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Systemic inflammation and sickness behaviour in Alzheimer's disease

by

Joseph Butchart

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ABSTRACT

FACULTY OF MEDICINE

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SYSTEMIC INFLAMMATION AND SICKNESS BEHAVIOUR IN ALZHEIMER'S DISEASE

Joseph William Butchart

Alzheimer's disease is the commonest cause of dementia in the UK. Inflammatory processes play a role in the pathology of the disease, and may trigger pathological neuroimmune sickness behaviour, manifesting as behavioural and psychological symptoms.

This thesis explores the influence of systemic inflammation on brain inflammation, neuroimmune sickness behaviour and disease progression in Alzheimer's disease.

First, I describe a study that examined the influence of age-related endocrine dyscrasia on systemic inflammation in Alzheimer's disease. Data are presented to demonstrate a relationship between altered hormonal status and systemic Tumour Necrosis Factor-alpha in men with Alzheimer's disease.

Second, I describe the construction of a scale to measure pathological neuroimmune sickness behaviour in people living with Alzheimer's disease. Data are presented for the reliability and validity of the resulting Sickness Behaviour Scale in a construction cohort and an independent validation cohort.

Third, I present a randomized, placebo-controlled, double-blind, clinical trial on the safety, tolerability and efficacy of the anti-inflammatory drug etanercept in Alzheimer's disease. Etanercept was safe, well-tolerated and significantly reduced behavioural, functional and cognitive decline in Alzheimer's disease.

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DECLARATION OF AUTHORSHIP

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

Systemic inflammation and sickness behaviour in Alzheimer's disease

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
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3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
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Definitions and Abbreviations

3xTgAD	Triple Transgenic Alzheimer's disease
Aβ	Amyloid-β
ACE	Angiotensin-Converting Enzyme
ACTH	Adrenocorticotropic Hormone
AD	Alzheimer's disease
ADAS-Cog	Alzheimer's Disease Assessment Scale: Cognitive Section
ANCOVA	Analysis of covariance
ANOVA	Analysis of Variance
ApoE, ApoJ	Apolipoprotein E, Apolipoprotein J
APP	Amyloid Precursor Protein
BACE-1	Beta-site APP Cleaving Enzyme-1 (or β-secretase)
BADLS	Bristol Activities of Daily Living Scale
BBB	Blood-Brain Barrier
BMI	Body Mass Index
BPSD	Behavioural and Psychological Symptoms of Dementia
C1q, C3, C4	Complement Protein 1q, 3, 4 etc.
CCL22	Chemokine C-C motif Ligand 22
CCR3, CCR5	Chemokine C-C motif Receptor 3, 5 etc.

CD40, CD200	Cluster of Differentiation 40, 200 etc.
CGIC	Clinical Global Impression of Change
CI	Confidence Interval
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
COX-1, COX-2	Cyclo-oxygenase enzyme-1, -2
CR-1	Complement Receptor type 1
CRH	Corticotropin-Releasing Hormone
CRP	C-reactive Protein
CSF	Cerebrospinal Fluid
DAMP	Damage-Associated Molecular Pattern
DHEA	Dehydroepiandrosterone
DI	Discrimination Index
DNA	Deoxyribonucleic Acid
DSM-IV-TR	Diagnostic & Statistical Manual of Mental Disorders, 4th Edition, Text Revision
ECG	Electrocardiogram
ELISA assay	Enzyme-linked immunosorbent assay
ER	Endorsement Rate
Fc	Fragment crystallizable tail region of immunoglobulin
FSH	Follicle Stimulating Hormone

FT	Free Testosterone
G proteins	Guanine nucleotide-binding proteins
GFAP	Glial Fibrillary Acidic Protein
GnRH	Gonadotropin Releasing Hormone
GWAS	Genome-Wide Association Study
HLA	Human Leukocyte Antigen
HPA axis	Hypothalamic-Pituitary-Adrenal axis
HPG axis	Hypothalamic-Pituitary-Gonadal axis
HRT	Hormone Replacement Therapy
ICAM-1	Intercellular Adhesion Molecule-1
ICC	Intraclass Correlation
IFN-α, IFN-γ	Interferon-α, Interferon-γ
IGF-1	Insulin-like Growth Factor-1
IgG, IgE	Immunoglobulin-G, Immunoglobulin E etc.
IL-1, IL-6	Interleukin-1, Interleukin-6 etc.
iNOS	inducible Nitric Oxide Synthase
IQR	Interquartile Range
IRR	Incident Rate Ratio
ITT	Intention to Treat
KASP	Kompetitive Allele Specific Polymerase chain reaction

kDa	Kilodaltons
KMO test	Kaiser-Maier-Olkin test
LH	Luteinizing Hormone
LOCF	Last Observation Carried Forward
LPS	Lipopolysaccharide
LREC	Local Research Ethics Committee
LSM	Least Squares Mean
M1, M2	Microglial phenotype 1, Microglial phenotype 2
MAC	Membrane Attack Complex
MAP	Mitogen-Activated Protein, as in MAP kinase
MARC	Memory Assessment & Research Centre, Southampton
MCI	Mild Cognitive Impairment
MCID	Minimum Clinically Important Difference
MCP1	Monocyte chemoattractant protein 1
MedDRA	Medical Dictionary for Regulatory Activities
MGUS	Monoclonal Gammaopathy of Unknown Significance
MHC	Major Histocompatibility Complex
MHRA	Medicines and Healthcare products Regulatory Agency
MIP-1α	Macrophage Inflammatory Protein-1α
MMSE	Mini-Mental State Examination
MRI	Magnetic Resonance Imaging

mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
MSD	Meso Scale Discovery
MWU	Mann Whitney U Test Statistic
NF-κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NICE	National Institute for Clinical Excellence
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association
NK	Natural Killer, as in NK cell
NMDA	N-methyl-D-aspartate
NPI	Neuropsychiatric Inventory
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
OR	Odds Ratio
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
PDAPP	Platelet-Derived growth factor-driven Amyloid Precursor Protein transgenic model

PET	Positron Emission Tomography
PGE_2	prostaglandin E_2
PiB	Pittsburgh B compound
poly I:C	Polyinosinic:polycytidylic acid
PPAR-gamma	Peroxisome Proliferator-Activated Receptor-gamma
RAGE	Receptor for Advanced Glycation End-products
RR	Relative Risk
S100B	S100 calcium-binding protein B
SAE	Serious Adverse Event
SBS	Sickness Behaviour Scale
SE	Standard Error
SEM	Standard Error of the Mean
SEMt	Standard Error of Measurement
SHBG	Sex Hormone Binding Globulin
sIL-6R	Soluble IL-6 Receptor
SLE	Systemic Lupus Erythematosus
SmPC	Summary of Product Characteristics
SNP	Single Nucleotide Polymorphism
STEADI-09	Safety and Tolerability of Etanercept in Alzheimer's Disease: Protocol Identification Number
TACE	TNF-α Converting Enzyme

TB	Tuberculosis
TGF-α, TGF-β	Transforming Growth Factor-alpha, -beta
TIGRA	Test for an Interferon-Gamma release Assay
Th1, Th2	Helper T-cell – Type 1, Helper T-Cell Type 2 etc.
TIA	Transient Ischaemic Attack
TNF-α	Tumour Necrosis Factor alpha
TNFR-I, TNFR-II	TNF-α Receptor-I, TNF-α Receptor-II
TREM2	Triggering Receptor Expressed on Myeloid Cells-2
TSPO-PET	Translocator Protein-Positron Emission Tomography
TT	Total Testosterone
TTR	Transthyretin (Transporter for Thyroxine and Retinol)
UK	United Kingdom
VCAM	Vascular Cell Adhesion Molecule

Chapter 1: Introduction

1.0 Research context

Alzheimer's disease is a progressive neurodegenerative disease. It is the most common underlying pathology in people diagnosed with dementia. Over 820 000 people have a diagnosis of Alzheimer's disease in the UK and the disease costs an estimated £23 billion each year.[1] The high prevalence, and huge social and economic costs associated with Alzheimer's disease mean that progress with research into the disease has now become a high priority for the UK government and the scientific community.[2] In this thesis I examine the subject of systemic inflammation and neuroimmune sickness behaviour in Alzheimer's disease, and present data on an effort to measure and ameliorate the effects of systemic inflammation in Alzheimer's disease. In this chapter I will introduce the research question that is the subject of this thesis, and outline the rationale for the research methods used to address this question.

The cause of late-onset Alzheimer's disease is unclear. The current prevailing hypothesis concerns the accumulation of amyloid-β protein in the brain, with subsequent amyloid-associated inflammation and neuro-toxicity.[3] However, amyloid deposition in the brain may be a necessary, but insufficient, factor in the pathophysiology of the disease. Post-mortem studies show that many older people have high levels of amyloid-β in the brain at death, but had no evidence of a clinical dementia during life.[4 5]

If amyloid-β alone is insufficient to cause Alzheimer's disease then what extra factor is necessary to trigger the disease? Recent evidence suggests that inflammatory processes are fundamental to the development and progression of Alzheimer's disease, and inflammatory processes may be the necessary factor that turns amyloid-β deposition into active Alzheimer's disease.[6-8] Factors that influence systemic and central nervous system inflammation may therefore influence the progression of neurodegeneration in Alzheimer's disease.[9 10] These influential inflammatory factors include age,[11] physical frailty,[12] alteration in the hypothalamic-pituitary-adrenal (HPA) axis,[13] and alteration in sex hormones.[14] In addition, the symptoms of Alzheimer's disease may result from more than the loss of synapses and neurons. Behavioural and

psychological symptoms in Alzheimer's disease have been associated with inflammatory factors, and some of these symptoms may be driven by inflammatory pathology more than by neuronal pathology.[15]

The behavioural and psychological symptoms of Alzheimer's disease that are associated with peripheral inflammation can, in part, be thought of as a pathological form of sickness behaviour. Sickness behaviours, such as social withdrawal or loss of appetite, are the normal, physiological behavioural responses that help an organism survive periods of sickness or trauma.[16] Animal models suggest that sickness behaviours are normally triggered by the production of inflammatory cytokines within the central nervous system, in response to peripheral cytokines.[17] However, in Alzheimer's disease, chronic activation of inflammation within the brain has been hypothesized to trigger inappropriate sickness behaviours, manifesting as behavioural and psychological symptoms of dementia, such as apathy and anorexia.[10] Furthermore, episodes of acute or chronic peripheral inflammation may exacerbate pathological sickness behaviour in Alzheimer's disease, by further induction of inflammatory cytokines within the central nervous system.[15] People living with dementia become more confused and delirious with apparently innocuous systemic inflammatory insults, such as mild chest or urinary tract infections. Sadly, the loss in cognition and function associated with these infections often fails to completely resolve after treatment of the precipitating event.[18] All geriatricians and those in regular contact with people living with dementia are aware of this phenomenon. Exaggerated neuroimmune sickness behaviour may provide an explanation for the sometimes severe behavioural and psychological symptoms that arise from apparently mild systemic inflammatory disturbance. That these systemic inflammatory episodes appear to cause irreversible cognitive and functional decline in some patients indicates the importance of systemic inflammation in the pathophysiology of Alzheimer's disease.

1.1 Research hypotheses

In this thesis I discuss and present evidence investigating the following hypotheses:

1. Systemic inflammation influences disease progression and neuroimmune sickness behaviour in Alzheimer's disease (Chapter 2).
2. In men with Alzheimer's disease, the degree of systemic inflammation is related in part to change in sex hormone levels (Chapter 4).
3. Neuroimmune sickness behaviour in Alzheimer's disease can be measured using an informant scale:
   a) based on psychometric analysis of commonly occurring sickness behaviour symptoms (Chapter 5).
   b) based on correlation of commonly occurring sickness behaviour symptoms with systemic inflammatory cytokines (Chapter 5)
4. In Alzheimer's disease, systemic circulating inflammatory cytokines are associated with neuroimmune sickness behaviour (Chapter 5)
5. Etanercept, a Tumour Necrosis Factor-alpha (TNF-α) blocking drug, is safe and well tolerated, as a potential treatment to reduce systemic inflammation in Alzheimer's disease (Chapter 6).
6. Drug blockade of systemic inflammation alleviates neuroimmune sickness behaviour and other symptoms in Alzheimer's disease (Chapter 6).

1.2 Research approach

These hypotheses are tested using the following approaches:

1. A study to examine the relationship between sex hormone levels and circulating systemic cytokines in a cohort of men with Alzheimer's disease
2. A study to develop and assess the reliability and validity of alternative sickness behaviour scales developed by psychometric analysis or by correlation with systemic cytokines, in a cohort of people with Alzheimer's disease
3. A study to assess the safety, tolerability and provisional efficacy of the TNF-α blocking drug, etanercept, in people with Alzheimer's disease

1.3 Rationale for approach

Study 1: Male sex hormones and systemic inflammation in Alzheimer's disease

Sex hormone levels may influence the incidence and progression of Alzheimer's disease.[19] In this cross-sectional study, we sought to demonstrate a relationship between sex hormone levels and systemic cytokines in men with Alzheimer's disease, and to explore the possibility that sex hormones may exert an influence on Alzheimer's disease by influencing systemic inflammation.

Study 2: Reliability and validity of a Sickness Behaviour Scale for Alzheimer's disease

At present, there is no validated scale for assessing and quantifying neuroimmune sickness behaviour in people with Alzheimer's disease. In this longitudinal cohort study, we developed alternative versions of a Sickness Behaviour Scale and tested reliability and validity in a cohort of people with Alzheimer's disease; and tested discriminant validity in cohorts that were cognitively normal or that had Lewy Body dementia. An independent validation cohort was then used to further assess the validity of the scales.

Study 3: Safety and Tolerability of Etanercept in Alzheimer's disease - STEADI-09

Tumour Necrosis Factor-alpha (TNF-α) is a key inflammatory cytokine and therefore is an ideal target for an attempt to reduce the effects of systemic inflammation in Alzheimer's disease. Drugs that block TNF-α are used widely for a variety of chronic inflammatory diseases. In the STEADI-09 study we use the TNF-blocker, etanercept, to reduce the effects of TNF-α in the periphery, in an attempt to reduce the exacerbating effects of systemic inflammation on chronic neurodegeneration. Etanercept has not been used previously in a randomized, placebo-controlled trial to treat Alzheimer's disease; therefore, we carried out a Phase 2 safety study, with the primary aim of establishing the safety and tolerability of etanercept in Alzheimer's disease. We examined efficacy measures as secondary outcomes, including the effect of etanercept treatment on the sickness behaviour scales we had developed in Study 2.

1.4 Summary

Alzheimer's disease is the commonest cause of dementia in the UK. Inflammatory processes play a role in the pathology of the disease, and may trigger pathological neuroimmune sickness behaviour, manifesting as behavioural and psychological symptoms. This thesis explores the role of systemic inflammation on neuroimmune sickness behaviour and disease progression in Alzheimer's disease. Within the thesis I review the evidence that links systemic inflammation, sickness behaviour and Alzheimer's disease. I present data on the effects of sex hormones on systemic inflammation in men with Alzheimer's disease. I describe alternative methods for the construction of a scale to measure neuroimmune sickness behaviour in people with Alzheimer's disease, and present evidence for the reliability and validity of the best-performing final scale. Finally, I present safety, tolerability and provisional efficacy data from a clinical trial of the anti-inflammatory drug, etanercept, used in people living with Alzheimer's disease with the aim of reducing the detrimental effect of systemic inflammation on the symptoms and progression of this devastating disease.

Chapter 2: Systemic inflammation and sickness behaviour in Alzheimer's disease

2.0 Introduction

In this chapter I review the evidence that has generated the overall hypothesis explored in this thesis: namely, that certain symptoms in Alzheimer's disease are related to systemic inflammation and factors that influence systemic inflammation, and that these symptoms can be ameliorated by treatment with an anti-inflammatory drug. I will examine four lines of evidence. First, I appraise the evidence for pathological sickness behaviour induction by systemic inflammation. Second, I examine the evidence for pathological sickness behaviour in Alzheimer's disease. Third, I examine the evidence that sex hormones have effects on Alzheimer's disease through their influence on systemic inflammation. Fourth, I review the literature on attempts to treat Alzheimer's disease with drugs that reduce central and systemic inflammation.

2.1 Pathological sickness behaviour and systemic inflammation

In this section I will define sickness behaviour and review the evidence that sickness behaviour is generated by the action of peripheral circulating cytokines on the central nervous system (CNS). I will examine evidence that inappropriate sickness behaviour can be pathological, and may manifest as behavioural and psychological symptoms, such as depression. Finally, I will introduce the concept that these inappropriate sickness behaviours have particular relevance in Alzheimer's disease, presenting as distressing behavioural and psychological symptoms of dementia (BPSD).

2.1.1 Definition of sickness behaviour

Humans, in common with other complex, multi-cellular organisms, have evolved a set of physiological, immune and behavioural responses to deal with the threat of infection by microorganisms. Non-specific symptoms of infection,

such as malaise or weakness, were previously thought to be merely secondary epiphenomena, related only to the debilitating effects of the causative microorganism. However, these non-specific symptoms of sickness are now recognized as evolved behavioural responses that help an organism to fight an infection, and are common across animal species.[20] During an infection, the immune system triggers a change in an organism's motivational state, to promote evolved behaviours that aid resistance to infection and recovery.[21] In humans, these evolved sickness behaviours include reduced activity, listlessness, malaise, poor concentration, lethargy and reduced appetite; which, by reducing metabolic demand, reducing exposure to external threats, and encouraging energy conservation, all aid in the immune response to infection. Thus, sickness behaviour may be defined as a set of adaptive behavioural responses to infection.[22]

2.1.2 Development of the concept of sickness behaviour

Early work conceptualized sickness behaviour as a by-product of the metabolic consequences of mounting a fever.[21] Fever is useful in the fight against infection because it stimulates the proliferation of immune cells and makes the body's internal environment less suitable for the multiplication of bacteria and viruses. However, fever comes with a high metabolic cost. Energy use must increase by 13% for every 1 ˚C rise in body temperature.[22] A feverish organism must therefore re-prioritize behaviour in favour of reducing energy expenditure and reducing thermal losses. Thus, feverish animals reduce foraging , reduce activity, curl up and rest.[22]

This work on fever and sickness behaviour was taken further after the discovery and purification of interleukin-1 (IL-1) in 1984, and the discovery that this cytokine induced fever.[16 23] This led to the hypothesis that cytokines were responsible not only for fever, but also for the sickness behaviour associated with fever. Subsequent animal experiments demonstrated diverse behavioural effects of IL-1, and led to a widening of the concept of sickness behaviour to encompass the adaptive behavioural response to the production of inflammatory cytokines.[16] Overall, there is a high degree of congruency between the effects of cytokines on the CNS and the non-specific symptoms of sickness seen in laboratory animals and in humans (Table 2.1.1).

Table 2.1.1 Comparison of non-specific symptoms of sickness with the behavioural and physiological effects of central or peripheral administration of cytokines (after Kelley et al. 2003)[20]

CNS effects of cytokines	*Non-specific symptoms of sickness*
General malaise	Feeling unwell
Decreased activity	Loss of energy, fatigue
Decreased social investigation	Loss of interest in usual activities
Decreased food and water intake	Poor appetite
Weight loss	Weight loss
Sleep changes	Sleep changes
Fever	Fever

The first evidence that cytokines influence sickness behaviour in humans came from early clinical trials using recombinant or purified forms of cytokines to treat various forms of cancer.[24] Patients developed flu-like symptoms such as malaise, listlessness and anorexia.[25] Administration of therapeutic interferon-α (IFN-α), interleukin-2 (IL-2) or tumour necrosis factor-α (TNF-α), caused many sickness behaviour symptoms, including depressed mood, anhedonia, helplessness, fatigue, anorexia, hypersomnia, psychomotor retardation, decreased concentration and confusion.[26] Similarly, laboratory animals become anorexic, lethargic and socially withdrawn after exposure to the recombinant cytokines interleukin-1β (IL-1β) and TNF-α.[27 28] In both human and animal studies, symptoms appear soon after cytokine administration, and often disappear shortly after termination of the cytokine treatment, implying that peripheral cytokine administration cause these behavioural symptoms.

Furthermore, in animal studies, pre-treatment with cytokine antagonists prevents the behavioural effects of an immune challenge.[29]

The hypothesis that sickness behaviour is a highly organized behavioural response to the threat of infection, suggests that sickness behaviour should have motivational properties.[22] That is, sick organisms should have the ability to select or alter behaviours, depending on the environment they are exposed to, and depending on the immune and metabolic consequences of the behaviour. Motivated behaviour is flexible, enabling an organism to select the most appropriate strategy, depending on the eliciting circumstances. Early conditioning experiments in rats showed that infection, or injection with bacterial endotoxin, altered motivational priorities, so that thirsty rats with induced sickness reduced drinking and eating, despite their thirst.[30] This view of sickness behaviour, as a change in motivational state, has been further tested in animal experiments that vary the level of motivational competition, demonstrating that the pattern of sickness behaviour alters depending on the relative level of competing motivational factors.[31] Similarly, in humans sickness behaviour may take more than one form, for example predominantly neuro-vegetative, with fatigue and anorexia, or predominantly affective, with depression and apathy, depending on an individual's circumstances.[25]

2.1.3 Communication between the immune system and the central nervous system

The relatively new discipline of psychoneuroimmunology deals with expanding knowledge about the various ways in which the immune system and the brain influence each other. The CNS communicates with the immune system in two main ways, neuroendocrine and direct neural innervation of immune tissues.

Neuroendocrine pathways include the release of cortisol by the zone fasciculata of the adrenal gland, and dehydroepiandrosterone (DHEA) from the zona reticularis of the adrenal gland, under the influence of pituitary adrenocorticotropic hormone (ACTH), and ultimately hypothalamic corticotropin-releasing hormone (CRH). Cortisol has many regulatory effects on the immune system. Additionally, activation of the sympathetic nervous system results in the release of noradrenaline and adrenaline from the adrenal medulla, with diverse modulatory effects on the immune system.

Direct neural innervations of lymphoid tissue by sympathetic and parasympathetic nerves allows a direct communication between the brain and individual lymphocytes.

In these ways the brain can influence the direction, duration and degree of an immune response, and the production and differentiation of immune-mediator cells, such as T-helper cell differentiation along the Th1-Th2 axis.[32]

Neuro-immune communication is bi-directional. Activation of innate immunity not only causes amplification of the appropriate immune response, and triggering of longer-term adaptive immunity, but is also registered in the brain, with consequent appropriate behavioural and motivational responses manifesting as sickness behaviour.

Systemic inflammation in the periphery must communicate with the CNS in order to generate appropriate behavioural effects. A number of routes of communication have been established:

i. Active transport across the blood brain barrier

ii. Activation, via Toll-like receptors, of macrophage-like cells in the circumventricular organs

iii. Activation of cytokine receptors on perivascular macrophages, and on vascular endothelial cells, with subsequent production of secondary messengers, such as prostaglandin E2

iv. Circulating cytokines can interact with endothelial cells causing the expression of immune-specific recognition molecules, such as intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule (VCAM). Expression of these recognition molecules allows circulating lymphocytes to cross the endothelium and enter the brain parenchyma.

v. Increased permeability across blood brain barrier in disease

vi. Migration of activated peripheral macrophages into CNS

vii. Stimulation of vagal nerve afferent fibres

All of these routes of communication are thought to result in the synthesis of cytokines in the brain parenchyma, which go on to effect behaviour by a variety of mechanisms.

The CNS is generally considered a site of "immune privilege"[33] but there is now a growing consensus that antigens arising from pathology in the brain parenchyma are presented to the immune system by both cellular antigen presentation and soluble antigen presentation in regional lymph nodes.[33-35] For example, cellular immune surveillance occurs in the CNS via circulation of the T-cells into the subarachnoid space from blood vessels in the choroid plexus. Within the subarachnoid space the T-cells can interact with choroid plexus macrophages, perivascular macrophages and meningeal macrophages, and antigen presentation and activation of T-cells can take place, before the T-cells return along cranial and spinal nerves to local afferent lymphatic vessels and then on to regional lymph nodes.[35] The best characterised pathway of this kind is the drainage of CSF along the sheath of the olfactory nerve to the olfactory bulb, and then via the lymphatics of the nasal mucosa on to the deep cervical lymph nodes.[34] Until recently there has been little evidence of any lymphatic vessels within the brain. However, a recent study demonstrated functional lymphatic vessels associated with the dura and dural sinuses in mice.[36] This intriguing finding suggests another possible route for re-circulation of antigen-presenting cells and T-cells from the subarachnoid space to regional lymph nodes.

2.1.4 Induction of cytokines within the brain

Specific receptors for cytokines are present throughout the brain.[17] However, cytokines are generally large hydrophilic molecules that are unable to easily cross the blood brain barrier. Consequently, the presence of cytokine receptors within the brain implies that cytokines are produced within the CNS itself. Indeed, induction of cytokines within the brain is consistently observed as a delayed result of increased peripheral cytokines.[22] There is a growing consensus that CNS microglia and perivascular macrophages produce cytokines within the CNS after activation by peripheral systemic inflammation along the pathways described in the previous section.[17]

Microglial cells, perivascular macrophages and meningeal macrophages are the main source for production of cytokines within the brain,[22 37] and, as in the periphery, induction of one cytokine leads to a molecular cascade with local production of other cytokines. For example, elevated systemic IL-1β, in response to peripheral lipopolysaccharide (LPS) injection, causes increased expression of mRNA for IL-1β, TNF-α and IL-6 within the hypothalamus, and this increased expression can be prevented by intra-ventricular injection of an IL-1 receptor antagonist, without altering circulating levels of IL-1β.[38]

2.1.5 Mechanisms for behavioural effects of CNS cytokines

Cytokines have a variety of actions within the CNS, providing mechanisms for their effects on behaviour:

i. Cytokine receptors exist on neurons in a variety of brain structures

ii. Cytokines have neuro-endocrine actions, for example within the hypothalamic-pituitary-adrenal (HPA) axis

iii. Cytokines act as neuronal growth factors

iv. Cytokines have effects on memory

v. Cytokines act as neurotransmitters and neuroregulators

2.1.6 Cytokine production within the CNS is tightly regulated

Importantly, microglial cells do not need to become activated to produce cytokines in response to peripheral cytokines.[39] Microglial activation is tightly regulated, as the effects of inflammation can be devastating within the delicate micro-architecture of the brain. It is vital that physiological triggering of sickness behaviour by peripheral inflammation does not cause tissue damage by the inflammatory effects of over-activated microglial cells. Once the episode of peripheral inflammation has subsided, the microglial cells need to be able to turn off cytokine production, so that cytokine-induced sickness behaviour itself subsides, without any attendant inflammation-associated tissue damage.[7]

2.1.7 Pathological sickness behaviour

Sickness behaviour can be thought of as a homeostatic system, where an inflammatory insult leads to behaviours that help to reduce and clear the inflammatory insult, returning the organism to equilibrium.[16] However, this physiological system can become pathological when it is triggered inappropriately. Thus, the symptoms of delirium may represent an exaggerated sickness behaviour response that is out of proportion to the original inflammatory insult. Furthermore, sickness behaviour can become pathological when the elicited behavioural response does not assist in the resolution of the perceived inflammatory insult. For example, the inflammation associated with chronic neurodegeneration may cause persistent sickness behaviour, but this altered behaviour does not reduce the triggering stimulus of chronic neurodegeneration. The persistence of these ineffective sickness behaviours becomes burdensome and obstructive. Thus, when it is inappropriately triggered, sickness behaviour may threaten, rather than defend, the health of the organism.

2.1.8 Ageing as a risk factor for pathological sickness behaviour

There is growing evidence to show that age is a risk factor for dysregulation of the sickness behaviour system, and for pathological sickness behaviour symptoms. In the brain, ageing is associated with increased expression of pro-inflammatory cytokines, such as IL-6,[40] and with decreased expression of anti-inflammatory cytokines, such as IL-10.[41] Markers of oxidative stress increase in the brain with age.[42] Additionally, mechanisms that normally regulate and terminate sickness behaviour become less effective with age. For example, microglia are normally down-regulated by interaction with cluster of differentiation-200 (CD200) molecules on neurons, but CD200 expression declines with age.[43] As a result of decline in regulatory mechanisms of this kind, microglia become increasingly activated with age, and grow more sensitive to peripheral immune signals.[44 45] In the periphery, ageing is associated with a variety of immunological changes, including up-regulation of pro-inflammatory cytokines, and a decline in immune-regulatory T-cell function.[46-48] There are also changes in the integrity of the blood brain barrier with age, allowing pro-inflammatory cytokines easier access to the CNS.[49]

Overall, age increases both the inflammatory signal from the periphery, and the sensitivity to this signal within the CNS, and together these effects of age lower the threshold for triggering sickness behaviour. Much of the data on these effects of age on the potential for pathological sickness behaviour are derived from animal models. Clinical studies of age and sickness behaviour in humans have been limited owing to the lack of a validated scale for measuring sickness behaviour in humans. However, there is clear clinical evidence for age-related increases in systemic and central inflammation in humans,[50] and age is an important risk factor for some forms of pathological sickness behaviour, such as delirium and the depression associated with chronic illness.[51 52]

2.1.9 Chronic neurodegeneration as a risk factor for pathological sickness behaviour

We have seen that sickness behaviour arises when activated microglia and other CNS cells produce cytokines within the CNS. Factors that activate microglia will therefore act as risk factors for pathological sickness behaviour. There is growing evidence that chronic neurodegeneration primes microglia to respond to an immune challenge in a more pro-inflammatory way. As a consequence, neurodegeneration is associated with exaggerated sickness behaviour. In animal models of neurodegeneration, extra-cellular deposits of abnormal proteins prime and activate microglia. Thus, in an animal model of prion disease, microglia are activated by the abnormal prion protein,[53] and in animal models of Alzheimer's disease microglia are activated by extra-cellular amyloid-β (Aβ) deposits.[54 55] Furthermore, degenerating synapses and neurons in chronic neurodegeneration may produce damage-associated molecular patterns (DAMPs) that trigger microglial activation via Toll-like receptors.[7] A vicious cycle may then develop where activated microglia produce neurotoxins that cause further neurodegeneration, and thus further microglial activation.[6] In humans, post mortem examination reveals microglial activation in many chronic neurodegenerative diseases. Thus, Alzheimer's disease, Parkinson's disease, Lewy Body dementia and motor neuron disease have all been associated with microglial activation.[6 56-58] Further evidence for microglial activation in Alzheimer's disease has been provided by nuclear imaging studies. These have demonstrated activated microglia in patients with

Alzheimer's disease in life.[59] Overall, there is clear evidence, in animal and human studies, of microglial activation in chronic neurodegenerative disease. As a result of this increase in microglial activation there may be a high risk of pathological sickness behaviour, especially in response to a precipitating peripheral immune challenge such as a systemic infection. Indeed, a recent study, using a mouse prion model of chronic neurodegenerative disease, showed that peripherally induced delirium, as a form of exaggerated sickness behaviour, was more severe, and more easily triggered, in mice with chronic neurodegeneration.[60]

2.1.10 Delirium as a form of exaggerated sickness behaviour

Delirium has been conceptualized as an abnormally exaggerated form of sickness behaviour.[61] Delirium is an acute disorder of consciousness and cognition, characterized by acute onset and a fluctuating course, inattention, perceptual and cognitive disturbance, altered consciousness level, and a perturbed sleep-wake cycle.[62] Delirium may be triggered by a large number of factors, although these may be broadly divided into two categories: direct insults to the brain and aberrant stress responses.[61] Examples of direct insults to the brain include acute stroke, brain hypo-perfusion, drug-induced alterations in neuro-transmitter levels, and metabolic disorders such as hyponatraemia or hypercalcaemia. Aberrant stress responses occur through dysfunction in normally adaptive stress response systems. These stress response systems include cytokine-induced sickness behaviour, and activation of the hypothalamic-pituitary-adrenal axis. Systemic infections are a major trigger for delirium in the elderly. Systemic infections are accompanied by increases in systemic cytokine levels, which normally induce adaptive sickness behaviour. However, in the presence of predisposing factors for delirium (age, dementia, drugs) it is thought that these increases in systemic cytokine levels can precipitate acute delirium.[61]

Several lines of evidence implicate exaggerated cytokine-induced sickness behaviour in the generation of delirium. In clinical studies, higher systemic levels of the pro-inflammatory cytokines IL-6 and IL-8, and lower systemic levels of the anti-inflammatory cytokine insulin-like growth factor-1 (IGF-1), are associated with delirium.[63-66] The incidence of delirium is associated with infections, carcinoma, surgery, and trauma; all of which are associated with

elevated cytokine levels.[18] Age and pre-existing dementia are important predisposing factors for delirium.[51] The impact of these risk factors in delirium may partly be explained because they increase the risk of exaggerated sickness behaviour. As outlined previously, age and chronic neurodegeneration are known to prime microglia, so that microglia respond to further activation in an exaggerated way.[45 60] In animal models, age and pre-existing chronic neurodegeneration both interact with a systemic inflammatory insult to cause more exaggerated sickness behaviour than is seen with a systemic inflammatory insult alone.[45 61] In sum, these data suggest that dysfunctional inflammatory mechanisms play a role in the generation of delirium, and that delirium can be seen as a form of pathological sickness behaviour.

2.1.11 Depression as a pathological form of sickness behaviour

Key elements of depression are also elements of cytokine-induced sickness behaviour. Social withdrawal, loss of concentration, apathy, lethargy and anorexia, are all common to both sickness behaviour and depression.[67] In 1999, Maes put forward an "inflammatory response system model of major depression".[68] This hypothesis states that depression may result from the action of peripheral cytokines on the brain in susceptible individuals. The hypothesis suggests that common mechanisms mediate the brain effects of cytokines in sickness behaviour and in depression. Thus, depression may represent a form of pathological sickness behaviour.

Several lines of evidence support the cytokine theory of depression. First, elevated cytokine levels, including IL-1, IL-6, IFN-γ, complement protein-3 (C3), complement protein-4 (C4), and C-reactive protein (CRP), are found in patients diagnosed with major depression.[67 69-71] Second, therapeutic administration of cytokines reproduces key symptomatic features of depression, such as low mood, anhedonia and fatigue; and these symptoms disappear when plasma cytokine levels return to normal.[72] Third, a peripheral immune challenge, with either LPS or rubella vaccination, causes depressive symptoms in healthy volunteers, and the severity of these symptoms correlates with plasma cytokine levels.[29] Fourth, clinical depression is associated with a number of physical illnesses known to cause chronic activation of the immune system, and elevated cytokine levels, such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and chronic obstructive pulmonary disease (COPD).[29]

Fifth, cytokines, especially IL-1, impair negative feedback in the HPA axis, and could therefore contribute to the hyper-adrenalism associated with major depression.[73 74] Sixth, peripheral cytokine levels can influence serotonergic transmission, possibly by immune-mediated depletion of tryptophan, contributing to serotonin depletion in depression.[67]

Much of the clinical data on inflammation and depression is cross-sectional and correlational. Consequently, there is still some debate about whether inflammatory cytokines play a causal role in the onset of depression, or are secondary phenomena that sustain, but do not initiate, depressive symptoms. However, the evidence, briefly outlined here, indicates that inflammation can pre-date depression, can cause depressive symptoms, and can contribute to the neuroendocrine and neurotransmitter changes observed in depression. Overall, the evidence described here suggests that clinical depression may be a form of pathological sickness behaviour arising in susceptible individuals in response to systemic inflammation.

2.1.12 Behavioural and psychological symptoms in Alzheimer's disease as a form of pathological sickness behaviour

We have seen that sickness behaviour is normally a self-limiting behavioural response to infection, mediated by the tightly regulated production of cytokines within the CNS. However, sickness behaviour can be pathological when it is triggered inappropriately or when it becomes un-coupled from the mechanisms by which it is normally controlled and limited. Chronic neuro-degeneration and age are risk factors for poorly regulated inflammation within the CNS, with an increased risk of pathological sickness behaviour. In this section I have presented evidence to show that delirium and depression can be thought of as pathological forms of neuroimmune sickness behaviour. In the next section I will present evidence that behavioural and psychological symptoms of dementia in Alzheimer's disease are also pathological forms of neuroimmune sickness behaviour.

2.2 Pathological sickness behaviour in Alzheimer's disease

2.2.1 Introduction

In this section I will summarize the evidence that demonstrates activation of innate immunity in the Alzheimer brain, and describe the possible influence of increased systemic inflammation on this process. The chronic brain inflammation associated with Alzheimer's disease may play an important role in the pathophysiology of the disease through damaging effects on synaptic plasticity and through pathways leading to neuronal cell death. However, chronic brain inflammation may also result in maladaptive neuroimmune sickness behaviour. It is a key hypothesis of this thesis that, in Alzheimer's disease, many behavioural and psychological symptoms are a form of pathological sickness behaviour. Systemic inflammatory insults may play a key role in precipitating these sickness behaviour symptoms in Alzheimer's disease; where age, chronic neurodegeneration and CNS inflammation interact to sensitize the brain to the effects of systemic inflammation. I conclude by describing the potential benefit of therapies that reduce systemic and CNS inflammation, alleviating both inflammation-associated neuronal damage and pathological neuroimmune sickness behaviour.

2.2.2 Challenges to the amyloid cascade hypothesis

In 1906 Alois Alzheimer described the hallmark histopathological features of dementia: senile neuritic plaques and neurofibrillary tangles. Neuritic plaques and neurofibrillary tangles are invariant features of Alzheimer's disease, and an intense research effort has therefore been made to identify the molecular and cellular constituents that form them. A pivotal finding came with the identification and cloning of the protein amyloid-β (Aβ).[75] Senile plaques were found to consist of a core of aggregated amyloid-β peptide, surrounded by degenerative pre-synaptic nerve endings, astrocytes and microglial cells.[6 76] The other hallmark feature of Alzheimer's disease, neurofibrillary tangles, were found to consist of hyper-phosphorylated aggregations of the microtubule-associated protein tau.[77]

The cloning of amyloid-β allowed further research into the unique properties of this peptide, and ultimately led to the amyloid hypothesis for the pathophysiology of Alzheimer's disease.[3] This states that the accumulation of excessive amyloid-β peptide is the initiating step in the pathology of Alzheimer's disease. An imbalance between the production and clearance of amyloid-β leads to the formation of insoluble plaques which, in turn, cause astrogliosis and activation of microglial cells. Pathological processes induced by amyloid-β plaques then cause the hyper-phosphorylation of tau, neurofibrillary tangle formation, synaptic loss and neuronal cell death.

Several observations have challenged the amyloid cascade hypothesis. First, the observation that plaque and tangle pathology is common in post mortem brains from people without dementia, suggests that amyloid-β might be necessary, but insufficient on its own, to trigger the disease.[4] Second, pharmacological and immunological therapies aimed at removing amyloid-β from the brain have not prevented on-going neurodegeneration.[78] Third, genome-wide association studies (GWAS) implicate pathways related to innate immunity, rather than amyloid metabolism, as genetic risk factors for late-onset Alzheimer's disease.[79-81]

2.2.3 Inflammation in the Alzheimer's brain

There is increasing evidence that differences in the inflammatory response to amyloid may explain why amyloid alone is insufficient to cause Alzheimer's disease. Researchers became interested in the role of inflammation in Alzheimer's disease when, in the early 1980s, markers of up-regulated inflammation were discovered in the brains of patients dying with Alzheimer's disease.[82] Differences were found in the degree of inflammation around amyloid-β aggregates between demented and non-demented people.[83] Microglial cells associated with amyloid plaques in patients with Alzheimer's disease expressed major histocompatibility complex class II (MHC class II) molecules on their cell surfaces, but microglial cells associated with amyloid plaques in non-demented controls did not express MHC class II molecules.[84] Subsequently, a wealth of genetic, neuropathological and epidemiological evidence has demonstrated an active role for the innate immune system and inflammation in the pathophysiology of Alzheimer's disease.[6] This data has led to a growing consensus to refine the amyloid hypothesis by suggesting that a

pathological inflammatory response to amyloid-β is also a necessary causative step in the pathophysiology of Alzheimer's disease.

2.2.4 Activated microglia in the Alzheimer brain

Microglial cells are strongly implicated as the principal inflammatory cell mediator in the Alzheimer brain. Typically, senile plaques are associated with activated microglial cells that have extensive ramified processes that deeply interdigitate with the plaques.[85] In addition to this activated morphology, plaque-associated microglia express up-regulated MHC class II molecules, complement proteins (C1q), cytokines (IL-1, IL-6, TNF-α) and chemokine receptors (CCR3, CCR5).[6] In vitro studies have shown that microglia are able to recognise amyloid-β through up-regulated RAGE, Toll-like receptors, and scavenger receptors,[86-88] and that amyloid-β stimulates microglial production of IL-1, IL-6, TNF-α, and free radicals.[6] Non-amyloid activation of microglia may also occur in neuritic plaques because of the co-localisation, within plaques, of other immune mediators, such as complement proteins, cytokines and chemokines. In addition, microglia phagocytose Aβ, possibly in an attempt to reduce toxic amyloid levels within the brain. However, such phagocytosis may be accompanied by a damaging release of reactive oxygen and nitrogen species, and pro-inflammatory cytokines such as TNF-α.[6]

Microglia can assume different activation states depending on the activating stimulus and the prior exposure of the microglial cell to various priming stimuli.[89] At one extreme microglia have an M1 phenotype, associated with activation by IFN-γ, characterized by increased expression of pro-inflammatory markers such as IL-1, phagocytosis and cytotoxic attack on pathogens. At the other extreme microglia have an M2 phenotype, associated with activation by IL-4, characterized by expression of anti-inflammatory markers such as IL-10, recruitment of Th2 cells and tissue repair. Between these extremes microglia can assume numerous levels of activation, with multiple phenotypes in response to varied activating influences.[89 90] In Alzheimer's disease, post-mortem studies suggest that microglia take on different phenotypes depending on the stage of the disease; with earlier pathological stages of Alzheimer's disease associated with a bias towards the M1 phenotype, and later-stage disease associated with a bias towards the M2 phenotype.[90] Microglial activation appears to be a critical step in the generation

of neuroinflammation in Alzheimer's disease. Working out how this critical microglial activation occurs is a key research priority. There is growing evidence to suggest that age-related change in microglial sensitivity, age-related change in the CNS micro-environment, neurodegeneration and the cumulative effects of a life-time of peripheral systemic inflammatory events, combine to generate harmful neuroinflammation by activating microglia.[89 91]

2.2.5 Complement proteins in the Alzheimer brain

An early indication of the importance of innate immunity in Alzheimer's disease came with the discovery of complement proteins within neuritic plaques.[82] Activation of the complement protein cascade plays a fundamental role in the initiation and amplification of the innate immune response in both the periphery and in the brain. Complement proteins are highly co-localised with Aβ plaques and neurofibrillary tangles in the Alzheimer's disease brain.[6 92] Furthermore, complement mRNA levels are up-regulated in the Alzheimer's disease brain, compared to non-demented controls.[93] In vitro and animal studies have shown that Aβ, neurofibrillary tangles and degenerating neurons can all activate complement pathways.[94] Although there is some evidence that complement activation may assist in the clearance of Aβ,[94] complement activation is thought, overall, to be a major contributor to pathological brain inflammation in Alzheimer's disease. Complement has direct cytotoxic effects on neurons through formation of the membrane attack complex (MAC) on dystrophic neuritis,[95] and indirect cytotoxic effects through the activation of microglial cells. Genetic evidence supports a role for complement activation in the pathogenesis of Alzheimer's disease; specifically, polymorphisms in the genes for the complement regulators CR1 and clusterin (ApoJ) were found to contribute to the risk of late-onset Alzheimer's disease in large genome-wide association studies.[80 81] However, clusterin may also have effects independent of complement, as it can bind Aβ, thereby facilitating transport of Aβ across the blood-brain barrier.[6]

2.2.6 Cytokines in the Alzheimer brain

Inflammation in the Alzheimer's disease brain is demonstrated not only by the presence of morphologically activated microglia and up-regulation of complement, but also by increased expression of inflammatory cytokines. Activated microglial cells are the principal source for pro-inflammatory cytokines in the Alzheimer's disease brain. Cytokines may contribute to Alzheimer's disease pathology by maintaining the activated state of microglia, initiating apoptotic mechanisms in vulnerable neurons, regulating the activity of complement proteins, and through direct inhibitory, or excitatory, effects on neurons.

IL-1, a key immuno-regulatory cytokine, is over-expressed in Alzheimer's disease.[6] The gene for the amyloid prescursor protein (APP) is on chromosome 21. Children with Down's syndrome, who have three copies of chromosome 21, therefore over-express APP, and amyloid-β begins to aggregate in plaques even during childhood. IL-1 is already present in diffuse, non-neuritic plaques in the brains of children with Down's syndrome, indicating that increased expression of IL-1 is an early event in the pathogenesis of Alzheimer's disease.[96] IL-1 has been implicated in the up-regulation of amyloid-β binding proteins (α1-antichymotrypsin, ApoE and C3) and in the up-regulation of the neurite growth-promoting protein S100B.[6]

Similarly, IL-6 and TNF-α are both over-expressed in the Alzheimer's disease brain.[97 98] Within the brain, IL-6 induces acute phase proteins and other cytokines, stimulates the pituitary-adrenal axis, and has a role in the regulation of neuronal survival and neuronal function.[6] TNF-α strongly induces NF-κ B, a transcription factor for other pro-inflammatory cytokines, complement proteins and cyclo-oxygenase (COX), and, overall, is thought to have a damaging role in the neuro-inflammation of Alzheimer's disease.[6] In addition, TGF-β, IL-8 and macrophage inflammatory protein-1α (MIP-1α) are up-regulated in Alzheimer's disease.[6]

2.2.7 The role of neuroinflammation in the Alzheimer's disease brain

Together, the evidence of increased microglial activation, high levels of complement expression, and high expression of pro-inflammatory cytokines, indicates heightened activity of the innate immune system in Alzheimer's disease. The role of these inflammatory processes has been controversial.[99] Inflammation may have some benefits in Alzheimer's disease through increased clearance of Aβ from the brain. However, the weight of evidence supports a pathological rather than physiological role for inflammation in Alzheimer's disease. First, the majority of inflammatory mechanisms observed in the Alzheimer brain are cytotoxic when they occur in the periphery, and are therefore presumed to be cytotoxic in the CNS.[6] Second, in post mortem Alzheimer's disease patients there is direct ultrastructural evidence of cytotoxic attack by complement on neurons.[100] Third, increased markers of inflammation are associated, macroscopically and microscopically, with areas of the most severe pathology.[6] Fourth, non-demented people with high levels of Aβ have less inflammation.[101] Fifth, epidemiological evidence suggests that long-term use of non-steroidal anti-inflammatory drugs (NSAIDS) is associated with a lower incidence of Alzheimer's disease.[102] Furthermore, patients with rheumatoid arthritis that are treated with anti-TNF-α drugs, have a markedly reduced incidence of Alzheimer's disease.[103] Overall, inflammatory mechanisms appear to be associated with increased neuronal damage, and accelerated Aβ-associated pathology. This can become a vicious cycle, as neuronal damage caused by inflammation can itself lead to increased inflammatory activity, perpetuating a cycle of inflammatory damage, even after the initial trigger has lost its influence.[6]

2.2.8 Triggering inflammation in the Alzheimer's brain

The trigger for inflammation in the Alzheimer's disease brain is unclear. Aβ and tau aggregates activate complement and microglia. However, Aβ is not always associated with damaging inflammation. Post mortem studies have shown that non-demented people can have very high levels of Aβ in the brain, but without marked inflammation.[101] In addition, APP and Aβ can both be induced by inflammatory mechanisms, and so excess amyloid may be a response, rather than an instigator, of inflammation, in late-onset Alzheimer's

disease.[6] Degenerating synapses and neuronal necrosis may be insufficient to cause robust activation of microglia, especially where these processes occur over a long period of time, allowing microglia to develop a degree of adaptative tolerance to the activating signals.[7]

Brain inflammation is very tightly regulated, as excessive inflammation in the delicate microstructure of the brain can be hugely destructive. Differences in the brain's immune response to Aβ may result from differences in anti-inflammatory regulation. Microglia are the principal inflammatory effector cell in the CNS. As such, microglial activation is tightly regulated, to prevent unnecessary or exaggerated neuroinflammation.[7] Several mechanisms exist to keep microglia in a quiescent state, and activation is only possible by concurrent activation along several pathways.[104] Unless Aβ-induced activation is also accompanied by other pro-inflammatory signals, microglia, in vivo, are unlikely to become sufficiently activated. Evidence from a prion model of neurodegeneration suggests that microglia are primed by neurodegeneration, and by aggregations of extra-cellular proteins, but require additional pro-inflammatory signals before they become fully activated.[105 106] These studies highlight the central role of peripheral systemic inflammation, and inflammatory communication across the blood brain barrier, in triggering neuro-inflammation, and subsequent neurodegeneration, in Alzheimer's disease.

2.2.9 Systemic inflammation in Alzheimer's disease

As we have seen, systemic inflammation during sickness induces CNS cytokines and thereby instigates changes in an organism's behaviour to better enable survival. However, CNS cytokines, induced by systemic inflammation, may also activate microglia, especially if they are already primed by on-going neurodegeneration. It follows that systemic inflammation could be a risk factor for the initiation and progression of Alzheimer's disease. In this section I will outline the evidence that systemic inflammation influences the initiation and progression of Alzheimer's disease.

Pro-inflammatory cytokines are elevated in the blood of patients with Alzheimer's disease. Specifically, IL-6, TNF-α, IL-1β, TGF-β, IL-12 and IL-18 were found to be significantly higher in Alzheimer's disease patients than in controls in a meta-analysis of peripheral cytokines in Alzheimer's disease.[107] The direction of causation is controversial, as elevated peripheral cytokine levels could be a response to Alzheimer's disease, rather than a causative factor.[108] However, there is growing evidence that elevated peripheral cytokines pre-date and increase the risk of Alzheimer's disease. First, longitudinal studies show that elevated peripheral cytokine levels predict cognitive decline and pre-date dementia diagnosis by several years.[109-111] In a large (n=5200) prospective longitudinal study, high IL-6 at baseline was associated with decline in cognition at 10 year follow-up.[112] High CRP in middle age is associated with a trebling of Alzheimer's disease risk up to 25 years later.[113] Second, the middle-aged children of parents with Alzheimer's disease produce higher levels of inflammatory cytokines than the middle-aged children of parents without Alzheimer's disease; implying that a genetic predisposition to higher systemic cytokines pre-dates and increases the risk of the disease.[114] Third, systemic infections occurring at any time over a 4 year period, which would be expected to be associated with transient increases in peripheral cytokine levels, are a predictive risk factor for Alzheimer's disease.[8] Fourth, periodontal infection and inflammation, which is also associated with a rise in peripheral cytokine levels, increases the risk of dementia.[115 116] Fifth, severe sepsis, and delirium, both associated with high peripheral cytokine levels, are associated with increased risk of cognitive decline and dementia.[117 118] Sixth, the syndrome of physical frailty, and the attendant increase in pro-inflammatory

cytokines, is associated with increased incidence and progression of Alzheimer's disease.[12 119] Lastly, increasing age is associated with both an exponential increase in the risk of Alzheimer's disease,[76] and with increased peripheral inflammation.[50] As we have seen, peripheral inflammation may accelerate neurodegeneration by causing inflammatory activation of primed microglia. Consequently, it is biologically plausible to ascribe some of the increased Alzheimer's disease risk associated with age to the increased peripheral inflammation associated with ageing.

Genetic evidence supports a role for inflammation in the pathogenesis of Alzheimer's disease. Genome-wide association studies consistently highlight the importance of immune pathways in Alzheimer's disease risk.[120-122] Pathway analysis shows that the majority of late-onset Alzheimer's disease risk genes are found in biological pathways involved in the immune response, after correcting for gene size, linkage disequilibrium and multiple testing.[79] The other biological pathways highlighted by risk genes involve regulation of endocytosis, cholesterol transport, and protein degradation, which all impact on the regulation of CNS and systemic immunity.[79] The innate immune system is also implicated in the significantly increased Alzheimer's disease risk conferred by a recently described genetic variant in the triggering receptor expressed on myeloid cells-2 (TREM2).[123 124] This signalling molecule is highly expressed on microglia in the CNS and is involved in the control of excessive pro-inflammatory microglial activation. Thus, the TREM2 mutation may increase Alzheimer's disease risk by reducing the threshold for microglial activation and increasing CNS inflammation.

Genome-wide association studies have implicated complement receptor type 1 (CR-1) pathways in Alzheimer's disease risk.[80 81 125] However, CR-1 is not expressed at high levels within the CNS and may therefore exert an effect on Alzheimer's disease risk through effects on peripheral inflammation.

Basal levels of peripheral cytokines differ between individuals due to genetic variation. If peripheral cytokines play a role in the pathogenesis of Alzheimer's disease then genetic variation in cytokine levels should affect Alzheimer's disease risk. Indeed, in some studies genetic polymorphisms for several cytokines do appear to increase the risk of Alzheimer's disease.[126-129] However, these polymorphisms were not found to be significant in large genome-wide

studies.[79-81 125] In the field of cardiovascular disease the role of CRP in disease risk was investigated using the concept of mendelian randomization in a genome-wide association study.[130] Mendelian randomization during meiosis means that assignment of genes for inflammatory risk should be independent of assignment of genes for other types of disease risk.[131] Despite a clear association between CRP and cardiovascular risk in cross-sectional studies, this large genome-wide study showed that CRP gene variants did not affect disease risk, therefore implying that elevated CRP is a marker of cardiovascular disease, but not implicated directly in disease risk.[130] There is a need for similar mendelian randomization studies to examine the issue of causation in the association between increased peripheral inflammation and Alzheimer's disease.

Although doubts remain concerning the direction of causation in the association between peripheral inflammation and Alzheimer's disease risk, there is also evidence that, once established, the progression and severity of Alzheimer's disease is affected by peripheral inflammation. Higher levels of serum TNF-α predict cognitive decline in Alzheimer's disease, and this effect is amplified by the occurrence of systemic inflammatory events such as chest or urinary tract infection.[9]

A further role for peripheral inflammation in the pathogenesis of Alzheimer's disease concerns the clearance of amyloid from the brain. Over a life time considerable amounts of amyloid-β are generated. In order to prevent pathological accumulation of this excess amyloid, clearance mechanisms exist allowing the amyloid to be transported across the blood brain barrier (BBB) and into the peripheral circulation. There is growing evidence that the immune system influences these clearance mechanisms. Specifically, peripheral infusion of Aβ-specific Th2 cells in transgenic mice that over-express APP resulted in increased plasma Aβ, reduced microglial activation, plaque-associated inflammation, and peripheral circulating cytokine levels.[132] In this experiment, Th2 cells did not infiltrate the CNS and so a peripheral mechanism for these beneficial effects was proposed. Perivascular macrophages may recruit circulating Aβ-specific Th2-cells at sites of cerebral amyloid angiopathy, facilitating clearance of vascular amyloid and improving the flow of Aβ out of the brain. Other work has indicated that RAGE on vascular endothelial cell surfaces may allow peripheral plasma Aβ to re-enter the brain parenchyma.[133]

Alteration in the Th1:Th2 ratio in the peripheral circulation may affect this mechanism, as reduced Th1 activity reduces the transcription of RAGE, thus improving Aβ clearance. It is interesting to note that the Th1:Th2 ratio can also be affected by the HPA axis, diurnal variation and psychological stress.[32]

In sum, systemic inflammation is a risk factor for the development and progression of Alzheimer's disease, and this effect is most likely mediated by the induction of CNS cytokines by systemic cytokines and systemic inflammation, with the subsequent pathological activation of microglia primed by dysregulated CNS inflammation, Aβ deposits, tau tangles, neuronal degeneration, or a combination of all of these factors.

2.2.10 Behavioural and psychological symptoms in Alzheimer's disease are a form of pathological sickness behaviour

I have presented evidence to show that systemic inflammation may be a necessary factor in the generation of pathological neuro-inflammation in Alzheimer's disease. I have also outlined evidence to show that systemic inflammation induces physiological neuro-inflammatory changes that result in adaptive sickness behaviour, and to show that pathological neuro-inflammatory changes can result in maladaptive, or excessive, sickness behaviour. Together, these observations have led to the hypothesis that in Alzheimer's disease, pathological neuro-inflammation, aggravated by systemic inflammation, results in maladaptive, pathological sickness behaviour. In Alzheimer's disease, this pathological sickness behaviour presents as behavioural and psychological symptoms.

A number of predictions arise from the hypothesis that certain behavioural and psychological symptoms of dementia are a form of exaggerated neuroimmune sickness behaviour:

1. Inflammatory pathology and cytokine production are invariant features of Alzheimer's disease, and occur early in the disease. Therefore, neuroimmune behavioural and psychological symptoms should be common, and should occur early in Alzheimer's disease.

2. Conditions normally associated with elevated peripheral cytokine levels and physiological sickness behaviour, such as systemic infections,

should be associated with an increased frequency of pathological sickness behaviour in patients with Alzheimer's disease.

3. Pathological sickness behaviour in Alzheimer's disease should respond to anti-inflammatory treatments.

4. Neuroimmune sickness behaviour would be predicted in other CNS diseases characterised by activation of the innate immune system and CNS cytokine production.

Prediction 1: Neuroimmune behavioural and psychological symptoms should be common, and should occur early in Alzheimer's disease

Post mortem studies suggest that microglial activation is an invariant feature of Alzheimer pathology, and occurs early in the progression of the disease.[134] Although post mortem studies show that typical Alzheimer pathology is associated with a neuroinflammatory response characterized by up-regulated microglia,[6] the strength of the association between microglial activation and Alzheimer's disease pathology declines with age.[135] As such, neuroinflammation may be more active at an early stage of the disease. In living patients with Alzheimer's disease, activated microglial cells can be imaged with positron emission tomography (PET) using the PK11195 ligand. Using this technique, microglial activation in Alzheimer's disease patients is found to occur early in the disease, prior to cerebral atrophy.[136] Furthermore, in a mouse model of neurodegenerative disease, microglial activation occurs early, prior to the onset of marked neurodegeneration.[137] Together these data suggest that microglial activation is an early event in Alzheimer's disease. As sickness behaviour is related to microglial activation, early microglial activation suggests that sickness behaviour symptoms should be present at an early clinical stage in Alzheimer's disease. As microglial activation is common in Alzheimer's disease, sickness behaviour symptoms should also be common.

Behavioural and psychological symptoms occur in nearly all patients with dementia.[138,139] One study found that 80% of patients with dementia had at least one symptom on the Neuropsychiatric Inventory (NPI) of behavioural and psychological symptoms; with 36% suffering apathy, and 32% suffering depression.[139] Importantly, this study also examined the prevalence of these symptoms in people with mild cognitive impairment (MCI), in many cases a

prodromal phase of Alzheimer's disease. In the MCI group, 50% of people had at least one symptom on the NPI; with 15% suffering apathy, and 20% suffering depression. The data from the MCI group support the assertion that sickness behaviour symptoms are common at an early stage in the progression of Alzheimer's disease. In patients with Alzheimer's disease, epidemiological studies show that apathy, fatigue and depression can be present at a pre-clinical stage, before the onset of cognitive deficits.[134 140]

Depression is common in people with Alzheimer's disease.[139] Although often considered merely a reactive psychological consequence of the disease, there is growing evidence that depression in Alzheimer's disease may be related to the actions of activated microglia.[141] Depressive symptoms are more severe in patients with mild dementia than in moderate to severe dementia,[142] indicating that depression is greatest when inflammatory activation is greatest, early in the disease. Indeed, in prospective studies of people with no evidence of dementia at baseline, depression predicts cognitive decline and a diagnosis of Alzheimer's disease,[140 143] implying that depressive symptoms may represent an early manifestation of the neuroinflammatory pathology found in Alzheimer's disease. Further evidence for depression as an early symptom of Alzheimer's disease is provided by a UK cohort in which the incidence of depression in dementia declined as age increased.[138] Of note, in this cohort, the incidence of depression increased in those with poor self-rated health, irrespective of age.[138] As poor self-rated health is associated with higher levels of peripheral pro-inflammatory cytokine levels,[144] the relationship between poor self-rated health and depression in this cohort suggests that peripheral inflammatory signals may play as important a role in pathological sickness behaviour in Alzheimer's disease as central neuroinflammation. Together, these findings support the view that depression occurs early in the natural history of Alzheimer's disease, when average is age is less, and when inflammatory activity is greatest.[135]

As discussed previously, delirium may represent a pathological form of neuroimmune, cytokine-related sickness behaviour. In Alzheimer's disease, episodes of acute delirium are common, and are triggered more easily than in non-demented elderly people,[145] supporting the hypothesis that pathological sickness behaviour is common in Alzheimer's disease. Dementia is the most common risk factor for delirium in hospitalised elderly people,[146] and

approximately 2/3 of all delirium cases occur in patients with dementia.[62] In non-demented people, an episode of delirium is a risk factor for the subsequent development of dementia, indicating that delirium may be an early presentation of dementia in some patients.[117 147] However, in contrast to this, the frequency of delirium increases as the severity of dementia progresses.[141] This runs contrary to the expectation that pathological sickness behaviour should be an early feature of Alzheimer's disease. Age may help to explain this discrepancy. Delirium is associated not only with neuroinflammation, but also with decreased cerebral metabolism and cholinergic deficiency. These factors are influenced by ageing, and may become more important as drivers of neurodegenerative delirium as age increases. Indeed, delirium is more common in late-onset Alzheimer's disease than in early-onset Alzheimer's disease, indicating that age plays an additional role in delirium risk in demented patients.[148]

Weight loss is a common physical sign in animal sickness behaviour.[16] Similarly, weight loss is a common sign in Alzheimer's disease, and may represent a risk factor for cognitive decline and dementia.[12] If weight change were a form of pathological sickness behaviour in Alzheimer's disease then peripheral inflammation would be expected to exacerbate the weight loss. Indeed, in a pilot study of patients with Alzheimer's disease, weight loss was common, and was related to serum levels of TNF-α.[149]

There is currently no validated scale for assessing sickness behaviour as a whole in Alzheimer's disease. I have presented evidence for individual sickness behaviour symptoms such as depression, delirium and weight loss. Despite the current lack of an overall sickness behaviour scale, the subjective experience of sickness behaviour in general would be predicted to result in lower scores of self-rated health. Indeed, as we have seen, poor self-rated health is related to higher levels of peripheral cytokines, in the way one would expect if it were a reflection of cytokine-related neuroimmune sickness behaviour.[144] Importantly, poor self-rated health is also a risk factor for incident dementia.[150] This finding demonstrates that, as predicted, cytokine-related decline in subjective health symptoms, as a proxy for cytokine-related neuroimmune sickness behaviour, is an early event in dementia pathology.[150]

Prediction 2: Conditions normally associated with elevated peripheral cytokine levels and physiological sickness behaviour, such as systemic infections, should be associated with an increased frequency of pathological sickness behaviour in patients with Alzheimer's disease.

In a mouse model of neurodegeneration a systemic challenge with lipopolysaccharide (LPS) mimics the effects of a bacterial infection, and causes exaggerated and prolonged sickness behaviour.[151] Similar exaggerated sickness behaviour is seen in this model following systemic challenge with the viral mimic polyinosinic:polycitidylic (poly I:C).[152] In these experiments, control mice, without neurodegeneration, do not exhibit the same level of sickness behaviour, and do not manifest the sickness behaviour for as long, as the mice with neurodegeneration. Thus, on-going neurodegeneration is thought to prime the brain to produce exaggerated sickness behaviour in response to a peripheral immune challenge.[10]

In Alzheimer's disease, symptoms characteristic of sickness behaviour are related to the proinflammatory cytokines TNF-α and IL-6.[15] Systemic inflammatory events that raise the levels of these cytokines would therefore be expected to increase the frequency of sickness behaviour symptoms in Alzheimer's disease.

Prediction 3: Pathological sickness behaviour in Alzheimer's disease should respond to anti-inflammatory treatments.

This hypothesis has not yet been properly tested in a clinical trial. Although several studies have examined the effects of anti-inflammatory drugs, none have used neuropsychiatric symptoms as a primary end-point. Anti-cholinesterase drugs have the best evidence for a beneficial effect on behavioural and psychological symptoms in dementia.[153-155] Although this beneficial effect may be related to cognitive effects, it is interesting to note that anti-cholinesterase drugs also have anti-inflammatory effects.[108] Of note, depression in Alzheimer's disease does not respond well to traditional anti-depressant treatment with serotonergic drugs, which do not have an anti-inflammatory action.[156] If the principal driver of depression in dementia is inflammation then this lack of effect is less surprising.

Prediction 4: Cytokine-related sickness behaviour is likely in other CNS diseases characterised by activation of the innate immune system and CNS cytokine production.

Neuropsychiatric symptoms are common in a number of neurodegenerative diseases associated with a neuroinflammatory response. Dementia in Parkinson's disease and Lewy Body dementia are both characterised by prominent neuropsychiatric disturbance, often occurring early in the course of the disease.[157] Apathy and appetite disturbance are common in fronto-temporal dementia.[157] In new variant Creutzfeldt-Jakob disease, psychiatric symptoms, including depression, precede other neurological symptoms, indicating that these symptoms may be more related to the degree of inflammation than to the degree of neurodegeneration.[134] Acute delirium is a frequent symptom after head trauma, and in these cases is associated with IL-1 and IL-6 CNS responses.[145] Overall, symptoms consistent with cytokine-related neuroimmune sickness behaviour are common in a number of inflammatory neurodegenerative conditions.

2.2.11 Conclusion

I have reviewed evidence to show that Alzheimer's disease is associated with a heightened sickness behaviour response. Sickness behaviour is driven by microglial cytokine production within the CNS. Microglia in Alzheimer's disease are primed by on-going neurodegeneration, and consequently are more sensitive to activating signals generated by systemic inflammatory events. As microglial activation is an early event in the pathology of Alzheimer's disease, sickness behaviour symptoms may occur prior to measurable cognitive decline. There is currently no established instrument for assessing sickness behaviour in Alzheimer's disease. However, measuring sickness behaviour in Alzheimer's disease is necessary as these symptoms are common, under-recognized and distressing. A scale for measuring sickness behaviour is also necessary as a primary outcome measure in therapeutic trials.

2.3 Sex hormones and systemic inflammation in Alzheimer's disease

In the preceding sections I have reviewed evidence concerning the role of systemic inflammation in the progression of pathology and symptoms in Alzheimer's disease. It follows that moderators of systemic inflammation may be important moderators of Alzheimer's disease. Here, I review evidence that age-related change in the hypothalamic-pituitary-gonadal axis (HPG axis), with subsequent effects on the levels of various sex hormones, may be a key moderator of the scale and consequences of systemic inflammation, with consequent diverse effects on behaviour and brain function.

Ageing is the principal risk factor for Alzheimer's disease.[158] There is a greater prevalence of Alzheimer's disease in women than in men because women have greater longevity and therefore live long enough to suffer the adverse effects of ageing on dementia risk.[159] However, there is a debate about whether women have additional risk for dementia, over and above that conferred by greater longevity. Epidemiological evidence suggests that female sex confers additional risk.[160-163] Sex hormone differences between the sexes, especially after menopause, are obvious candidates as an explanation for this additional risk.[164] However, several other factors may also contribute. First, there is evidence that the high dementia risk associated with the ApoE-e4 allele is increased further in women.[161] Second, structural and functional sexual dimorphism develops in embryonic life in the human brain and has effects across the life-course which have been implicated in the risk, progression and recovery of many neurological diseases.[162] Third, women over the age of 75 have a higher incidence of other risk factors for dementia when compared with men of a similar age; hypertension, hyper-cholesterolaemia and type 2 diabetes mellitus are all more common and confer increased dementia risk.[160] However, many of these factors are themselves associated with age and the apparent additional risk associated with gender becomes less apparent when the effect of age is carefully accounted for in epidemiological studies, causing some to question whether gender plays any part in Alzheimer's disease risk.[158] [159] Caution is needed however when controlling for age in this way. The effect of age on dementia risk is multi-factorial, with many risk factors associated with age combining to increase risk.[76] Controlling for age in studies of risk may

obscure significant age-dependent risk factors. Thus, many of the gender-related risk factors described above are also related to age and should not be discarded because their apparent importance is lessened by controlling for age. Overall, this evidence suggests that gender may affect Alzheimer's disease risk. As we have seen, several factors may be implicated in the mechanism underlying this gender-related risk, but there is growing evidence that age-related changes in sex hormones are a key component.[165]

Although there is debate about whether there are significant differences in Alzheimer's disease risk between the sexes, there is growing evidence, in both sexes, that age-related change in sex hormone levels increases neurodegenerative risk. Ageing is associated with menopause in women and andropause in men. Menopause and andropause occur when the usual production of androgens and oestrogens by the gonads declines; for women a sharp reduction in gonadal sex hormones occurs at menopause, for men a more gradual reduction in gonadal sex hormones occurs at andropause.[166] These hormonal changes disrupt the normal negative feedback system that regulates sex hormone levels.

In adult life a physiological negative feedback system regulates the levels of hormones within the hypothalamic-pituitary-gonadal axis (HPG axis). Gonadotropin releasing hormone (GnRH) is produced in the hypothalamus and acts at the level of the pituitary gland to increase the production and release of gonadotropic hormones: luteinizing hormone (LH) and follicular stimulating hormone (FSH). The gonadotropic hormones act at the level of the gonads to increase the production and release of sex steroids: oestrogens and androgens. The sex steroids, in turn, exert negative feedback on the release of GnRH in the hypothalamus, thereby maintaining balance within the HPG axis.[166] This regulated system begins to fail with age. Age-related change in the hypothalamus and pituitary gland results in reduced production of gonadotropins, exacerbating age-related reduction in sex steroid production in the gonads.[167] Consequently, there is significant decline in the gonadal production of the sex steroids, oestrogen and testosterone. As menopause and andropause progress there are reactive increases in gonadotropin release from the pituitary, in a failing effort to restore sex steroids to normal levels. Thus, after initial decline in gonadotropins with age, as age progresses,

gonadotropin levels rise to high levels; so that in women there is a 3-fold increase in LH, and in men there is a 2-fold increase in LH.[168]

These dramatic age-related changes in sex hormone levels have been postulated as potential risk factors for Alzheimer's disease.[169] In cross-sectional studies, men with AD have lower levels of testosterone than non-AD men.[170] A meta-analysis of prospective longitudinal studies in men has shown that lower serum testosterone levels at baseline are associated with increased risk developing of Alzheimer's disease.[171] In a small placebo-controlled trial, men with Alzheimer's disease treated with testosterone demonstrated improved cognition,[172] but there have not been further trials to corroborate this finding. Testosterone appears to play an early role in the risk of Alzheimer's disease; with evidence that men with low testosterone are at increased risk of amnestic mild cognitive impairment (MCI).[173] However, in men with MCI, treatment with testosterone resulted in only modest cognitive gains.[174] In post-menopausal women, lower oestrogen levels are associated with Alzheimer's disease,[175] and several studies have shown cognitive improvement in women treated with oestrogen replacement therapy.[176-178] Furthermore, hormone replacement therapy (HRT) at the onset of menopause in women is associated with reduced risk of Alzheimer's disease,[179 180] although delayed HRT, given after menopause, increases risk of Alzheimer's disease.[181]

The increased dementia risk associated with age-related decline in sex steroids is mirrored by an increased dementia risk associated with age-related elevation in the gonadotropins, FSH and LH. Higher levels of FSH and LH are associated with Alzheimer's disease.[19 182] High LH levels are also associated with poorer cognition in healthy community-dwelling men.[183] The high risk of Alzheimer's disease associated with ApoE-e4 is reduced by polymorphisms in the LH gene, and the LH-receptor gene.[184] In an Australian cohort, serum LH correlates with brain amyloid labelled by Pittsburgh B compound (PiB) in a positron emission tomography (PET) scan.[185] The correlation was strongest at the earliest stage of mild cognitive impairment, suggesting that LH may exert an effect on risk early in the pathogenesis of Alzheimer's disease. These findings point to a relationship between high gonadotropin levels and Alzheimer's disease, and it follows that therapeutic reduction of gonadotropin levels may reduce risk and symptoms in Alzheimer's disease. Accordingly, men with prostate cancer treated with leuprolide, a GnRH antagonist that reduces LH levels, have a 50%

reduction in Alzheimer's disease risk.[186] Women with Alzheimer's disease and already taking a cholinesterase inhibitor, treated with leuprolide, had reduced cognitive decline in a placebo-controlled trial, although there was no benefit in those not taking a cholinesterase inhibitor.[187]

Overall, this evidence suggests an association between age-related change in gonadotropins and sex steroids, cognitive decline and Alzheimer's disease. Researchers have therefore tried to understand the mechanisms by which sex hormones can influence cognition and neurodegenerative disease. Receptors for gonadotropins and sex steroids are widely distributed within the brain, and are thought to be involved in a number of physiological processes across the life-course.[166 168] In health, CNS receptors for gonadotropins, oestrogens and testosterone are involved in foetal development of the CNS, reproductive behaviour, learning and memory.[165 166 168] These normal physiological processes may become pathological when age-related endocrine dyscrasia begins to cause dramatic changes in the usual levels of sex hormones. The pathological mechanisms that may connect sex hormones and neurodegeneration include decreased neuroplasticity, increased pathological amyloid processing, and increased systemic and CNS inflammation.

Oestrogens and gonadotropins play a role in neuroplasticity; the ability of neurons to strengthen and change synaptic connections during learning and memory.[168] Loss of oestrogens with ageing may reduce neuroplasticity and reduce the compensatory mechanisms that prevent Alzheimer's disease.[165] In animal models, reduced oestrogen levels caused by ovariectomy are associated with reduced neuritic spine density and reduced synaptic maintenance in the prefrontal cortex and hippocampus.[168 188 189] In a model using rat hippocampal slices, oestrogens play a critical role in the maintenance of neuritic spines and synapses after activation of the N-methyl-D-aspartate (NMDA) receptor, a key receptor for long-term potentiation and memory consolidation.[190 191] In men, tissue aromatization of testosterone to oestrogen may confer neuro-protection by oestrogen-dependent mechanisms.[192] Moreover, testosterone and other androgens, may act directly on androgen receptors concentrated in the hippocampus, to promote maintenance of neuronal synapses in adult life.[193]

Gonadotropins, particularly LH, are also involved in neuroplasticity. Receptors for LH are widely expressed in brain areas associated with cognition, memory,

sensory processing, reproductive behaviour and autonomic function.[194] In animal models, the cognitive deficits and loss of synaptic plasticity induced by reduced oestrogens and ovariectomy are not completely reversed by oestrogen replacement.[168] However, leuprolide treatment, which reduces circulating LH, rescues ovariectomy-induced cognitive dysfunction in a transgenic mouse model of Alzheimer's disease.[195] Furthermore, in this model, leuprolide treatment is associated with increased signalling events in a number of pathways associated with synaptic plasticity.[168 195] There appears to be an inverse relationship between circulating LH and brain LH. High levels of circulating LH, as occurs with age, or after ovariectomy, are associated with reduced brain LH.[168] This may occur because of reduced endogenous neuronal production of LH as a response to high circulating levels of LH, by mechanisms that remain unclear. Nevertheless, in the same transgenic mouse model of Alzheimer's disease, therapeutic reduction in circulating LH with leuprolide normalizes LH levels in the brain after ovariectomy, and improves cognitive function.[195]

Gonadotropins may also be involved in neuroplasticity via effects on glial cell function. Glial cells also express the LH receptor, with activation of the receptor causing glial proliferation and increased synaptic maintenance. Activation of the LH receptor on glial cell is associated with a shift in the production of glial prostaglandins from prostaglandin E2, which reduces glial proliferation, towards production of prostaglandin D2, which promotes glial proliferation and increases synaptic maintenance.[196]

A further pathological mechanism that may connect sex hormones and neurodegeneration involves increased pathological amyloid processing. There is a great deal of evidence to show that age-related reductions in sex steroids, together with increases in circulating gonadotropins, are associated with increased production of pathological amyloid-β (Aβ) in rat, mouse and human cell lines and brain preparations.[197 198] Sex hormones may act in the brain partly through effects on the control of the cell cycle, with aberrant reactivation of the cell cycle causing increased pathological amyloid processing.[164 165] Activated microglia may play a role in sex hormone-related amyloidogenesis and reduced neurogenesis. In a mouse model of ageing, increased markers of hypothalamic inflammation, including increased hypothalamic TNF-α, were associated with

reduced production of GnRH, reduced sex steroids, elevated circulating gonadotropins, altered amyloid processing and reduced neurogenesis.[199]

In previous sections I have discussed the impact of systemic inflammation on neurodegeneration. Any effect of circulating sex hormones on systemic and CNS inflammation may therefore represent a further important pathological mechanism connecting sex hormones and neurodegeneration. Loss of oestrogen in post-menopausal women, and loss of testosterone in aged men, is linked to an increased incidence of many auto-immune, inflammatory diseases as age progresses, with an implication that age-related change in sex hormones is associated with non-specific systemic inflammation and reduced inflammatory control.[200] Several studies have shown correlations between low levels of testosterone and increased markers of systemic inflammation. In cross-sectional studies in men, there is an inverse relationship between testosterone and CRP.[201-203] In a large cross-sectional study in men, there was an inverse relationship between testosterone and white cell count.[204] In a longitudinal study of men aged 20 to 79 years, baseline levels of testosterone were inversely associated with increased markers of oxidative stress and inflammation after 5 years of follow-up.[205] However, the inverse relationship between baseline testosterone and CRP was less clear after controlling for potential confounders in a multivariate model.[205] The wide age range may have obscured any relationship as it seems likely that the relationship between sex hormones and inflammatory mediators changes with age, making a linear relationship unlikely across a wide age range. In young men, with a mean age of 21, a Mendelian randomization analysis of the cohort demonstrated no relationship between genetically-predicted testosterone level and CRP or white cell count.[206] However, in a study that examined older men, and excluded those under 65, there was a relationship between low testosterone and elevated levels of the soluble IL-6 receptor (sIL-6R), but not CRP or TNF-α after accounting for potential confounders.[207] The size of the correlations between sex hormones and individual components of the inflammatory system tend to be modest, reflecting the multi-factorial control of systemic inflammation; sex hormones contribute to variance in immune system activity, but are not expected to be the main driver of activity.

In vitro studies indicate a direct action of sex steroids on immune cells in the CNS and on immune cells in the systemic circulation. In the CNS, activation of

oestrogen receptors on rat microglial cells, reduces microglial activation in response to lipopolysaccharide (LPS), and reduces microglial production of TNF-α, IL-1β and COX-2.[208] In the systemic circulation, oestrogen inhibits the release of TNF-α from peripheral blood mononuclear cells (PBMCs) in post-menopausal women.[209] In men, testosterone undergoes tissue aromatization to oestrogen and may have anti-inflammatory effects via these oestrogen-dependent pathways.

Age-related elevation of gonadotropins may also have pro-inflammatory effects, with consequent inflammation-related neurodegeneration. In a mouse model investigating oestrogen-deficiency and osteoporosis, FSH directly stimulated TNF-α production by macrophages.[210] In men and women, reduced levels of LH, occurring during treatment with the GnRH antagonist leuprolide, were associated with reduced levels of IL-1β, IL-6 and MCP-1.[198] Both LH and FSH are positively associated with raised TNF-α, IL-1β and MCP-1 in men and women with rheumatoid arthritis, suggesting that the relationship between circulating cytokines and gonadotropins may become stronger in the presence of underlying systemic inflammation.[211]

The interaction of age-related change in sex hormones and the ageing immune system may be bi-directional; although there is less evidence for cytokine regulation of sex hormones than there is for sex hormone regulation of cytokines. Nevertheless, systemic illness and psycho-social stress are associated with reduced sexual drive and reduced reproductive behaviour, and this association is potentially mediated by down-regulation of sex steroids by systemic inflammatory markers. In health, infusion of recombinant human TNF-α into volunteers causes elevation of LH, but not FSH, and a subsequent reduction in testosterone.[212] Conversely, in vitro, in vivo and human studies investigating diabetes and obesity have demonstrated a relationship between TNF-α and sex hormone binding globulin (SHBG), such that TNF-α causes down-regulation of SHBG.[213] SHBG binds testosterone and oestrogens in the circulation and reduces biological availability of these hormones; therefore, in these pro-inflammatory states, TNF-α, by reducing SHBG, can increase the bio-availability of testosterone and oestrogens. It appears, then, that cytokines like TNF-α, can have different effects on sex hormones depending on the underlying inflammatory milieu.

The evidence reviewed here shows that age-related change in sex hormones may contribute to the pathogenesis of Alzheimer's disease. Potential mechanisms include the effects of age-related change in sex hormones on reducing neuroplasticity and neuronal maintenance; increasing amyloid production; and increasing systemic and CNS inflammation. As we have seen, there is growing evidence for the primacy of inflammatory mechanisms in the pathogenesis of Alzheimer's disease; therefore, the bi-directional relationship between inflammation and age-related sex hormone changes warrants further investigation.

2.4 Anti-inflammatory agents in Alzheimer's disease

I have presented evidence to show that some behavioural and psychological symptoms in Alzheimer's disease represent pathological sickness behaviour and are driven by the response of primed microglia within the CNS to systemic inflammation. Factors that modify systemic inflammation, such as age-related changes in sex hormones, may therefore also modify the symptoms and progression of Alzheimer's disease. I will now review the evidence for the effectiveness of anti-inflammatory agents in Alzheimer's disease. Although several studies have examined the effects of anti-inflammatory drugs in AD, the results have been discouraging. Few, if any, studies have examined the effects of anti-inflammatory drugs on neuropsychiatric symptoms as a primary outcome measure. Many of the anti-inflammatory agents used previously have had additional non-immune effects that may have affected their efficacy. I conclude that a randomized trial of a specific cytokine antagonist, such as our trial of the TNF-α antagonist etanercept, is warranted.

2.4.1 Non-steroidal anti-inflammatory drugs (NSAIDS)

The observation of inflammation in the brain of patients dying from Alzheimer's disease led to the hypothesis that reducing cerebral inflammation should slow the progression or decrease the incidence of the disease.[214] This hypothesis was first tested in epidemiological studies that sought to assess the effects of long-term anti-inflammatory drugs (NSAIDS) on the prevalence of Alzheimer's disease. Initially promising results led to further investigation of the effect of NSAIDS in animal models and in clinical trials. Although the potential benefit of NSAIDS was thought to arise from their anti-inflammatory effects, further work demonstrated other potential mechanisms, for example an anti-amyloid effect or a COX-independent effect via PPAR-γ receptors. Unfortunately, clinical trials have been unconvincing and the value of NSAIDS in the prevention and treatment of Alzheimer's disease remains controversial.

2.4.2 Epidemiological studies of NSAIDS

Epidemiological studies show an apparently protective effect of NSAIDS. The incidence of Alzheimer's disease in cohorts of older people has been examined in several large prospective studies.[102] Regular use of NSAIDS for at least 2

years is associated with a reduced relative risk of Alzheimer's disease in these studies. The Baltimore Longitudinal study of Ageing is the largest of the prospective trials to date, and found a relative risk of Alzheimer's disease of 0.35 for 10 years of NSAID use.[215]

Case-control studies have also shown reduced odds of Alzheimer's disease in people taking NSAIDS regularly. A meta-analysis of the case-control studies carried out up to 1996 (cases controls) found that regular NSAID use was associated with a 51% reduction in the odds of Alzheimer's disease (OR=0.496; P=0.0002).[216] However this did not include the largest case control study to date (49349 cases and 196850 matched controls) published by Vlad et al. in 2008.[217] Although the previous meta-analysis had shown a significant risk reduction after 2-3 years of regular NSAID use, the Vlad study found only a modest benefit at 2-3 years with a combined odds ratio for all NSAIDS of 0.93 (0.88 – 0.99). No individual NSAID had an odds ratio that differed significantly from 1.0 at 2-3 years. However, the study did show a significant effect of NSAIDS after 5 years of regular use, with a combined OR of 0.76 (0.68 – 0.85). Ibuprofen appeared to be the main driver of this significant risk reduction with an individual odds ratio of 0.56 (0.42 – 0.75), whereas none of the other NSAIDS had an individual OR that reached significance. This study is the only one large enough to allow comparisons of individual NSAIDS. The researchers looked for evidence that the NSAIDS known to lower beta-amyloid production in animal models and cell cultures provided better protection. They found no such evidence and concluded that the protective effect of NSAIDS was not due to an anti-amyloid effect.[217]

A meta-analysis of 25 epidemiological studies, including cross-sectional, prospective and case-control studies, showed that the risk reduction associated with NSAID use dropped from 50% (RR=0.51; 95% CI: 0.37-0.70) in studies examining prevalent dementia to 20% (RR=0.79; 95% CI: 0.68-0.92) in prospective studies examining incidence of dementia, and showed no benefit at all in reducing rates of cognitive decline (RR=1.23; 95% CI: 0.70-2.31). This sliding scale in benefit, with treatment effects shrinking as sources of bias decrease, raises the suspicion that bias may have confounded the result of some epidemiological studies. The authors hypothesise that the reported benefits of NSAIDS are likely to be due to a combination of recall, prescription and publication bias.[218] Recruitment bias may also play a part as patients with

Alzheimer's disease and arthritis, the principal indication for NSAIDS in this age group, may be less likely to participate in a study, than patients with Alzheimer's disease alone.

Contrary to the findings of many epidemiological studies, Breitner et al. found that frequent NSAID use at baseline increased, rather than decreased, the incidence of dementia, after up to 12 years of follow-up (adjusted Hazard Ratio 1.66, 95% CI: 1.24 to 2.24).[219] The cohort was older than many of the cohorts in other epidemiological studies, with a median age of 74.8 years. The authors have now published post mortem data on the cohort and have shown that frequent NSAID use at baseline is also associated with greater numbers of Aβ neuritic plaques at post mortem.[220] The results of this study are in marked contrast to other epidemiological studies of NSAID use. Given the higher median age of the cohort, the results raise questions about whether NSAIDS have detrimental effects in people aged over 65, and more beneficial effects in people aged under 65. Within the cohort, frequent NSAID users had higher co-morbidity. Medical co-morbidity is associated with higher levels of systemic inflammation, which may exacerbate dementia.[9] Therefore, higher systemic inflammation and higher co-morbidity in frequent NSAID users may have confounded the association between frequent NSAID use and dementia incidence in this cohort.

There is some epidemiological evidence that the protective effect of NSAIDS is only found in ApoE e4 allele carriers. In the Cache County cohort NSAIDS were found to slow rates of cognitive decline in e4 allele carriers only.[221] In a large prospective trial that used diagnosis of Alzheimer's disease as the outcome measure rather than cognitive decline, NSAIDS were shown to reduce the risk of diagnosis, but only in e4 allele carriers. There was no effect on the incidence of vascular dementia.[222]

In summary, there is some evidence from prospective and case-control studies that regular NSAID use for at least 2-5 years produces a 20-60% risk reduction in the incidence of Alzheimer's disease, although various sources of bias may have inflated the size of the effect. Ibuprofen is the most consistently beneficial NSAID in epidemiological studies. The benefit of NSAIDS may be greatest in ApoE e4 allele carriers.

2.4.3 NSAIDS in animal studies

NSAIDS were thought to be of potential benefit in Alzheimer's disease because of their anti-inflammatory effects. NSAIDS certainly reduce the inflammation associated with amyloid plaques in transgenic mouse models of Alzheimer's disease. Treatment of such animals with NSAIDS results in reduced ubiquitin-labelled dystrophic neurites, reduced numbers of activated microglia, and reduced expression of the inflammatory markers IL-1β and GFAP.[223]

NSAIDS inhibit the production of prostaglandins by cyclo-oxygenase enzymes. The anti-inflammatory effects of NSAIDS in the peripheral circulation are thought to be mediated through this mode of action. Cerebral inflammation in Alzheimer's disease is characterized by microglia activation, IL-1 and complement,[6] rather than by elevated prostaglandin levels or increased COX expression. However, elevated levels of prostaglandin E_2 (PGE_2) are found in the CSF of patients with Alzheimer's disease,[224] although these elevated CSF PGE_2 levels decrease in AD patients as the severity of dementia increases.[225] This reduction in prostaglandin levels may reflect the death of neurons that express COX-2 as Alzheimer's disease progresses. NSAIDS may be of benefit in AD because of a reduction in cerebral prostaglandin levels, but this benefit would reduce as the disease progressed and PGE_2 levels fell.

Some NSAIDS have a COX-independent anti-inflammatory effect by activating peroxisome proliferator-activated receptor gamma (PPAR-γ) that may be beneficial in reducing cerebral inflammation associated with Alzheimer's disease. PPAR-γ is a nuclear receptor that binds to DNA following activation and acts as a transcription factor, negatively regulating the expression of inflammatory genes. PPAR-γ is up-regulated in activated microglia and activation of the receptor with ibuprofen or indomethacin causes suppression of pro-inflammatory mediators (IL-1β, TNF-α, IL-6) in tissue culture experiments.[226]

NSAIDS have various anti-inflammatory effects that may be of benefit in Alzheimer's disease, but several groups, working with different transgenic mouse models of Alzheimer's disease, have shown that some NSAIDS also reduce the amount of amyloid present in the brain.[102]

NSAIDS have several potential modes of action, independent of their effects on cyclo-oxygenase enzymes. Evidence from cell culture and animal models has demonstrated that ibuprofen and some other NSAIDS modulate the activity of the γ-secretase membrane-bound enzyme complex that is, in part, responsible for Aβ production.[227] This amyloid-reducing effect may be mediated by an effect on the fluidity of cell membranes, making interactions between amyloid precursor protein (APP) and γ-secretase less frequent.[228] There is also evidence that some NSAIDS can reduce aggregation of amyloid fibrils into plaques, perhaps by direct interference with fibrillar binding or by inducing the expression of amyloid-binding proteins such as transthyretin (TTR).[223] A further anti-amyloid effect may be mediated through an effect on the expression of the principal β-secretase, BACE-1. Neuronal cells primed by IFN-γ show increased expression of BACE-1 in response to pro-inflammatory cytokines (IL-1β or TNF-α) and this amyloidogenic process can be blocked by treatment with ibuprofen.[229] It is possible that this effect of ibuprofen on BACE-1 expression is simply caused by a reduction in the triggering pro-inflammatory cytokines. However the effect of NSAIDS on some cytokines is unclear. NSAIDS can be shown to suppress expression of inflammatory cytokines,[226] but may paradoxically cause increased production of IL-1 and TNF-α.[230] Such an increase in IL-1 and TNF-α suggests a cytokine-independent mechanism must be responsible for the effect of ibuprofen on reduced BACE-1 expression.

2.4.4 Clinical trials of NSAIDS

Several clinical trials of NSAIDS have now been completed. Unfortunately, these have not borne out the promise of epidemiological and animal studies.

Although there have been several clinical trials of traditional NSAIDS most have been under-powered, of short duration and with a high drop-out rate. A meta-analysis of three trials of NSAIDS in 543 patients with Alzheimer's disease revealed no significant improvement in rates of cognitive decline.[218] A Cochrane review and meta-analysis of randomized clinical trials of aspirin (3 trials), NSAIDS (6 trials) and selective COX-2 inhibitors (5 trials) for the treatment of Alzheimer's disease in a total of 2445 patients, found no evidence of a beneficial treatment effect for any intervention.[231] Within the six randomized trials of traditional NSAIDS, only one trial, examining the effect of indomethacin, demonstrated any significant benefit.[232] In this small trial the

indomethacin group (n=14) had no significant change in scores on cognitive tests (+1.3%; +/-1.8%) but the placebo group (n=14) declined (-8.4%; +/-2.3%, P<0.003).[232] A more recent study examining the effect of ibuprofen found no significant effect overall but some improvement in the rate of cognitive decline in a sub-group analysis of patients that were ApoE e4 allele carriers.[233] This supports the epidemiological finding that ApoE e4 allele carriers benefit from greater protection with NSAIDS than non-carriers.

Trials using COX-2 specific NSAIDS have not shown any benefit in Alzheimer's disease patients or in mild cognitive impairment.[102 231]

NSAIDS were hypothesized to prevent dementia if given to people prior to significant neurodegenerative change.[214] The ADAPT Study examined the effect of the specific COX-2 inhibitor, celecoxib, or the traditional NSAID, naproxen, versus placebo, in healthy older adults with a family history of dementia.[234] The study was stopped early because of concerns about cardiovascular side effects from COX-2 inhibitors. Nevertheless, patients received a median of 1.5 years of treatment during the study, and continued to have follow-up assessments. The ADAPT Study group have now published long term follow-up data on the study participants. Neither celecoxib nor naproxen afforded any protection against cognitive decline, or any protection against dementia, over 10 years of follow up.[235]

2.4.5 Why don't NSAIDS work in clinical trials?

A potential reason for the failure of these trials is that the NSAIDS are being given too late, that once patients have severe enough neuropathology to make a clinical diagnosis of dementia the window of opportunity for a beneficial effect of NSAIDS has closed. As described above, the ADAPT Study was a primary prevention study using either naproxen, celecoxib or placebo to examine the effects of NSAIDS in non-demented people with a family history of Alzheimer's disease. The evidence gathered prior to the premature cessation of the trial revealed no benefit.[234] An attempt to prevent Alzheimer's disease by treating patients with mild cognitive impairment with rofecoxib was also negative.[236]

Both these studies used COX-2 inhibitors despite the fact that there is no convincing epidemiological evidence that COX-2 inhibitors are of any benefit for either prevention of treatment of AD. Recent evidence suggests that COX-1 and COX-2 have contrasting roles in the context of neurodegeneration.[237] In the normal brain COX-2 has physiological roles in the regulation of synaptic activity and long-term potentiation, and in neuroinflammation COX-2 activity is necessary for the resolution of inflammation.[237 238] In a model of neuroinflammation COX-2 inhibition increased neuronal damage, glial activation and brain levels of IL-1β and TNF-α, in addition to increasing blood-brain barrier permeability.[237] Furthermore, in mice treated with a COX-2 antagonist, a systemic inflammatory insult with LPS caused increased transcription of inflammatory genes in the brain parenchyma.[239] COX-1 and COX-2 expression changes in AD as the disease progresses. In early disease, when neurofibrillary tangle density is low, COX-2 expression is increased in neurons, and COX-1 expression is increased in microglia.[240] In these neurons COX-2 expression co-localizes with the expression of cell cycle proteins, suggesting that COX-2 may be part of a protective, regenerative pathway early in Alzheimer's disease. Further evidence to support this hypothesis comes from the finding that patients with higher CSF levels of PGE_2,the principal product of COX-2, live longer than those with lower levels.[225] Together these findings suggest that COX-2 inhibition could be detrimental rather than protective in early AD.

In light of these neuroprotective and anti-inflammatory effects of COX-2, these findings suggest that specific COX-1 inhibitors are more likely to be of benefit in Alzheimer's disease. Consistent with this hypothesis, indomethacin, which has greater COX-1 activity than COX-2, did show some promise in a small clinical trial.[232] More recently, triflusal, a COX-1 selective inhibitor, reduced the rate of conversion of amnestic MCI to dementia in a placebo-controlled trial.[241] Aspirin is COX-1 selective; however, aspirin was not used at a sufficient anti-inflammatory dose in the only trial of its use in Alzheimer's disease.[242]

Clinical trials have generally been of short duration, nearly all under 6 months. This may not be long enough for a beneficial effect of NSAIDS to become apparent.

A direct anti-inflammatory effect of NSAIDS in the brain requires them to cross the blood-brain barrier. However, penetration of NSAIDS into the brain is generally low; levels in the CSF are only 1-2% of the plasma levels required for a therapeutic effect in humans.[223] Ibuprofen and indomethacin, which interestingly show the most evidence for benefit in epidemiological studies, are the most lipophilic NSAIDS and are therefore likely to cross the blood-brain barrier more easily than other NSAIDS. This poor penetration of NSAIDS across the blood-brain barrier may mean that NSAID doses used in trials have been too small to cause a therapeutic anti-inflammatory effect in the brain.

Animal studies revealed non-COX modes of action for NSAIDS with both anti-inflammatory (PPAR-γ, NF-κ β) and anti-amyloid (γ-secretase, amyloid aggregation, BACE-1) effects. The clinical significance of these alternative pathways is questioned by the finding that brain levels of NSAIDS from clinically feasible dosing in animals is sufficient to inhibit COX but too low to effect other potential targets (PPAR-γ, NF-κ β, γ-secretase).[229] Epidemiological studies show that NSAIDS may be beneficial in reducing the risk of other neurodegenerative diseases such as CJD and Parkinson's disease.[243 244] This beneficial effect in non-amyloid disease implies that the anti-inflammatory action of NSAIDS is more relevant in neurodegeneration than an action that directly reduces amyloid.

Although NSAIDS reduce microglial activation in animal models, this effect appears to become attenuated in older animals subject to chronic pro-inflammatory stimuli.[223] Microglia that have become fully activated by chronic stimulation in Alzheimer's disease may be less susceptible to the down-regulating effect of NSAIDS. This observation may explain the epidemiological finding that NSAIDS reduce the incidence of AD, but that once the disease is established there appears to be little beneficial effect on progression.

In summary, there are several explanations for the poor performance of NSAIDS in clinical trials: the doses of NSAIDS in clinical trials may have been insufficient as penetration across the blood-brain barrier is poor; the wrong class of NSAID has often been used (i.e. COX-2 inhibitors); NSAIDS may not have been given early enough in the course of the disease; and chronically activated microglia in older patients may become resistant to the anti-inflammatory effects of NSAIDS.

Systemic inflammation may act as a driver of CNS inflammation in Alzheimer's disease.[9] NSAIDS reduce systemic inflammation, so the failure to demonstrate any benefit in clinical trials presents a challenge to the systemic inflammatory hypothesis. However, NSAIDS may actually increase some pro-inflammatory cytokines in peripheral systemic inflammation. NSAIDS increase TNF-α production in most experimental models.[245] In healthy human volunteers pre-treatment with ibuprofen caused augmented levels of TNF-α and IL-6 in response to intravenous endotoxin.[246] This paradoxical increase in systemic TNF-α in response to NSAID treatment might worsen inflammatory neuro-degeneration. However, the detrimental effects are mitigated because TNF-α has some effects that are themselves mediated via arachidonic acid metabolites.[247]

NSAID dosage in normal clinical practice reduces inflammation at locally inflamed sites, such as affected joints in rheumatoid arthritis, but has little effect on systemic inflammation and circulating cytokines.[248] Mortality in rheumatoid arthritis is 1.5-1.6 times higher than in the general population and much of this excess mortality is attributed to cardiovascular disease.[249] There is growing interest in the hypothesis that increased systemic inflammation increases the risk of coronary artery disease in rheumatoid arthritis.[250] The degree of inflammatory suppression associated with NSAID use appears to be enough to reduce localised joint inflammation, but not enough to reduce cardiovascular risk.[248] The same difficulty is likely to be the case in Alzheimer's disease; NSAIDS do not have a sufficient effect on systemic inflammation to reduce the on-going activation of primed microglia in the brain.

2.4.6 Corticosteroids

Corticosteroid drugs have potent and wide-ranging anti-inflammatory effects, and are used for the treatment of many inflammatory diseases. However, in a large epidemiological study of 7234 participants in 3 French cities, use of inhaled or oral corticosteroids did not reduce cognitive decline or reduce the incidence of dementia over 7 years of follow-up.[251] Moreover, inhaled corticosteroid use was associated with significant cognitive decline in women in the cohort, after accounting for potential confounders. Furthermore, a randomized clinical trial of corticosteroids for patients with Alzheimer's disease failed to show any beneficial cognitive effects and, unfortunately,

showed some decline in behavioural symptoms.[252] These negative findings present a difficulty for a hypothesis that places such importance on the role of systemic inflammation in the symptoms and progression of Alzheimer's disease.

However, several factors may explain the lack of efficacy of corticosteroid treatment in AD. First, glucocorticoid receptors are widely expressed within the CNS, and glucocorticoid receptor activation is associated with hippocampal toxicity.[253] Second, glucocorticoids inhibit the synthesis of protective neurotrophic factors, and may therefore prevent repair to damaged neural networks.[254] Third, chronic exposure to glucocorticoids increases microglial cell proliferation in the brain,[255] and primes the microglial response to a subsequent proinflammatory challenge.[256] Fourth, glucocorticoids may increase the expression of Aβ and of the amyloidogenic BACE enzyme, increase Aβ deposition, and decrease amyloid degradation.[257] Fifth, particularly in the context of CNS inflammation, glucocorticoids may have pro-inflammatory, rather than anti-inflammatory effects, including increased expression of pro-inflammatory transcription factors and increased expression of cytokines within the CNS.[258 259] Lastly, chronic neuroinflammation, stress and depression may lead to relative glucocorticoid resistance, and decreased glucocorticoid-mediated anti-inflammatory effects, in Alzheimer's disease, possibly because of down-regulation of glucocorticoid receptors by systemic cytokines.[260-262]

2.4.7 Amyloid vaccines

The amyloid hypothesis for the pathogenesis of Alzheimer's disease suggests that amyloid deposition in the brain is an initiating step, with subsequent inflammation, tau hyper-phosphorylation and eventual neurodegeneration. It follows that removal of amyloid and prevention of further amyloid deposition should be beneficial. The harmful cerebral inflammatory response in patients with AD may represent an inefficient attempt by the immune system to remove amyloid from the brain. Neurodegeneration could be reduced if the immune response to amyloid could be manipulated to maximize amyloid clearance and minimize excessive, harmful inflammation. Schenk and colleagues developed an anti-amyloid vaccine to test the hypothesis that immune-mediated removal of amyloid from the brain would reduce neurodegeneration. Vaccination of transgenic mice that over-expressed human APP with $A\beta_{1-42}$ reduced Aβ

deposition and reduced behavioural impairments.[263] The mice produced high titres of antibodies directed against Aβ following vaccination. In a separate experiment passive immunization with monoclonal antibodies to Aβ similarly reduced cerebral amyloid deposits implying that the beneficial effects of the vaccine were due to the generation of Aβ-specific antibodies.[264]

Aβ-specific antibodies could reduce cerebral amyloid load in several ways. Opsonization of amyloid deposits by specific IgG antibodies allows phagocytosis by microglia.[264] Anti-amyloid antibodies may directly bind to and dissolve amyloid deposits,[265] with the resulting soluble oligomers being removed via the blood stream.[266] Binding and sequestration of soluble amyloid species in the blood may provide a "peripheral sink" that draws soluble amyloid out of the brain parenchyma.[267] Antibodies may prevent the toxic effects of soluble oligomers by binding them and preventing toxic interactions.[268]

The success of the initial mouse vaccine studies led to human trials of AN1792, an anti-amyloid vaccine, in 2001. Eighty patients were enrolled in the initial phase 1 trial. 53% of the immunized participants developed detectable antibodies to Aβ.[269] The vaccine was altered slightly by the addition of the emulsifier polysorbate 80 and the subsequent larger phase 2 trial enrolled 372 patients.[270] This trial was halted after 18 out of 298 (6%) immunized patients developed symptoms of meningo-encephalitis.[271] Post mortem examination of the vaccine-treated patients revealed extensive plaque clearance from the cerebral cortex.[272 273] Microglia contained Aβ particles, implying phagocytosis as the method of clearance.[273] The degree of plaque removal was associated with mean antibody response.[78]

Although post mortem examination of AN1792-treated patients showed sustained and significant reductions in amyloid deposits within the brain, there were no beneficial therapeutic effects. Long term clinical follow-up and post mortem neuropathological examination of patients from the original phase 1 trial was reported in 2008.[78] Even in immunized patients with almost complete plaque removal there was no difference in the severity of dementia at death. Vaccination did not reduce the time until severe dementia (Hazard Ratio: 1.18, 95% CI: 0.45 to 3.11; P=0.73) and there was no evidence of improved survival (Hazard Ratio: 0.93, 95% CI: 0.43 to 3.11; P=0.86).[78]

An analysis in a small subset of the trial patients (n=30), all of whom had the active vaccine, compared patients who responded to the vaccine by producing stable antibody levels with those who did not produce detectable antibody titres. They found that antibody responders had a significantly slower rate of cognitive decline over 12 months.[274] Patients who failed to respond to the vaccine with a good antibody response may have instead had a more damaging cytotoxic inflammatory response. The apparent benefit in antibody responders may have been due to deterioration in the non-responders. Comparison with a placebo group would have allowed this hypothesis to be examined but no placebo group was included in this early analysis of the AN1792 trial. In the full analysis of the trial data, including the placebo group, there was no therapeutic effect on cognitive decline.[270]

Although the effect of the vaccine on amyloid deposits was clear, the effect on tau pathology was less obvious. Dystrophic neurites are one of the features of tau pathology and were reduced in vaccinated patients.[273] However, no effect was observed on neurofibrillary tangles, the principal neuropathological sign of hyper-phosphorylated tau. A possible explanation is that vaccination in patients took place too far into their disease, after amyloid has already triggered irreversible tau pathology. The mouse models used in the majority of vaccine studies lack tau pathology and have therefore been uninformative, but a recently developed transgenic mouse model that does show hyper-phosphorylated and aggregated tau has now been used to examine the effects of a vaccine on tau pathology.[275] The vaccinated mice demonstrated a 65-85% reduction in Aβ and a 50-60% reduction in hyper-phosphorylated tau.[275] Neuronal loss and cognitive deficits were partially reduced. Vaccination occurred at 12 months, after cognitive decline and neurodegeneration are already apparent in the mice. This work supports the view that a vaccine could have a beneficial effect on tau pathology in humans if the vaccine were given early enough in the disease, and that a beneficial effect is possible even after neurodegeneration has begun. However, micro-haemorrhage was seen in 100% of vaccinated animals in this model, raising concerns about unwanted vascular and inflammatory side effects.

Antibody production by B-cell lymphocytes in response to vaccination is facilitated by Th2 helper T-cells. Animal models show that vaccination with $A\beta_{1-42}$ produces a Th2 response that encourages B-cells to produce anti-Aβ

antibodies.[276] However, post mortem examination of the patients enrolled in the AN1792 trial showed that some patients had evidence of a pro-inflammatory Th1 T-cell reaction around some cerebral blood vessels.[272 273] The T-cell response to the vaccine in humans was further examined in experiments using peripheral blood mononuclear cells (PBMCs) taken from immunized patients.[277] PBMCs from many participants produced IL-2 and IFN-γ in response to challenge with Aβ, indicating a Th1 response. This excessive Th1-mediated response in some patients, possibly associated with the choice of adjuvant in the AN1792 trial, may have been the cause of the severe meningo-encephalitis seen in some patients.[278]

The failure of the AN1792 vaccine has led to further work in mouse models with re-designed vaccines. These vaccines have been designed to reduce the risk of stimulating Th1 lymphocytes and to encourage a purely humoral immune response mediated by Th2 cells. $A\beta_{1-42}$ is thought to have one major antibody binding site at the N-terminus and two major T-cell epitopes located at the central and C-terminal hydrophobic regions.[268] Newer vaccines therefore consist of short Aβ species containing the N-terminus region of $A\beta_{1-42}$ with the T-cell epitopes either altered or deleted altogether.[279] Other groups are working on vaccines using adjuvants less likely to elicit a cytotoxic Th1 response. A predominantly humoral antibody response can also be achieved by altering the route of administration of a vaccine, for example mucosal or transdermal.[11 280-282] DNA vaccines, which allow precise control of the immune reaction to the vaccine and have the advantage of not requiring adjuvants with unpredictable immune consequences, are also in development in animal models.[283] A DNA vaccine containing 3 copies of the Aβ B-cell epitope, a chemokine (CCL22) and a Th2-cell epitope to help drive a Th2 response reduces AD pathology in a transgenic mouse model.[284]

Even where the primary response to vaccination is antibody production rather than Th1-mediated inflammation there may be less desirable inflammatory consequences. In a microglial cell culture model opsonization of amyloid with anti-Aβ IgG increased microglial chemotaxis and phagocytosis of the Aβ.[285] However, the phagocytosis was also associated with secretion of TNFα and IL-6 from microglial cells. These pro-inflammatory cytokines are thought to be unhelpful with regard to damaging neuro-inflammation and generation of tau pathology. Interestingly, the authors found that the NSAID indomethacin

reduced TNF-α and IL-6 production in this model, without impairing Aβ clearance by the microglial cells.[285] It has therefore been suggested that NSAIDs might be a useful adjunct in clinical trials of Aβ vaccines.[286]

Passive immunization, using monoclonal antibodies to Aβ, may reduce the risk of a Th1 cell-mediated inflammatory response. Animal models using this approach have demonstrated similar effects on amyloid burden as with antigenic vaccination.[264] Despite disadvantages of this method including high cost, poor blood-brain-barrier penetration, and possible antibody cross-reactivity, a major research effort was undertaken to evaluate the potential benefit of passive immunotherapy with monoclonal antibodies raised against Aβ.[268] The most fully studied monoclonal anti-Aβ antibodies are bapineuzumab (Janssen-Pfizer) and solanezumab (Eli Lilly & Co.).

Bapineuzumab is a humanized, monoclonal antibody raised against the N-terminal region of Aβ. In mouse models, the murine form of the antibody bound fibrillar and soluble forms of amyloid, activated microglial phagocytosis of amyloid, and reduced amyloid burden in the brain.[264] A phase 2 trial of bapineuzumab had disappointing results.[287] 234 patients were enrolled in the study and treated with infusions over 18 months. No significant differences were found in the primary efficacy end-points of cognitive function and disability. A sub-group analysis showed some possible benefit in ApoE e4 non-carriers. 12/124 (10%) of the antibody-treated subjects had reversible vasogenic oedema on routine MRI imaging, particularly in ApoE e4 carriers. Despite these discouraging results, ambitious phase 3 trials were started in patients with mild to moderate Alzheimer's disease, with separate trials in ApoE e4 carriers and non-carriers.

Phase 3 trials of bapineuzumab in ApoE e4 carriers (n=1090) and in non-carriers (n=1114) were completed.[288] Participants were randomized to receive either 18 months of intravenous bapineuzumab or placebo, with infusions every 13 weeks. The results showed no improvement in any clinical outcomes in either e4-carriers or non-carriers. There was mixed evidence that bapineuzumab was engaging amyloid in the brain and effecting down-stream neurodegeneration. Specifically, in ApoE e4 carriers there was reduced Aβ on PiB-PET scanning, but this was not evident in non-carriers. Additionally, CSF levels of tau, a marker of neurodegeneration, were reduced both in e4-carriers

and in non-carriers, but only at higher doses of bapineuzumab.[288] Of note, 36% of ApoE e4 non-carriers had negative scans for amyloid at baseline, raising doubt about the diagnostic criteria used within the trials to recruit people with Alzheimer's disease.

In the phase 3 trials, bapineuzumab increased the incidence of MRI abnormalities within the brain. Patients on bapineuzumab had increased amyloid-related imaging abnormalities (ARIA) on MRI, a term describing intra-cerebral vasogenic oedema or micro-haemorrhages, with these abnormalities increasing with bapineuzumab dose and with the number of e4 alleles.

The negative results led to the premature closure of two further phase 3 bapineuzumab trials. However, data from these early-closure studies are now available.[289 290] The trials examined e4-carriers (n=683) and non-carriers (n=329) and found no benefit of bapineuzumab on cognitive and functional outcome measures.[289] In contrast to the completed trials, there was no evidence of reduced Aβ on PiB-PET scanning, and no evidence of reduced CSF tau, either in the e4-carriers or in the non-carriers.[289] Prior to their early closure, open-label extension studies were carried out on participants within these trials that had completed 18 months of initial treatment (e4-carriers: n=492; non-carriers: n=202). The results of these extension studies, with up to 3 years of treatment, confirm the lack of any clinical benefit for bapineuzumab.[290] The incidence of ARIA on MRI was 11% (76/694) in the group originally assigned placebo and going on to bapineuzumab during the open-label phase, compared with 4% (28/694) in the group originally assigned bapineuzumab and remaining on the drug during the open-label phase, suggesting that the side effects of intra-cerebral vasogenic oedema and micro-haemorrhage become more common as Alzheimer's disease progresses. Overall, the results for bapineuzumab are negative, with no evidence of clinical benefit, and mixed evidence of effects on brain amyloid burden and neurodegeneration. Further development of bapineuzumab has been halted.

Solanezumab is a humanized monoclonal antibody raised against the mid-domain of Aβ, and selected for an ability to recognize soluble monomeric Aβ, rather than plaque-based fibrillar Aβ. Mouse models demonstrated reduced Aβ deposition in the brain and increased levels of Aβ in the CSF.[267 291] The researchers hypothesized that targeting soluble Aβ would increase the

clearance of soluble monomeric Aβ from the brain, thereby reducing the toxic effects of monomeric Aβ on synapses and neurons, without disrupting the Aβ safely sequestered in stable plaques.

Phase 2 studies of solanezumab showed no clinical benefit over 12 weeks of treatment, but did demonstrate an increase in CSF Aβ, consistent with reduced soluble Aβ in the brain.[292]

Two phase 3 trials were carried out, recruiting a combined study population of 2052 people with mild to moderate Alzheimer's disease, and randomizing participants to monthly infusions of solanezumab or placebo for 80 weeks. Levels of total (bound and unbound) Aβ were significantly increased in the CSF of patients treated with solanezumab in both phase 3 studies, consistent with removal of soluble Aβ species from the brain. However, the trials found no benefit of solanezumab treatment in the cognitive and functional primary end-points.[293] One of the phase 3 trials (EXPEDITION 1) showed a small improvement in the rate of cognitive decline in a sub-group analysis of patients with mild Alzheimer's disease treated with solanezumab. However, this was not borne out in the other phase 3 trial (EXPEDITION 2) which used treatment effect in mild AD as a primary end-point.[293] There were no significant treatment effects on CSF tau, or on the rate of hippocampal atrophy on volumetric MRI. There was no change in amyloid plaque burden measured by amyloid PET scanning (^{18}F-florbetapir PET).

In a pooled sub-group analysis of the patients with mild Alzheimer's disease from both studies, there was evidence of small improvements in the rate of cognitive and functional decline; however, the size of the treatment effect, after 80 weeks of treatment, was very small; less than one point on the MMSE (adjusted mean difference: 0.93, P=0.001); less than two points on the ADAS-Cog (adjusted mean difference: 1.7, P=0.001); and just over one point on the ADCS-ADL (adjusted mean difference: 1.4, P=0.057).[294] The size of these effects brings into doubt the clinical significance of the findings.

Solanezumab was safe and well tolerated in the phase 3 trials.[293] In particular, there was no greater risk of ARIA on MRI in the pooled trial treatment groups (ARIA in solanezumab group: 5.8%, ARIA in placebo group: 6.0%). This result supports the view that higher rates of ARIA occur with antibody engagement of

fibrillar, plaque-based Aβ by bapineuzumab, and are not a concern for antibody engagement of soluble Aβ by solanezumab.

Overall, solanezumab appears safe but there is little evidence of any clinically significant benefit from treatment in mild to moderate Alzheimer's disease. Nevertheless, on-going studies are assessing the effect of solanezumab in different patient groups. One study will further examine patients with mild Alzheimer's disease and biomarkers of CNS amyloid by CSF sampling or PET scanning (EXPEDITION 3). Other studies are assessing solanezumab in asymptomatic people that have biomarkers of CNS amyloid, and in asymptomatic people with dominantly-inherited familial Alzheimer's disease.

Amyloid vaccines are designed to break up amyloid plaques, or to reduce amyloid deposition in the brain, and it is hoped that this will lead to reduced neurodegeneration. Despite the promising results in animal studies human trials of both active and passive immunisation have not demonstrated convincing clinical results. Failure of these studies may be due to a number of factors. Once dementia is diagnosed neurodegeneration in Alzheimer's disease is already well established. Interventions that reduce amyloid burden at this stage may be too late to significantly improve the disease. Breaking plaques up into soluble amyloid species may be more harmful than beneficial. Soluble amyloid oligomers are potentially more neurotoxic than amyloid sequestered in plaques. Increased cerebral amyloid angiopathy and consequent microhaemorrhages have been observed in post mortem examination of subjects from the AN1792 trial.[295] This finding may be explained by a movement of amyloid from plaques to cerebral blood vessels in vaccinated patients, with a failure of adequate perivascular drainage.[296] The side effects of meningo-encephalitis in the AN1792 trial, and of vasogenic oedema in the bapineuzumab trial, demonstrate the potential harm of unintended activation of pro-inflammatory pathways by vaccination.

Inflammatory pathways in the brain are very tightly regulated, and our understanding of these pathways remains limited. It should be no surprise that a therapeutic strategy that attempts to subvert immune processes in the brain produces unexpected inflammatory side effects. A future therapeutic vaccine for Alzheimer's disease will require precise control of attendant inflammation

in the brain, and delivery to patients with Alzheimer's disease before dementia and neurodegeneration becomes clinically apparent.

2.4.8 Intravenous immunoglobulin

Intravenous immunoglobulin (IV-IgG) is a therapeutic preparation of polyclonal human antibodies derived from the donated plasma of thousands of carefully selected healthy volunteers and rigorously treated to reduce the risk of transmissible disease. IV-IgG is derived from a wide population of donors and contains the majority of immunoglobulin-G (IgG) subtypes found in the human immune system. IV-IgG is used to replace IgG in deficiency syndromes and as an anti-inflammatory agent in B-cell mediated inflammatory diseases, such as chronic idiopathic demyelinating polyneuropathy and Guillain-Barre syndrome. The discovery that IV-IgG contains naturally occurring antibodies to various forms of monomeric and oligomeric Aβ raised the prospect of using IV-IgG as a potential anti-inflammatory treatment for Alzheimer's disease.[297] Epidemiological evidence supports the use of IV-IgG in Alzheimer's disease, with one study demonstrating a 42% risk reduction in Alzheimer's disease in patients treated with IV-IgG for other indications.[298]

Phase 1 and phase 2 clinical studies in patients with Alzheimer's disease showed that IV-IgG was safe and well tolerated, and provided some evidence of possible efficacy on cognitive measures.[299 300] However, in a larger phase 3 study in 390 patients over 18 months, IV-IgG showed no benefit on any primary or secondary end-points, including change on the ADAS-Cog, change in atrophy on volumetric MRI, and change in amyloid burden on amyloid-PET scanning.[299 301] In a sub-group analysis of ApoE e4 carriers with moderate severity Alzheimer's disease there was some evidence of reduced cognitive decline in the treatment group. The trial had a negative outcome despite evidence that IV-IgG was engaging target Aβ in the CNS. Treated patient had increased levels of IgG in the CSF, indicating IV-IgG penetration into the CNS; and treated patients had altered plasma Aβ levels, indicating an interaction between IV-IgG and Aβ.

The negative results of the clinical trials of IV-IgG in Alzheimer's disease raise questions about the rationale and methodology employed in these studies. The doses of IV-IgG used in the trials to date have been based on the doses used to

replace IgG in deficiency syndromes (doses up to 400mg/kg), and have been insufficient to produce a robust anti-inflammatory response (doses >1000mg/kg). Thus, failure of the trials to show any benefit for IV-IgG may be the result of failure to trigger anti-inflammatory processes. IV-IgG may have been given too late in the progression of Alzheimer's disease, after a point where binding and reducing Aβ can make any significant difference to disease progression. An on-going study of IV-IgG in people with mild cognitive impairment (MCI) may clarify whether the timing of IV-IgG treatment in the pathogenesis of Alzheimer's disease affects the efficacy of the treatment. Finally, IV-IgG contains antibodies predominantly against oligomeric and fibrillar Aβ,[299] and these antibodies may be less effective at reducing amyloid toxicity than antibodies against soluble monomeric Aβ.

2.4.9 Vitamin E

Vitamin E compounds comprise a group of 8 naturally-occurring, fat-soluble, dietary compounds related to tocopherol. Vitamin E acts as a physiological anti-oxidant by preferential interaction with toxic free radicals. The fat-soluble nature of Vitamin E means make it particularly active as an anti-oxidant within lipid cell membranes. These anti-oxidant effects within cell membranes make Vitamin E an attractive potential anti-inflammatory treatment for Alzheimer's disease. In transgenic mouse models of Alzheimer's disease, Vitamin E had beneficial effects on neurodegeneration and cognitive function.[302] However, epidemiological evidence was mixed; Vitamin E levels were inversely associated with cognitive decline in some studies, but not all. Vitamin E levels tend to decrease as dementia progresses, alongside many other vitamins and nutrients, and this decrease is likely to be a consequence, rather than a cause, of the behavioural change associated with worsening dementia.[302]

An early trial of Vitamin E demonstrated potential benefit, with a reduction in a combined end-point of change in global cognitive and functional status and institutionalization in 77 patients treated over 2 years with 2000 IU/day of alpha-tocopherol.[303] The results were corrected for a baseline difference in MMSE score between the Vitamin E and the placebo groups, with the Vitamin E group having a lower median MMSE score at baseline. A further study examined treatment with 800 IU/day in 57 patients over 6 months.[304] This study found no evidence of benefit on an end-point measure of change in

MMSE, despite evidence of improvement in oxidative stress markers.[304] Concerningly, cognition worsened in the treatment group patients that did not show improvement in oxidative stress markers. A larger study examined the effect of 2000 IU/day alpha-tocopherol over 3 years of treatment in 769 people with mild cognitive impairment (MCI).[305] This study found no evidence of any effect on the rate of progression from MCI to Alzheimer's disease.

Overall, the results of clinical trials of Vitamin E as an anti-inflammatory treatment for cognitive impairment and Alzheimer's disease have been negative. Vitamin E may not be an entirely benign treatment; higher doses are associated with increased symptoms of cardiac disease and with increased all cause mortality.[302] As described above, the study by Lloret et al. showed some evidence of cognitive decline in patients where treatment did not alter oxidative stress markers.[304] The studies so far have used alpha-tocopherol alone; however, there is evidence that neuroprotection may result from interaction between the 8 various forms of Vitamin E, and therefore treatment may be more effective if comprised of a combination of various forms of Vitamin E, rather than one form alone.[306]

2.4.10 TNF-α blockade

TNF-α is a pro-inflammatory cytokine that has been implicated in the progression of neuro-inflammation in Alzheimer's disease.[9] High TNF-α in the peripheral blood of older people is associated with an increased risk of Alzheimer's disease.[307] Treatment with drugs that reduce systemic TNF-α in patients with rheumatoid arthritis is associated with a marked reduction in the incidence of Alzheimer's disease.[103] Plasma levels of TNF-α are higher in patients with Alzheimer's disease.[308] Cerebral inflammation may progress in Alzheimer's disease because microglial cells that are stimulated by Aβ produce TNFα.[309 310] Genetic polymorphisms for TNF-α are associated with increased risk of Alzheimer's disease.[129] This risk is amplified if other pro-inflammatory polymorphisms are also present, for example IL-6 and IL-10.[126]

Work in animal models has lent support to the hypothesis that reducing TNF-α levels will reduce neurodegeneration in Alzheimer's disease. In a mouse model intra-cerebral injection of Aβ increased mRNA expression of TNF-α and inducible nitric oxide synthase (iNOS) in the hippocampus.[311] Intra-cerebral

injection of an anti-TNF-α antibody prevented the nitration of proteins in the hippocampus and the impairment of recognition memory induced by Aβ in this model. Chronic systemic inflammation accelerates Alzheimer's-like pathology in the 3xTgAD Alzheimer's disease mouse model.[312] Chronic inhibition of soluble TNF signaling, using specifically engineered antibodies delivered intra-cerebrally, prevented amyloid-associated neuropathology in 3xTgAD mice and reduced the deposition of intra-neuronal amyloid species.[313] In an APP/PS1 transgenic mouse model of Alzheimer's disease, intra-cerebral treatment with infliximab, an anti-TNF fusion protein, reduced amyloid plaque deposition, decreased tau phosphorylation and decreased brain TNF-α.[314]

The use of peripheral anti-TNF treatment is also effective in an animal model. Intra-peritoneal injection of an anti-TNF antibody for up to 30 weeks reduced abnormal behaviour in the PDAPP mouse model of Alzheimer's disease where amyloid precursor protein (APP) is over-expressed.[315] In a separate experiment the same group found that TNF-α (-/-) knock-out mice crossed with PDAPP mice did not have the behavioural deficits seen in TNF-intact PDAPP mice, demonstrating that TNF-α is necessary for the amyloid-induced pathology in this model. Etanercept is a fusion protein that links 2 identical TNF-receptor domains with the Fc portion of IgG, and acts to block the action of TNF-α. In a non-transgenic model of amyloid-induced cognitive deficit, etanercept rescues Aβ-induced cognitive decline and normalizes TNF-α in the hippocampus.[316] An oral medication that blocks TNF-α improved cognition and reduce tau and amyloid pathology in 3xTgAD mice.[317] In summary, there is evidence that TNF blockers given peripherally and centrally reduce symptoms and pathology in animal models of Alzheimer's disease.

There has only been exploratory use of anti-TNF recombinant proteins in human subjects. In an exploratory study, published as a conference poster, 9 patients with Alzheimer's disease received etanercept for 24 weeks.[318] The drug was well tolerated but the study was too small to reveal any clinical benefit, and there was only a non-significant trend towards lower plasma TNF-α levels from baseline. A recent case report described a patient with Alzheimer's disease that was treated with peripheral etanercept for concomitant rheumatoid arthritis, in whom cognitive scores stabilized or improved over six months of treatment.[319]

Etanercept has also been given as a peri-spinal injection to a small number of patients with Alzheimer's disease.[320 321] Cognitive scores improved after administration of etanercept, but the validity of the case report is questionable. No control group was used in this open-label study, the number of subjects was low (n=15) and the timescale of improvement (minutes to hours after a single injection) is inconsistent with changes in the genetic expression of neuro-inflammatory mediators. An independent group have reported similar positive results in a single case report describing the use of a different anti-TNF agent, infliximab, given as an intra-thecal infusion at the time of lumbar puncture.[322] In this case, the authors report rapid improvement in cognitive test scores after one dose of intra-thecal infliximab. Compared to CSF levels taken before administration of infliximab, there were increased levels of CSF $A\beta_{1-42}$ and p-tau but no change in CSF $A\beta_{1-40}$ seven days after administration of the drug.[322] However, there was no placebo control in this case report and so the results require caution – the CSF change from baseline may simply be the result of chance fluctuation in CSF markers of Alzheimer's disease rather than due to the infliximab infusion.

2.4.11 Thalidomide

Thalidomide was first used in the late 1950s as an anti-emetic. It has terrible teratogenic effects and its use is now strictly controlled. There has been renewed interest in the powerful anti-inflammatory effects of thalidomide in recent years and it is now used to treat inflammation in specific conditions such as multiple myeloma and erythema nodosum leprosum. One action of thalidomide is to decrease the stability of TNF-α mRNA.[311] As discussed, TNF-α is up-regulated in models of Alzheimer's disease and plays a role is inflammatory neurodegeneration.[9] Thalidomide has therefore been used in animal models to assess the effect of reduced TNF-α on neurodegeneration.

Intra-cerebral injection of Aβ causes up-regulation of mRNA for TNF-α and inducible nitric oxide synthase (iNOS), and subsequent neurodgeneration and behavioural change in mice.[311] Thalidomide treatment (20mg/kg) reduced TNF-α and prevented neurodegeneration. In this model Aβ-induced neurodegeneration is not seen in TNF-α (-/-) knock-out mice, supporting the hypothesis that neurodegeneration is dependent on TNF-α. Thalidomide was

neuroprotective in an animal model of the inflamed Alzheimer's disease brain, with reduced gliosis and vascular pathology and reduced levels of TNF-α.[323]

There is only one small pilot study (n=12) of thalidomide in patients with Alzheimer's disease.[324] The trial was too small to show any change in behaviour or cognition, although there was a non-significant trend towards lower serum levels of TNF-α. Thalidomide reduces the activity of TNF-α and this may be neuroprotective in Alzheimer's disease, but this hypothesis has not been properly tested in patients.

2.4.12 Statins

There has been considerable interest in statin therapy as a potential preventative or symptomatic treatment for Alzheimer's disease. Cholesterol modulates amyloid precursor protein (APP) processing in cell culture and animal models and has been implicated in the production of Aβ.[325] The increased risk of Alzheimer's disease associated with the ApoE e4 allele may be due, in part, to the interaction of ApoE and cholesterol, as the lipidation state of ApoE influences both the metabolism of Aβ and the ability of Aβ to form pathological amyloid fibrils.[326] Statins also have anti-inflammatory modes of action.[327] Researchers became interested in the possibility of using statins for the treatment and prevention of Alzheimer's disease because of a potential double-whammy effect of reduced amyloid burden and reduced inflammation.

Statins have various effects on the immune system. These effects are independent of any effect on cholesterol metabolism. Statins reduce isoprenylation (the addition of lipid moieties to cellular proteins) and therefore change the ability of certain molecules to be localized at cell membrane sites.[325] This effect prevents G-proteins from triggering the production of inflammatory cytokines for example.[327] These anti-inflammatory effects have been examined in animal models. Statin therapy results in decreased lymphocyte trafficking from the periphery to the CNS.[328] Inflammation stimulated by Aβ is reduced by statin therapy.[327] However, there are conflicting reports about the effects of statins on some aspects of CNS inflammation. One group has found reduced expression of inflammatory markers, including TNFα and Nitric Oxide,[328] while another group has found evidence of activated

microglia and increased TNF-α expression.[329] Atorvastatin had no effect on plasma CRP in patients with Alzheimer's disease.[330]

Case-control studies indicated large reductions in Alzheimer's disease risk among statin users, but prospective studies have failed to find evidence of benefit. A Cochrane review of randomized, double-blind, placebo-controlled studies aimed at preventing Alzheimer's disease concluded that there was no significant risk reduction.[331] A meta-analysis of trials that used statins as a treatment for existing Alzheimer's disease found no evidence of benefit.[332] The earlier retrospective studies were prone to indication bias; people with the mild cognitive changes of un-diagnosed Alzheimer's disease are less likely to demand statin therapy, tolerate statin therapy or have statins prescribed.[333] Li and colleagues analyzed the data from a prospective prevention trial as if it were a retrospective case-control study in order to demonstrate the potentially misleading results of cross-sectional analysis. Although the results, when properly analyzed, showed no evidence of risk reduction, the cross-sectional analysis produced a large treatment effect.[334]

Statin therapy does not appear to be of any benefit in Alzheimer's disease. Statins may have too small an effect on Aβ burden to prevent inflammation in the brain.[335] The anti-inflammatory effect of statins may not be robust enough to prevent damaging inflammation. The pro-inflammatory signals in AD that trigger neurodegeneration may be too powerful for the mixed anti-inflammatory effects of statin therapy. Statins may simply have been given too late in the course of the disease to prevent on-going neuro-degeneration in the clinical trials that have been carried out so far. Cholesterol is necessary for synaptogenesis and neural homeostasis and so cholesterol reduction may have detrimental effects on neuronal survival during chronic neurodegenerative stress.[336] Statins differ in their lipophilic properties and therefore in their ability to penetrate across the blood brain barrier. This may affect the ability of some stains to prevent neuro-inflammation and may therefore contribute to their failure in clinical trials.

2.4.13 Other anti-inflammatory agents

There are several other anti-inflammatory agents that have potential for use in Alzheimer's disease. Unfortunately the promise of many pre-clinical studies is often not realized when researchers carry out clinical trials (Table 2.4.1). Of these potential drugs, PPAR-γ agonists and RAGE inhibitors have clear anti-inflammatory mechanisms that make their clinical use attractive. Clinical trials of these agents are now taking place.

Elements of the renin-angiotensin-aldosterone system have pro-inflammatory properties in the periphery and within the CNS.[337] Additionally, within the CNS, angiotensin may be involved in amyloid processing, cholinergic transmission and cerebrovascular pathology. Drugs that antagonize angiotensin are therefore attractive in Alzheimer's disease, with potential benefit arising from reduced vascular risk and better blood pressure control, in addition to possible direct effects on CNS inflammation, amyloid processing and neurotransmission.[338] Angiotensin receptor blockers are now the subject of clinical trials in Alzheimer's disease.

2.4.14 Conclusions

Many clinical studies have attempted to treat inflammation, or manipulate the immune system, in an attempt to treat Alzheimer's disease. However, the promise of epidemiological studies, that have repeatedly provided evidence of benefit for anti-inflammatory drugs, has not been borne out in randomized, blinded clinical trials. What conclusions can be drawn concerning the failure of these anti-inflammatory strategies? First, epidemiological studies and clinical trials differ in the way the study participants are included or excluded. Epidemiological studies take all-comers, including those with significant systemic inflammatory co-morbidities. Conversely, clinical trials often specifically exclude such subjects, in an effort to reduce the noise to signal ratio in trial results. Yet the "noise" of co-morbid systemic inflammation may be very significant. Clinical studies that exclude participants with inflammatory co-morbidities run the risk of excluding the very people whom anti-inflammatory drugs are most likely to benefit. Second, most anti-inflammatory strategies thus far employed have paid little regard to the close relationship between systemic inflammation and central inflammation described in Section

2.1 and Section 2.2 of this chapter. Most studies have aimed to manipulate the central innate immune response, and have ignored the role of systemic inflammatory signals. This approach may be problematic because in some cases attempts to subdue central immunity alone may be drowned out by the neglected peripheral inflammatory signal, and in other cases attempts to subdue central immunity may have counteracting effects in the periphery that mitigate any potential central benefit. Thus, a more successful anti-inflammatory strategy in Alzheimer's disease is likely to require broad inclusion criteria that do not exclude sources of peripheral inflammation, and will seek to reduce both systemic and central inflammation. An anti-inflammatory strategy that specifically aims to disrupt the activating signal from periphery to brain has not been tried, but such a trial is necessary to properly test the hypothesis that systemic inflammation exacerbates brain inflammation in Alzheimer's disease.

Table 2.4.1 Anti-inflammatory drugs in Alzheimer's disease

Potential mechanisms of action	Clinical studies
NSAIDS COX inhibition causing reduced prostaglandin synthesis In vitro/animal models: Effects on amyloid pathways[6] PPAR-γ activation[226]	**Prevention:** Case-control/cohort studies: Modest reduction in risk,[216 217] greater risk reduction in ApoE e4 carriers[222] Clinical prevention trials: Overall no significant risk reduction[234] **Treatment:** Overall no significant benefit[218] COX2 inhibitors: No benefit[102] Some evidence of ibuprofen benefit in ApoE e4 carriers[233]
PPAR-γ agonists Decreased microglial activation in mouse model[339] Reduced COX-2 and iNOS expression[340]	**Treatment:** Overall no significant benefit[341]
RAGE Inhibitors Reduced generation of reactive oxygen species in response to Aβ[342 343] Reduced Aβ load in a mouse model[344]	**Treatment:** Phase II Study (n=67): well tolerated, not powered to show treatment effect[345]
Statins Reduced Aβ-induced inflammation[327] Decreased lymphocyte traffic between CNS and periphery[328] Modulation of APP processing[325]	**Prevention:** Cochrane review of prevention trials revealed no significant risk reduction[331] **Treatment:** No evidence of benefit in meta-analysis of clinical treatment trials[332]
Thalidomide Reduced TNF-α levels and neurodegeneration in response to Aβ (mouse model)[311] Reduced reactive gliosis and inflammatory vascular pathology in response to Aβ (mouse model)[323]	**Treatment:** Phase II pilot study (n=12): Too small to show changes in behaviour or cognition[324]
TNF-α Antagonists Reduced CNS inflammation[313] Intra-cerebral injection: reduced amyloid-associated neuropathology in 3xTgAD mice[313] Peripheral injection: Reduced behavioural deficits in PDAPP mouse model[315]	**Treatment:** Peripheral injection: Phase II pilot study (n=9): Well tolerated, too small to show clinical benefit[318] Perispinal injection: Open-label, no control group, n=15: improved cognitive scores in some subjects[320]

Table 2.4.1 Continued

Potential mechanisms of action	Clinical studies
Omega-3 Fatty Acids Found in fish oils Reduced arachidonic acid metabolites Prevents oxidative damage in mouse model[346] Several other putative neuro-protective and anti-amyloid effects[347]	**Prevention:** Epidemiological evidence that low levels of Omega-3 increase risk of AD[347] **Treatment:** A preliminary study (n=35) found some improvement in Clinician's Interview-Based Impression of Change Scale (CIBIC-plus) over the 24 week follow-up (P=0.008) but no change in formal cognitive scores[348] A pilot study (n=39) found no change in oxidative markers, but reduced decline in MMSE and IADL after 12 months of Omega-3 plus α-lipoic acid[349]
Curcumin The yellow pigment in turmeric Possible effects on innate immunity resulting in less oxidative damage and increased amyloid clearance[350] Decreases LPS-stimulated IL-1β and iNOS in a mouse model of AD[351]	**Treatment:** Exploratory trial (n=30) showed no effect on cognition over 6 months[352] On-going trials
Sodium valproate Short chain branched fatty acid, commonly used as an anti-convulsant, with anti-inflammatory and neuro-protective effects[353] Decreases LPS-induced microglia activation[354], although chronic use causes increased microglia activation[355]	**Treatment:** Anecdotal use for agitation in advanced dementia, but no clear evidence of benefit, and one Canadian trial showed worsening[356] National Institute for Ageing (NIA) clinical trial is on-going (VALID study)
Antibiotics Mutliple studies showing evidence for possible role of infective agents in AD[357]	**Treatment:** A trial of doxycycline and rifampicin in AD patients showed improved cognitive scores, despite no effect on Chlamydia burden[358] A trial of Helicobacter pylori eradication showed improved cognitive scores at 2 years[359]
Cholinesterase inhibitors Commonly used symptomatic drugs in AD – may have anti-inflammatory modes of action in addition to effects on cholinergic neuro-transmission Decrease Aβ-induced microglia activation[360] Alpha-7 nicotinic receptor agonists prevent LPS-induced microglia activation[361]	**Treatment:** Good evidence of symptomatic benefit[362] Possibility that some of the efficacy is due to anti-inflammatory effects regulated by alpha-7 nicotinic receptors[361]

2.5 Conclusions

The evidence presented in this chapter demonstrates that systemic inflammation may exacerbate neurodegeneration and pathological neuroimmune sickness behaviour in people with Alzheimer's disease. Sex hormone status may be an important moderator of this interaction between systemic inflammation and neurodegeneration. The evidence suggests that reducing systemic inflammation in people with Alzheimer's disease should reduce neurodegeneration and pathological neuroimmune sickness behaviour. However, there have been mixed results in previous attempts to harness the immune system using anti-inflammatory drugs or anti-amyloid vaccines in the treatment of Alzheimer's disease. No single agent has been fully successful and the effects of treatments on pathological sickness behaviour remain unclear, because no validated scale exists for measuring neuroimmune sickness behaviour in Alzheimer's disease.

The evidence described in this chapter provides a background and rationale for the three studies that are presented in the remainder of this thesis.

First, I present a cross-sectional study that examines the hypothesis that in men with Alzheimer's disease, systemic inflammation is related in part to change in sex hormone levels (Chapter 4).

Second, I present a longitudinal study that examines the hypothesis that pathological neuroimmune sickness behaviour can be measured using an informant scale constructed to have good reliability and validity in people living with Alzheimer's disease (Chapter 5).

Third, I present a clinical trial that examines the hypothesis that reducing systemic inflammation with the TNF-blocker etanercept is safe, well-tolerated and effective at reducing symptoms and pathological neuroimmune sickness behaviour in Alzheimer's disease (Chapter 6).

Chapter 3: General overview of the research

3.0 General overview

The research studies presented in this thesis all explore an overarching hypothesis that systemic inflammation and factors that affect systemic inflammation influence CNS inflammation, with consequent effects on pathological neuroimmune sickness behaviour, neurodegeneration and cognitive decline. In Chapter 2 I discussed evidence that examines this general hypothesis. In this thesis I describe three studies which we carried out in order to further examine several hypotheses arising from this overall hypothesis. I will now describe the experimental approach used in the three research studies described in this thesis.

3.1 Male sex hormones and systemic inflammation in Alzheimer's disease

Sex hormone levels influence the incidence and progression of Alzheimer's disease in epidemiological and clinical studies, and there is a wealth of evidence from animal models suggesting that sex hormones influence neuroplasticity, neurodegeneration, amyloid processing in the CNS, neuroinflammation, and systemic inflammation, as discussed in Chapter 2. The first study presented in this thesis therefore examines the hypothesis that sex hormones are altered in AD and the hypothesis that sex hormones have a relationship with markers of systemic inflammation in AD.

We carried out a cross-sectional study to examine sex hormone levels in a cohort of men with AD in comparison to an age-matched cohort without AD. In the men with AD we looked for evidence of a relationship between sex hormone levels and serum TNF-α as a key marker of systemic inflammation.

3.2 Reliability and validity of a Sickness Behaviour Scale for Alzheimer's disease

Behavioural and psychological symptoms in Alzheimer's disease are a source of considerable distress. We hypothesize that many of these symptoms are a manifestation of pathological neuroimmune sickness behaviour; in which the unremitting CNS inflammation associated with Alzheimer's disease triggers chronic, maladaptive, pathological activation of normally short-lived, adaptive, physiological sickness behaviour. Normal adaptive sickness behaviour is thought to be a CNS response to increased systemic inflammation. We hypothesize that this normal connection between adaptive behavioural change in the CNS and systemic inflammation becomes pathological in AD; with perturbations in systemic inflammation causing maladaptive, pathological sickness behaviour in AD, because of excessive microglial responses in the degenerating, inflamed brain.

There is no validated tool for measuring neuroimmune sickness behaviour in AD. The second study presented in this thesis therefore examines the hypothesis that neuroimmune sickness behaviour in AD can be measured using an informant scale, and the hypothesis that neuroimmune sickness behaviour in AD is related to the degree of systemic inflammation.

We carried out a longitudinal cohort study in which we developed alternative versions of a Sickness Behaviour Scale and tested reliability and validity in a cohort of people with Alzheimer's disease, including assessment of the relationship between the scales and serum cytokines, as a measure of systemic inflammation. We tested discriminant validity by comparing scale performance in AD with cohorts that were cognitively normal or that had Lewy Body dementia. An independent validation cohort was then used to further assess the reliability and validity of the scales.

3.3 Safety and tolerability of etanercept in Alzheimer's disease – STEADI-09

The pathology of Alzheimer's disease is characterized by CNS inflammation, amyloid plaques and neurofibrillary tangles. Amyloid deposition in the brain appears to be an invariant feature of Alzheimer's disease. However, there is evidence that amyloid may be necessary but insufficient to cause Alzheimer's disease. Animal models and clinical studies suggest that systemic inflammation plays a crucial role in initiating the CNS inflammation associated with amyloid plaques and that the resulting CNS inflammation causes neurodegeneration and initiates Alzheimer's disease. Furthermore, systemic inflammation appears to continue to aggravate the CNS inflammation associated with on-going Alzheimer's disease, with evidence in animal models that acute systemic inflammatory episodes increase CNS inflammation; and evidence in clinical studies that acute systemic inflammatory episodes increase the rate of cognitive decline and worsen the behavioural and psychological symptoms of Alzheimer's disease.

TNF-α as a key inflammatory cytokine in systemic inflammation; with diverse functions that serve to initiate and coordinate the immune response to a multitude of threats. TNF-α blockade is therefore an attractive target for an attempt to reduce the effects of systemic inflammation in Alzheimer's disease. Drugs that block TNF-α are used widely for a variety of chronic inflammatory diseases and are safe and well tolerated in those conditions.

The third study presented in this thesis therefore examines the hypothesis that the anti-TNF drug etanercept is safe and well tolerated in Alzheimer's disease, and the hypothesis that etanercept treatment may reduce the progression of symptoms in Alzheimer's disease.

We carried out a randomized, placebo-controlled, double-blind, phase 2 study, with the primary aim of establishing the safety and tolerability of etanercept in Alzheimer's disease. As a secondary aim, we examined the efficacy of etanercept on cognitive, behavioural and functional outcomes, including the effect of etanercept treatment on the sickness behaviour scales which we had previously developed.

3.4 General methods

3.4.1 Cognitive scales

3.4.1.1 Alzheimer's Disease Assessment Scale: Cognitive Section (ADAS-Cog)

The Alzheimer's Disease Assessment Scale: Cognitive Section (ADAS-Cog)[363] is the most widely used scale to measure cognitive change in Alzheimer's disease clinical trials. The scale was designed to measure cognition across a broad range of cognitive domains in people with Alzheimer's disease. The scale has been well validated and is generally accepted as the standard measure of cognitive function for patients with mild to moderate Alzheimer's disease in therapeutic trials. The scale contains 11 items relating to memory, language, orientation and praxis. The total scale score ranges from 0 to 70, with *higher* scores indicating more severe cognitive impairment. The ADAS-Cog is administered by an assessor using standard test materials (for example, objects for naming tests, flash cards for word memory tasks). All assessors received standardized training provided at the Memory Assessment & Research Centre (MARC) in Southampton, to ensure uniform administration of the scale tests.

3.4.1.2 Mini-Mental State Examination (MMSE)

The Mini-Mental State Examination (MMSE) is a well-established measure of cognitive function in elderly people.[364] It shows good test-retest and inter-rater reliability and performs satisfactorily against more detailed measures of cognitive function.[364] The utility of the MMSE as a means of assessing treatment response in Alzheimer's disease has been questioned,[365] but the status of the MMSE as a primary clinical outcome measure has been strengthened by National Institute for Clinical Excellence (NICE) guidance on the management of Alzheimer's disease.[366] The standardized Mini Mental State Examination has been developed to improve the reliability of the original instrument and is the version used in the studies described in this thesis.[367] The MMSE total score ranges from 0 to 30, with *lower* scores indicating more severe cognitive impairment.

3.4.1.3 Clinical Global Impression of Change (CGIC)

The Clinical Global Impression of Change (CGIC) is commonly used as an assessment of overall change in Alzheimer's disease clinical trials.[368] The validity of this type of measure is based on the ability of an experienced clinician to detect clinically worthwhile change in a patient's overall clinical state. There are several different versions of these scales, which have introduced varying degrees of structure to the interview process and guidance for rating change. Whilst these modifications improve reliability they may undermine the construct validity of the scale; consequently, in this research we used an unstructured CGIC. The patient's initial level of illness severity was rated on a seven-point scale (no impairment– extremely impaired), then subsequent change from baseline was rated again on a seven-point scale (marked improvement – no change – marked decline), where 1 is marked improvement, and 7 is marked decline. Rating clinicians based their assessment of change on general interview with the patient and their study partner to assess cognitive, behavioural and functional change.

3.4.2 Behavioural and psychological scales

3.4.2.1 Cornell Scale for depression in dementia (Cornell)

The Cornell Scale for Depression in Dementia (Cornell) is a 19 item scale that was developed to assess the signs and symptoms of depression in people with dementia. The scale has good internal consistency and validity, and is widely used in dementia clinical research.[369] Information is gathered by asking questions in a semi-structured interview with an informant, with the informant asked to consider symptoms occurring during the last week. Each of the 19 scale items is scored 0 to 2 (0=no symptoms, 1=mild or intermittent, 2=severe). The Cornell total scale score is scored from 0 to 38, with *higher* scores indicating more severe depression. Scores above 18 indicate major depression; scores below 6 indicate the absence of significant depressive symptoms.[369] The developers of the Cornell scale produce written administration and scoring guidelines to help ensure uniform administration. All raters in the studies described in this thesis received training in the administration of the Cornell scale at the Memory Assessment & Research Centre (MARC) in Southampton.

3.4.2.2 Neuropsychiatric Inventory (NPI)

The Neuropsychiatric Inventory (NPI) assesses 12 non-cognitive behavioural and psychiatric domains in dementia: delusions, hallucinations, agitation, depression, anxiety, elation, apathy, disinhibition, lability, aberrant motor symptoms, sleep disturbance and appetite disturbance.[370 371] The scale is administered by conducting a semi-structured interview with an informant who has frequent contact with the patient. Informants are asked to consider symptoms within the last four weeks. In order to reduce administration time, a screening question is asked for each of the 12 symptoms domains and more detailed questioning only occurs if the screening question indicates the presence of a problem. Each of the 12 symptom domains is then scored for frequency on a scale from 0 to 4 (0=not present, 4=once or more per day), and scored for severity on a scale from 1 to 3 (1=mild, 2=moderate, 3=marked). The frequency and severity scores are multiplied to give a total for each item, and the 12 item scores are added to provide a total scale score, with a range 0 to 144, with *higher* scores indicating more severe symptoms. The total scale score is not an interval scale and is not normally distributed. This occurs because multiplying the frequency and severity scores for each item results in a scale that is constrained never to score certain values (there are no multiples of 5, 7 and 11) – limiting the use of parametric statistics in the analysis of the NPI.[372] Nevertheless, the NPI is used widely in clinical trials for dementia and there is evidence for good reliability and validity.[370]

3.4.3 Functional and physical health scales

3.4.3.1 Bristol Activities of Daily Living Scale (BADLS)

The Bristol Activities of Daily Living Scale was designed specifically for use in patients with dementia.[373] The scale assesses functional ability and asks an informant to rate a patient on independence with 20 daily-living abilities. The scale has good reliability and construct validity and has been used as a measure of functional ability in therapeutic trials in dementia patients.[374] Principal component analysis demonstrated four underlying domains assessed by the BADLS: instrumental activities of daily living, self-care, orientation to time and place, and mobility.[373] The total scale score ranges from 0 to 60, with

higher scores indicating more severe dependency (0=totally independent, 60=totally dependent).

3.4.3.2 Fried Frailty Score

The Fried Frailty Score was developed to assess physical frailty in older patients.[375] The Fried Frailty Score has been widely used to categorize physical frailty, including studies in patients with cognitive impairment.[376]

The scale consists of five items:

1. Un-intentional weight loss in the previous 12 months (Yes=frail)
2. Self-reported fatigue (Score: 0 to 3, ≥2=frail)
3. Estimate of usual levels of physical activity (Score: 0 to 5, ≥4=frail)
4. Usual walking speed (time in seconds over 4.6 metres)
5. Hand-grip strength (measured with a dynamometer, in kg)

Walking speed is measured using a stop-watch to time a walk between marks placed 4.6 metres (15 feet) apart. The participant walks at their usual pace and uses their own walking aid if needed. Two measurements are taken and the fastest time is used for scoring. Walking speed cut offs, corrected for gender and height, are used to score for the presence of physical frailty (for all: walking speed >7 seconds= frail; for men with height >173cm: walking speed >6 seconds=frail; for women with height >159cm: walking speed >6 seconds=frail).[375]

Hand-grip strength is measured using a Jamar dynamometer – a hand-held device for measuring hand-grip strength. Readings are taken with the participant seated, their elbow by their side and flexed at a right angle, with a neutral wrist position, and provision of support underneath the dynamometer. Three readings are taken for each hand, asking the participant to make their best effort, and the highest grip strength recorded in either hand is used for scoring. Grip-strength cut-offs, corrected for gender and Body Mass Index (BMI), are used to score for the presence of physical frailty (for men with BMI>28: grip strength<32kg=frail; for men with BMI=24 to 28: grip strength<30kg=frail; for men with BMI<24: grip strength<29kg=frail; for

women with BMI>29: grip strength<21kg =frail; for women with BMI=26 to 29: grip strength<18kg=frail; for women with BMI=23 to 26: grip strength<17.3kg=frail; for women with BMI<23: grip strength<17kg=frail).[375 377] The five scale items are each score 0=not frail, or 1=frail and then added to give a final total Fried Frailty Score of 0 to 5, where 5 indicates a high degree of physical frailty.

3.4.3.3 Systemic inflammatory events questionnaire

A simple questionnaire was used to collect information on the occurrence of systemic inflammatory events within the two weeks prior to each study visit (Table 3.4.1)

Table 3.4.1 Questionnaire to assess presence of systemic inflammatory events in prior two week period

Systemic inflammatory event	Indicate if present in past 2 weeks
Infections/inflammatory disease	
Upper respiratory	
Lower respiratory	
Gastrointestinal	
Genitourinary	
Dental	
Rheumatoid arthritis flare	
Other infection or inflammation	
Fever for any reason	
Trauma	
Soft tissue injury (including burns)	
Surgical intervention	
Myocardial infarction	
Other trauma	
CNS events	
Stroke	
Other inflammatory CNS event	
Other events	
Influenza vaccination	

3.4.4 Cytokine analysis

3.4.4.1 Blood sampling

Blood was taken using a vacutainer system with plain serum tubes. Where samples were taken on home visits, the sample tubes were transferred back to the Memory Assessment & Research Centre in Southampton in an ambient temperature storage container. The samples were then centrifuged at 3000 rpm for 15 minutes. Immediately after centrifugation, the supernatant was pipetted into 5 ml aliquot storage tubes and stored at -80°C.

3.4.4.2 Multiplex cytokine analysis

1. Sex hormone study

TNF-α levels were assayed by Elina Zotova, in the Clincial Neurosciences laboratory at the University of Southampton, using a sandwich multiplex cytokine immunoassay (Meso Scale Discovery (MSD), Gaithersburg, MD). A protocol provided by MSD for custom assays was used with no major modifications (Table 3.4.2).

2. The Sickness Behaviour Study

Serum samples were used for assays of IFN-γ, TNF-α, IL-6, IL-1, IL-10, IL-12 and IL-8 using a sandwich multiplex cytokine immunoassay (Meso Scale Discovery (MSD), Gaithersburg, MD). A protocol provided by MSD for custom assays was used with no major modifications (Table 3.4.2). Assays were carried out in the Clinical Neurosciences laboratory at the University of Southampton. I carried out baseline assays for the sickness behaviour scale study, with assistance from Elina Zotova. Follow-up assays were carried out by James Fuller, with assistance from me. A custom ELISA assay for CD40-ligand was carried out by James Fuller in the Clinical Neuroscience laboratory at the University of Southampton.

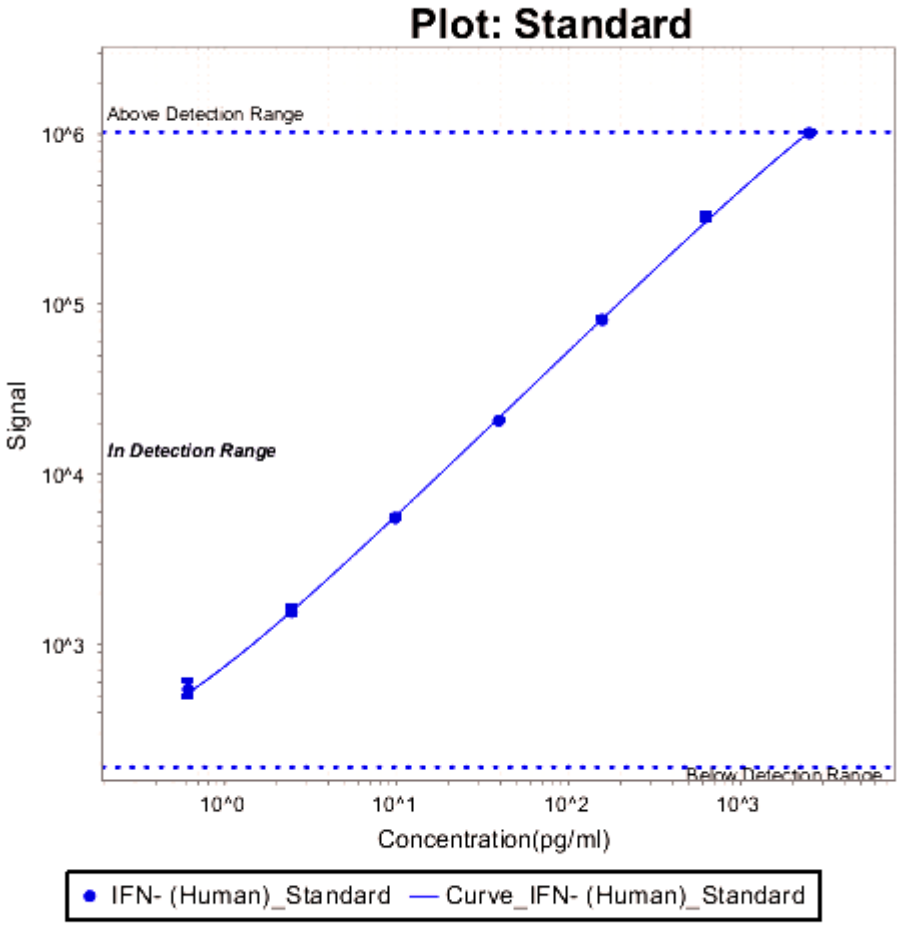
3. Safety and Tolerability of Etanercept in Alzheimer's disease (STEADI-09)

Serum samples were used for assays of IFN-γ, TNF-α, IL-6, CRP, IL-10, IL-12, IL-8 and MCP1 using a sandwich multiplex cytokine immunoassay (Meso Scale Discovery (MSD), Gaithersburg, MD). A protocol provided by MSD for custom assays was used with no major modifications (Table 3.4.2). Assays were carried out in the Clinical Neurosciences laboratory at the University of Southampton by Dr Ursula Puentener and Professor Jessica Teeling.

Table 3.4.2 Standard protocol for sandwich multiplex cytokine immunoassay (Meso Sclae Discovery)

MSD Protocol for multiplex cytokine assays
1. 25 µL of the provided assay diluent is dispensed into each well of a 96-well plate. An adhesive plate seal is used to seal the plate, which is then incubated for 30 minutes at room temperature with vigorous shaking on a plate-shaker (300–1000 rpm).
2. 25 µL of sample or calibrator is dispensed into separate wells of the MSD plate. Each sample or calibrator is dispensed twice, so that the mean of the paired samples can be calculated for improved reliability. The plate is then sealed with an adhesive plate seal and incubated for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
3. The plate is washed 3 times with PBS-Tween. Following the washes, 25 µL of the 1X Detection Antibody Solution is dispensed into each well of the MSD plate. The plate is sealed with an adhesive plate seal and then incubated for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
4. The plate is washed 3 times with PBS-Tween. Following the washes, 150 µL of 2X Read Buffer solution is added to each well of the MSD plate. The plate is then immediately analyzed on the MSD SECTOR Imager.
5. A standard plot is calculated for each cytokine using the signal generated by standard dilutions of the calibrator samples (Figure 3.4.1). The standard plot is used to calibrate the assay for each cytokine, and the average of the signal generated by paired samples is used to provide the cytokine levels for each sample.

Figure 3.4.1 A representative example of a standard plot for cytokine analysis using the MSD Sector imager. This plot is for IFN-γ in the baseline samples of participants in the Sickness Behaviour Scale study



3.4.5 ApoE genotyping

Whole blood was taken at baseline in the STEADI-09 study and stored at -80˚C. ApoE genotypes were determined by TaqMan genotyping of single nucleotide polymorphism (SNP) rs7412 and KASP genotyping of SNP re429358. Genotyping was carried out by Rachel Raybould and Rhodri Thomas in the MRC Centre for Neuropsychiatric Genetics and Genomics at Cardiff University.

3.4.6 Sex hormone assays

Serum samples were used for assays of total testosterone (TT), sex hormone binding globulin (SHBG), and luteinizing hormone (LH) by chemiluminescent immunoassay using a standard laboratory protocol (UniCel DxI 800, Beckman Coulter, Brea, CA). Assays were carried out by the staff of the clinical biochemistry laboratory at Southampton General Hospital. Bioavailable free testosterone (FT) was calculated using the Vermeulen formula.[378]

Chapter 4: Male sex hormones and systemic inflammation in Alzheimer's disease

4.0 Introduction

Sex hormones have been investigated as potential risk factors for Alzheimer's disease (AD), as discussed in Section 2.3 of this thesis. A number of studies show that men with AD have lower levels of testosterone than non-AD men.[170] [182] In addition, low testosterone levels make a diagnosis of AD more likely in longitudinal follow-up studies.[379] Testosterone may have direct neuro-protective effects via interactions with androgen receptors expressed on neurones and glial cells.[380] Furthermore, tissue aromatisation of testosterone to oestrogen may confer neuro-protection by oestrogen-dependent mechanisms.[192]

High luteinizing hormone (LH) levels are associated with poorer cognition in healthy community-dwelling men,[183] and higher levels of LH have also been postulated to play a role in the pathogenesis of AD.[14] However, although studies consistently find low levels of testosterone in men with AD, studies of LH levels are conflicting, with some groups finding elevated levels,[182] and others low levels.[170]

Recent evidence suggests that increased peripheral inflammation, and specifically increased TNF-α production, is a risk factor for the development of AD,[381] and for increased rate of cognitive decline in AD and non-AD subjects.[9] [382] Low testosterone levels are associated with increased peripheral inflammation in cross-sectional studies of aged men.[201] [202] Therefore, we hypothesize that male sex hormones may exert some of their influence on cognition in AD by modulating systemic TNF-α levels.

In this study we tested the hypothesis that sex hormone levels in men with AD differ from normal values and correlate with systemic TNF-α.

4.1 Male sex hormones and systemic inflammation in Alzheimer's disease: Methods

4.1.1 Design and setting

94 community-dwelling men with mild to severe AD living in Southampton, UK, were enrolled in a study examining the relationship between cognitive decline and peripheral cytokine levels.[9] Participants were recruited between November 2003 and May 2006 from clinical referrals to memory assessment services in Southampton, UK. After consent procedures, all subjects fulfilling National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria[383] for probable or possible AD had a blood sample taken for sex hormone and TNF-α analysis. Participants were further characterized by a full medical history, medication history, assessment of delirium and cognitive testing, as part of their involvement in the larger cytokine study.[9] Comparisons of androgen levels are made between the AD cohort we describe here, and a separate cohort of aged males, with no diagnosis of AD. Data on the cohort of non-AD men was obtained from a previously published study by Yeap et al.[384] Androgen levels are greatly affected by age, with normal values differing in different age groups. Therefore, we stratified the AD cohort into two age groups, <80 years, and ≥80 years. This cut point was chosen to allow comparison with normal, non-AD, age-specific androgen levels, derived from the Yeap study.[384] We did not have data on TNF-α levels in this non-AD cohort. Ethical approval for the study was obtained from the local NHS Research Ethics Committee.

4.1.2 Assays

Serum samples were immediately placed on ice and stored within 2 hours at -80˚C. Baseline serum samples were used for assays of total testosterone (TT), sex hormone binding globulin (SHBG) and LH by chemiluminescent immunoassay using a standard laboratory protocol (UniCel DxI 800, Beckman Coulter, Brea, CA). Bioavailable free testosterone (FT) was calculated using the Vermeulen formula.[378] Population values for FT and LH in men aged ≥80 and in men aged <80 were derived from a previously described cohort of 2938

community-dwelling men.[384] The same baseline serum samples used for sex hormone analysis were also used to assay TNF-α using a sandwich multiplex cytokine immunoassay (Meso Scale Discovery (MSD), Gaithersburg, MD). A protocol provided by MSD for custom assays was used with no major modifications, as described in the General Methods of this thesis (Section 3.4). The lowest detectable limit for TNF-α was 1.1 pg/mL. Severity of dementia was measured using the cognitive subsection of the Alzheimer's Disease Assessment Scale (ADAS-Cog), as described in Section 3.4 – General Mehtods.[363]

4.1.3 Statistical analysis

Normally distributed variables are summarized as mean±SD. Non-normally distributed variables are summarized as median and interquartile range. The study population was stratified by age (<80 or ≥80 years) for comparison with data on normal, non-AD, age-specific (<80 or ≥80 years) androgen levels, derived from a non-AD cohort.[384] One-sample t-tests were used to compare normally distributed variables with normal population means within a defined age range. Non-parametric data were compared to population medians using the Wilcoxon signed-rank test. Partial correlation statistics, with ranked data where necessary, were used to assess cross-sectional associations between hormone levels, TNF-α level, and age, using the STATA statistical package (StataCorp, version 11.0). We considered results significant at $P<0.05$.

4.2 Male sex hormones and systemic inflammation in Alzheimer's disease: Results

Of the 94 subjects, 58 were aged less than 80 years, and 36 were aged 80 years or older. Demographic characteristics, and ranges for sex hormones and TNF-α for the AD cohort are given in Table 4.1.1.

Free testosterone (FT) and luteinizing hormone (LH) levels differed between men with AD and the age-matched population cohorts (Table 4.1.2). FT levels were lower in men with AD (One-sample t-test for age<80 P=0.0002; age≥80 P<0.0001). LH levels were higher (Wilcoxon signed-rank test for age<80 P=0.0014; age≥80 P<0.0001).

Correlations between TNF-α, FT and LH are given Table 4.1.3. There was a significant positive correlation between LH and serum TNF-α levels in men with AD (Spearman's r=0.25, P=0.019) and this remained significant after correcting for age (partial r=0.21, P=0.05). LH in the highest quartile versus LH in the lowest quartile predicted TNF-α above the median in a logistic regression model, after adjusting for age, (OR 3.8, 95% CI 1.1 to 13.4, P=0.039, LR test for model: P=0.034, Hosmer-Lemeshow GOF test: χ^2 (2) = 0.04, P=0.98). There was no significant relationship between FT and serum TNF-α levels (Spearman's r=-0.03, P=0.8), or between FT and LH (Spearman's r=-0.17, P=0.11). Severity of dementia, as measured by the ADAS-Cog, was not related to FT, LH or TNF-α (Spearman's r=-0.11, P=0.29; r=0.03, P=0.80; r=0.04, P=0.74, respectively), and these correlations did not reach significance after adjusting for age.

Table 4.2.1 Characteristics of the Alzheimer's disease study population

		Total AD sample		Age group				P-value
		Age range: 59 to 98 (n = 94)		Age < 80 (n = 36)		Age ≥ 80 (n = 58)		
		n	Mean or %	n	Mean or %	n	Mean or %	
Age (SD)		94	81.2 (7.2)	36	74.3 (4.8)	58	85.5 (4.5)	N/A
Age at onset of AD (SD)		94	75.8 (8.0)	36	70.6 (5.3)	58	79.5 (7.5)	**<0.01**[a]
ADAS-Cog score percentiles	25% 50% 75%	94	17.3 23.3 33.7	36	16.5 24.7 36.8	58	18.7 23.3 31.3	0.71[b]
Years of schooling	5 to 8 years	19	20%	2	6%	17	29%	**<0.01**[c]
	9 to 12 years	55	59%	23	64%	32	55%	
	> 12 years	20	21%	11	30%	9	16%	
Previous history of depression	Yes	18	19%	6	19%	12	23%	0.64[d]
	No	66	70%	26	81%	40	77%	
	Unknown	10	11%					
Previous history of other psychiatric problem	Yes	7	8%	3	9%	4	8%	0.99[c]
	No	79	84%	30	91%	49	92%	
	Unknown	8	8%					
Use of cholinesterase inhibitor	Yes	47	50%	23	64%	24	41%	**<0.05**[d]
	No	47	50%	13	36%	34	59%	
Use of anti-inflammatory medication	Yes	25	27%	13	36%	12	21%	0.10[d]
	No	69	73%	23	64%	46	79%	

AD indicates Alzheimer's disease; SD, standard deviation; IQR, interquartile range; N/A, not applicable; LH, luteinizing hormone; SHBG, sex hormone binding globulin; TNF-α, tumour necrosis factor alpha

[a] P-value from two independent samples t-test; [b] P-value from Kruskal-Wallis rank test; [c] P-value from Fisher's exact test
[d] P-value from Pearson's chi-squared test

Table 4.2.2 Sex hormones and TNF-α in the study cohort of men with Alzheimer's disease, by age group

	Total AD sample	AD sample by age group		
	Age range: 59 to 98 (n=94)	Age<80 (n=36)	Age≥80 (n=58)	P-value
Age (SD)	81.2 (7.2)	74.3 (4.8)	85.5 (4.5)	N/A
ADAS-Cog score (IQR)	23.3 (17.3 to 33.7)	24.7 (16.5 to 36.8)	23.3 (18.7 to 31.3)	0.71[b]
Total testosterone nmol/L (SD)	12.63 (5.80)	14.58 (5.47)	11.42 (5.71)	**<0.01**[a]
Free testosterone [nmol/L] (SD)	0.19 (0.09)	0.23 (0.08)	0.17 (0.08)	**0.001**[a]
LH [IU/L] (IQR)	7.45 (4.19 to 12.91)	5.85 (3.75 to 9.52)	9.07 (5.50 to 14.55)	**0.02**[b]
SHBG [nmol/L] (IQR)	49.0 (38.0 to 63.0)	43.5 (37.5 to 54.0)	51.5 (38.0 to 64.0)	0.16[b]
TNF-α [pg/ml] (IQR)	3.21 (2.44 to 4.23)	3.05 (2.19 to 3.96)	3.43 (2.52 to 4.34)	0.26[b]

AD indicates Alzheimer's disease; SD, standard deviation; IQR, interquartile range; N/A, not applicable; LH, luteinizing hormone; SHBG, sex hormone binding globulin; TNF-α, tumour necrosis factor alpha

[a] P-value from two independent samples t-test; [b] P-value from Kruskal-Wallis rank test; [c] P-value from Fisher's exact test; [d] P-value from Pearson's chi-squared test

Table 4.2.3 Comparison of free testosterone and luteinizing hormone between men with Alzheimer's disease and values derived from a non-Alzheimer's population, stratified by age

		AD		Non-AD[‡]		
		N	Mean or median	N	Mean or median	P-value
Free testosterone [nmol/L] (SD)	Age < 80	36	0.23 (0.08)	2938	0.28 (0.10)	0.0002[a]
	Age ≥ 80	58	0.17 (0.08)	707	0.26 (0.10)	<0.0001[a]
Luteinizing hormone [IU/L] (IQR)	Age < 80	36	5.85 (3.75 to 9.52)	2938	4.20 (2.96 to 6.30)	0.0014[b]
	Age ≥ 80	58	9.07 (5.50 to 14.55)	707	5.15 (3.36 to 8.29)	<0.0001[b]

AD indicates Alzheimer's disease; SD, standard deviation; IQR, interquartile range
[‡] Calculated from Yeap et al.[384]
[a] One-sample *t* test
[b] Wilcoxon signed-rank test

Table 4.2.4 Spearman correlations between TNF-α, LH and Free testosterone, within age groups; and partial correlations to adjust for age within total Alzheimer's disease cohort

	Age groups					
	<80 N=36		≥80 N=58		Combined N=94	
	r	P-value	r	P-value	r	P-value
TNF-α vs LH[a]	**.34**	**0.05**	.20	0.15	**.25**	**0.019**
Adjusted for age[b]					**.21**	**0.05**
TNF-α vs Testosterone[a]	.05	0.78	.01	0.98	-.03	0.80
Adjusted for age[b]					.01	0.92
LH vs Testosterone[a]	-.13	0.44	-.08	0.53	-.17	0.11
Adjusted for age[b]					-.08	0.43

LH indicates luteinizing hormone; TNF-α, tumour necrosis factor alpha
[a] Spearman's rank correlation
[b] Partial correlation of ranked data

4.3 Male sex hormones and systemic inflammation in Alzheimer's disease: Discussion

In this study we sought to examine the relationship between sex hormone levels and systemic inflammation in men with Alzheimer's disease. We found lower than average free testosterone (FT) and higher than average luteinizing hormone (LH) in the blood of community-dwelling men with Alzheimer's disease (AD). This finding is consistent with several studies in men with AD that have also found lower FT levels than in non-AD men.[170 182 385] However, previous reports on LH levels in men with AD have been less consistent, with some showing increased levels,[182] and others low or normal levels.[19 170] High levels of LH imply primary hypogonadism, with testicular failure as the cause of low testosterone levels. Conversely, low levels of LH imply hypothalamic-pituitary-gonadal axis failure, with loss of the necessary stimulatory message to the testes. Our findings imply testicular failure as the cause for the cohort's lower than average testosterone levels, and thus suggest that changes in sex hormone levels in men with AD represent an exaggerated form of the usual pattern of change that is associated with ageing. Whether the sex hormone differences found in this study pre-date and increase the risk of AD, or are secondary to AD, is unknown.

We examined the relationship between these altered sex hormone levels in men with AD and systemic inflammation, as measured by serum levels of TNF-α. We found a significant positive correlation between LH and serum TNF-α. This finding has not been previously described in men with AD. There are few, if any, studies examining the relationship between peripheral cytokines and sex hormone status in elderly male cohorts. However, a positive correlation between LH and TNF-α has been described in people with rheumatoid arthritis,[211] and patients with prostate cancer treated with an LH suppressor have reduced TNF-α levels.[386] Hence, the relationship between LH and serum TNF-α in men with AD is consistent with findings in other populations.

LH has been postulated to modulate cognitive decline in AD.[14] Our study provides some evidence of an association between LH and TNF-α in men with

AD, and, given that TNF-α is associated with increased cognitive decline in AD,[9] this may suggest TNF-α as a possible mediator for the effect of LH in AD.

Whilst in this cross sectional study it is not possible to assess the direction of the modulation between LH and TNF-α, there is growing evidence that age-related changes in sex hormone levels have effects on immune regulation. Lower levels of testosterone, and of its aromatization product, oestrogen, and higher levels of LH, increase the sensitivity of peripheral monocytes to pro-inflammatory stimuli, with exaggerated production of pro-inflammatory cytokines such as TNF-α.[387] Testosterone can inhibit IL-6 production[388] and can affect induction of NO synthase.[389] As discussed in Section 2.3, aromatization of testosterone to oestrogen has anti-inflammatory effects in the central nervous system via oestrogen-dependent mechanisms.[192]

However, whilst sex hormones have various effects on the immune system, the immune system also affects hormone production at the hypophyseal, pituitary and gonadal level. Inflammatory cytokines increase aromatase activity, and therefore increase the metabolic degradation of testosterone. IL-6 induces persistent testicular resistance to LH, and directly suppresses testosterone production by Leydig cells in the testes.[390 391] Elevated TNF-α levels also induce testicular failure, due to down-regulating effects on Leydig cells.[212] High TNF-α in people with AD[107] may therefore reduce testosterone production by the testes and cause a physiologically-appropriate reflex increase in LH release from the anterior pituitary.

Elevated TNF-α levels predict cognitive decline in AD.[9] Our current finding, that TNF-α and LH are positively correlated, supports the view that sex hormones and the immune system influence each other in men with AD.

A limitation of this study is the lack of a formal control group. We used sex hormone data from a non-demented, age-matched, comparison cohort described by Yeap et al.[384] However, this cohort consists of community-dwelling men in Perth, Western Australia, randomly selected from the compulsory Australian electoral roll. Yeap does not report the ethnic composition of the cohort, and it is therefore possible that the ethnic composition of the Australia-based control group is significantly different to the ethnic composition of the UK-based AD group used in this study. Consequently, ethnic differences

between the UK and Australian cohorts may account for some of the differences in sex hormone levels which we observed in this study.

In addition, there may be significant differences in the procedures used for blood sample collection, processing, storage and sex hormone measurement between the two cohorts. Yeap reports that samples were taken in the morning, spun immediately, stored at -80℃, and that sex hormone assays were carried out using a chemiluminescent immunoassay on an Immulite 2000 analyser. This is very similar to the process for sample collection, processing and storage used in our AD cohort. However, in the UK-based AD cohort, sex hormones were measured using a different chemiluminescent immunoassay system on a UniCel DxI 800 analyser. Although the methodology was similar between the two cohorts, it is still possible that some of the observed differences in sex hormone levels between the AD cohort and the non-AD cohort are accounted for by differences in blood collection systems, different lengths of storage time between sample collection and de-frosting for assay completion, and differences between the chemiluminescent immunoassay systems that were used.

A further limitation due to the lack of a formal control group concerns a lack of data on several possible confounding variables. Therefore, except for age, we were unable to control for confounding variables in the comparison of sex hormone levels between AD and non-AD men in this study. Possible confounding factors which we could not account for include Body Mass Index, co-morbidities such as Type 2 diabetes mellitus, and medication history. However, age is the most relevant confounding factor and we were able to adjust for age in the analysis by comparing sex hormone levels in AD men with age-matched sex hormone levels in non-AD men, with age-matched normal values derived from the very large cohort described by Yeap et al.[384] Despite these limitations, it is hoped that this study will lead to prospective, longitudinal studies of sex hormones and cytokines in ageing men, designed to disentangle the relationship between sex hormones, cytokines and the risk and severity of Alzheimer disease.

Chapter 5: Reliability and validity of a neuroimmune Sickness Behaviour Scale in Alzheimer's disease

5.0 Introduction

The evidence presented in Chapter 2 of this thesis suggests that in Alzheimer's disease, systemic inflammation exacerbates the progression of neurodegeneration by aggravating inflammation within the CNS. Furthermore, systemic inflammation may exacerbate the symptoms of chronic, maladaptive sickness behaviour in Alzheimer's disease, by increasing CNS cytokine production in microglial cells primed by the on-going neurodegenerative disease. We hypothesize that many of the distressing behavioural and psychological symptoms of Alzheimer's disease arise because they represent chronic, maladaptive sickness behaviour. These symptoms include depression, poor concentration, anorexia, muscle weakness, poor coordination, altered sleep and delirium.

Despite the growing evidence for maladaptive sickness behaviour in animal models of neurodegeneration, and the exacerbation of such behaviour by increased systemic inflammation, there is no reliable, validated scale to assess sickness behaviour in people living with Alzheimer's disease. The lack of such a scale hinders progression of clinical research in this area. A validated sickness behaviour scale in Alzheimer's disease would have value in epidemiological studies, as a measure of the prevalence and severity of neuroimmune sickness behaviour; in clinical trials, as a measure of treatment efficacy; and in the memory clinic, as a measure of symptom progression and symptom burden.

Behavioural and psychological symptoms are well recognized in Alzheimer's disease. The burden of these symptoms is high for patients and carers and their amelioration with effective treatment is a high priority for clinical research. Although a number of instruments have been developed to assess various behavioural and psychological symptoms in Alzheimer's disease, these

instruments may be unsuitable as measures of neuroimmune sickness behaviour.

The Neuropsychiatric Inventory (NPI) is a widely used scale for measuring a broad spectrum of behavioural and psychological symptoms, as described in Chapter 3 (General Methods, Section 3.4). The variety of symptoms covered by the NPI gives the scale high sensitivity as a measure of any disturbance in neuropsychiatric symptoms.[370] However, the variety of symptoms covered by the NPI also gives the scale moderate internal consistency and reliability.[372 392] In the paper that reported the construction of the NPI, Cummings et al. reported that 78% of the scale items had no significant relationship with the other scale items, therefore limiting internal consistency, increasing the probability of measurement error, and limiting reliability.[371] The NPI appears to measure several underlying constructs at the same time, including emotional affect, personality traits, cognitive function, attachment behaviour, and neuroimmune sickness behaviour. Researchers interested in the relationship between the immune system and behavioural and psychological symptoms of dementia may fail to find important relationships between any single construct of interest and biological markers of neuroimmune status because of the noise generated by the other measured constructs.

Many of the symptoms of sickness behaviour are also associated with affective depressive illness; indeed, some would argue that affective depressive illness is itself a form of inappropriately triggered sickness behaviour.[68] This raises the prospect that scales measuring depressive illness in Alzheimer's disease may also serve as measures of maladaptive sickness behaviour. However, scales like the Cornell Scale for depression in dementia,[369] validated as measures of affective depression, will naturally tend to exclude or demote non-affective symptoms, such as hyperalgesia, malaise, gastrointestinal disturbance and dysexecutive function. However, these non-affective symptoms may be of great relevance in neuroimmune sickness behaviour.

In summary, none of the scales that assess behavioural and psychological symptoms in Alzheimer's disease have been validated as measures of neuroimmune sickness behaviour. This lack of validation makes the use of these instruments as measures of neuroimmune sickness behaviour inappropriate.

Sickness behaviour is thought to arise, at least in part, from elevated levels of circulating cytokines in the bloodstream communicating across the blood-brain barrier and inducing cytokine production in the brain. This increased cytokine production in the brain then generates the motivational and behavioural features of adaptive sickness behaviour. This hypothesis is partly generated by the observation that therapeutic cytokine administration in the treatment of cancer induces sickness behaviour symptoms in many patients, as discussed in Chapter 2 (Section 2.1). Thus, the extent to which a scale that aims to measure sickness behaviour correlates with blood cytokines may be considered a measure of the construct validity of that scale. However, many factors are likely to be involved in the generation of sickness behaviour under normal physiological conditions, including HPA axis activity, the degree of age-related change within the brain, immune senescence, the activation state and sensitivity of microglia, and the level of neuroprotective sex hormones. During neurodegenerative disease, change in the relationships between these many factors may demote the primacy of circulating cytokines in favour of other factors.

Given this uncertainty concerning the primary driver of pathological sickness behaviour in Alzheimer's disease, we propose using two competing methods of sickness behaviour scale construction. The first method does not assume the primacy of any one of the many factors that may be involved in the generation of pathological sickness behaviour. This method involves choosing scale items that share a high degree of co-variance, in an effort to unify the final scale items into a single underlying construct of neuroimmune sickness behaviour. The second method assumes the primacy of blood cytokine levels in the generation of pathological sickness behaviour. This method involves basing a sickness behaviour scale on those scale items that best correlate with levels of blood cytokines.

The study reported in this chapter therefore sought to construct a simple scale to measure neuroimmune sickness behaviour in Alzheimer's disease, using two competing methods of scale construction to produce a scale with the best reliability and validity.

5.1 Sickness Behaviour Scale: Methods

5.1.1 Sickness Behaviour Scale construction

The aim of the study was to develop a scale for measuring neuroimmune sickness behaviour in people with Alzheimer's disease. Following recruitment and consent, participants underwent psychometric and clinical evaluation at baseline and at a 4-6 month follow-up. A secondary aim of the study was to look at whether a measure of sickness behaviour could discriminate between people with Alzheimer's disease, people with Lewy Body dementia and people without dementia. Therefore, in addition to participants with Alzheimer's disease, further participants with Lewy Body dementia and cognitively normal controls were also recruited.

5.1.2 Study participants

Potential study participants with Alzheimer's disease and Lewy Body dementia were identified by clinicians at the Memory Assessment and Research Centre (MARC) at Moorgreen Hospital in Southampton, UK. Cognitively normal controls were volunteers recruited from the population of relatives and carers of patients with dementia known to the memory service at MARC.

5.1.2.1 Sample size

There is no clear agreement about the most effective way to estimate sample size for reliability and validation studies in psychometric research.[393 394] The most commonly used method is to generate a sample size estimate from a target ratio of subjects needed per number of scale items. However, Anthoine et al. report wide variation in reported estimates of numbers of subjects required per scale item, ranging from 2 to 20 subjects per scale item.[393] Assuming a middle ground of 10 participants per item, and a scale of 20 items, after excluding items with very high or very low endorsement rates, we initially planned to recruit 200 subjects with cognitive impairment. However, following initial ethical review, the ethics committee felt that this target was over ambitious for an intervention of no proven worth; therefore they recommended carrying out a pilot study with smaller numbers. We therefore aimed to recruit 50 subjects with cognitive impairment in order to develop the

scale. Although this number is considerably less than the estimate based on subjects per scale item, it is closer to the estimate of sample size given by Streiner and Norman, who recommend an approximate sample size of 85 based on determining Cronbach's alpha=0.70 with a 95% CI: 0.60 to 0.80, and 10 to 50 scale items.[394]

Subjects with Lewy Body Dementia were recruited between September 2007 and March 2008 (n=21). Cognitively normal controls were recruited between December 2008 and June 2009 (n=42). Subjects with Alzheimer's disease were recruited between July 2009 and August 2011 (n=64).

5.1.3 Inclusion and exclusion criteria

Inclusion criteria:

1. Age ≥ 65
2. English as first language
3. Participant has either a diagnosis of Alzheimer's disease, a diagnosis of Lewy body dementia, or is a person without known cognitive impairment

Exclusion criteria:
There were no exclusion criteria for the Sickness Behaviour Scale study.

5.1.4 Study measures

Following recruitment, data were collected at baseline and at 6 months. Test-retest data were collected in a subgroup of participants 7 days after administration of the Sickness Behaviour Scale.

Demographic data were collected at baseline. Participants were assessed using the Sickness Behaviour Scale under development, the Neuropsychiatric Inventory (NPI), the Cornell Scale for Depression in Dementia (Cornell), a checklist of systemic inflammatory events, current medication, and medical history, a Fried Frailty Score, ADAS-Cog, and blood sampling for cytokine analysis. These measures are described in the General Methods section of this thesis (Section 3.4).

5.1.5 Cytokine analysis

Serum cytokines were assayed at baseline and at 6 months using the MSD Meso Scale platform described in the General Methods (Section 3.4).

In brief, assays of IFN-γ, TNF-α, IL-6, IL-1, IL-10, IL-12 and IL-8 were performed using a sandwich multiplex cytokine immunoassay (Meso Scale Discovery (MSD), Gaithersburg, MD). A protocol provided by MSD for custom assays was used with no major modifications (Table 3.4.2). An assay of CD40-ligand was performed using a custom ELISA assay.

5.1.6 Ethical approval

The study protocol and the patient information/consent forms to be used in the study were submitted to the Southampton and South West Regional NHS Research Ethics Committee. Ethical approval for the study was obtained prior to commencement (LREC Number: 07/Q1704/78).

5.1.7 Initial scale construction

5.1.7.1 Initial selection of scale questions

A provisional scale containing 26 items was derived following a literature review of the evidence from animal and clinical studies of cytokine-related sickness behaviour. The full 26-item scale is presented in the Appendices. Two versions of the scale were produced. The first was an “informant” scale, where the questions about sickness behaviour in an individual are directed towards an informant, rather than to the individual themselves. A second version, the “participant” scale, re-worded the item questions so that the questions about sickness behaviour in an individual were directed towards that individual. The scale development analysis used the “informant” scale only, as this was the version used for study participants with dementia.

The wording of the items must be un-ambiguous and easily understood by the subjects for whom the scale is intended. For this reason, comprehension of the wording of the questions in the scales was pre-tested in a sample of 10 elderly subjects without dementia (relatives of the investigators) and 10 relatives of

subjects with dementia (volunteers from the Alzheimer's Society) in an attempt to ensure that the scale was easily understood.

5.1.7.2 Content validity

Content validity, or content relevance, refers to the extent that the Sickness Behaviour Scale covers the range of currently understood cytokine-induced sickness behaviours. Content relevance is usually assessed by asking experts in the field to examine the scale for an opinion on whether the scale adequately covers the domain it purports to, and to comment on what may be left out.

Table 5.1.1 shows the main domains of sickness behaviour[22] alongside the Sickness Behaviour Scale items that aim to assess each domain. The best covered domains are those that relate to the negative motivational effects of sickness behaviour, in keeping with the theory of sickness behaviour as an alteration in motivational priorities. All domains, with the exception of fever, are covered by several scale items. This level of redundancy was necessary to prevent a loss in content validity when items were excluded during the analysis of the scale.

5.1.7.3 Use of Likert-type scale

A 4-point Likert scale was chosen for the severity measure. There has been some debate in the psychometric literature concerning the relative benefits and deficiencies of 4-point or 5-point scales.[394] A 5-point scale provides a mid-point, but this can bias responding towards the midpoint. The advantage of a 4-point scale is that it forces a choice to be made. The Sickness Behaviour Scale relies on an informant to provide information on sickness behaviour in a person close to them who has dementia. A 4-point scale has the advantage in this context because it forces informant to make a judgment on behalf of the person they care for, where they might otherwise sit on the fence.

5.1.7.4 Informant scale

The informant scale questionnaire was administered by a study nurse or doctor. The interviewer read out the questions to the informant and listed the possible responses for each item. Further information was provided in cases where the informant required further clarification of the meaning of any particular question.

Table 5.1.1 Items in the 26-item Sickness Behaviour Scale categorised according to recognised domains of animal sickness behaviour

Sickness Behaviour domain	Sickness Behaviour Scale item	
Negative motivational effects (anxiety, loss of interest in surroundings, reduced grooming, listlessness, depression, lethargy)	Anxiety Depression Apathy Social interaction	Fatigue Listlessness Somnolence Helplessness
Inability to concentrate	Concentration Executive function Psychomotor speed	Short term memory Orientation in time Orientation in place
Anorexia and adipsia	Appetite Adipsia Weight loss	Nausea Diarrhoea Apathy
Weakness	Fatigue Listlessness	Malaise
Hyperalgesia and malaise	Malaise Myalgia	Headaches Hyperalgesia
Effects on circadian rhythm	REM sleep disturbance Somnolence	Orientation in time
Delirium	Visual hallucinations Orientation in time Orientation in place	Anxiety Concentration
Fever	Temperature regulation	

5.1.8 Statistical methods used in scale construction

Statistical analyses were carried out using the STATA software package (StataCorp, version 11.0) and the SPSS software package (IBM, version 22).

5.1.8.1 Endorsement rate

Scale items that are endorsed positively by nearly all, or by extremely few, participants are redundant – as they cannot contribute to overall scale variability. In the first phase of scale development we examined the endorsement rate of each scale item, with the aim of excluding items that were either endorsed very frequently, or very infrequently.

A simple Endorsement Rate (ER) for each item in the scale was generated using the following equation:

$$ER_i = \frac{x_i}{n} \qquad (5.1)$$

where

ER_i the endorsement rate for a particular scale item

x_i is the number of subjects endorsing that item

n is the total number of subjects.

Items with a low endorsement rate, indicated by an ER≤0.05, were discarded. The Endorsement Rate was calculated from the data using a short piece of code written by the author using the STATA statistical analysis package (StataCorp, version 11.0) (See Appendices).

5.1.8.2 Discrimination index

A Discrimination Index was generated using the following equation:

$$d_i = \frac{U_i - L_i}{median\ n} \qquad (5.2)$$

where

d_i is the discrimination index

U_i is the number of subjects endorsing a particular item who score above the median on the total scale score

L_i is the number of subjects endorsing that item who score below the median on the total scale score

median n is the number of subjects above or below the median.

This equation was used to calculate a discrimination index value for each scale item in order to identify any items that were poor discriminators between above median or below median total score. An item more commonly endorsed by people scoring below the median than above the median, is undesirable in the final scale as it will share little variance with the total scale and undermine the homogeneity of the total scale. A discrimination index differs from a simple endorsement rate because it examines endorsement in relation to all of the other items in the scale, whereas the endorsement rate treat items independently of each other.[394] The discrimination index varies from -1 (endorsing this item always predicts a below median total score) to +1 (endorsing this item always predicts an above median score). A discrimination index of 0 means endorsing this item has no predictive value either way. Our aim was to identify any items with a zero or negative discrimination index in order to discard these items.

5.1.9 Alternative scale construction methods were used and compared

Two alternative construction methods were used in scale construction, and the resulting scales compared for reliability and validity. The first method used principal components analysis to produce a scale of the items that contributed most to overall scale variance. The second method used correlation analysis to produce a scale of the items that correlated best with serum cytokines, with the assumption that sickness behaviour in Alzheimer's disease is principally driven by systemic cytokine levels. A principal aim of the study was to assess the strengths and weaknesses of these two alternative techniques for constructing a sickness behaviour scale. The two methods are described in more detail below.

A factor analysis-derived scale was constructed by carrying out a form of factor analysis on the initial scale items and then selecting those items accounting for the majority of the variance in the total scale score. Further iterations of this process were then performed until a scale with a logical factor

structure was generated. This method had the advantage of being initially independent of the baseline cytokine data, which could instead be used to help validate the scale. However, the disadvantage was that correlation with pro-inflammatory cytokines could not be guaranteed, risking the possibility of a scale with high internal consistency for various behavioural items, but no relation to the cytokines thought to be associated with that behaviour.

A cytokine-derived scale was constructed by examining the correlations between individual scale items and pro-inflammatory cytokines. The underlying strategy here was that it would be a better way to ensure the cytokine "relatedness" of the final scale. A further advantage is the simplicity of this method. However, there is the disadvantage that baseline cytokine data could not be used to validate the scale, as this data was used to choose the items used in the final scale.

These contrasting methods, employing scale construction with or without cytokine data, produced two alternative scales. Following construction, we then compared the two alternative scales by examining measures of reliability and construct validity. The principal measure of construct validity was correlation of the total scale scores and blood cytokine levels. In the case of the factor-analysis derived scale, baseline cytokine levels and follow-up cytokine levels could be used. In the case of the cytokine-derived scale, follow-up cytokine levels could be used, but not the baseline cytokine levels that had been used to construct the scale. Other measures of construct validity were correlations of the total scale scores with other measures of behaviour, psychological symptoms and physical frailty. Other tests of the two scales were test-retest reliability, change over time, and ability to discriminate between dementias and normal controls.

5.1.9.1 Factor analysis-derived scale: non-linear principal components analysis

Principal components analysis (PCA) is a form of factor analysis; a technique used to reduce large numbers of correlated variables into a smaller number of composite variables.[395] These new variables are called components or factors depending on the method employed. The aim is to generate a smaller number of new composite variables, made up of weighted combinations of the many

original variables, so that these composite variables (or components or factors) can then be used to simplify further analysis.[396 397] In this instance PCA is used to examine the relationship between items in an initial version of the scale, after endorsement rate analysis, to see if this number of items can be reduced further, whilst minimising the effect on total scale variance.[397] Traditional PCA assumes that variables are measured on an interval or ratio scale and that relationships between the variables are linear. Categorical scales such as the Likert-type scale used for the sickness behaviour variables are therefore unsuited to traditional PCA. Nonlinear categorical PCA is an alternative method suitable for categorical or ordinal variables that may not be linearly related.[398]

I will describe conventional linear PCA first, to make the discussion of nonlinear PCA clearer. The first step in a successful PCA is to obtain a correlation matrix of all the variables. In order to draw out common factors or components the correlations between variables must be sufficiently high. Bartlett's test uses the null hypothesis that the correlation matrix is an identity matrix, meaning that the off-diagonal elements are all zero, and that there are therefore no significant correlations between the variables. Similarly, partial correlation between two of the variables, after adjustment for the correlation effects of other variables, must be low. This is tested by the Kaiser-Maier-Olkin (KMO) test of sphericity.[395 399]

These conditions being met, each extracted component is then generated using a statistical procedure that works out a linear combination of the variables which maximises the variance accounted for by that component. Theoretically there are as many components as there are variables, but where variables are correlated it is likely that only a few components will be important.

The importance of each component is assessed by the amount of variance in the original data that is explained by that component. This is the "eigenvalue" of that component. For each person a component score can be calculated using their original or quantified variable score and the component weights for each variable that make up the linear combination of variables for that component. All subjects will then have a component score that can be correlated with the original or quantified variables. Component loadings in both linear and nonlinear PCA are the Pearson correlations between the

component scores and the observed or quantified variables. The square of the component loading for a particular variable on a particular component is the variance accounted for by that variable within that component. The sum of squared component loadings of all the variables on a single component is the eigenvalue of that component.

In categorical nonlinear PCA a process of optimal scaling is used to assign numeric values to the categories within a categorical variable.[398] The category quantifications are produced so that they retain as much as possible of the original variance in the categorical variables. The process of optimal scaling is performed simultaneously with the extraction of the principal components in a process that maximises the variance accounted for by the extracted components.

In PCA, an attempt to reduce higher number of variables to a smaller number of components causes a loss of information. Successful PCA requires that this loss of information is minimized as much as possible. For a given solution this loss of information can be quantified as the difference between the original data matrix and the product of the matrix containing the component scores of the subjects and the matrix containing the component loadings of the variables.

The best fitting PCA solution is one where the subjects' component scores multiplied by the variable component loadings reproduces the variance of the original data very closely. If X is the matrix of subject component scores, A is the matrix of variable component loadings and Q is the original matrix of subject variable scores, then:

$$X \times A \cong Q \qquad (5.3)$$

In categorical PCA the initial values of X are random. The procedure then seeks to minimize the difference between the original data Q, and the product of the component scores X and the component loadings A.

$$Q - (X \times A) = 0 \qquad (5.4)$$

Categorical PCA does this by using an iterative function that minimizes the sum of the squared differences between the original data and the product of

the component score and component loading across each subject and each variable.[398]

The categorical PCA procedure makes cyclical alterations in one of Q, X or A, while keeping the other two fixed, and while maximising the variance accounted for by a pre-specified number of components. This process continues until there is no further improvement in the fit of the model, which is when $Q - (X \times A)$ is closest to zero. From this it can be seen that optimal scaling or quantification of the categorical variables (Q) occurs simultaneously with the extraction of the principal components.

As quantification of the categorical variables occurs simultaneously with extraction of the principal components, the quantification values and component loadings may differ for solutions with different numbers of pre-specified components. The solutions are described as non-nested – meaning that the component loadings may differ between a solution containing m components, and a solution containing m+1 or m-1 components. This is in contrast to linear PCA where solutions are nested, and component loadings do not differ depending on the number of components extracted.

This discussion of nested versus non-nested solutions becomes important when describing the method employed in this study because it has an effect on the method used to decide the number of components to extract.

5.1.9.2 Choosing the number of components to extract

The choice of the number of components to retain after PCA can be guided by a number of different considerations: Kaiser's eigenvalue criterion,[400] use of a scree plot,[399] and parallel analysis.[401] In this study each of these methods was used to confirm component retention. These methods are now described.

Retention of components can be guided by the eigenvalue of each component. Components with an eigenvalue >1 can be considered important as they explain more of the variance than is explained by a single variable.[400]

Scree plots are graphical aids to assist researchers in the decision about the number of components to retain.[397] In a scree plot, the eigenvalues of consecutive components are plotted against the component number. The first few components tend to have a steep decline in eigenvalue until an inflexion is

reached, after which subsequent components have eigenvalues which are all similar and the plot plateaus out – the bottom of the scree slope which gives this plot its name. In general researchers do not retain the factor at the point of the inflexion, but retain all the factors with eigenvalues above that point.[399]

In linear PCA the inflexion in the scree plot can be unclear. However, in non-linear PCA the inflexion point is often clear because non-linear PCA maximises the sum of the first p eigenvalues, where p is a pre-specified number of components. It follows that the sum of the m-p residual eigenvalues is minimized, where m is the total possible number of components.[398] The inflexion in a non-linear PCA scree plot may therefore be clearer than in linear PCA.

As described previously non-linear PCA solutions are not nested and the eigenvalues for each component will differ across solutions, where each solution maximises the variance accounted for by a pre-specified number of components. The scree plots for each solution will therefore differ depending on the number of pre-specified components. The authors of the procedure for categorical PCA therefore recommend that scree plots for different solutions should be compared. Where the inflexion point in the scree plot is consistently at p or p+1, the p dimensional solution should be used.[398]

Finally, parallel analysis is also used to assist in the decision about the number of components to retain.[401] In parallel analysis the eigenvalues for a particular PCA solution are compared with eigenvalues obtained from an equally sized randomly generated data set. The only eigenvalues that are retained in the PCA solution are those greater than the corresponding values in the randomly generated data set. Parallel analysis can be used in both linear and non-linear PCA and is less likely to overestimate the number of factors to retain when compared to Kaiser's criterion or the scree plot.[402] In this study parallel analysis was performed using a Monte Carlo PCA for Parallel Analysis computer application. The application is used to generate a random data set for comparison with the eigenvalues generated from the data by categorical non-linear PCA (MW Watkins, 2000, from SPSS Survival Manual 5th Ed Julie Pallant McGraw Hill).[403]

5.1.9.3 Communality and factor complexity

Principal components analysis is used in this study for two purposes. First, during scale construction, to identify scale items that contribute poorly to overall scale psychometric performance. Second, during scale analysis, to identify underlying latent structure within the scale to assist in the assessment of construct validity. This section of the methods is concerned with scale construction. During scale construction using PCA, scale items must be dropped where they do not contribute significantly to the variance accounted for by the extracted components.[394] When such items are dropped from a solution the overall variance accounted for by that solution increases.

Items can contribute poorly to the overall variance of a solution in two ways. First, if a scale item within a model shares very little variance with other items then it will not contribute to the explanation of variance within that model. Second, if a scale item loads on to more than one component, where the model is straining to generate un-related orthogonal components, then eliminating these factorially complex items will improve the variance accounted for by the model.

In the first situation, the squared component loadings for each variable can be summed into an overall total, termed an item's "communality" – and this is a measure of the total variance accounted for by that variable within the particular PCA solution. Another way to view this is that an item's communality within a PCA solution is the amount of variance in that item that is explained by the retained factors.[399] If an item's communality is low then variance in that item is poorly explained by the retained factors, and the overall variance accounted for by the model will improve when that item is dropped from the solution.

In the second situation, after PCA the component loadings for each item can be examined in order to identify items that have a significant loading on more than one component.

In a 2-component PCA solution we might imagine the extracted components as the arms of a pair of drawing compasses, with an angle set between the arms. The PCA method attempts to pull apart the arms of the compass so that the angle between them is as near to 90 degrees as possible, indicating that the

components are uncorrelated. In an ideal solution, individual scale items should load on to only one or the other of the retained components. In a 2-component solution, an item that loads significantly on both components is like a constraining band preventing the opening up of the angle between the two arms of the compass. Eliminating the item removes this constraint within the model and allows the arms to be more fully opened out, nearer to the right angle that indicates the extracted components are unrelated. Eliminating these factorially complex items allows the maximum explanation of within model variance by the extracted components.

5.1.9.4 Factor correlation and factor rotation

In most real world situations the underlying constructs are likely to be related to each other. Traditional factor analysis and PCA does not allow for this and can therefore lose information about the way variables relate to the extracted factors. However, PCA and factor analysis solutions can be mathematically rotated in such a way that correlation between factors is allowed. Oblique rotation allows for the fact that in reality the extracted components are rarely truly orthogonal and allows the solution to be rotated within the vector space to maximize the clarity of the solution

In order to assess factor complexity for each scale item it is first necessary to rotate the solution to a position where the highest number of individual variables load maximally on to only one factor. Oblique rotation allows for some correlation between factors. In this study it is likely that any underlying components or factors relating to sickness behaviour are related to each other. Oblique rotation therefore provides the best way to rotate the PCA solution so that items are most likely to load on to single components.

After oblique rotation an item's relationship to a component can be assessed by examining two related statistics: pattern coefficients and structure coefficients. Structure coefficients are correlation coefficients between quantified variables and extracted components, similar in concept to component loadings in the un-rotated solution. Pattern coefficients are the weights applied to the measured variables to obtain component scores. Oblique rotation allows for correlation between components and an inter-component correlation matrix can therefore be generated. The pattern

coefficient matrix multiplied by the inter-component correlation matrix approximated the structure coefficient matrix. Structure coefficients therefore take into account the strength of the correlation between the components in an obliquely rotated solution, that is to say, an item's structure coefficient with a component includes the relationship provided through correlation of the item with other components correlated with the first.

Although rotation gives the best chance of a solution where each item loads on to only one component, and also allows for a degree of correlation between underlying components, it does create a difficulty. Namely, that there are now two sets of component loadings to consider when attempting to assess factor complexity: pattern coefficients and structure coefficients.

In practice pattern coefficients are most often used to examine how items load on to components in rotated models. This approach has the advantage of providing good information about item component loadings without the obscuring effects of the inter-component correlation used to help build the model. However, there is a danger in only looking at the pattern coefficients precisely because they do not take in to account the possible correlation between factors.[404] Several problems can arise and these are now discussed.

Factor complex items might be missed. If an item has a strong relationship with more than one component, and there is some correlation between components, then the item's structure coefficient will be larger and more significant than the pattern coefficient. An item with a low pattern coefficient, that appears to have no significant loading on a component, may have a high structure coefficient indicating significant component loading. Failing to look at both the pattern and the structure coefficients can result in a failure to appreciate the extent of this shared variance, and so factor complex items might be missed.

Items that appear to be factor complex may be falsely excluded. If the pattern coefficient is significant but the structure coefficient is not significant then there is a chance that the item's pattern coefficient over-estimates the true relationship with the component. This occurs when the item contributes to the factor indirectly, by changing or modifying the contribution of other items to the same factor. This type of effect modification, or interaction, is termed a suppression effect because inclusion of the item suppresses the error in the

pattern coefficients for other items. A suppressing item increases the chance of seeing a true relationship between other items and a component, so including it in the analysis improves or unmasks the true relationship between the other items and the component. These items should be retained.

Graham et al. therefore recommend a side by side comparison of the pattern and structure coefficients.[404]

To prevent over estimating factor complexity: both the pattern and structure coefficients must be >0.30 on more than one component for an item to be considered potentially complex.

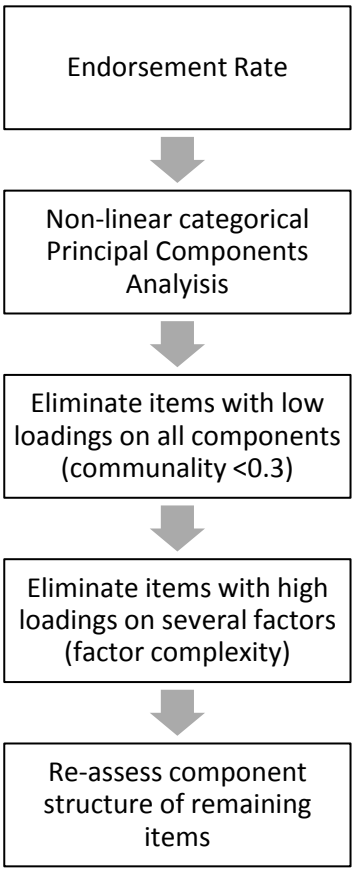
To prevent under-estimating factor complexity: in the surviving items a further check is made to make sure the structure coefficients are not significantly greater than the pattern coefficients in any of the other components.

5.1.9.5 Component analysis of the remaining scale items

The aim of these processes is to guide scale construction by identifying scale items that contribute little to the variance of the scale, or which decrease the overall variance accounted for by the model because of sharing variance across factors. The analysis provides structured guidance for eliminating less helpful scale items, but where items items have equivocal or contradictory signals with regards to their effect on the overall variance of the scale they are retained for further analysis.

After elimination of the unequivocal items the component structure and communalities of the remaining items is examined by a further round of categorical principal components analysis to eliminate any other items that do not contribute well to the variability of the final scale. The overall method using non-linear PCA is illustrated in Figure 5.1.1.

Figure 5.1.1 Scale construction method using non-linear principal components analysis



5.1.9.6 Cytokine-derived scale: correlation of individual scale items with IFN-γ

An initial examination of the cytokine data and correlations showed that IFN-γ was the cytokine most commonly correlated with individual scale items. As IFN-γ is a key mediator in the initiation of innate immune responses[405] it had good face validity as the cytokine to take forward into further analysis. The values for IFN-γ in individual study participants were not normally distributed. Spearman's rank correlation coefficient was used to assess the strength of correlation between individual scale items and cytokine levels. This assumed that there was a monotonic relationship (as X increases, Y increases) between scale items and IFN-γ levels. Each scale item was scored for severity on a 5-point scale, with a zero score for no symptoms, and a 4-point Likert score for increasing severity of symptoms. This 5-point scale contains the necessary

rank levels to satisfy the assumptions for using Spearman's rank correlation coefficient.[406] [407] As the variability in a 5-point scale is necessarily low, we decided to allow less restrictive values for significance level and correlation coefficient; correlations were considered significant if r>0.2 and results were considered significant if P<0.1.

5.1.10 Statistical methods used to assess scale reliability

5.1.10.1 Reliability: Internal consistency

Reliability concerns measurement error. Reliability lets us "know the extent that measurements are measuring anything".[408] Unreliable measurements are unlikely to correlate with other relevant variables, so an estimation of reliability provides a foundation for correlational tests of construct validity. A test cannot be valid if it is not reliable, although it is perfectly possible for a reliable test to measure the wrong thing and therefore to have no validity.[394] To quantify reliability we need to assess the amount of measurement error. A measure of reliability refers to the proportion of the variability in test scores which is due to true differences in an individual's test scores in different circumstances. This can be paraphrased as the ratio between the variability in an individual's test score in different circumstances (e.g. different raters, different occasions, differences over time, differences in total test score caused by measurement error in test items) to the total variability in test score across a population. This can be formalised as a "reliability coefficient", (where 0 = not reliable, 1 = totally reliable):

$$Reliability\ coefficient\ \alpha = \frac{\text{Variability in population scores due to true differences between individuals}}{\text{Total variability in scores across a population, including variability due to measurement error}}$$

Internal consistency is a way of measuring measurement error for a particular test when that test consists of several underlying items. A measure of internal consistency asks: for an individual how much of the variability in the total or composite test score is due to variability in the underlying test items?

If there is a lot of variability in the scores of underlying test items, together with poor correlations to the total test score, then we expect the variability in total score across individuals to be high, and that a high proportion of this

variability in total score will be due to differences in underlying test item responses that do not reflect true differences in the parameter that the test is meant to measure. This high level of variability in items and the total score is due to heterogeneity between items; that is items are unlikely to measure the same underlying trait or construct. This introduces measurement error if we are trying to construct a scale that does measure a single underlying trait. High internal consistency implies homogeneity between individual scale items; that is items all reflect the same underlying trait or construct. High homogeneity in a scale allows us to "interpret the composite score as a reflection of the test's items".[409] In other words, high homogeneity allows us to make assumptions about the probability of a particular response on an individual scale item based on the overall test score; it allows us to group people with a similar overall test score in the knowledge that they are likely to have had similar responses to the underlying scale items.

5.1.11 Cronbach's alpha as a measure of internal consistency

In this study Cronbach's alpha is used to assess internal consistency.[410] Cronbach's alpha can be calculated from the variance-covariance matrix of all items; where a diagonal element within the matrix is the variance within a particular item, and the off-diagonal elements are covariances between pairs of items. Cronbach's alpha is the ratio between an item by item matrix where all elements are the average covariance between items, and an item by item variance-covariance matrix.[399] Cronbach's alpha can be conceptualised as the proportion of the total scale variance that is due to the degree of covariance between items, rather than due to within item variance.

$$\alpha = \frac{N^2\,\overline{cov}}{\sum {s^2}_{item} + \sum cov_{item}} \tag{5.5}$$

where

N is the number of items

$\overline{cov}$ is the mean covariance between items

${s^2}_{item}$ is the variance for an item

cov_{item} is the covariance between an item-item pair.

From this equation we can see that internal consistency is supported by a high level of covariance or correlation between scale items, and that it is undermined by high variance within individual items. If Cronbach's alpha is high (nearer to 1) then the overall scale will be robust to random response differences in individual scale items.

Where an individual item has a large amount of un-shared variance with other items in a scale, that item will exert a bias on the overall score. We do not want a scale where one item has a greater effect on the total scale variance than other items, as this increases the chance of measurement error in the total score.

Cronbach's alpha is a useful measure of overall scale reliability. In general values >0.8 indicate good reliability, high internal consistency, and a scale robust to random error within individual items.[394 395 399] Item-level psychometrics are also examined by calculating Cronbach's alpha for a scale without each item in turn. Any significant increase in Cronbach's alpha due to elimination of a particular scale item means that the item is exerting a deleterious effect on internal consistency and reliability, and we should consider whether to eliminate the item from the scale. Item-rest correlations should be >0.3 to support internal consistency, and items with correlation values below this are unlikely to improve the internal consistency of the scale.[399]

Cronbach's alpha can be thought of as a "lower bound" for the reliability of a particular test, in situations where the scale is thought to be "congeneric". Congeneric scales contain items that are all correlated to each other and are thought to measure the same underlying construct.[394] The sickness behaviour scale developed in this work is a congeneric scale and so Cronbach's alpha can be thought of as an estimate of the lower bound for scale reliability.

However, some caution is needed when interpreting Cronbach's alpha. Principally, Cronbach's alpha is strongly influenced by the number of items within a scale – simply increasing the number of items causes an increase in Cronbach's alpha because of the way that it is calculated. Within the Cronbach's alpha equation (Equation 5.5) the signal that allows us to quantify reliability is the error term in the denominator, namely the sum of all the within item variances. However this vital signal can be swamped by noise in the numerator, which is the product of the number of items and the average item-

item covariance, which will be high when there are high numbers of items, even when the covariance between items is low.

Further caution is needed where Cronbach's alpha is very high. This may indicate that there is redundancy within the scale; items are so similar that fewer could be used with little effect on reliability. Furthermore, very high values of Cronbach's alpha (>0.9) may indicate that the overall scale is insensitive to differences among underlying scale items, so that important differences between individuals in the underlying construct being measured are not registered by the scale.[394]

Streiner and Norman remind us that Cronbach's alpha is a "parameter" and that it should therefore be reported with a confidence interval.[394] A parameter in this context refers to a constant term within a statistical model, calculated by applying that statistical model to sample data, and where the constant term within the statistical model is thought to estimate a true population constant. As Cronbach's alpha is an estimate of a true population constant, it is possible to construct a 95% Confidence Interval around it, related to the size of the sample, the number of scale items and the F-distribution. In this study, the Charter method was therefore used to calculate a 95% CI around Cronbach's alpha.[411]

5.1.12 Intraclass correlation as a measure of test-retest reliability

Test-retest reliability was assessed in a subset of study participants using a re-test score performed seven days after initial testing. A two-way, repeated-measures analysis of variance (ANOVA) was used to assess whether there was a significant difference between test scores at the two time points. The ANOVA is classed as a "two-way" analysis because there are two independent variables: trials and subjects. An intraclass correlation (ICC Type A) was calculated using a two-way random effects model for single measures,[412] to demonstrate the proportion of variance in test score results that was due to true variance among participants, rather than due to retest-related measurement error.

Test-retest reliability refers to the extent to which scores are stable across a time period. It is an estimate of the precision of a test, that is, the likelihood that an individual's test score is an accurate reflection of their underlying true

score. If a scale has low test-retest reliability then it implies a large degree of error around an individual's test score. This error might be due to practice effects, reduced psychological stress during repeat testing, high levels of random noise within the test etc. Test-retest reliability can be thought of as a further example of the general reliability equation (Equation 5.6).

$$Reliability = \frac{s^2true}{s^2true + s^2error} \qquad (5.6)$$

where

s^2true is the true score variance and

s^2error is the variance due to test error.

Error in test scores can be "systematic" or "random". Systematic error refers to error that has a consistent effect on the result in a particular direction. There is systematic error in a situation where individuals consistently score more points on a scale when it is re-administered, perhaps because of familiarity with the test. Increasing the sample size would not reduce the magnitude of this error. There is random error in a situation where individuals have random fluctuations in test responses, causing random change in the final test score in any direction. Increasing the sample size would help to reduce the magnitude of this type of error.

Test-retest reliability can be assessed by a number of statistics, including a Pearson correlation between the scores at two time points. However there are problems with this approach and an intraclass correlation has several statistical advantages. I will now discuss the definition and calculation of an intraclass correlation, and then discuss the advantages this has over a simple correlation between the scores at two time points.

An intraclass correlation is used for variables measuring the same factor, and is therefore suited to test-retest analysis. Although several different types of ICC can be calculated they all have the same general form as the reliability equation:

$$ICC = \frac{s^2true}{s^2true + s^2error} \qquad (5.7)$$

where

ICC is the intraclass correlation coefficient

s^2true is the true score variance and

s^2error is the variance due to test error.

The estimates of true score variance (s^2true) and error variance (s^2error) differ depending on the model being applied to the data.

A statistical model is a set of assumptions concerning the generation of observed data within a sample taken from a larger population, usually specified by a mathematical equation relating a dependant variable to independent variables.[395] Statistical models may be used to test hypotheses, to extract explanatory information, to account for the variance within the observed data.[407] A statistical model can be used to assess the test-retest reliability of a measure. We want to assess the significance of the effect of repeating the test, so our model needs to include a term which represents this. The ICC model most suitable is a two-way, random effects model, specified by:

$$x_{ij} = \mu + subject_i + trial_j + \varepsilon_{ij} \qquad (5.8)$$

where

x_{ij} is a subject's score

i is the subject

j is the time point

subject is the effect of the subject

trial is the effect of the trial

ε is the error or residual term.

This is a two-way model because two factors are involved: the effect of between subject variance, and the effect of between trial variance. The model is a random effects model because we do not want to confine inferences about the model to only those participants within this particular sample; we assume the particular participants are subject to random effects due to sampling from a larger population, so that inferences can be made to the larger population. This model can be applied to the sickness behaviour scale data and then tested to see how well, or how poorly, the model fits the data. This two-way, random effects model of the relationship between a subject's score and re-testing can be assessed by performing a one-way, repeated measures analysis of variance (ANOVA).[412] How can this two-way model be assessed using one-way, repeated measures ANOVA? A single factor, repeated measures is also a two-way factorial model because there are two independent variables: the time point factor and the participant factor. In a repeated measures ANOVA the subjects are considered a second factor; that is, each subject is considered a different factor level within the subject factor, with n=1. The subject factor gives rise to random effects because the subjects are a random sample from a population. Within the ANOVA analysis the F ratio is used to assess the degree to which a model describes the data compared to the degree that it does not describe the data (F=Mean Square(Model) / Mean Square (Residual). This assumes fixed effects; that is, inferences can only be made about the particular factor levels used in the design – acceptable if comparing particular drug dosages, but not acceptable if considering a random sample from a larger population. A different method for calculating F is used for random effects.[395]

This represents a version of Equation 5.7:

$$ICC = \frac{s^2true}{s^2true + s^2error}$$

For test-retest data within our study, a two-way, random effects model is used to generate estimates of true score variance and error. The appropriate ICC equation for a two-way, random effects model is (k=trials):[413]

$$ICC = \frac{MS_{Between\ Subjects} - MS_{Error}}{MS_{Between\ Subjects} + (k-1)MS_{Error} + \frac{k}{n}\left(MS_{trial\ effect} - MS_{Error}\right)} \quad (5.9)$$

We need four sources of variance to calculate the two-way, random effects ICC.[412] To calculate the necessary sums of squares for this two-way model, a one-way, repeated measures ANOVA is carried out on the data. This calculation provides the four sources of variance within the test-retest data that are needed to calculate the appropriate intraclass correlation.

In general terms, the between-participant variance is calculated from the participant means across trials compared with the overall mean. The within-participant variance is calculated from the amount of variation within each participant from trial to trial. Some of this within-participant variance will be due to any systematic effect arising from repetition of the test, termed the trial effect or model effect. The variance due to the trial effect is calculated from the difference between the mean score for each trial compared with the overall mean. Large differences between trial means indicates a large trial effect, and will decrease the resulting ICC. The rest of the within-participant variance, after the trial effect has been deducted, is the remaining residual variance or error variance. A large error variance will also decrease the resulting ICC, and reflects actual change in the measured construct within an individual over the time period between trials, in addition to measurement error, recording error and other unobserved biases.

The ICC provides us with a measure of the reliability of the scale within a particular sample, with the inference that the reliability will be similar in the population from which the sample is taken. The ICC represents the proportion of variance in a set of scores that is attributable to the true between subject variance rather than due to systematic and random error.[412] The ICC therefore reflects how well a test differentiates between different individuals, when the test is repeated. An ICC of 0.8 means 80% of the variance in scores can be attributed to real differences between individuals and 20% to noise arising from trial effects and random error. The interpretation of the ICC is therefore more like R^2 in regression analysis than like Pearson's *r*.

5.1.13 Standard Error of Measurement as a measure of scale precision

At the level of an individual the ICC does not provide information about the degree of trial to trial error in the scale. However, from the two-way, random effects model used to generate the ICC we can also calculate a further statistic: the Standard Error of Measurement (SEMt). The SEMt is the Standard Error in estimating observed scores from true scores.[396] The method used to calculate the SEMt in this study uses the square root of the mean square error term from the ICC ANOVA.

$$SEMt = \sqrt{MS_{error}} \qquad (5.10)$$

The SEMt can be thought of as the precision of an individual's score on a scale, or the "typical error" in a measurement.[412] The SEMt therefore reflects the reliability of a scale within individual subjects. The SEMt is not affected by between subject variability, whereas the ICC can be high when between subject variability is high. The SEMt allows us to quantify the precision of an individual's score by constructing a 95% Confidence Interval around the individual's score using the following equation:[412]

$$True\ Score = Observed\ Score \pm 1.96(SEMt) \qquad (5.11)$$

What are the advantages of the ICC over Pearson's correlation? As described, the ICC is used to correlate different measures of the same variable, which is the case when we are correlating scores on the same scale at different time points. Strictly, Pearson's correlation and Spearman's correlation are inter-class correlations and should be used when correlating variables measuring different things. Indeed, the test of significance for the value of a correlation coefficient comparing two trials may well show high significance, but it would be astonishing if two measurements of the same trait in the same individual 7 days apart were not related. The test of significance would be "irrelevant to the question of agreement" between the two trials.[414] The ICC arising from the test-retest model used here gives an indication of absolute agreement in scores, rather than relative consistency or correspondence. The ICC is therefore robust to systematic skewing errors from one time point to another, which would be missed by interclass correlations. The ICC allows us to carry out a significance test for the effect of the trials; so we can assess whether there is some

systematic error merely related to repetition of the scale. It has been argued that test-retest reliability is incompletely assessed without such a test.[415] Lastly, we can calculate a Standard Error of Measurement (SEMt) from the ICC, providing an index of the actual precision of the scale, or the "typical error", and allowing for calculation of a confidence interval around an individual's score.

To summarise, to assess test-retest reliability a one-way, repeated measures ANOVA is performed on the data and the results are cast as a two-way, random effects model (the effect of the trials is part of the model as well as the effect of participants). We can then examine the F ratio for the trials effects, to examine for a systematic error due to the repetition of the test. If the systematic error of the trial effect is not significant then the ICC and Standard Error of the Measurement can be calculated from the variances arising from the ANOVA and used to quantify the reliability and precision of the measure.[412]

5.1.14 Statistical methods used to assess validity

5.1.14.1 Construct validity: latent factor structure

Sickness behaviour is hypothesised as a single construct, namely the set of behaviours evolved to support immunity and repair. However, it is likely that sickness behaviour consists of related underlying factors. For example, sickness behaviours can be related to internal motivational states and protection.[21] The construct validity of the sickness behaviour scales developed in this study were therefore assessed by performing non-linear principal components analysis on the finalised scales. We examined the resulting factors to assess whether scale items were partitioned in groups that were congruent with the evolutionary and behavioural theory underpinning sickness behaviour. The method for non-linear principal components analysis has been described above (Section 5.7).

5.1.14.2 Construct validity: correlation with other measures

The construct validity of the scales was evaluated by examining correlations with other measures of functional, behavioural and psychological symptoms in Alzheimer's disease. Spearman correlations were used for non-parametric data. Convergent construct validity refers to the degree of association between

scales that measure similar constructs. Divergent construct validity refers to the degree of association, or lack of it, between scales that measure different constructs. In this study, convergent validity was assessed by examining correlations between the sickness behaviour scale and the NPI, Cornell scale and the Fried Frailty Score; and divergent validity was assessed by examining correlations between the sickness behaviour scale and the ADAS-Cog.

Biological construct validity was assessed by examining correlations between the sickness behaviour scale and serum cytokines, where this was appropriate to the construction method for the scale, using Spearman correlation for non-parametric data.

5.1.14.3 Discriminant validity

Discriminant validity refers to the ability of the scale to discriminate between populations thought to have different levels of the construct the scale purports to measure. We examined the total sickness behaviour score in three different groups; the original Alzheimer's cohort (n=64) from whom the scales were derived; a "control" cohort (n=42) and a group with Lewy Body Dementia (n=21). We hypothesised that the Lewy Body Dementia group would have the highest rates of sickness behaviour and the control group the least.

Differences in median score across the three groups were assessed using the Kruskall-Wallis Equality of Populations Rank Test.

5.1.15 Follow up at 6 months

Follow up data were available for 60 subjects at 6 months. Internal consistency was re-assessed by calculating Cronbach's alpha. The median change in total scale score was calculated to assess the degree of change in the total scale score over 6 months. The significance of the degree of change within individuals across 6 months was assessed using repeated measures ANOVA. Stability in the scale was assessed by examining the correlation between baseline score and 6-month score using an Intraclass Correlation (ICC).

Convergent, divergent and biological construct validity were assessed at the 6 month time point by examining correlations between the sickness behaviour scale, NPI, Cornell, ADAS-Cog and serum cytokines.

5.1.16 Validation in independent cohort

Following this analysis, the scales generated by the two methods described were taken forward for further validation in a separate cohort of patients with Alzheimer's disease. These patients were all of the enrolled participants in the STEADI study, with the observations of sickness behaviour taken at baseline, prior to drug administration. The scales were compared with NPI, BADLS, Fried Frailty Score, MMSE, ADAS-Cog and blood cytokines.

5.2 Sickness Behaviour Scale: Results

5.2.1 Baseline characteristics

In total there were 127 participants in the Sickness Behaviour Scale study: 64 participants with a diagnosis of Alzheimer's disease, 21 participants with a diagnosis of Lewy Body dementia, and 42 participants were recruited as cognitively normal controls. Sickness behaviour scale construction, validity and reliability analysis was carried out within the Alzheimer's disease group, and the baseline characteristics of this Alzheimer's disease group are detailed in Table 5.2.1. Baseline cognitive and functional measures across all 3 participant groups (Alzheimer's disease, Lewy Body dementia, controls) are shown in Table 5.2.2.

5.2.2 Endorsement rate

An endorsement rate was calculated for each scale item, in order to exclude items with an ER>0.9 or an ER<0.2, as described in the methods (Table 5.2.3). Nine items had an endorsement rate below 0.2, indicating less than 20% of subjects endorsed these items (Adipsia, Diarrhoea, Malaise, Nausea, REM sleep disturbance, Headache, Weight Loss, Temperature, Visual hallucinations). One item, short term memory loss, had an endorsement rate greater than 0.9. The actual value of the endorsement rate for short term memory was 0.95, indicating that 95% of subjects endorsed this item. These ten scale items were therefore excluded, and the remaining 16 scale items were taken forward for further analysis.

Table 5.2.1 Baseline characteristics of the Sickness Behaviour Scale in Alzheimer's disease construction cohort

		Participants (n=64)	
		n	Mean (SD) or Median [IQR} or %
Age (years)			82.0 (6.4)
Age at onset			77.8 (7.3)
Years since diagnosis			2 [1 to 4]
Gender	Male	28	44%
	Female	36	56%
Years of schooling	<9	1	1.6%
	9 to 12	50	78.1%
	>12	13	20.3%
Family history of dementia	No	40	63.5%
	Yes	23	36.5%
Previous history of depression	No	56	87.5%
	Yes	8	12.5%
Anticholinesterase medication	No	18	28.1%
	Yes	46	71.9%
Antidepressant medication	No	43	67.2%
	Yes	21	32.8%
Antipsychotic medication	No	59	90.6%
	Yes	5	9.4%
Anti-inflammatory medication	No	58	63.5%
	Yes	6	36.5%

Table 5.2.2 Baseline characteristics in different patient groups

	Controls N=42	Alzheimer's disease N=64	Lewy Body Dementia N=21	P-Value
Age	78 (72 to 83)	82 (78 to 87)	82 (78 to 85)	$X^2(2)=6$ P=0.06
NPI	0 (0 to 0)	17.5 (7 to 29)	11 (6 to 26)	$X^2(2)=62$ P<0.001
ADAS-Cog	6 (4 to 9)	23 (16.5 to 33.5)	18 (12 to 34)	$X^2(2)=57$ P<0.001
Cornell	Not assessed	7 (3 to 12)	6 (3 to 9)	$X^2(1)=0.9$ P=0.34
Fried	Not assessed	2 (1 to 3)	Not assessed	—

X^2 is Pearson's Chi-squared test for equality of medians

5.2.3 Discrimination index

A discrimination index was calculated for the remaining 16 scale items, as described in the sickness behaviour scale methods section (Section 5.1). The aim was to identify any items with a zero or negative discrimination index in order to discard these items (Table 5.2.4). No items had a discrimination index ≤0 so no items were discarded by this criterion.

Of note, the orientation in time scale item has a low discrimination index of 0.04, indicating that it is a low discriminator of individuals scoring above the median on the total scale score. However, the critical value for the discrimination index in this sample is 1/(n/2)=1/32=0.03, which is the value obtained when, of all the subjects endorsing an item, there is at least one more person in the above median group than in the below median group, indicating a slight bias towards the above median score in any item with a discrimination index above this critical figure. Orientation in time does have a value greater than this critical value; therefore, orientation was retained as a low positive discriminator of those scoring above the median on total score.

Table 5.2.3 Endorsement rates for items in the 26-item Sickness Behaviour Scale

Item	Endorsement rate
Short term memory	0.95
Orientation in time	0.89
Anxiety	0.67
Fatigue	0.66
Executive function	0.58
Hyperalgesia	0.53
Listlessness	0.52
Psychomotor speed	0.52
Depression	0.48
Apathy	0.48
Concentration	0.48
Orientation in place	0.42
Helplessness	0.42
Appetite	0.33
Somnolence	0.31
Myalgia	0.25
Social interaction	0.20
Adipsia	0.19
Diarrhoea	0.19
Malaise	0.14
Nausea	0.14
REM sleep disturbance	0.13
Headache	0.11
Weight Loss	0.08
Temperature	0.06
Visual hallucinations	0.05

Shading indicates endorsement rate >0.9 or <0.2

Table 5.2.4 Discrimination index for 16-item Sickness Behaviour Scale

Item	Discrimination Index
Social interaction	0.83
Concentration	0.66
Appetite	0.60
Somnolence	0.60
Depression	0.55
Apathy	0.53
Helplessness	0.52
Listlessness	0.52
Psychomotor speed	0.48
Fatigue	0.41
Anxiety	0.35
Executive function	0.31
Orientation in place	0.26
Hyperalgesia	0.21
Myalgia	0.20
Orientation in time	0.04

5.2.4 Scale construction by principal components analysis (SBS-9)

The 16 items retained after endorsement rate and discrimination index analysis were taken forward into factor analysis to build a scale using this method.

5.2.4.1 Initial PCA to identify factor complex items with low communality

Categorical Principal Components Analysis was carried out on the baseline data for all 16 retained items. The Kaiser-Meyer-Olkin measure verified the sampling adequacy for the analysis (KMO=.78; indicating acceptable sample size).[416] Bartlett's test for non-identity of the correlation matrix was significant (Bartlett's test of sphericity $\chi^2(120)=938.1$, $P<0.001$), indicating acceptable

item-item correlations for the analysis. Eigenvalues were obtained for each factor in the data. Four factors had eigenvalues greater than Kaiser's criterion of 1, and were also greater than the corresponding value generated by parallel analysis (Table 5.2.5). Examination of the scree plot supported the retention of 4 factors in the final model, with the inflexion point at the non-retained fifth factor (Figure 5.2.1).

This PCA solution was used to calculate total eigenvalues, or communality, for individual scale items, where the total eigenvalue is an indicator of the proportion of total possible variance (=1.0) accounted for by each item. All scale items had sufficient total eigenvalues >0.3 and were therefore retained in the model (Table 5.2.6).

Oblique rotation of the PCA solution was performed to allow for a degree of correlation between underlying factors. This generated pattern and structure coefficients which were compared as described in the methods (Section 5.1.8.4). Factor complex items (with significant loadings on more than one factor) were identified if both the pattern and structure coefficients were >0.30 on more than one component (Table 5.2.7). Seven items were identified: Apathy, Hyperalgesia, Listlessness, Social interaction, Helplessness, Orientation to time and Appetite. Except for Appetite, the other 6 factor complex items clustered together with total item communality ≤0.6 (Table 5.2.7).

Appetite had a total item communality of 0.76 indicating that this item shared a good proportion of its own variance with other scale items, and therefore contributed well to the overall variance accounted for by the model, despite significant loadings on more than one factor. Generally, items that load on to more than one factor contribute less to the shared variance within a model, and models tend to improve after removing these factor complex items. Therefore, the six items with significant loadings on more than one factor, and with total item communality ≤0.6, were dropped from the scale, leaving 10 items. The Appetite scale item was retained in the solution because of the high total item communality, despite significant loading on to Factor 2 and Factor 4, because removal of Appetite reduced the total variance accounted for by the model.

Table 5.2.5 Model summary for PCA of 16 item scale

	Variance accounted for	
Component	Eigenvalue (parallel analysis)	% of Variance
1	5.85 (1.8)	36.59
2	2.63 (1.6)	16.45
3	1.74 (1.5)	10.86
4	1.36 (1.3)	8.47
Total	11.58	72.36

KMO=.78. Bartlett's test of sphericity $\chi^2(120)=938.1$, $P<0.001$
Parallel analysis eigenvalues generated using MonteCarlo application[403]

5.2.1 Scree plot after principal components analysis of 16-item SBS

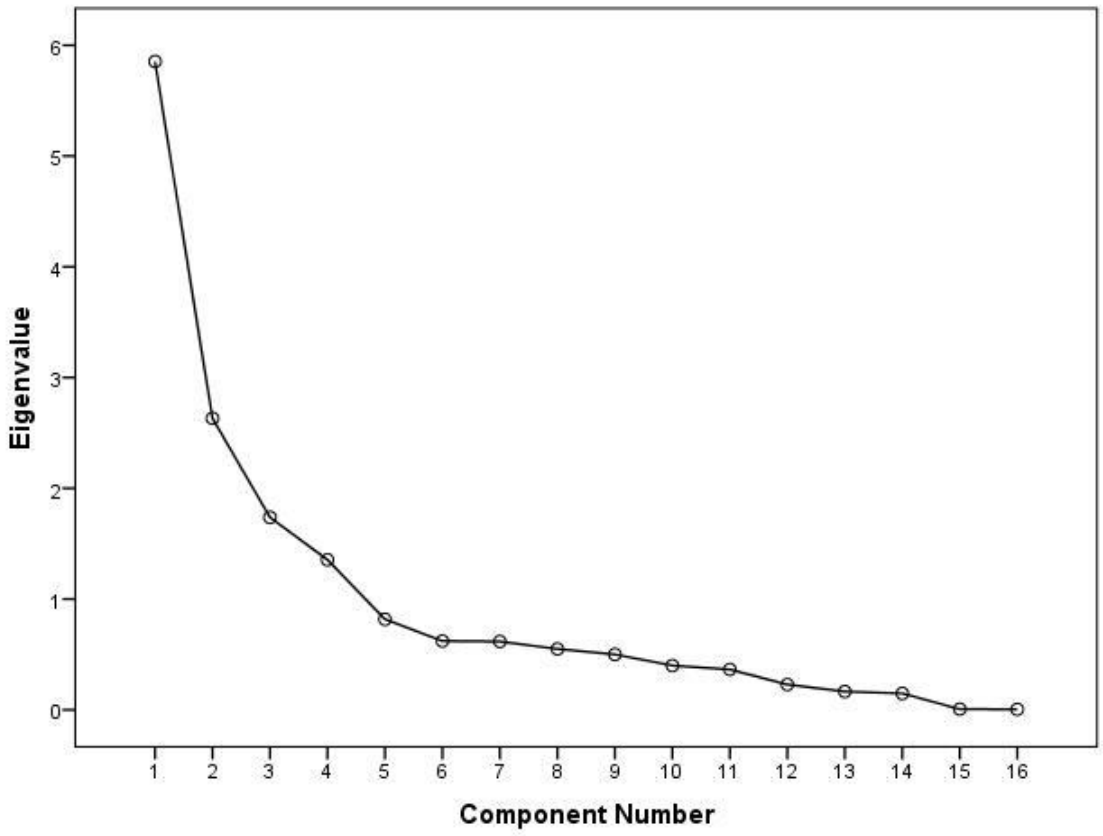


Table 5.2.6 Eigenvalues for factor analysis of 16-item scale, before rotation

	Variance accounted for					
	Components					
Item	**1**	**2**	**3**	**4**	**Mean**	**Total**
Depression	0.58	0.37	0	0	0.24	0.94
Somnolence	0.55	0.39	0	0	0.24	0.94
Orientation to place	0.55	0.39	0	0	0.23	0.94
Anxiety	0.36	0.01	0.33	0.15	0.21	0.85
Concentration	0.27	0.26	0.23	0.04	0.2	0.81
Appetite	0.42	0.06	0	0.28	0.19	0.76
Psychomotor speed	0.36	0.13	0.21	0.06	0.19	0.75
Myalgia	0.04	0.13	0.2	0.33	0.18	0.72
Fatigue	0.34	0.12	0.04	0.2	0.18	0.71
Executive function	0.36	0.1	0.21	0	0.17	0.67
Apathy	0.57	0.05	0.01	0.02	0.16	0.65
Hyperalgesia	0.36	0.06	0.17	0.06	0.16	0.65
Listlessness	0.35	0.18	0.07	0.05	0.16	0.65
Social interaction	0.13	0.27	0.22	0.02	0.16	0.64
Helplessness	0.33	0.05	0.03	0.13	0.13	0.53
Orientation to time	0.29	0.06	0	0.02	0.09	0.36
Total (Eigenvalue)	**5.85**	**2.63**	**1.74**	**1.36**		**11.6**
% of Variance	36.6	16.5	10.9	8.5		72.4

Item total = communality

Table 5.2.7 PCA pattern and structure coefficients, after oblique rotation, on the 16-item scale, sorted by initial item communality

	Component 1		Component 2		Component 3		Component 4		Comm.
Scale item	P	S	P	S	P	S	P	S	
Depression	0.02	0.25	**-0.92**	**-0.95**	0.19	0.36	-0.10	0.04	0.9
Somnolence	0.01	0.23	**-0.93**	**-0.95**	0.17	0.34	-0.10	0.03	0.9
Orientation to place	0.01	0.23	**-0.93**	**-0.95**	0.18	0.34	-0.11	0.03	0.9
Anxiety	-0.10	0.15	-0.19	-0.34	**0.88**	**0.90**	0.04	0.21	0.8
Concentration	**0.93**	**0.89**	0.13	-0.08	-0.01	0.17	-0.06	0.18	0.8
Appetite	0.29	0.50	**-0.32**	**-0.42**	-0.15	0.11	**0.67**	**0.75**	0.8
Psychomotor speed	**0.87**	**0.86**	-0.02	-0.22	0.06	0.24	-0.12	0.13	0.7
Myalgia	-0.22	0.01	0.07	0.03	0.02	0.13	**0.88**	**0.81**	0.7
Fatigue	0.08	0.27	**-0.77**	**-0.76**	-0.27	-0.04	0.27	0.31	0.7
Executive function	**0.80**	**0.81**	-0.11	-0.28	-0.07	0.14	0.00	0.21	0.7
Apathy	**0.48**	**0.66**	-0.27	-0.44	0.12	0.35	**0.34**	**0.52**	0.6
Hyperalgesia	-0.12	0.12	**-0.40**	**-0.50**	**0.66**	**0.71**	0.01	0.15	0.6
Listlessness	**0.35**	**0.52**	0.13	-0.09	**0.56**	**0.67**	0.25	0.44	0.6
Social interaction	0.05	0.25	0.26	0.10	**0.38**	**0.46**	**0.62**	**0.69**	0.6
Helplessness	**0.34**	**0.47**	0.01	-0.18	**0.58**	**0.65**	0.00	0.21	0.5
Orientation to time	**0.34**	**0.48**	-0.14	-0.27	0.09	0.26	**0.32**	**0.44**	0.4

KMO=.78. Bartlett's test of sphericity $\chi^2(120)=938.1$, $p<0.001$
Greyed out figures indicate items loading on to 2 or more factors
Boxed figures indicate the highest component loading for each item

5.2.4.2 PCA to re-assess factor structure and item communality of remaining items

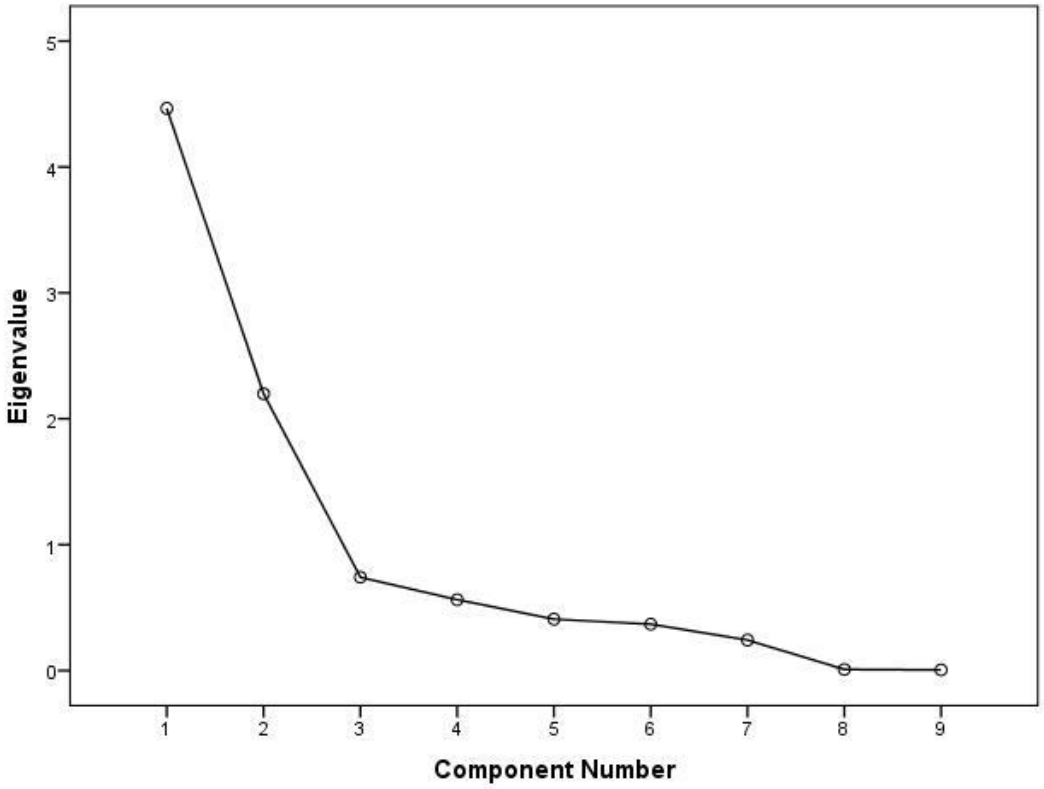
After exclusion of factor complex items with low total item communality, the remaining 10 items now formed a scale that was re-analysed with non-linear Principal Components Analysis, to assess the underlying factor structure and the communality of the remaining items (Table 5.2.8).

The Kaiser-Meyer-Olkin measure verified the sampling adequacy for the analysis (KMO=.81; indicating acceptable sample size).[416] Bartlett's test for non-identity of the correlation matrix was significant (Bartlett's test of sphericity $\chi^2(45)=706.9$, $P<0.001$), indicating acceptable item-item correlations for the analysis.

The Myalgia scale item had a total eigenvalue <0.3 and therefore could not be retained in the solution, leaving a 9-item scale. Without the Myalgia item the variance accounted for by the model increased from 67.3% to 74.2% (Table 5.2.8).

For the 9-item scale, eigenvalues were obtained for each factor in the data. Two factors had eigenvalues greater than Kaiser's criterion of 1, and were also greater than the corresponding value generated by parallel analysis (Table 5.2.9). Examination of the scree plot supported the retention of two factors in the final model, with an inflexion point at the non-retained third factor (Figure 5.2.2).

Figure 5.2.2 Scree plot for 9 item scale



Oblique rotation of the PCA solution was performed to allow for a degree of correlation between underlying factors, generating pattern and structure coefficients. There were no significant differences between the pattern and structure coefficients in the rotated solution, and all items loaded principally on to one or the other of the two retained factors (Table 5.2.10).

The Fatigue item loaded principally on the second factor, but did exhibit a degree of factor complexity, with pattern coefficients of 0.303 on the first component and 0.604 on the second component. However, for both of the retained factors, Fatigue had the lowest loading of all the retained items, indicating a low level of shared variance across factors. In support of retaining Fatigue within the scale, the internal reliability of the scale was not improved when Fatigue was excluded (Cronbach's alpha with Fatigue: 0.84; and without Fatigue: 0.80, Table 5.2.11).

Component loadings for the two retained factors are given in Table 5.2.10, and are presented as vectors in a component loading plot in Figure 5.2.3. In the loading plot (Figure 5.2.3) the coordinates of the end point of each scale item's vector are equal to the loadings of each item on the first and second components (Table 5.2.10). The length of the vector from the origin (0,0) is related to the eigenvalue of that item. Thus, a longer vector represents an item with a higher eigenvalue, and therefore accounts for a larger proportion of the total sample variance. A shorter vector, with end coordinates nearer to the origin, represents an item with a lower eigenvalue, and accounts for a smaller proportion of the total sample variance. The angle between the vectors represents the strength of the correlation between the items. Items with vectors that make a 180˚ angle with each other would be closely negatively related. Vectors making a 90˚ angle indicate items that are not related.

Overall, principal component analysis of the scale produced a 9-item scale (SBS-9) with two underlying latent factors. The loading plot shows two clusters of items, each cluster loading on to one of the two latent factors (Figure 5.2.3). Five items (orientation to place, somnolence, depression, appetite and anxiety) loaded principally on the first component factor, and together accounted for 49% of total scale variance. Four items (concentration, psychomotor speed, executive function and fatigue) loaded principally on the second component, and together accounted for 25% of total scale variance.

5.2.8 Factor analysis by categorical PCA of SBS, with and without myalgia

	Variance accounted for					
	Component 1		Component 2		Total eigenvalue	
Item	**10**	**9**	**10**	**9**	**10**	**9**
Depression	.849	.841	.105	.114	.954	.955
Orientation to place	.828	.822	.124	.132	.952	.954
Somnolence	.818	.813	.125	.131	.943	.944
Concentration	.150	.180	.671	.654	.821	.834
Psychomotor speed	.209	.230	.538	.562	.747	.791
Executive function	.281	.248	.350	.389	.631	.637
Appetite	.593	.592	.024	.029	.617	.621
Fatigue	.431	.407	.126	.144	.557	.551
Anxiety	.305	.309	.088	.083	.393	.392
Myalgia	.005	-	.113	-	.117	-
Total (Eigenvalue)	4.47	4.44	2.26	2.24	6.73	6.68
% of Variance	44.7%	49.4%	22.6%	24.8%	67.3%	74.2%

10-items: KMO=.81, Bartlett's test of sphericity $\chi^2(45)=706.9$, P<0.001
9-items: KMO=.82, Bartlett's test of sphericity $\chi^2(36)=704.1$, P<0.001

Table 5.2.9 Model summary for PCA of the 9 item scale

	Variance accounted for	
Component	Eigenvalue (parallel analysis)	% of Variance
1	4.44 (1.49)	49.366
2	2.24 (1.31)	24.849
Total	6.68	74.2

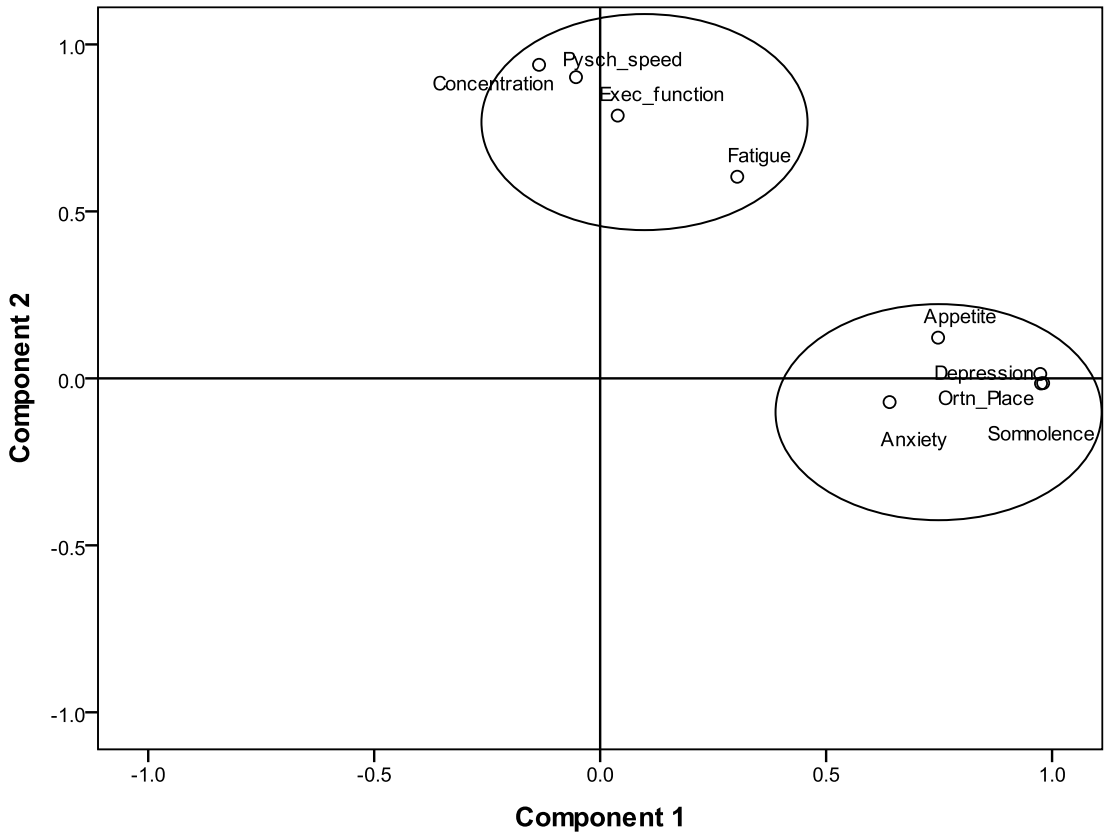
KMO=.82. Bartlett's test of sphericity $\chi^2(36)=704.1$, P<0.001
Parallel analysis eigenvalues generated using MonteCarlo application[403]

Table 5.2.10 Pattern and structure coefficients for 9-item scale after oblique rotation

	Component 1		Component 2	
Scale item	Pattern	Structure	Pattern	Structure
Orientation to place	**.980**	**.977**	-.014	.238
Somnolence	**.976**	**.972**	-.015	.236
Depression	**.974**	**.977**	.013	.264
Appetite	**.748**	**.779**	.122	.315
Anxiety	**.640**	**.622**	-.071	.094
Concentration	-.135	.107	**.939**	**.904**
Psychomotor speed	-.053	.179	**.902**	**.888**
Executive function	.039	.242	**.787**	**.797**
Fatigue	.303	.459	**.604**	**.682**

KMO=.82. Bartlett's test of sphericity $\chi^2(36)=704.1$, $P<0.001$

Figure 5.2.3 Loading plot after oblique rotation of the 9-item scale



5.2.4.3 PCA-derived scale (SBS-9): Reliability

Reliability was assessed by examining the internal consistency of the PCA-derived scale using Cronbach's alpha (Table 5.2.11). No scale items had item-rest correlations so low that they should not be included in the scale (item-rest correlations were all positive and >0.3). The overall scale had high internal consistency and good reliability (Cronbach's alpha=0.84 (95% CI 0.77 to 0.89). Cronbach's alpha was not significantly improved by eliminating any single item. Cronbach's alpha for the SBS-9 is <0.9 indicating that the scale is not so homogenous as to make constituent items redundant.

Table 5.2.11 Item level psychometrics for SBS-9

Item	Observations	Item-rest correlation	Cronbach's alpha[2]
Anxiety	64	0.38	0.84
Orientation to place	64	0.50	0.82
Executive function	64	0.52	0.82
Somnolence	64	0.53	0.82
Concentration	64	0.55	0.82
Appetite	64	0.57	0.82
Depression	64	0.58	0.82
Psychomotor speed	64	0.63	0.81
Fatigue	64	0.67	0.80
Test scale			0.84
		95% CI[1]:	0.77 to 0.89

[1]CI: Confidence interval. Calculated according to the Charter procedure[411]
[2]Cronbach's alpha for the scale without each item

5.2.4.4 PCA-derived scale (SBS-9): test-retest reliability at 7 days

For the PCA-derived scale (SBS-9), there were no significant differences between individuals' test scores at the two time points (one-way, repeated measures ANOVA: $F(1,12)=0.399$, $P=0.539$) (Table 5.2.12). Test-retest reliability over 7 days for the SBS-9 was very good, as indicated by the intraclass correlation (ICC), calculated using a two-way random effects model for single measures (ICC=.94 (95% CI .83 to .98), $F(12,12)=33.6$, $P<0.001$) (Table 5.2.13). An ICC=.94 indicates that 94% of the variance in test scores results from true variance among participants, rather than measurement error. There was a significant simple correlation between the total SBS-9 score at the two time points (Pearson's r=.94, $P<0.001$). The SBS-9 had good precision at 7 days, with an acceptable Standard Error of Measurement (SEMt=1.86), indicating that scores for the SBS-9 can be read as Score±1.9, with a total scale range from 0 to 36 (9 items, scored 0 to 4).

Table 5.2.12 Standard error of measurement assessed by repeated measures ANOVA of SBS-9 total score

		Sum of Squares	Degrees of Freedom	Mean Square	F	P value
Between Subjects		1396.38	12	116.37		
Within Subjects	Between T1 and T2	1.38	1	1.38	0.399	0.539
	Residual	41.62	12	3.47		
	Total	43.00	13	3.31		
Total		1439.38	25	57.57		
Standard Error of Measurement (SEMt)		0.86		√3.47 = 1.86		

T1=initial SBS-9 score, T2=Day 7 SBS-9 score
SEMt = √Mean Square Residual

Table 5.2.13 Test-retest reliability at 7 days, assessed by Intraclass Correlation Coefficient calculated after ANOVA for SBS-9

Intraclass Correlation	95% CI		F test			
	Lower Bound	Upper Bound	F	df1	df2	P value
.94	.83	.98	33.6	12	12	<0.001

Two-way random effects model where both people effects and measures effects are random
Type A intraclass correlation coefficients using an absolute agreement definition

5.2.4.5 Construct validity: correlation of PCA-derived scale with other measures

The PCA-derived scale (SBS-9) had good construct validity in correlation analysis against other psychological measures. Convergent validity was demonstrated by highly significant positive correlations with the Neuropsychiatric Inventory and the Cornell Scale for Depression (Table 5.2.14). Divergent validity was demonstrated by a lack of correlation between SBS-9 and the ADAS-Cog (Table 5.2.14).

The PCA-derived sickness behaviour scale correlated positively with Fried Frailty Score in patients with Alzheimer's disease (Table 5.2.15).

Construct validity for the PCA-derived scale (SBS-9) was assessed by examining the correlation between the SBS-9 score and serum cytokines, with a significant positive correlation between SBS-9 and IFN-γ at baseline: Spearman r=0.28, P=0.02 (Table 5.2.15).

Table 5.2.14 Correlations between SBS-9 and other measures

	SBS-9	NPI	ADAS-Cog	Cornell
NPI	**.66**	1		
	<0.0001			
ADAS-Cog	.11	**.30**	1	
	0.38	**0.02**		
Cornell	**.82**	**.79**	.13	1
	<0.0001	**<0.0001**	0.31	
Fried Score	**.64**	**.49**	.07	**.61**
	<0.0001	**0.0001**	0.57	**<0.0001**

Spearman correlations (n=64, except Fried correlations where n=58)

Table 5.2.15 Spearman correlations between SBS-9 and cytokines

	SBS-9	IFN-γ	IL-10	IL-12	IL-1	IL-6	IL-8	TNF-α	CD40
SBS-9	1								
IFN-γ	**.28**	1							
	0.02								
IL-10	.02	**.30**	1						
	0.88	**0.02**							
IL-12	-.21	**.24**	**.57**	1					
	0.09	**0.05**	**<0.01**						
IL-1	-.14	.06	**.27**	**.50**	1				
	0.28	0.65	**0.03**	**<0.01**					
IL-6	-.05	.22	**.39**	.23	.18	1			
	0.68	0.09	**<0.01**	0.06	0.16				
IL-8	.11	.22	.03	-.01	-.13	.17	1		
	0.38	0.08	0.81	0.96	0.32	0.18			
TNF-α	.16	**.43**	**.31**	.02	-.12	**.38**	**.27**	1	
	0.20	**<0.01**	**0.01**	0.89	0.33	**<0.01**	**0.03**		
CD40	.14	-.07	-.03	-.02	.02	-.11	-.23	-.04	1
	0.29	0.60	0.79	0.89	0.87	0.38	0.07	0.73	

Spearman correlations and P-values
Shading indicates correlations where P≤0.05

5.2.4.6 Construct Validity: Follow up at 6 months

Follow up data for 60 patients was available at 6 months. Reliability assessed by internal consistency was good (Cronbach's alpha=0.74 (95% CI 0.63 to 0.83).

The SBS-9 was stable over 6 months with highly significant positive correlations between baseline and follow up scores: ICC=0.60 (95% CI 0.42 to 0.74) ANOVA (two-way, random effects) F(59,59)=4.1 P<0.001, Spearman's r=0.47, P<0.001. Within subjects there was no significant change between baseline and follow-up at 6 months: the median change was 1.5 (IQR -3.75 to +4), ANOVA for the

difference between the two scores (one-way, repeated measures): F(1,59)=1.8, P=0.18).

Higher baseline scores in SBS-9, NPI and Cornell were associated with less change in the SBS-9 over 6 months (Table 5.2.16). There was no association between baseline cognition as measured by the ADAS-Cog and change in SBS-9 over 6 months (Table 5.2.16).

At six months, correlations between the SBS-9 and other psychometric measures were very similar to the baseline correlations, supporting the pattern of construct validity observed at baseline. Scores on the SBS-9 correlated with NPI (Spearman r=.62, P<0.0001), Cornell (Spearman r=.76, P<0.0001), Fried Score (Spearman r=.47, P=0.0002), but, as at baseline, there was no correlation with ADAS-Cog (Spearman r=.20, P=0.13) (Table 5.2.17).

Further corroborative evidence for the construct validity of the scale was provided by examining associations between SBS-9 and serum cytokines at 6 months, with significant positive correlations between SBS-9 and TNF-α and IL8 at 6 months (Table 5.2.18), but with a positive correlation for IFN-γ at 6 months failing to reach the 0.05 significance level: Spearman r=.22, P=0.085 (Table 5.2.18).

Table 5.2.16 Correlations of change in SBS-9 over 6 months with other measures

Baseline	Change in SBS-9	SBS-9 Baseline
SBS-9	**-0.62**	
	<0.001	
NPI	**-0.29**	**0.66**
	0.026	**<0.001**
ADAS-Cog	0.09	0.11
	0.49	0.38
Cornell	**-0.39**	**0.82**
	0.002	**<0.001**
Fried Score	**-.31**	**.64**
	0.021	**<0.0001**

Table 5.2.17 Correlations of SBS-9 with other measures at 6 months

Follow up (6 months)	SBS-9	NPI	ADAS-Cog	Cornell
NPI	**.62**			
	<0.0001			
ADAS-Cog	.20	.11		
	0.13	0.40		
Cornell	**.76**	**.78**	.12	
	<0.0001	**<0.0001**	0.35	
Fried Score	**.47**	**.43**	.10	**0.36**
	0.0002	**0.0006**	0.43	**0.0051**

Spearman correlations (n=60, except Fried correlations where n=59)

Table 5.2.18 Spearman correlations between SBS-9 and cytokines at 6 months

	SBS-9	IFN-γ	IL-10	IL-12	IL-1	IL-6	IL-8	TNF-α	CD40
SBS-9	1								
IFN-γ	**.22**	1							
	0.085								
IL-10	.19	**.45**	1						
	0.135	**<0.001**							
IL-12	.08	**.35**	**.53**	1					
	0.557	**0.005**	**<0.001**						
IL-1	.08	.16	**.29**	**.62**	1				
	0.561	0.217	**0.02**	**<0.001**					
IL-6	.21	**.32**	**.49**	**.34**	**.31**	1			
	0.110	**0.009**	**<0.001**	**0.006**	**0.012**				
IL-8	**.28**	**.44**	**.51**	.17	.20	**.41**	1		
	0.031	**<0.001**	**<0.001**	0.168	0.110	**0.001**			
TNF-α	**.27**	**.69**	**.55**	**.25**	.06	**.42**	**.47**	1	
	0.04	**<0.001**	**<0.001**	**0.045**	0.627	**<0.001**	**<0.001**		
CD40	-.04	.032	**-.23**	.15	.14	-.07	**-.38**	-.01	1
	0.781	0.816	**0.079**	0.267	0.292	0.583	**0.003**	0.918	

Spearman correlations and P-values

5.2.5 Scale construction by correlation analysis (SBS-6)

5.2.5.1 Correlation of individual scale items with IFN-γ

As described, following analysis of endorsement rate and discrimination index, there were 16 surviving scale items. To build a cytokine-derived scale, we assessed correlation between scale items and serum IFN- γ. In total, six scale items correlated with serum IFN-γ at baseline (Table 5.2.19). These scale items were: Listlessness, Anxiety, Social Interaction, Depression, Fatigue and Orientation in time (all with Spearman's r>0.2 and P<0.1). These six items formed the final 6-item Sickness Behaviour Scale (SBS-6), which was then taken forward to assess reliability and validity.

Table 5.2.19 Scale item correlations with IFN-γ

Scale item	Spearman r	P-value
Listlessness	0.38	0.002
Anxiety	0.30	0.017
Social Interaction	0.29	0.021
Depression	0.29	0.022
Fatigue	0.24	0.054
Orientation in time	0.23	0.066
Hyperalgesia	0.18	0.145
Executive Function	0.15	0.232
Psychomotor Speed	0.15	0.239
Appetite	0.15	0.244
Myalgia	0.14	0.259
Helplessness	0.14	0.273
Apathy	0.14	0.279
Orientation in place	0.11	0.376
Somnolence	0.14	0.394
Concentration	0.07	0.587

5.2.5.2 Cytokine-derived scale (SBS-6): Reliability

Reliability was assessed by examining the internal consistency of the cytokine-derived scale using Cronbach's alpha (Table 5.2.20). No scale items had item-rest correlations so low that they should not be included in the scale (item-rest correlations were all positive and >0.3). The overall scale had high internal consistency and good reliability (Cronbach's alpha=0.79 (95% CI 0.70 to 0.86). Cronbach's alpha was not significantly improved by eliminating any single item. Cronbach's alpha for the SBS-6 is <0.9 indicating that the scale is not so homogenous as to make constituent items redundant.

Table 5.2.20 Item-level and overall scale psychometrics for the IFN-γ derived 6-item Sickness Behaviour Scale

Item	Observations	Item-rest correlation	Cronbach's alpha[2]
Orientation in time	64	0.44	0.79
Social interaction	64	0.46	0.78
Anxiety	64	0.55	0.76
Fatigue	64	0.56	0.75
Depression	64	0.64	0.73
Listlessness	64	0.65	0.73
Test scale			0.79
		95% CI[1]:	0.70 to 0.86

[1]CI: Confidence interval. Calculated according to the Charter procedure[411]
[2]Cronbach's alpha for the scale without each item

5.2.5.3 Cytokine-derived scale: test-retest reliability at 7 days

For the cytokine-derived scale (SBS-6), there were no significant differences between individuals' test scores at baseline and Day 7 (one-way, repeated measures ANOVA: F(1,12)=2.7, P=0.129) (Table 5.2.21). Test-retest reliability over 7 days for the SBS-6 was poor, as indicated by the intraclass correlation (ICC), calculated using a two-way random effects model for single measures (ICC=.66 (95% CI .23 to .88), F(12,12)=5.39, P=0.003) (Table 5.2.22). An ICC=.66 indicates that 66% of the variance in test scores results from true variance among participants, and that 34% of the variance in test scores may

result from measurement error. There was a significant simple correlation between the total SBS-6 score at the two time points (Pearson's r=.69, P=0.009). The SBS-6 had good precision at 7 days, with an acceptable Standard Error of Measurement (SEMt=2.52), indicating that scores for the SBS-6 can be read as Score±2.5.

Table 5.2.21 Standard error of measurement assessed by repeated measures ANOVA of SBS-6 total score

		Sum of Squares	Degrees of Freedom	Mean Square	F	P value
Between Subjects		412.538	12	34.378		
Within Subjects	Between T1 and T2	16.962	1	16.962	2.659	0.129
	Residual	76.538	12	6.378		
	Total	93.500	13	7.192		
Total		506.038	25	20.242		
Standard Error of Measurement (SEMt)				√6.378 = 2.52		

T1=initial SBS-6 score, T2=Day 7 SBS-6 score
SEMt = √Mean Square Residual

Table 5.2.22 Test-retest reliability after 7 days, assessed by Intraclass Correlation Coefficient calculated after ANOVA for SBS-6

Intraclass Correlation	95% CI		F test			
	Lower Bound	Upper Bound	F	df1	df2	P value
.661	.225	.881	5.390	12	12	0.003

Two-way random effects model where both people effects and measures effects are random
Type A intraclass correlation coefficients using an absolute agreement definition

5.2.5.4 Construct validity of the cytokine-derived scale: latent factor structure

The construct validity of the scale was assessed using factor analysis. We found that items related to mood were clustered on a single factor accounting for more than 50% of the variance of the total scale. This result was obtained from non-linear principal components analysis conducted on the cytokine-derived scale (SBS-6) with oblique rotation. The Kaiser-Meyer-Olkin measure verified the sampling adequacy for the analysis (KMO=.84; indicating good (meritorious) sample size)[416] and Bartlett's test for non-identity of the correlation matrix was significant (Bartlett's test of sphericity $\chi^2(15)=114.7$, $P<0.001$) (Table 5.2.23). All items were retained in the solution after oblique rotation, as no items had significant differences between pattern and structure coefficients, and no items had significant loadings (>0.3) on to more than one component (Table 5.2.24). Eigenvalues were obtained for each factor in the data. Only one factor had an eigenvalue greater than Kaiser's criterion of 1, and this factor explained 52.6% of the variance (Table 5.2.23). The scree plot showed an inflection at the second component, providing further evidence for the retention of only one factor in the solution (Figure 5.2.4). Items related to mood clustered on the retained factor: Fatigue, Listlessness, Anxiety and Depression (Figure 5.2.5). These four items were the sickness behaviours that contributed most to overall variance in the combined construct of neuroimmune sickness behaviour, with disorientation in time and social withdrawal acting more independently on total scale variance.

Table 5.2.23 Model summary for PCA of 6 item scale

	Variance accounted for	
Component	Eigenvalue (parallel analysis)	% of Variance
1	3.157 (1.34)	52.611
2	0.748 (1.17)	12.460
3	0.713 (1.05)	11.884
Total	4.617	76.956

KMO=.84. Bartlett's test of sphericity $\chi^2(15)=114.7$, $P<0.001$
Parallel analysis eigenvalues generated using MonteCarlo application[403]

Figure 5.2.4 Scree plot after Principal Components Analysis of 6-item IFN-γ derived scale

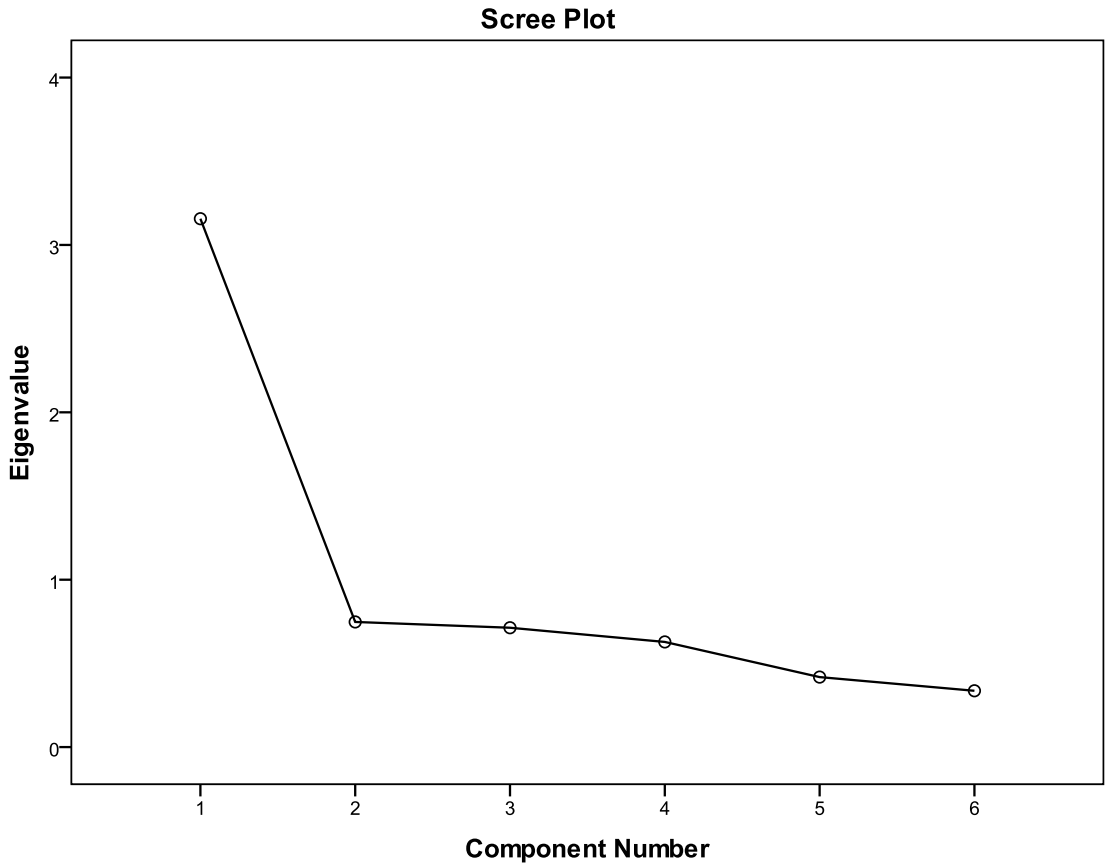
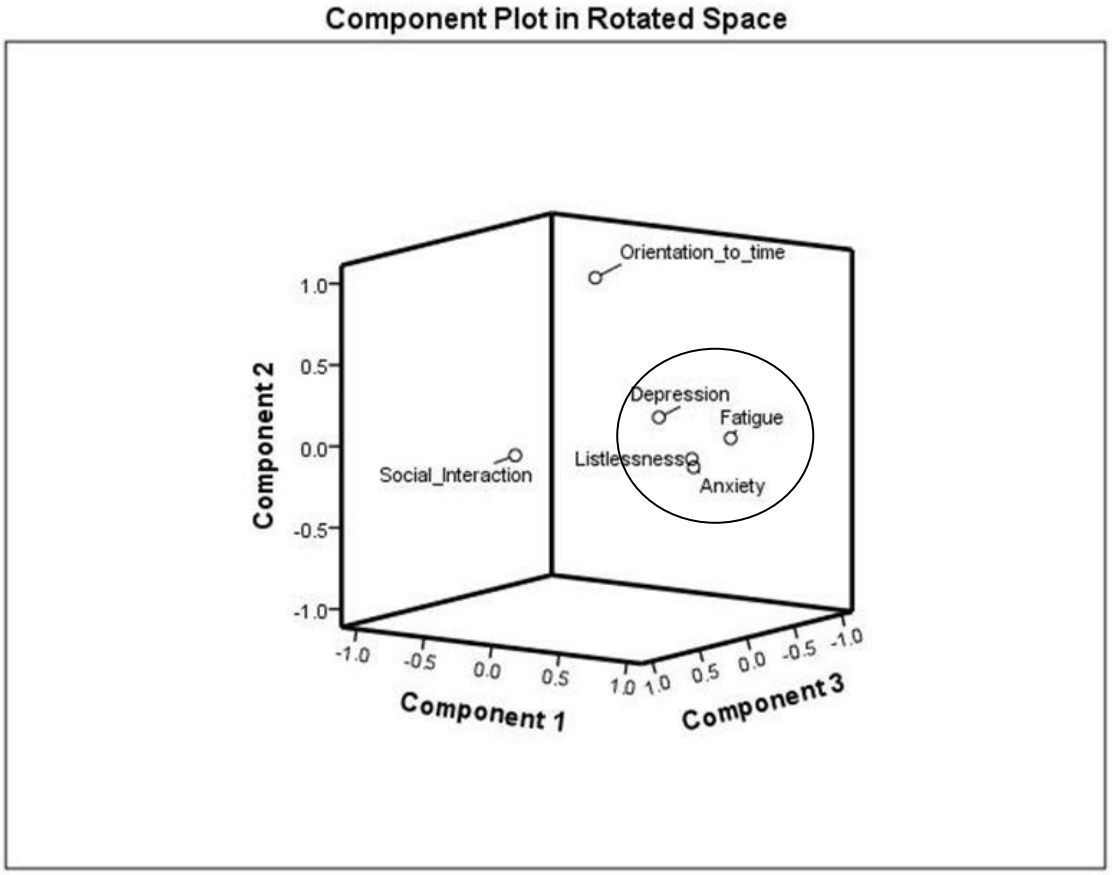


Table 5.2.24 Pattern and structure coefficients after PCA on the 6-item scale

	Component 1		Component 2		Component 3	
Scale item	Pattern	Structure	Pattern	Structure	Pattern	Structure
Fatigue	**.838**	.781	.055	.356	-.217	.104
Listlessness	**.812**	.860	-.021	.350	.156	.451
Anxiety	**.792**	.798	-.081	.272	.108	.382
Depression	**.591**	.750	.217	.503	.187	.453
Orientation to time	-.004	.410	**.989**	.989	.010	.224
Social interaction	.053	.415	.037	.264	**.938**	.966

KMO=.84. Bartlett's test of sphericity $\chi^2(15)=114.7$, $P<0.001$
Shading indicates significant loading on to a component

Figure 5.2.5 Component plot after principal components analysis of 6-item IFN-γ derived scale (SBS-6)



5.2.5.5 Construct validity: correlation of cytokine-derived scale with other measures

The cytokine-derived scale (SBS-6) had good construct validity in correlation analysis against other psychological measures. Convergent validity was demonstrated by highly significant positive correlations with the Neuropsychiatric Inventory and the Cornell Scale for Depression (Table 5.2.25). Divergent validity was demonstrated by a lack of correlation between SBS-6 and the ADAS-Cog (Table 5.2.25).

The cytokine-derived sickness behaviour scale correlated positively with Fried Frailty Score in patients with Alzheimer's disease (Table 5.2.25).

Items in the cytokine-derived scale were selected because of their correlation with IFN-γ, and, as expected, the overall scale correlated with IFN-γ. However, at baseline, other measured serum cytokines did not correlate well with the overall SBS-6 (Table 5.2.26).

Table 5.2.25 Correlations between SBS-6 and other psychological measures

	SBS-6	NPI	ADAS-Cog	Cornell
NPI	**.66**	1		
	<0.001			
ADAS-Cog	.14	**.30**	1	
	0.28	**0.02**		
Cornell	**.83**	**.79**	.13	1
	<0.001	**<0.001**	0.31	
Fried Score	**.65**	**.49**	.07	**.61**
	<0.0001	**0.0001**	0.57	**<0.0001**

Spearman correlations

5.2.5.6 Construct Validity: Follow up at 6 months

Follow up data for 60 patients was available at 6 months. Reliability assessed by internal consistency was acceptable (Cronbach's alpha=.64 (95% CI 0.48 to 0.77) (Table 5.2.27).

The SBS-6 was stable over 6 months with highly significant positive correlations between baseline and follow up scores: ICC=0.57 (95% CI 0.37 to 0.72) ANOVA (two-way, random effects) F(59,59)=3.7 P<0.001, Spearman's r=0.51, P<0.001. Within subjects there was no significant change between baseline and follow-up at 6 months: the median change was -1 (IQR -4 to +2), ANOVA for the difference between the two scores (one-way, repeated measures): F(1,59)=2.2, P=0.15).

Higher baseline scores in SBS-6, NPI and Cornell were associated with less change in the SBS-6 over 6 months (Table 5.2.28). There was no association between baseline cognition as measured by the ADAS-Cog and change in SBS-6 over 6 months (Table 5.2.28).

At six months, correlations between the SBS-6 and other psychometric measures were very similar to the baseline correlations. Scores on the SBS-6 correlated with NPI (Spearman r=.58, P<0.0001), Cornell (Spearman r=.74,

P<0.0001), Fried Score (Spearman r=.44, P=0.0004), and, as at baseline, there was no correlation with ADAS-Cog (Spearman r=-.09, P=0.13) (Table 5.2.29).

Further corroborative evidence for the construct validity of the scale was provided by examining associations between SBS-6 and serum cytokines at 6 months, with positive correlations between SBS-6 and IFN-γ, TNF-α, IL8 and IL10 at 6 months (Table 5.2.29).

Table 5.2.26 Spearman correlations between SBS-6 and systemic cytokines

	SBS-6	IFN-γ	IL-10	IL-12	IL-1	IL-6	IL-8	TNF-α	CD40
SBS-6	1								
IFN-γ	**.43**	1							
	<0.01								
IL-10	.13	**.30**	1						
	0.29	**0.02**							
IL-12	-.11	**.24**	**.57**	1					
	0.37	**0.05**	**<0.01**						
IL-1	-.07	.06	**.27**	**.50**	1				
	0.56	0.65	**0.03**	**<0.01**					
IL-6	-.01	.22	**.39**	.23	.18	1			
	0.95	0.09	**<0.01**	0.06	0.16				
IL-8	.08	.22	.03	-.01	-.13	.17	1		
	0.53	0.08	0.81	0.96	0.32	0.18			
TNF-α	.13	**.43**	**.31**	.02	-.12	**.38**	**.27**	1	
	0.32	**<0.01**	**0.01**	0.89	0.33	**<0.01**	**0.03**		
CD40	.10	-.07	-.03	-.02	.02	-.11	-.23	-.04	1
	0.42	0.60	0.79	0.89	0.87	0.38	0.07	0.73	

Spearman correlations and P-values
Shading indicates correlations where P≤0.05

Table 5.2.27 Item-level and overall scale psychometrics for the SBS-6 Sickness Behaviour Scale at 6 months follow up

Item	Observations	Item-rest correlation	Cronbach's alpha
Orientation to time	60	0.21	0.65
Listlessness	60	0.31	0.63
Social Interaction	60	0.38	0.60
Anxiety	60	0.40	0.59
Depression	60	0.44	0.58
Fatigue	60	0.53	0.53
Test scale			0.64
		95% CI[1]:	0.48 to 0.77

[1]CI: Confidence interval. Calculated according to the Charter procedure[411]

Table 5.2.28 Correlations of change in SBS-6 over 6 months with other measures

Baseline scores	Change in SBS-6 (n=60)	Baseline SBS-6 (n=64)
	r P-value	r P-value
SBS-6	**-.66**	
	<0.001	
NPI	**-.36**	**.66**
	0.005	**<0.001**
Cornell	**-.47**	**.83**
	<0.001	**<0.001**
ADAS-Cog	-0.16	.14
	0.21	0.28
Fried Score	**-.36**[†]	**.65**[‡]
	0.007	**<0.0001**

Data are Spearman correlations and P-values
[†] n=54 [‡]n=58

Table 5.2.29 Correlations of SBS-6 with other measures at 6 monthsCorrelations of SBS-6 with other measures at 6 months

Follow up (6 months)	SBS-6	NPI	ADAS-Cog	Cornell
NPI	**.58**			
	<0.0001			
ADAS-Cog	-.09	.11		
	0.13	0.40		
Cornell	**.74**	**.78**	.12	
	<0.0001	**<0.0001**	.35	
Fried Score	**.44**	**.43**	.10	**.36**
	0.0004	**0.0006**	0.43	**0.0051**

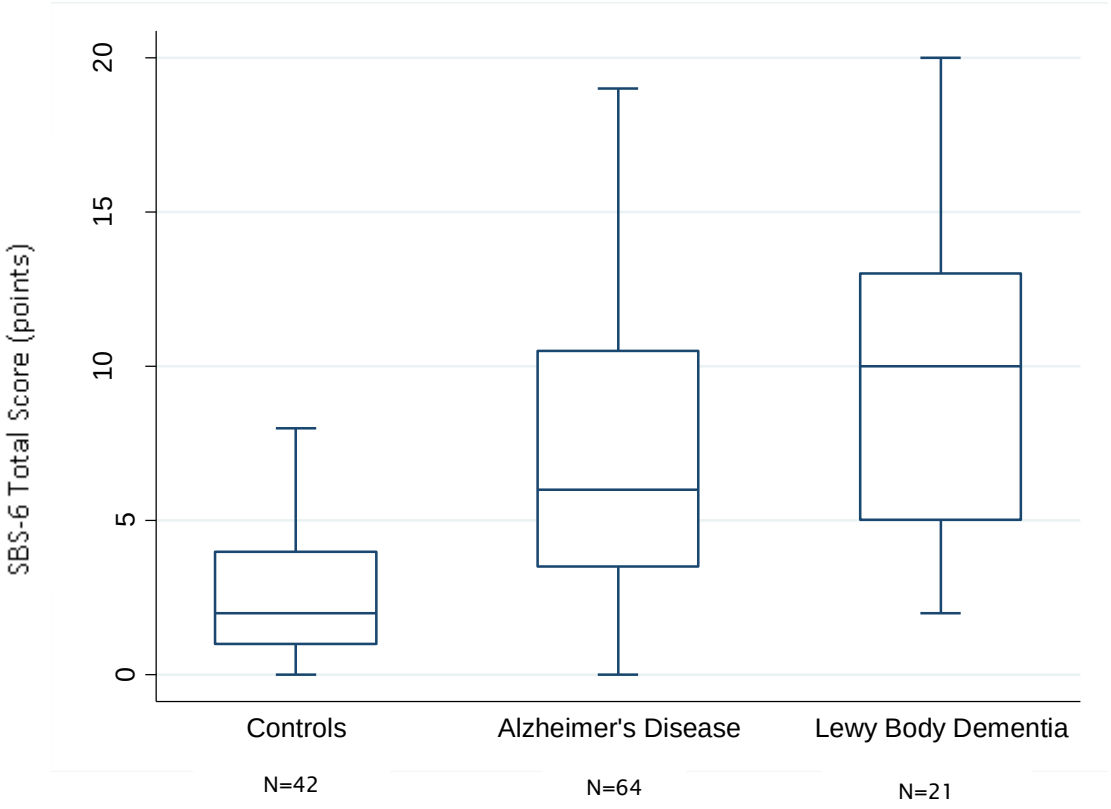
Spearman correlations (n=60, except Fried Score correlations where n=59)

Table 5.2.30 Correlations between SBS-6 and cytokines at 6 months

	SBS-6	IFN-γ	IL-10	IL-12	IL-1	IL-6	IL-8	TNF-α	CD40
	r P value								
SBS-6	1								
IFN-γ	**.36**	1							
	<0.01								
IL-10	**.28**	.25	1						
	0.03	0.06							
IL-12	-.05	.11	**.35**	1					
	0.72	0.40	**<0.01**						
IL-1	.00	-.02	.14	**.56**	1				
	0.99	0.88	0.31	**<0.01**					
IL-6	.11	.06	**.28**	.09	.17	1			
	0.42	0.68	**0.04**	0.49	0.22				
IL-8	**.35**	.23	**.31**	-.13	.04	.17	1		
	<0.01	0.09	**0.02**	0.33	0.78	0.22			
TNF-α	**.31**	**.58**	**.37**	-.02	-.15	.18	.25	1	
	0.02	**<0.01**	**<0.01**	0.88	0.27	0.17	0.07		
CD40	-.01	.03	-.23	.15	.14	-.07	**-.38**	-.01	1
	0.95	0.82	0.08	0.27	0.29	0.58	**<0.01**	0.92	

Spearman correlations and P-values
Shading indicates correlations where P≤0.05

Figure 5.2.6 Box Plot of SBS-6 in different patient groups

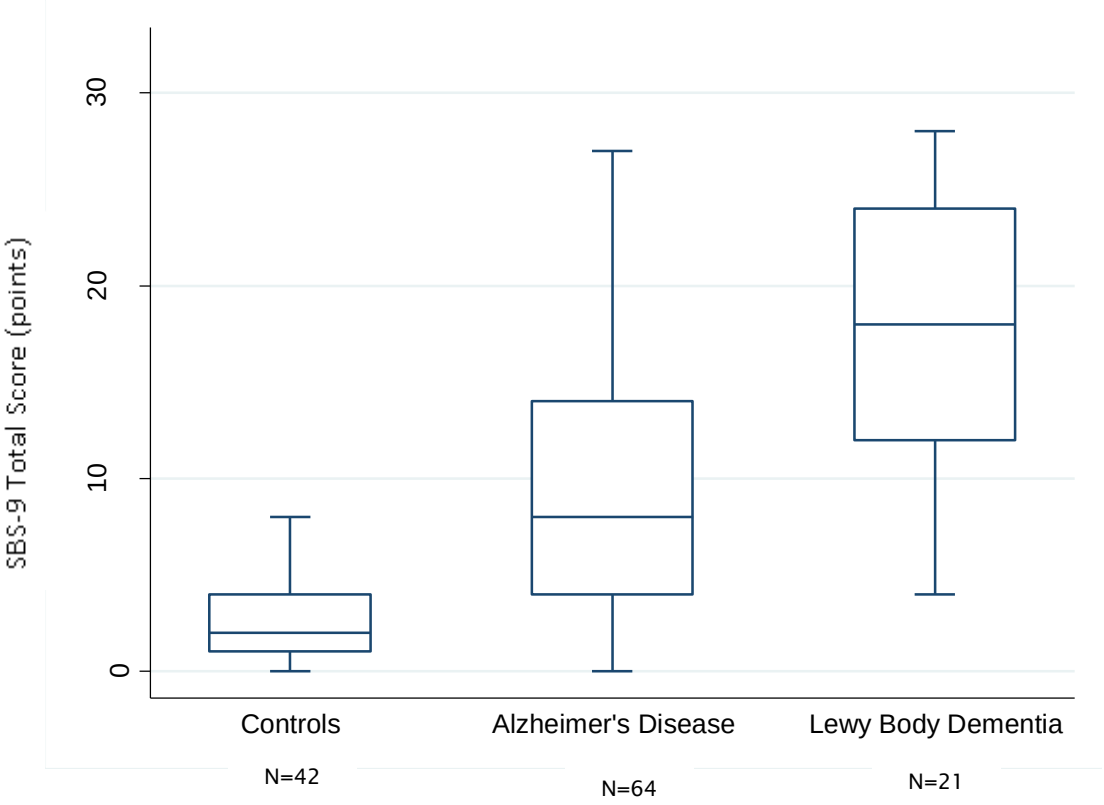


Kruskal-Wallis Equality of Populations Rank Test: $\chi^2(2, n=127)=36.2$, $P<0.001$
Box shows median, 25th and 75th centiles.
Whiskers show upper and lower adjacent values, the smallest and largest values within 1.5 x IQR of the 25th and 75th centiles. SBS-6 is scored in points out of 24.

5.2.6 Discriminant Validity of SBS-6 and SBS-9

Both SBS-6 and SBS-9 demonstrated good discriminant validity with median scale scores differing between participants with Alzheimer's disease, participants with Lewy Body dementia and participants with normal cognition; SBS-9 Kruskal-Wallis Equality of Populations Rank Test: $\chi^2(2, n=127)=52.0$, $p<0.001$ and SBS-6 Kruskal-Wallis Equality of Populations Rank Test: $\chi^2(2, n=127)=36.2$, $p<0.001$ (Figure 5.2.6 and Figure 5.2.7). For both scales participants with Lewy Body dementia had significantly higher scores than the participants with Alzheimer's disease.

Figure 5.2.7 Box Plot of SBS-9 in different patient groups



Kruskal-Wallis Equality of Populations Rank Test: $\chi^2(2, n=127)=52.0$, $P<0.001$
Box shows median, 25th and 75th centiles.
Whiskers show upper and lower adjacent values, the smallest and largest values within 1.5 x IQR of the 25th and 75th centiles. SBS-9 is scored in points out of 36.

5.2.7 Comparison of SBS-6 and SBS-9

A summary of the validity and reliability data for the two scales is given in Table 5.2.31.

Table 5.2.31 Comparison of reliability and validity data for the cytokine-derived sickness behaviour scale (SBS-6) and the principal components analysis-derived scale (SBS-9)

	SBS-6	SBS-9
Descriptives		
Possible score range	0 to 24	0 to 36
Range in data	0 to 19	0 to 32
Median (IQR)	6 (3 to 11)	8 (4 to 14)
Standard Error of Measurement	±2.5	±1.9
At six months:		
Median Change (IQR)	-1.0 (IQR -4 to +2)	+1.5 (IQR -3.75 to +4)
ICC (95% CI)	ICC=.57 (.37 to .72)	ICC=.60 (.42 to .74)
F-test	F(59,59)=3.7, P<0.001	F(59,59)=4.1, P<0.001
Reliability		
Cronbach's alpha	α=.79 (.70-.86)	α=.84 (.77-.89)
At six months: Cronbach's alpha	α=.64 (.48-.77)	α=.74 (.63-.83)
Construct validity		
NPI	r=.66, P<0.001	r=.66, P<0.0001
Cornell	r=.83, P<0.001	r=.82, P<0.0001
ADAS-Cog	r=.14, P=0.28	r=.11, P=0.38
Fried Score	r=.65, P<0.0001	r=.64, P<0.0001
IFN-γ	r=.43, P<0.01	r=.28, P=0.02
At T2		
IFN-γ	r=.36, P<0.01	r=.23, P=0.08
IL-8	r=.35, P<0.01	r=.30, P=0.02
TNF-α	r=.31, P=0.02	r=.28, P=0.03
Test-retest (7-day)		
ICC (95% CI)	ICC=.66 (.23 to .88) F(12,12)=5.4, P=0.003	ICC=.94 (.83 to .98) F(12,12)=33.6, P<0.001
Pearson correlation	r=.69, P=0.009	r=.94, P<0.001

	SBS-6	SBS-9
Factor structure		
Variance accounted for by model	Retained 1 factor model Model VAF=52.6% F1 VAF=52.6%	Retained 2 factor model Model VAF=74.0% F1 VAF=49.6% F2 VAF=24.4%
Factor 1 loadings	Depression Fatigue Anxiety Listlessness	Orientation to place Somnolence Depression Appetite Anxiety
Factor 2 loadings	Not applicable	Concentration Psychomotor speed Executive function Fatigue
No significant loadings	Orientation to time Social interaction	None
Is scale construction independent of cytokines?	No Stronger correlations with cytokines at baseline and at six months	Yes Psychometrically independent of cytokines, therefore cytokine correlation is a better test of construct validity
Do scale items contribute to overall variance?	2 scale items (orientation to time and social interaction) do not contribute significantly to overall scale variance	All scale items contribute to overall scale variance
Do scale items all correlate with IFN-γ?	Yes	No
Scale length	6 items	9 items

5.2.8 Validation of SBS-6 and SBS-9 in an independent cohort

Both the SBS-6 and SBS-9 versions of the Sickness Behaviour Scale were examined in an independent cohort of people with Alzheimer's disease. These were subjects enrolled in the STEADI-09 trial. Data from baseline visits, prior to administration of any study drug, were analysed to assess the validity of the SBS-6 and SBS-9 in a separate cohort.

The range of SBS-6 and SBS-9 scores were lower in the STEADI-09 cohort than in the original validation cohort (Table 5.2.32).

In support of the construct validity of the scales, within the independent STEADI cohort, both SBS-6 and SBS-9 had significant positive correlations with loss of functional ability as measured by BADLS, with neuropsychiatric symptoms as measured by the NPI, with depression as measured by the Cornell Scale, and with physical frailty as measured by the Fried Frailty Score (Table 5.2.33).

In this validation cohort there was a trend towards an association between the level of sickness behaviour as measured by the scales and poor cognition as measured by the ADAS-Cog and MMSE (Table 5.2.33). For the ADAS-Cog in particular this association was close to the 0.05 significance level: ADAS-Cog vs SBS-6: Spearman r=.27, P=0.08; ADAS-Cog vs SBS-9: Spearman r=.29, P=0.06 (Table 5.2.33).

There were no significant correlations between either of the sickness behaviour scales and serum cytokines in the baseline STEADI cohort (Table 5.2.34).

Table 5.2.32 SBS-6 and SBS-9 within a separate Alzheimer's disease cohort, compared with original scale construction cohort

	SBS-6		SBS-9	
	STEADI (n=41)	SBS (n=64)	STEADI (n=41)	SBS (n=64)
Median (pts)	4	6	6	8
IQR	(2 to 6)	(3 to 11)	(3.5 to 10.5)	(4 to 14)
Range	0 to 18	0 to 19	0 to 24	0 to 32
(Max range)	(0 to 24)	(0 to 24)	(0 to 36)	(0 to 36)

Table 5.2.33 SBS-6 and SBS-9 in a separate Alzheimer's disease cohort: comparison of correlations with other psychological and behavioural measures

		SBS-6 (n=41)	**SBS-9** (n=41)
SBS-6	Spearman r (n=41)	1	**.76**
	P-value	.	**<0.001**
BADLS	Spearman r	**.40**	**.54**
	P-value	**0.01**	**<0.001**
NPI	Spearman r	**.55**	**.62**
	P-value	**<0.001**	**<0.001**
MMSE	Spearman r	-.25	-.25
	P-value	0.12	0.12
ADAS-Cog	Spearman r	.27	.29
	P-value	0.08	0.06
Fried Scale	Spearman r	**.36**	**.36**
	P-value	**0.02**	**0.02**
CORNELL	Spearman r	**.72**	**.80**
	P-value	**<0.001**	**<0.001**

Table 5.2.34 SBS-6 and SBS-9 in a separate Alzheimer's disease cohort: comparison of correlations with serum cytokines

		SBS-6	SBS-9
SBS-6	Spearman r (n=41)	1	**.76**
	P-value	-	**<0.001**
CRP	Spearman r (n=40)	-.07	-.11
	P-value	0.68	0.50
IFN-γ	Spearman r (n=40)	-.00	-.01
	P-value	0.99	0.94
IL10	Spearman r (n=40)	-.05	-.09
	P-value	0.76	0.59
IL12	Spearman r (n=40)	-.03	-.24
	P-value	0.83	0.13
IL6	Spearman r (n=40)	.04	.21
	P-value	0.80	0.19
IL8	Spearman r (n=40)	-.02	-.08
	P-value	0.91	0.64
TNF-α	Spearman r (n=40)	.14	.15
	P-value	0.39	0.34

5.3 Sickness Behaviour Scale: Discussion

The work described here sought to answer whether a scale could be developed to measure maladaptive neuroimmune sickness behaviour in people with Alzheimer's disease. We developed the SBS-9 scale and the results show that this scale has good reliability and validity in a construction cohort and in an independent validation cohort. Several scales have previously been developed to measure various aspects of behavioural and psychological symptoms in dementia. However, the SBS-9 scale that we have developed is the first to draw together, into a single scale, those symptoms that are thought to arise from maladaptive neuroimmune sickness behaviour in dementia.

We also aimed to assess which of two alternative methods of scale construction produced the most valid and reliable scale. One method relied on the hypothesis that neuroimmune sickness behaviour symptoms tend to occur together, and that a scale based on factor analysis of the symptoms would produce the most reliable and valid scale. This method produced the SBS-9 scale. The second method relied on the hypothesis that systemic inflammatory cytokines are the principal driver of neuroimmune sickness behaviour, and that a scale based on the symptoms that correlate best with pro-inflammatory cytokines would produce the most reliable and valid scale. This method produced the SBS-6 scale. The results show that the SBS-9 scale has better psychometric properties than the SBS-6 scale on the majority of measures of scale performance (Table 5.2.31). The finding that the SBS-9 has better psychometric properties than the SBS-6 challenges the hypothesis underlying the construction of the SBS-6 scale; namely, that systemic inflammatory cytokines are the principal driver of neuroimmune sickness behaviour in Alzheimer's disease.

The SBS-9 had good reliability on assessment of internal consistency and test-retest reliability. As described in the methods, the reliability of a scale concerns the degree of measurement error, and Cronbach's alpha is a measure of reliability based on the internal consistency of a congeneric scale. Psychometric theory suggests that reliable scales have Cronbach's alpha greater than 0.80, but less than 0.90.[394] The SBS-9 has good reliability with Cronbach's alpha=0.84 (95% CI 0.77 to 0.89). This indicates that

measurement error due to poor co-variability between individual scale items is low for the SBS-9.

The SBS-9 contains a manageable number of items, and is straightforward to complete by an informant close to the person with dementia. The scale has good face validity, with retained scale items falling across several sickness behaviour domains (Table 5.1.1).

We assessed the construct validity of the SBS-9 using principal components analysis. Two underlying latent factors were obtained, and these factors can be visualized by inspection of the loading plot (Figure 5.2.3). The first factor, accounting for 49% of the total scale variance, is characterized by sickness behaviours associated with decreased motivation to interact and low emotional affect (orientation to place, somnolence, depression, appetite and anxiety). The second factor, accounting for 25% of the total scale variance, is characterized by sickness behaviours associated with decreased functional ability and decreased cognitive efficiency (concentration, psychomotor speed, executive function). Fatigue, the final scale item, had significant loadings on both factors (Factor 1 pattern coefficient=0.30, Factor 2 pattern coefficient=0.60, Table 5.2.10). Fatigue has aspects of both the latent factors and has previously been characterized as a state of reduced motivation (Factor 1) and reduced functional efficiency (Factor 2).[417]

Previous work on sickness behaviour supports the view that sickness behaviours reflect an altered state of motivation, directing energy towards repair and protection rather than towards exploration and interaction; and an altered state of functional ability, with efficiency and efficacy reduced because of diversion of energy towards repair and protection.[16 22] These altered states of motivation and functional ability are reflected well by the latent factors that are seen in the principal components analysis of the SBS-9, where the first factor represents a state of altered motivation, and the second factor represents a state of altered functional ability. Thus, the construct validity of the SBS-9 is supported by our finding of underlying latent factors that are consistent with the accepted understanding of sickness behaviour as a motivational state that allows for diversion of energy away from functional efficacy towards systems of repair and protection.

Further evidence for the construct validity of the SBS-9 was demonstrated by convergent and divergent validation against other previously validated psychometric measures. The SBS-9 had a significant positive correlation with the NPI in both the SBS construction cohort and in the STEADI-09 validation cohort (SBS cohort: r=0.66, P<0.0001; STEADI-09 cohort: r=0.62, P<0.001, Table 5.2.14 and Table 5.2.33). This positive correlation was stable across 6 months (SBS cohort at 6 months: r=0.62, P<0.0001, Table 5.2.17), increasing confidence in the finding of a positive association. The NPI consists of ten domains of behavioural and psychological symptoms, some of which are likely to be related to sickness behaviour (hallucinations, depression, anxiety, apathy), and some of which are less likely to be related to sickness behaviour (euphoria, aggression, disinhibition, motor symptoms, delusions).[371] Previous work has demonstrated a correlation between systemic pro-inflammatory cytokines (TNF-α and IL-6) and total NPI score in patients with Alzheimer's disease, together with significant correlations between systemic pro-inflammatory cytokines and the constituents of the NPI most likely to represent neuroimmune sickness behaviour: apathy, depression and anxiety.[15] The convergent construct validity of the SBS-9 is therefore supported by the finding of a correlation against the NPI. The correlation is highly significant but modest in size, as expected for scales that shared some common domains, but that measure different overall constructs.

The SBS-9 demonstrated convergent validity against the Cornell Scale for Depression in Dementia.[369] Affective symptoms are key features of neuroimmune sickness behaviour, reflecting decreased motivation to interact and explore when unwell.[17] We found a positive correlation between the SBS-9 and the Cornell in the SBS construction cohort at baseline and at 6 months, and in the independent STEADI-09 validation cohort (SBS cohort: r=0.82, P<0.0001, Table 5.2.14; SBS cohort at 6 months: r=0.76, P<0.0001, Table 5.2.17; STEADI-09 cohort: r=0.80, P<0.001, Table 5.2.33). The correlation is moderate (0.6 to 0.9), rather than strong (>0.9),[395] indicating that the SBS-9 shares some variability with a validated depression scale, but that not all the variability in the SBS-9 can be explained by a depression scale.

Some aspects of physical frailty share common features with neuroimmune sickness behaviour. Convergent construct validity for the SBS-9 was therefore

also examined by assessing the degree of correlation against the Fried Frailty Score.[375] This validated measure includes a grading of the statement "Everything I did was an effort, or, I could not get going" – reflecting a level of fatigue and loss of motivation that may reflect neuroimmune sickness behaviour. We found weak to moderate correlations between the SBS-9 and the Fried Frailty Score in the SBS construction cohort at baseline and at 6 months, and in the independent STEADI-09 validation cohort (SBS cohort: r=0.64, P<0.0001, Table 5.2.14; SBS cohort at 6 months: r=0.47, P=0.0002, Table 5.2.17; STEADI-09 cohort: r=0.36, P=0.02, Table 5.2.33). Thus, those aspects of maladaptive neuroimmune sickness behaviour that contribute to a phenotype of physical frailty appear to be captured by the SBS-9, providing further evidence for the construct validity of the scale.

Divergent construct validity for the SBS-9 was demonstrated by the lack of correlation between the SBS-9 and cognition measured with the ADAS-Cog (SBS cohort: r=0.11, P=0.38, Table 5.2.14; SBS cohort at 6 months: r=0.20, P=0.13, Table 5.2.17). This supports the divergent validity of the SBS-9 scale in that the scale does not purport to measure cognition and we would therefore not expect to find a strong correlation against a cognitive scale. However, patients with Alzheimer's disease have activated microglia, contributing both to neurodegeneration and to the triggering of sickness behaviour, and we therefore expected to find evidence for a weak relationship between maladaptive sickness behaviour and the degree of cognitive decline. We therefore need to consider why we did not find evidence for a relationship between cognition and the SBS-9 in the construction cohort.

As discussed in Chapter 2, there is evidence that CNS inflammatory processes occur early in the development of Alzheimer's disease. Thus, inflammatory markers predict cognitive decline in people without dementia, and predict conversion to Alzheimer's disease in people with mild cognitive impairment.[418-420] Given that CNS inflammation appears to precede symptomatic neurodegeneration, pathological sickness behaviour may occur early in Alzheimer's disease, before a clinical diagnosis of dementia is made. Therefore, in Alzheimer's disease, the relationship between pathological sickness behaviour and cognition is unlikely to be linear. In early Alzheimer's disease, sickness behaviour may be evident when cognition is still well

preserved, and sickness behaviour may decrease in severity when neurodegeneration becomes very severe. This stage-dependent relationship between pathological sickness behaviour and cognition may obscure a direct linear correlation between measures of sickness behaviour and measures of cognition.

A further reason for the lack of linear correlation between the SBS-9 and cognition arises because cognitive symptoms were excluded from the SBS-9 during scale development. The scale item relating to poor short term memory was excluded from the SBS-9 scale because of an endorsement rate greater than 95% in the construction phase of scale development. At that level of endorsement the short term memory item could not contribute to the total scale variance and its inclusion would weaken the psychometric strength of the scale.[394] However, exclusion of the short term memory item may decrease the ability of the scale to capture a relationship with a cognitive measure like the ADAS-Cog.

There may be advantages to a sickness behaviour scale in Alzheimer's disease that does not track cognition too closely, because the noise of the on-going cognitive decline may drown out the signal of the maladaptive sickness behaviour.

However, despite these considerations about sickness behaviour and cognition in Alzheimer's disease, we still expected to find a weak relationship between our measure of sickness behaviour and measures of cognition, because the two processes are hypothesized to have the common pathophysiology of activated microglia. In the independent STEADI-09 validation cohort there was a statistical trend towards a positive correlation between SBS-9 and ADAS-Cog (STEADI-09 cohort: r=0.29, P=0.06, Table 5.2.33), but not between the SBS-9 and MMSE (STEADI-09 cohort: r=-0.25, P=0.12, Table 5.2.33). Overall, there is no strong relationship between the SBS-9 and cognition, suggesting that the SBS-9 has divergent validity against cognitive measures, and that larger numbers of study participants would be necessary to demonstrate the expected weak relationship between poor cognition and sickness behaviour measured with the SBS-9 in people with Alzheimer's disease.

Sickness behaviour reduces efficacy in usual everyday activity, diverting energy towards homeostatic systems of repair and protection.[16 21] We examined the relationship between SBS-9 score and performance in everyday activities measured by the Bristol Activities of Daily Living Scale (BADLS).[373] Further evidence for the construct validity of the SBS-9 is therefore demonstrated by our finding of a highly significant positive correlation between the SBS-9 and BADLS in the independent STEADI-09 validation cohort ($r=0.54$, $P<0.001$, Table 5.2.33).

We hypothesized that there would be a relationship between systemic inflammatory cytokines and sickness behaviour in people with Alzheimer's disease. Evidence supporting this hypothesis would also add to the construct validity of the sickness behaviour scale. We found evidence for a weak correlation between SBS-9 and IFN-γ at baseline ($r=0.28$, $P=0.02$, Table 5.2.15), but no other correlation between SBS-9 and the other measured cytokines at baseline (Table 5.2.15). At six months we found significant correlations between the SBS-9 and TNF-α ($r=0.27$, $P=0.04$), and IL-8 ($r=0.28$, $P=0.03$), but the correlation with IFN-γ was less significant than at baseline ($r=0.22$, $P=0.08$) (Table 5.2.18). We did not find any evidence of a relationship between SBS-9 and peripheral cytokines in the independent STEADI-09 validation cohort. Together, these results suggest that the relationship between sickness behaviour, as measured by the SBS-9, and peripheral cytokines, measured at a single time point, is not a simple, linear relationship of cause and effect. Sampling of cytokines at a single time point may be an ineffective measure of immune system activation or quiescence. Thus, Holmes et al. found a relationship between sickness behaviour symptoms measured by the NPI and IL-6 and TNF-α in patients with Alzheimer's disease, by combining cytokine measurements at four time points across six months to give an average cytokine level across the six month time period.[15] However, this approach was not possible for a single measure of circulating cytokine levels taken at baseline in the STEADI-09 validation cohort. Cytokines produced by immune system cells are most effective at signalling to other immune system cells at or near their site of production, as autocrine or paracrine signalling molecules.[405] Therefore, levels of circulating cytokines in peripheral blood may simply represent escape of surplus cytokines into the circulation from the site of immune activation, and may have less functional significance than the levels

of circulating lymphocytes and granulocytes in peripheral blood, or the levels of CNS cytokines.[417]

The age distribution of study participants may provide a further reason for the lack of any correlation between the SBS-9 and cytokines in the independent STEADI-09 validation cohort. The sickness behaviour scale construction cohort was older than the STEADI-09 cohort (mean age in SBS cohort: 82 (SD 6.4), mean age in STEADI-09 cohort: 72 (SD 9.7)). Ageing is associated with increased variability in cytokine levels, with a tendency to higher cytokine levels as age increases.[50] In the absence of systemic inflammatory events, this increased variability with age may be necessary for correlation analysis against sickness behaviour. Furthermore, the STEADI-09 cohort had less severe sickness behaviour at the time of cytokine sampling, with a lower median SBS-9 score and lower variability than in the SBS construction cohort (Table 5.2.32). In the STEADI-09 cohort, this low level of sickness behaviour may have reduced the probability of finding a relationship between sickness behaviour severity and levels of circulating cytokines.

The hypothesis underlying this study is that systemic inflammation aggravates chronic sickness behaviour in the primed Alzheimer's disease brain. It follows that systemic inflammatory events in people with Alzheimer's disease are expected to cause marked increases in sickness behaviour, for example, suffering delirium during a respiratory infection. Systemic inflammatory events, such as infections, cause marked elevations in circulating cytokines, albeit transiently, with peak and decline usually occurring within hours to days of the start of the inflammatory event.[405] At these times, when circulating cytokine levels are high, there may be a greater chance of demonstrating the hypothesized relationship between sickness behaviour and circulating cytokine levels. We therefore recommend further studies to examine the relationship between the SBS-9 and peripheral cytokines in patients with Alzheimer's disease during inflammatory insults, for example, in hospitalized patients with systemic infection.

There was no significant within-subject change in the SBS-9 over six months (median change over 6 months=+1.5, IQR -3.7 to +4; ANOVA for the difference between the two scores: $F(1,59)=1.8$, $P=0.18$, Section 5.2.4.6). The intraclass correlation was 0.60 (95% CI 0.42 to 0.74, $P<0.001$, Section 5.2.4.6) indicating

good correlation between scores six months apart, and an acceptable level of measurement error over six months.[394 412] This suggests that the SBS-9 measures a basal level of sickness behaviour, subject to little change over six months; although, we predict that systemic inflammatory events occurring within a six month period would cause more obvious changes in sickness behaviour score on the SBS-9 at the time of the systemic insult.

Alzheimer's disease is a progressive illness in which cognition continues to worsen over time. However, it is less clear whether some behavioural and psychological symptoms in dementia progress in the same way. In one study, hallucinations and delusions in Alzheimer's disease increased in frequency year on year over four years of follow up.[421] However, patients that suffered delusions and depression in Alzheimer's disease did not have a longer duration of dementia, compared to patients without delusions and depression, although delusions and depression were associated with increased age at diagnosis and more severe cognitive impairment.[422] Furthermore, the incidence of psychosis in Alzheimer's disease increases over time, but then appears to plateau after three years of follow up.[423] Therefore, the incidence of some behavioural and psychological symptoms appears to increase over time, to be influenced by age and the degree of cognitive impairment, and to plateau after a time. Our finding of little change in sickness behaviour over six months suggests that sickness behaviour may not progress in a predictable linear way, but rather that sickness behaviour may progress in a stochastic way, with progression due to unpredictable systemic inflammatory events, or to other unexplained influences.

We found a significant correlation between change in the SBS-9 over six months and baseline scores in other psychometric tests. The NPI, Cornell, and Fried Frailty Score all had a significant negative correlation with change in the SBS-9 over six months (Table 5.2.16). Additionally, there was a highly significant negative correlation between baseline SBS-9 score and change in the SBS-9 over six months ($r=-0.62$, $P<0.001$, Table 5.2.16). At face value these results suggest that more severe behavioural, psychological and sickness behaviour symptoms, at baseline, are associated with less change in the SBS-9 over six months; or, conversely, that less severe behavioural, psychological and sickness behaviour symptoms, at baseline, are associated with greater

change in the SBS-9 over six months. However, these results must be interpreted with much caution because of the phenomenon of regression to the mean.[424] Francis Galton, the brilliant Birmingham polymath, first described "regression to mediocrity" in relation to the size of sweet pea seeds in successive generations; noticing that parent plants from large seeds tended to produce offspring plants with smaller seeds, and parent plants from small seeds tended to produce offspring plants with larger seeds. Galton went on to show the same phenomenon in people; observing that tall parents tended to have shorter children whose height was nearer to average, and that short parents tended to have taller children whose height was nearer to average.[425] Regression to the mean occurs when repeat measurements, or properties that occur across generations, are subject to a degree of random error.[426] By its nature, random error is unlikely to be the same when a measurement is repeated, or when a property recurs. A repeat measurement that is subject to random error will tend to be smaller if the first measurement was larger, and larger if the first measurement was smaller. Therefore, we should expect to find a negative correlation between the size of the first measurement and the size of the difference between the two measurements.[424] This is precisely what we see in the significant negative correlation between the SBS-9 at baseline, and change in SBS-9 between baseline and six months (Table 5.2.16). At baseline the SBS-9 has significant correlations with baseline NPI, Cornell and the Fried Frailty Score, so the significant negative correlations between baseline NPI, Cornell and Fried Frailty Score and change in the SBS-9 between baseline and six months, are very likely to be related to regression to the mean in the SBS-9 over six months, rather than to any non-random, causative relationship.

It seems likely that the severity of sickness behaviour should differ in different neurodegenerative conditions, because of different levels of neuroinflammation and microglial activation in different neurodegenerative conditions. We demonstrated good discriminant validity in the SBS-9 by assessing median SBS-9 score in Alzheimer's disease, Lewy Body dementia and in normal controls (Figure 5.2.7). There was a significant difference in median SBS-9 scores between subjects with Lewy Body dementia, Alzheimer's disease, and normal controls ($X^2(2,127)=52$, $P<0.001$). Furthermore, subjects with Lewy Body dementia had significantly higher SBS-9 scores than subjects with

Alzheimer's disease, but lower NPI scores (Table 5.2.2), demonstrating discriminatory power for the SBS-9 across different neurodegenerative diseases, and suggesting that the SBS-9 may be a more sensitive measure of neuropsychiatric symptoms in Lewy Body dementia than the NPI.

This study sought to contrast two sickness behaviour scales, constructed using different methods of scale production. Overall, the SBS-6 failed to capture aspects of neuroimmune sickness behaviour that we believe are captured by the SBS-9. The SBS-9 was produced by factor analysis of a large number of potential scale items related to sickness behaviour, and the SBS-6 was produced by choosing those items that correlated best with the circulating cytokine IFN-γ, where IFN-γ was selected because this cytokine had the highest number of significant correlations with individual scale items. The study results show several failings in the SBS-6, leading to the conclusion that the SBS-9 should be retained as a better measure of neuroimmune sickness behaviour.

A principal failing of the SBS-6 is that it has less face or content validity. This arises partly from the smaller number of scale items, covering only two domains of sickness behaviour - negative motivation and reduced concentration (Table 5.1.1). Five out of the six items within the SBS-6 relate to negative motivation, raising the possibility that the SBS-6 effectively measures the depressive aspect of sickness behaviour and does not capture other domains. Principal components analysis of the SBS-6 showed that items related to mood clustered on a single retained factor accounting for more than 50% of the variance of the total scale, supporting the contention that the SBS-6 is largely a scale measuring depressive symptoms (Figure 5.2.5). In contrast, as described above, principal components analysis of the SBS-9 showed that items clustered on two retained factors, the first related to reduced motivation, and the second related to reduced functional ability, in keeping with the theoretical construct of neuroimmune sickness behaviour.

The SBS-6 failed in reliability comparisons with the SBS-9. The SBS-9 has better internal consistency as assessed by Cronbach's alpha (SBS-9: Cronbach's alpha=0.84, 95% CI: 0.77 to 0.89; versus SBS-6: Cronbach's alpha=0.79, 95% CI: 0.70 to 0.86). Scales with larger number of items tend to have higher values for Cronbach's alpha; however, it is unlikely that this is the cause of the difference between the SBS-9 and the SBS-6, as the magnitude of the difference

between a scale containing six items and a scale containing nine items is insufficient to cause significant change in Cronbach's alpha.[394] Principal components analysis (PCA) demonstrated psychometric strength for the SBS-9, because all the constituent scale items clustered on the two retained factors in the PCA solution, and therefore all scale items contributed to the overall variance of the total scale score (Table 5.2.10); whereas, for the SBS-6, two out of six of the constituent scale items did not cluster on the single retained factor in the PCA solution, and therefore a third of the scale items did not significantly contribute to the overall variance of the total scale score (Table 5.2.24).

The SBS-6 also failed in test-retest reliability comparisons with the SBS-9. Test-retest reliability at seven days was very good for the SBS-9 (ICC=0.94, 95% CI: 0.83 to 0.98, P<0.001, Table 5.2.13), but poor for the SBS-6 (ICC=0.66, 95% CI: 0.23 to 0.88, P=0.003, Table 5.2.22). Furthermore, the Standard Error of Measurement, calculated from the test-retest scores at seven days was better for the SBS-9 (SEMt=±1.9) than for the SBS-6 (SEMt=±2.5) (Table 5.2.31), providing evidence of greater precision for the SBS-9 than for the SBS-6. This lack of measurement precision for the SBS-6 in comparison with the SBS-9 is influenced by reduced internal consistency at seven days, as assessed by Cronbach's alpha. For the SBS-6, Cronbach's alpha fell below the acceptable level of 0.70[394] on re-testing at seven days (Cronbach's alpha=0.64 (95% CI: 0.48 to 0.77), but remained acceptable for the SBS-9 (Cronbach's alpha=0.74 (95% CI: 0.63 to 0.83) (Table 5.2.31).

The SBS-6 performed poorly in the independent STEADI-09 validation cohort in comparison with the SBS-9. The SBS-9 had better correlations with other behavioural and psychological measures (Table 5.2.33). Although the SBS-6 had good correlations with circulating cytokines at baseline and at six months in the construction cohort (Table 5.2.31), it did not have any significant correlations with circulating cytokines in the independent validation cohort (Table 5.2.34). This failure of the SBS-6 to correlate with cytokines undermines the underlying assumption behind the construction of the scale; namely, that systemic cytokines are the principal driver of neuroimmune sickness behaviour in Alzheimer's disease. The SBS-9, however, was not constructed based on any assumptions about relationships with circulating cytokines, but on the

assumption that sickness behaviour symptoms will tend to occur together, because they have evolved together in response to the same selection pressures.

There are a number of potential weaknesses in the methodology used to develop the SBS-9. The sample size for the study was limited by the ethics committee that reviewed the original study design. Data from 64 subjects with Alzheimer's disease were used in the construction of the scale. This is some distance from the original power calculation that suggested that 200 subjects with dementia would be needed to assess a scale containing an estimated 20 items. Ultimately, the final scale has 9 items, which would require 90 study subjects by the original power calculation, so the shortfall in subject numbers was not as extreme as originally expected. Furthermore, the numbers recruited were sufficient to provide estimates of Cronbach's alpha with a good 95% confidence interval (Cronbach's alpha=0.84, 95% CI: 0.77 to 0.89, Table 5.2.11) and sufficient to carry out principal components analysis with a Kaiser-Meyer-Olkin measure indicating acceptable sample size (KMO=0.78, Table 5.2.5). However, given the small sample size and the exploratory nature of this work, we have not adjusted significance levels for multiple comparisons in the analysis of the data.

Another potential weakness of the SBS-9 concerns the use of an informant to provide information about the subject of the scale. This has advantages and disadvantages. An obvious advantage is that a person with dementia may be an unreliable reporter of their own symptoms, because of poor recall, confusion and confabulation. This was the reason for choosing an informant scale for use with people with dementia in the study design. However, an informant scale also has disadvantages. Although there is evidence that proxies provide accurate reports about more objective phenomena, such as pain and social isolation,[427] there is also evidence that proxies over-estimate the severity of more subjective phenomena, such as loss of vitality or emotional distress, especially in the elderly.[428 429] Furthermore, a scale subject may be rated more severely on health and psychological factors by an informant who themselves has poor psychological or physical health, as is often the case with the carers and relatives of people with Alzheimer's disease.[394]

A further weakness in the methodology used to develop the SBS-9 arises from the lack of data on objective markers of central nervous system inflammation. We did not have resources to examine inflammatory markers and cytokine levels within the cerebrospinal fluid (CSF), or to carry out imaging studies to assess microglial activation. Additionally, we did not collect peripheral blood mononuclear cells (PBMCs) and were therefore unable to assess PBMC activation and function in relation to sickness behaviour, which may have provided a more accurate assessment of systemic immune function than was provided by circulating serum cytokine levels.

Overall, the results provide evidence for the reliability and validity of the SBS-9 scale, which we developed in order to measure neuroimmune sickness behaviour in Alzheimer's disease. The development of the scale supports the view that maladaptive sickness behaviours can be thought of as a unified construct in dementia, and measured as a single construct. We recommend that a measure of sickness behaviour, such as the SBS-9 described here, should be used as an outcome measure in treatment trials of dementia. The distress caused by sickness behaviour symptoms is an important aspect of suffering in dementia and this aspect of the disease is an important target for therapeutic agents aimed at relieving suffering.

Further studies are needed to assess the performance of the SBS-9 in different cohorts. We found a trend towards an association between sickness behaviour and cognition in the independent validation cohort. Therefore, we hypothesize that in a larger cohort of people with Alzheimer's disease we would see a significant correlation between the severity of sickness behaviour measured by the SBS-9 and cognition. The SBS-9 should also be investigated in Mild Cognitive Impairment with the hypothesis that early cognitive disease is accompanied by sickness behaviour. We found higher levels of sickness behaviour in Lewy Body dementia, whereas NPI symptoms were higher in Alzheimer's disease; we therefore hypothesize that sickness behaviour in Lewy Body dementia is more severe, perhaps because of greater neurodegeneration in fronto-striatal pathways. This hypothesis should be tested in a Lewy Body dementia cohort, by assessing the degree of sickness behaviour and correlating with atrophy in fronto-striatal structures measured by MRI of the brain.

The failure to find a significant correlation between sickness behaviour on the SBS-9 and systemic cytokines in the STEADI validation cohort suggests that sickness behaviour in dementia is not a simple product of an exaggerated response to systemic inflammation but a product of several other additional factors. These factors may include the degree and vigour of neurodegeneration; life-course influences and epigenetic change within the immune system; the brain and immune effects of co-morbid disease and medication; the degree of change in sex hormone levels; the degree of physical frailty and the subsequent lack of immunological reserve within the CNS; the influence of psychological and social factors; and, not least, the level of activity within the HPA axis. Given these myriad influences on sickness behaviour, of which systemic inflammation is only one, it seems likely that the SBS-9 is a better marker of neuroinflammation than of systemic inflammation, and we therefore hypothesize that, in dementia, sickness behaviour will correlate better with markers of neuroinflammation than with markers of systemic inflammation.

We therefore suggest that researchers examine the hypothesized relationship between sickness behaviour and markers of neuroinflammation in different contexts. We hypothesize that CSF markers of inflammation and CSF markers of Alzheimer's disease will correlate with the degree of sickness behaviour measured by the SBS-9. We would expect to find a significant correlation between the SBS-9 and microglia activation in the CNS, as measured by TSPO-PET scanning.[430] Within a larger cohort, we hypothesize that increased activity in the HPA axis, associated with mid-life psycho-social stress, chronic systemic inflammation, ageing and progression of dementia,[13 431] will be associated with increased sickness behaviour on the SBS-9 in Alzheimer's disease. In addition, functional MRI should be used to examine the relationship between SBS-9 score in patients with dementia and fronto-striatal network signalling, which has been implicated in the activation of sickness behaviour.[417]

Our attempt to produce a sickness behaviour scale using correlational analysis against systemic cytokines produced a scale, SBS-6, consisting largely of symptoms related to depression. This finding suggests that systemic

inflammation in dementia may be more related to depressive symptoms than to other aspects of sickness behaviour. Previous studies have failed to show any benefit for traditional antidepressant treatments in people with Alzheimer's disease.[156 432] Our results suggest that a reason for this failure is that traditional antidepressants, affecting monoamine pathways, may have little effect on the neuroinflammatory basis for depressive symptoms in Alzheimer's disease. We therefore suggest future clinical trials should test anti-inflammatory approaches to the treatment of depression in dementia.

Lastly, measuring sickness behaviour may give us an insight into a person's subjective experience that is not evident in other tests of cognitive and behavioural function. The SBS-9 is an informant scale, but sickness behaviour is *felt* as well as *observed*.[417] The subjective experience of sickness behaviour, as it is felt by the person exhibiting the behaviour, has effects on a person's mental representation of themselves and their disease, and on their overall feeling of self-efficacy and personhood.[433] Unfortunately, as dementia progresses a person suffering with the disease may be unable to articulate the subjective distress related to these effects on self-efficacy and personhood. In those circumstances, measuring observed sickness behaviour may act as a proxy for the unmeasured feelings of distress associated with sickness behaviour. It is a major failing in clinical trials of drugs for Alzheimer's disease to fail to measure, or even to notice, this distress. We therefore recommend that sickness behaviour is measured in clinical trials of drugs in Alzheimer's disease, using a scale such as the SBS-9 described here.

In the next chapter I report a study which uses the SBS-9 scale described here as an outcome measure for maladaptive neuroimmune sickness behaviour. The study uses an anti-inflammatory approach to treat Alzheimer's disease with the TNF-α blocker etanercept.

Chapter 6: Safety and tolerability of etanercept in Alzheimer's disease (STEADI-09)

6.0 Introduction

Alzheimer's disease is characterized by increased CNS inflammation. There is growing evidence that CNS inflammation is exacerbated by systemic inflammation in the periphery, as discussed in Chapter 2 of this thesis. TNF-α is a key cytokine in the innate immune response and acts to orchestrate much of the on-going inflammation in many chronic inflammatory diseases. Drugs that specifically block TNF-α have been highly successful in treating many chronic inflammatory diseases. This chapter reports a randomized, double-blind, placebo-controlled trial using the TNF-α blocker etanercept in Alzheimer's disease. In the trial, we wished to test the hypothesis that TNF-α blockade reduces the exacerbating effect of systemic inflammation on CNS inflammation in Alzheimer's disease, thereby reducing the progression of neurodegeneration and reducing the symptoms of Alzheimer's disease.

6.0.1 Biology of TNF-α

TNF-α is a key cytokine in the immune response to bacterial, viral and parasitic infection.[434] The gene for TNF-α is phylogenetically ancient, appearing well conserved in wide range of biological species including *Acropora* corals, fish, birds and mammals.[435] The human TNF-α gene is within the HLA Class III region on chromosome 6; a region that also contains genes for other core components of the innate immune response, including heat shock proteins and many members of the complement cascade. The human TNF-α gene codes for a 233 amino acid transmembrane protein with a molecular weight of 26 kilodaltons (kDa). This transmembrane precursor protein is cleaved by a specific metalloproteinase, TNF-α converting enzyme (TACE), forming a soluble 157 amino acid, 17 kDa protein, which oligomerizes to form the active trimer. The trimer has three receptor recognition sites and interacts with two distinct TNF-α receptors, TNFR-I and TNFR-II. The receptors have no

enzymatic function; instead, when TNF-α binds with the receptor there is a conformational change in the TNF-receptor molecule that attracts other signalling proteins, including NF-κ B, p38 MAP kinase and various proteases. The different signalling proteins present in a particular cell then trigger secondary messenger cascades, which also differ depending on the expression profile of the cell. Furthermore, the affinity of binding between TNF-α and the TNF receptors is influenced by the activity of other signalling molecules within the cell. Thus, TNF-α can have widely different, sometimes opposing actions, depending on the expression profile of the target cell.

TNF-α is produced predominantly by tissue macrophages and circulating monocytes, but a wide variety of other immune cells also produce TNF-α, including CD4 T-cells, lymphocytes, NK cells and neutrophils.[436] TNF-α is also produced by neurons, astrocytes and microglia within the CNS.[434]

TNF-α has manifold actions in health and disease, and acts to produce all of the classical features of acute inflammation recognised by Galen: calor (fever), dolor (pain), rubor (erythema), tumor (oedema) and functio laesa (loss of function).[200 437] TNF-α acts on many immune cell sub-types, activating the cells and inducing the production of cytokines, including IL-1 and IL-6; and the production of chemokines, including IL-8. In endothelial cells, TNF-α acts to increase the expression of adhesion molecules and to increase endothelial permeability, thereby increasing leucocyte migration and oedema. In macrophages, TNF-α acts to increase phagocytosis and the expression of PGE_2.[436] Within the liver, TNF-α increases the production of acute phase proteins, such as CRP. In a variety of tissues, TNF-α increases insulin resistance.[438]

Within the CNS, TNF-α acts on the hypothalamus to stimulate the production of corticotropin-releasing hormone (CRH), thus activating the hypothalamic-pituitary-adrenal axis. Hypothalamic actions of TNF-α also induce fever and decrease the activity of orexin-producing neurons, with subsequent effects on sleep, diurnal variation, appetite and motivated behaviour.[439] In neurons,TNF-α has opposing functions, with differences depending partly on whether the cell expresses TNFR-I or TNFR-II.[438] Binding at TNFR-II causes predominantly protective effects, with increased synaptic transmission, increased synaptic plasticity, and promotion of long-term potentiation.[440] Whereas, binding at

TNFR-I can induce neuronal apoptosis, via NF-κ B, and can increase glial migration and activation.[441] In the foetus, TNF-α has important physiological functions during brain development, with binding at TNFR-I inducing programmed cell death, and binding at TNFR-II playing a role in neuronal survival.[438]

In summary, TNF-α is a key orchestrating cytokine within the immune system, with important functions in innate and adaptive immunity, and in the physiology of neuronal development, function and survival.

6.0.2 TNF-α in Alzheimer's disease

There is evidence that TNF-α can drive and potentiate the pathophysiology of Alzheimer's disease. In mouse models of Alzheimer's disease, amyloid production is decreased, and memory function is improved, by deletion the TNFR-I gene.[442] Furthermore, in mouse models, the TNFR-I receptor is necessary for Aβ-mediated neurotoxicity.[443] Microglia produce TNF-α as a response to Aβ.[309 310] Additionally, TNF-α produced by neurons acts to increase microglial activation and neurotoxicity at sites of amyloid deposition.[444]

In clinical studies, elevated plasma TNF-α, and certain TNF-α polymorphisms associated with elevated TNF-α, are associated with increased risk of Alzheimer's disease, as discussed in Chapter 2 of this thesis (Section 2.4.10). Moreover, TNF-α levels are increased in the blood and CSF of people with Alzheimer's disease.[435] There is some evidence for contrasting effects of TNFR-I and TNFR-II in Alzheimer's disease.[438] In post mortem studies, expression of TNFR-I is increased, and expression of TNFR-II is decreased, within Alzheimer's disease brains, compared to control brains. Furthermore, TNF-α had increased affinity for TNFR-I, and decreased affinity for TNFR-II, in Alzheimer's disease brains.[445] TNF-α is a key cytokine in the generation of adaptive sickness behaviour,[22] and is likely to play a role in the generation of pathological sickness behaviour in Alzheimer's disease (see Section 2.2). Together these studies demonstrate a key role for TNF-α in the progression of Alzheimer's disease.

6.0.3 Etanercept: an effective TNF-α blocker

Etanercept is an anti-TNF fusion protein manufactured using recombinant DNA technology to consist of two identical TNF-receptor domains (TNFR-p75) linked to the Fc portion of human IgG1 (2xTNFR-p75:IgG-Fc). Etanercept binds with high affinity and specificity to 2 of the 3 receptor binding sites that are found on the active TNF-α trimer.[446] Etanercept is an effective TNF-α antagonist that prevents TNF-α binding at both TNFR-I and TNFR-II, by neutralizing soluble TNF-α before it can interact with the membrane-bound TNF receptors. Etanercept has a half-life of 3-5 days, with a slow onset of action after subcutaneous administration, with peak plasma levels occurring 50 hours after dosing.[447]

Etanercept is used to effectively treat a number of chronic inflammatory conditions. The powerful anti-inflammatory effect of etanercept has made it a corner stone of treatment for rheumatoid arthritis,[447] psoriasis,[448] ankylosing spondylitis,[434] and Crohn's disease.[449] Etanercept was not effective as a treatment after the onset of acute severe sepsis,[447] reflecting the importance of TNF-α in the early initiation rather than continuing maintenance of acute inflammation, and in marked contrast to key role of TNF-α in maintenance of chronic inflammation.[434] Etanercept was not effective as a treatment for chronic cardiac failure, and worsened symptoms. This negative effect was related to the undesired block of cardiac protection by TNFR-II mechanisms, in addition to the desired block of inflammatory cardiac damage by TNFR-I mechanisms.[447] [450] Lenercept, a TNFR:IgG fusion protein very similar to etanercept, was ineffective in relapsing-remitting multiple sclerosis (MS), and increased the frequency and duration of exacerbations.[451] The mechanism by which peripheral TNF blockade enhanced the pathology of MS is unclear. One possibility involves pro-inflammatory activation of Fc receptors on lymphoid cells by the IgG-Fc component of the drug, with subsequent transit of the activated lymphoid cells into the CNS. Another possibility involves reduced TNFR-II-mediated neuro-protection. In the MS study, there was no MRI evidence, using gadolinium-enhancement, of increased blood-brain barrier permeability in the treatment group. Lenercept and etanercept have molecular weights of 150 kDa and are therefore unlikely to cross the intact blood-brain barrier; thus, a direct effect of lenercept or etanercept in the brain is unlikely.

However, peripheral sequestration of TNF-α by drugs like etanercept may prevent TNF-α crossing the blood-brain barrier and thereby reduce any detrimental or beneficial effects of TNF-α in CNS disease.

6.0.4 Anti-TNF treatment for Alzheimer's disease

In the light of the evidence implicating TNF-α in Alzheimer's disease, blocking the action of TNF-α is an attractive target for potential therapies. I have reviewed evidence relating to anti-TNF therapy in Chapter 2 (Section 2.4.10). Briefly, anti-TNF agents given both directly into the CNS, and given peripherally, have been of benefit in transgenic and non-transgenic mouse models of Alzheimer's disease.[311 313-317] In clinical Alzheimer's disease, there is anecdotal case report evidence for the benefit of anti-TNF treatment; however, there have been no randomized, placebo-controlled trials. We therefore carried out a double-blind, randomized, placebo-controlled trial of anti-TNF treatment using etanercept in Alzheimer's disease.

6.1 STEADI-09: Methods

6.1.1 Trial design

The STEADI-09 study was a phase 2, randomized, double-blind, placebo-controlled, single centre, 24 week trial of subcutaneous etanercept versus placebo, in patients with mild to moderate Alzheimer's Disease. The allocation ratio was 1:1 etanercept versus placebo. The primary aim was to assess the safety and tolerability of 50mg weekly subcutaneous etanercept in patients with Alzheimer's disease. The potential treatment benefit of subcutaneous etanercept was assessed using a range of cognitive, behavioural and functional secondary outcome measures.

6.1.2 Trial participants

Setting

Participants were recruited through the NHS memory service in Southampton, UK. Potential participants were identified by a clinician involved in their care and permission sought for contact by the research team. If permission was granted then the potential participant was contacted by the research team and invited to take part.

6.1.3 Inclusion criteria

Patients were required to meet all of the following criteria at screening to enter the study:

<u>Demographic criteria</u>

- Male or female patients aged >54 years
- Have a minimum of 7 years of education
- Be able to hear, read, write and perform study neuropsychological tests in English
- Have adequate visual and auditory acuity to allow neuropsychological testing based on the research clinician's judgement

Medical and therapeutic criteria

- Fulfil DSM-IV-TR criteria for diagnosis of dementia of the Alzheimer type
- Have a diagnosis of probable Alzheimer's Disease (NINCDS-ADRDA criteria)[383]
- Mini Mental State Examination (MMSE) score <27 and >10 points
- To be currently taking and have been taking a cholinesterase inhibitor, and/or memantine, for a minimum period of 3 months prior to the day of inclusion into the study or to have been not been taking a cholinesterase inhibitor, or memantine, for a minimum period of 3 months prior to the day of inclusion into the study
- Have an informant who spends at least 24 hours per week with the patient; this may be a close friend or a neighbour, not necessarily a close relative, spouse, son or daughter. He/she should be the same throughout the study and should be present at all visits. If it becomes necessary, a change of informant can be made but this must be clearly documented.

6.1.4 Exclusion criteria

Patients meeting any of the following criteria during screening or baseline evaluations were excluded from the study:

General criteria

- Inability or refusal to provide informed consent from patient or caregiver
- Absence of informant
- Unlikely to cooperate in the study, or not able to attend scheduled examinations and visits, or not able to follow study instructions
- Participation in another study with administration of any investigational drug in the previous 3 months or already enrolled in another study

Medical and therapeutic criteria

- Parkinson's Disease, Dementia with Lewy Bodies or clinically significant Parkinsonian symptoms
- Vascular disorder (modified Hachinski Ischaemic Scale score >4)

- Recent Transient Ischaemic Attack (TIA) – within the last 3 months
- Signs of major cerebrovascular disease on MRI or CT scan, if performed prior to entry into study (i.e. presence of infarction in greater than 25% of white matter, more than 1 lacune within basal ganglia, more than 2 lacunes in white matter)
- Any other previous or ongoing chronic or recurrent disease of the central nervous system, including demyelinating disease or psychiatric diseases, that may have an impact on cognitive performance, left to the research clinician's judgement
- Clinically significant Vitamin B12 levels less than the lower limit of normal
- Clinically significant folate levels less than the lower limit of normal
- Clinically significant thyroid-stimulating hormone (TSH) levels greater than the upper limit of normal and a clinically significant free thyroxine (FT4) level lower than the lower limit of normal
- Patients with previous or present history of severe or unstable medical conditions (e.g. hypertension, diabetes, left to the research clinician's judgement)
- Current alcohol >35 units per week for men, or >28 units per week for women, or drug abuse at the discretion of the research clinician
- Surgical intervention planned during the study period
- Treatment with immunosuppressive drugs and/or oral prednisone greater than 10mg/day within the past 90 days
- Vaccination or immunization with any live vaccine (eg: polio, rubella, yellow fever) or the pneumococcal vaccine within the past 30 days
- Pregnancy or breast feeding
- Severe hepatic, renal or cardiac disease
- Previous use of a TNF-α agent
- Known skin photosensitivity
- Infection in past 4 weeks or active infection
- Heart failure: New York Heart Association (NYHA) Grade 3-4
- History of blood disorders or current WCC≤3.5 x 10^9/l
- Platelet count≤100x10^9/l, Hb≤10g/dl
- Active or latent tuberculosis

- Rheumatoid arthritis, psoriasis, psoriatic arthritis or anklylosing spondylitis
- Septic arthritis in past 12 months
- Sepsis of prosthesis in past 12 months
- Chronic leg ulcers
- Indwelling urinary catheter
- Pulmonary fibrosis
- History of neoplasms/malignancies in past 5 years
- Pre-malignant conditions including Barrett's oesophagus, cervical dysplasia, large bowel polyps
- Any relevant acute or chronic abnormality detected during the physical and neurological examinations, ECG or laboratory tests likely to interfere with the study evaluations in the research clinician's judgement
- Previous exposure to amyloid vaccines, monoclonal antibodies or intravenous immunoglobulins meant to treat Alzheimer's disease

6.1.5 Change to eligibility criteria after commencement

The only change to the eligibility criteria after commencement of the trial was to allow patients on memantine to take part. Initially patients taking memantine were excluded from the study. However, local prescribing practice meant this limited the availability of potential participants considerably. The original justification for the exclusion of patients taking memantine was that these patients would tend to have more severe disease and be unable to consent to their own participation in the study. However, as local prescribing practices changed, many potential participants that would otherwise be eligible for screening were excluded because they were prescribed memantine. We therefore submitted a protocol amendment to the Medicines and Healthcare Products Regulatory Agency (MHRA) and NHS Ethics Committee removing the memantine exclusion criterion, and allowing inclusion of patients that had been on memantine for at least 3 months.

6.1.6 Interventions

All patients underwent initial screening tests for tuberculosis (TB). The TB screen comprised a chest radiograph, tuberculin skin test (Mantoux test) and a blood test for an interferon-gamma release assay for TB exposure (TIGRA).

Patients fulfilling the inclusion and exclusion criteria were randomized using a computer generated randomization protocol to placebo or weekly 50mg subcutaneous etanercept on a 1:1 ratio for 24 weeks followed by a 4 week washout phase.

Etanercept or placebo was administered by weekly subcutaneous injection by a study team health professional blinded to treatment allocation. Etanercept or placebo was supplied in 25mg matching vials.

Clinic visits took place at screening, baseline, week 12, week 24 and 4 weeks after washout (week 28). During these visits adverse event monitoring and psychometric evaluation took place. Demographic and medical information was gathered at baseline, including the date of dementia diagnosis, medical history, drug history, smoking and alcohol history, and markers of early-life deprivation (recalled birth weight, early life hospitalization, years of schooling and parental social class).

6.1.7 Outcome measures

6.1.7.1 Primary outcome measures

The primary outcome was the safety and tolerability of the study medication. Safety was assessed by recording full details of all adverse events occurring from baseline to the end of the study, and then comparing the rates of adverse events within the etanercept group and the placebo group.

Tolerability was assessed by comparing treatment groups for compliance with study medication. Compliance was measured as a percentage of the 24 total number of study drug doses. Tolerability was further assessed by comparing treatment groups for the rate of early discontinuation from the study.

6.1.7.2 Secondary outcome measures

Secondary outcome measures were used to assess the potential efficacy of etanercept treatment on cognitive, behavioural, psychological and functional outcome measures.

1. Cognitive measures

The ADAS-Cog,[363] standardized MMSE,[367] and Clinical Global Impression of Change[368] were used to assess cognition in the study. Full details of these measures are given in the General Methods (Section 3.4).

2. Behavioural and psychological measures

The Cornell scale for depression in dementia (Cornell)[369] was used to assess the severity of depressive symptoms. The 12-domain neuropsychiatric inventory (NPI)[370] was used to assess a comprehensive range of behavioural and psychological symptoms. Full details of these measures are given in the General Methods (Section 3.4).

The sickness behaviour scales (SBS-6 and SBS-9) developed from the research described in Chapter 5 were used to assess neuroimmune sickness behaviour.

3. Functional measures

The Bristol Activities of Daily Living Scale (BADLS)[373] was used to assess the level of dependency in a number of everyday functional domains. The Fried Frailty Score[375] was used to assess the degree of physical frailty. Full details of both these measures are given in the General Methods (Section 3.4).

6.1.8 Cytokine analysis

Serum samples for cytokine analysis were taken at baseline, week 12, week 24 and at week 28, after a 4-week drug-free washout period. Samples were obtained and processed as described in the General Methods (Section 3.4). Briefly, after phlebotomy and centrifugation serum was stored at -80^{o}C until assays were performed. Assays of IFN-γ, TNF-α, IL-6, CRP, IL-10, IL-12, IL-8 and MCP1 were performed using a sandwich multiplex cytokine immunoassay (Meso Scale Discovery (MSD), Gaithersburg, MD). A protocol provided by MSD for custom assays was used with no major modifications (Table 3.4.2). Assays

were carried out in the Clinical Neurosciences laboratory at the University of Southampton by Dr Ursula Puentener and Professor Jessica Teeling, who were blinded to treatment allocation.

6.1.9 Sample size

This study was powered to assess the tolerability of subcutaneous etanercept in Alzheimer's disease based on dropout rates. Previous therapeutic trials of patients with Alzheimer's disease studies have experienced dropout rates of between 29% and 41%.[153 452] We therefore estimated a dropout rate of 35%, and sought a sample size that would generate a 95% confidence interval that the true population dropout rate would be within a margin of error of ±15% of the estimated sample dropout rate of 35%.

Sample size can be calculated for a population proportion estimate with a 95% confidence level that the true population proportion is within a specified margin of error by the following equation (sample size estimation based on a population proportion with specified absolute precision):[395]

$$N = \frac{\pi(1-\pi) * (z\alpha_{/2})^2}{E^2} \qquad (6.1)$$

where

N=the estimated sample size

π=anticipated value of the proportion of the population

$z_{\alpha_{/2}}$ is the cut-off z-score from the Gaussian distribution, such that the probability between $-z_{\alpha_{/2}}$ and $+z_{\alpha_{/2}}$ is (1-α), where α is the probability of a Type I error (falsely rejecting the null hypothesis)

E is the absolute precision required on either side of the proportion (margin of error); which is the half-width of the 100(1-α) % confidence interval.

We estimated α (probability of Type I error)=0.05, E (margin of error)=±15%, and π (estimated dropout rate)=35%. For α=0.05, and therefore a probability of

0.95 for a z-score between –z and +z, the value of z from the standard Gaussian probability distribution is ±1.96.

Fitting these values to Equation 6.1, the estimated sample size was 39 (95% CI: 36 to 41) participants, to give a 95% confidence interval for a margin of error of ±15% around a dropout rate of 35%.

Thus, if 39 participants were in the study and the study dropout rate was 35% then the 95% CI around that population estimate would be 29% to 41%, which is ±15% of 35%.

6.1.10 Randomization

ACE Pharmaceuticals BV (PO Box 1262, NL-3890, BB Zeewolde, The Netherlands) manufactured the placebo and packaged both the study medication and placebo to ensure blinding. The company used a computer to generate a random allocation sequence to ensure 20 patients in the treatment group (etanercept) and 20 patients in the placebo group, in randomization blocks of 4. The investigators had no knowledge of the allocation sequence and the allocation sequence remained concealed throughout the study.

ACE Pharmaceuticals BV were responsible for packaging either etanercept or placebo vials into serially numbered containers according to the allocation sequence. The loaded containers, and the interventions inside them, were identical in appearance to ensure concealment of the allocation sequence from the investigators. Participants entering the study, and after successful screening, were assigned the container with the next available serial number in strict chronological order.

6.1.10.1 Blinding

All participants, study partners and research team members remained blinded throughout the study.

6.1.11 Statistical methods

For safety and tolerability outcomes, all enrolled patients that received at least one dose of study medication were included in the outcome analysis.

For secondary outcome measures of efficacy, the primary analysis was performed on study completers, defined as all study participants that received at least one dose of study medication, and who provided data at baseline and at week 24. An intention-to-treat (ITT) analysis was also carried out using Last Observation Carried Forward (LOCF); defined as all participants that received at least one dose of study medication, and who provided data at baseline and at least one post-baseline efficacy assessment.

Adverse event rates were counted across the study period allowing the calculation of adverse event incident rates per 1000 person-days. Treatment groups were compared by calculating the Incident Rate Ratio (IRR) and an associated 95% Confidence Interval, as the etanercept adverse event rate divided by the placebo adverse event rate.

Baseline characteristics and change in efficacy measures were compared between the etanercept and the placebo groups using independent samples T-tests for normally distributed variables, or using non-parametric tests for variables that were not normally distributed (Mann Whitney U Test (MWU), Wilcoxon signed-rank test). Differences in proportions between treatment groups, and between baseline and follow-up, were compared using Pearson's Chi-square test of independence (χ^2) or Fisher's exact test.

Spearman correlations were used to examine the linear relationship between non-normally distributed variables.

Linear regression was used to examine differences between the etanercept group and the placebo group for change in outcome variables, after correcting for age, gender and baseline outcome score. The un-standardized β-coefficient for the effect of treatment on change in outcome score obtained by linear regression was used to provide a corrected mean difference and an associated 95% confidence interval.

Further modeling of differences in change in outcome variables between the etanercept and placebo groups was performed using analysis of covariance

(ANCOVA). Mixed-effects, repeated measured ANCOVA models were used to control for age, gender, baseline outcome score, ApoE genotype, baseline cytokine profile, and to examine the interaction between treatment and time. The models produced adjusted least squares mean change values and associated 95% confidence intervals for the adjusted effect of treatment group on outcome measure change.

Two-way ANCOVA models were used to examine for any effect modification by ApoE genotype on change associated with treatment group.

All statistical analyses were carried out using the SPSS software package (IBM, version 22).

6.1.12 Regulatory approval and funding

Ethical approval for the study including the study protocol, patient information sheets and consent forms was granted by Southampton and South West Hampshire NHS Research Ethics Committee (A) (REC reference number 10/H0502). The study was registered with EudraCT (2009-013400-31) and at ClinicalTrials.gov (NCT01068353). The study was approved by the MHRA. The University of Southampton was the trial sponsor. The study was funded by researcher-initiated grants from Wyeth Pharmaceuticals and Pfizer Inc. to Professor Clive Holmes (University of Southampton).

6.2 STEADI-09: Results

6.2.1 Recruitment

Recruitment commenced on 13/01/2011. The final study participant completed the study on 30/08/2013.

6.2.2 Participant flow

Participant flow through the trial is shown in Figure 6.2.1. A total of 67 patients were screened of whom 41 entered the study and were assigned to either etanercept (N=20) or placebo (N=21).

6.2.3 Screen failures

Reasons for screen failure are given in Table 6.2.1. Almost half of the screen failures were due to prior exposure to TB or latent TB (12/26, 46%).

Figure 6.2.1 CONSORT Trial Profile

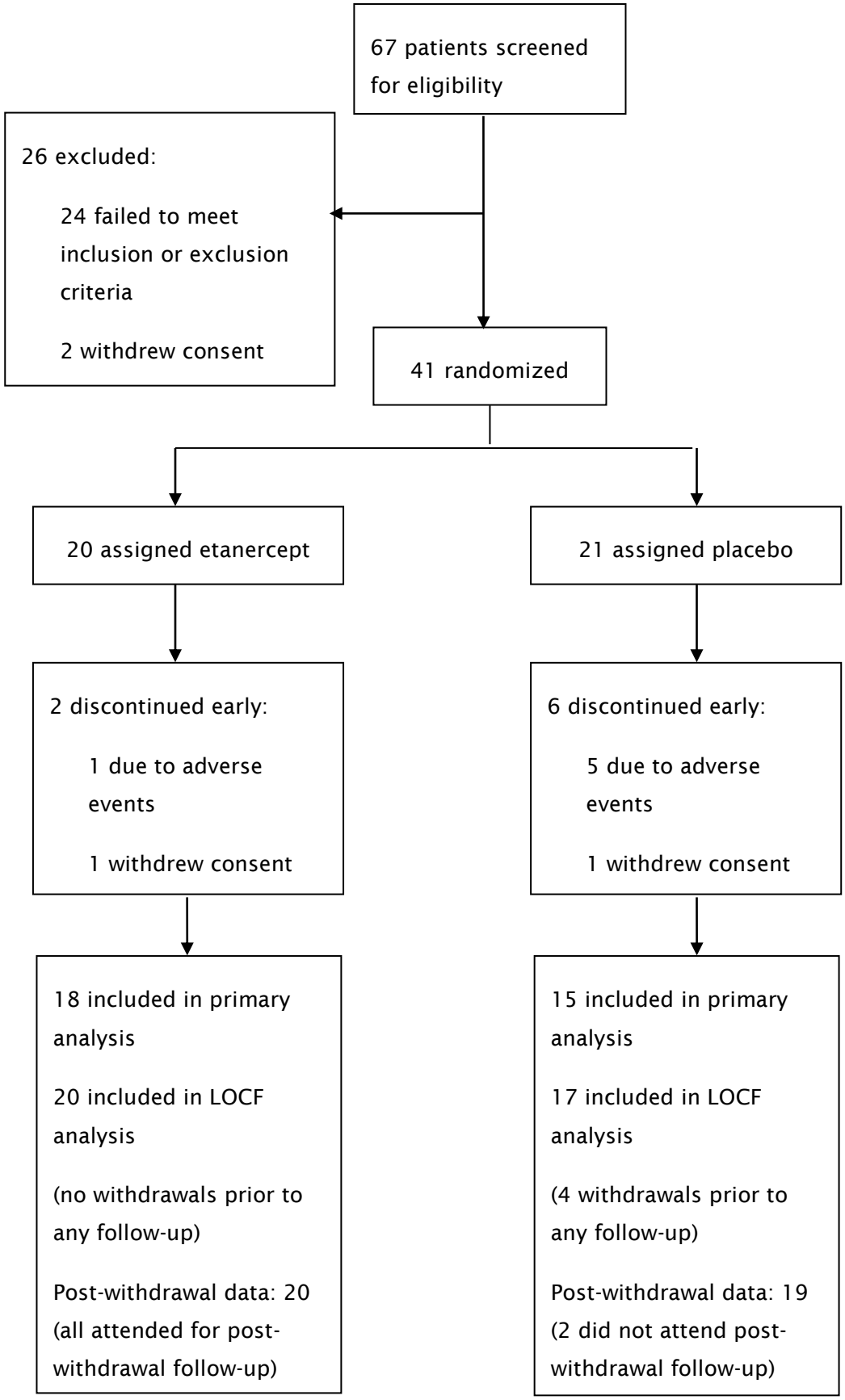


Table 6.2.1 Reasons for exclusion during screening

Reason for exclusion at screening	N
Prior exposure to TB or latent TB	12
Monoclonal gammaopathy of unknown significance (MGUS)	3
Abnormal chest radiology	2
Skin cancer	2
Withdrew consent during screening	2
Abdominal aortic aneurysm	1
Clinically significant anaemia	1
MMSE too high	1
Unable to complete cognitive tests	1
Lack of informant	1
Total excluded at screening	26
As a percentage of total screenings (N=67)	39% (26/67)

Table 6.2.2 Reasons for early withdrawal

Etanercept group (N=20)		Placebo (N=21)	
Event	**N**	**Event**	**N**
Chest infection	1	Urinary tract infection	1
Withdrew consent	1	MGUS	1
-	-	Required elective hip replacement	1
-	-	Blood in stools	1
-	-	Worsening behavioural symptoms	1
-	-	Withdrew consent	1
Total early withdrawals	2		6
Percentage withdrawal	10% (2/20)		29% (6/21)

Table 6.2.3 Baseline characteristics

	Etanercept N=20	Placebo N=21	Mean difference (95% CI)	Test	P-value
Age Mean years (SE)	72.0 (2.1)	72.9 (2.2)	0.9 (-5.3 to 7.1)	*t*(39)=0.3	P=0.8
Men N (%)	15 (75%)	10 (48%)	—	$X^2(1)=3.2$	P=0.1
White ethnicity N (%)	19 (95%)	21 (100%)	—	$X^2(1)=1.1$	P=0.3
Disease duration Mean years (SE)	5.1 (0.8)	4.1 (0.4)	-1.0 (-2.8 to 0.8)	*t*(29)=-1.2	P=0.2
ε 4 carriers N (%)	9 (45%)	11 (52%)	—	$X^2(1)=0.2$	P=0.6
Co-morbidities Median (IQR)	7 (4 to 9)	7 (5 to 9)	—	$X^2(1)=0.02$	P=0.9
Concomitant medications Median (IQR)	5 (4 to 9)	7 (5 to 11)	—	$X^2(1)=2.1$	P=0.1
MMSE Mean (SE)	20.0 (1.4)	20.3 (1.2)	0.3 (-3.3 to 4.0)	*t*(38)=0.2	P=0.9
ADAS-cog Mean (SE)	25.8 (2.9)	25.7 (2.5)	-0.1 (-7.8 to 7.7)	*t*(38)=-0.02	P=1.0
BADLS Mean (SE)	16.5 (3.0)	14.0 (1.7)	-2.5 (-9.3 to 4.3)	*t*(30)=-0.7	P=0.5
NPI Mean (SE)	16.4 (2.5)	12.0 (2.7)	-4.4 (-11.8 to 3.0)	*t*(39)=-1.2	P=0.2
Cornell Mean (SE)	6.4 (0.8)	5.6 (1.0)	-0.8 (-3.4 to 1.9)	*t*(37)=-06.	P=0.6
SBS-6 Median (IQR)	5 (IQR 2 to 6)	4 (IQR 2 to 5.5)	—	*MWU* (41)=228	P=0.6
SBS-9 Median (IQR)	6 (IQR 3 to 12)	5 (IQR 3.5 to 9.5)	—	*MWU* (41)=240	P=0.4
Fried Frailty Score N in each category	Not frail = 11 Pre-frail = 9 Frail = 0	Not frail = 8 Pre-frail = 11 Frail = 2	—	$X^2(2)=2.7$	P=0.3

ε 4 carriers = Apolipoprotein E ε 4 carrier status, *MWU*=Mann Whitney U Test, *t*=Independent samples t-test, X^2=Pearson Chi-square test of independence

6.2.4 Baseline characteristics

The mean age of the patients entering the study was 72.4 (SD 9.7) years, with the majority being men (61%). Twenty one participants (21/67, 51%) carried the ApoE e4 allele. Randomisation of patients at baseline led to two treatment groups that were similar with respect to demographic details, ApoE e4 status and baseline psychometric test scores (P-values in all cases >0.1 except gender P=0.1) (Table 6.2.3).

There was no significant difference between treatment groups in the frequency of subjects taking a cholinesterase inhibitor (16/20 (80%) etanercept versus 18/21 (86%) placebo; X^2= 0.2, P=0.6); memantine (3/20 (15%) etanercept vs 3/21 (14%) placebo; X^2= 0.004, P=0.9) or antidepressant medication (7/20 (35%) etanercept vs 8/21 (38%) placebo; X^2= 0.01, P=0.8).

6.2.5 Numbers analysed

After randomisation, 20 participants were assigned to the etanercept group, with 2 early withdrawals (1 withdrew consent, 1 related to adverse events), giving 18 participants in the primary analysis. 21 participants were assigned to the placebo group, with 6 early withdrawals (1 withdrew consent, 5 related to adverse events), giving 15 participants in the primary analysis. Reasons for early withdrawal are summarized in Table 6.2.2.

As indicated in the study protocol, a “last observation carried forward” (LOCF) analysis was also carried out. Follow up data were available for all 2 out of 2 early withdrawal participants in the etanercept group (N=20 in LOCF versus N=18 in the primary analysis). Follow up data were available for 2 out of 6 early withdrawal participants in the placebo group (N=17 in LOCF versus N=15 in the primary analysis).

Of the 8 subjects who discontinued study medication early, 6 agreed to an early termination visit to assess adverse events (2 out of 2 in the etanercept group, 4 out of 6 in the placebo group). Median time to the early termination visit for these seven subjects from last study injection was 42days (IQR 38 to 51 days).

6.2.6 Primary outcome

6.2.6.1 Adverse event frequency

Adverse events are summarised in Table 6.2.4 (all events) and in Table 6.2.5 (excluding events unrelated to the study intervention). Adverse events were considered definitely related, probably related, possibly related, unlikely to be related or unrelated to the study intervention. The decision on causality was based on the MHRA Summary of Product Characteristics (SmPC) for etanercept. Details of all adverse events for each study subject are given in the Appendix.

The incidence of adverse events, with any cause, was lower in the etanercept group. In the etanercept group, the adverse event incident rate was 11 per 1000 person-days (95% CI: 8 to 15 per 1000 person-days, 42 events/3720 person-days). In the placebo group the adverse incident rate was 17 per 1000 person-days (95% CI: 13 to 22 per 1000 person-days, 55 events/3274 person-days). The Incidence Rate Ratio (IRR) was 0.67 (95% CI: 0.44 to 1.02, P=0.05), indicating that subjects on etanercept had 0.67 times the rate of adverse events compared to those on placebo; the adverse event incidence rate was 33% less in the etanercept group (Figure 6.2.2). Visual inspection of the adverse incidence rates chart (Figure 6.2.2) shows that there was no increase in incidence during the withdrawal-phase of the study, with the slopes flattening after day 168 (Week 24), rather than steepening.

The incidence of adverse events, excluding those deemed unrelated to the study intervention, but including all events that were definitely, probably, possibly or unlikely to be related, was the same in both arms of the study. In the etanercept group, the adverse event incident rate was 9 per 1000 person-days (95% CI: 6 to 12 per 1000 person-days, 32 events/3720 person-days). In the placebo group the adverse incident rate was also 9 per 1000 person-days (95% CI: 6 to 13 per 1000 person-days, 31 events/3274 person-days). The Incidence Rate Ratio (IRR) was 0.91 (95% CI: 0.54 to 1.54, P=0.70), indicating no significant difference between the adverse incident rate in the etanercept and placebo groups for possible related adverse events, after excluding events deemed unrelated to the study intervention.

Injection site reactions were numerically more frequent in the etanercept group compared to the placebo group (4 events in 2 individuals compared to 1 event

in 1 individual). Infections were numerically greater in the etanercept arm (11 infections in 9 individuals the etanercept group versus 7 infections in 6 individuals in the placebo group).

6.2.6.2 Tolerability

Following randomization, 8 out of 41 (20%) subjects failed to complete the study (Table 6.2.2). Although there were a larger number of early withdrawals in the placebo group, there was no significant difference in the completion rates between those allocated placebo and those allocated etanercept: 6/21 (29%) from the placebo group were non-completers versus 2/20 (10%) from the etanercept group (Fisher's exact P=0.2).

From the etanercept group one subject contracted a chest infection and was withdrawn due to safety concerns and one subject withdrew consent due to drug delivery logistic problems.

From the placebo group five subjects were withdrawn due to safety concerns: one subject contracted a urinary tract infection; one subject contracted a monoclonal gammaopathy of unknown significance; one subject experienced blood in stools; one subject experienced worsening of behavioural symptoms; one subject required an elective hip replacement. One subject withdrew consent due to other family commitments.

6.2.6.3 Compliance

Compliance (number of injections given / number of planned injections) to medication was high over the six month trial period (overall median percentage compliance: 100% (IQR 87.5 to 100%). There was no significant difference in the median percentage compliance between treatment groups (etanercept 100% (IQR 95.8 to 100%) versus placebo 94% (IQR 62.5% to 100%), MWU P=0.2).

Table 6.2.4 Overall summary of adverse events including unrelated events

	Etanercept (N=20) Events [Participants]	Placebo (N=21) Events [Participants]
Blood-lymphatic system	0 [0]	3 [3]
Cardiac	2 [2]	2 [2]
Eye	0 [0]	2 [2]
Ear and labyrinth	0 [0]	2 [1]
Gastrointestinal	5 [5]	7 [5]
General fatigue	1 [1]	1 [1]
Injection Site Reaction	4 [2]	1 [1]
Infections	11 [9]	7 [6]
Injury (falls)	2 [2]	4 [4]
Investigations	6 [6]	5 [5]
Metabolic	2 [1]	1 [1]
Musculoskeletal	1[1]	5 [3]
Neoplasms	0 [0]	0 [0]
Nervous system	2 [2]	2 [2]
Psychiatric	3 [3]	6 [5]
Renal and urinary	1 [1]	1 [1]
Respiratory (yawning)	1 [1]	0 [1]
Skin	1 [1]	4 [3]
Vascular	0 [0]	2 [2]
All disorders	**42 [37]**	**55 [48]**

Related adverse events include definitely, probably, possibly, unlikely and not thought to be related to the study intervention.
Participants could report multiple events in any category.
Adverse drug reactions are coded by the MedDRA preferred term (Medical Dictionary for Regulatory Activities MedDRA 15.0)

Table 6.2.5 Adverse events, excluding unrelated events. Data are events (participants).

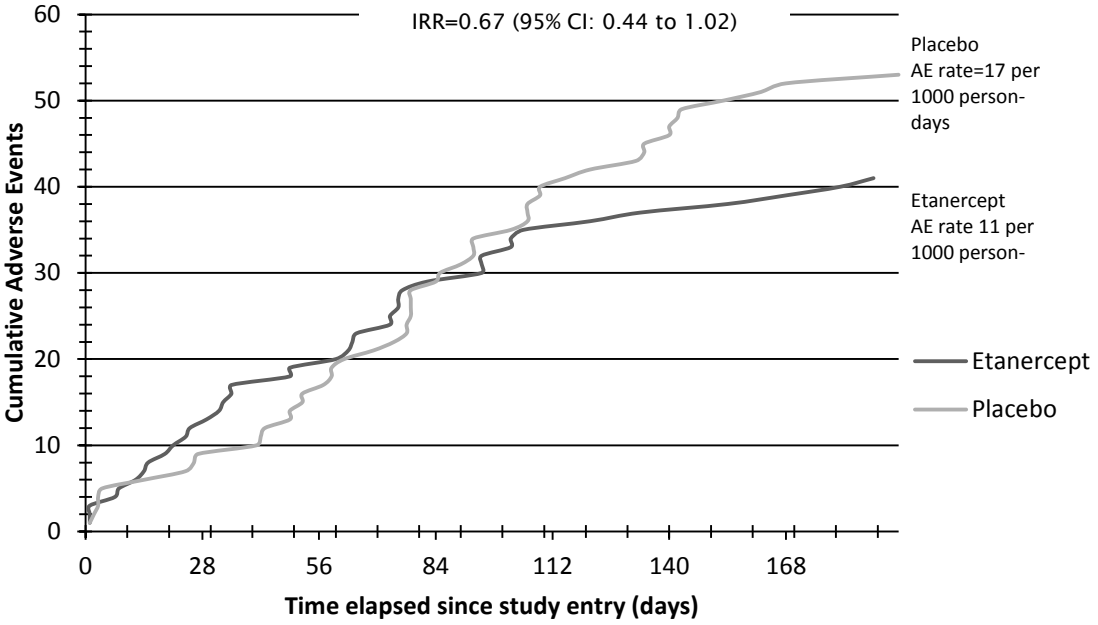
	Etanercept (N=20) Events (Participants)	Placebo (N=21) Events (Participants)
Blood and lymphatic disorders		
Normocytic anaemia	0	2
Eye disorders		
Eye pain	0	2
Gastrointestinal disorders		
Nausea and vomiting	1	1
Diarrhoea	2	1
Constipation	0	2
All gastrointestinal disorders	3	4
Administration site conditions		
Injection Site Reaction	4 (2)	1
General disorders		
Fatigue	1	1
Infections		
Gastroenteritis	2	1
Respiratory tract infection	8 (7)	2
Urinary tract infection	0	3 (2)
Pharyngitis	1	0
Cellulitis	0	1
All infections	11 (9)	7 (6)
Injury		
Fall	2	4
Investigations		
C-reactive protein increased	3	3
DNA antibody positive	1	1
All investigations	4	4
Metabolic disorders		
Hyperkalaemia	2	0
Nervous system disorders		
Parosmia (metallic taste)	1	0
Headache	1	0
All nervous system disorders	2	0

Table 6.2.5 continued

	Etanercept (N=20) Events (Participants)	Placebo (N=21) Events (Participants)
Psychiatric disorders		
Behavioural symptoms	1	1
Mood altered	0	2
Delusions	0	1
All psychiatric disorders	1	4
Renal and urinary disorders		
Urinary frequency	1	1
Respiratory disorders		
Yawning	1	0
Vascular disorders		
Hypertension	0	1
All adverse events	**32 (28)**	**31 (30)**

Adverse events definitely, probably, possibly and unlikely to be related to study intervention are reported.
Participants could report multiple events in any category.
Adverse drug reactions are coded by the MedDRA Preferred Term (Medical Dictionary for Regulatory Activities MedDRA 15.0)

Figure 6.2.2 Cumulative incidence of adverse events with any cause



6.2.7 Secondary outcomes

6.2.7.1 Analysis of psychometric outcomes

Change from baseline at Week 12 and Week 24 in psychometric and behavioural outcome measures, alongside the mean difference in change between the etanercept group and the placebo group are shown in Table 6.2.6, and graphically represented in Figures 6.2.3 to 6.2.11.

6.2.7.1.1 Cognitive outcome measures

At Week 24, the cognitive tests showed numerical worsening in the placebo group (negative change in the MMSE, positive change in the ADAS-Cog) and little change in the etanercept group, but the difference in change did not reach statistical significance.

The results for the MMSE showed a trend towards better outcome in the etanercept group (Mean change in placebo group: -1.9 (SEM=1.2) versus mean change in etanercept group: -0.1 (SEM=0.5), mean difference -1.8 (95% CI: -4.2 to 0.6), one-tailed P=0.07 (Figure 6.2.3). Further modelling, using linear regression to control for age, gender and baseline MMSE score, gave a corrected mean difference in MMSE change between the placebo and etanercept groups of -2.5 (95% CI: -5.2 to 0.2), P=0.07 (Table 6.2.8).

There was no statistically significant difference in change in the ADAS-Cog between the etanercept group and the placebo group (Figure 6.2.4, Table 6.2.6).

6.2.7.1.2 Behavioural and functional outcome measures

There was a significant difference in change in the Bristol Activities of Daily Living (BADLS) measure in favour of etanercept treatment. At week 24, the placebo group had worse BADLS scores, indicating increasing dependency on others for activities of daily living, while there was no evidence of worsening in the etanercept group (mean change in placebo group +6.0 (SEM=2.1) versus mean change in etanercept group: +0.8 (SEM=1.2), mean difference (placebo-etanercept) of +5.2 (95% CI: 0.3 to 10.2), P=0.04 (Figure 6.2.5). Further modelling, using linear regression to control for age, gender and baseline BADLS score, made no appreciable difference to the result, with a corrected mean difference (placebo-etanercept) of +5.6 (95% CI: 0.4 to 10.9), P=0.04 (Table 6.2.8).

There was a significant difference in change in the Neuropsychiatric Inventory (NPI) in favour of etanercept treatment. The placebo group had worse NPI scores, indicating higher neuropsychiatric symptom burden, at Week 24, with no evidence of worsening in the etanercept group (mean change in placebo group: +10.2 (SEM=3.6) versus mean change in etanercept group: -0.3 (SEM=3.3), mean difference (placebo-etanercept) of +10.5 (95% CI: 0.5 to 20.5), P=0.04 (Figure 6.2.6). Further modelling, using linear regression to control for age, gender and baseline NPI score, gave a corrected mean difference (placebo-etanercept) of +13.2 (95% CI: 2.4 to 24.0), P=0.02 (Table 6.2.8).

There were no significant differences between the treatment groups in other psychometric and behavioural measures (Table 6.2.6 for unadjusted mean

differences, and Table 6.2.8 for adjusted mean differences). The Cornell Scale, which assesses depression in dementia, showed numerical worsening in the placebo group compared to the etanercept group, but this difference did not reach statistical significance (Figure 6.2.7, Table 6.2.6). There was no difference in change from baseline between the etanercept group and the placebo group for Clinical Global Impression of Change (CGIC) (Figure 6.2.8), Fried Frailty Score (Fried) (Figure 6.2.9), or the Sickness Behaviour Scales (SBS-6, SBS-9) (Figure 6.2.10, Figure 6.2.11).

6.2.7.2 Last Observation Carried Forward (LOCF) analysis of psychometric outcomes

The results of an intention-to-treat analysis, using last-observation-carried-forward (LOCF) data to account for study participants that withdrew early, are shown in Table 6.2.7 (unadjusted) and Table 6.2.9 (adjusted) and in the graphical representations of difference in change in all secondary outcome measures contained in Figures 6.2.3 to 6.2.11.

The significant differences in change between the etanercept group and the placebo group for NPI and BADLS were largely unchanged in the intention-to-treat analysis, indicating no major withdrawal bias in the results.

For BADLS, the LOCF mean difference (placebo-etanercept) was +5.6 (95% CI: 0.8 to 10.3), P=0.02 (unadjusted) and +5.1 (95% CI: -0.1 to 10.4), P=0.05 (adjusted for age, gender and baseline BADLS score) (Table 6.2.7 and Table 6.2.9). This compares with an adjusted mean difference of +5.6 (95% CI: 0.4 to 10.9), P=0.04 in the primary analysis of study completers at Week 24 (Table 6.2.9).

For NPI, the LOCF mean difference (placebo-etanercept) was +8.6 (95% CI: -0.5 to 17.8), P=0.064 (unadjusted) and +9.8 (95% CI: -0.2 to 19.9), P=0.056 (adjusted for age, gender and baseline NPI score) (Table 6.2.7 and Table 6.2.9). This compares with an adjusted mean difference of +13.2 (95% CI: 2.4 to 24), P=0.02 in the primary analysis of study completers at Week 24 (Table 6.2.9).

For MMSE the trend towards a beneficial effect in the etanercept group was reduced in the LOCF analysis. The LOCF mean difference (placebo-etanercept) was -1.5 (95% CI: -3.8 to 0.8), P=0.19 (unadjusted) and -2.1 (95% CI: -4.5 to

0.3), P=0.09 (adjusted for age, gender and baseline MMSE score) (Table 6.2.7 and Table 6.2.9). This compares with an adjusted mean difference of -2.5 (95% CI: -5.2 to 0.2), P=0.07 in the primary analysis of study completers at Week 24 (Table 6.2.9).

Table 6.2.6 Unadjusted mean change in psychometric scores at Week 12 and Week 24 compared with baseline, by treatment group

	Week 12			Week 24		
	Etanercept N=20	Placebo N=17	Mean Difference† (95% CI) P-Value (two-tailed)	Etanercept N=18	Placebo N=15	Mean Difference (95% CI) P-Value (two-tailed)
MMSE (SE)	-0.6 (0.5)	-0.5 (0.7)	0.1 (1.6 to -1.9) P=0.8	-0.1 (0.5)	-1.9 (1.2)	-1.8 (-4.2 to 0.6) P=0.14
ADAS-Cog (SE)	1.3 (1.4)	1.4 (1.5)	-0.1 (-3.9 to 4.2) P=0.9	3.2 (1.8)	5.6 (2.0)	2.4 (-3.1 to 7.9) P=0.38
BADLS (SE)	-1.1 (2.1)	1.2 (0.9)	2.3 (-2.7 to 7.4) P=0.4	0.8 (1.2)	6.0 (2.1)	**5.2 (0.3 to 10.2)†** **P=0.04**
NPI (SE)	-2.0 (2.7)	3.8 (1.6)	5.8 (-0.97 to 12.6) P=0.09	-0.3 (3.3)	10.2 (3.6)	**10.5 (0.5 to 20.5)** **P=0.04**
Cornell (SE)	-0.2 (0.9)	0.1 (1.0)	0.3 (-2.5 to 3.0) P=0.8	0.4 (1.0)	1.9 (1.5)	1.5 (-2.0 to 5.1) P=0.38
CGIC (SE)	0.4 (0.2)	0.5 (0.2)	0.1 (-0.5 to 0.6) P=0.8	0.6 (0.2)	0.9 (0.3)	0.3 (-0.5 to 1.1) P=0.42
Fried (SE)	0.3 (0.1)	0 (0.2)	-0.3 (-0.8 to 0.2) P=0.3	0.4 (0.2)	0.3 (0.3)	-0.1 (-0.8 to 0.5) P=0.70
SBS-6 (SE)	-0.2 (0.7)	0.2 (0.8)	0.4 (-1.8 to 2.6) P=0.7	0.2 (0.8)	0.3 (0.8)	0.1 (-2.2 to 2.4) P=0.92
SBS-9 (SE)	-0.3 (1.3)	0.5 (1.2)	0.8 (-2.8 to 4.4) P=0.7	0.6 (1.1)	1.0 (1.5)	0.4 (-3.2 to 4.1) P=0.81

MMSE=Mini-Mental State Examination, ADAS-Cog=Alzheimer's Disease Assessment Scale cognitive section, BADLS=Bristol Activities of Daily Living Scale, NPI=Neuropsychiatric Inventory, Cornell=Cornell Scale for Depression in Dementia, CGIC=Clinical Global Impression of Change, Fried=Fried Frailty Score, SBS=Sickness Behaviour Scale. 95% CI and P-value from independent samples T-test with equal variances assumed (Levene's Test for Equality of Variances was met unless stated otherwise).
†Equal variances not assumed as Levene's Test for Equality of Variances was not met.

Table 6.2.7 Unadjusted mean change in psychometric scores at Week 24 and Last Observation Carried Forward (LOCF) compared with baseline, by treatment group

	Week 24			Last Observation Carried Forward (LOCF)		
	Etanercept N=18	Placebo N=15	Mean Difference† (95% CI) P-Value (two-tailed)	Etanercept N=20	Placebo N=17	Mean Difference (95% CI) P-Value (two-tailed)
MMSE (SE)	-0.1 (0.5)	-1.9 (1.2)	-1.8 (-4.2 to 0.6) P=0.14	0.05 (0.5)	-1.5 (1.1)	-1.5 (-3.8 to 0.8) P=0.19
ADAS-Cog (SE)	3.2 (1.8)	5.6 (2.0)	2.4 (-3.1 to 7.9) P=0.38	2.5 (1.7)	5.4 (1.8)	2.9 (-2.1 to 7.9) P=0.25
BADLS (SE)	0.8 (1.2)	6.0 (2.1)	**5.2 (0.3 to 10.2)† P=0.04**	-0.4 (1.4)	5.2 (1.9)	**5.6 (0.8 to 10.3) P=0.023**
NPI (SE)	-0.3 (3.3)	10.2 (3.6)	**10.5 (0.5 to 20.5) P=0.04**	0.3 (3.1)	8.9 (3.3)	8.6 (-0.5 to 17.8) P=0.064
Cornell (SE)	0.4 (1.0)	1.9 (1.5)	1.5 (-2.0 to 5.1) P=0.38	0.6 (0.9)	1.8 (1.3)	1.2 (-2.0 to 4.4) P=0.44
CGIC (SE)	0.6 (0.2)	0.9 (0.3)	0.3 (-0.5 to 1.1) P=0.42	0.5 (0.2)	0.8 (0.3)	0.3 (-0.4 to 1.0) P=0.43
Fried (SE)	0.4 (0.2)	0.3 (0.3)	-0.1 (-0.8 to 0.5) P=0.70	0.4 (0.2)	0.3 (0.2)	-0.1 (-0.7 to 0.5) P=0.85
SBS-6 (SE)	0.2 (0.8)	0.3 (0.8)	0.1 (-2.2 to 2.4) P=0.92	0.5 (0.8)	0.4 (0.7)	-0.1 (-2.3 to 2.0) P=0.89
SBS-9 (SE)	0.6 (1.1)	1.0 (1.5)	0.4 (-3.2 to 4.1) P=0.81	0.9 (1.2)	0.8 (1.3)	-0.1 (-3.8 to 3.5) P=0.95

MMSE=Mini-Mental State Examination, ADAS-Cog=Alzheimer's Disease Assessment Scale cognitive section, BADLS=Bristol Activities of Daily Living Scale, NPI=Neuropsychiatric Inventory, Cornell=Cornell Scale for Depression in Dementia, CGIC=Clinical Global Impression of Change, Fried=Fried Frailty Score, SBS=Sickness Behaviour Scale. 95% CI and P-value from independent samples T-test with equal variances assumed (Levene's Test for Equality of Variances was met unless stated otherwise).
†Equal variances not assumed as Levene's Test for Equality of Variances was not met.

Table 6.2.8 Corrected mean difference in psychometric scores at Week 12 and at Week 24, compared with baseline, corrected for age, gender and baseline psychometric score, by treatment group

	Week 12			Week 24		
	Etanercept N=20	Placebo N=17	Corrected Mean Difference† (95% CI) P-Value	Etanercept N=18	Placebo N=15	Corrected Mean Difference† (95% CI) P-Value
MMSE (SE)	-0.6 (0.5)	-0.5 (0.7)	0.004 (-1.9 to 1.9) P=1.0	-0.1 (0.5)	-1.9 (1.2)	**-2.5 (-5.2 to 0.2) P=0.07**
ADAS-Cog (SE)	1.3 (1.4)	1.4 (1.5)	0.2 (-4.3 to 4.6) P=0.9	3.2 (1.8)	5.6 (2.0)	1.2 (-5.1 to 7.5) P=0.7
BADLS (SE)	-1.1 (2.1)	1.2 (0.9)	0.6 (-4.6 to 5.8) P=0.8	0.8 (1.2)	6.0 (2.1)	**5.6 (0.4 to 10.9) P=0.04**
NPI (SE)	-2.0 (2.7)	3.8 (1.6)	4.4 (-3.0 to 11.7) P=0.2	-0.3 (3.3)	10.2 (3.6)	**13.2 (2.4 to 24.0) P=0.02**
Cornell (SE)	-0.2 (0.9)	0.1 (1.0)	0.1 (-2.8 to 3.0) P=0.9	0.4 (1.0)	1.9 (1.5)	1.6 (-2.3 to 5.5) P=0.4
CGIC (SE)	0.4 (0.2)	0.5 (0.2)	0.2 (-0.5 to 0.8) P=0.6	0.6 (0.2)	0.9 (0.3)	0.3 (-0.6 to 1.1) P=0.3
Fried (SE)	0.3 (0.1)	0 (0.2)	-0.02 (-0.6 to 0.5) P=0.9	0.4 (0.2)	0.3 (0.3)	-0.1 (-0.8 to 0.7) P=0.9
SBS-6 (SE)	-0.2 (0.7)	0.2 (0.8)	0.02 (-2.3 to 2.3) P=1.0	0.2 (0.8)	0.3 (0.8)	-0.3 (-2.7 to 2.0) P=0.8
SBS-9 (SE)	-0.3 (1.3)	0.5 (1.2)	-0.3 (-4.1 to 3.6) P=0.9	0.6 (1.1)	1.0 (1.5)	-0.8 (-4.9 to 3.3) P=0.7

MMSE=Mini-Mental State Examination, ADAS-Cog=Alzheimer's Disease Assessment Scale cognitive section, BADLS=Bristol Activities of Daily Living Scale, NPI=Neuropsychiatric Inventory, Cornell=Cornell Scale for Depression in Dementia, CGIC=Clinical Global Impression of Change, Fried=Fried Frailty Score, SBS=Sickness Behaviour Scale.
† Corrected mean difference is the unstandardized B-coefficient obtained by linear regression, corrected for baseline age, gender and baseline psychometric score.

Table 6.2.9 Corrected mean difference in psychometric scores at Week 24 and Last Observation Carried Forward, compared with baseline, corrected for age, gender and baseline psychometric score, by treatment group

	Week 24			Last Observation Carried Forward		
	Etanercept N=18	Placebo N=15	Corrected Mean Difference† (95% CI) P-Value	Etanercept N=20	Placebo N=17	Corrected Mean Difference† (95% CI) P-Value
MMSE (SE)	-0.1 (0.5)	-1.9 (1.2)	**-2.5 (-5.2 to 0.2) P=0.07**	0.05 (0.5)	-1.5 (1.1)	**-2.1 (-4.5 to 0.3) P=0.09**
ADAS-Cog (SE)	3.2 (1.8)	5.6 (2.0)	1.2 (-5.1 to 7.5) P=0.7	2.5 (1.7)	5.4 (1.8)	2.7 (-2.9 to 8.2) P=0.3
BADLS (SE)	0.8 (1.2)	6.0 (2.1)	**5.6 (0.4 to 10.9) P=0.04**	-0.4 (1.4)	5.2 (1.9)	**5.1 (-0.1 to 10.4) P=0.055**
NPI (SE)	-0.3 (3.3)	10.2 (3.6)	**13.2 (2.4 to 24.0) P=0.02**	0.3 (3.1)	8.9 (3.3)	**9.8 (-0.2 to 19.9) P=0.056**
Cornell (SE)	0.4 (1.0)	1.9 (1.5)	1.6 (-2.3 to 5.5) P=0.4	0.6 (0.9)	1.8 (1.3)	0.8 (-2.6 to 4.2) P=0.6
CGIC (SE)	0.6 (0.2)	0.9 (0.3)	0.3 (-0.6 to 1.1) P=0.3	0.5 (0.2)	0.8 (0.3)	0.3 (-0.4 to 1.1) P=0.4
Fried (SE)	0.4 (0.2)	0.3 (0.3)	-0.1 (-0.8 to 0.7) P=0.9	0.4 (0.2)	0.3 (0.2)	-0.1 (-0.8 to 0.6) P=0.7
SBS-6 (SE)	0.2 (0.8)	0.3 (0.8)	-0.3 (-2.7 to 2.0) P=0.8	0.5 (0.8)	0.4 (0.7)	-0.5 (-2.8 to 1.7) P=0.6
SBS-9 (SE)	0.6 (1.1)	1.0 (1.5)	-0.8 (-4.9 to 3.3) P=0.7	0.9 (1.2)	0.8 (1.3)	-0.6 (-4.6 to 3.4) P=0.7

MMSE=Mini-Mental State Examination, ADAS-Cog=Alzheimer's Disease Assessment Scale cognitive section, BADLS=Bristol Activities of Daily Living Scale, NPI=Neuropsychiatric Inventory, Cornell=Cornell Scale for Depression in Dementia, CGIC=Clinical Global Impression of Change, Fried=Fried Frailty Score, SBS=Sickness Behaviour Scale.
† Corrected mean difference is the unstandardized B-coefficient obtained by linear regression, corrected for baseline age, gender and baseline psychometric score.

Figure 6.2.3 Mean change in MMSE (±SE) at Weeks 12, 24 and Last Observation Carried Forward (LOCF) by treatment

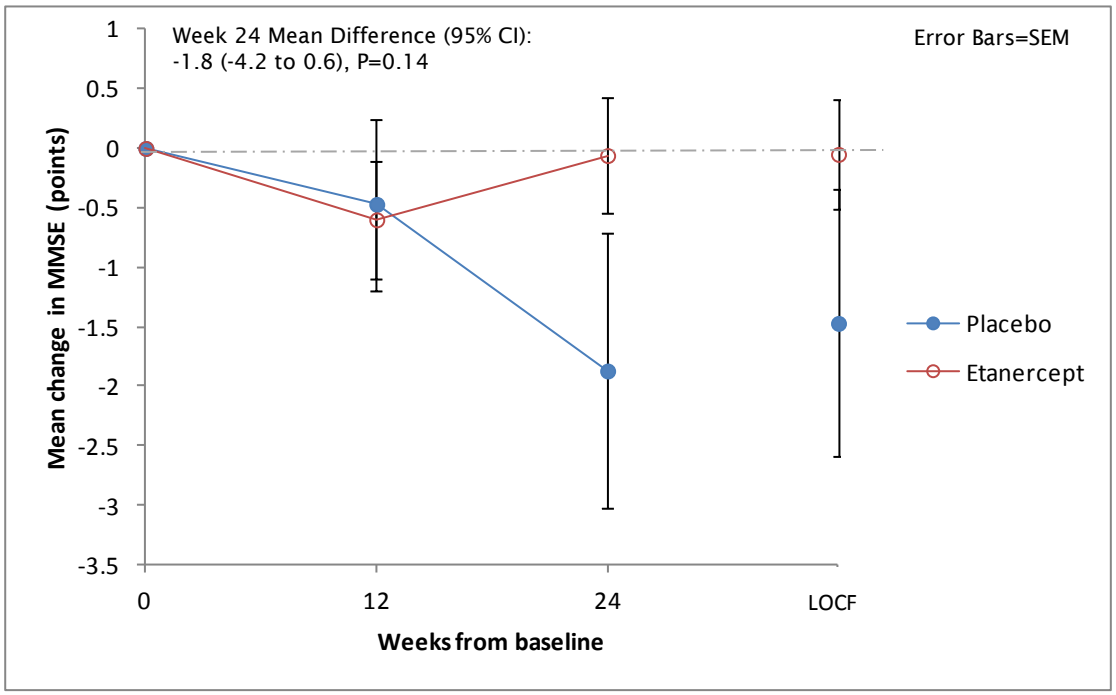


Figure 6.2.4 Mean change in ADAS-Cog (±SE) at Weeks 12, 24 and Last Observation Carried Forward (LOCF) by treatment

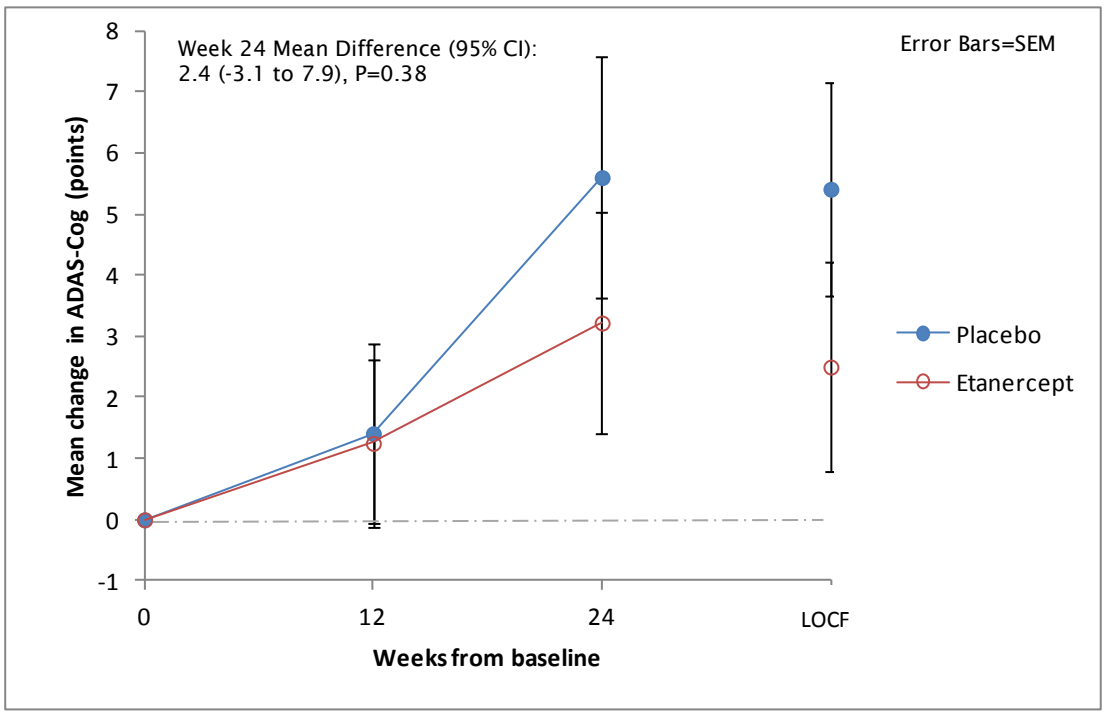


Figure 6.2.5 Mean change in BADLS (±SE) at Weeks 12, 24 and Last Observation Carried Forward (LOCF) by treatment

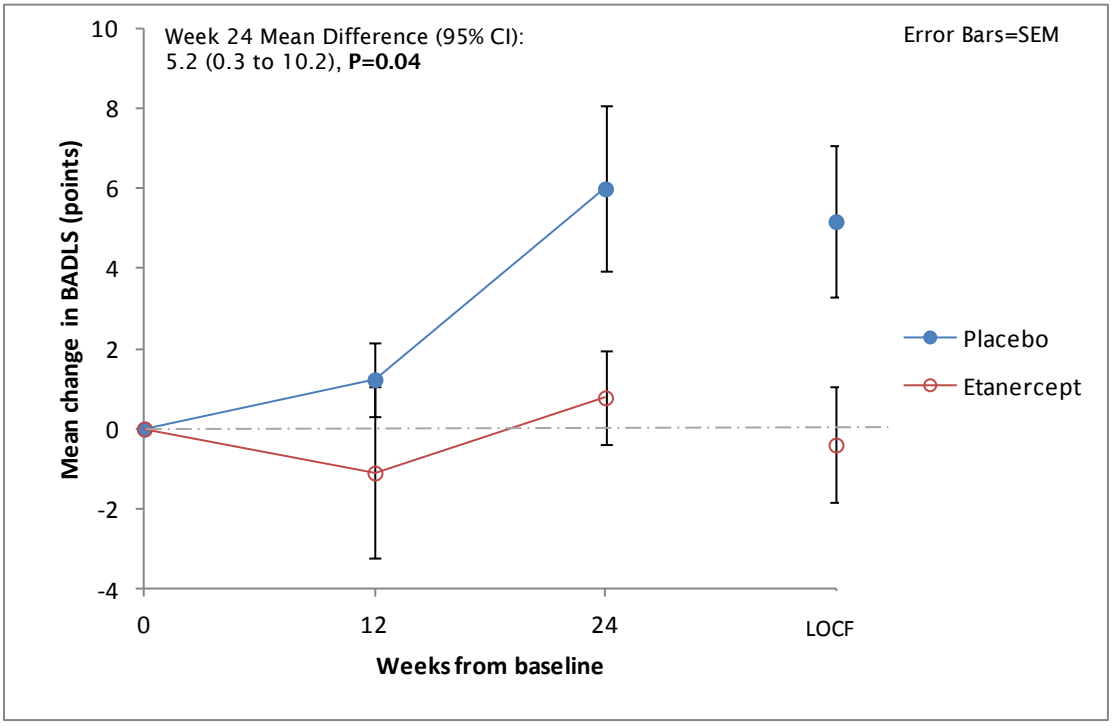


Figure 6.2.6 Mean change in NPI (±SE) at Weeks 12, 24 and Last Observation Carried Forward (LOCF) by treatment

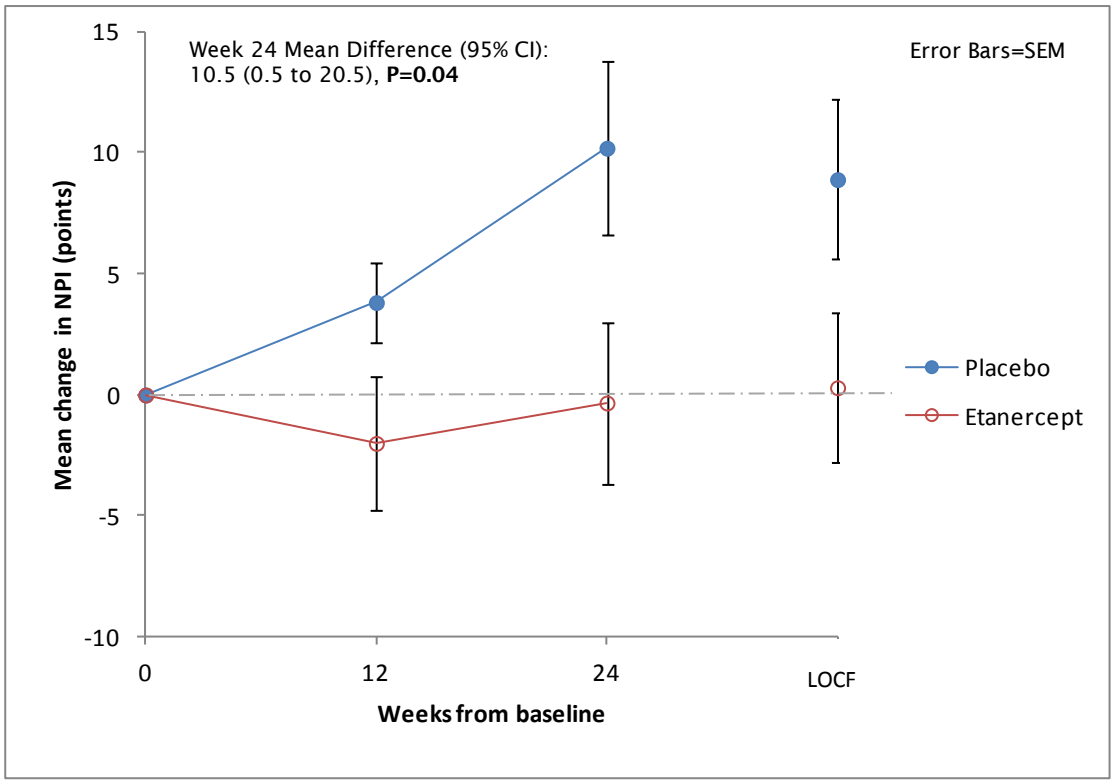


Figure 6.2.7 Mean change in Cornell (±SE) at Weeks 12, 24 and Last Observation Carried Forward (LOCF) by treatment

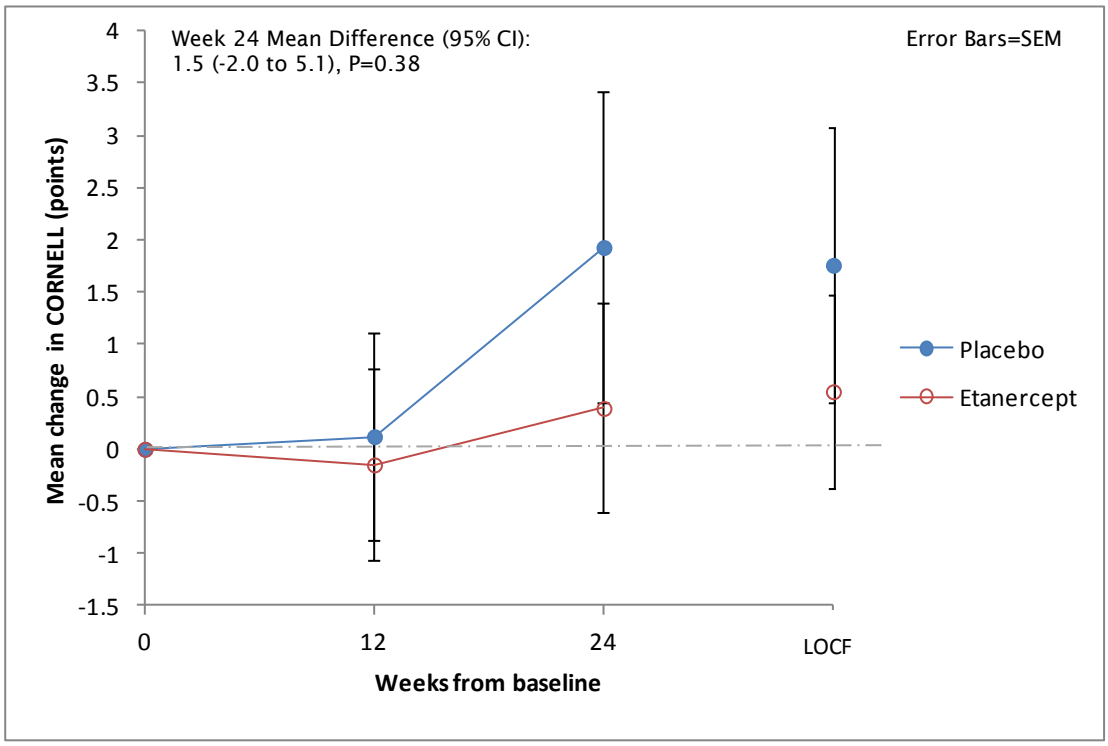


Figure 6.2.8 Mean change in CGIC (±SE) at Weeks 12, 24 and Last Observation Carried Forward (LOCF) by treatment

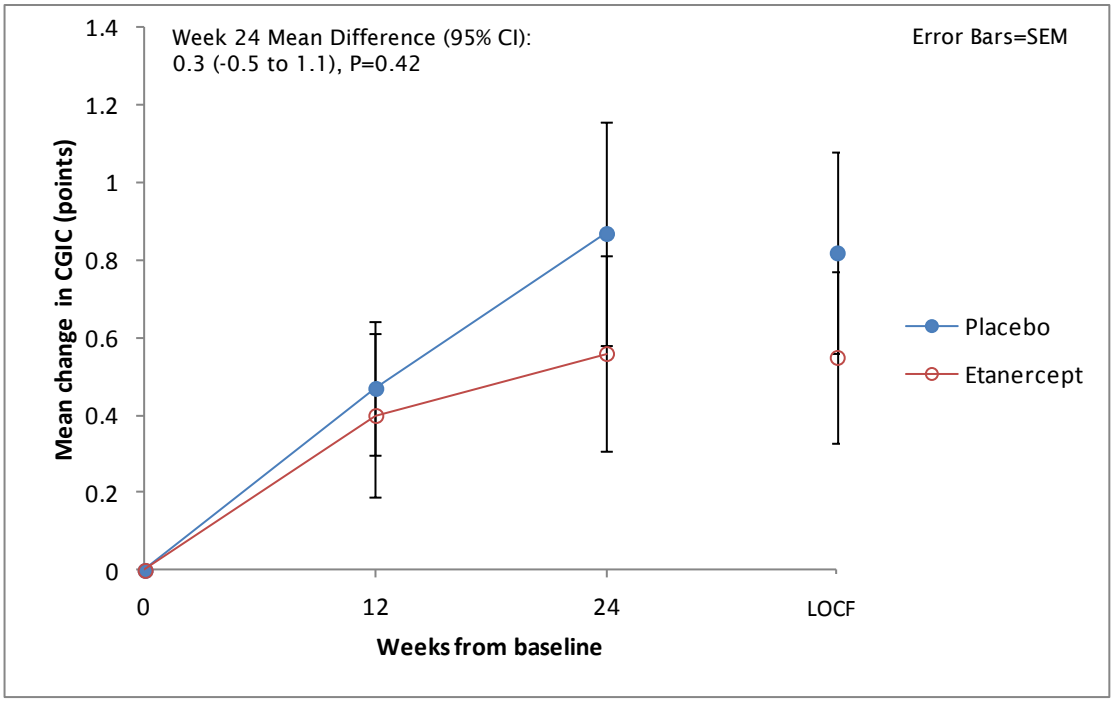


Figure 6.2.9 Mean change in Fried Frailty Score(±SE) at Weeks 12, 24 and Last Observation Carried Forward (LOCF) by treatment

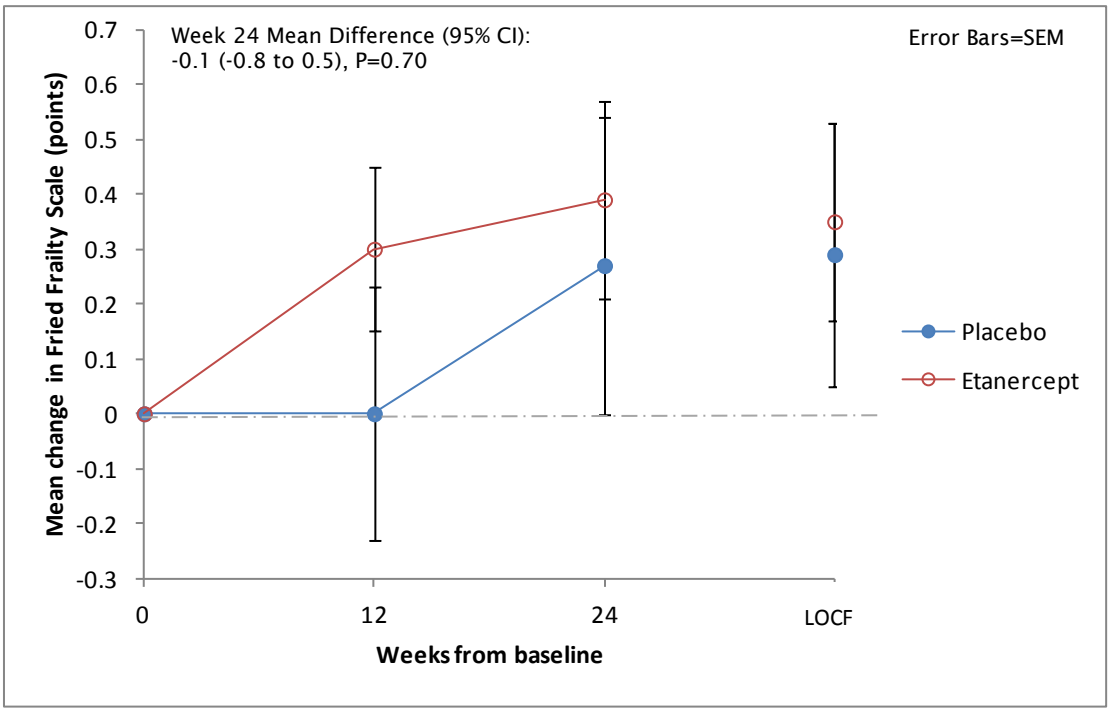


Figure 6.2.10 Mean change in SBS-6 (±SE) at Weeks 12, 24 and Last Observation Carried Forward (LOCF) by treatment

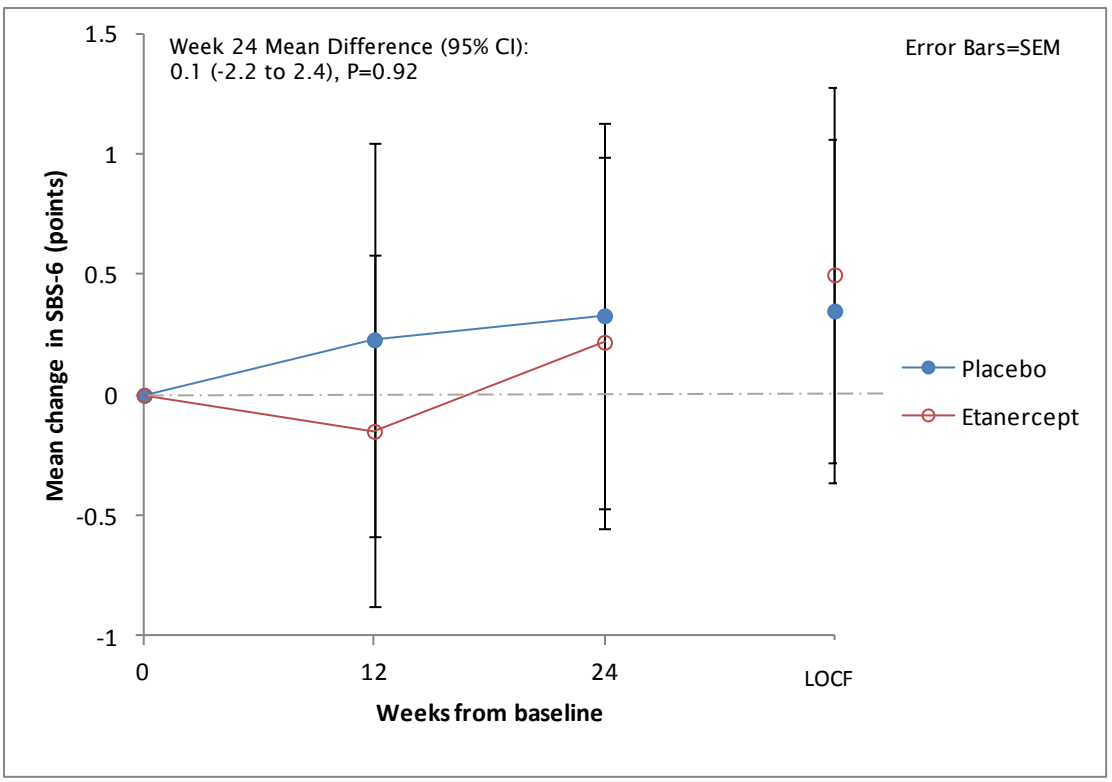
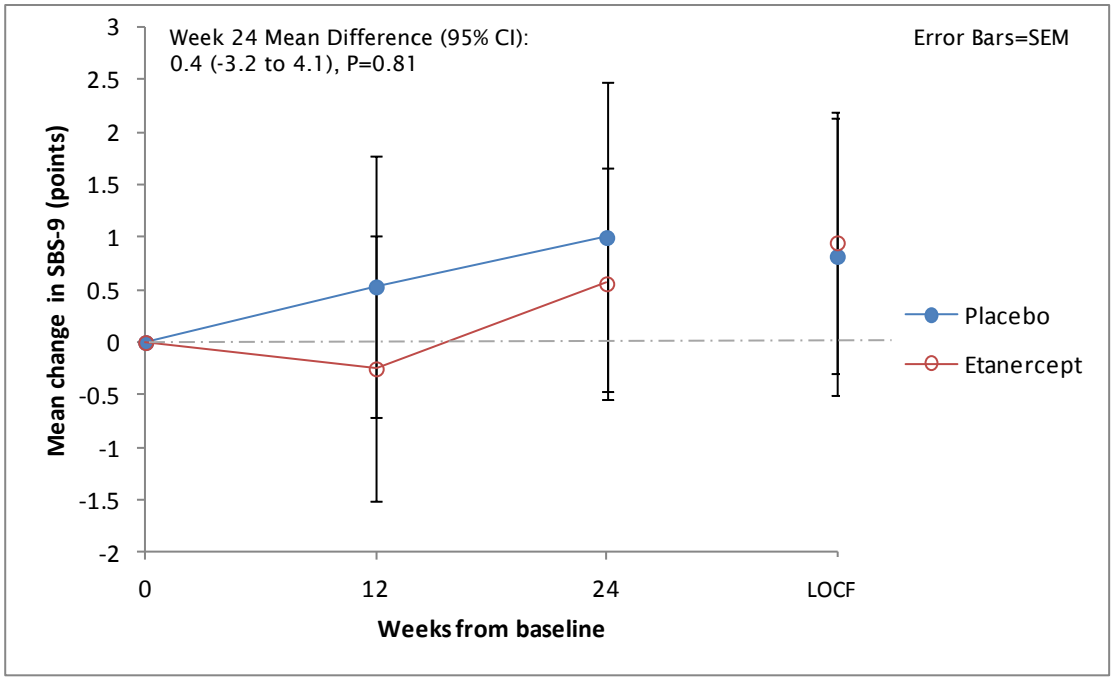


Figure 6.2.11 Mean change in SBS-9 (±SE) at Weeks 12, 24 and Last Observation Carried Forward (LOCF) by treatment



6.2.7.3 Analysis of psychometric outcome treatment modification by ApoE status

The Neuropsychiatric Inventory (NPI) showed the most significant difference in change from baseline between the etanercept group and the placebo group at Week 24, after adjusting for age, gender and baseline score. In order to further analyse this treatment effect across the entire study period, with special regard to any effect of ApoE status, and to look at the different main effects of treatment, time, and the interaction of treatment and time, we used a mixed-effects, repeated-measures ANCOVA model. The model included the categorical variables treatment group, gender and ApoE e4 carrier status, the continuous variables age and baseline NPI score, and an interaction term for time and treatment (Table 6.2.10).

The overall treatment effect was significant ($F(1,27)=6.0$, $P=0.02$, Partial Eta Squared=0.18), indicating that overall there was a significant difference in change in NPI score between treatment groups across the course of the study, after adjusting for the effects of time, age, gender, baseline NPI score and ApoE e4 carrier status.

The interaction term between time and treatment was also significant ($F(1,27)=6.4$, $P=0.017$, Partial Eta Squared=0.19) – indicating that the effect of time on change in NPI score was different between the etanercept and placebo groups (Figure 6.2.12). The significant between treatment differences were observed at Week 24, when the least squares mean change in NPI was greater in the placebo group after adjusting for age, gender, baseline NPI score and ApoE e4 carrier status (+11.7 in placebo group compared with -1.6 in etanercept group, least squares mean difference: +13.4, 95% CI: 2.5 to 24.2, $P=0.017$, Partial Eta Squared=0.19) The change at Week 12 did not reach significance (+3.9 in placebo group compared with -2.5 in etanercept group, least squares mean difference: +6.4, 95% CI: -1.3 to +14.2, $P=0.099$, Partial Eta Squared=0.10).

ApoE e4 carrier status did not have a significant effect on change in NPI ($F(1,27)=0.5$, $P=0.50$, Partial Eta Squared=0.02). In a separate analysis, using a two-way ANCOVA model including e4 carrier status as a between-subject variable, we investigated whether the treatment effect differed between e4 carriers and non-carriers. In this model, the overall difference in change in NPI

score remained significantly different between the treatment group and the placebo group, after adjusting for ApoE carrier status (F(1,26)=7.3, P=0.012, Partial Eta Squared=0.22). The interaction of ApoE e4 carrier status and treatment effect was not significant (F(1,26)=2.9, P=0.10, Partial Eta squared=0.10), indicating no significant difference in the treatment effect between ApoE e4 carriers and non-carriers.

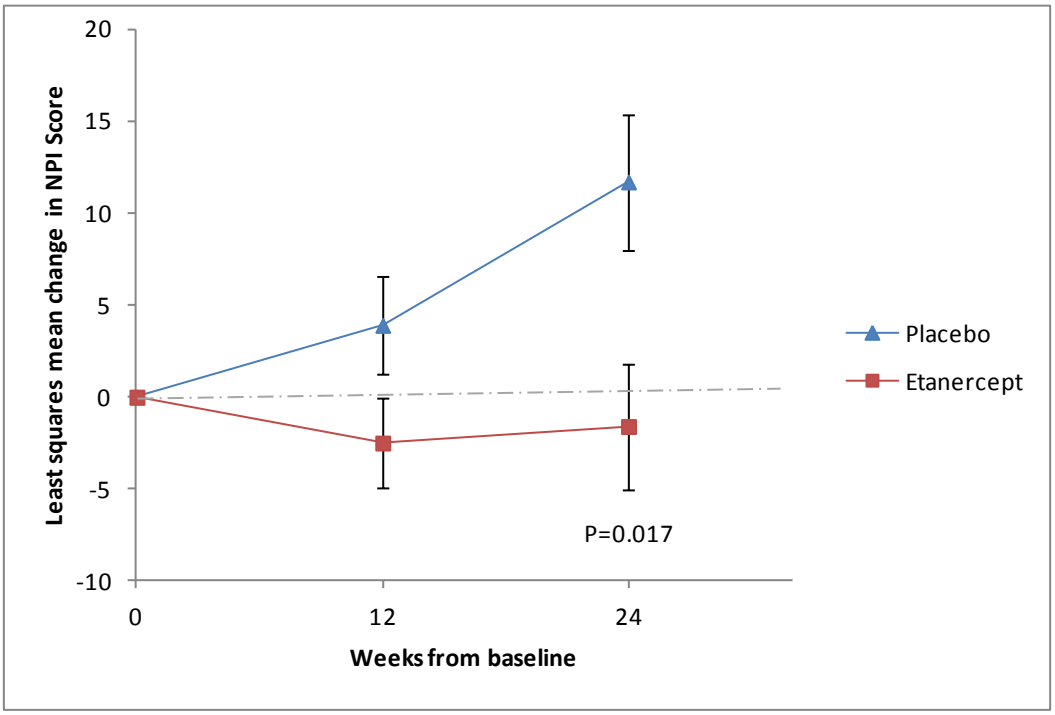
Considering other secondary outcome measures, the presence or absence of the e4 allele did not have any significant relationship with change from baseline in any of the other clinical measures in either the placebo or the etanercept groups (P-value in all cases >0.1).

Table 6.2.10 Adjusted mean change in NPI score during the study period

	Adjusted mean change in NPI score		Least squares mean difference [Placebo-Etanercept]
	Placebo	Etanercept	
Baseline			
LSM change	0	0	0
Week 12			
LSM change	+3.9	-2.5	+6.4
(SE or 95% CI)	(2.7)	(2.4)	(-1.3 to +14.2)
P-value			P=0.099
Week 24			
LSM change	+11.7	-1.6	+13.4
(SE or 95% CI)	(3.7)	(3.4)	(+2.5 to +24.2)
P-value			**P=0.017**
Overall effect of treatment (adjusted for effect of time)			
LSM change	+5.2	-1.4	+6.6
(SE or 95% CI)	(1.9)	(1.7)	(+1.1 to +12.1)
P-value			**P=0.021**
Overall effect of treatment x time			
F-test for overall effect of interaction of treatment x time	F(1,27)=6.4, Partial Eta Squared=0.19, **P=0.017**		

Data are Least Squares Means derived from mixed-effects, repeated-measures ANCOVA, adjusting for age, gender, ApoE e4 carrier status and baseline NPI score
LSM=Least squares mean, SE=Standard error, NPI=Neuropsychiatric inventory

Figure 6.2.12 Corrected mean change in NPI (±SE) at Weeks 12 and 24, by treatment, adjusted for age, gender, baseline NPI score and ApoE status



Data points show least squares means (standard error). NPI=Neuropsychiatric inventory
P-value from mixed-effects model, repeated-measures ANCOVA, analyzing change from baseline to each post-baseline visit, adjusted for age, sex, APO-E e4 carrier status and baseline NPI score

6.2.7.4 Analysis of treatment outcome modification by baseline cytokine levels

We hypothesized that baseline levels of pro-inflammatory and anti-inflammatory cytokines would influence the treatment effect of etanercept. The most significant treatment effects or trends were observed for the MMSE, NPI and BADLS outcome measures. We therefore used a mixed-effects, repeated-measures, ANCOVA model to examine treatment effects for the MMSE, NPI and BADLS after adjusting for baseline TNF-α, as a principal pro-inflammatory cytokine, and IL-10, as a principal anti-inflammatory cytokine. The models included the categorical variables treatment group, gender and ApoE e4 carrier status, the continuous variables age, baseline psychometric score, baseline TNF-α, baseline IL-10, and an interaction term for time and treatment.

MMSE:

A model that adjusted for baseline TNF-α and IL-10 as potential confounders demonstrated a more significant overall treatment effect for the MMSE, in favour of etanercept treatment, than a model that did not include the cytokines (cytokines included in model: overall treatment effect (placebo-etanercept) least squares mean difference: -3.0 points (95% CI: -5.3 to -0.6), F(1,24)=6.9, P=0.015, Table 6.2.11; cytokines not included in model: overall treatment effect (placebo-etanercept) least squares mean difference: -2.5 points (95% CI: -5.2 to 0.2), P=0.07, Table 6.2.8).

Baseline levels of both TNF-α and IL-10 were significantly related to MMSE change across the course of the study, after accounting for variation due to treatment group and other co-variates (TNF-α: F(1,24)=4.3, P=0.048, Partial Eta Squared=0.15; IL-10: (F(1,24)=6.2, P=0.02, Partial Eta Squared=0.20). The most significant effect of baseline cytokines on MMSE change was seen at Week 24, with higher TNF-α at baseline associated with greater decline in MMSE score at Week 24, and higher IL-10 associated with less decline in MMSE score at Week 24 (for every 1.0 pg/ml in baseline TNF-α: adjusted change in MMSE at Week 24: -0.2 (95% CI: -0.4 to 0.0), P=0.05, Partial Eta Squared=0.15. For every 0.1 pg/ml in baseline IL-10: adjusted change in MMSE from baseline at Week 24: +1.0 (95% CI: 0.04 to 2.0), P=0.05, Partial Eta Squared=0.15).

The interaction term between time and treatment was also significant (F(1,24)=7.2, P=0.013, Partial Eta Squared=0.23) – indicating that the effect on MMSE score was different between the etanercept and placebo groups across time, with little change in score in the etanercept group across time, and decline in score in the placebo group across time (Table 6.2.11, Figure 6.2.13).

The effect of etanercept treatment on MMSE score change was highly significant at Week 24, when the least squares mean change from baseline in MMSE score was greater in the placebo group than in the etanercept group (placebo group: -3.5, etanercept group: +1.1, least squares mean difference: -4.7 (95% CI: -7.8 to -1.6), P=0.004, Partial Eta Squared=0.29, after adjusting for age, gender, baseline MMSE score, ApoE e4 carrier status, and baseline TNF-α and IL-10).

The difference in MMSE score change at Week 12 did not reach significance (placebo group: -1.4, etanercept group: -0.1, least squares mean difference: -1.3, (95% CI: -3.5 to +1.0), P=0.26, Partial Eta Squared=0.05).

NPI and BADLS:

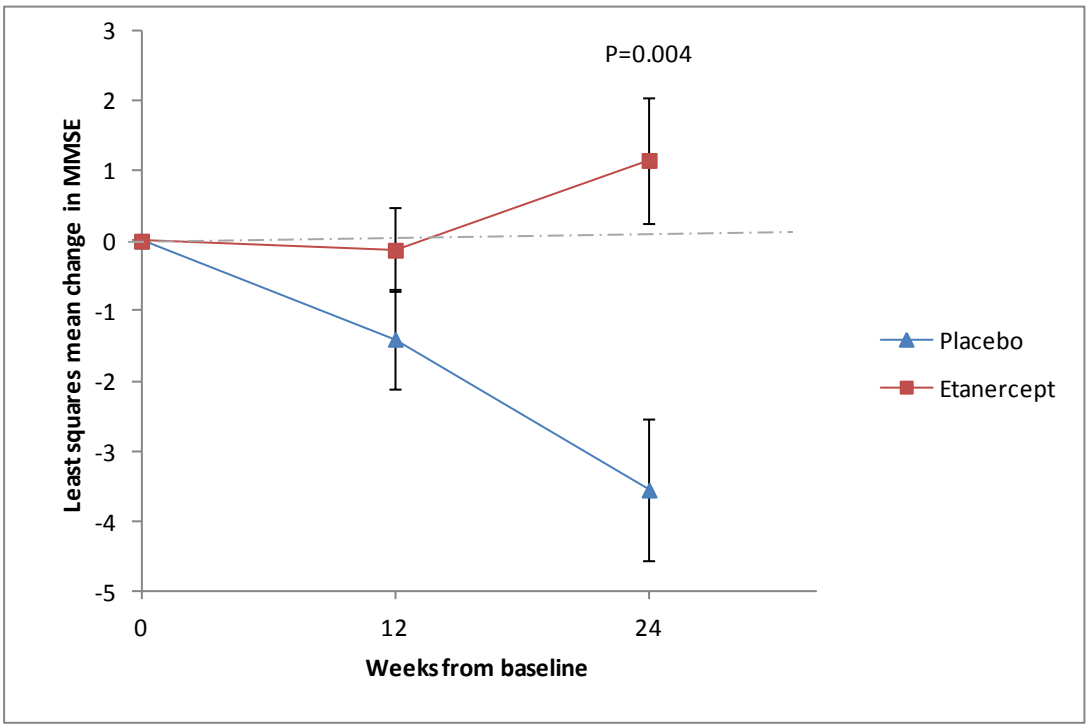
There was no significant change in the treatment effects for the NPI and BADLS outcome measures after including baseline TNF-α and IL-10 in the ANCOVA models.

Table 6.2.11 Adjusted mean change in MMSE score during the study period

	Adjusted mean change in MMSE score		Least squares mean difference [Placebo-Etanercept]
	Placebo	Etanercept	
Baseline LSM change	0	0	0
Week 12 LSM change (SE or 95% CI) P-value	-1.4 (0.7)	-0.1 (0.6)	-1.3 (-3.5 to +1.0) P=0.26
Week 24 LSM change (SE or 95% CI) P-value	-3.5 (1.0)	1.1 (0.9)	-4.7 (-7.8 to -1.6) **P=0.004**
Overall effect of treatment (adjusted for effect of time) LSM change (SE or 95% CI) P-value	-2.5 (0.8)	0.5 (0.7)	-3.0 (-5.3 to -0.6) **P=0.015**
Overall effect of treatment x time F-test for overall effect of interaction of treatment x time	F(1,24)=7.2, Partial Eta Squared=0.23, **P=0.013**		
Overall effect of baseline TNF-α F-test for overall effect on change in MMSE score	F(1,24)=4.3, Partial Eta Squared=0.15, **P=0.048**		
Overall effect of baseline IL-10 F-test for overall effect on change in MMSE score	F(1,24)=6.2, Partial Eta Squared=0.20, **P=0.02**		

LSM=Least squares mean, SE=Standard error, MMSE=Mini Mental State Exam. Data are Least Squares Means derived from mixed-effects, repeated-measures ANCOVA, adjusting for age, gender, baseline IL-10, baseline TNF-α, ApoE e4 carrier status and baseline MMSE score

Figure 6.2.13 Corrected mean change in MMSE (±SE) at Weeks 12 and 24, by treatment, adjusted for age, gender, baseline MMSE score, baseline TNF-α, baseline IL-10 and ApoE status



Data points show least squares means (standard error). MMSE=Mini Mental State Examination
P-value from mixed-effects model, repeated-measures ANCOVA, analyzing change from baseline to each post-baseline visit, adjusted for age, sex, APO-E e4 carrier status, baseline IL-10, baseline TNF-α and baseline MMSE score

Table 6.2.12 Corrected mean difference in psychometric score change between baseline and Week 28, and between Week 24 and Week 28 (washout phase) , corrected for age, gender and baseline psychometric score, by treatment group

	Week 28 (change from baseline)			Washout Phase (change from Week 24 to Week 28)		
	Etanercept N=18	Placebo N=15	Corrected Mean Difference† (95% CI) P-Value	Etanercept N=18	Placebo N=15	Corrected Mean Difference† (95% CI) P-Value
MMSE (SE)	-0.3 (0.7)	-2.0 (0.9)	-1.9 (-4.5 to 0.7) P=0.15	-0.3 (0.4)	-0.1 (0.9)	0.2 (-1.9 to 2.3) P=0.82
ADAS-Cog (SE)	4.5 (2.1)	1.3 (1.8)	-4.7 (-11.5 to 2.0) P=0.16	1.3 (1.2)	-4.3 (1.2)	**-5.7 (-9.6 to -1.9) P=0.005**
BADLS (SE)	3.2 (1.3)	4.3 (1.9)	1.7 (-3.2 to 6.5) P=0.48	2.4 (1.0)	-1.7 (1.0)	**-4.0 (-7.2 to -0.8) P=0.017**
NPI (SE)	-2.8 (3.0)	6.7 (3.1)	**10.0 (0.3 to 19.7) P=0.04**	-2.4 (2.3)	-3.5 (2.3)	0.1 (-6.4 to 6.7) P=0.97
Cornell (SE)	-0.4 (1.0)	-0.1 (1.4)	0.5 (-3.2 to 4.2) P=0.78	-0.8 (0.6)	-2.1 (0.7)	-0.8 (-2.7 to 1.1) P=0.38
CGIC (SE)	0.4 (0.3)	0.4 (0.2)	-0.2 (-1.0 to 0.6) P=0.55	-0.2 (0.2)	-0.5 (0.4)	-0.3 (-1.1 to 0.4) P=0.34
Fried (SE)	0.2 (0.2)	0.3 (0.3)	0.1 (-0.6 to 0.9) P=0.71	-0.2 (0.2)	0.0 (0.1)	0.1 (-0.4 to 0.6) P=0.61
SBS-6 (SE)	-0.5 (0.8)	-1.1 (0.8)‡	-0.2 (-2.1 to 1.7) P=0.85	-0.7 (0.7)	-0.9 (0.6)‡	0.0 (-1.7 to 1.6) P=0.99
SBS-9 (SE)	0.3 (1.3)	0.6 (1.7)‡	0.6 (-4.1 to 5.3) P=0.80	-0.2 (0.6)	-0.7 (0.8)‡	-0.6 (-2.8 to 1.6) P=0.57

MMSE=Mini-Mental State Examination, ADAS-Cog=Alzheimer's Disease Assessment Scale cognitive section, BADLS=Bristol Activities of Daily Living Scale, NPI=Neuropsychiatric Inventory, Cornell=Cornell Scale for Depression in Dementia, CGIC=Clinical Global Impression of Change, Fried=Fried Frailty Score, SBS=Sickness Behaviour Scale. † Corrected mean difference is the unstandardized B-coefficient obtained by linear regression, corrected for baseline age, gender and baseline psychometric score. ‡ N=14 for SBS-6 & SBS-9 in placebo group

6.2.7.5 Changes in psychometric outcomes at Week 28

We examined data at Week 28 to establish whether any treatment effects persisted after the 4-week washout phase (Table 6.2.12).

At Week 24 there was a trend towards a treatment effect favouring etanercept for the MMSE, but this did not persist at Week 28 (Week 28 corrected mean difference (placebo-etanercept) of -1.9 (95% CI: -4.5 to 0.7), P=0.15, compared to Week 24 corrected mean difference (placebo-etanercept) of -2.5 (95% CI: -5.2 to 0.2), P=0.07).

The treatment effect favouring etanercept persisted for the NPI at Week 28 (Week 28 corrected mean difference (placebo-etanercept) of +10.0 (95% CI: 0.3 to 19.7), P=0.04, compared to Week 24 corrected mean difference (placebo-etanercept) of +13.2 (95% CI: 2.4 to 24.0), P=0.02).

The treatment effect favouring etanercept did not persist for the BADLS at Week 28 (Week 28 corrected mean difference (placebo-etanercept) of +1.7 (95% CI: -3.2 to 6.5, P=0.48, compared to Week 24 corrected mean difference (placebo-etanercept) of +5.6 (95% CI: 0.4 to 10.9), P=0.04).

We examined the change from Week 24 to Week 28 to establish whether there was a rebound effect after withdrawal of the study drug (Table 6.2.12).

There was a significant improvement in the ADAS-Cog score in the placebo group between Week 24 and Week 28, with little change in the etanercept group on this measure (mean change in placebo group: -4.3 (SEM=1.2) versus mean change in etanercept group: +1.3 (SEM=1.2), corrected mean difference (placebo-etanercept) of -5.7 (95% CI: -9.6 to -1.9), P=0.005. At Week 24 there was no significant difference in change in ADAS-Cog score between the etanercept group and the placebo group, with numerical worsening in change in the placebo group (Week 24 mean change in placebo group: +5.6 (SEM=2.0) versus Week 24 mean change in etanercept group: +3.2 (SEM=1.8), corrected mean difference (placebo-etanercept) of +1.2 (95% CI: -5.1 to 7.5), P=0.70). There was no similar improvement in the MMSE score in the placebo group in the washout phase (washout phase corrected mean difference (placebo-etanercept) of +0.2 (95% CI: -1.9 to 2.3), P=0.82).

There was a worsening in the BADLS score in the etanercept group during the washout phase, after withdrawal of the drug (mean change in placebo group: -1.7 (SEM=1.0) versus mean change in etanercept group: +2.4 (SEM=1.0), corrected mean difference (placebo-etanercept) of -4.0 (95% CI: -7.2 to -0.8), P=0.017.

There was no evidence of rebound worsening for the NPI during the washout phase, after withdrawal of the drug, with the beneficial treatment effect of etanercept persisting into Week 28 (mean change in placebo group: -3.5 (SEM=2.3) versus mean change in etanercept group: -2.4 (SEM=2.3), corrected mean difference (placebo-etanercept) of 0.1 (95% CI: -6.4 to 6.7), P=0.97).

6.2.7.6 Analysis of treatment effects on serum cytokines

6.2.7.6.1 Correlations between baseline cytokines

Baseline correlations between serum cytokines were examined as a quality check on the validity of the measurements. Cytokine levels measured at baseline, prior to randomisation, were examined within the complete study population (N=40) (Table 6.2.13).

The correlation table shows that cytokines were frequently correlated with patterns consistent with their expected function (Table 6.2.13). TNF-α levels correlated with CRP (Spearman r=0.49, P<0.01) and IL-10 (Spearman r=0.37, P=0.02) and were weakly associated with IFN-γ (Spearman r=0.27, P=0.09), MCP1 (Spearman r=0.28, P=0.08) and IL-6 (Spearman r=0.25, P=0.12). CRP levels correlated with IL-6 (Spearman r=0.49, P<0.01), MCP1 (Spearman r=0.51, P<0.01) in addition to TNF-α (Spearman r=0.49, P<0.01), and CRP was weakly associated with IL-12 (Spearman r=0.30, P=0.06) and IL-10 (Spearman r=0.27, P=0.09).

The anti-inflammatory cytokine IL-10 was correlated with IFN-γ (Spearman r=0.43, P=0.01) and IL-12 (Spearman r=0.31, P=0.05), in addition to TNF-α as described above (Spearman r=0.37, P=0.02). IL-10 had weak associations with CRP (Spearman r=0.27, P=0.09) and MCP1 (Spearman r=0.26, P=0.11).

6.2.7.6.2 Pro-inflammatory cytokines (TNF-α, IL-6, CRP, IFN-γ, IL-12)

TNF-α:

Baseline levels of TNF-α did not differ between the etanercept group and the placebo group (etanercept group: 2.2 pg/ml (IQR 1.8 to 3.2), placebo group: 2.7 pg/ml (IQR 2.1 to 3.2), MWU=150, P=0.18, Table 6.2.14).

A mixed-effects, repeated-measures ANCOVA model showed an association between baseline TNF-α and increased cognitive decline as measured by the MMSE (Section 6.2.7.4 above) within the combined etanercept and placebo groups, after adjusting for treatment group and other co-variates (for every 1.0 pg/ml in baseline TNF-α: adjusted change in MMSE at Week 24: -0.2 (95% CI: -0.4 to 0.0), P=0.05, Partial Eta Squared=0.15, Table 6.2.11).

However, in the placebo group alone, the relationship between baseline TNF-α and MMSE change was not significant, and there were no associations between baseline TNF-α and decline in any of the other psychometric measures (all measures P>0.1).

Only one subject in the placebo group had TNF-α levels in the lowest quartile found at baseline (<1.8pg/ml) throughout the study. At 6 months this subject declined 1 point on the MMSE, ADAS-Cog and CGIC, showed no change (0 points) on the NPI and Cornell, and showed improvement on the BADLS (+4 points).

In the etanercept group alone, there was a significant positive correlation between baseline TNF-α levels and change in NPI score at Week 12 (Spearman=0.6, P=0.01) and Week 24 (Spearman=0.6, P=0.01).

The significant relationship between TNF-α and increased cognitive decline in the MMSE that was observed within the combined etanercept and placebo groups (Section 6.2.7.4 above) did not reach significance within the etanercept group alone, and there were no associations between baseline TNF-α and decline in any of the other psychometric measures within the etanercept group (all measures P>0.1).

The assay for TNF-α recognises TNF-α bound to etanercept, which has a longer half-life in serum than the unbound cytokine, and therefore etanercept treatment causes apparent high levels of TNF-α in treated subjects.

As expected therefore, measured TNF- α increased significantly in the etanercept group over the course of the study period, but levels remained stable in the placebo group (Table 6.2.14, Figure 6.2.14).

In the etanercept group, there was a drop in measured TNF-α between Week 24 and Week 28, as expected after cessation of treatment because of the loss of TNF-α bound to etanercept (median change in TNF-α between Week 28 and Week 24: etanercept group: -28 pg/ml (IQR -44 to -22), placebo group: 0.1 pg/ml (IQR -0.3 to 0.6), MWU<100, P<0.0001, Table 6.2.22).

IL-6, CRP and IFN-γ:

There were no significant baseline differences between the etanercept group and the placebo group in serum levels of IL-6, CRP or IFN-γ, and the levels of these cytokines did not change significantly across the course of the study in either treatment group (Tables 6.2.15 to 6.2.17, Figures 6.2.15 to 6.2.17).

IL-12:

At baseline, there was no significant difference in serum IL-12 levels between the etanercept group and the placebo group (etanercept group: 0.10 pg/ml (IQR 0.04 to 0.24), placebo group: 0.16 pg/ml (IQR 0.04 to 0.33), MWU=171, P=0.45, Table 6.2.18, Figure 6.2.18).

There was a significant increase in IL-12 in the etanercept group at Week 12 and at Week 24, compared to baseline, but no increase in IL-12 in the placebo group (Wilcoxon Signed Rank test for Week 12 compared to baseline in etanercept group: z=3.6, P<0.001, placebo group: z=1.0, P=0.31; Week 24 compared to baseline in etanercept group: z=2.6, P<0.01, placebo group: z=0.2, P=0.86, Table 6.2.18, Figure 6.2.18). At Week 28, after the 4-week wash-out phase, IL-12 levels had reverted to baseline in the etanercept group (Table 6.2.18, Figure 6.2.18).

At Week 12, there was a significant difference in IL-12 levels between the etanercept group and the placebo group (Week 12: etanercept group: 0.31 pg/ml (IQR 0.23 to 0.47), placebo group: 0.20 pg/ml (IQR 0.08 to 0.29), MWU=221, P=0.02, Table 6.2.18, Figure 6.2.18). However, at Week 24, although IL-12 levels remained significantly elevated from baseline in the etanercept group, and remained numerically higher than in the placebo group,

the difference between the etanercept group and the placebo group did not reach statistical significance (Week 24: etanercept group: 0.23 pg/ml (IQR 0.19 to 0.32), placebo group: 0.19 pg/ml (IQR 0.04 to 0.23), MWU=185, P=0.15, Table 6.2.18, Figure 6.2.18).

6.2.7.6.3 Chemokines (IL-8, MCP1)

There were no significant baseline differences between the etanercept group and the placebo group in serum levels of IL-8 and MCP1, and the levels of these chemokines did not change significantly across the course of the study in either treatment group (IL-8: Table 6.2.19, Figure 6.2.19, MCP1: Table 6.2.20, Figure 6.2.20).

6.2.7.6.4 Anti-inflammatory cytokine (IL-10)

At baseline, the anti-inflammatory cytokine IL-10 was lower in the etanercept group than in the placebo group (etanercept group: 0.25 pg/ml (IQR 0.19 to 0.33), placebo group: 0.35 pg/ml (IQR 0.25 to 0.46), MWU=117, P=0.02, Table 6.2.21, Figure 6.2.21).

A mixed-effects, repeated-measures ANCOVA model showed an association between baseline IL-10 and reduced cognitive decline as measured by the MMSE (Section 6.2.7.4 above) within the combined etanercept and placebo groups, after adjusting for treatment group and other co-variates (for every 0.1 pg/ml in baseline IL-10: adjusted change in MMSE from baseline at Week 24: +1.0 (95% CI: 0.04 to 2.0), P=0.05, Partial Eta Squared=0.15, Table 6.2.11). However, within the separate placebo and etanercept groups, this association between baseline IL-10 and MMSE change across the study did not reach significance, and there were no associations within the separate etanercept and placebo groups between baseline IL-10 and change in any other psychometric outcome measure (all measures P>0.1).

There was a significant increase in IL-10 in the etanercept group at Week 12 and at Week 24, compared to baseline, but no increase in IL-10 in the placebo group (Wilcoxon Signed Rank test for Week 12 compared to baseline in etanercept group: z=2.7, P=0.008, placebo group: z=0.4, P=0.69; Week 24 compared to baseline in etanercept group: z=2.8, P=0.005, placebo group: z=-0.3, P=0.79, Table 6.2.21, Figure 6.2.21).

At Week 28, after the 4-week wash-out phase, IL-10 levels had reverted to baseline in the etanercept group and remained constant in the placebo group (median change in IL-10 between Week 28 and Week 24: etanercept group: -0.12 pg/ml (IQR -0.2 to -0.07), placebo group: 0.02 pg/ml (IQR -0.06 to 0.18), MWU<100, P<0.0001, Table 6.2.22). At Week 28, this reduction in IL-10 in the etanercept group meant there was a re-appearance of the significant difference between the etanercept group and the placebo group seen at baseline (Week 28: etanercept group: 0.26 pg/ml (IQR 0.20 to 0.29), placebo group: 0.34 pg/ml (IQR 0.28 to 0.55), MWU=59, P=0.005, Table 6.2.21, Figure 6.2.21).

Table 6.2.13 Correlations between pre-treatment serum cytokines at baseline within the combined study population (N=40)

	Spearman r P-value							
	TNF-α	**IL-6**	**CRP**	**IFN-γ**	**IL-12**	**IL-8**	**MCP-1**	**IL-10**
TNF-α	1	.25	**.49**	.27	.25	.09	.28	**.37**
		0.12	***<0.01***	*0.09*	*0.12*	*0.59*	*0.08*	***0.02***
IL-6	.25	1	**.49**	.15	-.11	.06	.18	.14
	0.12		***<0.01***	*0.35*	*0.49*	*0.71*	*0.27*	*0.38*
CRP	**.49**	**.49**	1	.05	.30	-.10	**.51**	.27
	<0.01	***<0.01***		*0.77*	*0.06*	*0.53*	***<0.01***	*0.09*
IFN-γ	.27	.15	.05	1	.15	-.09	.02	**.43**
	0.09	*0.35*	*0.77*		*0.36*	*0.57*	*0.89*	***0.01***
IL-12	.25	-.11	.30	.15	1	-.19	.26	**.31**
	0.12	*0.49*	*0.06*	*0.36*		*0.24*	*0.10*	***0.05***
IL-8	.09	.06	-.10	-.09	-.19	1	-.05	.11
	0.59	*0.71*	*0.53*	*0.57*	*0.24*		*0.78*	*0.49*
MCP-1	.28	.18	**.51**	.02	.26	-.05	1	.26
	0.08	*0.27*	***<0.01***	*0.89*	*0.10*	*0.78*		*0.11*
IL-10	**.37**	.14	.27	**.43**	**.31**	.11	.26	1
	0.02	*0.38*	*0.09*	***0.01***	***0.05***	*0.49*	*0.11*	

Data are Spearman correlations and associated P-values. Significant correlations in bold.

Table 6.2.14 TNF-α in serum: within-group change and between-group differences from baseline to Week 28

	Treatment group		
	Etanercept	Placebo	Comparison of treatment groups
Baseline TNF-α, pg/ml (IQR)	2.2 (1.8 to 3.2) N=20	2.7 (2.1 to 3.2) N=20	MWU=150, z=-1.3 P=0.18
Week 12 TNF-α, pg/ml (IQR)	36.4 (32.4 to 44.1)	2.7 (2.1 to 3.1)	MWU=304, z=5.0 **P<0.001**
P-value for comparison with baseline†	N=19, z=3.8 **P<0.001**	N=16, z=-0.5 P=0.59	
Week 24 TNF-α, pg/ml (IQR)	38.3 (32.4 to 54.5)	2.9 (2.1 to 3.4)	MWU=261, z=4.1 **P<0.001**
P-value for comparison with baseline†	N=19, z=3.8 **P<0.001**	N=15, z=0.1 P=0.93	
Week 28 TNF-α, pg/ml (IQR)	9.4 (7.4 to 11.1)	2.8 (2.4 to 3.7)	MWU=252, z=4.2 **P<0.001**
P-value for comparison with baseline†	N=19, z=2.9 **P=0.003**	N=15, z=0.4 P=0.68	

IQR=Interquartile range, MWU=Mann Whitney U Test Statistic
†Wilcoxon Signed Rank Test

Figure 6.2.14 TNF-α in serum at different time points in etanercept and placebo groups

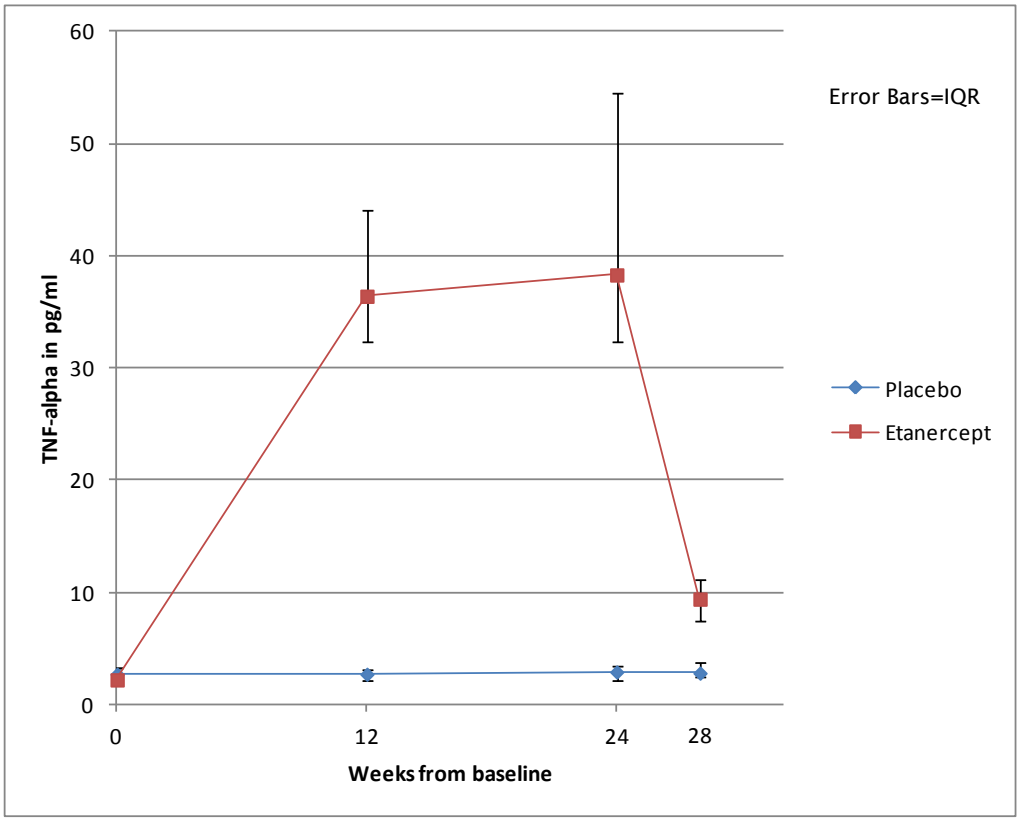


Table 6.2.15 IL-6 in serum: within-group change and between-group differences from baseline to Week 28

	Treatment group		
	Etanercept	Placebo	Comparison of treatment groups
Baseline IL-6, pg/ml (IQR)	0.89 (0.52 to 1.76)	0.93 (0.66 to 1.65)	MWU=200, z=0.0 P=1.00
	N=20	N=20	
Week 12 IL-6, pg/ml (IQR)	0.92 (0.58 to 1.59)	0.78 (0.49 to 1.43)	MWU=168, z=0.5 P=0.61
P-value for comparison with baseline†	N=19, z=-1.0 P=0.32	N=16, z=-0.7 P=0.50	
Week 24 IL-6, pg/ml (IQR)	0.96 (0.60 to 1.68)	0.67 (0.48 to 1.57)	MWU=165, z=0.8 P=0.45
P-value for comparison with baseline†	N=19, z=-0.4 P=0.72	N=15, z=-0.03 P=0.97	
Week 28 IL-6, pg/ml (IQR)	1.01 (0.49 to 1.71)	0.86 (0.66 to 1.92)	MWU=140, z=0.2 P=0.87
P-value for comparison with baseline†	N=18, z=-0.4 P=0.71	N=15, z=1.1 P=0.27	

IQR=Interquartile range, MWU=Mann Whitney U Test Statistic
†Wilcoxon Signed Rank Test

Figure 6.2.15 IL-6 in serum at different time points in etanercept and placebo groups

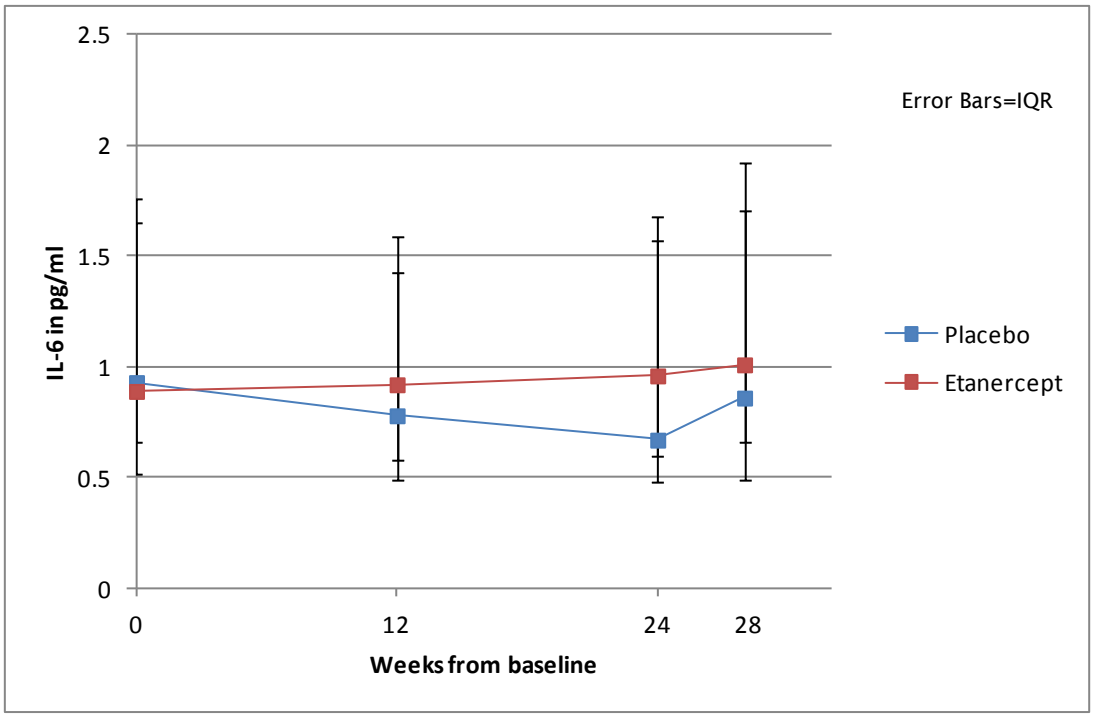


Table 6.2.16 CRP in serum: within-group change and between-group differences from baseline to Week 28

	Treatment group		
	Etanercept	Placebo	Comparison of treatment groups
Baseline CRP, mg/L (IQR)	1.61 (0.61 to 3.44) N=20	1.47 (0.60 to 6.69) N=20	MWU=197, z=-0.1 P=0.95
Week 12 CRP, mg/L (IQR)	1.03 (0.54 to 3.08)	1.40 (0.45 to 4.95)	MWU=143, z=-0.3 P=0.78
P-value for comparison with baseline†	N=19, z=-1.7 P=0.10	N=16, z=-1.9 P=0.06	
Week 24 CRP, mg/L (IQR)	1.37 (0.58 to 3.13)	2.09 (0.50 to 5.14)	MWU=131, z=-0.4 P=0.71
P-value for comparison with baseline†	N=19, z=-1.2 P=0.21	N=15, z=0.1 P=0.87	
Week 28 CRP, mg/L (IQR)	1.68 (0.73 to 5.60)	2.78 (0.45 to 7.19)	MWU=130, z=-0.2 P=0.87
P-value for comparison with baseline†	N=18, z=1.3 P=0.18	N=15, z=0.60 P=0.55	

IQR=Interquartile range, MWU=Mann Whitney U Test Statistic
†Wilcoxon Signed Rank Test

Figure 6.2.16 CRP in serum at different time points in etanercept and placebo groups

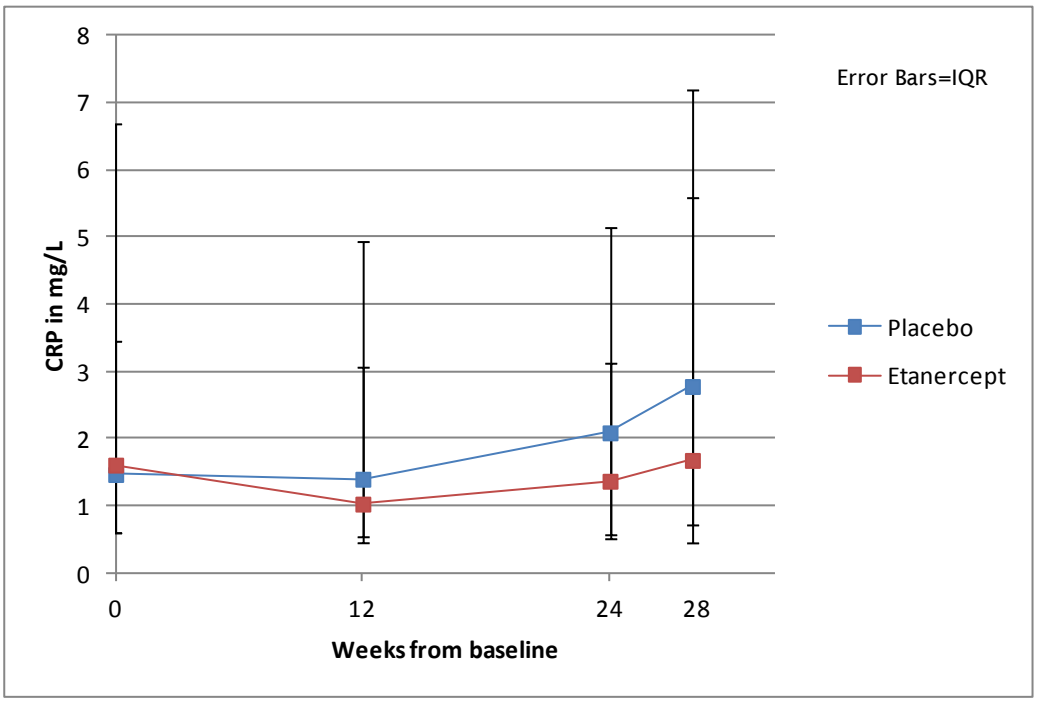


Table 6.2.17 IFN-γ in serum: within-group change and between-group differences from baseline to Week 28

	Treatment group		
	Etanercept	Placebo	Comparison of treatment groups
Baseline IFN-γ, pg/ml (IQR)	6.83 (4.43 to 9.04)	8.33 (4.55 to 12.33)	MWU=179, z=-0.6 P=0.58
	N=20	N=20	
Week 12 IFN-γ, pg/ml (IQR)	7.60 (6.48 to 13.80)	5.53 (4.27 to 9.07)	MWU=209, z=1.9 P=0.06
P-value for comparison with baseline†	N=19, z=0.93 P=0.36	N=16, z=-0.79 P=0.43	
Week 24 IFN-γ, pg/ml (IQR)	7.22 (5.11 to 11.40)	8.63 (5.70 to 13.20)	MWU=127, z=-0.5 P=0.58
P-value for comparison with baseline†	N=19, z=-0.52 P=0.60	N=15, z=-0.60 P=0.55	
Week 28 IFN-γ, pg/ml (IQR)	6.68 (5.29 to 10.73)	8.00 (4.54 to 12.10)	MWU=122, z=-0.5 P=0.66
P-value for comparison with baseline†	N=18, z=0.33 P=0.74	N=14, z=0.72 P=0.47	

IQR=Interquartile range, MWU=Mann Whitney U Test Statistic
†Wilcoxon Signed Rank Test

Figure 6.2.17 IFN-γ in serum at different time points in etanercept and placebo groups

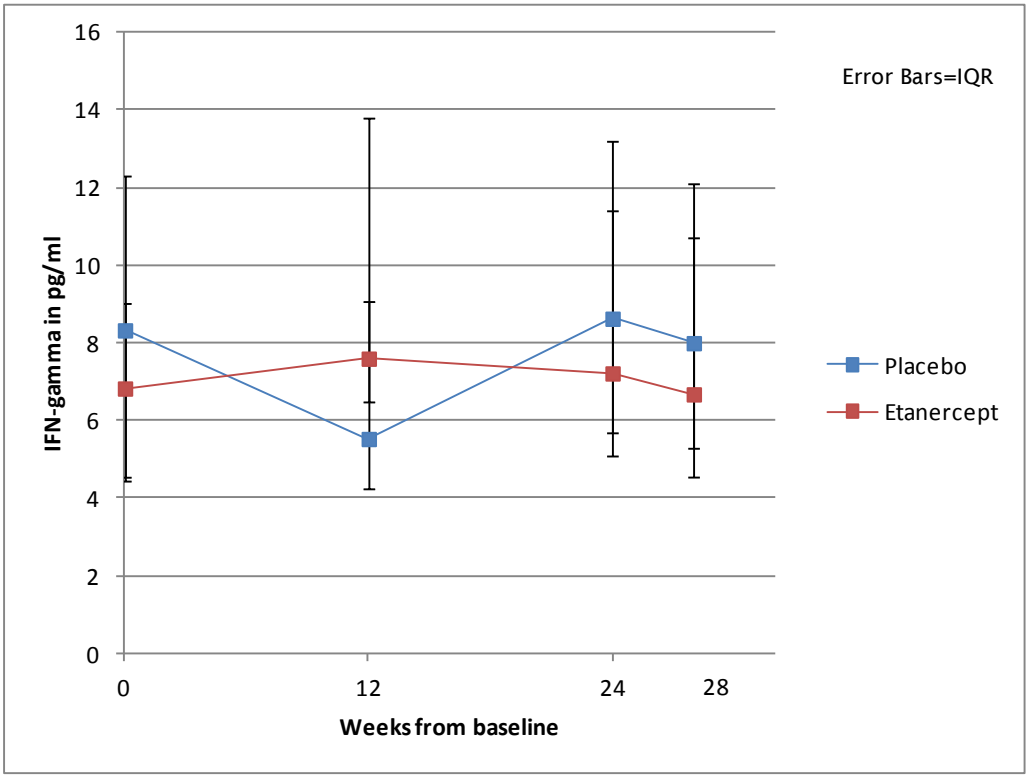


Table 6.2.18 IL-12 in serum: within-group change and between-group differences from baseline to Week 28

	Treatment group		
	Etanercept	Placebo	Comparison of treatment groups
Baseline IL-12, pg/ml (IQR)	0.10 (0.04 to 0.24) N=20	0.16 (0.04 to 0.33) N=20	MWU=171, z=-0.8 P=0.45
Week 12 IL-12, pg/ml (IQR)	0.31 (0.23 to 0.47)	0.20 (0.08 to 0.29)	MWU=221, z=2.3 **P=0.02**
P-value for comparison with baseline†	N=19, z=3.6 **P<0.001**	N=15, z=1.0 P=0.31	
Week 24 IL-12, pg/ml (IQR)	0.23 (0.19 to 0.32)	0.19 (0.04 to 0.23)	MWU=185, z=1.5 P=0.15
P-value for comparison with baseline†	N=19, z=2.6 **P=0.009**	N=14, z=0.2 P=0.86	
Week 28 IL-12, pg/ml (IQR)	0.15 (0.06 to 0.28)	0.18 (0.09 to 0.28)	MWU=113, z=-0.8 P=0.42
P-value for comparison with baseline†	N=18, z=0.2 P=0.85	N=14, z=0.2 P=0.83	

IQR=Interquartile range, MWU=Mann Whitney U Test Statistic
†Wilcoxon Signed Rank Test

Figure 6.2.18 IL-12 in serum at different time points in etanercept and placebo groups

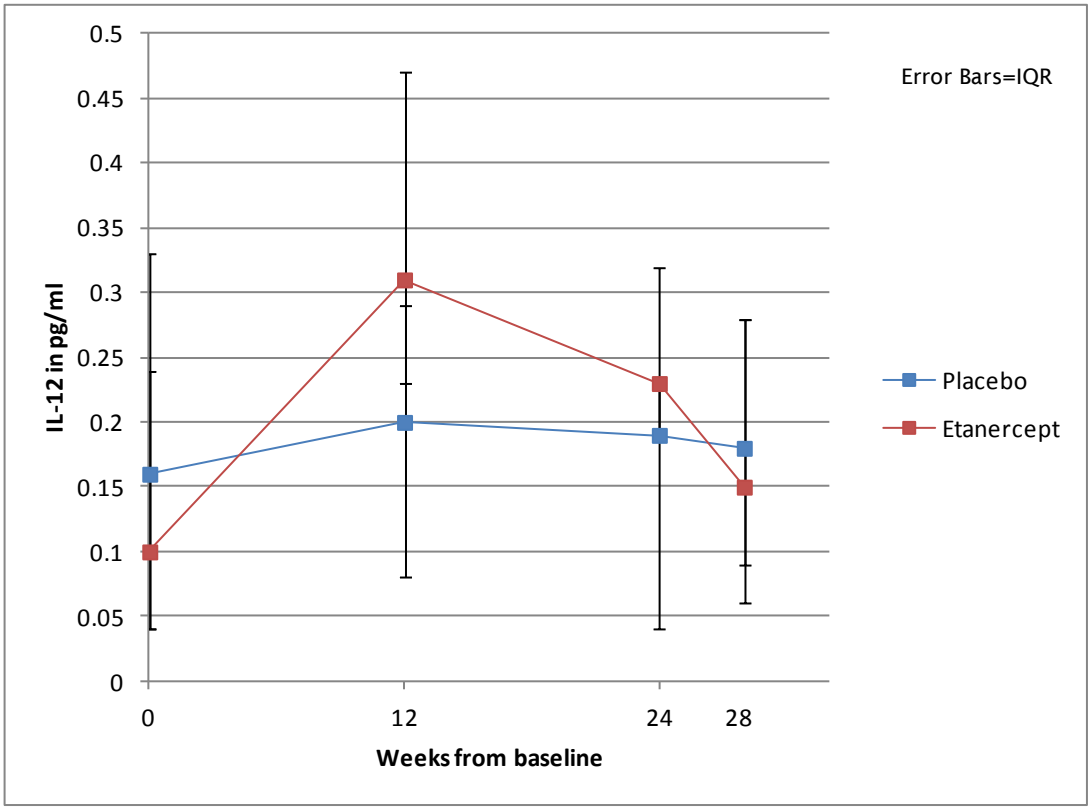


Table 6.2.19 IL-8 in serum: within-group change and between-group differences from baseline to Week 28

	Treatment group		
	Etanercept	Placebo	Comparison of treatment groups
Baseline IL-8, pg/ml (IQR)	12.0 (10.2 to 15.3)	13.1 (9.7 to 15.7)	MWU=193, z=-0.2 P=0.86
	N=20	N=20	
Week 12 IL-8, pg/ml (IQR)	13.2 (10.4 to 17.2)	13.5 (10.0 to 15.6)	MWU=161, z=0.3 P=0.78
P-value for comparison with baseline†	N=19, z=1.0 P=0.29	N=16, z=0.9 P=0.36	
Week 24 IL-8, pg/ml (IQR)	13.9 (9.1 to 18.9)	15.2 (10.4 to 20.2)	MWU=131, z=-0.4 P=0.68
P-value for comparison with baseline†	N=19, z=0.4 P=0.68	N=15, z=1.3 P=0.18	
Week 28 IL-8, pg/ml (IQR)	11.5 (8.9 to 18.9)	14.7 (8.5 to 19.7)	MWU=119, z=-0.6 P=0.58
P-value for comparison with baseline†	N=18, z=-0.9 P=0.35	N=15, z=1.1 P=0.29	

IQR=Interquartile range, MWU=Mann Whitney U Test Statistic
†Wilcoxon Signed Rank Test

Figure 6.2.19 IL-8 in serum at different time points in etanercept and placebo groups

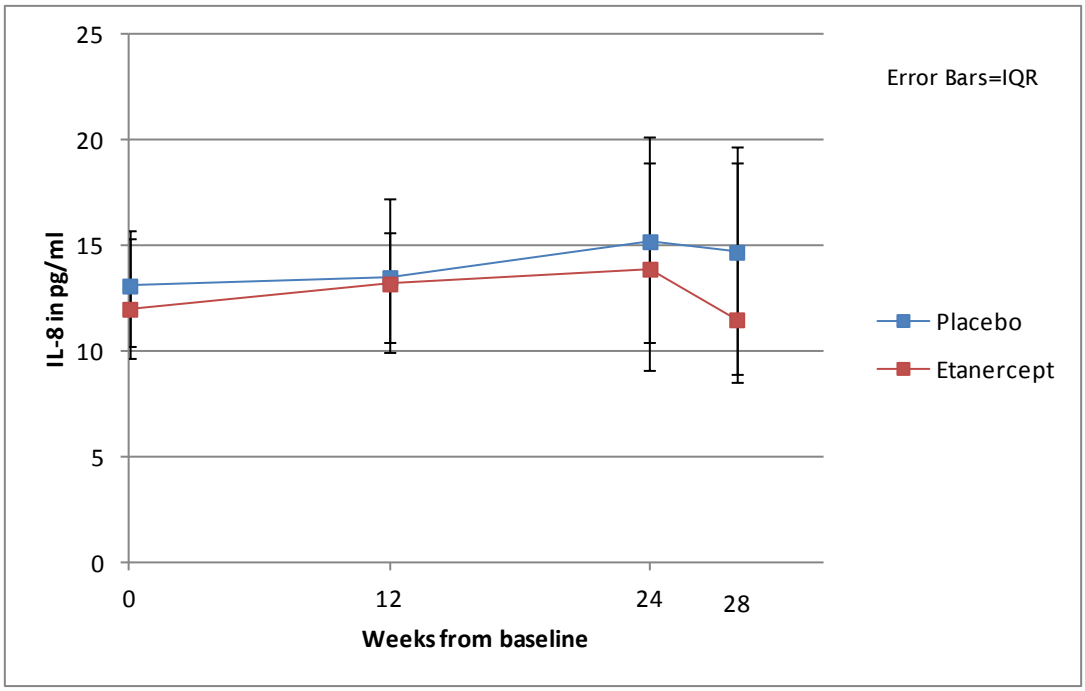


Table 6.2.20 MCP1 in serum: within-group change and between-group differences from baseline to Week 28

	Treatment group		
	Etanercept	Placebo	Comparison of treatment groups
Baseline MCP1, pg/ml (IQR)	353 (293 to 452)	353 (311 to 475)	MWU=193, z=-0.2 P=0.86
	N=20	N=20	
Week 12 MCP1, pg/ml (IQR)	353 (290 to 428)	387 (314 to 441)	MWU=132, z=-0.7 P=0.52
P-value for comparison with baseline†	N=19, z=-0.8 P=0.44	N=16, z=1.0 P=0.33	
Week 24 MCP1, pg/ml (IQR)	366 (337 to 444)	317 (248 to 391)	MWU=189, z=1.6 P=0.11
P-value for comparison with baseline†	N=19, z=0.6 P=0.52	N=15, z=-1.8 P=0.07	
Week 28 MCP1, pg/ml (IQR)	360 (265 to 419)	342 (302 to 404)	MWU=135, z=0.0 P=1.0
P-value for comparison with baseline†	N=18, z=-1.8 P=0.08	N=15, z=-0.9 P=0.40	

IQR=Interquartile range, MWU=Mann Whitney U Test Statistic
†Wilcoxon Signed Rank Test

Figure 6.2.20 MCP1 in serum at different time points in etanercept and placebo groups

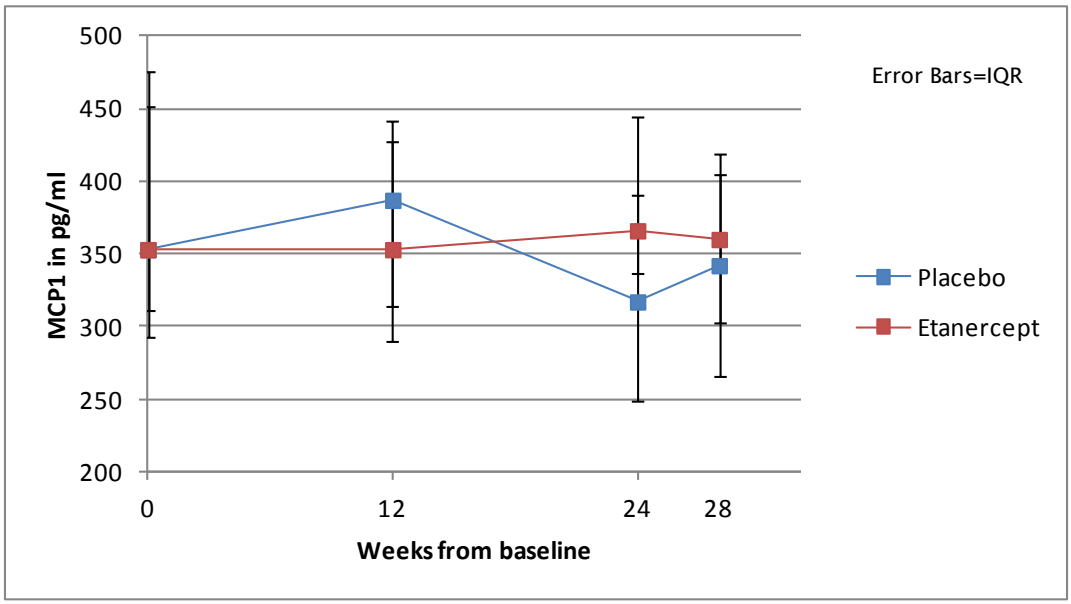


Table 6.2.21 IL-10 in serum: within-group change and between-group differences from baseline to Week 28

	Treatment group		
	Etanercept	Placebo	Comparison of treatment groups
Baseline IL-10, pg/ml (IQR)	0.25 (0.19 to 0.33) N=20	0.35 (0.25 to 0.46) N=20	MWU=117, z=-2.3 **P=0.02**
Week 12 IL-10, pg/ml (IQR)	0.37 (0.29 to 0.43)	0.33 (0.20 to 0.47)	MWU=153, z<0.1 P=1.0
P-value for comparison with baseline†	N=19, z=2.7 **P=0.008**	N=15, z=0.4 P=0.69	
Week 24 IL-10, pg/ml (IQR)	0.37 (0.27 to 0.50)	0.33 (0.28 to 0.45)	MWU=158, z=0.5 P=0.61
P-value for comparison with baseline†	N=19, z=2.8 **P=0.005**	N=14, z=-0.3 P=0.79	
Week 28 IL-10, pg/ml (IQR)	0.26 (0.20 to 0.29)	0.34 (0.28 to 0.55)	MWU=59, z=-2.7 **P=0.005**
P-value for comparison with baseline†	N=18, z=0.5 P=0.65	N=14, z=-2.2 **P=0.03** Sign Test r=1.9, P=0.06	

IQR=Interquartile range, MWU=Mann Whitney U Test Statistic
†Wilcoxon Signed Rank Test

Figure 6.2.21 IL-10 in serum at different time points in etanercept and placebo groups

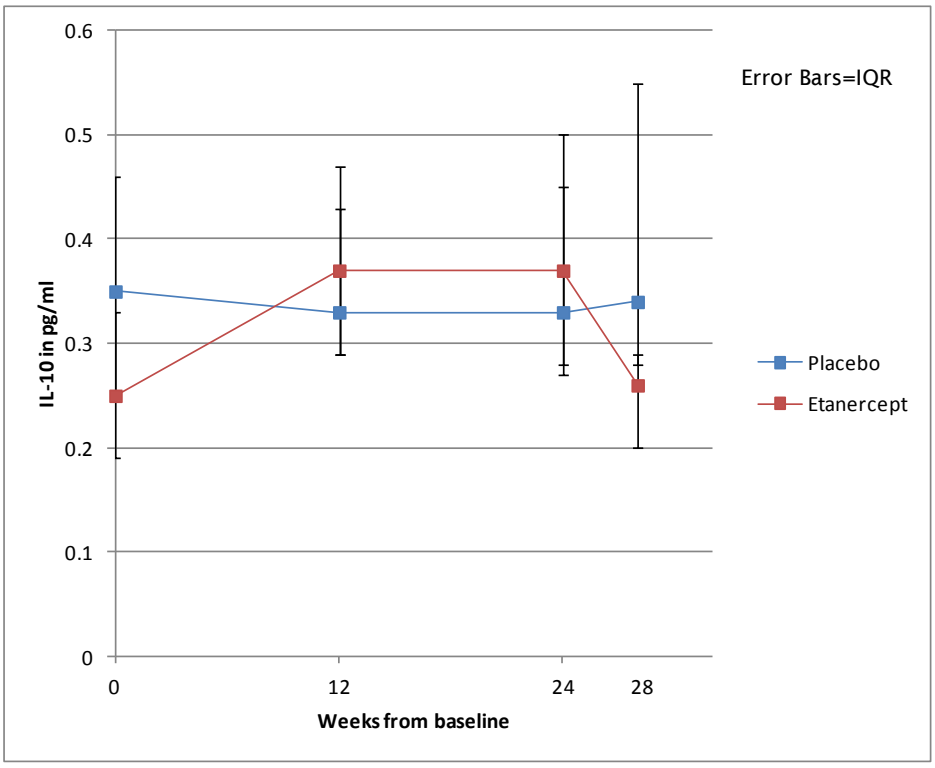


Table 6.2.22 Median change in serum cytokine levels between last study injection and 4-week follow-up (4 week wash-out phase)

	Treatment group		
	Etanercept (N=18)	Placebo (N=14)	Comparison of treatment groups
TNF-α (pg/ml) (IQR)	-28.0 (-44 to -22)	0.1 (-0.3 to 0.6)	**P<0.0001**
IL-6 (pg/ml) (IQR)	0 (-0.3 to 0.4)	0.2 (-0.2 to 0.3)	P=0.7
CRP (mg/L) (IQR)	0.3 (-0.4 to 1.2)	0.04 (-1.1 to 2.1)	P=0.6
IFN-γ (pg/ml) (IQR)	-0.13 (-3.0 to 3.3)	1.7 (-1.6 to 4.5)	P=0.5
IL-12 (pg/ml) (IQR)	-0.11 (-0.21 to 0.08)	0.06 (-0.09 to 0.12)	P=0.2
IL-8 (pg/ml) (IQR)	-1.5 (-5.0 to 3.9)	1.3 (-3.8 to 2.4)	P=0.6
MCP1 (pg/ml) (IQR)	-12 (-66 to 32)	16 (-16 to 104)	P=0.1
IL-10 (pg/ml) (IQR)	-0.12 (-0.2 to -0.07)	0.02 (-0.06 to 0.18)	**P<0.0001**

IQR=Interquartile range, MWU=Mann Whitney U Test Statistic

6.3 STEADI-09: Discussion

This study was carried out to establish whether treatment with subcutaneous etanercept is safe and well tolerated in patients with mild to moderate Alzheimer's disease, and to explore the effect of etanercept treatment on cognitive, behavioural and functional secondary outcome measures. Our results show that subcutaneous etanercept is both safe and well tolerated in this population.

Safety is demonstrated by the finding that, for adverse events with any cause, the incidence rate was lower in the etanercept group than in the placebo group (IRR=0.67 (95% CI: 0.44 to 1.02), P=0.05, Figure 6.2.2). Furthermore, there were no Serious Adverse Events (SAEs) in the etanercept group.

Tolerability is demonstrated by the finding that withdrawal rates were similar between the etanercept group and the placebo group, with a numerically lower withdrawal rate in the etanercept group (etanercept withdrawal: 10% of those enrolled; placebo withdrawal: 29% of those enrolled, P=0.2, Table 6.2.2). The observed withdrawal rate of 10% seen in the etanercept group was favourably low compared to withdrawal rates in other Alzheimer's disease drug trials (20% to 30%).[362 453] The low withdrawal rate may be related to the frequent contact participants had with the study team – injections were given each week by a study team member, often in the participant's own home. This frequent contact meant that adverse events and tolerability issues could be addressed efficiently and without delay by the study team.

In addition to the primary outcomes of safety and tolerability, we also found evidence that treatment with subcutaneous etanercept is effective in treating the symptoms of Alzheimer's disease measured with cognitive, functional and behavioural secondary outcome measures.

On cognitive measures, we found that treatment with subcutaneous etanercept results in reduced cognitive decline, as measured by the MMSE at Week 24, after adjusting for baseline levels of TNF-α and IL-10 (overall treatment effect (placebo-etanercept) least squares mean difference: -3.0 points (95% CI: -5.3 to -0.6), F(1,24)=6.9, P=0.015, Table 6.2.11, Figure 6.2.13). Adjusting for baseline levels of TNF-α and IL-10 is necessary because we found that baseline levels of both TNF-α and IL-10 were significantly related to MMSE change

across the course of the study, after accounting for variation due to treatment group and other co-variates (Table 6.2.11). This finding of a relationship between baseline cytokine levels and cognitive change on the MMSE supports the hypothesis that systemic cytokines affect neuro-inflammation and neuro-degeneration. Indeed, as expected from the hypothesis, baseline levels of the pro-inflammatory cytokine TNF-α were associated with increased decline in the MMSE score, and baseline levels of the anti-inflammatory cytokine IL-10 were associated with reduced decline in the MMSE score. These findings are consistent with previous studies that demonstrate a correlation between Alzheimer's disease and levels of TNF-α[454] and IL-10,[455] and support the overall hypothesis that peripheral cytokines play a role in the progression of neuro-degeneration in Alzheimer's disease.

This finding of reduced cognitive decline in patients treated with subcutaneous etanercept compares well with the reduced cognitive decline seen in patients treated with donepezil. Our finding of a 3 point difference in change in MMSE score at 24 weeks is greater than the 1 to 2 point differences in change in MMSE score at 24 weeks seen in donepezil studies.[153 362] Although the MMSE response to donepezil treatment is small it is nevertheless judged to be clinically significant,[153] and this suggests that the etanercept treatment effect seen in our study is also of a magnitude that should be considered clinically significant.

On behavioural measures, we found that treatment with subcutaneous etanercept results in reduced decline in behavioural and psychological symptoms measured by the NPI (least squares mean difference (placebo-etanercept): +13.4, 95% CI: 2.5 to 24.2, P=0.017, Partial Eta Squared=0.19, Table 6.2.10). Behavioural and psychological symptoms are an important target for putative treatments for Alzheimer's disease because these symptoms are common, distressing and difficult to manage.[456 457] Indeed, families and carers of people living with dementia find that these symptoms are often more troubling and difficult than cognitive symptoms.[458] Existing treatments for behavioural and psychological symptoms have limited efficacy, and are associated with significant unwanted side effects. Memantine treatment is of limited benefit over 24 weeks.[459] Anti-psychotic medication is commonly used to treat these symptoms but the evidence showing benefit is poor, and the use of antipsychotic medication is associated with significant cardiac side effects

and an increase in mortality.[460 461] The extent of this increase in mortality is such that regulatory bodies now recommend withdrawing anti-psychotic treatment if possible.[462] In this study we have shown that subcutaneous etanercept treatment halts decline in behavioural and psychological symptoms measured by the NPI. The degree of this change compares favourably with the effects seen in studies of existing treatments for these symptoms. Meta-analysis demonstrates that memantine treatment reduces NPI change at 24 weeks by 1 to 3 points, compared to 13 points in this study.[459 463] The decline in NPI score in the placebo group in this study is larger than in the placebo group in many of the memantine trials within the meta-analysis. One reason for this difference is that we had broad and inclusive inclusion criteria and few exclusion criteria in comparison with many dementia studies. Our study was set up in this way as we did not want to exclude those with multi-comorbidity and frailty, as we consider these Alzheimer's disease patients the most likely to have dysregulation of systemic inflammation and therefore the most likely to experience worsening of pathological sickness behaviour symptoms. Many dementia studies exclude such patients and therefore progression of symptoms may be less marked in those selected populations.

This beneficial behavioural effect of etanercept supports the overall hypothesis concerning systemic inflammation and Alzheimer's disease. We hypothesised that many behavioural and psychological symptoms in Alzheimer's disease can be considered forms of pathological sickness behaviour, triggered by the effect of systemic inflammation on a brain sensitized by on-going neuro-degeneration. We therefore hypothesized that reduced systemic inflammation, after treatment with etanercept to reduce peripheral pro-inflammatory signalling, would be particularly associated with reduced decline in behavioural and psychological symptoms. Our findings support this hypothesis. In the study, out of all secondary outcome measures, we found the largest and most significant treatment effects were for the NPI assessment of behavioural and psychological symptoms. Animal models have previously supported the hypothesis that reducing systemic inflammation with peripheral etanercept can reduce sickness behaviour symptoms associated with neuro-degenerative disease,[464] but this study is the first to demonstrate a similar finding in humans.

On functional measures, we found that treatment with subcutaneous etanercept results in reduced decline in functional ability as measured by the Bristol Activities of Daily Living (BADLS) scale (corrected mean difference (placebo-etanercept): +5.6 points (95% CI: 0.4 to 10.9), P=0.04, Table 6.2.8). This finding is greater than the Minimum Clinically Important Difference (MCID) of 3.5 points that has previously been recommended for BADLS treatment effect size in Alzheimer's disease studies.[374 465] This reduction in decline in activities of daily living did not persist after withdrawal of etanercept treatment and there was evidence of decline in the etanercept group between completion of etanercept treatment in Week 24 and wash-out follow up in Week 28 (mean change in placebo group: -1.7 (SEM=1.0) versus mean change in etanercept group: +2.4 (SEM=1.0), P=0.017, Table 6.2.12).

This post-withdrawal decline in the etanercept group on the BADLS functional measure may be considered a withdrawal reaction. For all other measures that had a significant treatment effect, there was a reduction in the treatment effect after the 4-week wash-out phase, although this withdrawal effect did not reach statistical significance for measures other than the BADLS. This failure to sustain the beneficial effects of etanercept, after withdrawal of the treatment, is consistent with the hypothesized active pathophysiological role of systemic inflammation in Alzheimer's disease. It is also consistent with the experience of etanercept use in other active inflammatory conditions, such as psoriasis and rheumatoid arthritis, where symptoms recur soon after cessation of treatment.[448 466]

Etanercept treatment had effects on peripheral cytokine levels in the study population. There was a significant increase in the anti-inflammatory cytokine IL-10 by Week 12, sustained at Week 24 (Baseline: 0.25 pg/ml, Week 12: 0.37 pg/ml, P=0.008, Week 24: 0.37 pg/ml, P=0.005, Figure 6.2.21). There was a similar increase in IL-12 (Baseline: 0.10 pg/ml, Week 12: 0.21 pg/ml, P<0.001, Week 24: 0.23 pg/ml, P=0.009, Figure 6.2.18).

The effect of anti-TNF treatment on cytokine profiles varies with the inflammatory pathology and severity of the underlying condition which the drug is used to treat. The effect of anti-TNF treatment on cytokine profiles can be different for patients with the same underlying condition because of differences in concurrent treatment with other potent immuno-modulatory

drugs. In rheumatoid arthritis some patients experience a decrease in peripheral IL-6,[467] and others experience no change,[468] while others have a fall in CRP and an increase in IL-4 and IFN-γ.[469] In ankylosing spondylitis, the number of IFN-γ and IL-2 positive T-cells increases, but the number of IL-10 positive T-cells falls,[470] and peripheral blood mononuclear cells (PBMCs), contrary to expectation, have an increased capacity to produce TNF-α and IFN-γ.[471] In psoriasis, some patients experience a decrease in IL-6,[472] and some experience falls in IL-1, IL-8 and IL-17.[473] Furthermore, in contrast to the cytokine findings in our study of Alzheimer's disease, in psoriasis, IL-10 and IL-12 levels decreased during etanercept therapy, rather than increased.[474] In Crohn's disease there is a decrease in CRP and IL-6, and an increase in PBMC production of IFN-γ, TNF-α and IL-10.[475] Overall, anti-TNF treatment has diverse effects on cytokine profiles, with the effect varying from one condition to another, and often also varying within the same condition. In general it appears that drugs like etanercept help to restore balance within the immune system when this balance has been disturbed by an underlying inflammatory condition. So, in ankylosing spondylitis, rheumatoid arthritis, Crohn's disease and Behcet's disease, anti-TNF treatment tends to increase IFN-γ, whereas there is no effect on IFN-γ in healthy controls.[469 470 475 476] In ankylosing spondylitis, low IL-2 levels are increased by anti-TNF treatment,[470] whereas in Crohn's disease, high IL-2 levels are decreased by anti-TNF treatment.[477] Etanercept is therefore not thought to have significant effects on peripheral cytokines in healthy controls, but does have profound effects on peripheral cytokines when these are dysregulated by an underlying inflammatory condition. Therefore, the significant effects on IL-10 and IL-12 in this study imply an underlying dysregulation in peripheral cytokines in patients with mild to moderate Alzheimer's disease, and are consistent with the evidence demonstrating differential cytokine levels between patients with Alzheimer's disease and healthy controls.[107 110 114]

6.3.1 Limitations in the study design

There are limitations in the design and implementation of the study that must be considered when interpreting the results. The sample size in the study was small (N=41) and the study was not statistically powered to detect effect sizes in the secondary outcome measures. With this small numbers of participants, the significant effect sizes in the study may be the result of chance, rather than due to etanercept. However, the pattern of benefit in favour of etanercept was similar across different cognitive, behavioural and functional outcome measures, giving some confidence in the validity of the results. The small sample size also means that the results are more susceptible to bias from early withdrawal, especially if early withdrawal is greater in one group than in another. Overall, withdrawal rates were low compared to other Alzheimer's disease drug studies.[362 453] There were more early withdrawal subjects in the placebo group than in the etanercept group, raising the possibility of bias in the analysis of study completers. However, the intention-to-treat, last-observation-carried-forward data show similar findings in outcome measures as the main analysis, although the statistical significance is less. Furthermore, early withdrawals in the placebo group, especially where these are due to worsening dementia, would tend to reduce, rather than increase, the chance of seeing benefit in the etanercept group.

Although the sample size was small, the placebo group and the etanercept group were well matched on baseline characteristics. However, an important difference occurred in the baseline levels of the anti-inflammatory cytokine IL-10, with lower levels in the etanercept group. Higher levels of the anti-inflammatory cytokine IL-10 in the placebo group might reduce the degree of inflammation-driven neuro-degeneration in this group, concealing any beneficial anti-inflammatory effect of treatment. We were able to correct for this baseline difference in IL-10 in the analysis, and found that adjusting for baseline IL-10 increased the size and significance of the effect size for the MMSE, but did not change the size and the significance of effect sizes in other outcome measures.

The duration of the study (6 months) may be considered insufficiently long for marked cognitive decline in patients with mild to moderate Alzheimer's disease, so there is a risk of Type II error, missing a beneficial effect for

etanercept because of lack of decline in the placebo group. However, despite this expectation, there was measurable cognitive and functional decline in the placebo group, perhaps due to the deliberately unrestrictive exclusion criteria in the study.

A further limitation in the design of the study is that we did not measure sex hormone levels in study participants, and, as suggested by the results discussed in Chapter 4 of this thesis, sex hormone levels may confound the interaction between systemic inflammation and neuro-degeneration.[478] In Chapter 4, I reported results that show sex hormones in men with Alzheimer's disease differ from a cognitively normal cohort, and that the higher than expected levels of luteinizing hormone (LH) correlate with systemic TNF-α. Age-related change in sex hormone status in both men and women may have pathological effects on systemic inflammation and on neuro-protective mechanisms.[165] The results of the study might be biased if there was a significant difference in sex hormone levels between the etanercept group and the placebo group; apparent beneficial treatment effect in the etanercept group might be due to higher testosterone, higher oestrogen, or lower luteinizing hormone in that group. However, randomization in the study produced two groups that were well matched across baseline characteristics and it therefore seems unlikely that there should be a significant difference in sex hormones between the two groups.

In addition to gender effects on systemic inflammation, there is also evidence that the rate of progression of cognitive decline in Alzheimer's disease is faster in women than in men.[479 480] There was a non-significant higher proportion of men in the etanercept group compared to the placebo group (75% (15/20) versus 48% (10/21), $X^2(1)=3.2$, $P=0.1$), and there is therefore a possibility that some of the benefit in the etanercept group is related to sex differences in rates of decline rather than a true treatment effect. However, we have attempted to control for this potential bias by including gender as a co-variate in the analyses of change in outcome measures – and in these analyses gender did not have any significant effect on change.

A further limitation of the study concerns other variables that might affect systemic inflammation and neuro-inflammation. There is growing evidence for long term effects of intra-uterine disadvantage on many diseases of ageing,

including effects on many diseases that are risk factors for late-onset Alzheimer's disease, namely hypertension, metabolic syndrome, hypercholesterolaemia and cardiovascular disease, mediated in part by epigenetic mechanisms.[481-484] We collected data on recalled birth weight, early life hospitalization, years of schooling and parental social class in an effort to control for variation due to differing early life circumstances. However, within the small sample size of this study, there was insufficient early life data to control for confounding in the statistical analyses. We were therefore unable to control for these early-life factors within the study. Broadly, intra-uterine disadvantage increases the risk of co-morbidities and the need for concomitant medication later on in adult life. Although we were unable to use the data we collected on early life disadvantage, we did not find any significant differences between the etanercept group and the placebo group on median number of co-morbidities (7 (IQR 4 to 9) versus 7 (IQR 5 to 9), P=0.9, Table 6.2.3) or on median number of concomitant medications (5 (IQR 4 to 9) versus 7 (IQR 5 to 11), P=0.1, Table 6.2.3), and this suggests that any early-life effects were distributed equally between the etanercept group and the placebo group.

6.3.2 Negative findings

Some of the secondary outcome measures did not show evidence of benefit for subcutaneous etanercept treatment. In Chapter 5, I reported the development of two alternative Alzheimer's disease Sickness Behaviour Scales: SBS-6 and SBS-9. In the current study we wished to test the hypothesis that treatment with a peripheral cytokine antagonist would ameliorate cytokine-related pathological sickness behaviour. However, we did not find any evidence of benefit on either the SBS-6 or SBS-9, suggesting that etanercept may not be an effective treatment for sickness behaviour in Alzheimer's disease.

There are a number of issues which may help to explain the lack of any obvious treatment effect on the Sickness Behaviour Scales. Baseline scores on the SBS-6 and SBS-9 were low in the STEADI cohort (SBS-6: median score=4 out of 24, SBS-9: median score 6 out of 36, Table 5.2.32) and there were no significant changes over 6 months in either the placebo or etanercept groups. This finding is consistent with the stability of the SBS measures over 6 months in the scale construction cohort, where there was no significant change over 6 months in either scale, and both had good test-retest reliability at 6 months

(ICC=0.6 for both SBS-6 and SBS-9, Table 5.2.31). The SBS is therefore not sensitive to change over 6-months and seems to measure more stable Alzheimer's disease symptoms than the NPI over this period of time. This lack of change in the SBS means there is not enough variation in the data to see any beneficial effects from treatment in this measure.

The lack of a treatment benefit on the SBS measures does not exclude the possibility of improvement in pathological sickness behaviour symptoms during subcutaneous etanercept treatment. We found a beneficial treatment effect on behavioural symptoms as measured by the NPI, which measures several symptoms that can be thought of as possible sickness behaviours. Furthermore, there was a positive correlation between baseline NPI and both the SBS-6 ($r=0.5$, $P<0.001$, Table 5.2.33) and the SBS-9 ($r=0.6$, $P<0.001$, Table 5.2.33), similar to the correlation between the SBS and the NPI in the scale construction cohort, suggesting that the NPI does measure some aspects of the sickness behaviour measured by the SBS-6 and SBS-9.

The SBS measures were constructed in an older Alzheimer's disease population than the STEADI cohort (mean age in SBS cohort: 82 (SD 6.4); mean age in STEADI cohort: 72 (SD 9.7)). We would expect sickness behaviour to increase with age, because of age-associated increase in systemic inflammation, and age-associated decline in the threshold for exaggerated sickness behaviour. Thus, the STEADI cohort may not have been sufficiently aged for measurable decline in the symptoms measured by the SBS over the short 6-month study period.

We did not find evidence of a beneficial treatment effect for subcutaneous etanercept on the Fried Frailty Score. The degree of frailty was low in the STEADI cohort, and there was no significant increase in frailty in the placebo group against which to measure any potential treatment effect. Frailty is generally insidious and gradual in progression, as cumulative insults are slowly acquired.[485] It follows that we would not expect a large degree of change in frailty over 6-months. One concern about etanercept treatment in an older population is that the anti-immune effect of the drug would leave people more vulnerable to infection and therefore increase the risk of burgeoning frailty. This was not borne out by the results of this study, with no increased frailty in

the etanercept group, despite a numerically higher number of infections during the treatment phase of the study (11 infections versus 7 infections, Table 6.2.5).

For the ADAS-Cog cognitive scale, Cornell depression scale and CGIC global change scale, there was numerical benefit in the etanercept group but the effect did not have statistical significance. The pattern of change seen in these measures in Figures 6.2.4, 6.2.7 and 6.2.8, mirrors the significant change seen in MMSE, NPI and BADLS, and therefore these results do not conflict with the finding of benefit in other measures.

An unexpected finding was the significant improvement in the placebo group on the ADAS-Cog score between Week 24 and Week 28. Within the placebo group, there were no similar rebound effects in other secondary outcome measures, suggesting that the effect seen with the ADAS-Cog may have been random error, rather than a real effect. There were a greater number of early withdrawals from the placebo group, causing a potential bias because of retention of the more cognitively able. This potential bias provides another explanation for the rebound improvement in the ADAS-Cog during the withdrawal period, with those remaining in the study more likely to exhibit a practice effect in the repetition of the ADAS-Cog between Week 24 and Week 28, than those left in the etanercept group. A further explanation is that there was a “nocebo” effect in the placebo group[486] – where symptoms are exacerbated in a placebo group by expectation of potential drug side effects – with the implication that release from this nocebo effect caused improvement in the ADAS-Cog after withdrawal of the placebo injection.

6.3.3 Conclusions

The results described here show that treatment with subcutaneous etanercept in mild to moderate Alzheimer's disease is safe and well-tolerated, and reduces decline in cognitive, behavioural and functional outcomes over 24 weeks.

These findings in a small safety study warrant further research on peripheral TNF-α blockade in a larger study, over a longer period of time. In addition to cognitive, behavioural and functional outcome measures, a future study should collect data on baseline cytokine profiles, sex hormone status, cortisol levels, and epigenetic markers, as these factors may all influence the impact of systemic inflammation on the brain. In our study we were unable to collect cerebrospinal fluid (CSF) to assess the effect of etanercept treatment on CNS cytokines, and on CSF markers of amyloid and tau pathology. However, data of that kind would be useful to help confirm whether the effect of etanercept on peripheral cytokines is mirrored by an effect on CSF cytokines; and whether cytokine modulation with subcutaneous etanercept alters amyloid and tau processing and excretion into the CSF in Alzheimer's disease.

The results support a recommendation that future studies do not exclude those with multiple co-morbidities and frailty, as these patients have perhaps the most to gain from a systemic anti-inflammatory approach to Alzheimer's disease treatment. Despite a higher degree of co-morbidity in this study, withdrawal rates were low and there were no treatment-associated serious adverse events. As discussed, study participants had frequent contact with a stable study team who were able to identify and manage possible adverse events quickly and effectively. Based on the benefits seen in this study, we therefore recommend similar frequent contact with a stable study team in future studies in this area of challenging clinical research.

In addition to carrying out a larger study, these results have some more immediate clinical implications. The results emphasize the importance of systemic inflammation in the progression of Alzheimer's disease. Our findings suggest that any measure that reduces the degree of systemic inflammation in Alzheimer's disease is likely to have beneficial effects. Therefore, attention should be paid to the rapid diagnosis and treatment of inflammatory events in

patients living with Alzheimer's disease: there should be a low threshold for treating infections with antibiotics; and chronic inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, periodontitis and chronic limb ulceration should be treated effectively, using a laboratory measure of inflammation such as the CRP as a guide to effective therapy.

There is an emerging consensus that potential treatments for Alzheimer's disease are more likely to be effective if administered in the very early or prodromal stages of the disease, when neurodegeneration is in progress but before significant symptoms develop. Efforts to identify patients with Mild Cognitive Impairment secondary to Alzheimer's disease, using CSF and radiological bio-markers, provide an opportunity to test etanercept as a potential treatment in this group, when the benefit of reducing systemic inflammatory exacerbation of CNS inflammation is likely to be greatest.

A principal aim of the current study was to test the hypothesis that reducing systemic inflammatory signalling would have a beneficial effect on maladaptive sickness behaviour symptoms in Alzheimer's disease. The results show some beneficial effect on behavioural symptoms, although there was no change in sickness behaviour measured by the SBS measures, with possible explanations for this outlined above. These mixed results suggest that treating maladaptive sickness behaviour is not as simple as merely reducing systemic inflammatory signalling by cytokines. The emergence of pathological sickness behaviours in Alzheimer's disease is likely to be affected by several interacting patho-physiological systems – examples including the activity of the HPA axis, sex hormone levels, afferent vagal tone, the degree of immuno-senescence in the periphery and in the CNS, neurological reserve, the activity of other psychological constructs including the attachment behaviour system and long-standing personality traits, and the level of inflammation within the degenerating CNS. It is likely that several concurrent treatment strategies, each operating within a different modulating system, will be necessary to effectively treat sickness behaviour in Alzheimer's disease. A comparison can be made with the successful treatment of hypertension. Blood pressure is controlled by several interacting systems, including the sympathetic nervous system, inherent muscular tone within blood vessels and the renin-angiotensin system. Treating high blood pressure with a single agent, with effects on only one of these systems, is rarely successful, as the un-affected systems increase their

activity in response to any drop in blood pressure. Successful treatment of hypertension nearly always requires use of at least two agents, working on different systems; so a good combination might include a calcium channel blocker to reduce inherent muscular tone, alongside an ACE-inhibitor to reduce activity in the renin-angiotensin system. A parallel can be drawn with the treatment of sickness behaviour, where reducing systemic inflammation using a drug like etanercept may need to be accompanied by treatment to antagonise cortisol, or to reduce afferent vagal tone, in order to successfully treat maladaptive sickness behaviour.

In summary, we found that in patients with mild to moderate Alzheimer's disease, treatment with subcutaneous etanercept is safe, well tolerated and reduces decline in a number of cognitive, behavioural and functional secondary outcome measures. We recommend larger studies, in cohorts at different stages of cognitive decline, in order to further explore the promising potential for anti-TNF treatment that we have observed in this study.

Chapter 7: General Discussion

7.0 General discussion

This thesis has explored several hypotheses relating to the connection between systemic inflammation, sickness behaviour and Alzheimer's disease. The overarching hypothesis is that systemic inflammation communicates across the blood-brain barrier to increase the pathological activation of microglial cells that have been primed into a potentially pro-inflammatory state by the on-going neurodegeneration of Alzheimer's disease. This increased pathological activation of primed microglia causes increased production of pro-inflammatory cytokines within the brain, which exacerbates the pathology of Alzheimer's disease and increases neurodegeneration, and induces maladaptive neuroimmune sickness behaviour. Maladaptive sickness behaviour occurs when there is chronic activation of microglia, with no sustained counteracting anti-inflammatory mechanism to bring the behaviour to an end. In this concluding discussion I shall review the extent to which the work described in this thesis supports this over-arching hypothesis, and I will discuss recommendations for future research to test new hypotheses that have been generated by this work.

In Chapter 4, I presented the results of a cross-sectional study examining the hypothesis that sex hormones may influence Alzheimer's disease by influencing systemic inflammation. First, we showed that sex hormone levels are different in men with Alzheimer's disease compared to men without Alzheimer's disease; in the study, men with Alzheimer's disease had lower testosterone and higher luteinizing hormone (LH) than age-matched men without Alzheimer's disease. This finding is consistent with other studies that have demonstrated low testosterone as a risk factor influencing the incidence and progression of Alzheimer's disease. However, the mechanism for this influence is unclear. Second, we demonstrated a positive correlation between LH and the key inflammatory cytokine, TNF-α. This positive correlation has not previously been described in men with Alzheimer's disease. LH is known to be a risk factor for the incidence and progression of Alzheimer's disease, and there is accumulating evidence in animal models showing that elevated LH

affects amyloid processing, neural plasticity and microglial activation. The relationship that we have demonstrated between LH and TNF-α is evidence for a further mechanism by which LH could influence Alzheimer's disease; by increasing systemic TNF-α with a consequent increase in neuroinflammation. The work described in Chapter 4 therefore supports the overarching hypothesis that systemic inflammation contributes to Alzheimer's disease, by showing that a factor known to influence Alzheimer's disease, sex hormone status, may have a mechanism for this influence that involves effects on systemic inflammation.

In Chapter 5, I presented the results of a longitudinal study that explored the hypothesis that pathological neuroimmune sickness behaviour in Alzheimer's disease can be measured using an informant scale. This study explored the underlying hypothesis that pathological neuroimmune sickness behaviour in Alzheimer's disease is generated by neuroinflammation in the Alzheimer's disease brain and is therefore influenced by systemic inflammation. The results described the construction of the SBS-9 scale, and demonstrated good internal reliability, test-retest reliability, construct validity and discriminant validity for the final scale. I contrasted competing construction methods for the scale. The first method was based on factor analysis of sickness behaviour symptoms. The second method was based on correlation between sickness behaviour symptoms and levels of circulating systemic inflammatory cytokines. The results showed that a scale that did not assume the primacy of systemic cytokines in the generation of maladaptive sickness behaviour in Alzheimer's disease was more reliable and valid.

These results suggest that sickness behaviour can be measured as a unified construct in Alzheimer's disease. Sickness behaviour symptoms are thought to represent a suite of behavioural responses to inflammation and injury that occur together because they have evolved together, in response to the same selective evolutionary pressures. The SBS-9 scale was built with the assumption that the most important sickness behaviour symptoms should therefore vary together. Factor analysis of the final SBS-9 scale produced two principal factors relating to decreased motivation and decreased functional ability. These two underlying factors are consistent with theoretical views about adaptive sickness behaviour as a motivational state that alters psychological priority in favour of rest and recuperation; and a functional state

that diverts energy from other systems in support of systems of repair and protection.

We used the baseline observations of participants in the STEADI-09 study to re-validate the SBS-9 in an independent cohort. The results showed that the SBS-9 had good internal reliability, convergent construct validity and divergent construct validity within a cohort that was independent of the construction cohort. However, there was no correlation between the SBS-9 and systemic cytokines in the STEADI-09 cohort. This result suggests that systemic cytokines may not be the principal driver of neuroimmune sickness behaviour in Alzheimer's disease. Other factors, possibly in addition to systemic inflammation, may influence neuroimmune sickness behaviour in Alzheimer's disease, including neurodegeneration, life-course influences on the immune system, neuro-plastic reserve, co-morbid disease, medication use, frailty, personality factors, attachment behaviour, social factors, HPA axis activity and sex hormone status. Given this diversity of influencing factors, it is unsurprising that the SBS-6 failed to achieve acceptable reliability and validity, when compared to a scale based on the behavioural pattern arising from these numerous influences, rather than on any single influence.

Sex hormones are partly responsible for the motivating drive to engage in reproductive behaviour. Sex hormone change associated with Alzheimer's disease, as demonstrated in the work reported in Chapter 4, may reduce the drive to engage in reproductive behaviour. This reduced drive to engage in reproductive behaviour may lower the threshold for the emergence of sickness behaviour in Alzheimer's disease, altering the balance of motivation away from reproductive behaviour and towards health maintenance and protection. This hypothesis could be tested by observing whether declining interest in reproductive behaviour predicts more severe sickness behaviour in established Alzheimer's disease, or predicts conversion from MCI to Alzheimer's disease.

A criticism of the SBS-9 may be that it simply measures depressive symptoms, or unrelated behavioural and psychological symptoms in Alzheimer's disease, akin to the NPI. However, the correlation between the SBS-9 and the Cornell scale, which is a validated scale for depression in Alzheimer's disease, was moderate; only 67% of the variance in the SBS-9 is explained by performance on the Cornell, suggesting that the SBS-9 measures an underlying construct

that shares some, but not all, features with affective depression. Negative affect is an expected and necessary part of maladaptive neuroimmune sickness behaviour; therefore, some relationship between SBS-9 and depression is expected. Furthermore, in the STEADI-09 study described in Chapter 6, etanercept had a significant treatment effect on the NPI, but not on the SBS-9, reinforcing the view that the NPI and the SBS-9 measure different underlying constructs.

The work described in Chapter 5 therefore supports the overarching hypothesis that systemic inflammation contributes to Alzheimer's disease, by showing that several behavioural and psychological symptoms of Alzheimer's disease, grouped together because they may arise from maladaptive neuroimmune sickness behaviour, co-vary to the extent that they can be formed into a single scale and measured as a single construct.

In Chapter 6, I presented the results of a randomized, double-blind, placebo-controlled trial designed to test the hypothesis that TNF blockade using etanercept is safe and well tolerated in Alzheimer's disease, with secondary outcome measures examining the efficacy of etanercept in Alzheimer's disease on several cognitive, behavioural and functional outcomes, including the SBS scales discussed in Chapter 5. The results showed that etanercept was safe and well tolerated in Alzheimer's disease. Moreover, etanercept showed evidence of reduced decline compared to the placebo group on several of the secondary outcome measures. The size of these effects appears to be clinically significant, and is larger than the effect sizes seen for many existing treatments. We found evidence for the efficacy of etanercept despite the fact that the study was not powered to show significant differences in the efficacy measures. In the measures where the treatment effect was not statistically significant the numerical difference was in favour of etanercept for the large majority of outcomes. This consistent pattern across outcome measures gives some credence to the results, despite the small sample size.

Etanercept did not appear to be effective at reducing decline in sickness behaviour, as measured by the SBS-9. There are several reasons that this may have been the case, as discussed more fully in Chapter 6. Briefly, the sample size may have been too small to detect a difference because baseline levels of

sickness behaviour were low. Furthermore, there was very little decline in the placebo group over six months, against which to judge any effect.

The positive findings of the STEADI-09 study support the overarching hypothesis linking systemic inflammation and Alzheimer's disease; treatment with a drug that ameliorates systemic inflammation appears to have a beneficial effect on the rate of decline in Alzheimer's disease. The treatment effect was better for behavioural and psychological symptoms, as assessed by the NPI, than for cognitive symptoms, supporting the overall hypothesis that systemic inflammation aggravates CNS inflammation, with CNS inflammation aggravating pathological sickness behaviour, neurodegeneration and cognitive decline.

Overall, the research presented in this thesis supports the overarching hypothesis that systemic inflammation, and factors that influence systemic inflammation such as sex hormones, exacerbate neuroinflammation with the result that neurodegeneration worsens and neuroimmune sickness behaviour worsens. The results suggest that the SBS-9 is a valid measure of sickness behaviour. Treatment with an anti-inflammatory agent was good at preventing decline in many symptoms but the effect on sickness behaviour as a whole was less clear. The SBS-9 did not correlate well with systemic cytokines in the construction cohort, and not at all in the STEADI-09 validation cohort. Circulating cytokines may not be the best measure of systemic inflammation, or others factors may play a more dominant role in sickness behaviour in Alzheimer's disease. Basal levels of cytokine production, in a person with Alzheimer's disease that is not suffering an acute inflammatory event, may not correlate well with the basal level of pathological neuroimmune sickness behaviour. In fact, we would expect a lower threshold for triggering neuroimmune sickness behaviour in Alzheimer's disease, with low cytokine levels inducing sickness behaviour, whereas in people without Alzheimer's disease we would not expect sickness behaviour to emerge at such a low cytokine level. However, during acute inflammatory episodes such as infections, the relationship between the SBS-9 and cytokines may be more obvious.

7.1 Conclusions and future research

The work described in this thesis has generated new hypotheses that should be tested in future studies, and the work has implications for the current care of people living with Alzheimer's disease.

Our finding of a positive correlation between LH and TNF-α should be confirmed in a larger longitudinal study in men and women with Alzheimer's disease. We also recommend prospective studies examining the relationship between sex hormones and systemic cytokines and whether this relationship influences the risk of Alzheimer's disease, or the risk of conversion from MCI to Alzheimer's disease.

The results suggest that the SBS-9 may be a better measure of neuro-inflammation than systemic inflammation. This hypothesis can be tested by examining the performance of the SBS-9 against measures of CNS inflammation, such as CSF cytokines or microglial activity on PET scanning. We expect the SBS-9 to correlate better with systemic cytokines during periods of acute infection or injury, therefore studies should be performed in Alzheimer's disease patients in hospital with systemic infections or undergoing elective surgery. We recommend that the SBS-9 is used as a measure of neuroimmune sickness behaviour in future clinical trials of Alzheimer's disease.

The STEADI-09 study generated further hypotheses that should be tested in future studies. It is clear that a larger study of anti-TNF treatment is warranted, preferably for a longer duration of treatment, taking place in several centres and sufficiently powered to detect clinically meaningful treatment effects as primary outcome measures. The experience from the STEADI-09 study suggests that a larger study should not exclude people with multiple co-morbidity, as these people may have the most to gain from an anti-inflammatory approach to treating Alzheimer's disease. Furthermore, a future study should seek to reproduce the regular contact with a stable study team which we suggest helped to reduce the withdrawal rate in the STEADI-09 study. Our finding of a relationship between TNF-α and LH suggests that sex hormones should be measured as a potential confounder in a larger study of an anti-TNF agent. A larger anti-TNF study, over a longer period of time, may detect a beneficial effect on sickness behaviour symptoms, measured by the

SBS-9. Moreover, we hypothesize that a trial of anti-TNF agents in people recruited because of more severe sickness behaviour symptoms at baseline will demonstrate a beneficial effect on sickness behaviour, measured by the SBS-9. Additionally, we recommend a clinical trial to test anti-TNF agents in a cohort of people with mild cognitive impairment in an effort to reduce or delay the conversion to Alzheimer's disease.

Finally, the work described in this thesis has implications for the current care of people living with Alzheimer's disease. The results suggest that we must be alert to the detrimental effects of systemic inflammation on the symptoms and progression of Alzheimer's disease. We therefore recommend a low threshold for early treatment of infections with antibiotics in people with dementia. We should actively treat co-morbid chronic inflammatory conditions such as rheumatoid arthritis, or chronically infected leg ulcers. Importantly, measuring pathological neuroimmune sickness behaviour may help us to understand better the distress that someone experiencing these symptoms suffers. Measuring these symptoms allows us to recognize and acknowledge the distress they cause, and simply recognizing and acknowledging the distress may go some way to relieving the symptoms and improving quality of life.

Appendix A

A.1 The Sickness Behaviour Scale: SBS-9

Q1. Anxiety In the past 2 weeks has X been anxious, worried about minor matters, unable to relax or feeling tense?
NO □
YES □ If YES complete question below
On the days when it was present how bad was X's anxiety?
1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q2. Depression In the past 2 weeks has X been feeling low in mood, sad or tearful?
NO □
YES □ If YES complete question below
On the days when it was present how bad was X's low mood?
1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q3. Fatigue In the past 2 weeks has X become easily tired after mild exertion?
NO □
YES □ If YES complete question below
On the days when it was present how bad was X's fatigue?
1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q4. Somnolence In the past 2 weeks has X been excessively drowsy or sleepy?
NO □
YES □ If YES complete question below
On the days when it was present how bad was X's drowsiness?
1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q5. Concentration In the past 2 weeks has X found it difficult to concentrate on more complicated jobs or tasks? (e.g. do they easily give up on reading a newspaper article or a TV programme)
NO □
YES □ If YES complete question below
On the days when it was present how bad was X's difficulty concentrating on a task?
1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q6. Executive function In the past 2 weeks has X found it difficult to perform or execute more complicated jobs or tasks that they used to do? (e.g. cooking or filling in forms)
NO □
YES □ If YES complete question below
On the days when it was present how bad was X's difficulty performing more complex tasks?
1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q7. Psychomotor speed In the past 2 weeks has X appeared to take a long time to think things through or to perform simple tasks?
NO □
YES □ If YES complete question below
On the days when it was present how bad was X's difficulty with thinking quickly?
1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q8. Orientation in place In the past 2 weeks has X had difficulty remembering where they are, or become lost?
NO □
YES □ If YES complete question below
On the days when it was present how bad was X's difficulty remembering where they are?
1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q9. Appetite In the past 2 weeks has X lost interest in food? (i.e. lost their appetite)
NO □
YES □ If YES complete question below
On the days when it was present how bad was X's poor appetite?
1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Appendix A

A.2 The Sickness Behaviour Scale: 26-item scale

Q1. Anxiety In the past 2 weeks has X been anxious, worried about minor matters, unable to relax or feeling tense?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had anxiety?	On the days when it was present how bad was X's anxiety?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q2. Depression In the past 2 weeks has X been feeling low in mood, sad or tearful?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had low mood?	On the days when it was present how bad was X's low mood?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q3. Apathy In the past 2 weeks has X lost interest in their usual activities or in the activities of others?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had a lack of interest in their usual activities?	On the days when it was present how bad was X's lack of interest in their usual activities?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q4. Helplessness In the past 2 weeks has X said that there is nothing they can do to get better?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X felt helpless?	On the days when it was present how bad was X's feeling of helplessness?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q5. Social interaction In the past 2 weeks has X not wanted to meet family, friends or others socially?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X been reluctant to mix with people socially?	On the days when it was present how bad was X's reluctance to mix with people socially?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q6. Listlessness In the past 2 weeks has X become disinclined to do anything even mildly exerting?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X been reluctant to exert themselves?	On the days when it was present how bad was X's reluctance to exert themselves?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q7. Fatigue In the past 2 weeks has X become easily tired after mild exertion?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had fatigue?	On the days when it was present how bad was X's fatigue?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q8. Malaise In the past 2 weeks has X complained of feeling generally unwell? (i.e. bodily discomfort with a feeling of unease)	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had a feeling of malaise?	On the days when it was present how bad was X's feeling of malaise?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q9. Somnolence In the past 2 weeks has X been excessively drowsy or sleepy?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X been drowsy?	On the days when it was present how bad was X's drowsiness?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q10. REM sleep disturbance In the past 2 weeks has X had disturbed sleep with vivid nightmares or violent movements? (i.e. not just disturbed sleep)	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had nightmares or violent sleep movements?	On the days when they were present how bad were X's nightmares or violent sleep movements?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q11. Visual hallucinations In the past 2 weeks has X seen things that other people can't see? (i.e. actually seen rather than *thought* they could see a person or object)	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had visual hallucinations?	On the days when they were present how bad were X's visual hallucinations?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q12. Concentration In the past 2 weeks has X found it difficult to concentrate on more complicated jobs or tasks? (e.g. do they easily give up on reading a newspaper article or a TV programme)	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had difficulty concentrating on a task?	On the days when it was present how bad was X's difficulty concentrating on a task?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q13. Executive function In the past 2 weeks has X found it difficult to perform or execute more complicated jobs or tasks that they used to do? (e.g. cooking or filling in forms)	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had difficulty performing complex tasks?	On the days when it was present how bad was X's difficulty performing more complex tasks?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q14. Psychomotor speed In the past 2 weeks has X appeared to take a long time to think things through or to perform simple tasks?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had difficulty with thinking quickly?	On the days when it was present how bad was X's difficulty with thinking quickly?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q15. Short term memory In the past 2 weeks has X forgotten conversations, day to day events or where they have put things?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had difficulty with short term memory?	On the days when it was present how bad was X's difficulty with short term memory?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q16. Orientation in time In the past 2 weeks has X had difficulty remembering what day of the week it is?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had difficulty remembering the day of the week?	On the days when it was present how bad was X's difficulty remembering the day of the week?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q17. Orientation in place In the past 2 weeks has X had difficulty remembering where they are, or become lost?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had difficulty remembering where they are?	On the days when it was present how bad was X's difficulty remembering where they are?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q18. Appetite In the past 2 weeks has X lost interest in food? (i.e. lost their appetite)	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had poor appetite?	On the days when it was present how bad was X's poor appetite?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q19. Adipsia In the past 2 weeks has X reduced the amount of fluids they are drinking? (i.e. refusing or leaving fluids)	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had poor fluid intake?	On the days when it was present how bad was X's poor fluid intake?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q20. Weight loss In the past 2 weeks has X lost any weight?	
NO □	
YES □	If YES complete questions below
Intentionally blank	How bad was X's weight loss?
Intentionally blank	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q21. Nausea In the past 2 weeks has X vomited or said they feel as if they are about to vomit?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had a feeling of nausea?	On the days when it was present how bad was X's feeling of nausea?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q22. Diarrhoea In the past 2 weeks has X had excessive loose stools?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had diarrhoea?	On the days when it was present how bad was X's diarrhoea?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q23. Temperature regulation In the past 2 weeks has X had a fever or experienced chills or shivering?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had a fever or a chill?	On the days when they were present how bad were X's fevers or chills?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q24. Myalgia In the past 2 weeks has X complained of muscle aches or pains? (not joint related)	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had muscle aches?	On the days when they were present how bad were X's muscle aches?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Q25. Hyperalgesia In the past 2 weeks has X complained of any other aches or pains? (i.e. in other areas of the body, or seeming very sensitive to pain)	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had any other aches or pains?	On the days when they were present how bad were X's other aches or pains?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad
Q26. Headaches In the past 2 weeks has X complained of any headaches?	
NO □	
YES □	If YES complete questions below
Over the last two weeks how often has X had headaches?	On the days when they were present how bad were X's headaches?
1 Occasionally – *(one day or less)* 2 Often – *(two days to seven days)* 3 Frequently – *(eight days to thirteen days)* 4 Very frequently – *(every day for two weeks)*	1 Slightly bad 2 Moderately bad 3 Very bad 4 Extremely bad

Appendix A

Appendix B

B.1 STATA code for Endorsement Rate and Discrimination Index

```
* Endorsement Rate: discover items identified by ER>0.9 or ER<0.2
egen sevTOTAL = rowtotal(V1SBSEV*)
summ sevTOTAL, detail
foreach var of varlist V1SBSEV1-V1SBSEV26 {
quietly egen subj`var' = count(`var') if `var' < 5
quietly egen endo`var' = count(`var') if `var' > 0 & `var' < 5
quietly gen endorate`var' = endo`var' / subj`var' if endo`var' > 0
quietly summ endorate`var'
display  "`var'" _newline r(mean)
        if r(mean) < 0.2 | r(mean) > 0.9 {
        table endorate`var'
        }
}
* Discrimination Index: discover items identified by DI<0.03
summ sevTOTAL, detail
return list
gen denominator = r(N) / 2
gen ActualMedianSBS = r(p50)
gen medianSBS=1 if sevTOTAL > ActualMedianSBS
replace medianSBS=0 if sevTOTAL < ActualMedianSBS

foreach var of varlist V1SBSEV1-V1SBSEV26 {
quietly egen below`var' = count(`var') if `var' > 0 & `var' < 5 & medianSBS == 0
quietly summ below`var', detail
gen belowcount`var' = r(N)
quietly egen above`var' = count(`var') if `var' > 0 & `var' < 5 & medianSBS == 1
quietly summ above`var', detail
gen abovecount`var' = r(N)
gen index`var' = (abovecount`var' - belowcount`var') / (abovecount`var' +
belowcount`var')
quietly summ index`var', detail
display "`var'" _newline r(mean)
if r(mean) < 0.03 {
        di "low: `var'" _newline r(mean)
        }
`
```

Appendix B

Appendix C

Table C.1 All adverse events by treatment group, classified by disease or event category, patient number, severity, relatedness and resolution status at the end of the study

Etanercept	Placebo
Blood and lymphatic system disorders	
	Benign monoclonal hypergammaglobulinaenia P06. Mild. Unrelated. Continuing.
	Normocytic anaemia P25. Mild. Unlikely. Resolved. P18. Severe. Possible. Unexpected. Resolved.
Cardiac disorders	
Split 2nd heart sound (abnormal heart sounds) P11. Mild. Unrelated. Resolved.	**Heart rate irregular** P03. Moderate. Unrelated. Resolved.
Angina pectoris P20. Moderate. Unrelated. Continuing.	**Atrial fibrillation** P08. Moderate. Unrelated. Continuing.
Congenital, familial and genetic disorders	
Ear and labyrinth disorders	
	Ear wax impaction P18. Mild. Unrelated. Resolved.
	Deafness P18. Moderate. Unrelated. Continuing.
Endocrine disorders	
Eye disorders	
	Eye pain P09. Mild. Unlikely. Resolved. P08. Mild. Unlikely. Unexpected. Resolved.
Gastrointestinal disorders	
Vomiting P13. Mild. Unrelated. Resolved.	**Nausea** P16. Mild. Unrelated. Resolved.
Haematochezia (blood in stool) P11. Mild. Unrelated. Resolved.	**Haematochezia (blood in stool)** P36. Mild. Unrelated. Resolved. P36. Severe. Unrelated. Resolved.
Diarrhoea P27. Mild. Possible. Expected. Resolved. P19. Mild. Unlikely. Resolved.	**Diarrhoea** P03. Mild. Unrelated. Resolved.
Epigastric discomfort P30. Mild. Unrelated. Resolved.	**Colonic polyp** P36. Mild. Unrelated. Resolved.
	Constipation P08. Mild. Unlikely. Unexpected. Resolved. P23. Mild. Unrelated. Resolved.

Appendix C

Table C.1 continued

Etanercept	Placebo
General disorders and administration site conditions	
Injection Site Reaction	**Injection Site Reaction**
P02. Mild. Possible. Expected. Resolved.	P18. Mild. Definite. Expected. Resolved.
P34. Mild. Probable. Expected. Resolved.	
P34. Mild. Probable. Expected. Resolved.	
P34. Mild. Probable. Expected. Resolved.	
Fatigue	**Fatigue**
P11. Mild. Unrelated. Resolved.	P03. Moderate. Unlikely. Unexpected. Continuing.
Hepatobiliary disorders	
Immune system disorders	
Infections and infestations	
Gastroenteritis	**Gastroenteritis**
P12. Mild. Possible. Expected. Resolved.	P14. Moderate. Possible. Expected. Resolved.
P22. Moderate. Possible. Expected. Resolved.	
Respiratory tract infection	**Respiratory tract infection**
P30. Mild. Unrelated. Resolved.	P09. Mild. Possible. Expected. Resolved.
P38. Moderate. Unrelated. Resolved.	P25. Mild. Unrelated. Resolved.
P33. Mild. Unrelated. Resolved.	
P38. Moderate. Possible. Expected. Resolved.	**Urinary tract infection**
P02. Mild. Possible. Expected. Resolved.	P03. Moderate. Possible. Expected. Resolved.
P34. Mild. Possible. Expected. Resolved.	P17. Severe. Probable. Expected. Resolved.
P19. Mild. Unlikely. Resolved.	P03. Moderate. Possible. Expected. Resolved.
P11. Mild. Possible. Expected. Resolved.	
	Cellulitis (facial)
Pharyngitis	P08. Moderate. Possible. Expected. Resolved.
P19. Mild. Possible. Expected. Resolved.	
Injury, poisoning and procedural complications	
Fall	**Fall**
P10. Mild. Unrelated. Resolved.	P03. Moderate. Unrelated. Resolved.
P20. Moderate. Unrelated. Stabilised not resolved.	P25. Mild. Unrelated. Resolved.
	P15. Mild. Unrelated. Resolved.
	P23. Severe. Unrelated. Resolved.
Investigations	
C-reactive protein increased	**C-reactive protein increased**
P04. Mild. Possible. Expected. Resolved.	P08. Mild. Unrelated. Continuing.
P05. Mild. Unrelated. Resolved.	P14. Mild. Unrelated. Continuing.
P12. Mild. Possible. Expected. Resolved.	P15. Mild. Possible. Expected. Resolved.
DNA antibody positive	**DNA antibody positive**
P11. Mild. Unrelated. Continuing.	P17. Mild. Probable. Expected. Continuing.
Blood creatinine increased	**Colonoscopy**
P19. Mild. Unrelated. Resolved.	P37. Mild. Unrelated. Resolved.
Transaminases increased	
P33. Mild. Unrelated. Resolved.	

Table C.1 continued

Etanercept	Placebo
Metabolism and nutrition disorders	
Hyperkalaemia P34. Mild. Unlikely. Resolved. P34. Mild. Unlikely. Resolved.	**Dehydration** P15. Mild. Unrelated. Resolved.
Musculoskeletal and connective tissue disorders	
Sciatica P02. Mild. Unrelated. Resolved.	**Osteoarthritis** P08. Moderate. Unrelated. Resolved. **Joint stiffness** P15. Moderate. Unrelated. Stabilised not resolved **Back pain** P03. Moderate. Unrelated. Stabilised not resolved. **Thoracic vertebral fracture** P03. Moderate. Unrelated. Stabilised not resolved. **Muscle weakness** P08. Mild. Unrelated. Continuing.
Neoplasms benign and malignant	
Nervous system disorders	
Parosmia (metallic taste) P27. Mild. Unlikely. Resolved. **Headache** P02. Mild. Possible. Expected. Resolved.	**Parkinsonism** P23. Severe. Unrelated. Continuing. **Balance disorder** P08. Mild. Unrelated. Continuing.
Pregnancy, puerperium and perinatal conditions	
Psychiatric disorders	
Poor quality sleep P01. Mild. Unrelated. Continuing. **Confusional state** P12. Moderate. Unrelated. Resolved. **Behavioural symptoms** P20. Severe. Unlikely. Continuing.	**Poor quality sleep** P14. Moderate. Unrelated. Continuing. **Mood altered** P32. Mild. Unlikely. Unexpected. Resolved. P15. Mild. Unrelated. Resolved. **Behavioural symptoms** P40. Moderate. Unlikely. Continuing. **Hallucinations** P23. Moderate. Unrelated. Continuing. **Delusions** P23. Severe. Unlikely. Unexpected. Resolved.
Renal and urinary disorders	
Urinary frequency (pollakiuria) P20. Mild. Possible. Expected. Resolved.	**Urinary Incontinence** P17. Mild. Unrelated. Resolved.

Appendix C

Table C.1 continued

Etanercept	Placebo
Reproductive system and breast disorders	
Respiratory disorders	
Yawning P02. Mild. Unlikely. Resolved.	
Skin and subcutaneous tissue disorders	
Varicose eczema (stasis dermatitis) P12. Moderate. Unrelated. Continuing.	**Hyperhidrosis (excess sweating)** P23. Mild. Unrelated. Continuing. **Seborrhoeic dermatitis** P09. Mild. Unrelated. Resolved. **Seborrhoeic keratosis** P18. Mild. Unrelated. Resolved. **Eczema** P18. Mild. Unrelated. Resolved.
Social circumstances	
Surgical and medical procedures	
Vascular disorders	
	Hypertension P41. Mild. Unlikely. Continuing. **Lymphoedema** P08. Mild. Unrelated. Resolved.

Disease or event

Patient number (Px). Severity (Mild, Moderate or Severe). Relatedness (Definitely related, Probably related, Possibly related; Unlikely related or Unrelated); Resolution (Resolved at study end or Continuing).

Adverse drug reactions are coded by the Medical Dictionary for Regulatory Activities Version 15.0 (MedDRA 15.0)

Appendix D

D.1 Butchart et al. *Alzheimer Disease & Associated Disorders.* 2013; 27(2): 153-156

ORIGINAL ARTICLE

Male Sex Hormones and Systemic Inflammation in Alzheimer Disease

Joe Butchart, MA, MRCP, Brian Birch, MD, FRCS,† Ramy Bassily, BM,* Laura Wolfe, BSc,* and Clive Holmes, PhD, MRCPsych**

Abstract: Several studies have shown that the levels of sex hormones in men with Alzheimer disease (AD) differ from men without AD. Therefore, male sex hormones have been postulated as risk modifiers in AD, possibly through immunomodulatory effects on known inflammatory AD risk factors, such as tumor necrosis factor α (TNF-α). We conducted a cross-sectional study of sex hormones and TNF-α levels in 94 community-dwelling men with AD. Comparisons were made with normal values derived from the literature. Men with AD had lower free testosterone levels than non-AD men (1-sample *t* test: age <80, $P = 0.0002$; age ≥ 80, $P < 0.0001$), and higher luteinizing hormone (LH) levels (Wilcoxon signed rank test: age <80, $P = 0.001$; age ≥ 80, $P < 0.0001$). Within the cohort of men with AD, there was a positive correlation between LH and TNF-α (Spearman $r = 0.25$, $P = 0.019$), and this remained significant after correcting for age (partial $r = 0.21$, $P = 0.05$). These data support the hypothesis that sex hormones and the immune system influence each other in AD. Furthermore, modulatory effects between LH and TNF-α may provide a mechanism for an effect of male sex hormones on AD risk.

Key Words: Alzheimer disease, testosterone, luteinizing hormone, TNF-α

(*Alzheimer Dis Assoc Disord* 2013;27:153–156)

Sex hormones have been investigated as potential risk factors for Alzheimer disease (AD). A number of studies show that men with AD have lower levels of testosterone than non-AD men.[1,2] In addition, low testosterone levels make a diagnosis of AD more likely in longitudinal follow-up studies.[3] Testosterone may have direct neuroprotective effects through interactions with androgen receptors expressed on neurons and glial cells.[4] Furthermore, tissue aromatization of testosterone to estrogen may confer neuroprotection by estrogen-dependent mechanisms.[5]

High luteinizing hormone (LH) levels are associated with poorer cognition in healthy community-dwelling men,[6] and higher levels of LH have also been postulated to play a role in the pathogenesis of AD.[7] However, although studies consistently find low levels of testosterone in men with AD, studies of LH levels are conflicting, with some groups finding elevated levels[1] and others low levels.[2]

Recent evidence suggests that increased peripheral inflammation, and specifically increased tumor necrosis factor α (TNF-α) production, is a risk factor for the development of AD[8] and for an increased rate of cognitive decline in AD and non-AD subjects.[9,10] Low testosterone levels are associated with increased peripheral inflammation in cross-sectional studies of aged men.[11,12] Therefore, we hypothesize that male sex hormones may exert some of their influence on cognition in AD by modulating systemic TNF-α levels.

In this study, we tested the hypothesis that sex hormone levels in men with AD differ from normal values and correlate with systemic TNF-α levels.

METHODS

Design and Setting

Ninety-four community-dwelling men with mild to severe AD living in Southampton, UK, were enrolled in a study examining the relationship between cognitive decline and peripheral cytokine levels.[10] Participants were recruited between November 2003 and May 2006 from clinical referrals to memory assessment services in Southampton, UK. After consent procedures, all subjects fulfilling the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria[13] for probable or possible AD had a blood sample taken for sex hormone and TNF-α analysis. Participants were further characterized by a full medical history, medication history, assessment of delirium, and cognitive testing, as part of their involvement in the larger cytokine study.[10] Comparisons of androgen levels are made between the AD cohort we describe here and a separate cohort of aged males with no diagnosis of AD. Data on the cohort of non-AD men were obtained from a previously published study by Yeap et al.[14] Androgen levels are significantly affected by age, with normal values differing in different age groups. Therefore, we stratified the AD cohort into 2 age groups: <80 years and 80 years or older. This cut point was chosen to allow comparison with normal, non-AD, age-specific androgen levels, derived from the Yeap et al study.[14] We did not have data on TNF-α levels in this non-AD cohort. Ethical approval for the study was obtained from the local NHS Research Ethics Committee.

Assays

Serum samples were immediately placed on ice and stored within 2 hours at $-80°C$. Baseline serum samples were used for assays of total testosterone, sex hormone-binding globulin (SHBG), and LH by chemiluminescent

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From the *Memory Assessment and Research Centre, Clinical Neurosciences, Faculty of Medicine, University of Southampton; and †Department of Urology, Southampton University Hospitals NHS Trust, Southampton, UK.

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The authors declare no conflicts of interest.

Reprints: Joe Butchart, MA, MRCP, Memory Assessment and Research Centre, Clinical Neurosciences, Faculty of Medicine, University of Southampton, Moorgreen, Botley Road, Southampton SO30 3JB, UK (e-mail: j.w.butchart@soton.ac.uk).

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immunoassay using a standard laboratory protocol (UniCel DxI 800, Beckman Coulter, Brea, CA). Bioavailable free testosterone (FT) was calculated using the Vermeulen formula.[15] Population values for FT and LH in men aged 80 years or older and in men aged <80 years were derived from a previously described cohort of 2938 community-dwelling men.[14] The same baseline serum samples used for sex hormone analysis were also used to assay TNF-α using a sandwich multiplex cytokine immunoassay [Meso Scale Discovery (MSD), Gaithersburg, MD]. A protocol provided by MSD for custom assays was used with no major modifications. The lowest detectable limit for TNF-α was 1.1 pg/mL. The severity of dementia was measured using the cognitive subsection of the Alzheimer's Disease Assessment Scale (ADAS-cog).[16]

Statistical Analysis

Normally distributed variables are summarized as mean ± SD. Non-normally distributed variables are summarized as median and interquartile range. The study population was stratified by age (less than 80 yrs or 80 yrs or older) for comparison with data on normal, non-AD, age-specific (less than 80 yrs or 80 yrs or older) androgen levels, derived from a non-AD cohort.[14] One-sample *t* tests were used to compare normally distributed variables with normal population means within a defined age range. Nonparametric data were compared with population medians using the Wilcoxon signed rank test. Partial correlation statistics, with ranked data where necessary, were used to assess cross-sectional associations
between hormone levels, TNF-α level, and age, using the Stata statistical package (v.11, Statacorp). We considered results significant at $P < 0.05$.

RESULTS

Of the 94 subjects, 58 were aged less than 80 years, and 36 were aged 80 years or older. Demographic characteristics and ranges for sex hormones and TNF-α for the AD cohort are given in Table 1.

FT and LH levels differed between men with AD and the age-matched population cohorts (Table 2). FT levels were lower in men with AD (1-sample *t* test for age group less than 80 y: $P = 0.0002$; age group 80 y and above: $P < 0.0001$). LH levels were higher (Wilcoxon signed rank test for age group less than 80 y: $P = 0.0014$; age group 80 y and above: $P < 0.0001$).

Correlations between TNF-α, FT, and LH are given in Table 3. There was a significant positive correlation between LH and serum TNF-α levels in men with AD (Spearman $r = 0.25$, $P = 0.019$) and this remained significant after correcting for age (partial $r = 0.21$, $P = 0.05$). LH in the highest quartile versus LH in the lowest quartile predicted TNF-α above the median in a logistic regression model, after adjusting for age [odds ratio 3.8; 95% confidence interval, 1.1-13.4;

TABLE 1. Characteristics of the Alzheimer Disease Study Population

	AD Cohort: All Ages Age Range: 59-98 (n = 94)		AD Cohort: In 2 Age Groups				
			Age <80 (n = 36)		Age ≥80 (n = 58)		*P* for Difference
	n	Mean or %	n	Mean or %	n	Mean or %	Between Age Groups
Age (SD)	94	81.2 (7.2)	36	74.3 (4.8)	58	85.5 (4.5)	NA
Age at onset of AD (SD)	94	75.8 (8.0)	36	70.6 (5.3)	58	79.5 (7.5)	< 0.01*
ADAS-cog score (IQR)	94	23.3 (17.3-33.7)	36	24.7 (16.5-36.8)	58	23.3 (18.7-31.3)	0.71†
Years of schooling							
5-8	19	20%	2	6%	17	29%	< 0.01‡
9-12	55	59%	23	64%	32	55%	
> 12	20	21%	11	30%	9	16%	
Known history of depression							
Yes	18	19%	6	19%	12	23%	0.64§
No	76	81%	26	81%	40	77%	
Known history of other psychiatric problem							
Yes	7	7%	3	9%	4	8%	0.99‡
No	87	93%	30	91%	49	92%	
Use of cholinesterase inhibitor							
Yes	47	50%	23	64%	24	41%	< 0.05§
No	47	50%	13	36%	34	59%	
Use of anti-inflammatory medication							
Yes	25	27%	13	36%	12	21%	0.10§
No	69	73%	23	64%	46	79%	
Total testosterone (nmol/L) (SD)	94	12.63 (5.80)	36	14.58 (5.47)	58	11.42 (5.71)	< 0.01*
Free testosterone (nmol/L) (SD)	94	0.19 (0.09)	36	0.23 (0.08)	58	0.17 (0.08)	0.001*
LH (IU/L) (IQR)	94	7.45 (4.19-12.91)	36	5.85 (3.75-9.52)	58	9.07 (5.50-14.55)	0.02†
SHBG (nmol/L) (IQR)	94	49.0 (38.0-63.0)	36	43.5 (37.5-54.0)	58	51.5 (38.0-64.0)	0.16†
TNF-α (pg/mL) (IQR)	94	3.21 (2.44-4.23)	36	3.05 (2.19-3.96)	58	3.43 (2.52-4.34)	0.26†

**P*-value from 2 independent samples *t* test.
†*P*-value from Kruskal-Wallis rank test.
‡*P*-value from the Fisher exact test.
§*P*-value from the Pearson χ^2 test.
AD indicates Alzheimer disease; IQR, interquartile range; LH, luteinizing hormone; NA, not applicable; SHBG, sex hormone-binding globulin; TNF-α, tumor necrosis factor α.

TABLE 2. Comparison of Free Testosterone and Luteinizing Hormone Between Men With Alzheimer Disease and Values Derived From a Non-Alzheimer Population, Stratified by Age

	AD		Non-AD*		
	N	Mean or Median	N	Mean or Median	P
Free testosterone (nmol/L) (SD)					
Age < 80	36	0.23 (0.08)	2938	0.28 (0.10)	0.0002†
Age ≥ 80	58	0.17 (0.08)	707	0.26 (0.10)	< 0.0001†
Luteinizing hormone (IU/L) (IQR)					
Age < 80	36	5.85 (3.75-9.52)	2938	4.20 (2.96-6.30)	0.0014‡
Age ≥ 80	58	9.07 (5.50-14.55)	707	5.15 (3.36-8.29)	< 0.0001‡

*Calculated from Yeap et al.[14]
†One-sample *t* test.
‡Wilcoxon signed rank test.
AD indicates Alzheimer disease; IQR, interquartile range.

$P = 0.039$; LR test for model: $P = 0.034$; Hosmer-Lemeshow GOF test: $\chi^2_2 = 0.04$, $P = 0.98$]. There was no significant relationship between FT and serum TNF-α levels (Spearman $r = -0.03$, $P = 0.8$), or between FT and LH (Spearman $r = -0.17$, $P = 0.11$). Severity of dementia, as measured by the ADAS-cog, was not related to FT, LH, or TNF-α (Spearman $r = -0.11$, $P = 0.29$; $r = 0.03$, $P = 0.80$; $r = 0.04$, $P = 0.74$, respectively), and these correlations did not reach significance after adjusting for age.

DISCUSSION

We found lower than average FT and higher than average LH in community-dwelling men with AD. Lower FT levels in men with AD are consistent with other studies.[1,2,17] However, previous reports on LH levels in men with AD have been less consistent, with some showing increased levels[1] and others low or normal levels.[2,18] High levels of LH imply primary hypogonadism, with testicular failure as the cause of low testosterone levels. Low levels of LH imply hypothalamic-pituitary-gonadal axis failure, with loss of the necessary stimulatory message to the testes. Our findings imply testicular failure as the cause for the cohort's lower than average testosterone levels, and thus suggests that changes in sex hormone levels in men with AD represent an exaggerated form of the usual pattern of change that is associated with aging.

TABLE 3. Correlation* Between TNF-α, LH, and Free Testosterone, Within Age Groups, and in the Total Alzheimer Disease Cohort, Adjusted for Age†

	Age Groups					
	< 80 n = 36		≥ 80 n = 58		Combined n = 94	
	r	P	r	P	r	P
TNF-α	0.34	0.05	0.20	0.15	0.25	0.019
LH (adjusted for age†)					(0.21)	(0.05)
TNF-α	0.05	0.78	0.01	0.98	−0.03	0.80
Free testosterone (adjusted for age†)					(0.01)	(0.92)
LH	−0.13	0.44	−0.08	0.53	−0.17	0.11
Free testosterone (adjusted for age†)					(−0.08)	(0.43)

*Spearman rank correlation.
†Partial correlation of ranked data.
LH indicates luteinizing hormone; TNF-α, tumor necrosis factor α.

The significant positive correlation between LH and serum TNF-α has not been described previously in men with AD. There are few, if any, studies examining the relationship between peripheral cytokines and sex hormone status in elderly male cohorts. However, a positive correlation between LH and TNF-α has been described in people with rheumatoid arthritis,[19] and patients with prostate cancer treated with an LH suppressor have reduced TNF-α levels.[20] Hence, the relationship between LH and serum TNF-α in men with AD is consistent with findings in other populations.

LH has been postulated to modulate cognitive decline in AD.[7] Our study provides some evidence of an association between LH and TNF-α in men with AD, and given that TNF-α is associated with increased cognitive decline in AD,[10] this may suggest TNF-α as a possible mediator of the effect of LH in AD.

Although it is not possible to assess the direction of the modulation between LH and TNF-α in this cross-sectional study, there is growing evidence that age-related changes in sex hormone levels have effects on immune regulation. Lower levels of testosterone, and of its aromatization product estrogen, and higher levels of LH increase the sensitivity of peripheral monocytes to proinflammatory stimuli, with exaggerated production of proinflammatory cytokines such as TNF-α.[21] Testosterone can inhibit IL-6 production[22] and can affect the induction of NO synthase.[23] Aromatization of testosterone to estrogen has an anti-inflammatory effect in the central nervous system through estrogen-dependent mechanisms.[5]

However, while sex hormones have various effects on the immune system, the immune system also affects hormone production at the hypophyseal, pituitary, and gonadal levels. Inflammatory cytokines increase aromatase activity, and therefore increase the metabolic degradation of testosterone. IL-6 induces persistent testicular resistance to LH, and directly suppresses testosterone production by Leydig cells.[24,25] Elevated TNF-α levels also induce testicular failure, because of the downregulating effects on Leydig cells in the testes.[26] High TNF-α in people with AD[27] may therefore reduce testosterone production by the testes and cause an increase in physiologically appropriate reflex in LH release from the anterior pituitary.

We have previously shown that elevated TNF-α levels predict cognitive decline in AD.[10] Our current finding that TNF-α and LH are positively correlated supports the view that sex hormones and the immune system influence each other in men with AD.

Whether the sex hormone differences found in this study pre-date and increase the risk of AD, or are secondary to AD, is unknown. A limitation of this study is the use of comparison sex hormone values from published data on a non-AD cohort. We were unable to control for possible confounding in the comparison of sex hormone levels between AD and non-AD men because we did not have data on possible confounding variables in the non-AD

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group described by Yeap et al.[14] However, the normal values for sex hormone levels in the non-AD group are derived from a large cohort, and we have used matched age groups in the analysis, to control for age as the most likely confounding factor. It is hoped that this study will lead to prospective, longitudinal studies of sex hormones and cytokines in aging men, designed to disentangle the relationship between sex hormones, cytokines, and the risk and severity of AD.

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Etanercept in Alzheimer disease

A randomized, placebo-controlled, double-blind, phase 2 trial

Joseph Butchart, MRCP
Laura Brook, BSc
Vivienne Hopkins, RMN
Jessica Teeling, PhD
Ursula Püntener, PhD
David Culliford, MSc
Richard Sharples, MD
Saif Sharif, MRCPsych
Brady McFarlane, MRCPsych
Rachel Raybould, PhD
Rhodri Thomas, BSc
Peter Passmore, FRCP
V. Hugh Perry, DPhil
Clive Holmes, FRCPsych

Correspondence to
Prof. Holmes:
c.holmes@soton.ac.uk

ABSTRACT

Objectives: To determine whether the tumor necrosis factor α inhibitor etanercept is well tolerated and obtain preliminary data on its safety in Alzheimer disease dementia.

Methods: In a double-blind study, patients with mild to moderate Alzheimer disease dementia were randomized (1:1) to subcutaneous etanercept (50 mg) once weekly or identical placebo over a 24-week period. Tolerability and safety of this medication was recorded including secondary outcomes of cognition, global function, behavior, and systemic cytokine levels at baseline, 12 weeks, 24 weeks, and following a 4-week washout period. This trial is registered with EudraCT (2009-013400-31) and ClinicalTrials.gov (NCT01068353).

Results: Forty-one participants (mean age 72.4 years; 61% men) were randomized to etanercept (n = 20) or placebo (n = 21). Etanercept was well tolerated; 90% of participants (18/20) completed the study compared with 71% (15/21) in the placebo group. Although infections were more common in the etanercept group, there were no serious adverse events or new safety concerns. While there were some interesting trends that favored etanercept, there were no statistically significant changes in cognition, behavior, or global function.

Conclusions: This study showed that subcutaneous etanercept (50 mg/wk) was well tolerated in this small group of patients with Alzheimer disease dementia, but a larger more heterogeneous group needs to be tested before recommending its use for broader groups of patients.

Classification of evidence: This study shows Class I evidence that weekly subcutaneous etanercept is well tolerated in Alzheimer disease dementia. *Neurology*® 2015;84:2161–2168

GLOSSARY

AD = Alzheimer disease; **ADAS-cog** = Alzheimer's Disease Assessment Scale–cognitive; **BADLS** = Bristol Activities of Daily Living Scale; **CGI-I** = Clinical Global Impression–Improvement; **CI** = confidence interval; **CRP** = C-reactive protein; **IL** = interleukin; **IQR** = interquartile range; **ITT-LOCF** = intention to treat–last observation carried forward; **NPI** = Neuropsychiatric Inventory; **RPCT** = randomized placebo-controlled trial; **sMMSE** = standardized Mini-Mental State Examination; **TNF-α** = tumor necrosis factor α.

Acute and chronic systemic inflammation is characterized by the production of proinflammatory cytokines including tumor necrosis factor α (TNF-α) from immune cells. TNF-α has a role in systemic immune-to-brain communication by activating the central immune response.[1] In humans, low levels of chronic systemic inflammation are associated with evidence of microglial activation.[2] In animals, experimentally induced acute systemic inflammation results in an exaggerated central immune response leading to exacerbated neurodegeneration.[3] In participants with Alzheimer disease (AD) dementia, we have shown that modestly increased serum TNF-α levels are associated with an increased rate of cognitive decline[4] and an exaggeration of neuropsychiatric symptoms.[5]

Peer-reviewed published data on the use of the TNF-α inhibitor etanercept in AD dementia is limited to small open-label studies[6–8] purporting to deliver etanercept centrally[9] through a perispinal administration route. However, we have hypothesized that peripheral administration

Supplemental data at Neurology.org

From the Faculty of Medicine, Clinical Experimental Sciences (J.B., L.B., D.C., C.H.), and Faculty of Natural and Environmental Science, Centre for Biological Sciences (J.T., U.P., V.H.P.), University of Southampton; Memory Assessment and Research Centre (J.B., L.B., V.H., R.S., S.S., C.H.), Moorgreen Hospital, Southern Health Foundation Trust, Southampton; Becton Health Centre (B.M.), Southern Health Foundation Trust, New Milton; Centre for Public Health (P.P.), Queens University Belfast; MRC Centre for Neuropsychiatric Genetics and Genomics (R.R., R.T.), Institute of Psychological Medicine and Clinical Neurosciences, Cardiff University, UK.

Go to Neurology.org for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

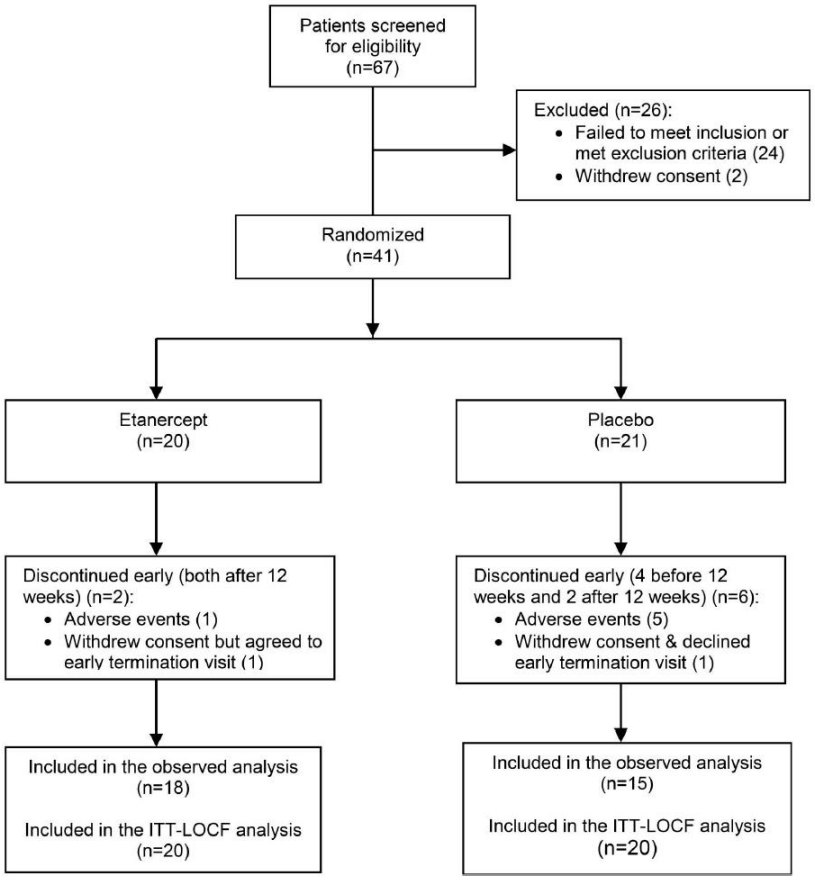
of a TNF-α inhibitor with high affinity and specificity could, if well tolerated and safe, have long-term beneficial cognitive and behavioral efficacy in an AD dementia population through inhibition of peripheral signaling to the brain.[10] Peripheral administration of TNF-α inhibitors is licensed for a wide range of rheumatologic and skin conditions in the elderly but there are no randomized placebo-controlled trials (RPCTs) of these drugs in AD dementia that give comparative data regarding tolerability, safety, or its effects (beneficial or otherwise) on measures of clinical outcome. We report the findings of a double-blind, phase 2 RPCT examining the tolerability, safety, and clinical effects on secondary clinical psychometric measures of subcutaneous etanercept in a mild to moderate AD population.

METHODS Standard protocol approvals, registrations, and participant consents. The protocol and consent forms were approved by a multicenter research ethics committee (Southampton and South West Hampshire REC [A], reference number 10/H0502). All participants provided informed consent before screening procedures. The study was registered with EudraCT (2009-013400-31) and ClinicalTrials.gov (NCT01068353).

Study design and participants. Safety and Tolerability of Etanercept in Alzheimer's Disease was an investigator-initiated, 24-week, single-center, phase 2, double-blind RPCT to assess the tolerability and safety of weekly 50 mg subcutaneous etanercept in participants with AD dementia including cognitive, behavioral, and functional outcomes.

The study was performed in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice. An independent data and safety monitoring board monitored adverse events.

Figure 1 Trial profile



ITT-LOCF = intention to treat–last observation carried forward.

At screening, eligible participants had to be aged 55 years or older, be diagnosed with probable AD defined by the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria[11] (diagnostic accuracy approximately 75%[12,13]), have a modified Hachinski Ischemic Scale score[14] of less than 5 points, have a standardized Mini-Mental State Examination (sMMSE)[15] score above 10 and below 27 points, have an informant spending at least 24 hours per week with the participant, and be capable of giving informed consent. Patients receiving a cholinesterase inhibitor, memantine, or antidepressant medication were required to have been on medication for a minimum period of 90 days before baseline. Patients with prior exposure to amyloid vaccines, monoclonal antibodies, or IV immunoglobulins for the treatment of AD were excluded. Patients with rheumatoid arthritis, psoriasis, psoriatic arthritis, or ankylosing spondylitis, or those taking anti–TNF-α agents, immunosuppressive drugs, and/or oral prednisone >10 mg/d within the past 90 days were excluded. Participants with known contraindications (active infections) or cautions (previous significant exposure to tuberculosis, herpes zoster, hepatitis B, heart failure [New York Heart Association grades 3 and 4], demyelination disorders, and active malignancy within past 5 years) to the use of etanercept were excluded.

Randomization and masking. ACE Pharmaceuticals BV (Zeewolde, the Netherlands) manufactured the placebo and packaged both the study medication and placebo to ensure blinding. They used a computer to generate a simple random allocation sequence (1:1), stratified in blocks of 4, to ensure 20 patients in the treatment group (subcutaneous etanercept 50 mg) and 20 patients in the placebo group (water for injection). The investigators had no knowledge of the allocation sequence, which remained concealed throughout the study. ACE Pharmaceuticals loaded etanercept or placebo vials into serially numbered containers according to the allocation sequence. The loaded containers, and the interventions inside them, were identical in appearance and consistency to ensure concealment of the allocation sequence from the investigators. After successful screening, participants were assigned the container with the next available serial number in strict chronological order. Study drug was administered by weekly subcutaneous injection at home or in the clinic by study team health professionals who were blinded to treatment allocation.

Procedures. Following consent, patients underwent a screening period including initial tuberculosis and infectious disease screen (i.e., a chest radiograph, tuberculin skin, and an interferon gamma release test). Participants fulfilling the inclusion and exclusion criteria received etanercept 50 mg or placebo subcutaneously once per week for 24 weeks. This was followed by a 4-week washout period in which no injections were given but in which blinding was maintained. Clinic visits took place at screening, baseline, week 12, week 24, and 4 weeks after the last study drug injection (week 28). Patients who withdrew from the study before scheduled clinic visits were seen within 1 week of withdrawal for an early termination visit. During these visits, adverse event monitoring and psychometric evaluation took place. Adverse events were recorded as definitely related, probably related, possibly related, unlikely to be related, or unrelated to the blinded study intervention. Patients experiencing a serious adverse event or recurrent infections were withdrawn from the study. Psychometric measures included the sMMSE,[15] Alzheimer's Disease Assessment Scale–cognitive section (ADAS-cog),[16] Bristol Activities of Daily Living Scale (BADLS),[17] Clinical Global Impression–Improvement (CGI-I) scale,[18] Cornell Scale for Depression in Dementia (Cornell),[19] and the Neuropsychiatric Inventory (NPI).[20]

At screening, blood samples were taken for routine clinical laboratory assessments with additional measures of immunoglobulins, anti-nuclear antibody, anti-cardiolipin, and a midstream urine test for infection. Blood for DNA (principally for *APOE* ε4 analysis) was taken at baseline. *APOE* genotypes were determined by TaqMan genotyping of single nucleotide polymorphism (SNP) rs7412 and KASP genotyping of SNP rs429358. Blood for routine laboratory assessments and serum inflammatory markers were taken at baseline, week 12, week 24, and week 28 between 9 AM and 12 noon. Serum samples for inflammatory markers were immediately placed on ice and stored within 2 hours at −80°C. Samples were analyzed blind to the treatment allocation using a V-PLEX assay (Meso Scale Discovery [MSD]). A protocol provided by MSD for custom assays was used with no major modifications. Five serum inflammatory markers were measured: TNF-α, interleukin (IL)-6, IL-10, IL-12p70, and C-reactive protein (CRP).

Outcomes. The primary outcomes of the study were tolerability and safety. Tolerability was measured by compliance (number of injections given/number of planned injections) over the 24-week trial period. Associated safety was measured by the number of serious adverse events and adverse events during the study treatment period coded using the *Medical Dictionary for Regulatory Activities* (version 15.0) preferred term (http://www.meddra.org). A symptom checklist was used at each visit to probe for adverse events.

Secondary outcomes were differences in the change from baseline of the psychometric measures (sMMSE, ADAS-cog, BADLS, CGI-I, Cornell, and NPI) at 12 weeks and 24 weeks between intervention groups for observed cases and intention to treat–last observation carried forward (ITT-LOCF). In addition, emergent adverse events and psychometric changes following a 4-week washout phase were measured.

Statistical analysis. This study was powered to assess tolerability (dropout rates) of weekly etanercept 50-mg subcutaneous injections and to identify adverse events associated with poor tolerability in an AD dementia population. The study also

Table 1 Characteristics of patients entering the randomization phase

Characteristic	Etanercept 50 mg (n = 20)	Placebo (n = 21)	Mean difference (95% CI) or X^2, *p* value
Mean age, y (SE)	72.0 (2.1)	72.9 (2.2)	0.9 (−5.3 to 7.1), *p* = 0.8
Men, n (%)	15 (75)	10 (48)	X^2 3.2, *p* = 0.07
White, non-Hispanic, %	1 (5)	0 (0)	X^2 1.1, *p* = 0.3
Disease duration, y (SE)	5.1 (0.8)	4.1 (0.4)	−1.0 (−2.8 to 0.8), *p* = 0.2
ε4 carriers, n (%)	9 (45)	11 (52)	X^2 0.2, *p* = 0.6
sMMSE pts (SE)	20.0 (1.4)	20.3 (1.2)	0.3 (−3.3 to 4.0), *p* = 0.9
ADAS-cog pts (SE)	25.8 (2.9)	25.7 (2.5)	−0.1 (−7.8 to 7.7), *p* = 1.0
BADLS pts (SE)	16.5 (3.0)	14.0 (1.7)	−2.5 (−9.3 to 4.3), *p* = 0.5
NPI pts (SE)	16.4 (2.5)	12.0 (2.7)	−4.4 (−11.8 to 3.0), *p* = 0.2
Cornell pts (SE)	6.4 (0.8)	5.6 (1.0)	−0.8 (−3.4 to 1.9), *p* = 0.6

Abbreviations: ADAS-cog = Alzheimer's Disease Assessment Scale-cognitive; BADLS = Bristol Activities of Daily Living Scale; CI = confidence interval; Cornell = Cornell Scale for Depression in Dementia; NPI = Neuropsychiatric Inventory; pts = points; SE = standard error; sMMSE = standardized Mini-Mental State Examination.

Table 2 Incidence of adverse events by system organ class category

Disorder	Adverse events, no. of events (no. of participants)	
	Etanercept (n = 20)	Placebo (n = 21)
All disorders	42 events	55 events
Blood and lymphatic system (normocytic anemia, benign monoclonal hypergammaglobulinemia)	0 (0)	3 (3)
Cardiac (abnormal heart sounds, angina pectoris, irregular heart rate, atrial fibrillation)	2 (2)	2 (2)
Eye (eye pain)	0 (0)	2 (2)
Ear and labyrinth (wax impaction, deafness)	0 (0)	2 (1)
Gastrointestinal (nausea and vomiting, hematochezia, epigastric discomfort, diarrhea, constipation, colonic polyp)	5 (5)	7 (5)
General fatigue	1 (1)	1 (1)
Injection-site reaction	4 (2)	1 (1)
Infections (gastroenteritis, respiratory tract, urinary tract, pharyngitis, cellulitis)	11 (9)	7 (6)
Injury (falls)	2 (2)	4 (4)
Investigations (DNA antibody positive, increase serum CRP, creatinine, transaminase, colonoscopy)	6 (6)	5 (5)
Metabolic (hyperkalemia, dehydration)	2 (1)	1 (1)
Musculoskeletal (sciatica, osteoarthritis, joint stiffness, back pain, thoracic vertebral fracture, muscle weakness)	1 (1)	5 (3)
Neoplasms	0 (0)	0 (0)
Nervous system (parosmia, headache, parkinsonism, balance disorder)	2 (2)	2 (2)
Psychiatric (poor sleep, confusional state, behavioral symptoms, mood alteration, hallucinations, delusions)	3 (3)	6 (5)
Renal and urinary (urinary frequency, urinary incontinence)	1 (1)	1 (1)
Respiratory (yawning)	1 (1)	0 (1)
Skin (varicose eczema, hyperhidrosis, seborrheic dermatitis, seborrheic keratitis, eczema)	1 (1)	4 (3)
Vascular (hypertension, lymphedema)	0 (0)	2 (2)

Abbreviation: CRP = C-reactive protein.
Adverse events include definitely, probably, possibly, unlikely, and not thought to be related to the study intervention. Participants could report multiple events in any category. Adverse drug reactions are coded by the MedDRA preferred term (*Medical Dictionary for Regulatory Activities*, MedDRA 15.0).

aimed to inform a potential phase 3 study of the variance and mean differences in clinical outcome measures. To measure dropout such that a 95% confidence interval (CI) about the estimated rate had a margin of error of ±15%, normal approximation about a proportion showed that 36 to 41 participants would be required for a dropout rate commensurate with previous AD studies of between 29% and 41%.[21,22] This sample size also fell between 24 and 50 recommended as necessary to estimate SDs for a future phase 3 study.[23–25] Efficacy analyses were performed on observed cases, defined as all patients who received at least one dose of study medication, and who provided data at baseline, week 12, and week 24, and on ITT-LOCF cases, defined as all patients who received at least one dose of study medication, and had at least one postrandomization assessment. Study demographic characteristics, efficacy measure outcomes, and serum inflammatory proteins were assessed for normality using Q-Q plots. Changes in psychometric measures and serum inflammatory protein levels between the 2 intervention groups were measured by unpaired *t* test and linear regression for parametric variables or Mann–Whitney *U* test for nonparametric variables. Clinical psychometric outcomes were adjusted for baseline age, sex, and baseline psychometric score, and were conducted at $p < 0.0017$ significance to allow for 30 multiple comparisons.

RESULTS **Participant disposition.** Participant disposition is detailed in figure 1. Between January 2011 and February 2013, a total of 67 patients were screened at the Memory Assessment and Research Centre, Southampton, UK, of whom 41 entered the study and were assigned to either etanercept or placebo. Reasons for screen failure included prior exposure to tuberculosis or latent tuberculosis (46% [12/26]), monoclonal gammopathy of unknown significance (12% [3/26]), MMSE screen failure (8% [2/26]), abnormal chest radiology (8% [2/26]), skin cancer (8% [2/26]), consent withdrawn before baseline (8% [2/26]), abdominal aortic aneurysm requiring surgery (4% [1/26]), clinically significant anemia (4% [1/26]), and lack of adequate informant time (4% [1/26]).

Randomization phase. The mean age of the patients entering the study was 72.4 (SD 9.7) years, and the majority (61%) were men. Randomization of patients at baseline led to 2 treatment groups that were similar in demographic details, *APOE* ε4 carrier status, and psychometric test scores (p values in all cases >0.1 except sex, $p = 0.07$) (table 1). There was no significant difference between treatment groups in the frequency of participants taking a cholinesterase inhibitor (16/20 [80%] etanercept vs 18/21 [86%] placebo; X^2 0.2, $p = 0.6$), memantine (3/20 [15%] etanercept vs 3/21 [14%] placebo; X^2 0.004, $p = 0.9$), or antidepressant medication (7/20 [35%] etanercept vs 8/21 [38%] placebo; X^2 0.01, $p = 0.8$).

Tolerability and safety. Compliance to medication was high over the 6-month trial period: overall median 100% (interquartile range [IQR] 87.5%–100%). There was no significant difference in the median compliance frequency between treatment groups (etanercept 100% [IQR 95.8%–100%] vs placebo 94% [IQR 62.5%–100%]; MWU $p = 0.2$). Eight participants (20%) (2 on etanercept and 6 on placebo) failed to complete the study following randomization. Of the 8 noncompleters, 4 participants (all taking placebo) failed to complete the study to 12 weeks, of whom one declined an early termination visit, and 4 participants (2 taking placebo and 2 taking etanercept) failed to complete the study to 24 weeks. Of the 2 noncompleters in the etanercept group, one participant contracted a chest infection and was withdrawn because of safety concerns and one participant withdrew consent because of drug

delivery logistic problems. Of the 6 noncompleters in the placebo group, 5 participants were withdrawn because of safety concerns (one participant contracted a urinary tract infection, one developed a monoclonal gammopathy of unknown significance, one experienced blood in stools, one experienced worsening of behavioral symptoms, and one participant required an elective hip replacement). One participant withdrew consent because of family commitments.

The overall study completion was 81% (33/41). There was no statistical difference in the completion rates between those allocated to etanercept and those allocated to placebo (90% [18/20] from the etanercept group were completers vs 71% [15/21] from the placebo group; Fisher exact test, $p = 0.2$).

A total of 97 adverse events occurred during the 24-week randomization phase of the study with one serious adverse event (one participant in the placebo arm was admitted to hospital with a urinary tract infection). Adverse events grouped by system are summarized in table 2. There were 42 (43%) adverse events in 20 participants in the etanercept group and 55 (57%) in 21 participants in the placebo group. Infections, injection site reactions, and investigational abnormalities were present in a greater number of participants in the etanercept arm compared with the placebo arm but no statistical differences were found between groups (X^2, all $p > 0.1$).

Secondary clinical outcomes. Changes in psychometric scores for observed cases and ITT-LOCF at 12 and 24 weeks after randomization are shown in table 3 and figure 2. One participant randomized to placebo withdrew from the study at 4 weeks and declined an early termination assessment of clinical outcomes. Increases in psychometric scores from baseline indicate a worsening in outcomes except for the sMMSE for which an increase indicates an improvement. None of the clinical outcomes were statistically significant between treatment groups following Bonferroni correction.

Washout phase. No serious adverse events occurred during the 4-week washout phase of the study. Six participants experienced a total of 7 adverse events: 3 adverse events (fall, upper respiratory tract infection, gastric irritation) in 3 participants taking etanercept previously and 4 adverse events (disturbed sleep, fall, constipation and fall) in 3 participants taking placebo previously.

Allowing for Bonferroni correction, there were no significant differences in the change of psychometric scores for the sMMSE, ADAS-cog, BADLS, NPI, Cornell, or CGI-I during the 4-week washout phase of the study between those participants previously taking etanercept compared with those previously taking placebo: sMMSE: etanercept −0.3 vs placebo −0.1 points (mean difference −0.2 points [95% CI 1.7 to

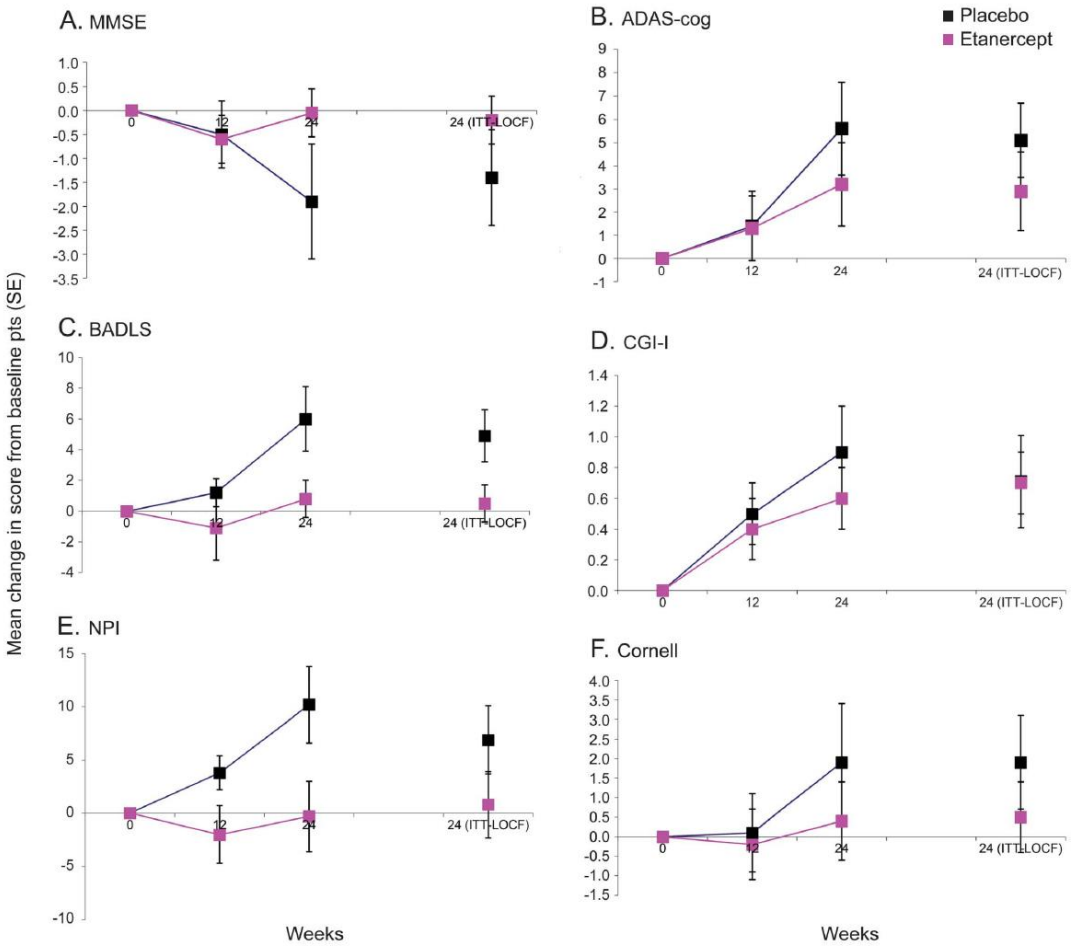
Table 3 Changes in psychometric scores 12 weeks and 24 weeks for observed and ITT-LOCF after randomization compared with baseline

	Week 12				Week 24			
	Etanercept (n = 20) (SE)	Placebo (n = 17) (SE)	Mean difference corrected[a] (95% CI)	p Value	Etanercept (n = 18) (SE)	Placebo (n = 15) (SE)	Mean difference corrected[a] (95% CI)	p Value
Observed cases								
sMMSE	−0.6 (0.5)	−0.5 (0.7)	0.004 (−1.9 to 1.9)	1.0	−0.1 (0.5)	−1.9 (1.2)	−2.5 (−5.2 to 0.2)	0.07
ADAS-cog	1.3 (1.4)	1.4 (1.5)	0.2 (−4.3 to 4.6)	0.9	3.2 (1.8)	5.6 (2.0)	1.2 (−5.1 to 7.5)	0.7
BADLS	−1.1 (2.1)	1.2 (0.9)	0.6 (−4.6 to 5.8)	0.8	0.8 (1.2)	6.0 (2.1)	5.6 (0.4 to 10.9)	0.04
NPI	−2.0 (2.7)	3.8 (1.6)	4.4 (−3.0 to 11.7)	0.2	−0.3 (3.3)	10.2 (3.6)	13.2 (2.4 to 24.0)	0.02
Cornell	−0.2 (0.9)	0.1 (1.0)	0.1 (−2.8 to 3.0)	0.9	0.4 (1.0)	1.9 (1.5)	1.6 (−2.3 to 5.5)	0.4
CGI-I	0.4 (0.2)	0.5 (0.2)	0.2 (−0.5 to 0.8)	0.6	0.6 (0.2)	0.9 (0.3)	0.3 (−0.6 to 1.1)	0.3
ITT-LOCF								
sMMSE	−0.6 (0.5)	−0.5 (0.7)	0.05 (−1.7 to 1.9)	0.9	−0.2 (0.5)	−1.4 (1.0)	−1.4 (−3.8 to 1.0)	0.2
ADAS-cog	1.3 (1.4)	1.9 (1.4)	0.6 (−3.6 to 4.8)	0.8	2.9 (1.7)	5.1 (1.6)	1.6 (−3.5 to 6.6)	0.5
BADLS	−1.1 (2.1)	1.4 (0.9)	0.5 (−4.2 to 5.2)	0.8	0.5 (1.2)	4.9 (1.7)	3.7 (−0.9 to 9.3)	0.1
NPI	−2.0 (2.7)	3.7 (1.5)	4.2 (−2.6 to 11.1)	0.2	0.8 (3.1)	6.9 (3.2)	6.5 (−3.4 to 16.5)	0.2
Cornell	−0.2 (0.9)	0.4 (0.8)	0.2 (−2.4 to 2.9)	0.9	0.5 (0.9)	1.9 (1.2)	0.8 (−2.4 to 3.9)	0.6
CGI-I	0.4 (0.2)	0.5 (0.2)	0.1 (−0.5 to 0.7)	0.7	0.7 (0.2)	0.7 (0.3)	0.1 (−0.8 to 0.6)	0.8

Abbreviations: ADAS-cog = Alzheimer's Disease Assessment Scale-cognitive; BADLS = Bristol Activities of Daily Living Scale; CGI-I = Clinical Global Impression-Improvement; CI = confidence interval; Cornell = Cornell Scale for Depression in Dementia; ITT-LOCF = intention to treat-last observation carried forward; NPI = Neuropsychiatric Inventory; SE = standard error; sMMSE = standardized Mini-Mental State Examination.
All p values are 2-sided.
[a] Corrected for baseline age, sex, and psychometric score.

Appendix D

Figure 2 Mean change in outcome scores (observed cases weeks 12 and 24) and ITT-LOCF (week 24) from baseline



ADAS-cog = Alzheimer's Disease Assessment Scale–cognitive; BADLS = Bristol Activities of Daily Living Scale; CGI-I = Clinical Global Impression–Improvement; Cornell = Cornell Scale for Depression in Dementia; ITT-LOCF = intention to treat–last observation carried forward; MMSE = standardized Mini-Mental State Examination; NPI = Neuropsychiatric Inventory.

−2.0]), $p = 0.9$; ADAS-cog: etanercept 1.3 vs placebo −4.2 points (mean difference 5.5 points [95% CI 9.0–2.0]), $p = 0.003$; BADLS: etanercept 2.4 vs placebo −1.7 points (mean difference 4.1 points [95% CI 7.0 to −1.4]), $p = 0.004$; NPI: etanercept −2.4 vs placebo −3.5 points (mean difference 1.1 points [95% CI 7.7 to −5.6]), $p = 0.7$; Cornell: etanercept −0.8 vs placebo −2.1 points (mean difference 1.2 points [95% CI 3.1 to −0.6]), $p = 0.6$; CGI-I: etanercept 0 vs placebo 0 points (mean difference 0 points [95% CI 0.7 to −0.8]), $p = 1.0$.

Serum inflammatory markers. Serum was not available in one (placebo randomized) participant. Serum levels for TNF-α, IL-6, IL-10, IL-12p70, and CRP for all other participants completing the study (18 randomized to etanercept and 14 to placebo) are shown at baseline (week 0), week 12, week 24, and after the 4-week washout phase (week 28) in table e-1 on the *Neurology*® Web site at Neurology.org. At baseline, there were no statistical differences in serum TNF-α or IL-12 between the treatment and placebo groups. Following randomization, serum TNF-α was higher in the treatment compared with the placebo group at weeks 12 and 24 and was still significantly increased, although diminished, at week 28. Serum IL-12 was also higher in the treatment compared with the placebo group at week 12 but not significantly different from placebo at week 24

or week 28. At baseline, serum IL-10 was statistically lower in the treatment compared with the placebo group. Following randomization, serum IL-10 was no longer significantly different from placebo at week 12 or 24, but following withdrawal was lower in the treatment arm at week 28. There were no significant differences between the serum inflammatory markers IL-6 or CRP between the treatment and placebo groups at baseline or weeks 12, 24, and 28.

DISCUSSION Of the 67 patients screened for this study, 26 failed to meet the inclusion or exclusion criteria. The 2-year recruitment period was the result of screening failures, the majority of which (n = 12 [46%]) were attributable to prior exposure to tuberculosis, of high frequency in the United Kingdom aged population,[26] and to drug delivery delay following Pfizer's acquisition of Wyeth. Compliance was high over the 24-week trial period with no significant differences between treatment groups. Ninety percent of patients (18/20) completed the etanercept arm of the study. There were no serious adverse events in the etanercept arm. Adverse events, including infection rates and injection-site reactions, were in keeping with the known, and potentially serious, side effects of etanercept,[27] but no new safety concerns were found regarding the use of etanercept in patients with AD dementia.

Whereas the psychometric changes in the placebo arm are in keeping with observed changes reported elsewhere in similar populations,[28,29] the change in ADAS-cog was double than anticipated.[4] While every attempt was made to improve internal validity by ensuring raters received the same psychometric rating training and, wherever possible, not change between visits, small numbers in the study cannot rule out the random allocation of a more rapidly declining group to the placebo arm and differences between groups should be viewed with caution. There was no statistical difference between the cognitive, functional, and behavioral assessments in the etanercept compared with the placebo group (or worsening following withdrawal) after Bonferroni correction. Serum TNF-α showed a marked increase in the etanercept-treated group compared with the placebo group, reflecting the increased half-life of the inert dimeric fusion protein after binding of TNF-α.[30,31]

The use of subcutaneous etanercept in this study is based on the hypothesis of modifying long-term, low-grade peripheral systemic inflammation,[1,4,10,32] a different concept than the hypothesized rapid modification of central TNF-α through a periventricular approach,[7] which has not been participant to an RPCT and remains highly controversial.[33]

The current study should not be seen to support the use of unlicensed subcutaneous etanercept for the treatment of AD dementia. Etanercept has recognized potentially serious adverse effects in the population,[27] and independent validation is needed in a larger more heterogeneous AD dementia population to fully assess the long-term safety and clinical effects of this approach.

AUTHOR CONTRIBUTIONS

J.B. made a substantial contribution to the design of the work, the acquisition and interpretation of the data, drafting the work, and reviewing it for important intellectual content. L.B. made a substantial contribution to the design of the work, the acquisition of the data, and reviewing it for important intellectual content. V.H., J.T., U.P., R.S., S.S., R.R., R.T. made a substantial contribution to the acquisition of the data and reviewing it for important intellectual content. P.P., V.H.P., D.C., B.M. made a substantial contribution to the interpretation of the data and reviewing it for important intellectual content. C.H. made a substantial contribution to the conception of the study, the design of the work, the acquisition and interpretation of the data, drafting the work, and reviewing it for important intellectual content. All authors had full access to all of the data in the study and C.H. had final responsibility for the decision to submit for publication.

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DISCLOSURE

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