 3.1: Time course of serum cortisol during alcohol detoxification



Legend: ** refers to results of Bonferroni corrected pair-wise comparisons showing a significant difference between early and late detoxification cortisol ($p = 0.007$).

Figure 3.2: Proportion of samples that fell within and outside the normal range



Legend: The proportion of samples that fell within the normal range increased significantly during detoxification ($p < 0.001$).

3.4.2 *The cortisol awakening response*

Samples from each time point was considered separately as they were slightly different samples and the proportion of missing values was too great to make imputation or use of mixed methods feasible alternatives. The Cortisol Awakening Response showed a mean increase in cortisol of 31% of the initial value (range -31% – 178%) during early detoxification, 42% (range -17% – 161%) during mid and 31% (range -39% – 80%) during late detoxification. However a normal or abnormal morning serum cortisol did not predict whether a patient's Cortisol Awakening Response was in the normal range.

3.4.3 *Relationship between cortisol and cytokines*

There were no relationships between serum cortisol and the cytokines IL-6, IL-10 and IL-12. There was a statistically significant moderate positive correlation between IL-6 and the Free Cortisol Index at the middle time point only, shown in Figure 3.3a ($r = 0.328$; 95% C.I.: 0.089 – 0.534; $p = 0.03$). There was also a statistically significant moderate positive correlation between the increase in cortisol during the Cortisol Awakening Response and IL-6 at the middle time point, shown in Figure 3.3b ($r = 0.462$; 95% C.I.: 0.129 – 0.685; $p = 0.03$). Neither the Free Cortisol Index nor the mean Cortisol Awakening Response increase were correlated with IL-10 or IL-12, at any time point.

Figure 3.3a: Relationship between Free Cortisol Index and IL-6 during mid-detoxification

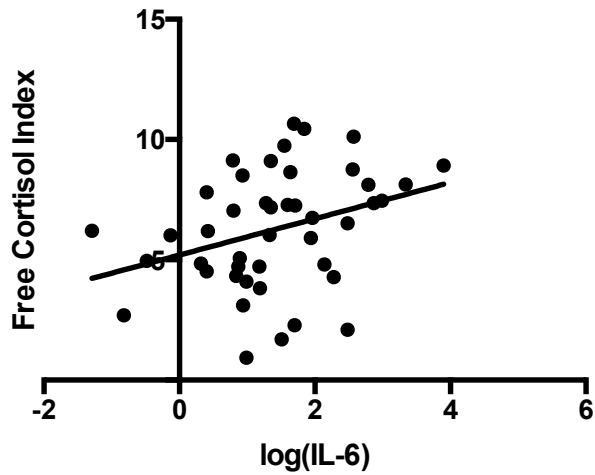
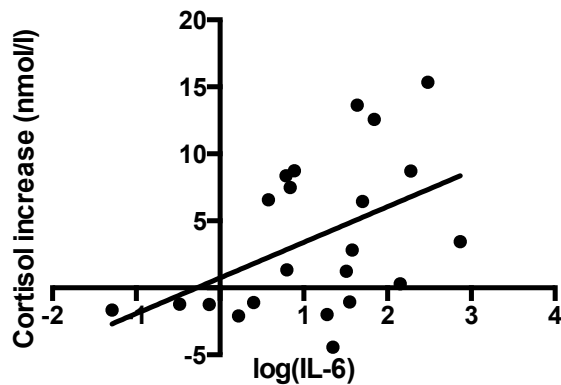


Figure 3.3b: Relationship between Cortisol Awakening Response and IL-6 during mid-detoxification



3.5 Discussion

I found that morning serum cortisol decreased significantly during alcohol detoxification, though there was no significant change in the Free Cortisol Index. There was a substantial minority of patients with abnormally high and abnormally low morning cortisol levels, and this proportion declined too during detoxification. In those patients who were able to provide appropriate samples for the Cortisol Awakening Response, the mean response was within the normal population range. I was unable to compare proportions of patients

with normal or abnormal Cortisol Awakening Response over the course of detoxification because of the high numbers of missing values. Both the Free Cortisol Index and Cortisol Awakening Response were positively correlated with IL-6 during mid-detoxification. No other relationships between HPA measures and cytokines were found.

My study has replicated previous reports of a decrease in cortisol during detoxification (Adinoff et al., 1991) (Kruger et al., 2006) (Keedwell et al., 2001) in a larger cohort of UK alcohol-dependent patients treated with benzodiazepines. In addition, I have found that some participants had both abnormally high and abnormally low cortisol measures, but that these were likely to normalise over time. Of note is that while all means fell within the clinical normal range as defined by the local hospital laboratory, the mean value in late detoxification was above 300nM, which corresponds to a level considered high in the psychiatric literature, for example, in depression (Christensen et al., 1989).

Even though the first measurement of cortisol in this study occurred after participants had received medication for withdrawal symptoms, early detoxification cortisol was elevated relative to late detoxification. This, together with the lack of relationship between cortisol and withdrawal severity, supplies additional support for the theory that benzodiazepines do not modify the HPA axis response to detoxification, despite their effect on withdrawal symptoms (Keedwell et al., 2001). This is in contrast to gamma-

hydroxybutyrate, which was associated with a decrease in cortisol relative to placebo when used to treat alcohol withdrawal (Nava et al., 2007).

Although there was a decrease in serum cortisol there was no change in the Free Cortisol Index. This implies that the changes seen in serum cortisol do not change significantly the amount of biologically available cortisol. However given that there were no changes in Cortisol Binding Globulin during detoxification, it is possible that the variability of Free Cortisol Index contributed to lack of association. Thus a study with a larger sample is required to appropriately investigate their inter-relationships.

The mean Cortisol Awakening Response was within normal limits (30% – 75%) at all time points. This is in contrast to reports of hypo-responsiveness to psychological or pharmacological challenge in other alcohol-dependent populations (Sinha et al., 2011) (Lovallo et al., 2000) (Errico et al., 2002) (Junghanns et al., 2005) (Fox et al., 2012) (Merry and Marks, 1972) (Coiro and Vescovi, 1999) (Adinoff et al., 2005) (Bailly et al., 1989). The single previous study of the Cortisol Awakening Response (Junghanns et al., 2007) which reported a blunted awakening response in ADPs in early abstinence, did not define this in terms of the normal range; rather they were compared to an alcohol-dependent group with a mean abstinence duration of several months. Stressful events are known to affect the Cortisol Awakening Response and are associated with a greater increase (Wust et al., 2000a) so it could be that the acute physiological stress of withdrawal and psychological

stress of admission to hospital influenced our findings; in other studies detoxification had been completed prior to testing.

There was a significant positive correlation between IL-6 and both the Free Cortisol Index, and the increase in cortisol during the Cortisol Awakening Response during mid-detoxification. This is consistent with previous pre-clinical reports that IL-6 stimulates the HPA axis (Bethin et al., 2000). It suggests that while the HPA axis in ADPs has been reported to show blunted responses to physiological stressors e.g. CRH or waking, it may still be responsive to an inflammatory stress such as IL-6. Importantly, this relationship is seen only transiently during alcohol detoxification, which suggests that at early and late time points, other factors are more important in determining cortisol concentrations and the Cortisol Awakening Response.

3.5.1 Limitations

Despite the large number of samples, only IL-6 was consistently detectable in my samples out of all the pro-inflammatory cytokines reported to stimulate the HPA axis. It may be that other HPA axis markers and cytokines are more sensitive to changes in endocrine-immune relationships: for example, TNF- α and ACTH were negatively correlated following the dexamethasone-CRH stimulation test when no relationships between cortisol and cytokines were found (Schuld et al., 2003). Studies using more sensitive techniques to detect TNF- α and measurement of ACTH would be of interest to investigate relationships further. The major limitation of the Cortisol Awakening Response is the difficulty in obtaining samples – only 36% of our participants

were able to provide at least two adequate sample sets despite verbal and written instructions and prompting by ward staff. In a healthy epidemiological cohort the proportion of adequate Cortisol Awakening Response samples returned was around 45%, suggesting that this is a problem inherent to the method (Carnegie et al., 2014). The Cortisol Awakening Response is susceptible to timing errors, which is likely to have affected our results despite all the measures taken to ensure timely collection. It is also reported that 25% of healthy people do not show a Cortisol Awakening Response (Wust et al., 2000b) (Fries et al., 2009), so a proportion of participants may ordinarily show no response. Further investigation of the Cortisol Awakening Response in later abstinence may prove informative in this regard. Lastly, I did not have a control group. Given that cortisol levels in ADPs have been reported to be higher than control groups sampled in the same study, but still within clinical laboratory limits, a control group may have provided additional information.

In summary, I found that mean morning cortisol levels were within a clinical laboratory normal range throughout detoxification, but one that is considered high in the psychiatric literature. Morning serum cortisol during early detoxification was raised relative to late detoxification, which replicates previous findings. However neither Cortisol Binding Globulin nor the Free Cortisol Index changed. Similarly, the mean cortisol awakening response was within the normal range at each time point in detoxification. I found a significant, though moderate, association between IL-6 and both morning serum cortisol and increase in cortisol during the Cortisol Awakening Response, both at the mid-detoxification point. This suggests that the HPA

axis is sensitive to inflammatory stimulation during mid-detoxification. That this association was not present at other points in detoxification suggests that other factors are important in variations in both cortisol and cytokines. Further investigations using validated suppression and stimulation tests, where researchers have more control over the samples collected, including measurement of ACTH and more sensitive methods for measuring pro-inflammatory cytokines with a greater effect on HPA axis function, such as TNF- α , could be pursued to characterise the relationship between the HPA and the immune system during alcohol withdrawal in greater detail.

CHAPTER 4

***IN VITRO* AFFINITY OF BENZODIAZEPINES FOR THE TRANSLOCATOR PROTEIN (18KDA)**

This chapter describes *in vitro* experiments conducted in preparation for the imaging study to investigate whether clinical doses of benzodiazepines could compete with the radioligand for binding to the TSPO. Given that my imaging study was to take place as early in abstinence from alcohol as practically possible, participants might be taking prescribed benzodiazepines. Even if prescription had ceased, long-lived active metabolites may still have been present. The TSPO was originally known as the Peripheral Benzodiazepine Receptor (see (Papadopoulos et al., 2006)). Diazepam, one of the most commonly prescribed benzodiazepines, has been reported to have an affinity for the TSPO of either $27.43 \pm 8.92\text{nM}$ (Rao and Butterworth, 1997) or 432nM (Doble, 1983) in *in vitro* assays conducted on human brain tissue at 4°C. Concerns about an interaction of benzodiazepines with the TSPO have led to the withdrawal of benzodiazepine treatment from participants prior to scanning with a TSPO radioligand (Doorduyn et al., 2009).

It was therefore important to investigate whether these potential doses of diazepam or chlordiazepoxide, benzodiazepines commonly used for alcohol detoxification, could compete with the PET tracer for TSPO binding. The affinities of midazolam and lorazepam for the PET tracer for TSPO were also investigated. The former is of interest in TSPO-PET research as it is a short-acting benzodiazepine potentially useful as a sedative in easily distressed

participant populations; and the latter because it is commonly prescribed in psychiatric populations that are increasingly investigated using TSPO-PET (e.g. (Doorduyn et al., 2009)).

Previous estimates of benzodiazepine affinity for the TSPO in human brain have been conducted at a low temperature and in Tris-HCl buffer, which does not reflect ionic or temperature conditions found *in vivo* (Rao and Butterworth, 1997) (Doble, 1983). Furthermore, the binding site for the novel TSPO PET ligand, [¹¹C]PBR28, may differ from that of the earlier ligands [³H]PK11195 and the benzodiazepine [³H]Ro5-4864 (4-chlorodiazepam), since PBR28 has been shown to block only ~80-90% of [³H]PK11195 specific binding (Owen et al., 2010). This may result from PK11195 and PBR28 having overlapping binding sites, as is thought to be the case for PK11195 and Ro5-4864 (Scarf and Kassiou, 2011). Thus earlier studies which used [³H]PK11195 and [³H]Ro5-4864 (Rao and Butterworth, 1997) (Doble et al., 1987) may not fully reflect the interaction between benzodiazepines and [¹¹C]PBR28.

4.1 Aims and hypotheses

I aimed to characterise the affinity of diazepam, chlordiazepoxide and their common metabolites desmethyldiazepam and demoxepam, and oxazepam for the PK11195 binding site on the TSPO, in human brain tissue under conditions designed to approximate the *in vivo* cerebral environment. The TSPO affinities of PK11195 and diazepam were investigated to see if they were affected by changes in ionic concentrations reflecting the extracellular and intracellular environment. The affinity of midazolam and diazepam for the

PBR28 site on the TSPO was measured to establish whether there was a significant difference in benzodiazepine affinity for the PK11195 site and the PBR28 site.

Of secondary interest was whether the rs6971 polymorphism in the gene encoding the TSPO, which dramatically alters binding affinity of most TSPO radioligands producing 'High Affinity Binders' (HABs) and 'Low Affinity Binders' (LABs) (Owen et al. 2010), (Owen et al. 2011) (discussed in Chapter 1, Section 1.6.3), also influences the binding affinity of benzodiazepines.

I hypothesised that:

1. The affinity of benzodiazepines for the TSPO would be low, such that they would not affect TSPO ligand binding at clinical doses.
2. The affinity of benzodiazepines for the TSPO would not be affected by ionic conditions.
3. There would be no significant difference in affinity for the PK11195 binding site and the PBR28 binding site, as PBR28 is able to block 80% of all PK11195 binding.
4. That binder status (HAB or MAB) would have an effect on the affinity of benzodiazepines to the TSPO.

4.2 Methods

4.2.1 Samples

Post-mortem human brain tissue from donors without an ante-mortem neurological diagnosis, previously characterised as High Affinity Binders

(HABs) (n=4) or Mixed Affinity Binders (MABs) (n=4) were obtained from the Multiple Sclerosis Brain Bank and stored at -80 °C until use (see Table 4.1 for details). Brain sample procedures all met the requirements of the Human Tissue Act (2004).

Table 4.1: Demographic details of brain samples

Donor number	Binding status	Age at death	Sex	Cause of death
1	HAB	82	M	Not recorded
2	HAB	92	M	Congestive cardiac failure, old age
3	HAB	84	F	Congestive cardiac failure, ischaemic heart disease, atrial fibrillation
4	HAB	69	F	Lung cancer
5	LAB	90	M	Old age
6	LAB	78	F	Acute myeloid leukaemia
7	LAB	68	M	Cor pulmonale, cryptogenic fibrosing alveolitis, cardiac failure
8	LAB	84	M	Bladder cancer, pneumonia

4.2.2 Competition assays

I performed all procedures in this experiment independently. Brain tissue was homogenised as described previously (Owen et al., 2010). Tissue membranes were resuspended in assay buffer (50mM Tris-Base, 140mM NaCl, 1.5mM MgCl₂, 5mM, KCl, 1.5mM CaCl₂, pH7.4, 37°C) at a protein concentration of ~500 µg/ml and incubated with racemic [³H]PK11195 (5nM) and benzodiazepine compounds at a range of concentrations (diazepam, chlordiazepoxide, lorazepam, desmethyldiazepam, oxazepam: 10nM - 1mM; midazolam: 3nM – 100µM) for 60 minutes (37°C). Each point was determined in triplicate or quadruplicate.

Specific binding was defined using unlabelled racemic PK11195 (10 μ M). Assays were terminated via filtration through GF/B filters followed by three washes with ice-cold wash buffer (50mM Tris-HCl, pH 7.4, 4°C), prior to transfer to vials. Scintillation fluid (3ml) was added to each vial and bound radioactivity determined using a Beta liquid scintillation counter. Midazolam and diazepam competition assays were repeated with [³H]PBR28 (5nM) with unlabelled PBR28 (10 μ M) to define specific binding. Only HAB tissue (n=4) was used in these studies since [¹¹C]PBR28 does not provide a measurable signal in PET studies involving LABs.

The effect of ionic composition of the assay buffer was investigated by comparing the IC₅₀ of PK11195 when the assay was performed in intra-cellular buffer (50mM Tris-Base, 10mM NaCl, 140mM KCl, 0.5mM MgCl₂, pH 7.0, 37°C) with the IC₅₀ when it was performed in extra-cellular buffer (50mM Tris-Base, 140mM NaCl, 1.5mM MgCl₂, 5mM, KCl, 1.5mM CaCl₂, pH7.4, 37°C) in HABs (n=2) and LABs (n=2). This was repeated using diazepam.

Experiments were also undertaken to ascertain whether the affinity of diazepam and PK11195 changed according to assay temperature, by conducting parallel experiments using the method above at 37°C and 4°C in assay buffer (50 mM Tris-Base, 140 mM NaCl, 1.5 mM MgCl₂, 5 mM, KCl, 1.5 mM CaCl₂, pH 7.4), using tissue from 3 HABs, with 1 hour of incubation at 37°C and 2 hours of incubation at 4°C (to account for a slower time to equilibrium at lower temperatures and render the results comparable with published *in vitro* studies at 4°C).

4.2.3 Statistical analysis

Competition data were analysed using non-linear regression curve-fitting software (GraphPad Prism 6.0). Affinities were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) with K_{iS} for PK11195 (29 nM) and PBR28 (3.4 nM) previously determined by Owen and coworkers (2010). Two-tailed Student's t-tests, also performed in GraphPad Prism 4.0 were used to ascertain whether there was a difference in affinity for the PK11195 site and PBR28 site.

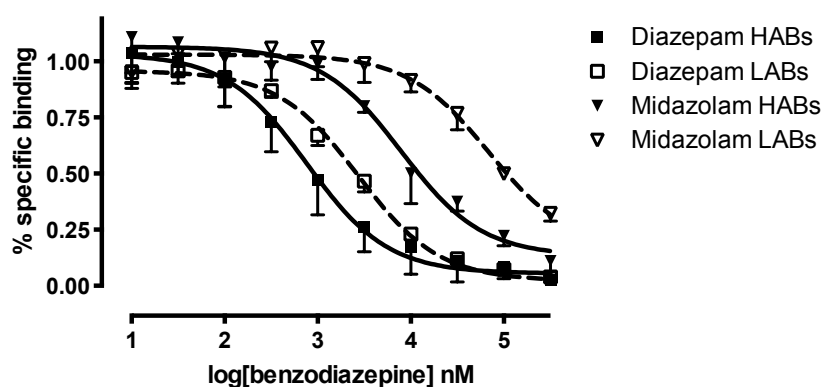
4.3 Results

Binding affinities of benzodiazepines for the [³H]PK11195 binding site on the TSPO are given in Table 4.2. The highest affinity was obtained with diazepam ($K_i = 719\text{nM}$). Chlordiazepoxide and midazolam showed much lower affinity ($K_i > 2\mu\text{M}$). The affinities of PK11195 and diazepam for the TSPO were not affected by changes in ionic conditions (see Table 4.3), implying that previous estimations of affinity of ligands for the TSPO using an extracellular buffer can be applied to *in vivo* conditions in which the ligand binds intracellularly. Contrary to my hypothesis, there was no effect of temperature on affinity of diazepam (K_i at 37°C = 992 ± 557 ; K_i at 4°C = 1008 ± 148 ; $p = 0.959$) or PK11195 (K_i at 37°C = 15.2 ± 6.0 ; K_i at 4°C = 14.9 ± 5.1 ; $p = 0.877$) for the TSPO.

The affinities for lorazepam, desmethyldiazepam, demoxepam, and oxazepam were too low to be estimated accurately ($> 50\mu\text{M}$). All compounds

for which accurate estimates of affinity were possible, displayed a ~3.5-10 fold higher affinity in the HABs compared to the LABs (Table 4.2 and Figure 1) which was consistent with my hypothesis. The affinities (K_i) of diazepam and midazolam for the PBR28 binding site on the TSPO were $943 \pm 58\text{nM}$ and $2,240 \pm 136\text{nM}$, respectively. These did not differ significantly from the affinity at the PK11195 site (Diazepam $p = 0.202$; Midazolam $p = 0.956$).

Figure 4.1: Competition curves showing TSPO affinity of diazepam and midazolam (PK11195 site)



Legend: Graph of data obtained from competition assays performed using human brain at pH 7.4; 37 °C using [^3H]PK11195 (5 nM). Curves represent the fraction of specific TSPO binding blocked by increasing concentrations of diazepam and midazolam. Diazepam points are squares and midazolam triangles. Closed symbols denote data points in the assay performed using HAB tissue (n=4) and open symbols denote data points from the LAB assay (n=4). The solid curves represent HAB data and the dashed curves LABs data.

Table 4.2: Affinity of commonly-prescribed benzodiazepines for the PK11195 site on the TSPO

Compound	K_i HABs (nM) \pm SEM	K_i MABs (nM) \pm SEM	Fold difference
Diazepam	719 ± 169	$2,141 \pm 307$	3.4
Midazolam	$2,270 \pm 620$	>50,000	>10
Chlordiazepoxide	$5,700 \pm 1,270$	>50,000	>10
Lorazepam	>50,000	>50,000	N/A
Desmethyldiazepam	>50,000	>50,000	N/A
Demoxepam	>50, 000	>50, 000	N/A
Oxazepam	>50,000	>50,000	N/A

Legend: SEM = Standard error of the mean; N/A = Not applicable

Table 4.3: Effect of ionic conditions on affinity of diazepam and PK11195 for the TSPO

Specimen no.	Diazepam		PK11195	
	Intracellular IC ₅₀ (95% CI)	Extracellular IC ₅₀ (95% CI)	Intracellular IC ₅₀ (95% CI)	Extracellular IC ₅₀ (95% CI)
HAB1	1527 (991.3-2353)	1867 (1277-2730)	11.1 (6.1-20.2)	10.2 (7.3-14.2)
HAB2	1547 (1018-3619)	1317 (796.9-2176)	13.3 (9.1-19.4)	10.5 (6.5-16.8)
LAB1	3318 (2203-4999)	4395 (2968-6508)	7.1 (5.2-9.7)	9.0 (5.3-15.6)
LAB2	2389 (1577-3619)	2847 (1923-3217)	8.9 (6.2-12.7)	10.6 (6.9-16.3)

4.4 Discussion

To determine whether benzodiazepine treatment leads to significant occupancy of the TSPO, the binding affinities need to be considered in conjunction with the brain free concentrations of these compounds. Diazepam, which has the highest TSPO affinity, is highly protein-bound with a free fraction of 2-3% following a single dose, and the free plasma concentration has been shown to be equal to cerebrospinal fluid concentration in man (Kanto, 1975), (Kanto et al., 1975), implying an equilibration of the free concentrations across the blood-brain barrier. A single oral dose of 10mg diazepam produces a peak total plasma concentration of 720nM (Dundee et al., 1979), and hence a peak free concentration of 21nM in plasma and brain. Such concentrations would be expected to produce ~1% occupancy of the TSPO, based on the affinity estimated in this study.

A cross-sectional study of diazepam concentrations in chronically treated patients suggested an increase in the free fraction with chronic treatment (3-

4%; (Kanto et al., 1974)), and induction of metabolism producing lower steady state plasma concentrations than may be expected: 147 to 1482nM (Kanto et al., 1974), implying a free concentration between 6 and 60nM. However, another study in chronically treated population reported a steady state concentration of 2580 nM – 3547nM in those taking 30mg/day dose (Rutherford et al., 1978) implying a free concentration of up to 106nM. Out-patients were included in both studies with compliance assessed by self-report, so may reflect inaccurate reporting. However, (Rutherford et al., 1978) also included in-patients, whose intake was monitored by nursing staff, so these results may be more accurate. Thus, at maximum recommended doses of diazepam TSPO occupancy is estimated to be 9%.

Unlike diazepam, TSPO occupancy by midazolam and chlordiazepoxide, the two other benzodiazepines with measurable affinity, would be expected to be negligible if clinical pharmacokinetic data are used to calculate likely competition at the TSPO at clinically relevant doses. A single dose of 30mg chlordiazepoxide ($K_i \sim 5700\text{nM}$ in HABs) orally produces a maximum concentration of 2mg/l (equivalent to 6.67 μM (Boxenbaum et al., 1977)). Repeated dosing has been shown to produce a lower steady-state of around 1.5mg/l (Boxenbaum et al., 1977). However, 93% of chlordiazepoxide was found to be protein bound, suggesting that the free fraction is around 7% (equivalent to 473nM (Boxenbaum et al., 1977)). When this value was interpolated in the curve generated using our *in vitro* data, there was no block of specific binding expected to be observed. Similarly, midazolam ($K_i \sim 2270\text{nM}$ in HABs), which is usually given acutely for anxiolysis, at a dose of 5

mg produces a maximum concentration of 559ng/l (equivalent to 170nM (Schwagmeier et al., 1998)). Like other benzodiazepines, midazolam is highly protein bound, leaving a free fraction of 3.9%, equivalent to a peak free concentration of 0.59nM (Vinik et al., 1983), which is also a concentration demonstrated not to produce any block when interpolated into our data.

The implications for my imaging study were that, provided that participants taking diazepam were scanned following the end of their medication regime, prescribed benzodiazepines were unlikely to compete with the radioligand at the TSPO. Patients taking chlordiazepoxide could be scanned during active treatment, and residual metabolites were unlikely to be a problem. More broadly, the implications for TSPO-PET research depended on study design. In studies where intra-subject comparisons are made, for example in a blocking study or pre- and post-treatment scans, chronic doses of benzodiazepines are unlikely to affect the findings providing the benzodiazepine dose is maintained between assessments. However, between-group comparisons where there are differences in benzodiazepine doses between the groups, a 9% difference in TSPO availability may be a consequence of doses of diazepam exceeding 30mg per day. However, sedation of patients likely to become distressed by the scan with standard doses of midazolam is unlikely to affect TSPO availability.

CHAPTER 5

INVESTIGATION OF MICROGLIAL ACTIVATION IN ALCOHOL DEPENDENCE: A [¹¹C]PBR28 PET-CT STUDY

5.1 Introduction

This chapter describes findings from a group comparison study investigating the presence of microglial activation in alcohol-dependent men in early abstinence. As discussed in Chapter 1, Section 6, microglia are the resident macrophages of the central nervous system. They have an array of highly branched ramifications that are acutely sensitive to the extracellular environment. This morphology enables them to respond quickly to pathogen invasion and become activated, which involves retraction of ramifications and assumption of a more amoeboid appearance, often with proliferation and migration to the site of injury.

It has been recognised that microglia which appear activated in morphology may in fact express a range of different cell surface molecules and secrete a range of mediators. So-called 'M1' activation is particularly of interest as this phenotype is inflammatory, associated with production of inflammatory cytokines and prostaglandins, and putatively associated with increased neuronal death. *In vitro* studies have demonstrated that alcohol can activate both microglia and astroglia via Toll-Like Receptor 4. The relevance of this receptor is that it is a receptor for bacterial products such as lipopolysaccharide, which is associated with an M1 inflammatory response.

Activated microglia have been emphasised in clinical research into neuroinflammation, as it is possible to detect them using Positron Emission Tomography (PET). This is because expression of a mitochondrial protein known as the Translocator Protein 18kDa (TSPO), which has low background levels of expression in the brain parenchyma, is increased in activated microglia. Increased TSPO expression has been demonstrated pre-clinically using [³H]PK11195 autoradiography in alcohol withdrawal (Obernier et al., 2002), (Marshall et al. 2013).

Only one post mortem study has been published which investigated microglial activation in alcohol dependence. He and Crews (2008) studied samples from the cingulate cortex of five alcohol dependent patients and from the ventral tegmental area, midbrain, and amygdala of eight alcohol dependent patients. Three cases died from cirrhosis. In other cases the listed cause of mortality was less specific and could not be related with certainty to advanced end organ damage as a result of alcohol. The mean age of the subjects – 60 - was higher than in my imaging study described below. Antibodies for the microglial proteins ionised calcium binding adaptor 1 (Iba1) and glucose transporter 5 (GluT5) were used to identify microglia via immunohistochemistry. Microglial morphology was also inspected. There was an increase relative to controls of both Iba1 and GluT5 immunoreactivity in the cingulate cortex, and of GluT5 but not Iba1 in the ventral tegmental area and midbrain of samples from alcohol dependent subjects, consistent with

increased microglial numbers. There were no differences in the amygdala. Numbers of activated microglia identified according morphology were not different between groups.

There is firmer evidence of microglial activation in clinical samples of patients with alcohol-related complications. TSPO-PET using [¹¹C]PK11195 has also been used to show increased TSPO in small patient cohorts with hepatic encephalopathy (Cagnin et al., 2006) in the context of alcohol dependence. A single case report of increased [¹¹C]PBR28 binding in a heavy drinker was reported in a conference abstract during my study, but the clinical details of the case including whether actively drinking or in early abstinence, and importantly TSPO binder genotype, were not specified (Hannestad et al., 2012). Given clinical evidence relating microglial activation to prognosis in acute and chronic neurodegenerative processes, and pre-clinical evidence suggesting its presence in alcohol dependence, further investigation of microglial activation in an alcohol-dependent clinical population is warranted.

5.2 Aims and hypotheses

The aim of this study was to investigate whether microglial activation is detectable by TSPO-PET in recently detoxified alcohol-dependent men, and whether there was a relationship between microglial activation and cognitive function. I hypothesised that:

1. TSPO expression would be increased in the brains of alcohol-dependent patients, particularly in those areas associated with microglial activation or

increased microglial numbers in pre-clinical or post-mortem samples (Obernier et al., 2002), (Riikonen et al. 2002), (He and Crews, 2008): cerebellum, hippocampus, midbrain, thalamus and cingulate cortex.

2. There would be a relationship between TSPO expression and performance on tests of verbal and spatial memory, and frontal lobe tests, which are all affected by alcohol (Davies et al., 2005).

Further exploratory analyses were performed to ascertain the relationship between TSPO expression and other clinical symptoms: depression, anxiety and craving.

5.3 Methods

5.3.1 Participants and recruitment

Alcohol-dependent patients (ADPs) were recruited through local statutory and non-statutory addiction services. Inclusion criteria comprised: meeting DSM-IV criteria for alcohol dependence; involvement in alcohol treatment services; and being within one month of detoxification. Some flexibility was allowed for availability of scanning slots and the participants. Exclusion criteria included major physical or psychiatric illness (though history of depression and anxiety were permitted); dependence on other substances of abuse, excluding nicotine (though use was allowed); alanine transaminase (ALT) exceeding five times the upper limit of normal; abnormal clotting parameters; and consumption of steroids or non-steroidal anti-inflammatory medication within two weeks of the scan. Although a blood alcohol concentration of 0.0mg/dl on the day of the scan was stipulated, a positive drug screen was not an exclusion criterion, as patients close to detoxification were likely to test

positive to benzodiazepines, the mainstay of treatment during detoxification, or other drugs used prior to detoxification, for example, cannabis, which can remain in urine for up to a month after use.

Healthy control participants were shared between this and a concurrent [¹¹C]PBR28 PET study. Additional healthy control participants were recruited via local volunteer databases. Exclusion criteria included history of physical or psychiatric illness (though history of depression or anxiety was allowed); harmful alcohol consumption; use of illicit substances; and consumption of steroid or non-steroidal anti-inflammatory medication within two weeks of the scan. Additional general exclusion criteria for both groups included: Low Affinity Binder (LAB) TSPO genotype; exposure to radiation such that the total dose would exceed 10mSv within three years; ulnar artery insufficiency; and blood donation within two months.

Women were excluded from the study in alcohol dependence because of concerns regarding radiation risk to the foetus in the case of undiagnosed pregnancy. However, in order to minimise radiation exposure, data from healthy control participants were shared with another study in healthy volunteers (Owen, Guo, et al, in preparation, n = 9) that included women. All participants gave informed consent about data sharing and both studies received approval from local NHS Research Ethics Committees and the Administration of Radioactive Substances Committee (ARSAC).

It was difficult to decide on a sample size as pre-clinical autoradiography data did not quantify the increase in TSPO seen in alcohol withdrawal, which meant that there was uncertainty regarding the magnitude of increase in signal anticipated. Furthermore, the study was to be done using novel TSPO-PET tracers that had not been used in the imaging centre previously. Therefore, Dr Qi Guo calculated a tentative sample size based upon mathematical simulation informed by in vitro binding data (see Guo et al. 2012). It was calculated that in order for the study to have 80% power to detect a 50% difference in signal between groups with a 95% probability, that 8 High Affinity Binders and 8 Mixed Affinity Binders in each group would need to be recruited.

5.3.2 Clinical evaluation

Participants were evaluated for alcohol dependence using DSM-IV criteria, and total lifetime alcohol consumption assessed by the modified Skinner's questionnaire (Skinner and Sheu, 1982). The Mini-International Neuropsychiatric Interview, 6th edition (MINI-6) (Sheehan et al., 1998) was administered by a doctor with at least three years' experience in psychiatry, to assess psychiatric co-morbidity and substance use. A full medical history was taken, and a physical examination was performed. Screening blood tests were performed, including urea and electrolytes, liver function tests, clotting function, and full blood count. In addition, blood was taken and analysed for TSPO genotype. Participants were breathalysed, and a urine drug screen performed.

5.3.3 Questionnaires

Participants completed the Spielberger Trait Anxiety Score (STAI) (Spielberger, 1983). Physiological dependence in alcohol-dependent participants was quantified using the Severity of Alcohol Dependence Questionnaire (SAD-Q) on the day of screening. A score of greater than 31 indicates severe dependence, 16 – 30 moderate dependence, and below 16 mild dependence (Stockwell et al., 1983). The Clinical Assessment of Withdrawal from Alcohol (CIWA-Ar) was completed by the attending doctor in the alcohol study, to establish that participants were not in need of treatment of withdrawal symptoms (score <10) (Sullivan et al., 1989). All participants in both studies completed the Spielberger State Anxiety Score (SSAI), Beck Depression Inventory (BDI), and the Fatigue Severity Scale (FSS). A score on the STAI or SSAI of above 40 is considered high (Spielberger, 1983). A score above 9 on the BDI is considered indicative of mild depression, 19 moderate depression and 30 severe depression (Beck et al., 1961). The FSS was developed to quantify fatigue in chronic inflammatory disorders such as multiple sclerosis (MS), and has been validated in MS, systemic lupus erythematosus, hepatitis, and chronic fatigue syndrome. A score of 4 or above is consistent with significant fatigue (Krupp et al., 1989).

Those enrolled in the study investigating alcohol dependence also completed the Alcohol Urge Questionnaire (AUQ) (Bohn et al., 1995), Obsessive Compulsive Drinking Scale (OCDS), but those controls derived from the other study, did not. The AUQ is an eight item, eight question Likert-style questionnaire, which measures acute craving (range: 8 – 64). It is

predominantly used in clinical populations to assess progress, and correlates with the SAD-Q and OCDS (Bohn et al., 1995). The OCDS is derived from obsessive compulsive disorder symptom scores and measures intrusive thoughts and compulsions around alcohol over a period of weeks (range: 0 – 56). Like the AUQ, there is no normal range; rather a higher score indicates greater preoccupation with alcohol and has been reported to relate to lapses and relapse (Anton et al., 1996).

5.3.4 Cognitive tests

Cognitive tests were completed on the day of scanning in the case of the alcohol dependence study, and on the day of screening in the other study. They were derived from a battery sensitive to the effects of alcohol dependence on cognition developed previously by the research group (Davies et al., 2005). Frontal lobe function assessments including digit span, and Trail Making Task A and B (Reitan, 1958), tests of spatial memory – Rey-Osterrieth Figure (ROCF) (Osterrieth, 1944), and verbal memory – and Weschler Memory Scale paragraph version (WMS) (Weschler, 1987) were administered.

5.3.5 Blood tests

Participants in the alcohol study had blood taken immediately before the scan for serum and plasma. High sensitivity CRP (0.3 – 500mg/dl) was analysed by clinical biochemistry services at the Hammersmith Hospital. Plasma samples, where available (n = 6), were sent to the Toxicology Laboratory at

Charing Cross Hospital for analysis of diazepam, chlordiazepoxide and their common metabolite desmethyldiazepam levels, using high-performance liquid chromatography with a detection threshold of 4µg/ml.

5.3.6 Scanning procedures: Positron Emission Tomography

Participants were scanned at the Imanova Centre for Imaging Science, London. Each participant received a [¹¹C]PBR28 PET scan in a Siemens Biograph 6 PET-CT with Truepoint gantry (resolution 4.6mm centre Full Width Half Maximum field of view). A low dose Computed Tomography transmission scan was administered initially to facilitate attenuation and scatter correction. Then, a dynamic 3-D PET image was acquired over 90 minutes. A bolus of [¹¹C]PBR28 (controls: mean 330.4MBq (range: 312.4 – 347.3); ADPs: mean 328.9MBq (range: 302.7 – 346.5) was injected over twenty seconds at the beginning of the scan. Arterial blood samples were collected from the radial artery to generate the plasma input function. A continuous sampling system (ABSS Allogg, Mariefred, Sweden) was used to measure whole blood activity for the first fifteen minutes. Discrete blood samples were drawn for measurement of whole blood and plasma counts, at five-minute intervals for the first 30 minutes, then at ten-minute intervals for the remainder of the scan. Arterial blood samples taken at 5, 10, 20, 30, 50, 70 and 90 minutes were analysed using High Performance Liquid Chromatography (HPLC) to determine the activity attributable to parent plasma fraction.

5.3.7 Scanning procedures: MRI

Participants received a high-resolution T1 MRI scan in a Siemens Verio 3T scanner (Siemens Healthcare, Erlangen, Germany). All structural MRI images were inspected by a clinical neuroradiologist, for unexpected findings of clinical significance, or features that might confound PET analysis.

5.3.8 Imaging analysis

PET images were reconstructed via filtered back projection with attenuation and scatter correction by PET technicians working at Imanova Limited. Dynamic images were separated into twenty-six frames (8 x 15s, 3 x 1 min, 5 x 2 min, 5 x 5 min, 5 x 10 min). A metabolite-corrected plasma input function was generated by Dr Qi Guo, who performed all pre-processing prior to statistical analysis, using a method described previously (Owen et al., 2014). Briefly, the total plasma time activity curve (TAC) was calculated by multiplying the whole blood curve by plasma-over-blood ratio, and the parent fraction data were fitted to a sigmoid model:

$$f = \left(\left(1 - \frac{t^3}{t^3 + 10^a} \right)^b + c \right) / (1 + c)$$

where t is time and a , b and c are fitted parameters. The fitted parent fraction profile was multiplied by the total plasma curve and then smoothed post-peak using a tri-exponential fit to derive the required parent plasma input function. A time delay correction was applied to each scan to account for delays between blood sample measurement and tomographic measurement of tissue.

PET data were corrected for motion via frame-by-frame co-registration to each participant's T1 MRI, using SPM5 (Wellcome Trust Centre for Neuroimaging, <http://www.fil.ion.ucl.ac.uk/spm>). Anatomical regions of interest (ROIs) – hippocampus, midbrain, cerebellum, thalamus and cingulate cortex – were delineated by the application of an in-house anatomical atlas, which was warped to the participant's structural MRI scan. ROIs were applied to the PET data to derive regional TACs.

Published data suggest that a two tissue compartmental model provides the optimal model fit for [¹¹C]PBR28 PET data (Fujita et al., 2008). A two tissue compartmental model using a metabolite corrected input function, with blood volume fixed at 5%, was therefore applied to dynamic PET data, using Matlab R2008b (Mathworks, Natick, MA, USA). Volume of distribution (V_T) was estimated according to the following equation:

$$V_T = K_1/k_2(1 + \frac{k_3}{k_4})$$

where V_T is the regional volume of distribution, K_1 and k_2 are rate constants for the movement of [¹¹C]PBR28 from plasma to brain parenchyma and parenchyma to plasma respectively, and k_3 and k_4 are rate constants for the movement of [¹¹C]PBR28 from free in the tissue to bound to the specific target, and from bound to free, respectively.

5.3.9 Hippocampal volume analysis and partial volume correction

Hippocampal atrophy is commonly described in imaging studies of alcohol dependence (Sullivan et al., 1995), (Sullivan et al., 1996), (Agartz et al., 1999), (Laakso et al., 2000). The volume of the hippocampus was therefore calculated during pre-processing of the structural MRI using SPM5. After warping the Montreal Neurological Institute template to the participant's T1 image, the transformation parameters were applied to the in-house atlas so that the atlas and the participants T1 was aligned. The volume was calculated as resolution per voxel (2x2x2mm) x number of voxels in the hippocampal region in T1 defined by the warped atlas x the probability map of grey matter generated using SPM5. Hippocampal volume was then compared between HCs and ADPs using a two-tailed t-test. I established that there was evidence of hippocampal atrophy in the alcohol dependent group (HCs mean = 7853 + 1004.9; ADPs mean = 7132 + 823; $p = 0.026$). Partial volume correction was therefore undertaken, as described below.

The relatively poor spatial resolution of the PET scanner (4.6mm) and procedures during image reconstruction mean that a PET image consists of overlapping 3D Gaussian distributions from each point radiation source. This blurring is known as the 'partial volume effect' (PVE). The partial volume effect can be expressed as a linear relationship between the measured intensity, g , at a given voxel, r , and the a distribution of uptake, f .

$$g(r) = (psf * f)(r) + \eta(r)$$

where psf is the non-variant point spread function of the scanner, η is additive noise, $*$ refers to the convolution of psf and f , and r is a 3-D vector.

PVEs may cause underestimation of signal in areas of high binding located next to areas of low binding e.g. cerebrospinal fluid, and are greater in small regions and atrophic brain. Partial volume correction using LoReAN, a hybrid voxel-region-based algorithm (Coello et al., 2013), was undertaken by Dr Guo. The point spread function (psf) of the scanner was established using a cylindrical phantom with a known dose of Carbon-11.

5.3.10 Parametric analysis

Exploratory voxel-based analysis was undertaken by Dr Guo to establish whether other brain regions than those identified a priori were affected in the patient group. A Logan plot graphical method (Logan et al., 1990) using the plasma input function, 5% fixed blood volume, and a linear start time at 35 minutes was applied to each voxel to generate a parametric V_T map for each participant.

5.3.11 Statistical analysis

I performed the statistical analysis aside from the parametric analysis. Median substitution used for missing values in questionnaires and cognitive tests. Demographic and questionnaire data was graphed to assess distribution. Differences between groups were assessed using unpaired 2-tailed t tests applied to parametric data, and Mann-Whitney tests to non-parametric data. Analyses of Covariance (ANCOVA), implemented in SPSS 20.0 were performed to examine the effect of patient group on [^{11}C]PBR28 V_T across regions of interest identified *a priori*. Genotype was included as a fixed factor in the analysis as it is known to influence V_T . Age was included as a

covariate as TSPO expression is reported to vary with age (Kumar et al., 2012). Parametric images were compared using an ANCOVA with age and genotype as covariates, with correction for multiple comparisons implemented in Randomise (FSL, Oxford, 2004). Two-tailed correlations, accounting for age and genotype, were performed between hippocampal V_T and performance on the Weschler Memory Scale, Rey-Osterrieth Complex Figure, and frontal V_T and performance on Trail Making Tasks.

5.4 Results

5.4.1 Demographic characteristics of the sample

Twenty-one ADPs and ten healthy controls were recruited for the study as shown in Figure 5.1. Two healthy participants were ineligible because of binder status or co-morbidity at screening. A number of participants dropped out in both groups between screening and the scan because of other commitments or withdrawal of consent. Four ADPs were later unfit to scan because of a fractured jaw, intolerance of arterial line insertion and an unexpected finding of pontine myelinolysis on MRI scan. Three ADPs had unusable scan data because of movement or small cerebrovascular lesions detected by the neuroradiologist that were not apparent on neurological examination. The final sample included 9 alcohol-dependent patients and 20 healthy controls.

Demographic characteristics of the final sample are shown in Table 5.1. There were no significant differences in age ($p=0.590$) but there was a trend towards controls having spent more time in education ($p = 0.09$). Compared with healthy controls (HCs), ADPs unsurprisingly drank significantly more in the

month prior to detoxification ($p = 0.000$) and in their lifetime ($p = 0.004$), and had a longer lifetime duration of drinking ($p = 0.036$). The mean duration of abstinence at the time of the scan for the ADP group was 24 days (range 13 to 38 days). The median number of previous detoxifications was 1 (range 0 – 7). The mean Severity of Alcohol Dependence Questionnaire score in the ADP group was 29 ± 9 , corresponding to moderate to severe dependence on alcohol.

Fewer control participants were smokers than the ADP group (controls: 4 current smokers, 3 ex-smokers; ADPs: 8 current smokers). Similarly, no control participants abused substances or tested positive for drugs of abuse, whereas three ADP participants abused cannabis and two cocaine, and tested positive for these at the time of scanning. Four control participants and four ADP participants had a past history of depression requiring medication. There was no other psychiatric co-morbidity. Neither group suffered from clinically apparent medical co-morbidity.

One control participant was taking amlodipine. Two ADPs were taking both acamprosate and disulfiram to prevent relapse, and one had been prescribed acamprosate during detoxification. Three other ADPs were taking antidepressants. All were taking thiamine and vitamin B complex tablets. Seven patients were treated with chlordiazepoxide to manage their withdrawal symptoms during detoxification prior to the study. One received diazepam, and one underwent an unmedicated detoxification. No ADP participants were still being prescribed benzodiazepines at the time of scanning, but three had a

positive urine screen for benzodiazepines. The mean duration since last benzodiazepine dose was 14 days (range 6 – 29 days).

Figure 5.1: Recruitment Flowchart

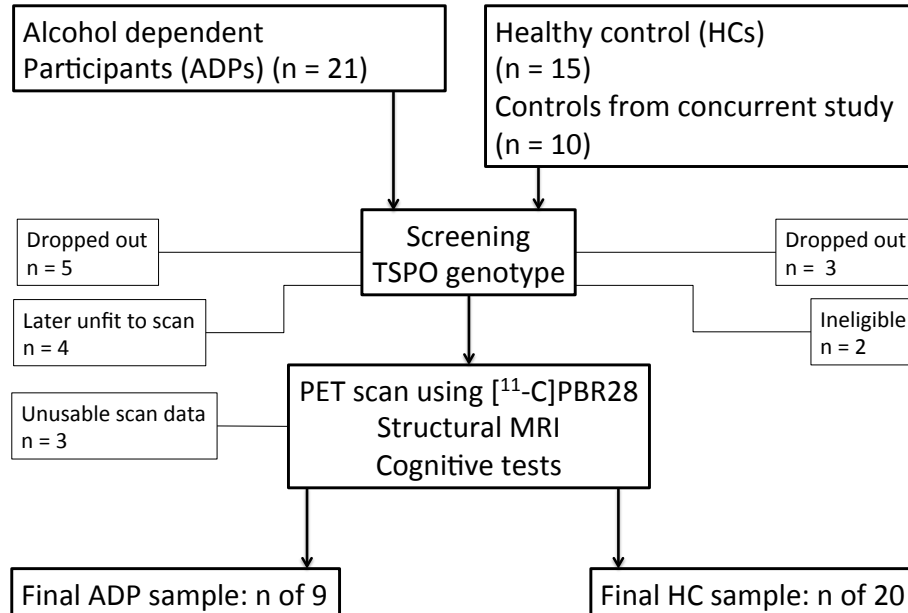


Table 5.1: Demographic characteristics of the sample

	Alcohol-dependent	Control	<i>p</i>
<i>n</i>	9	19	
Age	45 ± 13	45 ± 7	ns
Male:Female	9:0	14:5	ns
TSPO genotype (HAB: MAB)	3:6	10:10	ns
Years of drinking	26 (18 – 34)	15 (0 -24)	0.036*
Lifetime dose (kg)	83.29 (14.57 – 273.96)	10.91 (0 – 39.92)	0.004*
Cigarette smoking (n)	8	5	0.004**
Illicit drug use	4	0	0.006**
Past history of depression	4	2	ns

Legend: Parametrically distributed data are presented as mean ± standard deviation. Non parametrically distributed data are presented as median (range). Proportions are presented as ratios. **Differences between groups are calculated using 2-tailed t-tests for parametric data, the Kolmogorov-Smirnov test for non parametric data, and Fisher’s exact test for proportions. * *p* < 0.05; ** *p* < 0.01.**

5.4.2 Clinical questionnaires and cognitive function

Performance on clinical questionnaires and cognitive tests is tabulated in Table 5.2. Alcohol-dependent patients scored higher on the Obsessive Compulsive Drinking Scale but not the Alcohol Urge Questionnaire. They also scored higher on measures of depression and anxiety: the Beck Depression Inventory, Spielberger State Anxiety Inventory and Spielberger Trait Anxiety Inventory. Their scores on the Fatigue Severity Scale, a scale used in chronic inflammatory conditions, were also significantly increased relative to healthy controls.

Alcohol-dependent patients performed more poorly than healthy control participants on both immediate and delayed verbal recall. Delayed spatial memory, but not immediate spatial memory, was significantly worse in the ADP group. There were no differences in frontal lobe tasks.

Table 5.2: Psychological questionnaires and cognitive tests

Clinical questionnaires	Alcohol-dependent	Control	<i>p</i>
Obsessive Compulsive Drinking Scale	12.4 ± 8.0	3.9 ± 2.4	0.003**
Alcohol Urge Questionnaire	16 ± 9.5	12 ± 6.6	0.326
Beck Depression Inventory	11 ± 6	4 ± 4	0.002**
Spielberger Trait Anxiety Inventory	44 ± 6	35 ± 11	0.015*
Spielberger State Anxiety Inventory	33 ± 10	29 ± 9	0.013*
Fatigue Severity Scale	4 ± 0.7	2 ± 1.2	0.004**
Cognitive Tests			
Weschler Memory Scale Immediate	8 ± 2.9	11 ± 2.8	0.027*
Weschler Memory Scale Delayed	6 ± 2.5	9 ± 3.6	0.013*
Rey-Osterrieth Complex Figure Immediate	14 ± 11.2	20 ± 8.4	0.147
Rey-Osterrieth Complex Figure	14 ± 9	21 ± 7.9	0.048*

Delayed			
Trails A (time to complete)	30.3 ± 9.30	29.32 ± 16.9	0.861
Trails B (time to complete)	62.8 ± 36.48	57.7 ± 30.25	0.700
Digit span	16.0 ± 3.12	17.6 ± 4.52	0.340

Legend: * p <0.05; ** p < 0.01

5.4.3 *Peripheral blood measures*

All screening blood parameters were within normal limits in both groups, apart from mean cell volume (MCV), gamma glutamyl transferase (GGT) and alanine transaminase (ALT) in the alcohol-dependent group (see Table 5.2), that is, consistent with recent alcohol intake. Bilirubin, albumin and clotting values were normal, demonstrating that hepatic synthetic and metabolic functions were preserved in the patient group. Mean high sensitivity C-reactive protein (hsCRP) was within normal range in both groups and there was no significant difference between groups. No diazepam or chlordiazepoxide was detectable in any pharmacokinetic samples, but desmethyldiazepam was detectable in three samples (370 ± 148nM).

Table 5.3: Blood parameters

	Alcohol-dependent	Control	p
Haemoglobin	14.8 ± 1.2	14.6 ± 1.2	0.731
Mean cell volume	96.1 ± 6.1	84.9 ± 13.6	0.007**
White cell count	9.3 ± 2.0	6.5 ± 1.3	0.002**
Bilirubin	8 ± 5	12 ± 8	0.161
Alanine Transaminase	56 ± 20	26 ± 8	0.110
Alkaline Phosphatase	71 ± 50	68 ± 19	0.702
Gamma glutamyl transferase	134 (range 15 to 466)	30 ± 20	0.064
Albumin	40 ± 3	44 ± 3	0.004**
Adjusted Partial Thromboplastin Time	26.6 ± 2.3	28 ± 2	0.086

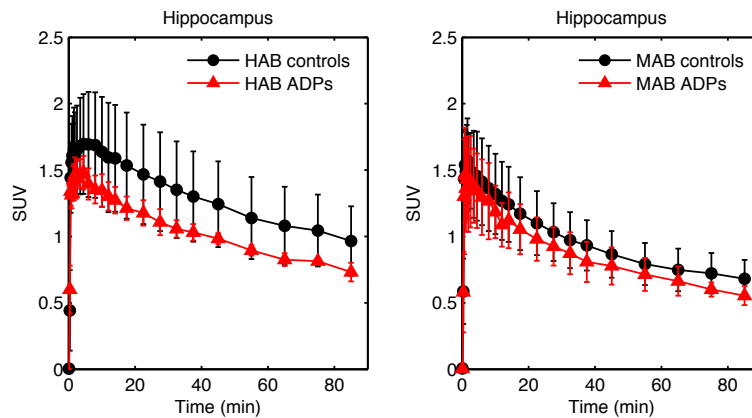
Legend: * p < 0.05; ** p < 0.01

5.4.4 [¹¹C]PBR28 Volume of distribution: ROI analysis

TSPO expression was significantly decreased by about 20% in the hippocampus in alcohol-dependent patients relative to healthy controls (F (1, 24) 5.694; p = 0.025; effect of patient group -1.196 (95% C.I.: -0.162 to -2.231)), corrected for the effects of genotype and age. Time activity curves for the hippocampus are shown in Figure 5.2, with HABs and MABs shown separately. Mean V_T s in the hippocampus for all genotypes and groups are shown in Figure 5.3. Differences in other regions of interest – midbrain, thalamus, cerebellum and anterior cingulate cortex – did not reach statistical significance, although a trend towards lower V_T was detected in the midbrain (F (1, 24) = 3.879; p = 0.061), as illustrated in Figure 5.4. As anticipated, age and genotype were significantly associated with variation in [¹¹C]PBR28 V_T in all regions tested (for example, in the hippocampus: genotype F (1, 24) = 8.190; p = 0.009; age: F (1, 24) = 5.370; p = 0.029). In the hippocampus, the effect of genotype was 1.459 (95% C.I.: 0.407 – 2.512) (F = 8.190; p = 0.009).

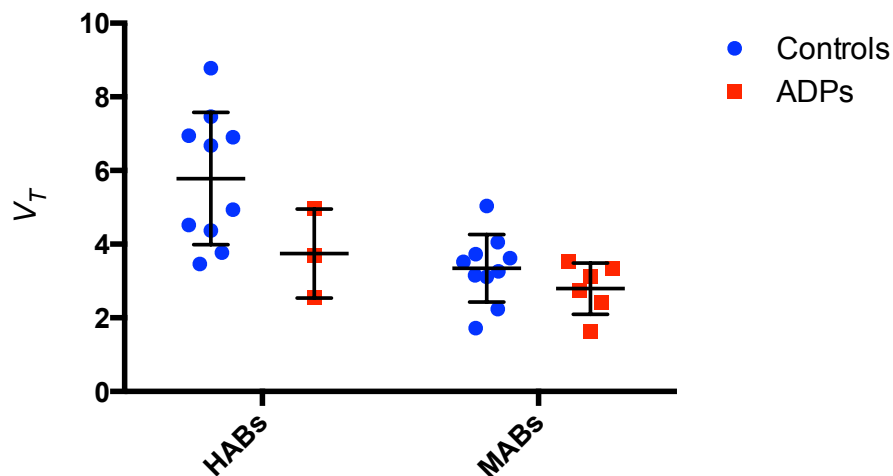
There was no patient group x genotype interaction ($p > 0.05$). V_T was not associated with duration of abstinence in alcohol-dependent group, controlling for age and genotype in any region.

Figure 5.2: Time activity curves in the hippocampus



Legend: This figure shows mean time activity curves with error bars representing standard deviation, from the hippocampus in healthy controls, shown in black, and ADPs, shown in red.

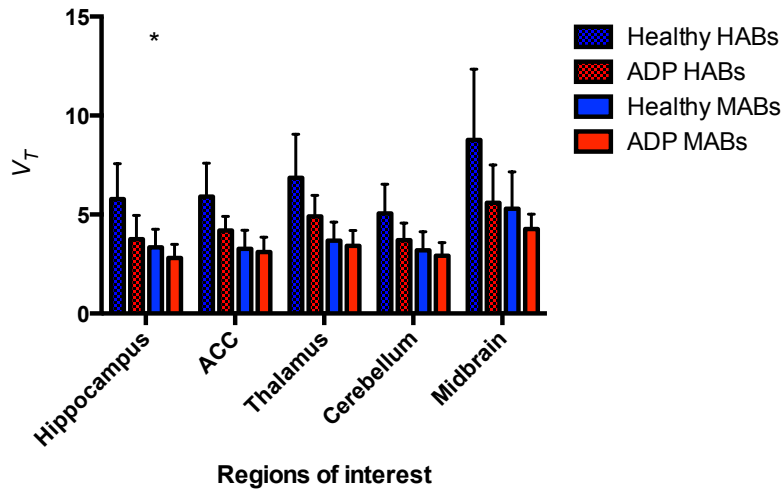
Figure 5.3: [^{11}C]PBR28 V_T is lower in the hippocampi of ADPs than in controls (mean \pm sd)



Legend: Blue circles reflect healthy control values and red squares alcohol dependent values. There is a larger standard deviation in the healthy control sample especially in the HAB subset. This reflects the greater age range in the health control group, as age

was positively associated with [^{11}C]PBR28 V_T in all ROIs studied (see Table 5.1 for relative age ranges and page 137 for effect of age on V_T).

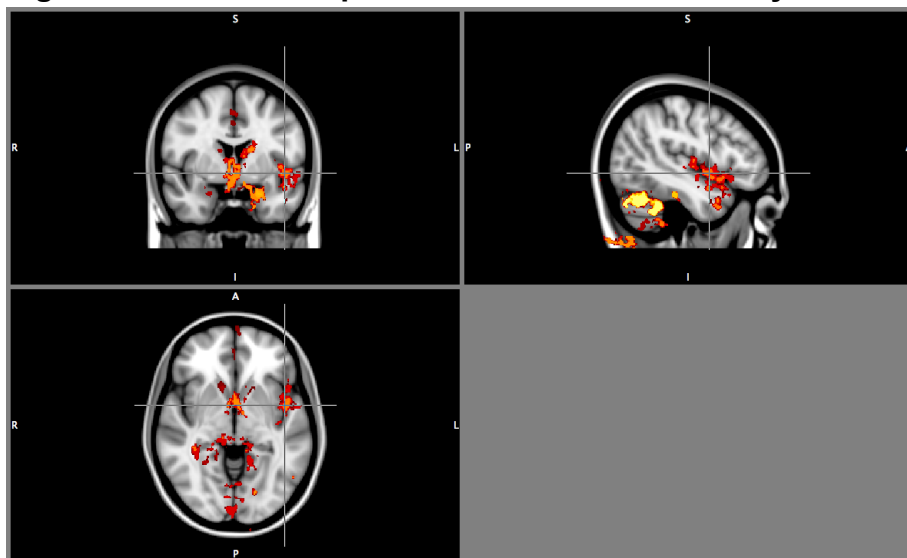
Figure 5.4: Lower V_T in ROIs apart from the hippocampus did not reach significance (mean + SD)



5.4.5 [^{11}C]PBR28 Volume of distribution: parametric analysis

Parametric analysis, controlling for the effects of age and genotype, did not yield any clusters meeting the multiple comparison threshold. At a less stringent threshold of $p = 0.13$, several clusters were identified: the left hippocampus, left insula, left caudate, and thalamus and cerebellar cortex, bilaterally exhibited lower V_T in the alcohol-dependent group. These are shown in Figure 5.5.

Figure 5.5: Results of parametric voxel-based analysis



5.4.6 Relationship between hippocampal [^{11}C]PBR28 V_T and memory

Correlations, controlling for the effects of age and genotype, were found between hippocampal V_T and scores on the delayed Weschler Memory Scale (WMS), a measure of verbal memory, and delayed Rey-Osterrieth Complex Figure (ROCF) test, a measure of spatial memory, when the patient group and the healthy group were combined (WMS: Pearson's $r = 0.720$, $p = 0.000$; ROCF: Pearson's $r = 0.004$), as shown in Figure 5.6 and 5.7. When the patient and control groups were separated, the association between verbal memory and hippocampal V_T remained in the healthy control group (Pearson's $r = 0.738$; $p = 0.001$), but not the alcohol-dependent group (Pearson's $r = 0.331$; $p = 0.468$). A trend toward an association remained between spatial memory and hippocampal V_T in the healthy group (Pearson's $r = 0.445$; $p = 0.073$), but not in the alcohol-dependent group (Pearson's $r = 0.058$; $p = 0.902$). There were no associations between hippocampal V_T and

performance on digit span or the Trail Making Task, nor any associations between frontal cortex V_T and performance on digit span or the Trail Making Task.

Figure 5.6: Correlation between hippocampal V_T and verbal memory

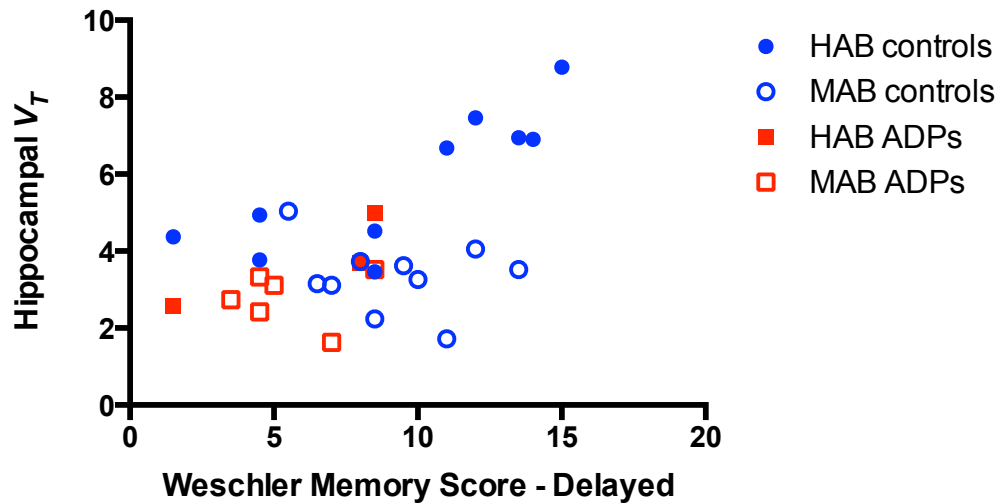
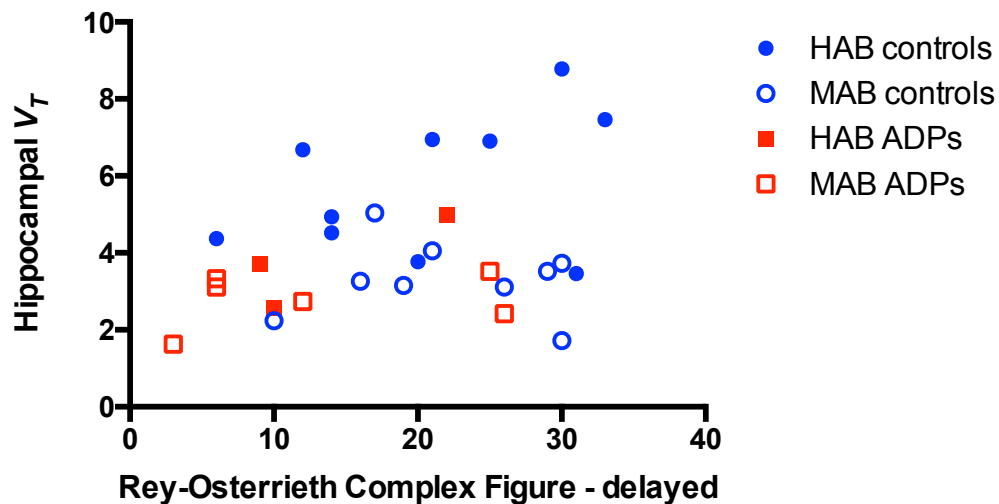


Figure 5.7: Correlation between V_T and spatial memory



5.5 Discussion

This is the first study to report changes in [^{11}C]PBR28 V_T in a cohort of otherwise healthy, alcohol-dependent patients within the first few weeks of abstinence. Contrary to my hypothesis, the main finding is that TSPO V_T is

significantly decreased in the hippocampus in alcohol-dependent patients, relative to healthy control participants. There was also a trend towards a decrease in the midbrain. In the study population taken as a whole there was a direct relationship between both verbal and spatial memory and hippocampal [¹¹C]PBR28 V_T . In the healthy control group, the correlation between verbal memory and hippocampal V_T remained significant and a trend towards a correlation between spatial memory and hippocampal V_T remained. These correlations were not present in the alcohol-dependent group. Published findings regarding the effect of genotype and age on V_T (Kumar et al., 2012) were replicated.

The finding of a regional, hippocampal decrease in alcohol-dependent patients shortly after detoxification may be of clinical significance, as lower binding was associated with poorer memory. Visuo-spatial and verbal learning and memory deficits associated with the hippocampus are well-recognised in pre-clinical models of alcohol dependence, as well as in clinical populations (e.g. (Matthews and Morrow, 2000) (Fama et al., 2009) (Davies et al., 2005)). Alcohol-related hippocampal atrophy has also been described in structural imaging studies (Sullivan et al., 1995), (Sullivan et al., 1996), (Agartz et al., 1999), (Laakso et al., 2000). There is debate about the underlying pathology causing the atrophy. Loss of neurons has been inconsistently reported, and loss of glia and microglia (Korbo, 1999), dendrites, or synapses, or the cessation of neurogenesis, have been proposed (reviewed in (Harper, 1998)). The finding of this study, that is, a decrease in [¹¹C]PBR28 V_T following partial volume correction in the setting of

hippocampal atrophy suggests is consistent with Korbo's histological study showing loss of glia and microglia in this brain region post-mortem (Korbo, 1991).

Structural MRI studies have failed to demonstrate an association between hippocampal volume and hippocampal memory function (Sullivan et al., 1995), (Ratti et al., 1999). Smaller hippocampal white matter volume, however, has been related to withdrawal seizures (Sullivan et al., 1996). Three patients had a history of seizures; however I did not investigate a relationship to seizures owing to sample size. A single PET study found that the density of the alpha-5-receptor subtype of the GABA receptor was associated with improved memory function (Lingford-Hughes et al., 2012a). This, taken together with my finding, suggests that PET may be of more use than structural imaging, to characterise alcohol-related changes of relevance to cognitive function.

Based on pre-clinical data, I had hypothesised that higher TSPO binding, reflecting microglial activation, would be present in alcohol dependence rather than lower levels compared with healthy controls. However it may be that the changes occurring after prolonged alcohol intake are different from those occurring after brief, binge exposure, which might account for the lower level in practice. Pre-clinical binge paradigm data published during my study found that hippocampal microglial activation during withdrawal was associated with an increase in TSPO (Marshall et al. 2013). However, this was associated

with an increase in the anti-inflammatory IL-10, and no changes in pro-inflammatory cytokines such as TNF- α or IL-6 concentrations. It has been suggested that this microglial response supports neurogenesis, suppressed during chronic drinking, in early abstinence (Nixon et al., 2008). Microglia in this context may therefore be neurotropic rather than neurotoxic.

In post-mortem human brain samples representing more chronic alcohol intake, loss of microglia and glia in the hippocampus has been reported (Korbo, 1999). Thus it is possible that microglial loss in the hippocampus either exacerbates damage, or delays the return of neurogenesis. Alternatively, it has been reported that TSPO is also expressed by hippocampal neural stem cells (Varga et al., 2009). Pre-clinical findings regarding the effect of alcohol on neurogenesis have yet to be replicated in human post-mortem samples and human neurogenesis in adulthood is reported to be rare (Sutherland et al., 2013), so it is less likely that lower TSPO binding reflects loss of neural progenitors in the hippocampus.

A difference between pre-clinical and clinical data is the delay between the initiation of detoxification and the scan, raising the possibility that short-lived microglial activation may be missed. Nixon and colleagues describe increased microglial activation as measured by [³H]PK11195 autoradiography on the first day and the seventh day of withdrawal but not at one month post withdrawal (Marshall et al. 2013). Other animal models describe shorter-lived activation of microglia as measured by immunohistochemistry (Zhao et al.,

2013). However, microglial activation following alcohol binge dosing has been reported to persist at twenty-one days of abstinence (Obernier et al., 2002), which suggests that microglial activation, if present, may have been detectable at this duration of abstinence. In my study there was no relationship between duration of abstinence and V_T , corrected for age and genotype.

The delay between the beginning of alcohol detoxification and scanning was unavoidable for several practical reasons. Patients presenting to local alcohol services for detoxification, were either detoxified as out-patients within days of presenting, or were admitted to an in-patient unit. Those who were detoxified as out-patients were usually recruited after their first detoxification appointment and screened within the next day or so. Those who were admitted for in-patient detoxification were either recruited on a local ward, or were recruited and screened prior to detoxification if they were going to a third-sector detoxification unit in Portsmouth. If the latter was the case, the earliest they could be scanned was on discharge from the unit, usually around two weeks after detoxification. TSPO genotyping took a minimum of seventy-two hours. Scan days needed to be booked a month in advance.

An additional and important concern was the potential impact of benzodiazepines on [^{11}C]PBR28 binding. As described in Chapter 4, this was unlikely to be the cause for concern after the first two days of detoxification, and only if patients were treated with diazepam (Kalk et al. 2013 2013). Only

one of the patients received diazepam and pharmacokinetic data from my study found detectable levels only of the metabolite, desmethyldiazepam, which does not bind the TSPO. TSPO may be affected by medication, such as the anaesthetic agent propofol (Hines et al., 2012). Further, anti-relapse medication has been reported to have anti-inflammatory effects (Franchi et al., 2010). However if this were the case, a general effect on binding, rather than regional differences, would have been anticipated. I was also unable to compare the effects of smoking, which is reported to affect hippocampal damage in the context of alcohol dependence (Gazdzinski et al., 2008), as all but one patients smoked, compared to a minority of controls who did.

Although this finding was contrary to my hypothesis, it is not one without precedent. When TSPO PET was undertaken using [^{11}C]PK11195 little specific signal was detected in healthy brain. This is not the case with [^{11}C]PBR28, which means that it is, in theory, more sensitive to decreases from healthy expression levels, as well as increases. Recent pre-clinical and clinical data support this. Individual age-matched depressed patients showed a decrease in global cerebral [^{11}C]PBR28 V_T relative to their matched controls, though there was no difference in *mean* V_T between groups (Hannestad et al., 2013). A longitudinal study in primates of the microglial response to lipopolysaccharide showed that in one subject the binding decreased by 40% relative to baseline, suggesting that microglial activation and/or TSPO expression is more dynamic than previously appreciated.

The study is affected by limitations mainly around the patient sample: a small sample size, with varied prescribed medications, a few weeks from cessation of alcohol intake. To an extent these limitations reflect the logistics of recruitment and PET scanning patients at high risk of disengagement from services as well as from research, in a setting where patients are not on-site and delivery of care occurs through a combination of statutory and voluntary organisations.

5.6 Conclusion

This study found a statistically significant decrease of 20% in [^{11}C]PBR28 V_T in the hippocampus of alcohol-dependent patients in early abstinence. Hippocampal V_T was inversely correlated with performance on a delayed memory task, which suggests that this may be of clinical relevance. Previously reported relationships between genotype and age, and V_T were replicated. Importantly, no increase in binding was found in the alcohol-dependent participants, raising questions about the function of microglia in this context and how this may translate into a treatment target. Certainly the pre-clinical literature initially suggested that anti-inflammatory treatment may be useful (Pascual et al., 2007). It remains to be established whether microglial activation occurs during drinking or more acutely during the first forty-eight hours of detoxification.

CHAPTER 6

BRAIN GLUTAMATE AND GLUTAMINE AND THEIR RELATIONSHIP TO MICROGLIAL ACTIVATION AND COGNITIVE FUNCTION

6.1 Introduction

In Chapter 5, microglial activation was investigated using [¹¹C]PBR28 PET, and significantly lower TSPO binding was found in the hippocampus and midbrain in alcohol-dependent (ADP) men compared with control participants. This chapter describes an experiment undertaken to investigate the relationship between microglial activation detected by TSPO PET and brain glutamate concentrations measured with Magnetic Resonance Spectroscopy (MRS). This is of interest because increased brain extracellular glutamate concentrations (Dahchour and De Witte, 2003) and increased glutamatergic transmission contribute to the pathogenesis of the symptoms and complications associated with alcohol withdrawal.

As described in the Chapter 1, Section 7, glutamate is involved in neurotransmission as well as metabolic pathways in the brain. Chronic alcohol intake results in upregulation of NMDA receptors and an increase in NMDA receptor sensitivity to glutamate. Sudden withdrawal of alcohol is associated with an increase in extra-cellular glutamate, which in the presence of upregulated, sensitive NMDA receptors, can lead to glutamatergic excitotoxicity (Mayer et al., 2002). Clinically, increased glutamate transmission is important in the pathogenesis of alcohol withdrawal, as pre-clinical studies (Stepanyan et al., 2008) and clinical studies support the use of NMDA

antagonists to manage withdrawal symptoms (Krupitsky et al., 2007). A pre-clinical alcohol model demonstrated concurrent glutamate excess and microglial activation in the hippocampus, so the two may be related (Ward et al., 2009). Pre-clinical research in other acute brain insults suggests that microglia may be protective against glutamatergic excitotoxicity (Vinet et al., 2012) (Palazuelos et al., 2009) (Lambertsen et al., 2009). I therefore undertook an MRS study of frontal and occipital cortex glutamate concentrations in conjunction with the [^{11}C]PBR28 PET study as I hypothesised that both [^{11}C]PBR28 V_T and glutamate would be increased in the alcohol dependent group, and that there may be a correlation between the two.

Brain glutamate can be measured in clinical populations using Magnetic Resonance Spectroscopy (MRS), though this measures both intracellular/metabolic and synaptic glutamate. MRS uses a succession of radiofrequency pulses in a strong magnetic field in order to disrupt the axes of hydrogen nuclei. It exploits the effects of the immediate molecular environment on the frequency with which the nuclei move from the flipped axis to the pre-existing axis, a process which generates electric currents. These are detected by the scanner and used to generate a spectrum of peaks, which correspond to the concentration of different molecules in the voxel studied. Clinical studies using MRS during alcohol withdrawal and early abstinence (see Table 1.3), have found that brain glutamate concentrations ([Glu]) are raised during the early phase of alcohol withdrawal (Yeo et al., 2013) (Hermann et al., 2012). During early abstinence, both increased and

decreased [Glu] have been described and may be modulated by brain region studied or clinical factors such as smoking (Mason et al., 2005) and relapse prevention treatment (Umhau et al., 2010).

6.2 Aims and hypotheses

Based on previous evidence available when I designed the PET and MRS studies, I hypothesised the following:

1. Brain glutamate, glutamine, and [Glx] (glutamate and glutamine concentrations combined) concentrations would be raised in alcohol-dependent patients (ADPs) from controls.
2. Glutamate, glutamine, or [Glx] would be positively correlated with [¹¹C]PBR28 binding.
3. Glutamate, glutamine, or [Glx] would be negatively correlated with cognitive test performance.

6.3 Methods

6.3.1 Recruitment and sample

Recruitment and demographic characteristics were as for the [¹¹C]PBR28 PET study in alcohol dependence described in Chapter 3, as the MRS scan was performed in the same group comparison study.

6.3.2 Magnetic Resonance Spectroscopy

Participants were scanned with a 32-channel head coil and a Siemens Verio 3 Tesla MRI scanner on the same day as the PET scan. The Magnetisation

Prepared Rapid Gradient (MPRAGE) (Repetition Time (TR)/Echo Time (TE) = 2300/2.98ms; 9° flip angle; resolution 1mm³) was used to acquire a 3-D sagittal T1-weighted image. The T1 images were also used for segmentation of the voxel into grey matter, white matter and cerebrospinal fluid using SPM8 (Ashburner and Friston, 2005).

A 20mm x 20mm x 20mm voxel was placed over the anterior cingulate cortex (ACC), defined as anterior to the genu of the corpus callosum and superior to the anterior commissure to posterior commissure line, centred on the median line (shown in Figure 6.1a). The ACC was chosen, as it was an area where more numerous microglia were found post-mortem in alcohol-dependent patients (He and Crews, 2008). Unlike deeper brain structures such as the hippocampus or nucleus accumbens, it is possible to obtain analysable spectra from the ACC in a 3 Telsa magnetic field.

A 30mm x 30mm x 30mm voxel was placed over the occipital cortex (OCC) parallel to the cerebellar tentorium, and as posterior as possible within the limits of the brain, again centred on the median line (see Figure 6.1b). The occipital cortex was chosen since this is an area with optimal signal to noise ratio, due to its location close to the radiofrequency coils and good static magnetic field homogeneity. As a result, robust, diurnally-stable measurements can be produced (Evans et al., 2010). It is also the only region that can accommodate the 30mm x 30mm x 30mm voxel necessary to capture GABA. 3-D shimming was performed to correct for inhomogeneities in the magnetic field.

Figure 6.1a Voxel placement in the Anterior Cingulate Cortex

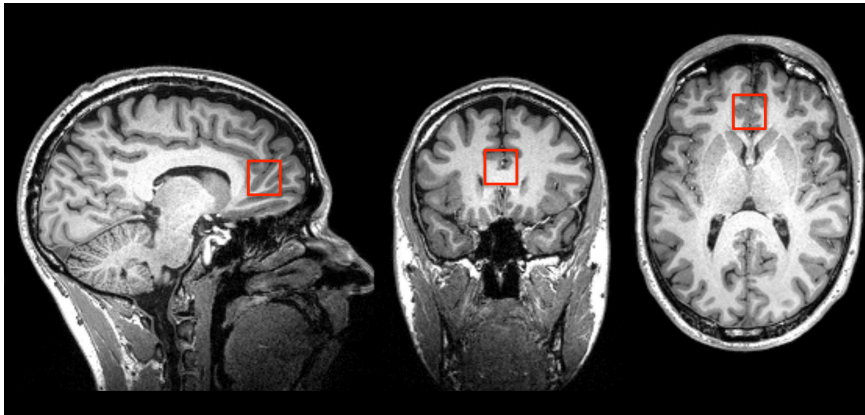
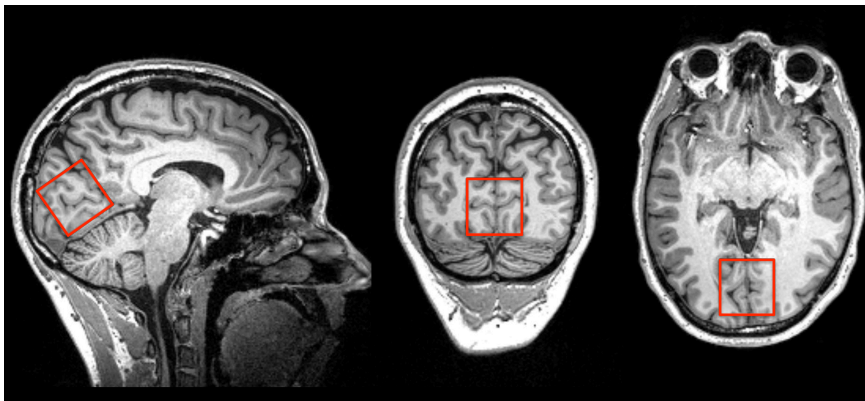


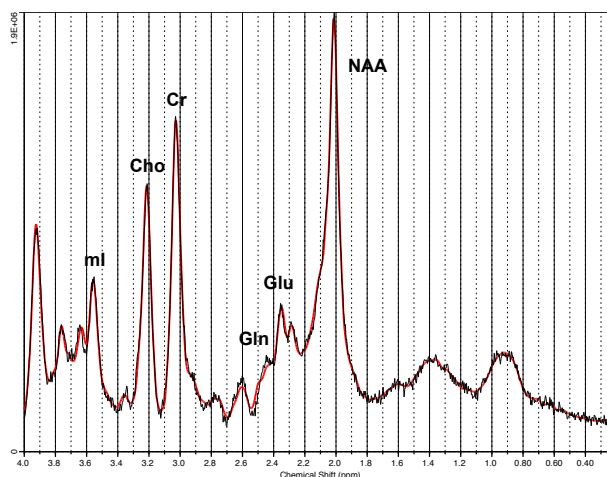
Figure 6.1b Voxel placement in the Occipital Cortex



Both water-unsuppressed and water-suppressed sequences were captured from the ACC and OCC. In the ACC, a Point Resolved Spectroscopy (PRESS) sequence was applied (96 averages; TR/TE: 3000/30ms; 90° flip angle; 2000Hz bandwidth) to measure a range of metabolite concentrations including glutamate ([Glu]), glutamine ([Gln]), gamma-hydroxy-butyric acid ([GABA]), creatine ([Cre], a measure of cellular metabolism), N-acetyl aspartate ([NAA], a marker of neuronal integrity), choline ([Cho], a marker of membrane turnover), and myoinositol ([ml], a glial marker) (see Figure 6.2). PRESS is a technique whereby magnetic gradients are applied along three orthogonal axes in the during each radiofrequency pulse to provide spatial localisation of the signal. A typical spectrum derived from a single subject

during my study, showing the characteristic spectra of brain metabolites, is shown in Figure 6.2.

Figure 6.2: PRESS sequence spectrum



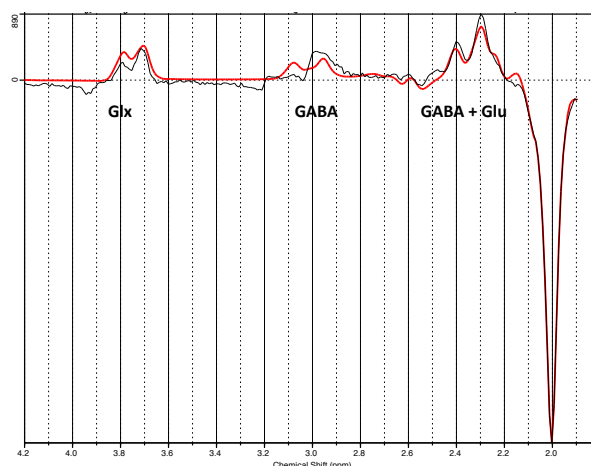
Legend: Spectra are presented with increasing frequency from right to left by convention. The black spectrum is the raw data and the red line the model fit generated by LCModel. The main spectra of the metabolites are labelled with the following abbreviations: Cho = choline; Cr = creatine; Gln = glutamine; Glu = glutamate; ml = myoinositol; NAA = N-acetyl aspartate.

In the OCC, a Mescher-Garwood PRESS (MEGA-PRESS) sequence was applied in order to detect GABA as well as [Glx], and [NAA] (240 averages; TR/TE: 1500/68ms; 90° flip angle; edit pulse frequency -1.9ppm; edit pulse bandwidth 45Hz; edit centre frequency: 4.70ppm). MEGA-PRESS is a method that uses a pulse at a specific frequency to differentiate the GABA peak from others at a similar frequency, although some contaminations with similar structures such as homocarnosine and certain macromolecules remain (Mescher et al., 1998). As a consequence, the GABA signal resolved by this edited spectrum is referred to as [GABA+]. A typical spectrum derived from MEGA-PRESS in a single subject is displayed in Figure 6.3.

6.3.3 Image processing

Image analysis was conducted using LCModel Version 6.3-1H (Provencher, 1993) using a standard basis set of metabolites acquired on a Siemens scanner of the same brand and field strength (3T). LCModel makes use of full spectra generated from *in vitro* metabolite solutions to inform analysis of the *in vivo* spectrum as a linear combination of separate spectra. Provided the same sequence is used, Basis sets, that is spectra acquired from a phantom containing a specified concentration of a metabolite, from a similar scanner can be used (Provencher, 1993). 'Water scaling', that is, adjustment of the area under the curve of each metabolite to area under the curve for water in each participant, was undertaken. However as water-scaled metabolite concentrations differ from mM by a factor of ~20% they are reported as 'institutional units' rather than mM (Provencher, 2014).

Figure 6.3: MEGA-PRESS sequence spectrum



Legend: Spectra are presented with increasing frequency from right to left by convention. The black spectrum is the raw data and the red line the model fit generated by LCModel. The main spectra of the metabolites are labelled with the following abbreviations: GABA = Gamma amino butyric acid; Glu = glutamate; Glx = a joint measure of glutamate and glutamine which is calculated if their peaks cannot be resolved adequately.

In the MRS literature it is commonplace to use either water scaling or a ratio of other metabolites as a way of normalising the measurement to account for differences in voxel composition. The validity of creatine as a reference rests on the assumption that creatine concentrations are constant across brain regions and are unaffected by brain pathology (Maddock and Buonocore, 2012). The ratio is uninformative if creatine differs between groups. My primary analysis therefore used water scaling and an adjustment for percentage of cerebrospinal fluid in the voxel. To allow comparison with previous findings, an analysis of metabolite:creatin ratios was conducted from the PRESS data. The concentrations supplied by MEGA-PRESS output in LCModel did not provide creatine concentrations, so no creatine ratios are reported for the occipital cortex metabolites.

Those concentrations where the standard deviation produced by LCModel exceeded Cramer-Rao standard error lower bounds of 25%, were excluded from analysis. For this reason, some metabolites could not be analysed: [GABA+] in the ACC, and glutathione in the OCC, as their standard error bounds for the majority of analyses exceeded 25%. Those analyses with non-random residuals or a 'wild baseline' (Provencher, 2014) were not included in statistical analysis (for ACC: 2 ADPs, 1 HC; for OCC: 2 ADPs, 1 HC).

6.3.4 Statistical analysis

All statistical procedures were performed using SPSS 20.0. *A priori* hypotheses regarding group differences were tested using independent-sample t-tests, with Bonferroni correction for multiple comparisons. For other

metabolites, exploratory analysis with no correction for multiple comparisons was undertaken. Pearson's correlations, corrected for genotype, were conducted to characterise relationships between [^{11}C]PBR28 V_T in the anterior cingulate cortex and [Glu] and [Glx] in the MRS cingulate voxel. Pearson's correlations were also undertaken to interrogate the relationship between glutamate and [Glx] with cognitive test performance. A more sophisticated statistical method such as MANOVA, which would take into account the correlation between performances on different cognitive tests, was not used here since the sample size was too small.

6.4 Results

The mean metabolite concentrations are presented in Table 6.2. Anterior cingulate cortex (ACC) [Gln] was significantly lower in Alcohol-dependent Patients (ADPs) than controls ($p = 0.016$). There were no significant differences in ACC glutamate. Although both ACC and occipital [Glx] were also significantly lower in ADPs (ACC: $p = 0.03$; occipital: $p = 0.044$), these differences disappeared after correction for multiple comparisons. Exploratory analysis revealed that ADPs had significantly lower choline ([Cho]) and creatine concentrations ([Cr]) in the ACC, without differences in ACC N-acetylaspartate ([NAA]), or myoinositol concentrations ([mI]), and with no differences in occipital γ -hydroxy-butyric acid concentrations ([GABA+]) and [NAA].

Table 6.2: Comparison of metabolites between ADPs and HCs (mean + SD) in Institutional Units

Metabolite	ADPs	HCs	<i>p</i>
Anterior cingulate cortex			
[Glu]	7.23 ± 1.277	7.47 ± 0.919	0.661
[Glx]	7.82 ± 3.239	10.74 ± 1.676	0.033*
[Gln]	1.93 ± 0.388	2.97 ± 0.987	0.016*
[Cr]	5.98 ± 0.578	6.88 ± 0.814	0.017*
[NAA]	6.32 ± 1.271	7.08 ± 0.714	0.147
[Cho]	1.39 ± 0.218	1.88 ± 0.181	0.000**
[ml]	4.87 ± 1.277	5.15 ± 0.615	0.540
ACC metabolite:Cr ratios			
[Glu]:[Cr]	1.20 ± 0.114	1.12 ± 0.244	0.402
[Glx]:[Cr]	1.50 ± 0.127	1.61 ± 0.434	0.528
[NAA]:[Cr]	1.06 ± 0.183	1.06 ± 0.210	0.970
[Cho]:[Cr]	0.23 ± 0.027	0.28 ± 0.041	0.023*
[ml]:[Cr]	0.81 ± 0.128	0.75 ± 0.086	0.336
Occipital cortex			
[Glx]	5.27 ± 1.245	6.61 ± 1.260	0.044*
[GABA+]	2.19 ± 0.350	2.44 ± 0.795	0.423
[NAA]	9.43 ± 1.420	9.68 ± 2.091	0.778

Legend: ADPs = alcohol-dependent patients; [Cho] = choline concentration; [Cr] = creatine concentrations; DLPFC = dorsolateral prefrontal cortex; [GABA+] = Gamma amino butyric acid concentration; [Gln] = glutamine concentration; [Glu] = glutamate concentration; HCs = healthy controls; [ml] = myoinositol concentration; [NAA] = N-acetyl aspartate

ACC [Cr] was lower in the alcohol-dependent patient group. As explained above, this renders other metabolites corrected for [Cr] concentrations, uninformative. These were calculated only to allow comparison with other studies in the same population that only used the [Glu]:[Cr] ratio. There were no significant differences in [Glu]:[Cr] or [Glx]:[Cr] between groups. The decrease in choline seen when absolute concentrations were considered remained significant.

There were no significant correlations between ACC [Glu] or [Glx] and ACC [^{11}C]PBR28 V_T , or between occipital [Glx] and occipital [^{11}C]PBR28 V_T . There were no significant correlations between ACC [Glu] or [Gln], or occipital [Glx] and cognitive performance on any test.

6.5 Discussion

There was significantly lower [Gln] in ACC of alcohol-dependent patients in early abstinence compared with healthy controls. [Glx] was also lower in both the ACC and occipital cortex, although not significantly so after correcting for multiple comparisons. These findings contradicted my hypothesis, as I expected to find higher [Glu] and [Glx] in ADPs. There was significantly lower [Cho] which remained after Bonferroni correction, and significantly lower [Cr], which did not. There was a significantly decreased [Cho]:[Cr] ratio in the ACC (though this disappeared after Bonferroni correction) but no other significant differences in metabolite:creatine ratios between groups. I found no association between MRS measures of [Glu], [Gln] or [Glx] and [^{11}C]PBR28 V_T . The absence of significant correlations in the ACC between [Glu], [Gln] or [Glx] and [^{11}C]PBR28 V_T was perhaps unsurprising given the small sample numbers and the need to take genotype into account. In addition, the PET study found no differences between ADPs and controls, which suggested an absence of microglial activation in this region. Finally, in contrast to a previous study which found an inverse association between the glutamate creatine ratio and short term memory, I found no association between performance on cognitive tests and ACC [Glu] or [Glx], [Glu]:[Cr] or [Glx]:[Cr], or occipital [Glx].

The finding of a significant decrease in [Gln] in the ACC and the suggestion that there may be decreased [Glx] in the ACC and occipital cortex, are novel findings in alcohol dependence. Glutamine has been suggested to be a proxy for synaptic glutamate, as it is formed from glutamate absorbed by astrocytes (Yuksel and Ongur, 2010). However, changes in widely distributed metabolites may not represent changes in neurotransmission (Myers et al., 2014). Decreased [Glu] and [Gln] may also represent diversion of glutamate towards other metabolic pathways. For example, glutamate is the precursor of glutathione, an antioxidant reported to be depleted in alcohol dependence (Peter, 2013). Decreased [Glu] or [Gln] could therefore represent diversion of glutamate to replenish glutathione stocks in early abstinence. Although glutamate is also a precursor of GABA, additional synthesis of GABA is unlikely, as occipital concentrations in my sample did not differ between ADPs and controls. Of interest is that the findings are opposite to those in hepatic encephalopathy, a common complication of alcohol dependence, where [Glx] is reported to be increased (van der Graaf, 2010).

As observed in the introduction, findings with respect to [Glu], [Gln] and [Glx] in alcohol dependence are variable according to population and brain region studied. Even in other studies considering only the anterior cingulate cortex with a similar duration of abstinence to my study, increases (Lee et al., 2007), decreases (Mon et al., 2012), or no differences (Bauer et al., 2013) in [Glu], and no differences in [Glx] (Bauer et al., 2013) have been reported. A single previous study considering occipital [Glx] found no differences (Mason et al.,

2005). In Lee and colleagues' study, a different way of quantifying glutamate, using creatine as a reference compound, was used (Lee et al., 2007). This could account for differences between their finding and mine, as they also found a significant decrease in creatine in their alcohol-dependent participants; the implication is that this decrease, rather than raised glutamate, is likely to explain the increase in glutamate:creatinine ratio.

Other findings such as the decrease in choline are consistent with previous reports in early abstinence (Ende et al., 2005) (Lee et al., 2007) (Mon et al., 2012). The MRS choline signal is a composite of choline and choline-containing compounds, such as phosphocholine, a component of cell membranes. Choline is an essential nutrient, with many roles in the brain including membrane component synthesis (Kennedy, 1956). It has been found to promote glutathione synthesis (Adibhatla and Hatcher, 2005), increase glial glutamate uptake (Hurtado et al., 2008) and promote hippocampal neurogenesis (Diederich et al., 2012) following acute brain insults such as stroke. It has been demonstrated to reduce ischaemic volume in pre-clinical models of stroke (Hurtado et al., 2013); however clinical trials of choline have failed to demonstrate neuroprotection, perhaps because of poor bioavailability (Davalos et al., 2012).

MRS studies of choline in relation to alcohol consumption have highlighted the importance of the point at which measures are taken in alcohol misuse and dependence. Choline has been reported to rise in association with drinking in both pre-clinical and clinical studies (Zahr et al., 2010) (Meyerhoff et al.,

2004), yet in early abstinence, choline has consistently been reported to be lower in ADPs than in healthy controls. It has been proposed that choline rises as a compensatory measure during heavy drinking but that chronic drinking causes decompensation (Ende et al., 2006). In other clinical contexts, choline has been proposed as a possible MRS marker of inflammation because of an association with demyelination (De Stefano and Filippi, 2007). The finding here of decreased choline in the same sample as decreased hippocampal [^{11}C]PBR28 binding raises the possibility that decreased choline is related to the suppression of neurogenesis. This could be investigated in a larger sample of patients with a single TSPO genotype.

6.5.1. Limitations

A limitation of this study in the light of the PET study findings was that I was unable to image the hippocampus using MRS, as this requires higher magnetic field strength than that available to me to generate interpretable spectra. Nonetheless, analysis of ACC and occipital voxels allows comparison with other studies in early abstinence. Another limitation was that the MRS sample size was much smaller than the main imaging study, as only controls that I recruited for the alcohol study had MRS measures taken. The sample size was further diminished by poor spectra from several participants, either because of movement or because of the proximity of the ACC voxel to the frontal sinus. This substantially limited my ability to investigate correlations with clinical variables or [^{11}C]PBR28 V_T . Additionally, the logistics of scanning precluded measurement of glutamate during the very first days of detoxification, when other studies have found increased concentrations (Yeo

et al., 2013) (Bauer et al., 2013) (Hermann et al., 2012). Finally, MRS measures both synaptic and metabolic pools of glutamate, making it difficult to distinguish the two (Stagg et al., 2011) (Myers et al., 2014).

In summary, the findings of decreased [Cho], [Glu] and [Glx] in cerebral voxels of alcohol-dependent patients are consistent with depletion owing to increased glutathione metabolism. There was no relationship between glutamate, glutamine or [Glx] and [¹¹C]PBR28 V_T though this may relate to the very small sample size and the additional variability related to genotype.

CHAPTER 7

DISCUSSION AND FUTURE DIRECTIONS

The main findings of my thesis are as follows:

- The pro-inflammatory cytokine IL-6 is associated with more severe withdrawal symptoms and with depression throughout detoxification.
- The pro-inflammatory chemokine is associated with better performance on cognitive tests at the end of detoxification.
- IL6 is positively associated with morning serum cortisol concentrations and with the magnitude of the Cortisol Awakening Response during detoxification.
- [^{11}C]PBR28 V_T is decreased in the hippocampi of alcohol dependent patients in early abstinence relative to controls.
- There is a positive association between hippocampal [^{11}C]PBR28 V_T and performance on a test of verbal memory.
- Prescribed benzodiazepines, other than diazepam at very high doses, are unlikely to interfere with interpretation of [^{11}C]PBR28 scanning data.
- There is no significant association between brain glutamate concentrations as measured by MRS and [^{11}C]PBR28 V_T .

The aim of my thesis was to characterise peripheral and central nervous system markers of inflammation during alcohol withdrawal and early abstinence in alcohol dependence. A secondary aim was to describe their relationship to clinical measures, particularly cognitive function, in order to generate hypotheses regarding their clinical relevance. Thirdly, I investigated

their relationships to neurochemical and neuroendocrine derangements previously described in withdrawal and early abstinence.

In order to address these aims, I conducted a series of experiments using a range of approaches including peripheral cytokine and cortisol assay, Positron Emission Tomography (PET), and Magnetic Resonance Spectroscopy (MRS), as well as the administration of psychological questionnaires and cognitive tests, in two populations of alcohol-dependent men. In this chapter, I will outline the implications of my findings and possible avenues of further study.

First, I have described, for the first time, the profile of twenty-five cytokines and chemokines during alcohol detoxification. I have established that macrophage associated cytokines and chemokines IL-6 (pro-inflammatory), IL-10 (anti-inflammatory), and CCL-2 (pro-inflammatory) are raised during early detoxification relative to later in detoxification, and that IL-6 in particular is associated with the severity of withdrawal symptoms and depressive symptoms throughout withdrawal.

The Th1-associated cytokine IL-12 and chemokine CXCL-10 increased during detoxification, with an increase in C-reactive protein, a common inflammatory marker. IL-12 was not related to depressive symptoms, in contrast to its relationship with depressed populations.

Further work could clarify the relative contributions of gut-derived lipopolysaccharide and sympathetic nervous system hyperactivity. The

relationship between the sympathetic nervous system and cytokines during detoxification could be investigated by measuring the cytokine response to administration of an alpha-2-receptor agonist such as lofexidine or clonidine during detoxification. The contribution of bacterial translocation from the gut and consequent hepatic inflammation could be assessed initially by investigating the relationship between circulating lipopolysaccharide and withdrawal severity. If such a relationship exists, there would be rationale for testing the effect of early probiotic therapy during alcohol detoxification. Probiotic therapy has shown promise in prevention of hepatic encephalopathy (McGee et al., 2011) (Lunia et al., 2014), which, like alcohol dependence, involves small bowel bacterial overgrowth and bacterial translocation. Finally, the cytokine profile during chronic intoxication would also be of interest. A recent study showed that a single bout of binge drinking was associated with a significant increase in peripheral LPS (Bala et al., 2014).

The relationship of peripheral cytokines and chemokines to cognitive function was complex. There was no association between IL-6, or CXCL-8, and cognitive function. Contrary to my hypothesis, CCL-2 was associated with better cognitive function in a range of cognitive tests. CCL-2 was chosen *a priori* as it is a cytokine associated with microglial function, related to poorer cognitive function in mild cognitive impairment and Alzheimer's Disease, and had been found to be increased in brain tissue of alcohol-dependent patients post-mortem (He and Crews, 2008). However evidence published since the inception of my study supports the hypothesis that CCL-2, in the presence of alcohol, may be protective. Transgenic mice overexpressing CCL-2 do not

display suppression of Long Term Potentiation following acute alcohol administration (Bray et al., 2013) and show additional up-regulation of synaptic proteins, but not those associated with cell death, following chronic alcohol administration (Gruol et al., 2014).

Second, I investigated whether there was evidence of microglial activation in early abstinence using [¹¹C]PBR28 PET. The findings in the PET study were also not as hypothesised. For the first time, I have characterised TSPO binding in healthy alcohol-dependent men in early abstinence. I found that [¹¹C]PBR28 V_T was decreased in the hippocampus, which was contrary to my hypothesis. There were no areas of increased V_T in the brains of the patient group. This was not consistent with microglial activation in alcohol dependence, in that microglial activation has been detected in other conditions as increased binding of TSPO-PET tracers (reviewed in (Politis et al., 2012)). Further, there was a positive correlation between performance on verbal memory and hippocampal V_T , which invites two possible explanations, as both microglia and hippocampal neural progenitors have been reported to express TSPO. If [¹¹C]PBR28 V_T in the hippocampus is a predominantly microglial signal, it suggests that microglia may serve a protective, rather than destructive, function in the hippocampus in the healthy brain and in alcohol withdrawal. This is supported by recent pre-clinical literature showing that microglia are activated to an anti-inflammatory phenotype during alcohol withdrawal (Marshall et al. 2013) and may support resumption of neurogenesis (Nixon et al., 2008).

My *in vitro* study showed that the findings of the PET study would not have been affected by conducting scans during the first twenty-four to forty-eight hours of detoxification, provided patients were treated with chlordiazepoxide not diazepam (Kalk et al. 2013 2013). Pre-clinical evidence suggested that this would have been the most likely period during which microglial activation peaked (Nixon et al., 2008). It was not logistically possible to scan the moderately to severely dependent population available for recruitment during this early period, however, as they either required in-patient detoxification which took place remotely or, if treated as out-patients, were reluctant to be scanned during the early, most difficult phase of their withdrawal. Such a scanning study could be undertaken in a mildly physiologically dependent population who would be willing to tolerate scanning in early detoxification, and would be of interest to investigate whether short-lived microglial activation occurs during these phases of alcohol dependence.

It may also be that hippocampal microglial activation and damage are early events in alcohol dependence, as supported by reports of hippocampal cognitive deficits and atrophy in both rodent and human binge-drinking adolescents (Medina et al., 2007) (De Bellis et al., 2000) (Nagel et al., 2005). A recent study demonstrated an increase in LPS following a single alcohol binge (Bala et al., 2014). Given the report of microglial activation in response to peripheral LPS in non-human primates (Hannestad et al., 2012), it would be of interest to investigate whether such activation occurs in the setting of an alcohol binge, and whether there is a relationship between the degree of activation and cognitive function.

The findings described in this thesis demonstrated peripheral inflammation during alcohol detoxification, but did not demonstrate neuroinflammation in early abstinence. The relationships between inflammatory processes in the periphery and microglial function in the brain, require further investigation. Both the positive relationship between CCL-2 concentrations and cognitive function in the cytokine study and the positive correlation between [¹¹C]PBR28 V_T and verbal memory, are more consistent with a protective role for at least certain aspects of the immune function. However, the findings have significantly added to the understanding of interacting processes occurring during withdrawal and early abstinence, and may provide an avenue of enquiry into the phenomenon of extended withdrawal, and into depression in alcohol dependence. It has long been recognised that after the acute alcohol withdrawal symptoms subside, symptoms of insomnia, anxiety and depression persist for the several weeks defined as early abstinence (Schuckit and Hesselbrock, 1994) (Brown et al., 1995). Even during prolonged abstinence, abnormalities in stress responsiveness remain and contribute to relapse (reviewed in (Heilig et al., 2010)). This, together with relative insensitivity to reward, following induction of substance dependence, is known as 'allostasis' (Koob and Le Moal, 2001). It would be interesting to investigate, therefore, the relationship between cytokines and relapse following detoxification, and whether this is mediated by relationships between cytokines and withdrawal and mood symptoms described in Chapter 2.

Past literature has emphasised HPA axis dysfunction (Koob and Le Moal, 2001) (Heilig et al., 2010) in the pathogenesis of allostasis. My thesis has extended pre-clinical work showing interactions between peripheral inflammatory stimulus and the HPA axis (Whitman et al., 2013) (Breese et al., 2008) by describing for the first time the relationship between peripheral cytokines and HPA axis function during alcohol detoxification in man. This was the third experiment listed in my introduction (page 15). I found a positive correlation between IL-6 and both serum cortisol and Cortisol Awakening Response, suggesting that the peripheral inflammatory drive exacerbates the stress response during detoxification. The relationship between HPA axis function and cytokines in this context could be extended using validated endocrine challenge and suppression studies during early abstinence. Measurement of additional hormones, such as ACTH, and cytokines, such as TNF- α , may be more sensitive to the endocrine-immune relationship (Schuld et al., 2003) and would provide information about interactions at various anatomical locations.

The findings from the study described in Chapters 2 and 3 are also of relevance to co-morbidity in alcohol dependence. The relationship of IL-6 to Visual Analogue Scale Depression, adjusted for withdrawal severity, found throughout detoxification was described for the first time in my study. Raised IL-6 has been consistently reported in depression and is may predict response to antidepressants (Yoshimura et al., 2009) (Yoshimura et al., 2013). Another study has found an inverse correlation between anti-inflammatory IL-10 and depression in alcohol-dependent patients (Leclercq et

al., 2012). I also replicated the finding that morning serum cortisol decreased during detoxification. Of interest is that the levels seen in my study were similar to morning cortisol seen in depressed cohorts, even at the later time points. The decrease in [Glx] found in the MRS study – the fourth experiment listed on page 15 of the Introduction - is also similar to findings in depressed subjects (for a review, see (Luykx et al., 2012)). Disappointing findings in early stage clinical trials of anti-inflammatory treatments in depression (e.g. (Inamdar et al., 2014)) have been attributed to heterogeneity in the depressed population – that is, the depressed patients in the trial had normal cytokine levels. It has been proposed that identification of sub-groups with an inflammatory response should be targeted for treatment. The evidence from my study suggests that alcohol-dependent patients with co-morbid depression would be such a sub-group. Further studies characterising the cytokine profile in patients with alcohol dependence and co-morbid depression would potentially provide a novel treatment rationale in this co-morbidity.

In summary, the findings presented in this thesis have characterised the role of peripheral inflammation and neuroinflammation during alcohol detoxification and early abstinence and placed them in the context of known neurochemical and neuroendocrine derangements related to detoxification. Their relationship to cognitive function in alcohol dependence is complex and warrants further investigation, in binge drinking cohorts. However, my findings suggest that cytokines may contribute to clinical symptoms in detoxification and early abstinence and may be important in the pathogenesis of allostasis in alcohol dependence, and in co-morbid mood disorders. This

provides a promising avenue of enquiry to develop more effective interventions targeting relapse prevention and depression.

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APPENDIX: OUTPUTS

Presentations/Posters

2014 Kalk NJ, Guo Q, Owen D, Waldman A, Dar K, Gunn RN, Nutt DJ, Lingford-Hughes AR, Rabiner EA: Hippocampal microglial dysfunction in alcohol dependence: a [11-C]PBR28 Positron Emission Tomography (PET) study – presented at Neuroreceptor Mapping, at which it won a poster prize, and the joint ISBRA/RSA conference.

2013 Kalk NJ, Cherian R, Naveed M, Cavanagh J, Dar K, McInnes IB, Lingford-Hughes AR: The peripheral cytokine profile in alcohol detoxification: relationship to withdrawal severity and affective symptoms; Royal College of Psychiatrists International Conference, at which it won a poster prize.

2012 Kalk NJ, Owen DR, Reynolds R, Tyacke RJ, Rabiner EA, Lingford-Hughes AR, Parker CA: 'Affinity of commonly-prescribed benzodiazepines for the 18kDa Translocator Protein (TSPO): implications for imaging studies' *J Cereb Blood Flow and Metab* 32(S52-53) Poster presented at 9th International Symposium on Functional Neuroreceptor Mapping of the Living Brain (NRM), Baltimore.

Publications

2014 Royal College of Psychiatrists, Royal College of General Practitioners and Association of British Neurologists. 'Alcohol and Brain Damage in Adults: with reference to high risk groups. *In: Wilson, P. K. (ed.) CR185*. London: Royal College of Psychiatrists

2014 Kalk NJ, Lingford-Hughes AR: 'Acamprosate: clinical pharmacology' *Br J Clin Pharmacol* 77(2): 315-23

2013 Kalk NJ, Owen DR, Reynolds R, Tyacke RJ, Rabiner EA, Lingford-Hughes AR, Parker CA: 'Are prescribed benzodiazepines likely to affect the availability of the 18kDa Translocator Protein (TSPO) in PET studies?' *Synapse* 67(12): 909-12.

2013 Orban C, McGonigle J, Kalk NJ, Erritzoe D, Waldman AD, Nutt DJ, Rabiner EA, Lingford-Hughes AR: 'Resting state synchrony in anxiety-related circuits of abstinent alcohol-dependent patients' *Am J Drug Alcohol Abuse* 39(6): 433-40.