TOWARDS A MECHANISTIC UNDERSTANDING OF HOW AGE INCREASES SUSCEPTIBILITY TO INFLAMMATION-INDUCED BEHAVIOURAL AND COGNITIVE DISTURBANCE

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ABSTRACT

Accumulating evidence implicates inflammation and the innate immune system in the pathophysiology of age-related cognitive decline and neurodegenerative disorders such as Alzheimer's disease. However, we have a limited understanding of the mechanisms by which age may increase the susceptibility to the behavioural and cognitive impairments associated with inflammation. In this thesis, I developed and validated a new experimental model of mild acute inflammation (IFN-β injection) and integrated it with two different MRI methodologies, behavioural, cognitive, physiological and immunology data to investigate how age modulates the effects of inflammation on the brain.

To achieve this, healthy young and old participants underwent two experimental sessions in which they each received IFN-β-1b (EXTAVIA® [100 μg]) and saline in randomised order. Two neuroimaging techniques were used to assess actions on the brain. 1) Resting-state functional magnetic resonance (rsfMRI) which was used to investigate functional connectivity architecture with a particular focus on the efficiency of information transfer. 2) Diffusion-Weighted Magnetic Resonance Spectroscopy (DW-MRS) which was used to explore whether this novel MRI-based method was sensitive to detecting changes in glial cells using a model of mild inflammation. Fatigue, sickness and mood questionnaires, as well as a battery of cognitive tasks (reward/punishment reinforcement learning task, psychomotor retardation and visuospatial memory tasks), were used to assess behavioural and cognitive changes. Physiological monitoring and serial blood draws were used to assess physiological and immunological responses.

Key findings include demonstrating that: 1) IFN-β is a safe and robust new experimental model of mild acute inflammation, as shown by the changes observed in the physiological, immune and behavioural responses. 2) That DW-MRS is sufficiently sensitive to detect changes in glial morphometry induced by a model of mild inflammation (IFN-β). 3) That DW-MRS is sensitive to previously reported age-related differences in glial and neuronal densities and glial morphometry. 4) That IFN-β altered global brain functional connectivity architecture (rsfMRI data). Here, IFN-β particularly affected highly connected nodes (as

has been reported in Alzheimer's disease) and, the intensity of the effect varied with respect to age.

Together, these data provide support for IFN-β as a model of mild acute inflammation and confirm the effectiveness of employing non-invasive imaging methods, in conjunction with a range of behavioural and cognitive tasks to investigate the impact of experimentally induced neuroinflammation in young and old individuals.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AChR	Acetylcholine receptor
AD	Alzheimer's disease
ADC	Apparent diffusion coefficient
ALS	Amyotrophic lateral sclerosis
AMI	Apathy motivation index
APC	Antigen presenting cell
APP	Antimicrobial proteins and peptides
BBB	Blood brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
BIS/BAS	Behavioural approach/avoidance scale
BMI	Body mass index
BOLD	Blood-oxygen-level-dependent
BP	Blood pressure
BWC	Betweenness centrality
Cho	Choline
CLRB	Cramer-Rao lower bound
CNS	Central nervous system
Cr	Creatine
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CUBRIC	Cardiff University Brain Research Imaging Centre
DA	Dorsal attention
dACC	Dorsal anterior cingulate cortex
DAMPs	Damage associated molecular patterns
DASS	Depression anxiety and stress questionnaire
Dc	Dendritic cell
DCE	Dynamic contrast enhancement
DDR	DNA damage response
DEPTOR	DEP domain-containing mTOR-interacting protein

DMN	Default mode network
DNA	Deoxyribonucleic acid
DW-MRI	Diffusion MRI
DW-MRS	Diffusion weighted magnetic resonance spectroscopy
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
ESS	Epworth Sleepiness Scale
FC	Functional connectivity
FD	Framewise displacement
FDG-PET	Fluorodeoxyglucose-position emission tomography
FDR	False discovery rate
fMRI	Functional magnetic resonance imaging
fVAS	fatigue visual analogue scale
FWHM	Full width half maximum
GABA	γ-aminobutyric acid
GFR	Glomerular Filtration Rate
HAMDI	Hamilton depression rating scale
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPA	Hypothalamic-pituitary-adrenal
ICA	Independent component analysis
ICC	Interclass correlation coefficient
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFNAR	Interferon α/β receptor
IL	Interleukin
Ins	Myo-inositol
IPA	Ingenuity pathway analysis

ISG	Interferon stimulated genes
JAK-STAT	Janus Kinase/signal transducer and activators of transcription
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MDD	Major depressive disorder
MHC	Major histocompatibility complex
MINI	Mini international neuropsychiatric interview
MMSE	Mini mental state examination
MPRAGE	Magnetization prepared rapid acquisition gradient echo
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRS	Proton magnetic resonance
MS	Multiple sclerosis
MTL	Medial temporal lobe
mTOR	Mammalian target of rapamycin
NAA	N-acetyl aspartate
NARI	Noradrenaline reuptake inhibitor
NDI	Neurite density index
NFkB	Nuclear factor k-light-chain
NK-T	Natural killers T cell
NLR	Nod-like receptor
NLR	Neutrophil to lymphocyte ratio
NOD	Nucleotide Oligomerization domain
NODDI	Dispersion and density imaging
NSA	Number of signals averages
NSAIDs	Non-steroid anti-inflammatory drugs
NTS	Nucleus tractus solitarius
PAMPs	Pathogen associated molecular patterns
PCA	Principal component analysis
PD	Parkinson's disease
PET	Positron emission tomography

POMS	Profile of mood states
PPI	Protein-to-protein interaction
PRR	Pattern recognition receptor
qMT	Quantitative magnetization transfer
RMA	Robust multi-array
RP	Ribosomal proteins
RPL	Ribosomal proteins large subunit
RPS27A	Ubiquitin-40S Ribosomal protein S27a
rsfMRI	Resting state functional magnetic resonance imaging
sACC	Subgenual anterior cingulate cortex
SASP	Senescence-associated secretory phenotype
SEM	Standard error of the mean
Semi-LASER	Semi-localization by Adiabatic Selective Refocusing sequence
SHAPS	Smith-Hamilton pleasure scale
SicknessQ	Sickness behaviour questionnaire
SLS	Systemic lupus erythematosus
SNRI	Serotonin and noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
STAI	State and trait anxiety inventory
sTNF	TNF soluble receptor
STRING	Search tool for the retrieval of interacting genes/proteins
Tc	Cytotoxic T lymphocyte
TCA	Tricyclic antidepressants
TCR	T-cell receptor repertoire
TE	Echo time
TEDANA	TE dependency Analysis
TEDICA	TE-dependent independent component analysis
TEDPCA	TE-dependent principal component analysis
Th	Helper T cell
TLR	Toll-like receptor
TNF-α	Tumour necrotic factor

Treg	Regulatory T cell
TR	Repetition time
TSPO	18kDa translocator protein
UV	Ultraviolet
UWH	University Wales hospital
VA	Ventral attention
VOI	Volume of interest
WCB	White cell blood
WM	White matter
XCP	Extensible connectivity pipeline
Δ	Delta value

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AUTHOR'S DECLARATION

I declare that the research presented in this thesis is unless explicitly stated in the text, my original contribution. This thesis has not been previously submitted for a degree at this or any other institution, nor does it include any content previously submitted for a degree.

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STATEMENT OF CONTRIBUTION

I oversaw all aspects of my PhD project: I wrote and prepared all IRAS documentation and study protocol for NHS ethics approval.

I managed participant recruitment and data collection phases. This included: running screening sessions: (including blood draws, ECG, and cognitive and psychological screening), organising experimental sessions: coordinating medic covers, radiographers and MRI sessions, administering behavioural questionnaires and cognitive tasks and processing blood samples for immunological analysis. I also managed purchases and the financial aspects associated with the project.

I was responsible for the data analysis of DW-MRS, physiological, behavioural, cognitive data as well as for the laboratory work to process and analyse cytokine data. I used PsychoPy to design and implement two of the cognitive tasks. I processed and analysed rsfMRI in collaboration with fellow PhD student Tamas Foldes and I provided interpretation of all the results.

I also collected mRNA data for transcriptomics analysis, heart rate variability and imaging data for Dynamic Contrast Enhancement (DCE), however due to time constraints associated with the pandemic, the analysis process of these data was not completed and therefore not included in this thesis.

During Covid, I provided interpretation and partial analysis of secondary data from a study using mRNA transcriptomics and I am the principal author of the paper (under revision) that includes the results I present in Chapter 7.

PUBLICATIONS DERIVED FROM THIS WORK

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CHAPTER OVERVIEW

Chapter 1

In this chapter, I first offer an overview of the immune system and detail its function in maintaining normal CNS homeostasis, along with its involvement in psychiatric and neurodegenerative disorders. I then considered ageing-related alterations of the immune system from an evolutionary perspective with and special focus on the network theory of ageing and the concept of inflammageing. I also outline the principal mechanisms through which the immune system and the brain interact and highlight how these interactions can potentially lead to behavioural and mood impairment. Finally, I describe the neuroimaging methods employed to study the impact of inflammation on the brain focusing on human studies using clinical samples and immune challenges in healthy individuals.

Chapter 2

This chapter outlines the main methodology of the study relevant to the following experimental chapters (chapters 3, 4, 5 and 6). This includes the experimental design, the protocol followed during the screening and experimental sessions, inclusion and exclusion criteria and the recruitment process.

Chapter 3

Here, I introduce and validate IFN-β as a new model of acute experimental inflammation. I highlight the physiological and immune responses to IFN-β demonstrating an expected immune activation characterised by rapid changes in vital signs, immune response (white cells) and cytokine plasma concentrations. I then contrast these reactions to those induced by other frequently employed immune challenges. Additionally, I explore and discuss the evidence indicating similar or varying responses to IFN-β as a function of age and underscore the additional research needed to fully validate the model.

Chapter 4

In this chapter, I report the behavioural and cognitive responses to inflammation.
I further validate the model by showing that IFN-β induces mood and fatigue symptoms, similar to other immune challenges. I then use a reinforcement learning task to assess the

effects of IFN-β on reward and punishment sensitivity. My results show a trend that replicates previous evidence suggesting that inflammation induces motivational reorientation, as evidenced by an increased sensitivity to punishment compared to reward. I explore two more cognitive domains, psychomotor retardation and visuospatial memory and relate my findings to prior evidence. Additionally, I discuss the design of the tasks and their potential challenges, contrasting them with tasks previously utilised in similar experimental designs.

Chapter 5

Here, I present findings indicating that DW-MRS (an innovative neuroimaging method that allows for the measurement of intracellular metabolite diffusion) can detect microstructural brain changes induced by inflammation. This is evidenced by the increased apparent diffusion coefficient (ADC) of the metabolite Choline. I also present results showing that DW-MRS can be sensitive to age-related changes, as shown by differences in N-Acetylaspartate (NAA) ADCs between young and old participants. I describe the technical limitations of this method, the steps necessary for further validation and how this technique could potentially emerge as a valuable clinical tool applicable across a spectrum of disease.

Chapter 6

In this chapter, I use resting state fMRI and apply graph-theoretic analysis on a functional connectivity matrix to investigate the acute effects of inflammation on the efficiency of brain information transfer. I present results showing that IFN-β leads to a rapid reduction in global network efficiency and the strength of connections between nodes, with highly connected nodes being more affected by inflammation. I also provide novel results demonstrating that the effects of IFN-β in high and low-connected nodes vary with respect to age. Finally, I conclude by considering future designs and the use of multimodal analysis approaches.

Chapter 7

In this final experimental chapter, I analyse secondary data from a sample of patients initiating IFN-α therapy to treat Hepatitis C with the aim of identifying acute immune-

response signatures to IFN-α that may predict the later development of fatigue. Using whole blood transcriptomics and protein-to-protein interaction network analysis, I first show a rapid transcriptomic response to IFN-α within 4 ½ hours of injection. In the second part of the analysis, I pinpoint the genes significantly positively correlated with fatigue and identify a novel network of cytosolic and ubiquitin proteins that may predispose to the experience of fatigue. I conclude by addressing the technical limitations of the methods employed and outlining the steps required to further investigate these findings.

Chapter 8

In this final section, I provide a summary of the results from earlier chapters, and I detail the advantages and challenges of the methods employed. I propose additional experiments to complement my findings to offer a more comprehensive understanding of the effectiveness of each technique.

CHAPTER 1. INTRODUCTION

OVERVIEW OF THE IMMUNE SYSTEM

The immune system is the body's defence against infections and other potentially damaging threats. These threats generally have an exogenous origin (e.g., virus, bacteria or parasites), however, they can also arise from the body's own tissue (e.g., damaged or dying tissue).

The immune system can be broadly classified into two categories: innate immunity and adaptive immunity. Innate or natural immunity consists of a series of non-specific defence mechanisms designed to fight infection in the early hours after exposure. It recognises many classes of pathogens and mounts the same response on repeated exposure.

The innate immune system comprises first-line natural anatomical and physiological barriers (e.g., skin, and mucosal epithelium), complement activation, secretion of cytokine and chemokine inflammatory mediators, antimicrobial peptides and proteins and immune cells (e.g., neutrophils, macrophages, monocytes). Innate immunity also plays a role in activating cells that support adaptive immunity.

The adaptive immune system is mediated by B and T lymphocyte cells' clonal selection following exposure to a specific antigen. The adaptive immune response mechanisms include (i) specificity, (ii) self/non-self -recognition and (iii) immunological memory which lead to a stronger response with each subsequent exposure. Both arms of the immune system interact and work together to fight infection and preserve bodily integrity. In this section, I aim to provide a brief introduction to the immune system as well as a descriptive summary of its role in normal CNS homeostasis and its contribution to psychiatric and neurodegenerative conditions.

INNATE IMMUNITY

BARRIERS TO INFECTION

The epithelial layers of the skin and the gastrointestinal, respiratory and genitourinary tracts are the first line of defence against invading agents. They provide a mechanical barrier that prevents microbial entrance. Cellular components of these physical barriers produce a wide variety of cytokines, chemokines, pathogen recognition receptors (PRRs) and antimicrobial proteins and peptides (APPs) that act as mediators of the inflammatory responses to infection (McDonald & Levy, 2019)

Within the central nervous system (CNS), the blood-brain-barrier (BBB) and the blood-cerebrospinal-barrier (BCSFB) are the main structures providing anatomical and physiological protection. Both systems carefully regulate and restrict the passage of specific molecules such as cytokines, amino acids, hormones and immune cells and block entry of microbes into the brain parenchyma.

The BBB is a diffusion barrier that consists of differentiated cellular elements and active transport mechanisms that facilitate the transport of nutrients and carefully controls the movement of specific molecules that could potentially be detrimental to neural function. It is a complex system that includes highly specialised endothelial cells and an elaborated network of tight junctions, astrocyte end-feet, pericytes and perivascular antigen-presenting cells (APCs) (Engelhardt & Sorokin, 2009). The strict control of movement of molecules allows the BBB to tightly regulate CNS homeostasis. However, the BBB is not a complete uninterrupted system and some areas such as the circumventricular organs (median eminence, subfornical organ, area postrema and pineal gland) contain fenestrated vessels that are highly permeable to solutes (Ufnal & Skrzypecki, 2014). This dynamic characteristic of the BBB is important for the correct function of different neural circuitries and seems to be a potential route by which some substances (natural and therapeutic) may gain access to the brain (Daneman & Prat, 2015).

The BCSFB (blood-cerebrospinal-barrier) is another interface between the CNS and the blood and it is formed by the choroid plexus, where most of the CSF is formed, and the arachnoid. The BCSFB is composed of a monolayer of epithelial cells and tight junctions and because capillaries in the choroid plexus are formed by fenestrated endothelial cells (permeable to blood-borne molecules), the BCSFB has an important role in regulating the flux of molecules between the blood and the CSF (Ueno et al., 2016).

CELLS

The innate leukocytes within the peripheral immune system include granulocytes (eosinophils, basophils, neutrophils), mast cells, dendritic cells (Dcs) and macrophages as well as innate lymphoid cells, natural killer (NK) cells and NK-T cells.

Within the CNS, immune responses are mainly mediated by resident innate immune cells (microglia and astrocytes). Astrocytes descend from neuroepithelial cells and are categorized as glia. They provide physical and metabolic support to neurons but also have a role in innate immune reactions by releasing inflammatory mediators such as cytokines, chemokines and complement components (Ransohoff & Brown, 2012).

Microglia are the resident mononuclear phagocytes within the CNS and are a unique myeloid cell population. They are highly dynamic, and their processes are constantly sensing the environment and local tissue damage as well as carrying out typical phagocytic functions such as clearing cellular debris. Microglial functions go beyond the immune response and have an important role in shaping neural circuitry, influencing neuronal activity and monitoring the integrity of synaptic function (Wake et al., 2012). Both microglia and astrocytes also provide protective and restorative responses to CNS insults (Ransohoff & Brown, 2012).

Astrocytes and microglia are not the only immune cells in the CNS. Myeloid macrophages, Dendritic and mast cells reside in the linings of the brain parenchyma (perivascular spaces,

meninges and choroid plexus) where they sample debris and potentially harmful molecules from the CSF and blood and shield the CNS from invaders (Herz et al., 2017).
A finely regulated immune system helps the host fight infection and regain homeostasis. However, altered activation of innate immune cells and over-expression of pro-inflammatory cytokines, may be harmful for the organism. For instance, in the periphery, changes in the number or activation status of macrophages have been linked with psychiatric disorders (Marin & Kipnis, 2013), and in the CNS, increased microglial activation has been associated with neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD) Major Depressive Disorder (MDD) and Schizophrenia (Q. Li & Barres, 2018)

ACTIVATING INNATE IMMUNITY

The innate immune response is initiated when pattern recognition receptors (PRRs) expressed by innate immune cells recognise and bind to microbial molecules (known as pathogen-associated molecular patterns-PAMPs) or molecules secreted by damaged or dying host cells (damaged associated molecular patterns-DAMPs). The innate immune system expresses a wide variety of PRRs in order to combat pathogens. Some of the PRRs important to neuropsychiatry include Toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) and non-NLR inflammasomes.

TLRs are expressed on or within immune cells and the epithelia. They initiate innate immunity and mediate and facilitate the adaptive immune response by activating antigen-presenting cells such as Dcs and macrophages. In the CNS, TLRs are also expressed by glial cells, neurons, BBB endothelial cells and neural progenitor cells. Toll signalling within the CNS goes beyond its traditional immune role and also influences multiple processes such as structural plasticity, axonal growth and neurogenesis (Okun et al., 2011). Furthermore, heightened activation of individual TLRs has been shown to contribute to neurological and neuropsychiatric conditions such as neuropathic pain and MDD (Hung et al., 2014; Tanga et al., 2005).

NOD-like receptors, (NLRs) are cytoplasmatic PRRs that sense PAMPs and DAMPs in the cytoplasm of host cells. Following activation, Nod-like receptors trigger the nuclear factor κ-light-chain enhancer of activated B cells complex (NFκB) leading to the expression of pro-inflammatory cytokines.

NRLs also play an important role in activating signalling cascades that trigger the inflammasome, a multiprotein complex responsible for pro-inflammatory cytokine 1L-1ß maturation and secretion. Activation and subsequent enhanced production of IL-1ß, and IL-18 by NLRP3, one of the prototypical inflammasomes, has been linked to the pathophysiology of neuropsychiatric disorders such as MDD, bipolar disorder and Alzheimer's Disease (Alcocer-Gómez et al., 2014; Heneka et al., 2013; Kim et al., 2016; Y. Zhang et al., 2015).

Another system that contributes to innate defence is the complement cascade. This network of membrane-associated serum proteins produced by the liver and a variety of cell populations, including brain cells, elicits a cascade of highly regulated inflammatory responses to infectious organisms. The complement system is also actively involved in several aspects of brain development, homeostasis, injury and degeneration. The alteration of this finely regulated network balance can lead to aberrant complement activation and exacerbation of the inflammatory response, which has also been implicated in the pathology of traumatic brain injury, AD or PD (Orsini et al., 2014).

ADAPTIVE IMMUNITY

The adaptive immune system is highly specific and characterised by the generation of memory responses that are carried out by B and T lymphocytes. Adaptive immunity can be classified into two categories: humoral immunity, mediated by B cells and their antibodies and cellular immunity mediated by T cells.

T AND B CELLS

T cells develop and reach maturation in the Thymus. They emerge from the Thymus as 'naive' T cells and initiate migration mainly to the spleen and lymph nodes. T lymphocytes are classified into CD4+ and CD8+ cells, which express T cell receptors that recognize antigens bound to the Major histocompatibility complex (MHC) class II or I respectively. CD4+ are mainly divided into two subsets T helper (Th) and T regulatory (Treg) cells: Th cells induce proliferation of CD8+ and B cells in response to antigens presented by APCs. Treg cells negatively regulate the responses of B cells and other T cells.

The three main types of Th cells are Th1, Th2 and Th17 which display specific cytokine expression and functions. Th1 cells express large amounts of IFN-γ and IL-2, are involved in the clonal expansion of CD8+ cells and help increase macrophage phagocytic efficiency. Th2 cells are activated during allergy and parasitic infection and secrete IL-4, IL-5 and IL-13 while Th17 have a role in fungal or bacterial infections and express IL-17 and IL-22. Imbalance and expression changes among different subsets of Th are thought to play a role in autoimmune diseases like multiple sclerosis (Stromnes et al., 2008) and psychiatric disorders (Harrison et al., 2009, 2014).

CD8+ cells become cytotoxic T lymphocytes (Tc) when activated, killing infected cells and expressing cytokines that attract other inflammatory cells to the site of infection. Once the infection has cleared, a fraction of Tc and Th cells become long-lived (CD45RO) memory cells and circulate throughout the lymphoid organs, mucosa, peripheral tissue and bloodstream where they will display a strong and rapid response when repeatedly

exposed again to their antigen. Experimental data have shown that the role of T cells in the CNS goes beyond immune responses in pathological conditions. For example, in the healthy rodent brain T cells have been shown to have pro-cognitive properties and appear to have a role in maintaining homeostasis and the regulation of physiological aspects of brain function (Kipnis et al., 2012).

B cells develop in the bone marrow and after maturation migrate through the secondary lymphoid organs. They are able to recognize different types of macromolecules without MHC presentation and their activation starts when they bind to a specific antigen via their B cell receptors. B cells can also recognize self-antigens however, they display a certain degree of tolerance (low antigen recognition) in order to limit unwanted immune responses. Autoimmune disorders can result from abnormal B cell recognition of self-antigens with evidence suggesting that certain autoantibodies to neuronal antigens may be associated with the psychiatric and behavioural manifestation associated with psychotic disorders (Brudek et al., 2017)

CYTOKINES

Cytokines are key modulators of inflammation that mediate cellular responses and intracellular signalling control mechanisms of both innate and adaptive immunity. Most cytokines are produced by macrophages, mast cells and Dendritic cells in the innate immune system and by T helper cells in the adaptive immune system. The cytokine family includes chemokines (which act as chemoattractants for immune cells), interleukins, interferons, tumour necrosis factors, (tumour) transforming growth factors and colony-stimulating factors. Cytokines can have pro- or anti-inflammatory effects, and usually display a high degree of interaction and pleiotropy. Examples of commonly investigated pro-inflammatory cytokines include 1L-1ß, TNF-α, IFN-γ, IL-6 and IL-12, while IL-4 and IL-10 are considered anti-inflammatory cytokines and promote tissue repair and healing.

Within the CNS, pro-inflammatory cytokines such as 1L-1ß, TNF-α, IL-6 and Chemokine ligand 2 (CCL2) are produced during microglial activation. Besides their immunological role, cytokines are also believed to play a role in processes like sleep (Krueger et al., 2008) and hippocampus-dependent learning and memory (Rachal Pugh et al., 2001). Furthermore, some, particularly 1L-1, have been linked to the pathogenesis of neurodegenerative disorders (Allan et al., 2005).

TYPE I INTERFERONS

Type I interferons comprise a fundamental category of cytokines pivotal for orchestrating immune responses, predominantly against viral infections. IFN-α is mainly produced by plasmacytoid dendritic cells, however many different types of cells can produce IFN-α when responding to a viral infection. The production of IFN-β is more restricted and is predominantly produced by fibroblasts and certain immune cells such as macrophages and dendritic cells. Type I interferons IFN-α and IFN-β are instrumental in hindering viral replication and facilitating the transcription of Interferon-stimulated genes (ISGs) through the JAK-STAT signalling (Janus Kinase/signal transducer and activators of transcription) pathway by binding to type I interferon receptor (IFNAR) (Ivashkiv & Donlin, 2014). While IFN-α has demonstrated therapeutic efficacy in treating viral infections like Hepatitis-C and certain forms of cancer such as melanoma (Antonelli et al., 2015), IFN-β has been used in the treatment of Multiple Sclerosis (MS), a chronic inflammatory demyelinating disorder of the central nervous system, marked by immune-mediated destruction of myelin. In the context of MS, IFN-β is believed to modulate the immune response primarily by downregulating inflammatory processes and thus reducing the frequency and severity of relapses.

While the exact mechanisms by which IFN-β exerts its anti-inflammatory and immunomodulatory impacts are still not fully understood, various mechanisms of action include the suppression of T-cell activation and growth; initiating apoptosis of autoreactive T cells; promoting regulatory T cells; reducing the activation and migration of leukocytes across the BBB, and modulating cytokine activity (Dhib-Jalbut & Marks, 2010).

Clinical evidence supports the efficacy of IFN-β in decreasing the rate of relapses, mitigating the formation of new lesions, and potentially decelerating the progression of disability in relapsing forms of MS (Jakimovski et al., 2018). IFN-β preparations, such as IFN-β-1a or IFN-β-1b are administered via subcutaneous or intramuscular injections and are generally well-tolerated, with side effects such as flu-like symptoms and injection site reactions being the most common.

SICKNESS BEHAVIOUR

Infection anywhere in the body triggers, in addition to immune, metabolic and physiological responses, a series of behavioural changes collectively known as 'sickness behaviour' (Hart, 1988). Typical adaptive behaviours include lethargy, inability to concentrate, fatigue and somnolence, decreased social activity, depressed behaviour and anhedonia. This motivational reorientation is considered crucial as the coordination of immune and behavioural responses helps prioritize resources to fight infection and preserve bodily integrity.

In response to a peripheral infection, for instance, after the experimental administration of Lipopolysaccharide (LPS is an endotoxin found in the outer membrane of gram-negative bacteria), immune cells express pro-inflammatory cytokines IL-1β and TNF-α that in turn act on the brain and give rise to the full spectrum of behavioural signs of sickness behaviour (Dantzer & Kelley, 2007). During infection, a delicate balance between the expression of pro and anti-inflammatory mediators also takes place. It has also been shown that anti-inflammatory cytokines such as IL-10 act on the brain to regulate the intensity of sickness behaviour and reduce the motivational and behavioural effects triggered by LPS stimulation (Bluthé et al., 1999).

Symptoms such as physical and social withdrawal accompanied by decreased reactivity and anhedonia seen in cytokine-induced sickness behaviour are very similar to symptoms of some neuropsychiatric disorders, particularly depression. In humans, depressed patients have been found to have higher levels of pro-inflammatory mediators and

approximately one-third of patients chronically treated with IL-2 and IFN-α develop Major Depressive Disorder (MDD) (Capuron & Miller, 2004; Raison et al., 2006). These findings suggest a role for inflammation in the pathogenesis of some neuropsychiatric disorders such as depression as well as offering a potential target for cytokine-mediated therapy.

AGEING AND THE IMMUNE SYSTEM: AN EVOLUTIONARY PERSPECTIVE

Evolutionary theory suggests that ageing is a complex process that results from the accumulation of molecular and biological damage. This damage occurs as a by-product of time and is attributed to an evolved limitation of the body's maintenance and repair mechanisms, which in turn increases the probability of dysfunction and, ultimately, death of the organism. One evolutionary theory, The Network Hypothesis of Ageing (Kirkwood & Kowald, 1997), integrates and extends the concept of unrepaired somatic defects to postulate that a variety of the organism's processes and anti-stress responses act globally and in parallel with each other as anti-ageing mechanisms. The nature of the stressors/antigens threatening the organism are diverse and range from chemical (e.g., oxygen-free radicals and reducing sugars) and physiological (e.g., ultraviolet (UV) radiation, heat) to biological agents (e.g., virus, bacteria). The efficiency of this network, which includes (but is not limited to) DNA repair processes, antioxidants, heat shock and other stress proteins, and the immune system, varies across species and individuals and could account for the observed differences in the life span. (Kirkwood & Franceschi, 1992).

Comparative and phylogenetic studies from invertebrates to mammals have shown that organisms' inflammation, immune and stress responses belong to a group of highly conserved and efficient evolutionary mechanisms pivotal for preservation, and which are aimed at neutralising harmful and infectious agents that may potentially impact the organism (Ottaviani et al., 2008).

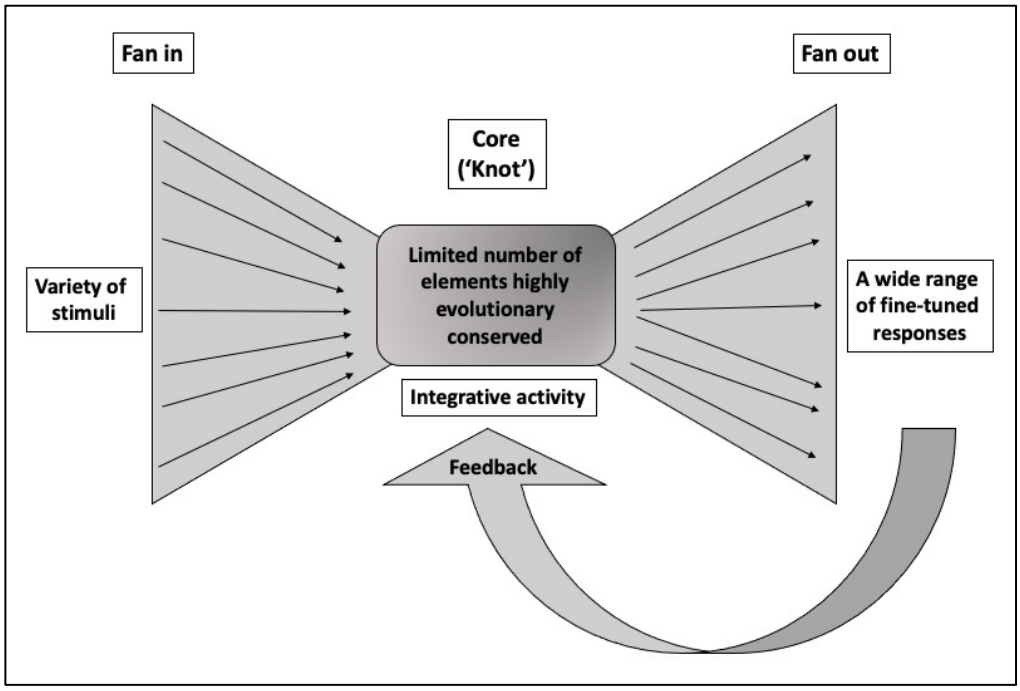
With age, however, the ability of the immune system to mount an appropriate immune response seems to decline (a process known as immunosenescence), which results in increased susceptibility to pathological conditions relating to inflammation (e.g., Alzheimer's or cardiovascular disease) (Caruso et al., 2009).

In 2000, Franceschi and colleagues, extended the network theory of ageing and proposed that there are two major characteristics of the ageing process: the global reduction of the immune system's ability to cope with stressors, and the development of inflammageing, a phenomenon characterised by a progressive and chronic low-grade inflammation status which is thought to be triggered by continuous antigenic exposure and stress (Franceschi et al., 2000). They also argue that the concept of inflammageing can only be explained within the framework of the Antagonistic Pleiotropy Theory of Ageing. This evolutionary theory suggests that the organism's ageing is caused by genes with more than a phenotypic effect. Antagonistic pleiotropic genes would then increase the odds of survival and reproduction early on in life but express a deleterious phenotype in old age (Ungewitter & Scrable, 2009). Based on that premise, the inflammatory response, essential and beneficial for survival during childhood and adulthood will become detrimental later in life, during a period largely unforeseen by evolution.

THE ARCHITECTURE OF INFLAMMAGEING

The inflammageing theory conceptualises the immune system as a network of 'Bow-ties' working at different levels of the immune response. According to this theory, the integrated activity of a reduced core or 'knot' (a limited set of evolutionarily conserved damage sensors such as TLRs and NOD-like receptors), is able to 'fan in' a variety of stressors (chemical, endocrine, immune, physical, emotional, etc.) and convert them into a wide range of fine-tuned responses ('fan-out') through the expression of a large number of pro-inflammatory molecules (Ottaviani et al., 2008**) (Figure 1).** According to this theory, this 'knot' of receptors has been evolutionarily optimized to increase inflammation and insulin resistance as a strategy to best combat pathogens and fight nutrient deprivation. It has been shown that their activation may be critical for beneficial (low inflammation) or detrimental (high inflammation) effects (Mathur et al., 2017). It would seem then that the evolutionary-driven pleiotropy of these sensors may be a factor that would lead, after years or decades of constant exposure to stressors, to the low-grade inflammatory status that defines inflammageing (Franceschi et al., 2018).

Figure 1. Inflammageing and the immune system as a network of bow-ties.



The bow-tie architectural framework converts a variety of stimuli (fan-in) into a range of finely calibrated responses (fan-out) which is mediated by the core (knot) integrative activity. The core is formed of a restricted number of conserved immune and endocrine elements. These elements possess the capacity to integrate a broad range of stimuli (e.g., immune, endocrine, physical, chemical) and provide a substantial diversity of responses. The core handles the vast number of various stimuli that may impact the immune and neuro-endocrine systems and releases a wide range of fine-tuned outputs along with a feedback regulatory mechanism. (Image adapted from Ottaviani et al., 2008).

There is not a clear mechanistic understanding of what causes this chronic, low-grade inflammation status but its development is influenced by changes at molecular, cellular and systemic level.

The main alterations that have been associated with the development and propagation of inflammageing are (i) Mitochondrial dysfunction, as damaged mitochondria that cannot be repaired and recycled release DAPMs and trigger the NLRP3 inflammasome (Ferrucci & Fabbri, 2018), (ii) Activation of the inflammasome by an imbalance between the production and disposal of cellular debris and misplaced self-molecules (known as Garba-ageing) (Franceschi et al., 2017), (iii) Perturbation of endoplasmic reticulum (ER) homeostasis by a variety of physiological conditions which disrupt protein folding and trigger ER stress (Brown & Naidoo, 2012), (iv) Defective disposal of misfolded and/or oxidized proteins by the proteasome system (Franceschi et al., 2017), (v) Activation of the

DNA damage response (DDR) in senescent cells. DDR promotes a senescence-associated secretory phenotype (SASP) and triggers DDR and SAPS paracrine activation leading to a local and eventually systemic proinflammatory environment (Olivieri et al., 2015), (vi) Change in the T cells repertoire (decrease in naive T cells due to thymic involution and increase in memory T cells) and their SASP (Fulop et al., 2018), and (vii) Changes of the composition of the gut microbiota and its role in the mediation of physiological and pathological nutrition-related inflammation (Franceschi et al., 2018).

INFLAMMAGEING AND ANTI-INFLAMMAGEING

One of the main observations about the ageing immune system is its inability to mount an appropriate immune response and the general consensus has been that immunosenescence or an immunodepressed response is a generalised state in older people.

However, in the early 90's a study challenged this idea and showed that mononuclear cells from older people release higher amounts of pro-inflammatory cytokines compared to their younger counterparts (Fagiolo et al., 1993). Findings from this study and the experiments that followed in the early 90's questioned the idea of generalised immunodepression and argued that not all responses decline with age in healthy older adults. On the contrary, years of evolutionary unpredicted exposure to stressors lead to the upregulation of the innate immune response as well as to changes in adaptive immunity. The inflammageing theoretical framework would then consider that with ageing, a maintained and up-regulated innate response, more ancestrally preserved and mainly macrophage-centred, would override a more altered adaptive immunity, which is mainly characterised by thyme involution and a decrease in naïve T cells as well as an increase of less efficient T memory cells (Franceschi et al., 2007).

The low-grade inflammatory status that defines inflammageing is characterized by higher levels of pro-inflammatory mediators as well as latent infections with viruses (e.g. Citalomegavirus) (Singh & Newman, 2011). This chronic pro-inflammatory state is considered a significant risk factor for morbidity and mortality in older adults and it has been implicated in the pathogenesis of a variety of conditions such as type-2 diabetes, Alzheimer's disease, osteoporosis and cardiovascular disease (Frasca & Blomberg, 2016).

However, how is It possible that this pro-inflammatory status, a significant risk factor, has been found present in long-living healthy individuals (e.g., centenarians)? The answer seems to be a fine-tuned balance between the expression of inflammatory and anti-inflammatory agents and the adaptive and remodelling processes put in place to counteract inflammation (Fulop et al., 2018).

In general, the main approach to studying inflammageing has been to determine plasma levels of pro-inflammatory markers. Studies on centenarians have shown that healthy long-living individuals are highly inflamed and show increased plasma levels of cytokines IL-18, IL-6, CRP (C-reactive protein), the acute-phase protein serum-amyloid A, blood clotting proteins such as fibrinogen and Von Wilebrand Factor and resistin (an adipose derived hormone involved in obesity and type II diabetes) (Franceschi et al., 2007). However, other studies show that pro-inflammatory molecules are only one part of the complex immune changes associated with age. Findings from a study comparing serum levels from young, aged healthy subjects and centenarians found higher levels of TNF soluble receptors I and II (sTNF-RI and RII), which have been shown to have anti-inflammatory potential, in older adults and centenarians compared to their younger counterparts (Gerli et al., 2010).

Another study looking into the complex relationship among inflammatory markers applied principal component analysis (PCA) to 19 inflammatory biomarkers in an Italian sample of

1010 individuals aged 21-96. The component that explained the higher percentage of variance was composed by sTNF-RI and RII, IL-6, TNF-alpha, high sensitivity CRP, IL-18 and the IL-1 receptor antagonist (IL-1RA) and was highly correlated with age. Interestingly, this component was driven by high levels of both pro and anti-inflammatory molecules which suggests a more activated (but not necessarily more inflamed) immune system (Morrisette-Thomas et al., 2014). These findings support the notion that inflammageing may be flanked by anti-inflammageing and both play a major role in the ageing and longevity processes. However, it is worth noticing that the PCA component found in this study represented only 19% of the total variance, suggesting that even though cytokine network plays an important role in the pathophysiology of inflammageing and anti-inflammageing, there is a need to explore further and investigate additional relationships among different pathways and biomarkers.

Evidence shows that other types of molecules also contribute to the global changes associated with the immune system during ageing. For instance, changes in lipid and amino acid metabolism have been reported as key regulatory processes involved in human longevity. Findings from a study of 396 participants including young individuals, centenarians and their elderly offspring described a distinct metabolic phenotype of ageing and longevity involving amino acids (e.g. tryptophan, tyrosine and phenylalanine), alterations of specific cell membrane lipids (i.e. glycerophospholipids and sphingolipids) and an increase in both pro-inflammatory (i.e. leukotrienes) and anti-inflammatory metabolites (i.e. 15-hydrozy-eicosa-tetraeinoic and 8,9-epoxyeicosatrienoic acid) (Collino et al., 2013). Another study including 294 elderly and centenarian individuals also found unique changes in lipids biosynthesis, with 41 lipids species (mostly phosphor/sphingolipids) in centenarians compared to the elderly group. These results suggest that a complex metabolic remodelling process takes place in the healthy ageing phenotype.(Biagi et al., 2010; Montoliu et al., 2014; Rampelli et al., 2013)

Another system that deserves special attention is the composition of the gut microbiota and the age-related differences between young adults, older individuals and centenarians. The evidence shows a difference between the gut ecosystem of young and older adults compared to centenarians. Specifically, centenarians show an increase in pro-inflammatory bacteria, generally present in low numbers in the adult gut system (e.g. phatobionts) (Biagi et al., 2010; Rampelli et al., 2013) as well a decrease in symbiotic species that possess anti-inflammatory properties (e.g. Faecalibacterium prauznitzii). Furthermore, strong correlations between increased levels of pro-inflammatory cytokines IL-6 and IL-8 and specific bacterial families were also found, suggesting a contribution of the gut microbiota to the development of inflammageing (Biagi et al., 2010).

Further evidence from Biagi (2016), looking into the longest human microbiota trajectory (22 to 109 years old), found a similar pattern. A progressive core microbiota shrinkage was found with age (i.e. Bacterioidaceae, Lachnospiraceae and Ruminocccaceae) as well as a negative correlation between age and the abundance of 'good bacteria' (i.e. Coprococcus and Faecalibacterium) However, the data also showed that the microbial ecosystem in extreme long-lived individuals was enriched with health-associated bacteria such as Akkermansia, Biffidobacterium and Christensenellaceae which are known to support healthy metabolic homeostasis and immunomodulation (Biagi et al., 2016).

Considering the important link between the gut microbiota and the immune system, this age-associated remodelling process, especially in extreme longevity, may have a large influence on inflammageing and immunosenescence as well as in the complex and delicate balance between the pro- and anti-inflammatory forces that drive healthy ageing.

ARE AGEING-RELATED ALTERATIONS IN THE IMMUNE SYSTEM SOLELY DETRIMENTAL?

There are several changes in the immune system that occur during the ageing process, however, it is not clear whether they are solely detrimental. Within the adaptive immune system, we observe a decrease in naïve T cells, mainly due to thymic involution, which leads to the shrinkage of the T-Cell receptor repertoire (TCR) and the increase of T memory cells primed by specific stressors (Pawelec et al., 2005). Thymic involution is one of the most basic age-associated changes in the adaptive immune system. However, there may be an evolutionary reason for this: The thymus is a very metabolically active organ, and its involution may be needed to reduce energy consumption that is not absolutely essential for survival. With age, the organism has already been exposed to a large and extensive number of harmful elements, and resources may be more efficiently directed to fighting more 'usual' cognate pathogens via the memory cell repertoire (Fulop et al.,2018). Given that a life-long exposure to pathogens may reduce direct infectious causes of death later in life, thymic down-regulation and less capacity to fight novel pathogens would seem to be a reasonable 'trade-off' in order to extend longevity.

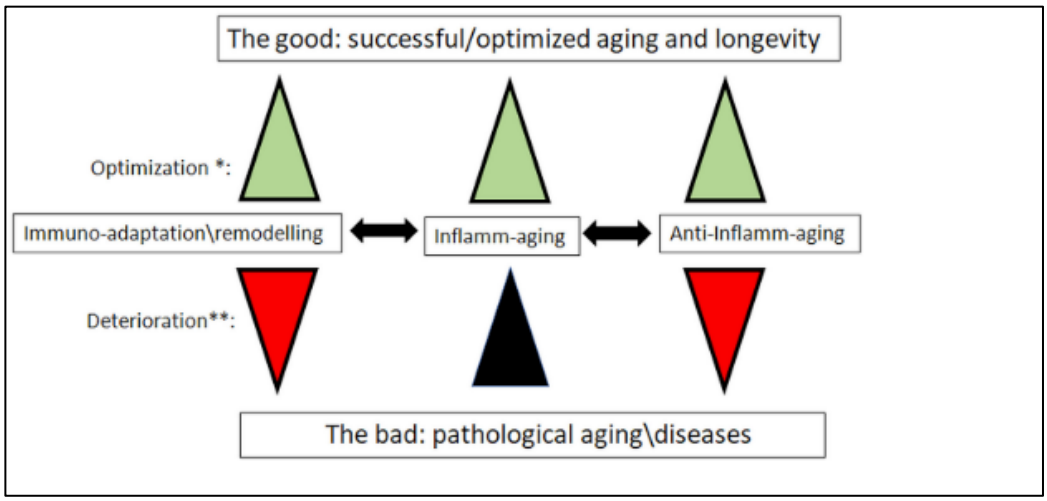
There are also some alterations within the innate immune system that may not necessarily be detrimental and may indeed have an evolutionary advantage. For instance, with age innate cells especially macrophages and monocytes seem to be in a state of constant activation. It is thought these cells sustain a certain 'trained memory' of the initial pathogen and are able to react even in the absence of the initial or other specific stimulation (Franceschi et al., 2017). This higher number of trained memory cells seems to be one of the factors that lead to the low-grade inflammation observed in aged individuals. Nevertheless, it may also increase the 'readiness' of the system to efficiently fight pathogens, which would confer, if well regulated, an advantage later in life.

HOW DO WE UNDERSTAND THE AGEING-RELATED ALTERATIONS OF THE IMMUNE SYSTEM FROM AN EVOLUTIONARY PERSPECTIVE?

The immune system is one of the physiological structures most affected by the ageing process with a large number of parameters declining in older adults compared to their young healthy counterparts. These series of alterations, generally termed immunosenescence have been considered detrimental since they seem to contribute to the low-grade chronic pro-inflammatory status that constitutes inflammageing. Furthermore, both inflammageing and immunosenescence are also considered two of the main risk factors that increase the susceptibility to pathological conditions such as cancer, neurodegenerative disorders or chronic inflammatory diseases. However, the notion of unidirectionality, meaning immune functions only decrease and the whole system becomes more inefficient, have been challenged. In fact, the general picture is more complex than that, with evidence pointing out that while some functions do decrease with age, some other markers are indeed increased. (Franceschi et al., 2017; Fulop et al., 2016)

Based on the idea that immune changes can be viewed as adaptive or remodelling rather that solely detrimental, a new paradigm was proposed. This approach considers on one side, that successful and optimized ageing and longevity take place when the three main forces: immune adaptation/remodelling, inflammageing and anti-inflammageing balance each other and increase in concert. On the other side, pathological ageing is characterised by inflammageing increases and a decrease of the other two forces, anti-inflammageing and immune adaptation/remodelling, which lead to an unstable and unbalanced system unable to compensate and cope with a hyperactive chronic pro-inflammatory estate **(Figure 2)** (Fulop et al., 2018).

Figure 2. Dynamic immune changes during ageing.



The role of inflammageing and immunoadaptation/remodelling in the ageing organism (image taken from Fulop et al., 2018)

From an evolutionary perspective, it may be argued that ageing leads to modulated/modified immune response adaptations that allow the organism to keep coping with pathogens and optimise the resources of the ageing body to increase longevity, even though it might, eventually, lead to disease and death.

CROSS-TALK BETWEEN THE BRAIN AND THE IMMUNE SYSTEM

In recent decades, the concept of the CNS as a system that enjoys 'immune privilege' has been extensively questioned and reassessed. Accumulating evidence now suggests regular interactions occur between the brain and the immune system in illness and health. For instance, the immune system can assist in recovery processes after brain damage and contribute to the brain's ability to manage stress (Kipnis, 2018b). Microglial cells (the resident macrophages of the CNS), predominantly studied within the context of disease, play a crucial role in maintaining tissue homeostasis within the healthy CNS, are involved in synaptic pruning (Schafer et al., 2012), and contribute to neurogenesis in the hippocampus (Ziv et al., 2006).

The immune system has been shown to facilitate vital brain functions like learning and social interactions and it has been considered a form of monitoring organ that senses microorganisms within and surrounding the body, conveying that information to the brain (Kipnis, 2018a). Furthermore, recent evidence suggests the brain, via the insular cortex, possesses the ability to store and retrieve immune-related information and determine its relevance to immune regulation (Koren et al., 2021). All this evidence indicates that the interaction between the brain and the immune system is not merely occasional or coincidental as once believed; instead, they are intricately intertwined.

IMMUNE PATHWAYS TO THE BRAIN

The immune system communicates with the brain through 3 main different parallel routes: humoral, neural and cellular.

In the Humoral Pathway, cytokines gain entry to the brain by binding to saturable transporters located on the blood-brain barrier (BBB) (Banks, 2005); numerous cytokines, including IL-1 and IL-6, are actively transported across the BBB (Banks et al., 1994; Plotkin et al., 1996). This passage, however, is notably selective, and some cytokines (e.g., IL-2) are not able to access the brain through it (Banks et al., 2004). Another pathway is via the circumventricular organs where circulating cytokines can infiltrate the brain due to the

highly fenestrated vasculature that characterises these organs, permitting the uptake and release of large molecules, that are typically unable to traverse the BBB (Maness et al., 1998).

Cytokines show variability in brain uptake across different regions. Some can access specific areas exclusively, while others can permeate every region, albeit with differing efficiency (Banks et al., 2001; Maness et al., 1998). Cytokines, especially IL-1 and IL-6, promote the release of prostaglandin E2 by binding to endothelium receptors, inducing fever (Dinarello, 1999; Evans et al., 2015). Furthermore, within the choroid plexus, phagocytic cells liberate IL-1 allowing it to diffuse through the brain (Quan et al., 1998). Additionally, in the circumventricular organs, macrophage-like cells residing perivascularly expressing Toll-like receptors (TLRs), can detect circulating pathogen-associated molecular patterns (PAMP) and respond by releasing cytokines (Dantzer et al., 2008).

In the Neural Pathway, pro-inflammatory cytokines released by activated monocytes and macrophages activate primary afferent nerve fibres located in the vagus nerve. These fibres possess endings that exhibit 1L-1β and Toll-like receptors that can be activated following LPS challenges or during systemic infections (Pavlov & Tracey, 2012). The activation of these fibres has been critical in understanding the neurobiological responses to inflammation.

When peripheral inflammation occurs, it triggers the expression of the immediate early gene c-Fos, a notable marker for neuronal activation, both in primary and secondary projections of the vagus nerve, such as the nucleus tractus solitarius (NTS), ventrolateral medulla, hypothalamic paraventricular and supraoptic nuclei, parabrachial nucleus and the central amygdala (Wan et al., 1994). This is indicative of the comprehensive neuronal response initiated by the presence of peripheral inflammation. Further emphasising the significant role of the vagus nerve in the body's response to inflammation, studies using endotoxemia (LPS) in mice as a model for systemic infection have proposed that vagal afferent fibres may contribute to an elevation in the production of 1L-1β within the hippocampus and hypothalamus (Laye et al., 1995). In line with this, studies using subdiaphragmatic vagotomy in rats have demonstrated an attenuation in the impairing

effects that peripheral inflammation has on social exploration further highlighting the involvement of the vagus nerve in modulating behavioural responses to inflammation (Luheshi et al., 2000).

The suggested mechanism behind the 3rd route, the Cellular Pathway, is the infiltration of activated monocytes in the brain. This notion comes from studies highlighting the role of monocyte recruitment in the modulation of depressive-like behaviours in mouse models of systemic inflammation. Mouse models of hepatic inflammatory disease have observed that peripheral TNF-α can stimulate the release of Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) from microglia, serving as a cerebral monocyte chemoattractant protein and leading to the infiltration of activated monocytes into the brain. By intercepting the recruitment of monocytes mediated by activated microglia, depressive-like behaviours induced by peripheral inflammation were significantly alleviated in mice (D'Mello et al., 2009).

Furthermore, the induction of peripheral myeloid cell invasion is not confined to instances of bacterial and viral infections or axonal injuries (Babcock et al., 2003; Mildner et al., 2008). It has also been reported that the recruitment of peripheral monocytes, which enhances inflammatory signalling within the brain, can be initiated by psychological stressors, and not just physical trauma or pathologies (Wohleb et al., 2013). Indeed, the recruitment of myeloid cells within the brain has been implicated in the modulation of behaviours, with evidence suggesting its critical role in instigating anxiety and depressive-like behaviour after Repeated Social Defeat stress exposure (Wohleb et al., 2015). In line with this, Wohleb et al. (2013) showed that a mouse model deficient in the monocyte chemotactic system (CCR2 knockout) did not exhibit stress-dependent anxiety behaviour, reinforcing the crucial role of monocyte infiltration in stress-induced behavioural alterations. While there is limited evidence for this system in humans, Nusslock et al's (2019) study findings corroborated this assumption and showed that higher level of circulating classical monocytes (CD14++/CD16 ++) negatively correlated with functional connectivity in emotion-regulation and central executive networks. **Figure 3** illustrates the three different routes by which cytokine signals access the brain

BRAIN TO IMMUNE PATHWAYS

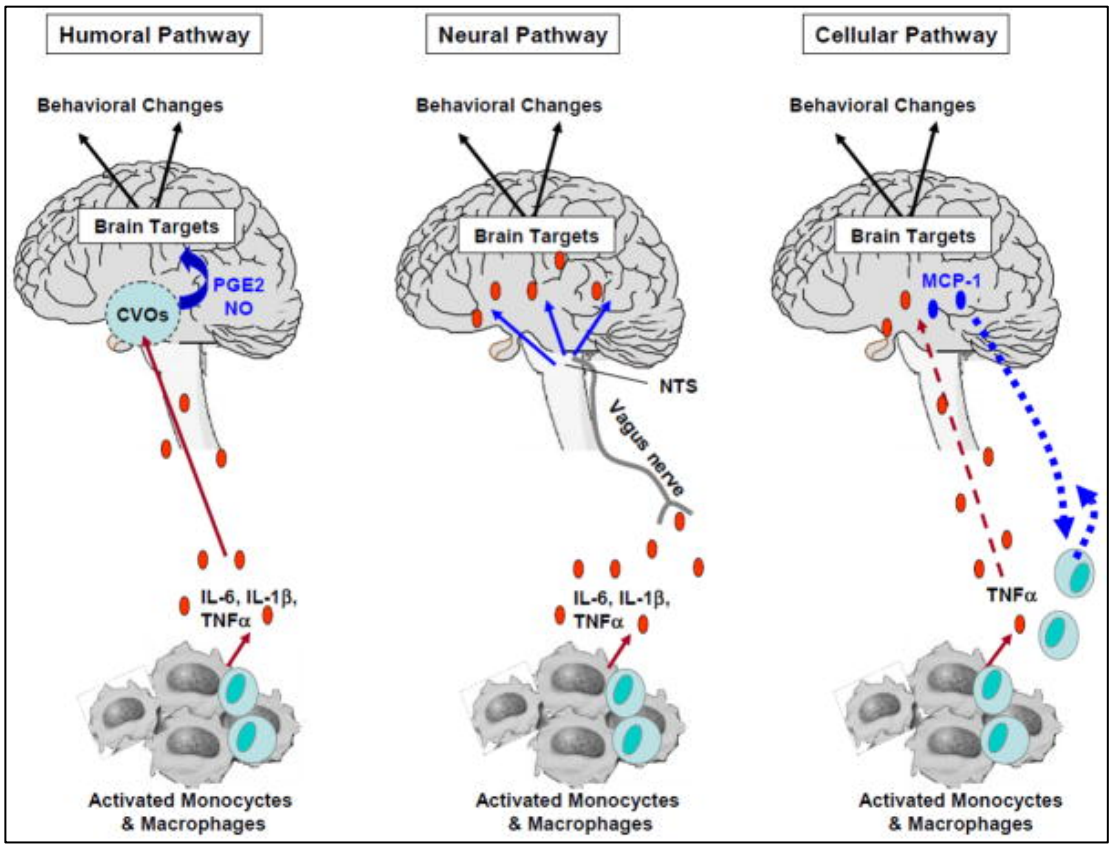
The CNS communicates with the immune system through two major pathways. The first one is the hormonal response, predominantly facilitated by the hypothalamic-pituitary-adrenal (HPA) axis.

On stimulation, the HPA triggers the production and release of glucocorticoids from the adrenal glands. Glucocorticoids regulate the expression and functionality of a broad variety of immune cells (e.g., cytokines, chemoattractants, adhesion molecules and other inflammatory mediators) (Adcock & Ito, 2000; Barnes, 1998). They have the capability to shift the immune response from pro-inflammatory to anti-inflammatory patterns (DeRijk et al., 1997; Elenkov & Chrousos, 1999) and are routinely used as immunosuppressant agents (Webster & Sternberg, 2004).

The second pathway is the autonomic nervous system, through the release of catecholamines and acetylcholine from the sympathetic and parasympathetic nerves. Immune organs such as the thymus, spleen or lymph nodes are innervated by sympathetic nerves (Ackerman et al., 1989; Felten et al., 1988). Moreover, immune cells can express neurotransmitter receptors (e.g., adrenergic receptors on lymphocytes) and are able to respond to neurotransmitters released by those nerves. It has been shown that catecholamines possess an immunosuppressant effect, for instance, they are able to reduce the production of pro-inflammatory cytokines (e.g., IL-12 or TNF-α) and boost the expression of anti-inflammatory cytokines such as IL-10 and growth factor-β (Elenkov & Chrousos, 1999)

The CNS modulated the secretion of cytokines at the site of injury through the 'inflammatory reflex' of the vagus nerve, mitigating the potential damage induced by abnormal cytokine release. This modulation is achieved by the activation of the parasympathetic system, specifically impacting the cholinergic fibres of the efferent branch of the vagus nerve (Pavlov & Tracey, 2012; Tracey, 2002). Consequently, this leads to the inhibition of cytokine synthesis by macrophages, a process mediated through the α subunit of the acetylcholine receptor AChR (Tracey, 2007).

Figure 3. Immune pathways to the brain.



Humoral pathway: pro-inflammatory cytokines released by activated monocytes and macrophages enter the brain via the circumventricular organs (CVOs) and the choroid plexus or through saturable transporters. Neuronal pathway: pro-inflammatory cytokines activate the primary afferent nerve fibres of the vagus nerve which convey information to various brain regions. Cellular pathway: Cytokines such as TNF-α induce the release of monocyte chemoattractant protein-1 (MCP-1) by microglial cells which promotes the leakage of activated monocytes into the brain (image taken from Capuron & Miller, 2011).

AGEING IN THE BRAIN

As previously described, ageing has been defined by a persistent low-grade inflammatory status (inflammageing) (Franceschi et al., 2000, 2007), marked by imbalances in pro- versus anti-inflammatory regulatory mechanisms (Fagiolo et al., 1992, 1993).

In the brain, inflammageing primarily manifests through the persistent activation of perivascular and parenchymal macrophages/microglia releasing proinflammatory cytokines and increased astrocyte number, along with increased reactive oxygen species (ROS) which in turn heightens the vulnerability to neuronal damage and death (Coyle & Puttfarcken, 1993; Floyd, 1999; Ye & Johnson, 1999).

The evidence also suggests that ageing sets a 'priming' condition for microglial cells, rendering them more responsive to harmful stimuli and immune challenges (Dilger & Johnson, 2008) . In mice, ageing has been associated with increased markers of reactive microglia and increased inflammatory markers. Following LPS administration primed microglial cells were identified as the source of excess pro-inflammatory cytokines within the CNS (Godbout et al., 2005). Behaviourally, the LPS challenge was associated with an increase in sickness behaviours, cognitive disturbance and depressive-like behaviour in aged mice (Godbout et al., 2005, 2008). This inflammatory shift or inflammageing in the ageing brain and the subsequent reactive state of microglial cells highlights a significant aspect of the ageing process, contributing to enhanced susceptibility to neurodegenerative conditions and potential cognitive alterations.

IMAGING THE EFFECTS OF INFLAMMATION ON THE BRAIN: NEUROIMAGING TECHNIQUES

A range of neuroimaging methodologies has been employed to explore the influence of inflammation on brain structure, function, neurochemistry, and its resultant cognitive implications. This exploration has served as a crucial link in translating foundational knowledge from pre-clinical investigations to applications in human models. Despite the insights offered by these different techniques, challenges still persist, especially relating to the specificity and accessibility of immune markers.

TSPO PET (Positron Emission Tomography) is considered the gold standard methodology for in-vivo quantification of neuroinflammation, specifically focusing on microglial activation. This technique has been used across an extensive array of neurological and psychiatric disorders (Colasanti et al., 2014; Kreisl et al., 2020; Meyer et al., 2020) and has also been integrated into experimental models of inflammation (Hannestad et al., 2012; Sandiego et al., 2015). Its methodology relies on the quantification of 18kDa translocator protein (TSPO), a protein found in the outer membrane of mitochondria. The low expression of TSPO in the brain under normal physiological conditions along with its relatively selective expression within glial cells, has positioned it as a potential biomarker for neuroinflammation, specifically indicative of microglial activation. Nevertheless, TSPO is associated with multiple functions (e.g., cholesterol transport into mitochondria, mitochondria respiration, calcium homeostasis, control of apoptosis or proliferation and regulation of inflammatory pathways) (Lee et al., 2020) and its exact role in immune functionality remains a subject of discussion.

Additionally, the precise contributions of microglia and astrocytes to the TSPO signals observed in human PET studies continue to be a matter of academic debate (Venneti et al., 2006). Several challenges persist in the application of TSPO PET imaging which question its viability as a reliable clinical tool: notably is an invasive modality that involves exposure to radioactive tracers and, as TSPO is expressed in other glial cells and the endothelium, its cell-specificity has been questioned (Guilarte, 2019). Moreover, increased expression of TSPO has been documented in neurons after different types of neuronal stimulation

(e.g., physiological or pharmacological) (Notter et al., 2021). Furthermore, recent data suggest that TSPO expression in humans corresponds to different events than in rodents (Nutma et al., 2023). This finding highlights that the increase in TSPO signal in humans indicates the number of inflammatory cells, rather than their reactivity status.

Other types of imaging studies, such as MRI-based techniques have already been used to explore the associations between inflammation markers and inflammation-related cognitive and behavioural disturbances. Structural neuroimaging studies have observed associations between immune markers and volumetric changes in specific brain regions. For instance, higher peripheral inflammation has been associated with cognitive alterations (e.g., decreased spatial reasoning, short-term memory or verbal proficiency) and lower grey and white matter volumes, hippocampal volume and cortical surface area in midlife adults (Marsland et al., 2015)

In patients with depression, the sACC (subgenual anterior cingulate cortex), caudate and hippocampal volumes have been corralled with the expression of immune genes (Savitz et al., 2013). Furthermore, a subsequent study highlighted an inverse correlation between striatal volume and the activation of the kynurenine pathway (a metabolic pathway considered a key regulator of the immune system and frequently associated with depression) (Savitz et al., 2015).

Research has explored the impact of systemic inflammation on neuronal networks through the use of functional magnetic resonance imaging (fMRI). They use circuit and network-based approaches to understand associations between low-grade inflammation and altered functional connectivity in psychiatric patients and healthy participants.

The effects of peripheral inflammation on brain areas associated with diminished motivation and psychomotor slowing have been consistently identified through fMRI studies using different inflammatory stimuli. For instance, actions on the ventral striatum associated with impaired reward sensitivity have been reported in experimental studies after controlled administration of inflammatory challenges (i.e. typhoid vaccine and endotoxin) (Eisenberger et al., 2010; Harrison et al., 2016) as well as in patients undergoing

IFN-α treatment for Hepatitis C (Capuron et al., 2012). Furthermore, typhoid vaccine impact of task-based activity in the substantia nigra, has been correlated with elevated plasma concentrations of IL-6 and psychomotor slowing (Brydon et al., 2008; Harrison et al., 2015). The effects of systemic inflammation on these dopamine-rich brain areas that mediate motivation and motor activity functions have been shown to be impaired also in depressed patients (Brydon et al., 2008; Eisenberger et al., 2010).

Resting-state functional magnetic resonance imaging (rsfMRI) data analysed using conventional or graph theoretic approaches, has also been utilised to investigate the associations between peripheral inflammation and functional connectivity. For instance, studies using research employing whole-brain connectomic analysis have shown alterations in the connectivity within the Default mode network -DMN- (subgenual anterior cingulate cortex and medial prefrontal cortex) associated with higher IL-6 (Marsland et al., 2017), increased cortico-subcortical connectivity following LPS administration (Labrenz et al., 2016) and decrease global network connectivity induced by IFN-α (Dipasquale et al., 2016). Moreover, increased subcortical connectivity as well as decreased cortical connectivity associated with TNF-α has been reported in adolescents (Swartz et al., 2021), and in depressed patients, increased CRP levels have been linked to impaired functional connectivity in the corticostriatal reward circuitry and within the Default mode network (Felger et al., 2016; Kitzbichler et al., 2021). Further evidence has shown correlations between peripheral inflammation markers (using a composite measure of several cytokines) and decreased functional connectivity within emotion regulation and central executive networks (Nusslock et al., 2019) and dorsal attention and DMN in older individuals (Walker et al., 2020) as well as higher levels of CRP associated with decreased frontotemporal functional connectivity in older adults (Bang et al., 2019).

While structural and functional magnetic resonance have provided consistent findings for inflammation-associated brain areas, they cannot reveal precise information about the mechanistic biological foundations of the effects of inflammation on the brain.

MRI techniques such as diffusion MRI (DW-MRI) have proven to be powerful methods for non-invasively measuring brain microstructure, as water diffusion is restricted by the cellular membranes (Alexander et al., 2019). Tissue integrity and complexity impact water diffusion by restricting the free movement of water molecules. Consequently, in scenarios of tissue damage or oedema, free water increase is observed. Neurite Orientation Dispersion and Density imaging (NODDI) offer insights regarding water diffusion within intracellular and extracellular spaces. In the context of inflammation, patients undergoing IFN-α based treatment for hepatitis-C showed an acute increase in striatal NDI (neurite density index), which may indicate alterations in water motion within the intracellular space, that predicted the onset of fatigue symptoms, a common symptom associated to acute inflammation (Dowell et al., 2019).

Another technique that has been used to explore the potential effect of inflammation on tissue microstructure is quantitative magnetization transfer (qMT). qMT can probe the exchange of magnetization between free and bound water and derive quantitative values that give us information about the physical and chemical properties of the tissue. For instance, qMT has revealed microstructural changes in the striatum, which predicted the onset of fatigue symptoms in patients initiating IFN-α therapy for Hepatitis C (Dowell et al., 2016). Moreover, qMT identified microstructural alterations in the insula that correlated with inflammation-induced fatigue in healthy volunteers after typhoid vaccination (Harrison et al., 2015). While the exact cellular and molecular mechanism is not fully understood, it is believed that qMT signal in grey matter areas are influenced by protein density and the presence of hydrophilic molecules like lactate. This suggests that qMT may offer insights into biochemical and metabolic shifts during inflammation (Harrison et al., 2015).

DWI and qMT have proven to be powerful methods for non-invasively measuring brain microstructure, however, their cellular specificity is limited. Proton Magnetic Resonance (MRS) is a powerful non-invasive method that offers insights into brain neurochemistry by enabling the quantification of metabolites within the brain (e.g., N-Acetyl-Aspartate (NAA), choline, myo-inositol, γ-aminobutyric acid (GABA) or Glutamate).

The evidence shows that MRS is a valuable method for identifying alterations in glutamate signalling during inflammation and could be used as a tool to test prospective drugs aimed at targeting the glutamatergic system in the context of mood disorders.

For instance, IFN-α has been shown to increase glutamate concentrations in the basal ganglia and the dorsal anterior cingulate cortex (dACC) of patients undergoing therapy for Hepatitis C. This glutamate concentration was associated with decreased motivation, a common symptom observed in depressive patients and frequently connected with inflammation-associated depression (Haroon et al., 2014). Moreover, depressed patients with high CRP (>3mg/L) displayed a higher glutamate concentration in the left basal ganglia compared to those with lower CRP (<1mg/L). Findings from this study also identified a positive correlation between plasma CRP and myo-inositol (considered a marker of astrocytic reactivity) which potentially could indicate irregular glial activity (Haroon et al., 2016). Furthermore, older patients undergoing IFN-α therapy displayed an increase in glutamate in the left basal ganglia in comparison to both older controls and younger subjects, whether treated with IFN-α or not (Haroon et al., 2015).

However, when interpreting MRS results, it is important to consider that the majority of glutamate is located within the intracellular space, and MRS cannot differentiate between intra- and extracellular space. Moreover, MRS enables the acquisition of data from only one voxel at a time and cannot supply information about microstructural and morphometric cellular changes.

Currently, there is no clear consensus regarding which imaging method, sensitive to peripheral inflammation, is best suited to evaluate the target engagement of emerging immunomodulating drugs. However, a novel method that combines DWI and MRS, Diffusion-Weighted Magnetic Resonance Spectroscopy (DW-MRS) has the potential to overcome some of the limitations previously described.

In this thesis, I will present in chapters 5 and 6, novel findings from DW-MRS and rsfMRI data using, for the first time, IFN-β as a new model of acute experimental inflammation.

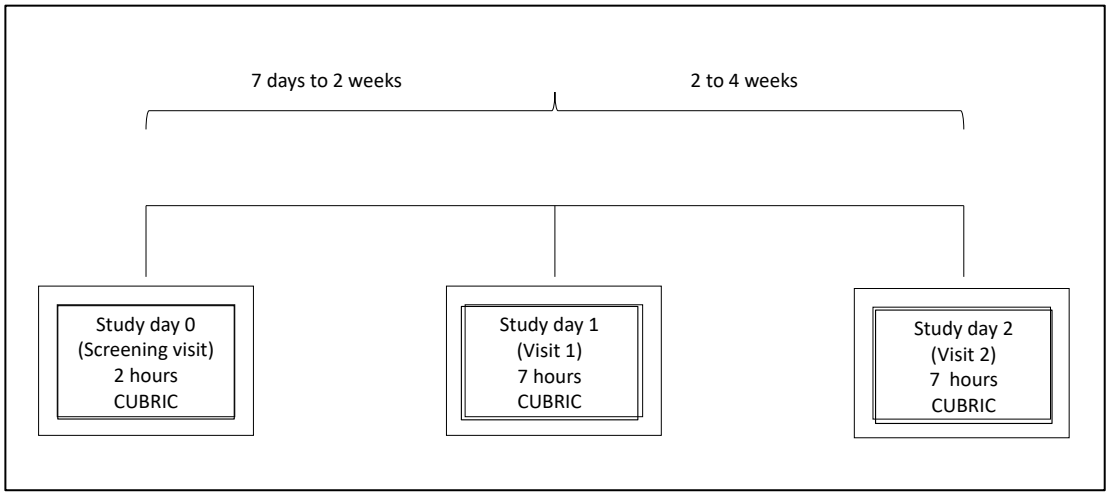
CHAPTER 2. GENERAL METHODS

STUDY DESIGN

I adopted a randomised, placebo-controlled, repeated measure cross-over study design in a cohort of health young 18-34 years (inclusive) and older 60-75 years (inclusive) adults. All participants were tested on two separate occasions and received a subcutaneous injection in the abdomen on each occasion. In one session this was 4mL of reconstituted IFN-β EXTAVIA® (100 µg), and the other (placebo session) 4mL of 0.9% saline. The order of intervention was randomised with half of the participants receiving IFN-β on their first study session and half placebo **(Figure 4).**

The Study took place at CUBRIC (Cardiff University Brain Research Imaging Centre) and was approved by the London - Camden & Kings Cross NHS National Research Ethics Committee (reference 20/LO/0239).

Figure 4. Study design



PARTICIPANT RECRUITMENT

A total of 35 participants were recruited. Among those 30 (15 young and 15 old) completed the study. Five participants did not return for the second session and were excluded from further analysis. Participants were volunteers who responded to electronic or emailed advertisements around the Cardiff area. Potential participants received the study information sheet by email before arranging a screening session to assess eligibility.

To participate in the study, subjects had to meet all the following criteria:

- Male or female aged 18-34 (inclusive) or 60-75 (inclusive)
- Non-smokers
- Participants had to be in good health as determined by medical history, physical and psychiatric screening, vital signs and clinical laboratory test results including ECG, renal, liver, thyroid function and full blood count
- Fluent in English

Participants meeting any of the criteria below were excluded from participation in the study:

- In the opinion of the principal investigator, participants with a history of cancer, diabetes or other clinically significant cardiovascular, respiratory, metabolic, renal, hepatic, gastrointestinal, haematological, neurological, psychiatric or other major disorders
- Participants who had a clinically significant illness within 4 weeks of testing
- Subjects taking regular medicines including NSAIDs (non-steroid anti-inflammatory drugs), tricyclic antidepressant (TCA), noradrenaline reuptake inhibitor (NARI), serotonin and noradrenaline reuptake inhibitor (SNRI) antibiotics, aspirin or anticoagulant therapy. Of note, use of: 1) selective serotonin reuptake inhibitors (SSRI); 2) anti-hypertensives; 3) statin lipid-lowering agents was NOT a contra-indication to enrolment

- Any clinically significant abnormal laboratory test results at screening
- Participants with a supine blood pressure at screening after resting for 10 minutes, higher than 149/89 mmHg or lower than 106/66 mmHg
- Participants with a heart rate at screening after resting for 10 minutes outside the range 50-90 beats per minute
- Participants who had received any prescribed systemic or topical medication within two weeks prior to the study
- Limited used of paracetamol or non-steroidal anti-inflammatory drugs (NSAIDs) prior to initiation of the study did not necessarily require exclusion unless there was on-going requirement for these medications
- Contraindications to MRI scanning as assessed by the MRI safety questionnaire (e.g., cardiac pacemaker, metal implants or fragments from previous injury, history of claustrophobia)
- Participants with mental incapacity or language barriers which precluded adequate understanding

During the screening session, the following assessments were performed:

- Full informed consent, including completion of the written informed consent form.
- Inclusion/exclusion criteria including MRI safety questionnaire
- Demographic data
- Medical History Pro-forma and record regular medications taken
- Weight and height to calculate BMI (Body mass index)
- Vital signs (Blood pressure, heart rate and temperature -auditory canal)
- 12 lead Electrocardiogram (ECG)
- Blood sampling for haematology (full blood count) and clinical chemistry (kidney, liver and thyroid function including Glomerular Filtration Rate estimation (GFR))
- Completion of MMSE (Mini Mental State Examination)
- Completion of MINI (Mini International Neuropsychiatric Interview)

- Questionnaires assessing depression anxiety and stress (DASS), anhedonia (SHAPS), behavioural approach/avoidance (BIS/BAS), apathy (AMI), early-life stress (child trauma questionnaire) and sickness behaviour (SicknessQ)
-

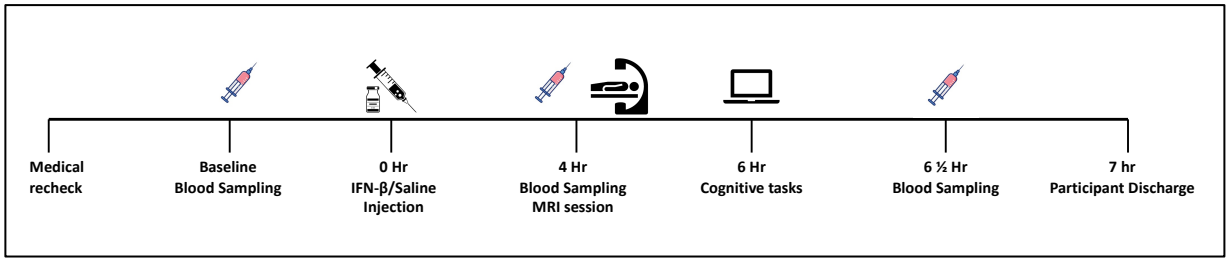
Participants received financial compensation after each session (£20 after screening, £65 after each testing session, a total of £150).

STUDY PROTOCOL

Eligible participants were invited to CUBRIC for both testing sessions. Upon arrival, participants were checked to ensure there had not been any change in health since the screening visit.

Temperature, heart rate, systolic and diastolic blood pressure were measured at baseline and 6 further time-points in both study sessions (1 hr, 2 hr, 3 hr, 4 hr, 5½ and 6½ hour post-IFN-β/saline injection). Blood samples were collected at baseline, 4 hr and 6½ hr for haematological and plasma cytokine analysis. Self-report questionnaires were administered at baseline, 1 hr, 2 hr, 3 hr, 4 hr and 5½ post-injection. An MRI scanning session of 75 min was completed at 4 hr after the injection. Participants completed a series of laptop-based cognitive tasks at around 6 hr post-injection and were monitored for another hour prior to discharge. **Figure 5** provides a schematic overview of the experimental protocol.

Figure 5. Experiment session flowchart



SAMPLE SIZE CALCULATION

The sample size for this study was based on the published literature.

There are currently no data looking into differences in metabolite diffusion in young and older adults after IFN-β. However, a preliminary study on 7 young participants (pre and 5 hours post-LPS) of a more potent pro-inflammatory challenge showed a significant increase in Choline diffusion (mean ± SEM) of 5.025±1.28% (De Marco et al., 2022) giving an estimated effect size (d) of 1.48. Our sample of 30 MRI participants will have >95% power to detect a similar main effect of IFN-β at alpha < 0.05 and 75% power to detect a condition (interferon/placebo) x age (young/older) interaction effect size (f) of 0.25 at alpha < 0.05.

Similarly, there are no published data investigating the effects of age in functional connectivity using IFN-β as an immune challenge. A preliminary study showed a significant reduction in global functional connectivity after IFN-α injection in a sample of 20 Hepatitis-C patients. Brain network alterations were correlated with associated changes in mood (r = -0.59) (Dipasquale et al., 2016). This gives an estimated effect size (d) of 0.54. Our sample of 30 participants will have 95% power to detect a similar main effect of Interferon. Using an estimated effect size (f) of 0.3 our sample will be powered >90% to detect any possible interaction between age and condition.

CHAPTER 3. INF-B: A NEW MODEL OF ACUTE EXPERIMENTAL INFLAMMATION

INTRODUCTION

Main antiviral cytokines, the type I IFNs (IFN-α and IFN-β) are important in controlling the innate immune response to viral infections.

Natural Type I interferon, IFN-β is secreted by fibroblasts. Both type I interferons (alpha and beta) regulate inflammatory responses by signalling to the heterodimeric IFNAR1/IFNAR2 receptor and activating the JAK-STAT family of signal transducers which in turn associate with IRF 3 (interferon regulatory factor). This process triggers the activation of ISG (Interferon stimulated genes) which results in the secretion of several cytokines such as IL-6, TNF-α or IL-1ra as well as other antiviral, antitumor and antiproliferative agents (Kasper & Reder, 2014; Kümpfel et al., 2000).

IFN-α has been widely administered as a treatment for Hepatitis C (McHutchison et al., 1998) while IFN-β therapies were the first major therapeutic class of medications to be developed for the treatment of MS, offering patients a therapy that resulted in lower recurrence rates and delayed disability onset (Jacobs et al., 1981, 1982; Jacobs et al., 1996).

IFN- α works against Hepatitis C (HCV) in multiple ways. The primary action of IFN- α is to impede the replication of the virus. It achieves this by signalling to infected cells and neighbouring uninfected cells to adopt an antiviral state which in turn leads to the production of proteins that block viral replication (Samuel, 2001). IFN- α also enhances the body's immune response to the virus by promoting the activity of natural killer (NK) cells, a type of immune cell capable of destroying HCV-infected cells. This helps reduce the viral load in the body and limit the spread of the virus (Rehermann, 2013). Additionally, IFN- α can influence the processing and functioning of HCV proteins, leading to the disruption of the virus lifecycle (Gale & Katze, 1998).

The exact mechanism by which IFN-β is believed to work against MS is not entirely understood, but there are several theories based on its known immunomodulatory effects. Some of the proposed mechanisms are: (i) Inhibition of T-cell activation and proliferation. T-cells are a type of immune cell implicated in the pathogenesis of MS. IFN-β is thought to modulate T-cell responses, reducing their activation and proliferation, thus preventing these cells from causing damage to myelin (Cheng et al., 2015; Teige et al., 2006). (ii) Suppression of pro-inflammatory cytokine production. IFN-β has been shown to suppress the production of various pro-inflammatory cytokines such as TNF- α, IL-12, and IFN-γ (Mirandola et al., 2009). (iii) Enhancement of anti-inflammatory cytokine production. IFN-β promotes the production of anti-inflammatory cytokines, like IL-10 and IL-4, which may help counteract the pro-inflammatory state in MS (Mirandola et al., 2009; Wiesemann et al., 2008). (iv) It may reduce the expression of adhesion molecules and other enzymes (i.e. matrix metalloproteinases), which are involved in the migration of immune cells across the BBB into the CNS. This is believed to limit the infiltration of immune cells in the brain parenchyma, thus reducing inflammation and damage (Kieseier, 2011). (v) IFN-β may promote the induction and expansion of regulatory T-cells, a subtype of T-cell that helps control immune responses and prevent autoimmune reactions (Windhagen et al., 1995).

Animal data indicate that peripherally injected cytokines such as Type I interferons may access the brain parenchyma through different routes. These routes include (i) passing through areas of the BBB that have increased permeability (Banks, 2016; Pan et al., 1997), (ii) actively transporting through saturable transport systems (Banks & Erickson, 2010; Erickson & Banks, 2018), (iii) triggering inflammatory mediators release from cells lining in the cerebral vasculature (e.g. endothelial cells) (Ericsson et al., 1994), (iv) binding to cytokine receptors present on peripheral afferent nerve fibres such as the vagus nerve, which then transmit signal to relevant brain regions (Goehler et al., 2000). This is supported by data in rodents, where the upregulation of ISGs is found in the brain parenchyma of mice following intra-peritoneal IFN administration (Wang, 2009; Wang & Campbell, 2005). In humans, one way to investigate the access and action of peripheral blood cytokines on the brain has been to adopt an experimental medicine approach in patients who are receiving IFN-α medication for therapeutic reasons. It has been shown

that IFN-α administration stimulates the production of IL-6, TNF-α as well as IL-1 beta in peripheral blood mononuclear cells and in a number of cell lines from relevant patient populations (Capuron et al., 2003; Taylor & Grossberg, 1998). Furthermore, raised IFN-α and IL-6 have been found in the CSF of patients undergoing IFN-β-based treatment for HCV (Raison et al., 2009).

The effects of type I interferons on the brain parenchyma may be mediated by microglial cells which induce the release of proinflammatory cytokines such as IL-6 which further the inflammatory response. This would support the role of CNS cells in the propagation of the immune response. In rodents, peripheral administration of IFN-α has been shown to induce a unique transcriptome profile (i.e. upregulation of ISGs and the complement component C4b) and phenotypic changes in microglial cells, primarily reliant on direct signalling via microglial type I interferon receptor IFNAR (Aw et al., 2020). However, it is not entirely clear how interferon signalling may have an impact on CNS function and modulate the neuro-behavioural effects associated with some of the most common neurodegenerative and neuropsychiatric disorders.

Routes of administration and frequency of injection vary among treatment strategies, nonetheless, in all cases, the IFN injection elicits a systemic inflammatory response that mimics a viral infection and commonly induces a physiological response marked by a variety of flu-like symptoms such as chills, fever, headache, fatigue and muscle pain (G. L. Davis et al., 1989; Filipi & Jack, 2020). In healthy volunteers, IFN-β symptoms start between 3-4 hours post-infusion with temperature and heart rate displaying a slow and steady increase that peak at around 6-8 hrs post injection (Exton et al., 2002; Salmon et al., 1996). For this study and based on IFN-β EXTAVIA® half-life (~4.3 to 5 hr) and the observations listed above, participants underwent a 1 ½ hr scan session 4 hr post-injection and were monitored during the whole duration of the study (~7 hr). IFN-β symptom presentation is generally mild which allowed subjects to sit comfortably and be still for the whole duration of the scanner session. Participants were back in a more comfortable clinical environment when more pronounced symptoms started developing (~5 ½ hr post-injection). In terms of adverse side effects, only one participant developed an injection site

reaction (itchiness and redness) which resolved on its own approximately 24 hr post-challenge.

For years, IFN-α therapies for Hepatitis C have provided strong empirical evidence on the impairing effects of inflammation on mood and sickness behaviour, however, IFN- α commercial availability (non-pegylated form) ceased due to market-related considerations. While other inflammation models are already described in the literature (e.g. typhoid or endotoxin immune challenges), the decision was to develop a new model of acute experimental inflammation using IFN-β. The rationale behind that decision was to put into practice a design that was not overly invasive yet highly effective (for instance, more effective than the typhoid model but not as strong and invasive as endotoxin challenges) and could be intended for wider experimental application (could be used in young as well as in older individuals)

In this chapter, I will focus on the physiological response to the peripheral administration of IFN-β in healthy young and old adults and investigate how age may modulate the effects of IFN-β and its impact on physiological responses (mood and behavioural responses will be discussed in the next chapter). Additionally, I will compare and discuss the IFN-β response in relation to other experimental challenges.

METHODS

PARTICIPANTS

Vital signs, haematology and cytokine data were obtained from 15 young (6 male, mean age 25.2 ± 5.1 (std) years, mean BMI: 26 ± 5.7 (std) kg/m^2) and 15 older (6 male, mean age 65.6 ± 4.5 9 (std) years, mean BMI: 28.4 ± 5.9 (std) kg/m^2) healthy participants

Participants attended a first screening visit which included an assessment of whether they met inclusion and exclusion criteria and to obtain written informed consent. A physical health check was performed including blood pressure, temperature heart rate and an electrocardiogram (ECG). Blood samples were taken for full blood count, differential white blood cell (WBC) count (lymphocytes, monocytes, neutrophils, eosinophils, basophils), and thyroid, liver, and renal function. All were screened to exclude any undergoing neurological or psychiatric conditions. The use of anti-hypertensives, selective serotonin reuptake inhibitors (SSRI) and statin lipid-lowering agents was not a contraindication to enrolment. Among the older group, 1 participant was on anti-hypertensives, while two were on SSRIs. There were no reports of medication use within the younger cohort.

In order to describe a broader psychological profile, participants also completed questionnaires assessing apathy, anhedonia, depression and anxiety as well as behavioural approach and avoidance and early-life stress. In the younger cohort (n=15), the majority of participants identified as belonging to a white ethnic background (n=13), with two participants identifying as Chinese. The older cohort (n =1 5) was composed of participants who identified as being of white ethnicity.

Participants were asked to avoid heavy exercise and the use of alcohol 24 hours prior to the start of each session. The study was approved by the London - Camden & Kings Cross Research Ethics Committee (reference 20/LO/0239).

VITAL SIGNS AND HAEMATOLOGY

Temperature, heart rate, systolic and diastolic blood pressure were measured at baseline and 6 further time-points for each condition (1 hr, 2 hr, 3 hr, 4 hr, 5 ½ and 6 ½ hour post-IFN-β/saline injection). Cardiovascular parameters heart rate and blood pressure were monitored via a finger probe and arm cuff connected to a vital signs monitor (IMEC 10, Mindray, China). Body temperature was measured using a digital tympanic thermometer (Braun ThermoScan 5, Braun, Germany)

A heart rate measuring device (Firstbeat bodyguard 2, Firstbeat Technologies, Finland) was attached to the skin and used to calculate heart rate variability throughout each 7-hour testing session. Upon arrival and after a medical recheck, a venous cannula was inserted in each session and removed after the last blood draw. Blood samples for total and differential WBC and cytokine analysis were collected into BD Vacutainer plastic EDTA tubes with lavender hemogard closure (4 mL, 13x75mm) (Becton, Dickson and Company, Franklin Lakes, New Jersey, United States) at baseline, 4 hr and 6 ½ hr post-injection. Blood bottles were labelled after collection and samples for WBC delivered to University Wales Hospital (UWH) pathology lab for analysis.

IMMUNE MODULATORS: CYTOKINE ANALYSIS

EDTA vacutainer tubes were centrifuged at 2000 rpm for 20 min: plasma was removed, aliquoted and stored at -80 ^{o}C on university premises. IFN-β plasma concentration was measured using VeriKine-HS™ Human IFN Beta Serum High Sensitivity ELISA Kit (PBL Assay Science, NJ, USA). Detection limit was 2.3 pg/mL and intra and inter-essay coefficients of variation were 3.6% and 7.9%.

Plasma levels of IL-6, TNF- α and IL-10 were quantified with Quantikine™ High Sensitivity (R&D Systems inc., Minneapolis, USA). Detection limits were 0.156 pg/mL, 0.156 pg/mL and 0.78 pg/mL respectively and coefficients of variation were 3.6% and 4.9% (IL-6), 2% and 6.7% (TNF- α) and 5.8% and 7.8% (IL-10). Standards and samples were tested in

duplicate. Samples with measurements below the lowest standard were given a value of half the lower limit of detection for IL-10 and IFN-β cytokines (Breen et al., 2011).

STATISTICAL ANALYSIS

Main effects of INF-b and age and their interaction were analysed using repeated measures mixed factorial ANOVAs: within-subject factors: condition (IFN-β/placebo) and time (pre- and post-injection times as described), between-subject factor: age (young/old) with significant interactions assessed using t-tests at each time points between conditions. Pearson's correlations were used to assess associations between cytokines and total and differential cell counts (computed as peak change minus baseline for the IFN-β condition). Data analysis was carried out using SPSS 27 statistical package.

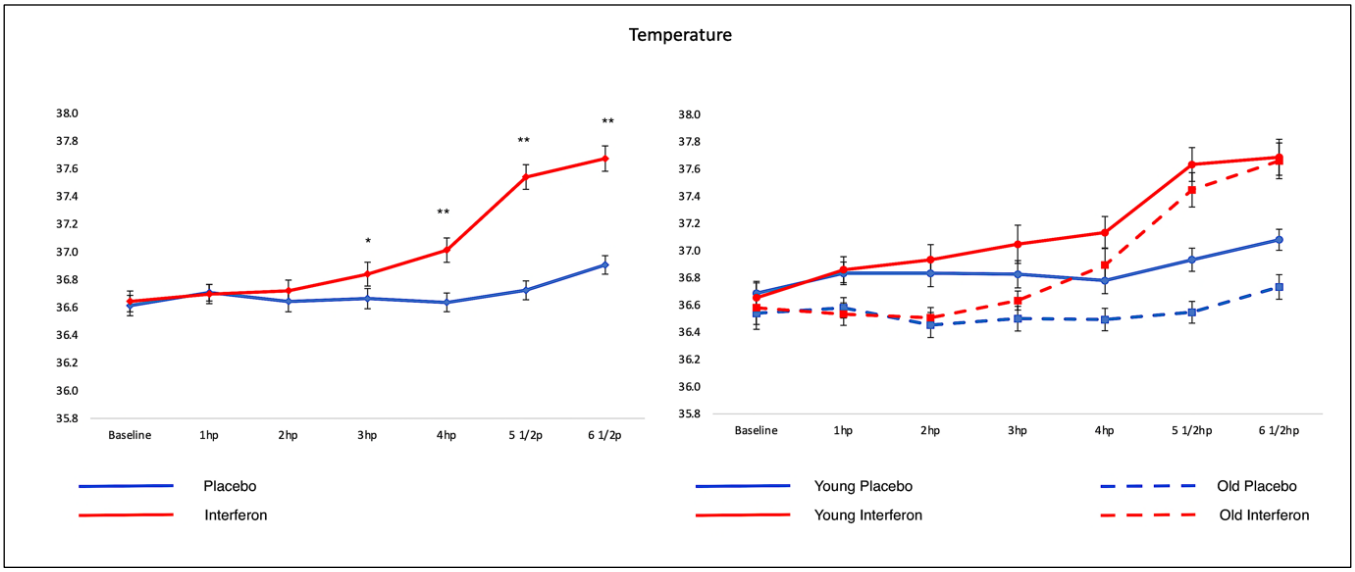
RESULTS

VITAL SIGNS

TEMPERATURE

Repeated measures ANOVA showed a significant main effect of condition (placebo/IFN-β) ($F_{(1,28)}$ = 45.5, p<0.001) and condition x time interaction ($F_{(3.3,94.48)}$ = 30.88, p<0.001). IFN-β significantly increased temperature (compared to placebo) from 3 hours post-injection ($t_{(28)}$= -2.87, p = 0.008), and peaked 6 ½ hours post-injection ($t_{(28)}$= -8.32, p<0.001). Between subject effects revealed a significant main effect of age ($F_{(1,28)}$=6.79, p=0.014) with older individuals' temperature being on average 0.271 °C (SE=0.104) lower than the younger participants regardless of condition or time **(Figure 6).** No statistically significant condition x age interactions were observed.

Figure 6. Effects of IFN-β on temperature



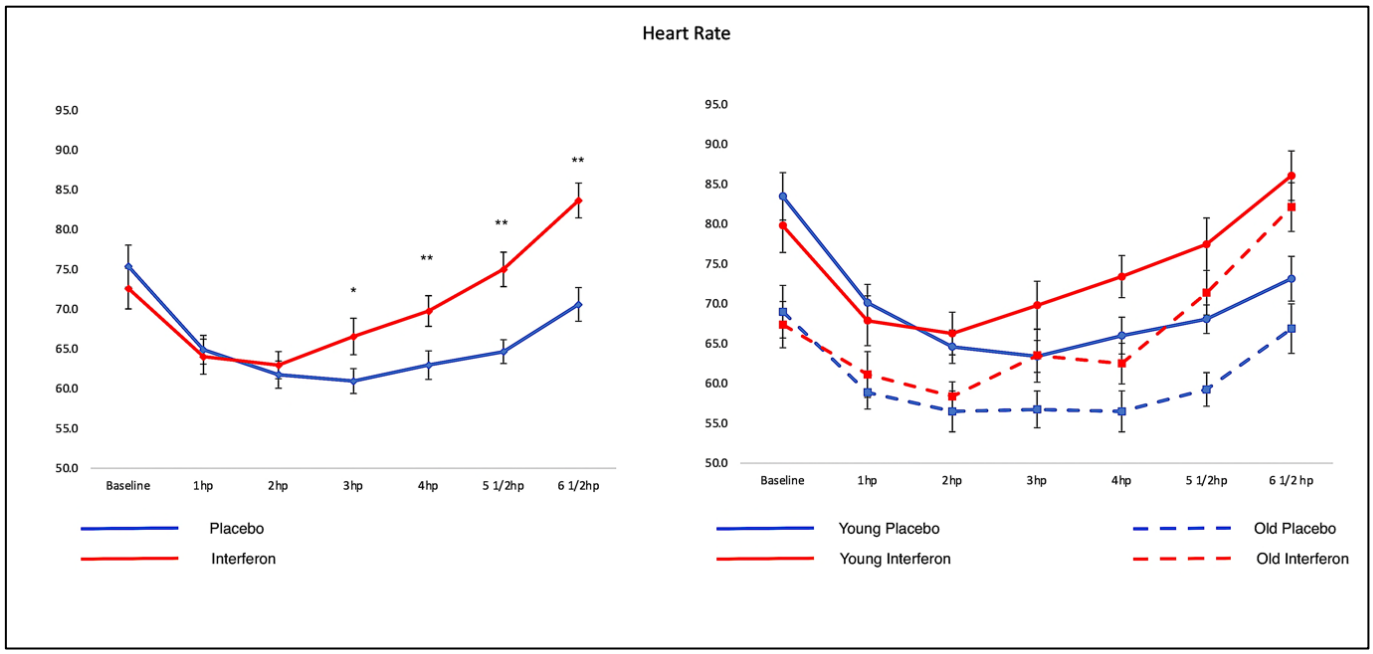
Effects of IFN-β on temperature (°C) (left), and age-associated effects on temperature (right). Red lines represent IFN-β and blue lines placebo. Dotted lines represent older individuals. Error bars denote SEM; significant values show the main effect of IFN-β compared to placebo (*p<0.05 **p<0.001).

HEART RATE

There was a significant main effect of condition ($F_{(1,28)}$=21.34, p<0.001) and condition x time interaction ($F_{(4.14,115.93)}$=16.62, p<0.001) for heart rate. IFN-β significantly increased heart rate relative to placebo from 3 hours post-challenge ($t_{(28)}$=-5.6, p=0.005) and peaked at 6½ hours post-injection ($t_{(28)}$=-10.33, p<0.001) **(Figure 7**). No condition by age interactions were observed.

Similar to temperature, there was a significant between-subject effects of age on heart rate $F_{(1,28)}$=6.27, p=0.018) with older individuals having a lower resting heart rate compared to the young group (mean difference=7.657 bpm, SE=3.05) (**Figure 7**)

Figure 7. Effect of IFN-β on heart rate



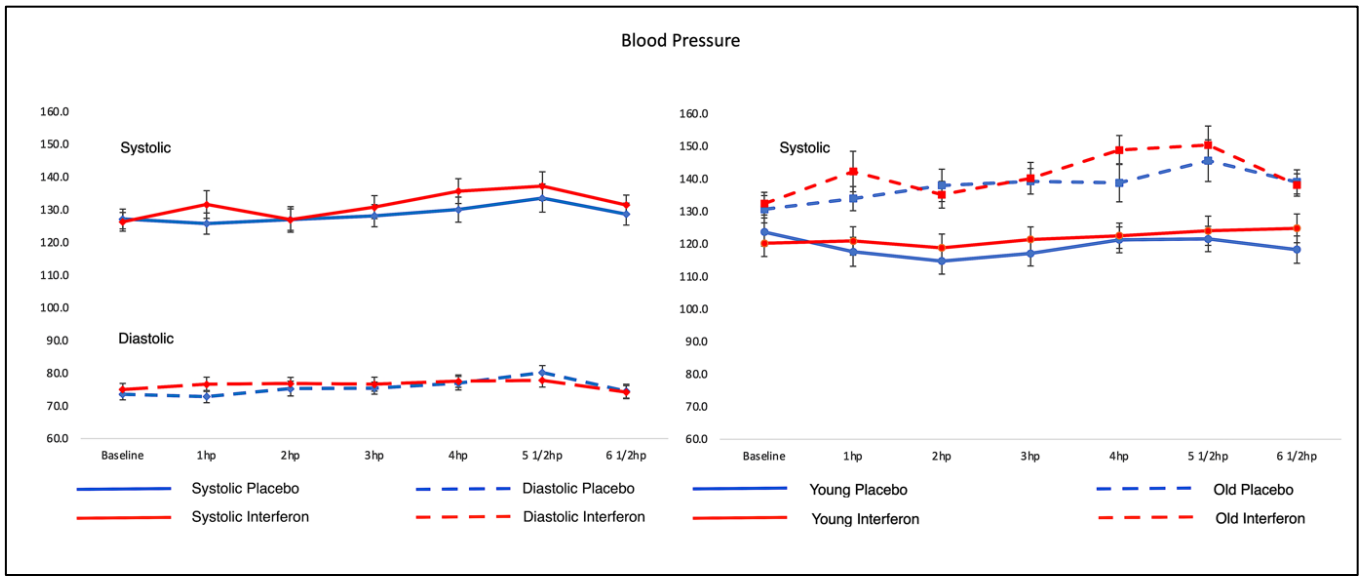
Effect of IFN-β on heart rate (bpm)(left) and age-associated differences on heart rate (right). Red lines represent IFN-β and blue lines placebo. Dotted lines represent older individuals. Error bars denote SEM; significant values show the main effect of IFN-β compared to placebo (*p<0.01 **p<0.001).

BLOOD PRESSURE

Repeated measures ANOVAs revealed a marginal main effect of condition on systolic BP ($F_{(1,28)}$= 4.15, p=0.051) but no condition x time or age interactions. No differences in diastolic blood pressure or interactions were observed **(Figure 8).**

Between subject effects also showed a significant age-associated difference in systolic BP ($F_{(1,28)}$=14.58, p<0.001) with systolic BP being on average higher in older individuals compared to their younger counterparts (mean difference=-19.086 mmHg, SE=4.9) (**Figure 8**). No age-associated effects were found in diastolic BP.

Figure 8. Effects of IFN-β on Blood pressure



Effects of IFN-β on Blood pressure (mmHg) (left) and age-associated effect of systolic BP (right). Left: Red lines represent IFN-β, blue lines placebo, dotted lines represent diastolic BP. Right figure: dotted lines represent older individuals. Error bars denote SEM.

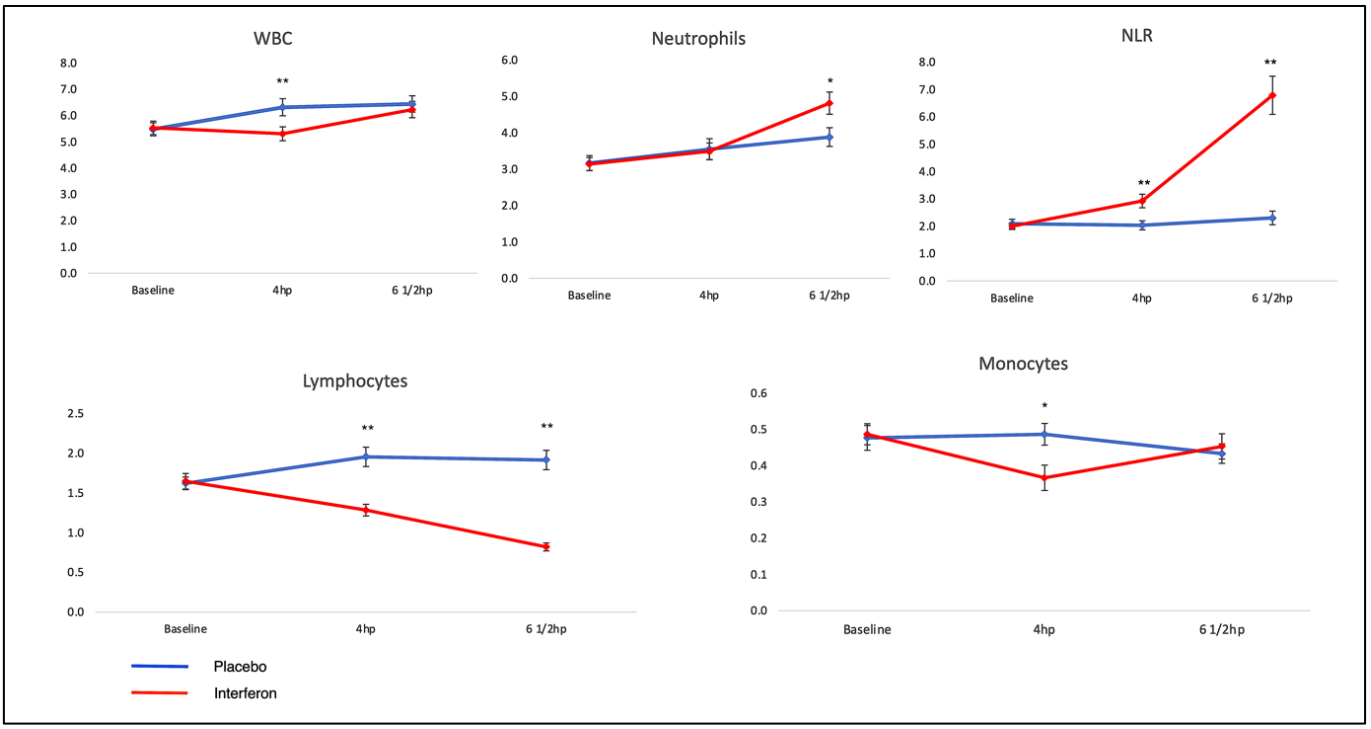
HAEMATOLOGY: CELLULAR IMMUNE RESPONSE

Significant condition x time interactions were found for total WBC ($F_{(1.49,40.47)}$=10.92, p<0.001) and each differential white blood cell count (10^9/L) : Monocytes: ($F_{(1.52,42.79)}$=7.22, p<0.001), Neutrophils: ($F_{(1.52,42.43)}$=15.9, p<0.001) and lymphocytes ($F_{(1.33,37.32)}$=72.46, p<0.001) We also observed a main effect of condition ($F_{(1,28)}$=112.26, p<0.001) for lymphocytes. A main effect of condition ($F_{(1,28)}$=35.95, p<0.001) and condition x time interaction ($F_{(1.06,29.7)}$=45.78, p<0.001) was also observed for Neutrophil to Lymphocyte ratio NLR **(Figure 9).**

Post INF-b cell count (expressed as relative delta percentage ± CI) increased 59.2 ± 20.4% at 6 ½ hours post-injection (compared to baseline) for Neutrophils. NLR increased both at 4 hrs (43.8 ± 11.6%) and 6 ½ hrs (245.4 ± 67.2%) post-IFN. Lymphocytes showed a decrease of 21.2 ± 4.8% and 49.3 ± 4.8% at 4 and 6 ½ hrs respectively. Monocytes displayed a decrease 4 hours after IFN-β of 29.6 ± 8.5% with WBC also decreasing (3.5 ± 5.8%) 4 hours post-IFN injection (relative to baseline).

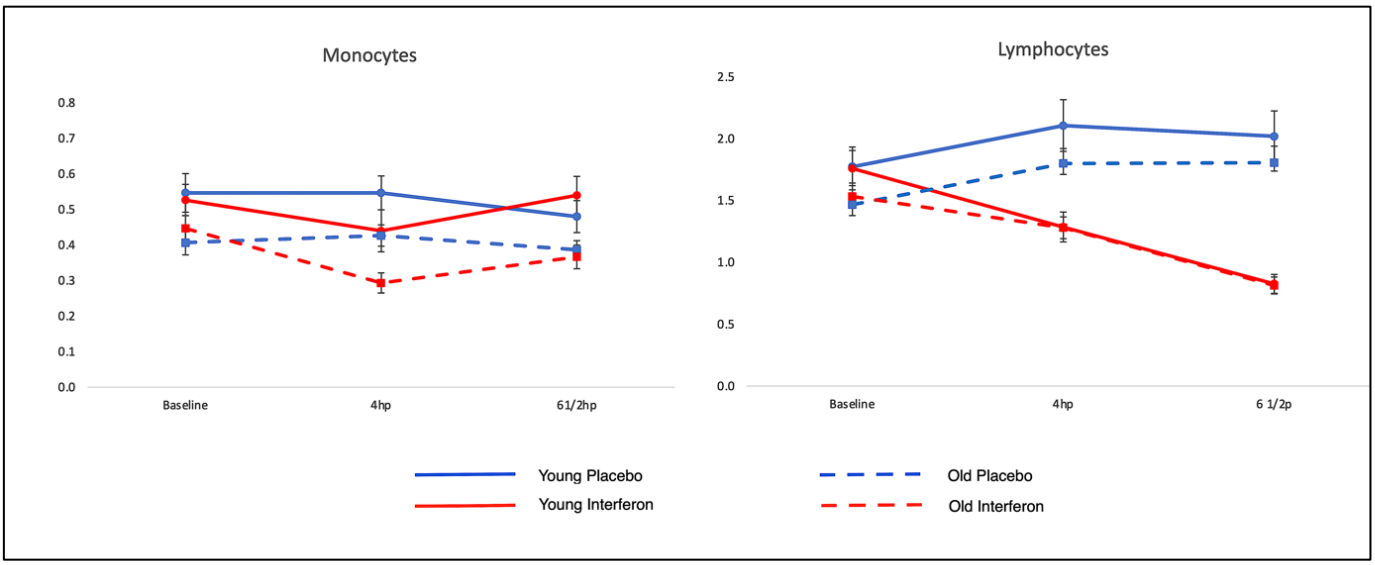
Furthermore, a significant between-subject effect was found for Monocytes ($F_{(1,28)}$=7.93, p=0.026). Older individuals showed lower Monocyte cell count regardless of time and condition (mean difference=0.126 (10^9/L), SE=0.045). In addition, I observed a condition x age interaction non-significant trend for Lymphocytes (10^9/L) ($F_{(1,28)}$=3.13, p=0.088) **(Figure 9).** No other interactions or age-associated differences were observed for any of the other WBC. Haematology data are described in **Table 1.**

Figure 9. Effects of IFN-β on total and differential WBC and Neutrophil to Lymphocyte ratio



Effects of IFN-β on total and differential WBC and Neutrophil to Lymphocyte ratio (NLR) (10^9/L). Red lines represent IFN-β, blue lines placebo. Error bars denote SEM; significant values show the main effect of IFN-β relative to placebo (*p<0.01,**p<0.001).

Figure 10. Age-associated effects on Monocytes and condition x age interaction on Lymphocytes



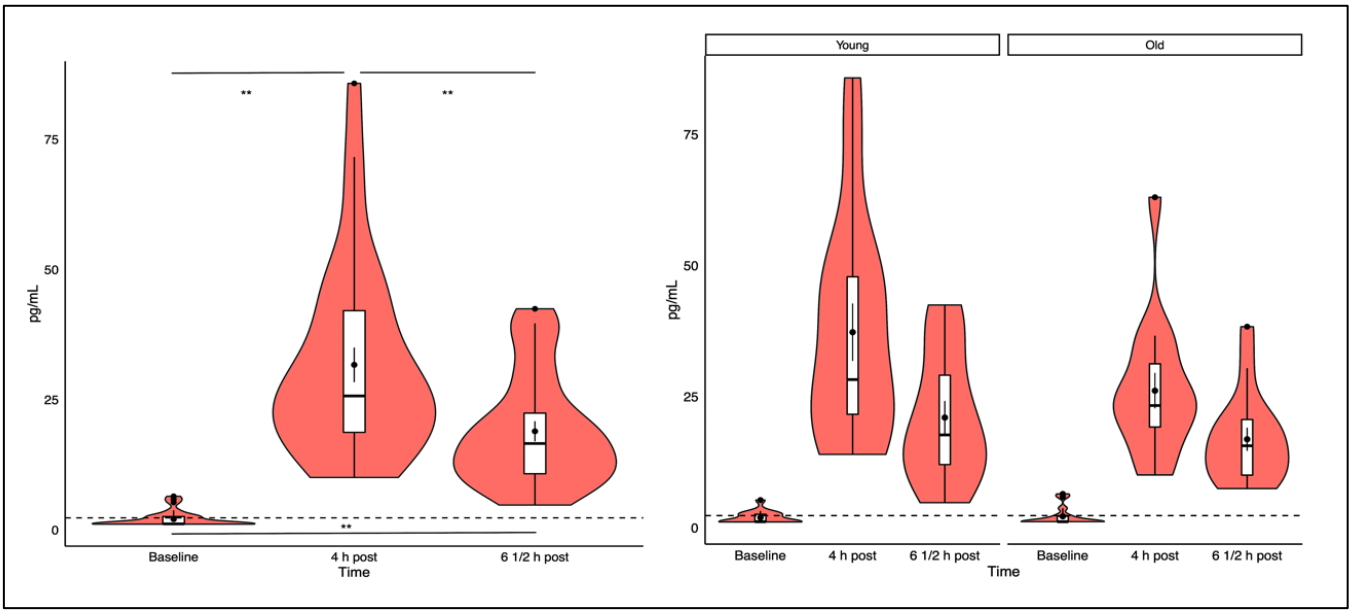
Age-associated effects on Monocytes (left) and condition x age interaction on Lymphocytes (right) (10^9/L). Red lines represent IFN-β, blue lines placebo. Dotted lines represent older participants. Error bars denote SEM.

Table 1. Haematology data ([10^9/L] ± SEM)

	Time post-injection (hrs)	Saline	IFN-β	p-values (Saline vs. IFN-β)
Total WBC	0	5.48 (0.24)	5.53 (0.25)	.789
	4	6.32 (0.32)	5.31 (0.26)	<.001
	6 ½	6.44 (0.31)	6.23 (0.31)	.478
Monocytes	0	0.47 (0.03)	0.48 (0.02)	.759
	4	0.48 (0.02)	0.34 (0.02)	<.001
	6 ½	0.43 (0.02)	0.42 (0.02)	.849
Lymphocytes	0	1.62 (0.08)	1.64 (0.09)	.699
	4	1.95 (0.12)	1.28 (0.07)	<.001
	6 ½	1.91 (0.12)	0.82 (0.05)	<.001
Neutrophils	0	3.17 (0.2)	3.14 (0.170	.848
	4	3.67 (0.25)	3.49 (0.23)	.414
	6 ½	3.88 (0.25)	4.81 (0.3)	.003
NLR	0	2.08 (0.16)	2.0 (0.11)	.536
	4	2.03 (0.16)	2.92 (0.24)	<.001
	6 ½	2.3 (0.25)	6.79 (0.69)	<.001

As anticipated, following the IFN-β challenge, plasma concentrations (pg/mL) of IFN-β significantly increased across time ($F_{(1.19,32.19)}$=10.14, p<0.001). Relative to baseline (pre-injection), plasma concentrations peaked at 4 hours ($t_{(29)}$=-8.74, p<0.001) and showed a decrease at 6½ hours post-challenge ($t_{(29)}$=-8.55, p<0.001). Significant differences in IFN-β plasma concentration were also observed between 4 and 6½ hours post-injection ($t_{(29)}$=6.98, p<0.001). No significant age effects were found, however, there is a trend towards a time x age interaction with higher levels of circulating IFN-β found in young individuals compared to their older counterparts ($F_{(1.18,33)}$=2.8, p=0.098) (**Figure 11).**

Figure 11. Distribution of IFN-β plasma concentrations for the interferon condition

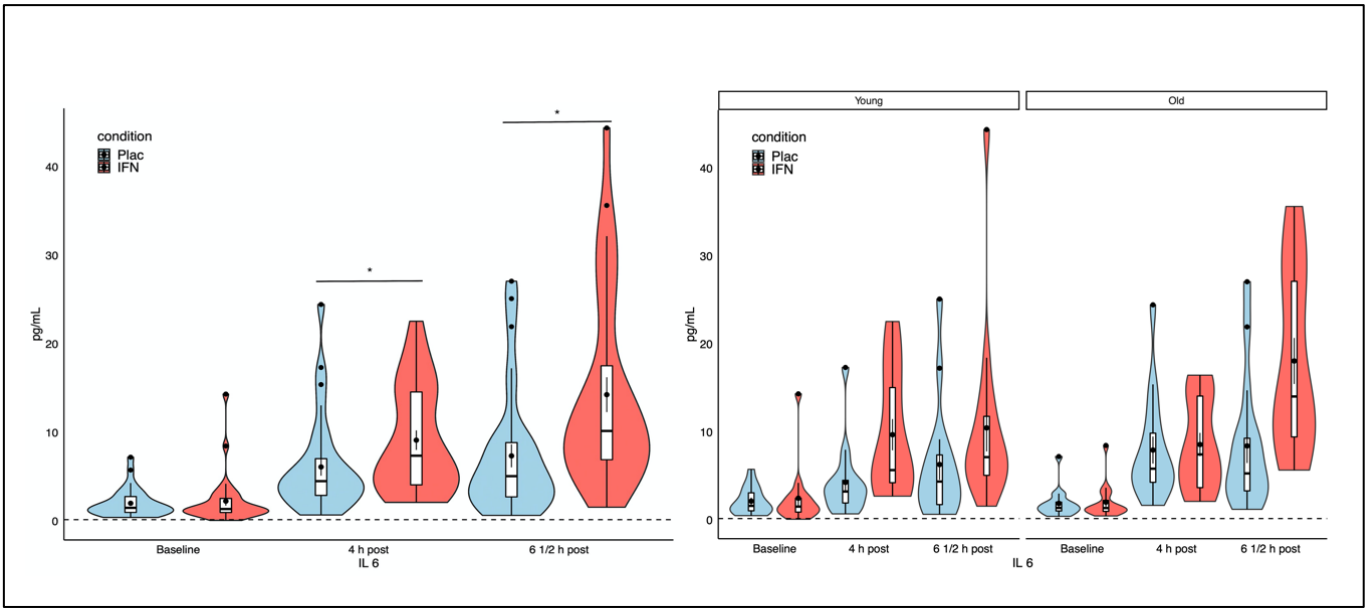


Distribution of IFN-β plasma concentrations (left) and split by age (right) for the interferon condition. Significant values show main paired sample t-test results (**p<0.001). Dashed line shows lower limit of detection (2.3 pg/mL).

IFN-β induced a significant increase in circulating cytokine IL-6 shown by significant condition (placebo/IFN-β) ($F_{(1.28)}$=10.14, p=0.004) and condition x time interaction ($F_{(1.45,40.78)}$=4.49, p=0.027). A time x age interaction ($F_{(1.85, 51.83)}$= 4.16, p=0.024). The mean ± SEM IL-6 concentration was for young and old respectively: 6.98 ± 1.1 and 8.25 ± 1.1 at 4 ½ hr post and 8.38 ± 1.4 and 13.24 ± 1.4 at 6 ½ hr post-injection.

Furthermore, I observed a trend towards a condition x time x age interaction with a higher increase of IL-6 plasma concentrations at 6 ½ post-injection in older individuals ($F_{(1.45,40.78)}$=2.65, p=0.097) **(Figure 12).**

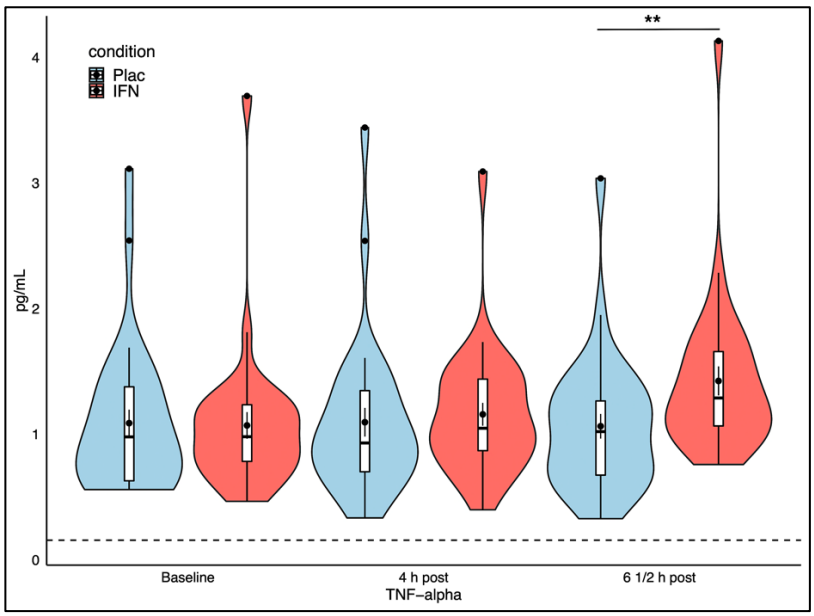
Figure 12. Distribution of IL-6 plasma concentrations



Distribution of IL-6 plasma concentrations (left) and IL-6 plasma concentrations split by age (right). Blue plots denote placebo, red plots interferon. Significant values show paired sample t-test results (*p<0.05). Dashed line shows lower limit of detection (0.156 pg/mL).

Condition x time interaction effects were observed also for TNF-α ($F_{(1.56,43.94)}$=22.07, p<0.001) **(Figure 13).** No condition x age interactions or age-associated differences were observed.

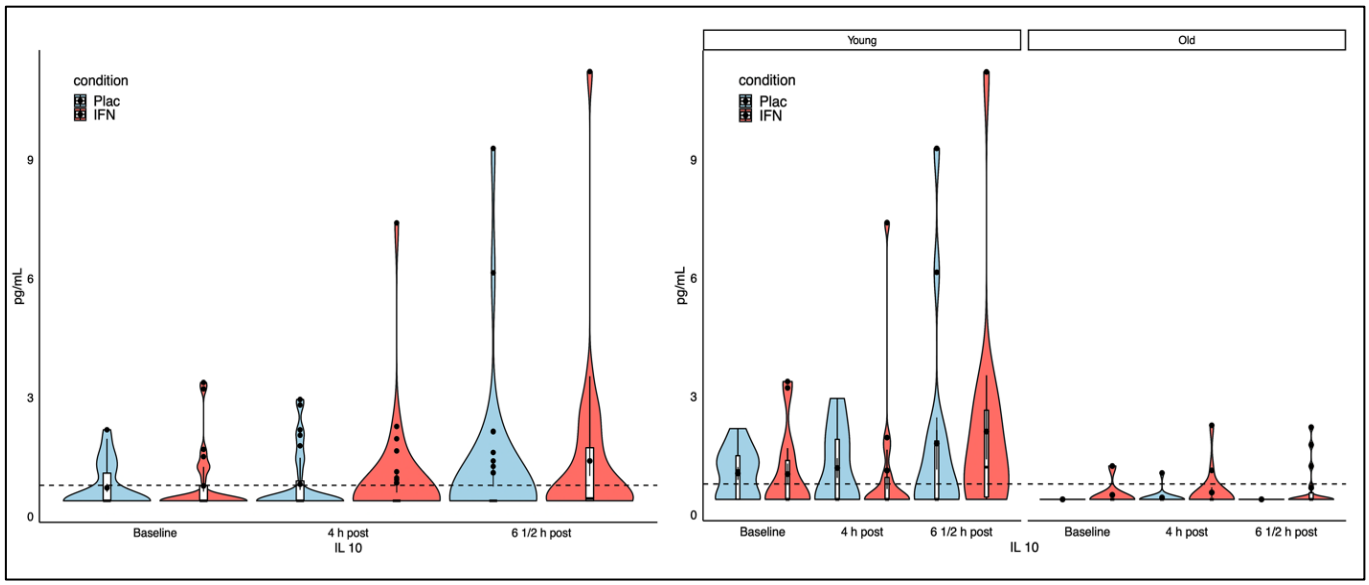
Figure 13. Distribution of TNF-α plasma concentrations



Red bars represent IFN-β, blue placebo Significant values show paired sample t-test results (**p<0.001). Dashed lines show lower limit of detection (0.156 pg/mL).

No Significant main effect of IFN-β was found in concentrations of the anti-inflammatory cytokine IL-10 (p>0.1) (However, between-subjects effects showed a significant age-associated difference ($F_{(1.28)}$=6.48, p=0.017). Compared to the young group, older individuals had lower plasma concentrations of IL-10 (mean difference=0.889 (pg/mL), SE=0.349) (**Figure 14**). Cytokine data and cytokine correlations are described in **Tables 2 and 3** respectively.

Figure 14. Distribution of IL-10 plasma concentrations

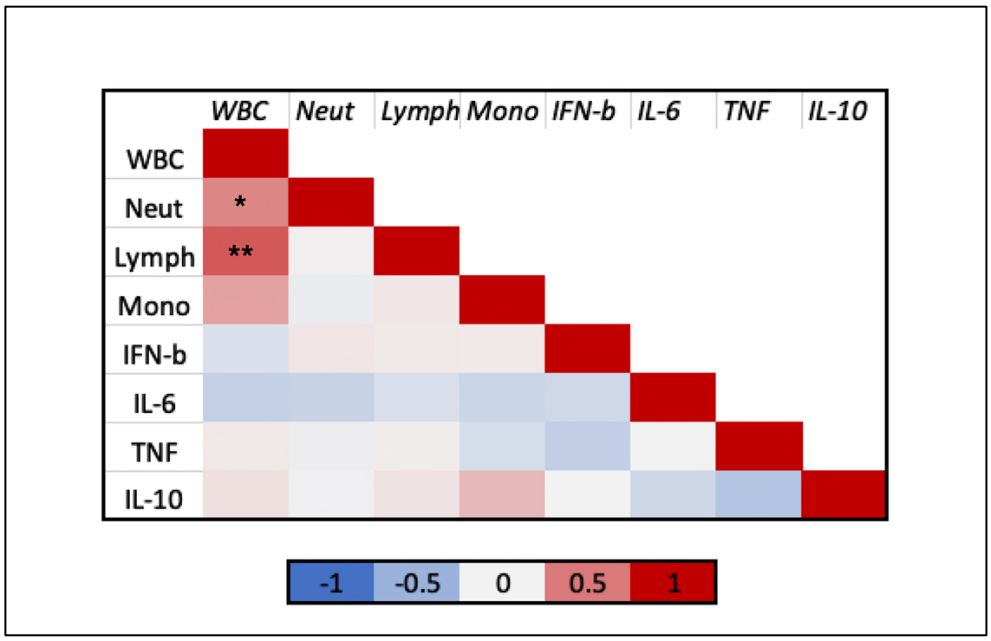


Distribution of IL-10 plasma concentrations (left) and IL-10 plasma concentrations split by age (right). Blue plots denote placebo, red plots interferon. Dashed line shows lower limit of detection (0.78 pg/mL).

Table 2. Cytokine data ([pg/mL] ± SEM)

IFN-β	0	4	6 ½	p-values (relative to baseline)	
				(4 hr)	(6 ½ hr)
	2.05 (0.27)	31.69 (3.32)	18.92 (1.92)	<.001	<.001
	Time post-injection (hrs)	Saline	IFN-β	p-values (Saline vs. IFN-β)	
IL-6	0	2.0 (0.28)	2.24 (0.51)	.609	
	4	6.1 (0.98)	9.13 (1.09)	.036	
	6 ½	7.35 (1.29)	14.26 (1.96)	.011	
IL-10	0	0.71 (0.1)	0.76 (0.14)	.744	
	4	0.8 (0.14)	0.84 (0.24)	.891	
	6 ½	1.09 (0.35)	1.39 (0.37)	.225	
TNF- α	0	1.08 (0.1)	1.06 (0.1)	.828	
	4	1.09 (0.11)	1.15 (0.09)	.512	
	6 ½	1.06 (0.98)	1.42 (0.11)	<.001	

Table 3. Cytokine correlations with immune cells



Colour map represents Pearson's correlation coefficients (peak change minus baseline) for the IFN-β condition (*p<0.05, **p<0.001).

DISCUSSION

Based on the rationale that IFN-β causes a temporary systemic immune response, Type-I interferons can be used as a model to evaluate neuroinflammation in healthy individuals. In this chapter, I investigated the physiological response of a new immune challenge: IFN-β.

Below, I will highlight physiological, and immune reactions to interferon beta and contrast these responses with other commonly used experimental immune challenges including endotoxin (LPS 0.4-2.0ng/Kg), Interferon-α and typhoid vaccination. Furthermore, I will explore and discuss evidence for similar and differential responses to IFN-β as a function of age and highlight further work required to fully validate the model.

As shown by the changes observed in vital signs, cellular immune response and circulating cytokines, IFN-β induced a significant systemic inflammatory response, IFN-β led to significant increases in body temperature and heart rate that started at 3 hrs and peaked 6 ½ hrs after injection with results in line with evidence found in IFN-β studies on healthy individuals and MS patients (Exton et al., 2002; Kümpfel et al., 2000; Salmon et al., 1996). Overall, and as anticipated, the IFN-β transient changes in temperature (+1.1 °C) and heart rate (+11 bmp) observed in our study were similar to data obtained from Type I interferon (IFN-α) inflammation models. Furthermore, IFN-β elicited a milder and stronger response compared to LPS and typhoid models respectively (Fukuhara et al., 1999; Glue et al., 2000; Han et al., 2013; Harrison et al., 2009, 2014b; Hijma et al., 2020; Lasselin et al., 2017).

IFN-β also significantly increased circulating cytokine IL-6 (~ 6 fold) following the pattern observed also in Interferon-based therapies for Hepatitis C patients (Capuron & Miller, 2004; Davies et al., 2020) LPS (Lasselin et al., 2017; Peters van Ton et al., 2021; Sandiego et al., 2015) and Typhoid vaccination studies. (Harrison et al., 2014, 2016).

TNF-α was also raised after IFN-β (~1.3 fold). TNF-α increased plasma concentrations are commonly observed in endotoxin studies, however, does not seem to be a common feature in typhoid vaccine and IFN- α models (Davies et al., 2020; Harrison et al., 2009). Nevertheless, acutely, IFN-β has been shown to contribute to the inflammatory response by increasing TNF-α and IL-6 in MS patients (Kümpfel et al., 2000).

Though IFN- α and IFN-β are both part of the Type I interferon family, their mechanism of action and their effects on other cytokines such as TNF- α can differ due to their different binding affinities (although both bind to the same receptor, they display different affinities for their subunits) and therefore a distinct influence on the cellular signalling pathways that control TNF-α production during an acute challenge could explain the differences (de Weerd et al., 2013; Ivashkiv & Donlin, 2014).

While IL-10 concentrations have been shown to be increased in IFN-α and LPS models, we did not observe any significant transient changes in IL-10 after IFN-β. In the context of MS, IFN-β has been demonstrated to enhance the production of IL-10. This boost in IL-10 leads to an anti-inflammatory response, providing an explanation for the therapeutic use of IFN-β in managing MS (Kvarnström et al., 2013; Özenci et al., 2000). IFN-β dosage variation may explain the different levels of response for IL-10. After administering IFN-β subcutaneously to healthy volunteers at a dose of 250 μg every other day for a week, IL-10 concentration significantly increased above baseline between 6 and 12 hr after the first IFN-β injection and peaked between 40 and 120 hrs (Williams & Witt, 1998). The variance in the IL-10 response could potentially be attributed to the use of a lower dose (100 μg) in this study.

The cellular reaction to IFN-β closely mirrored the response seen in IFN-α challenges. IFN-β increased Neutrophil count (~50%) decreased Lymphocytes (~50%) and monocytes showed a pattern of decrease after 4 hr post-challenge (~30%) increasing again (~35%) at 6 ½ hr. The observed effects were milder than in the Endotoxin model, however the pattern of change followed the exact same trend.

Overall, the physiological and cellular immune response to IFN-β was similar to those seen with IFN-α, more intense than those observed in the typhoid model but milder when compared to the endotoxin challenge (refer to **Table 4** for a comparison of the model with the other three immune challenges). Taken together, the data show that IFN-β can be used to induce a transient inflammatory state with consistent symptom development across participants.

The plasma concentration of IFN-β dramatically increased 4 and 6 ½ hours (~ 15 and 9-fold respectively compared to baseline) after the administration of IFN-β with participants displaying a significant rise in side effects from 3 hr post-injection.

Though the pharmacokinetics profile of IFN-β may display some variations that will depend on the route of administration, dosing regime and therapeutics (pegylated and non-pegylated forms), overall, in healthy individuals, IFN-β has shown a half-life and bioavailability of approximately 4-5 hr and 30-50% respectively, reaching peak concentrations between 1-8 hours post-injection. Presentation and intensity of common clinical side effects are very similar for the different administrations (flu symptoms such as chills, fever, fatigue, sickness) and usually start ~3-4 hr post-infusion. (Hu et al., 2016; Salmon et al., 1996).

Due to technical issues with one of the ELISA kits, it was not possible to obtain IFN-β plasma concentrations from the placebo condition. However, evidence from previous literature and human IFN-β detection studies show that serum and plasma levels of IFN-β in healthy individuals are commonly below the level of detection (which is consistent with our baseline data) and do not appear to display a circadian rhythm or be sensitive to stress factors such as the insertion of the cannula (*Human IFN-Beta ELISA Kit, High Sensitivity (Serum, Plasma, TCM)*, n.d.; *Human IFN-Beta ELISA Serum, Plasma Performance Characterization*, n.d.; Salmon et al., 1996). The above evidence supports the notion that the robust changes in the concentration of circulating cytokine IFN-β in our sample were explained by the administration of recombinant IFN-β.

After the injection of saline in the placebo condition, IL-6 plasma concentration data showed a slight increase, which may have been attributable to factors associated with minor inflammation (e.g., cannula insertion). Nonetheless, the condition x time interaction and post hoc paired t-test comparing conditions at matching time periods revealed significant differences indicating IFN-β has a robust effect. IFN-β increased IL-6 by ~6-fold (relative to baseline). At 4 and 6 ½ hr IFN-β raised IL-6 by ~2 fold compared to placebo.

I tested volunteers from two age groups (young and old) in this study. One of the project's primary goals was to investigate if age can modulate the effects of IFN-β and its impact on physiological, behavioural and cognitive responses. Based on the premise that older individuals often experience greater behavioural responses to infection and severe infections can lead to irreversible cognitive decline even in previously healthy older people (Iwashyna et al., 2010), my hypothesis was that the impairing effects of inflammation would have a differential effect on the physiology of older individuals. More precisely, I anticipated observing significant differences in the progression of transient changes associated to the physiological and immune cellular responses.

The data did not show any significant condition x age interactions for any of the physiological or immune markers. Different reasons may explain the findings: (i) The study (n=30) was estimated to have an 80% power to detect a medium interaction effect size (f) of ~0.25, therefore there is a 20% chance we could have missed a true effect due to sampling variability. However, I did observe a time x age interaction for IL-6 and trends, such as interactions for lymphocytes (condition x age) IL-6 (condition x time x age) as well as IFN-β (time x age). These data suggest that IFN-β, as a model of acute inflammation can be sensitive to detect age-related changes. Nevertheless, the capacity of the study to detect subtle effects may have been limited due to statistical power, and therefore a larger sample size could be considered, as it might reveal other possible interaction effects.

(ii) Immune senescence and inflammageing processes are considered two of the main explanatory factors associated with a less efficient immune response in older individuals (Franceschi et al., 2018; Pawelec, 2018). While older age has been clearly associated with an increase in inflammation markers such as proinflammatory cytokines (Cohen et al., 2003; Walston et al., 2002), the evidence of how age influences inflammation-induced changes remains limited.

For instance, evidence from sepsis studies comparing cytokine responses in young and elderly patients have reported increased IL-6 and TNF-α levels in the elderly after discharge or after few days of hospitalisation, suggesting an age-dependent delay in the resolution of inflammation (Bruunsgaard et al., 1999; Kale et al., 2010).

The study data show that physiological markers mostly peaked at 6½ hours after injection. However, for older people, the peak change may have happened after the session ended, and therefore it is not possible to establish if IFN-β would have had a greater effect on their response. Compared to their younger counterparts, most older individuals reported more intense symptoms after the session ended, with some effects remaining persistent overnight and the next morning which may suggest a prolonged inflammatory response.

Exploring this could be achieved by implementing a follow-up period. However, due to various factors, monitoring participants beyond the testing sessions presents challenges (e.g. organisational, financial), not to mention the additional burden it could put on participants, such as having to wear monitoring devices overnight and into the following day, or requiring additional blood draws. A possible solution to that could be to employ statistical approaches like time series analysis, which would allow the observation and forecast of changes in physiological signals.

Another potential explanation to why I did not observe significant condition x age interactions (iii) might be related to differences in IFN-β absorption and metabolic rates between the groups. I observed a time x age trend ($p=0.098$) for IFN-β plasma concentrations. At 4 hr post-challenge, I saw that there is ~30% more circulating IFN-β in the young group compared to the old, which may indicate a higher rate of absorption. Young participants also showed increased elimination rates. Roughly 4 to 6 ½ hr after the challenge, their plasma concentration of IFN-β had dropped by approximately 75% in contrast to a decrease of about 35% seen in older individuals. It appears that older people absorbed less IFN-β, but the cytokine remained in their bodies for extended periods. This effect may have had an influence in other cytokines, (e.g., the data shows a trend for condition x age x time interaction for IL-6) and potentially contribute to differences in the progression and resolution of the inflammatory response in young and old groups.

To the best of my knowledge, this study is pioneering in its application of IFN-β within an acute experimental inflammation model. It also represents the first investigation into the effects induced by age-related inflammation. The significant main effects of IFN-β observed in the physiological and immune response show that the model can be used as

a minimally invasive and effective experimental design able to induce transient changes in systemic inflammation in healthy individuals. As IFN-β elicits a mild but robust response (more effective than the typhoid model but not as strong and invasive as endotoxin challenges), It is suitable for wider experimental applications and can be used in more challenging and complex populations (i.e., older individuals). While I did not observe any condition x age interaction the data showed a few trends which suggest the model is sensitive to age-related changes.

A few considerations about the model: (i) A larger sample would increase statistical power and the capacity to potentially detect subtle interaction effects. For this study, the timeframe and overall constraints imposed by the covid-19 pandemic greatly impacted the recruitment process and added another layer of complexity to an already challenging interventional study. (ii) As project management was one of my main responsibilities (e.g. coordination of testing sessions, medic covers and radiographers), I could not be blind to condition and so knew which condition participants were assigned. However, because the physical reaction to IFN-β is mild and symptom presentation shows a steady but slow pattern of rise, most participants were unaware of which condition they were in until they completed the second session and were able to compare. (iii) For the study, I collected data for whole-blood mRNA analysis at baseline and 6 ½ post-challenge. Once these data are analysed, we will have a full IFN-β transcriptomic profile in healthy young and old individuals which will provide specific information about changes in gene expression and how they might influence the inflammatory response across time and age groups.

Table 4. Comparison of the IFN-β model with typhoid, IFN-a, and Endotoxin challenges for the physiological and immune response

Human systemic inflammation models

	Typhoid Vaccination (Typhim)	IFN-α2a (3MU)	Our model: IFN-b-1b (EXTAVIA® 3MU)	Endotoxin (1ng/kg NIH reference)
Physiological	Temperature: No change Heart Rate: + (~1-2 bmp) Blood Pressure: dBP +4 mmHg Heart rate variability: +LF/HF	Temperature: + (~0.08 °C) Heart Rate: ++ (5~6 bmp) Blood Pressure : +/- Heart rate variability: +LF/HF	Temperature: + (~1.1 °C) Heart Rate: ++ (~11 bmp) Blood Pressure : +/- *Heart rate variability: expected +LF/HF	Temperature: ++ (~1.4 °C) Heart Rate: +++(~25 bmp) Blood Pressure : +/- Heart rate variability: ++LF/HF
Immune/Inflammatory	Cellular: 25% ↑ WBC Cytokine: = ~3x IL-6, IL1ra Transcriptomics: Not known	Cellular: 40% ↑ Neutrophil, 50% ↓ Lymphocytes, Monocytes: biphasic ↓ 60% (3hrs) ↑ 50% (6hrs) Cytokine: +~3x IL-6, IL1ra, 50% ↑ IL-10 Transcriptomics: well-described	Cellular: 50% ↑ Neutrophil, 50% ↓ Lymphocytes, Monocytes: biphasic ↓ 30% (3hrs) ↑ 35% (6hrs) Cytokine: +~6x IL-6, ~1.3x TNF-α, +/- IL-10 *Transcriptomics: Described in MS patients only	Cellular: 230% ↑ Neutrophil, 60% ↓ Lymphocytes, Monocytes: biphasic ↓ 60% (3hrs) ↑ 50% (6hrs) Cytokine: +++ >10x IL-6, IL1ra, TNF, IL-8, ~5x IL-10 Transcriptomics: TLR-4 described

CHAPTER 4. EFFECTS OF IFN-B ON MOOD AND BEHAVIOUR

INTRODUCTION

In healthy mammals, when systemic inflammation occurs, it induces a range of behavioural changes referred to as 'sickness behaviour' (Hart, 1988). The manifestation of this behaviour includes motivational changes such as fatigue, loss of appetite lack of thirst or lethargy as well as, psychomotor slowing, shifts in cognition and mood, confusion, memory deficits and low mood (Capuron et al., 1999; Dantzer, 2001, 2009; Reichenberg et al., 2001).

Empirical evidence for the impairing effects of inflammation on mood and behaviour comes from clinical samples (where patients are chronically exposed to interferon as part of their clinical management) and experimental models of inflammation (where healthy participants are acutely exposed to a pro-inflammatory challenge).

In patients treated for Hepatitis C and some malignancies, the effects of chronic administration of IFN-α have been associated with the subsequent development of depressive episodes in one-third of the treated patients (Capuron et al., 2002; Udina et al., 2012). These occurrences of depressive episodes have been linked to the amygdala (Davies et al., 2020) and hypothalamus-pituitary axis (HPA) stress responses (Capuron et al., 2003).

Experimental studies in healthy humans have also demonstrated that various immune challenges such as LPS, vaccines and pro-inflammatory cytokines can quickly induce disturbances in mood, motivation and cognition that have remarkable similarities to the symptoms of depression (Dantzer et al., 2008). Two common self-rating questionnaires that have been used to assess the impact of systemic inflammation on mood and fatigue are the POMS (McNair et al., 1971) and the fVAS (Gift, 1989). Transient behavioural changes measured with these scales have been shown to be associated with microstructural and functional outcomes in neuroimaging studies using LPS and typhoid

vaccine (Eisenberger et al., 2010; Harrison, Cooper, et al., 2015). Moreover, these immune challenges seem to affect many of the same networks that are thought to play a role in the development of depression (Capuron et al., 2012; Harrison et al., 2009; Kitzbichler et al., 2021).

Impairment in reward and punishment-orientated behaviours are a key characteristic of the motivational reorientation observed in both murine and human studies investigating the effects of inflammation (Dantzer & Kelley, 2007; De Marco et al., 2023; Harrison et al., 2016) and have highlighted the impact of actions of inflammation dopamine in reward-related reorientation of behaviour. For instance, evidence from a human PET study (using radiolabelled fluorodopa F 18) has shown a reduction in dopamine turnover in the ventral striatum in Hepatitis-C patients undergoing chronic (4 weeks) treatment with IFN-α (Capuron et al., 2012). Similarly, a significant reduction in the ventral striatum activity to reward cues also has been reported following acute LPS challenge, altogether suggesting that this region is particularly sensitive to the impairing effects of inflammation (Eisenberger et al., 2010). Further evidence comes from milder immune challenges (i.e., typhoid vaccine) (Harrison et al., 2016). This study which combined computational modelling and fMRI demonstrated inflammation was associated with decreased ventral striatal reward outcomes and, simultaneously an increase in sensitivity to punishment associated with heightened right insula activation in response to punishment cues. These findings are in line with prior evidence that suggests dopaminergic neurons in the ventral striatum and insula are involved in reinforcement learning related to reward and punishments, respectively (Pessiglione et al., 2006).

Other observed cognitive alterations during inflammation include psychomotor slowing and memory impairments. During an infection, reduced psychomotor activity may help save energy (e.g., inflammation-induced pyrexia consumes a lot of the body's energy resources), potentially boosting the immune response. In humans, patients undergoing IFN-α therapy often experience reduced psychomotor slowing and fatigue (Capuron & Miller, 2004). These symptoms have been linked to changes in glucose metabolism in the striatum, indicating a potential dysregulation in dopamine signalling. Higher plasma concentration levels of circulating IL-6, psychomotor retardation and changes in striatal

dopaminergic neurotransmission have also been observed in individuals with depression (Maes et al., 1997; Martinot et al., 2001). Furthermore, additional data from experimental inflammation models have revealed that individuals with greater IL-6 response to the typhoid vaccine exhibited longer reaction times and enhanced substantia nigra activity during the performance of a task designed to assess the ability to inhibit cognitive interference (Stroop task), suggesting a role for this dopaminergic midbrain region in the psychomotor effects of inflammation (Brydon et al., 2008).

Cells traditionally associated with the immune system are (e.g., microglia) crucially involved in several fundamental and beneficial neuronal processes (e.g., long-term potentiation, neurogenesis or synaptic plasticity) which are essential for learning and memory (Ekdahl et al., 2003; Katsuki et al., 1991; Schafer et al., 2012; Schneider et al., 1998; Yirmiya & Goshen, 2011). When inflammation occurs and disrupts these beneficial regulatory processes, it can result in acute memory impairment. Severe infection can cause lasting cognitive deterioration in previously healthy older individuals and accelerate the decline in individuals with neurodegenerative diseases such as Alzheimer's (Iwashyna et al., 2010; Perry et al., 2007; Tynan et al., 2010; Weaver et al., 2002).

Most of our understanding of inflammation-induced memory impairment comes from murine models which highlight medial temporal lobe (MTL) structures as especially sensitive to the effects of inflammation. For example, direct administration of inflammatory cytokines, especially IL-1 into the hippocampus specifically disrupted spatial and contextual memory abilities in rodents performing the radial arm and Morris water maze (Barrientos et al., 2002; Oitzl et al., 1993; Yirmiya & Goshen, 2011). In humans, there is limited data available to support this claim. However, a study using Fluorodeoxyglucose PET showed a decrease in glucose metabolism in the MTL following typhoid vaccination. This inflammatory response specifically affected human spatial memory, without influencing procedural memory (which does not depend on the MTL) suggesting that MTL structures may have a particular vulnerability to inflammation, in turn leading to functional deficits (Harrison et al., 2014).

The relationship between inflammation, mood and cognitive disturbance is particularly important in the older population. Older adults seem to be more sensitive to the cognitively and behaviourally impairing effects of inflammation and infection, which when severe or chronic, can even lead to permanent cognitive impairment even in previously healthy individuals. (Iwashyna et al., 2010; Jorge-Ripper et al., 2017; Naughton et al., 1995). However, to the best of my knowledge, there have not been any studies examining, in healthy individuals, how age may modulate the effects of inflammation following a controlled immune challenge.

In this chapter, I evaluate the effects of experimental IFN-β administration on behaviour and cognition in a cohort of young and older healthy individuals. I hypothesised that IFN-β (i) would increase sickness symptoms as well as fatigue and negative mood, (ii) would lead to a relative reduction in sensitivity to rewards compared to punishments, (iii) would have detrimental psychomotor effects and (iv) would acutely impair spatial memory. I further hypothesised that (v) the impairing effects of inflammation on behaviour and cognition would be greater in the older group.

MATERIALS AND METHODS

The same cohort of participants (as described in previous chapters) completed POMS, fVAS and SicknessQ questionnaires at baseline and 1, 2, 3, 4 and 5 ½ hr after the injection and 3 different cognitive tasks 6 hr post-injection.

SUBJECTIVE QUESTIONNAIRES

PROFILE OF MOOD STATES (POMS)

The POMS was formulated as a 65-statement validated psychological rating state (McNair et al., 1971). For this study, we used a modified version of 45 statements to measure transient, distinct mood state dimensions over a period of time. The seven different

dimensions included: Tension, Tiredness, Vigour, Confusion, Depression, Anger and Somatic symptoms as well as negative mood (sum of Tension, Tiredness, Confusion, Depression and Anger scales) and total mood scales (computed as Vigour – (Tension + Depression + Anger + Fatigue + Confusion). Participants were asked to give a self-report for each question by using a 5-point Likert scale ranging from “Not at all”, to “Extremely” (1=Not at all, 2=A little, 3=Moderately, 4=Quite a bit, 5=Extremely).

FATIGUE VISUAL ANALOGUE SCALE (FVAS)

The VAS was created as a self-report scale to simply measure subjective phenomena such as pain, nausea or fatigue (Gift, 1989). For fatigue, the fVAS is traditionally used as a 100-mm-long horizontal line with two vertical anchoring lines labelled ‘no fatigue’ and ‘extremely fatigued’ at the left (0 mm) and right ends (100 mm) respectively. Participants were asked to rate their fatigue level by marking the point on the line that best represented their perception of fatigue at that moment.

SICKNESSQ

The SicknessQ is a self-report questionnaire created in the context of experimentally induced inflammatory activation to assess characteristics of sickness behaviour across time (Andreasson et al., 2018). It is composed of 10 statements that are rated on a 4-level Likert scale ranging from agree to disagree (0=Disagree, 1=Agree somewhat, 2=Mostly agree, 3=agree).

Questionnaire data were analysed using a mixed-design repeated measures ANOVA (within factors: condition (Placebo/IFN-β), Time (pre- and post-injection times as described); between factor: (Age category)). Questionnaires administered to participants can be found in Appendix A.

COGNITIVE TASKS

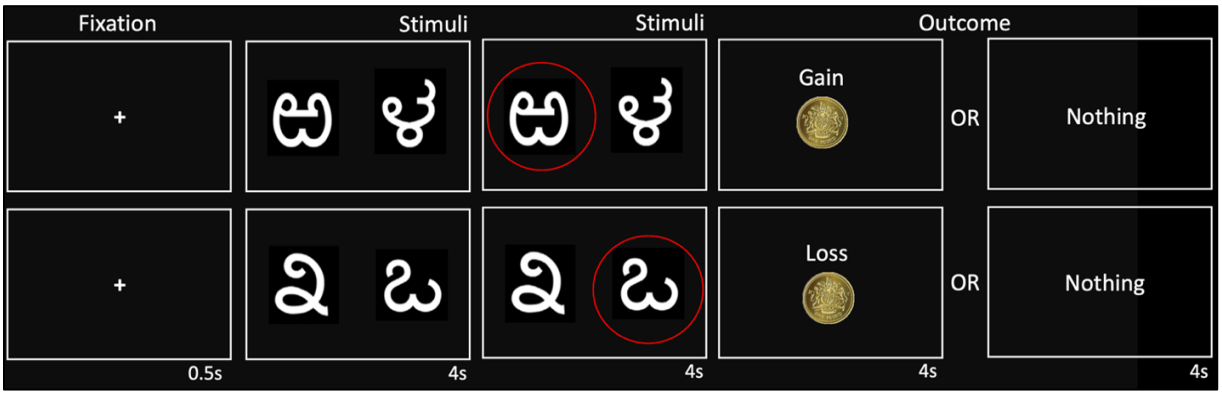
REWARD VERSUS PUNISHMENT

Six hours post-administration of either IFN-β or saline injection, participants completed a single run of a computerised probabilistic instrumental learning task that has been shown to detect inflammation-induced changes in sensitivity to reward versus punishments (De Marco et al., 2023; Harrison et al., 2016). In this task, participants were shown three pairs of abstract stimuli. Each pair (gain, loss, neutral) was associated with a different pair of outcomes: chance of winning money (gaining £1 or gain nothing), risk of losing money (losing £1 or lose nothing) and neither win nor lose money (look at £1 or nothing) **(Figure 15)**. A probability factor was added, ensuring that each of the two stimuli had a reciprocal probability (0.8/0.2 and 0.2/0.8) of leading to the designated outcome for every trial category (gain, loss, neutral). For example, in the win condition, one stimulus has an 80% chance of winning £1 and a 20% chance of winning nothing and the other stimulus of the pair has a 20% chance of winning £1 and an 80% chance of winning nothing. In each trial, one pair of stimuli was presented randomly. Each condition (gain, lose, neutral) consisted of 24 trials, and they were presented in randomized order. The two stimuli appeared on either side of a central fixation cross and their positions -left or right- were randomly determined for every trial. To choose the stimulus on the right, participants pressed a button ("go response"). Selecting the stimulus on the left required no action ("no-go response"). The selected option was highlighted in a red circle and the result was shown on the screen after 4 seconds.

A trial run (a shorter and different version) was conducted to confirm that the mechanics of the task were clear. Participants used trial and error in order to learn stimulus-outcome associations. Their objective was to boost their winnings by choosing the stimulus with a higher chance of winning and reduce losses by avoiding the stimulus with a higher likelihood of losing. At the end of the task, participants were informed of their overall winnings. Performance was calculated as the proportion of the last 50% of the trials (last 12 trials) where participants chose the high-probability gain stimulus for the reward trials

and successfully avoided the loss stimulus in the punishment trials. Data from two participants were excluded due to persistent 'no-go' responses indicating they did not engage in any button-press activity. To avoid reward/punishment learning effects, there were 2 different versions of the task which were randomly administered for the placebo and IFN-β conditions. Data were analysed using a mixed-design repeated measures ANOVA (within factors: condition (Placebo/IFN-β), Valence (Gain/Loss); between factor: (Age category))

Figure 15. Reinforcement learning task



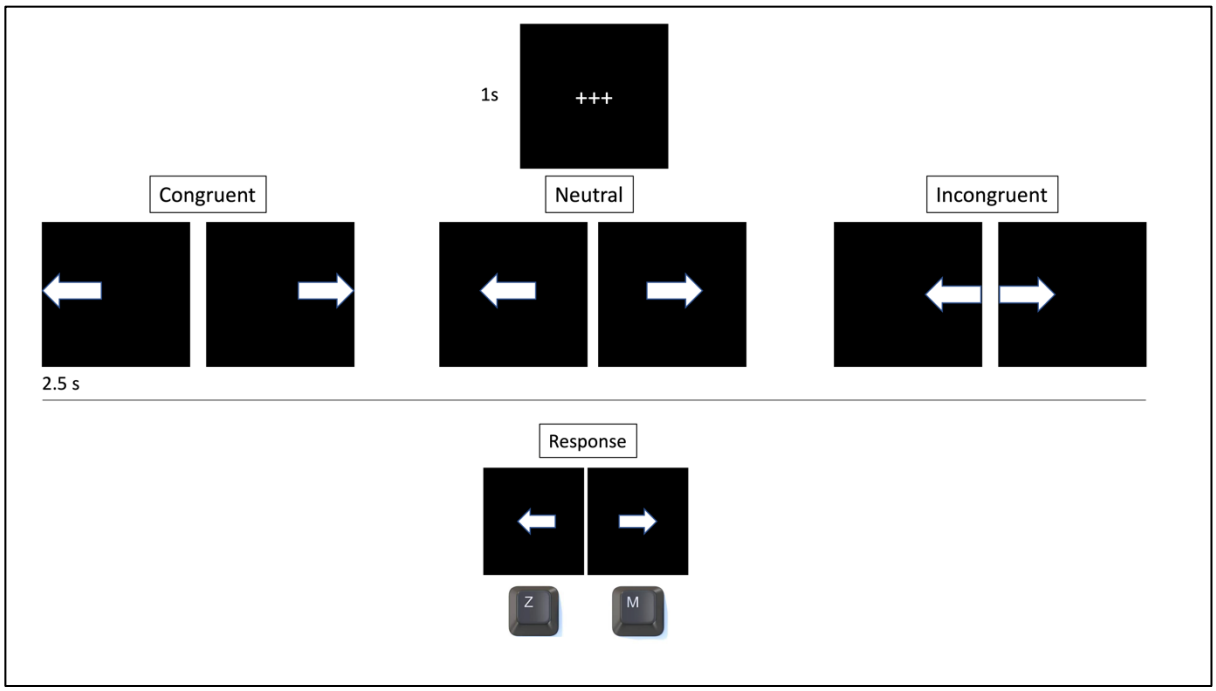
In each trial, participants chose a visual stimulus from one of the three conditions: gain, loss or neutral, then they saw the result. In the example, there is an 80% chance of gaining £1 (upper image) and an 80% chance of losing £1 (lower image). There is a 20% probability of obtaining nothing. The neutral set of stimuli (not shown in the figure) led to neutral results ("look £1" or nothing). The position of the stimuli either left or right was randomly determined for every trial.

PSYCHOMOTOR SLOWING

Participants also completed an adapted version of the Simon task, a well-established experimental framework created to explore high-demand processes such as attention, interference and cognitive control by examining how stimulus conflict impacts psychomotor reactions in humans (Simon, 1969). The task consisted of a series of trials: congruent, incongruent and neutral (120 in total) in which, after a fixation cross, a white arrow appeared on either the right, the middle or the left of the screen for a duration of 2.5 seconds. Participants had to press "Z" (situated on the left side of the keyboard) and "M" (on the right side of the keyboard) when the arrows were pointing left or right respectively **(Figure 16).** Congruent trials were those where the arrow direction matched its associated response (i.e., an arrow pointing left appearing on the left side of the screen). In incongruent trials, the arrow appeared on the opposite side of the screen (i.e., an arrow pointing left appearing on the right side of the screen). This creates a competition between the correct response associated with the direction of the arrow (left) and the incorrect response associated with the location of the stimulus (right side of the screen). Generally, incongruent trials result in increased errors and longer reaction times. Finally, for the neutral trials, the arrow was displayed in the centre of the screen. Congruent, incongruent and neutral trials were randomly presented. Reaction time (from correct trials) and accuracy were computed. If no response was produced within the allocated time (2.5 s) a no response was registered. A practice run was conducted prior to the main task. Data from two participants were excluded due to consistent no response.

Repeated measures ANOVA (within factor: condition (Placebo/IFN-β) x trial (congruent/Incongruent); between factor (Age category)) were used to investigate the main effect of IFN-β as well as condition x age interactions and age-associated differences. Pearson's correlation analysis was used to look at associations between reaction times and plasma concentration levels of cytokines IFN-β, IL-6 and TNF-α.

Figure 16. Simon Task



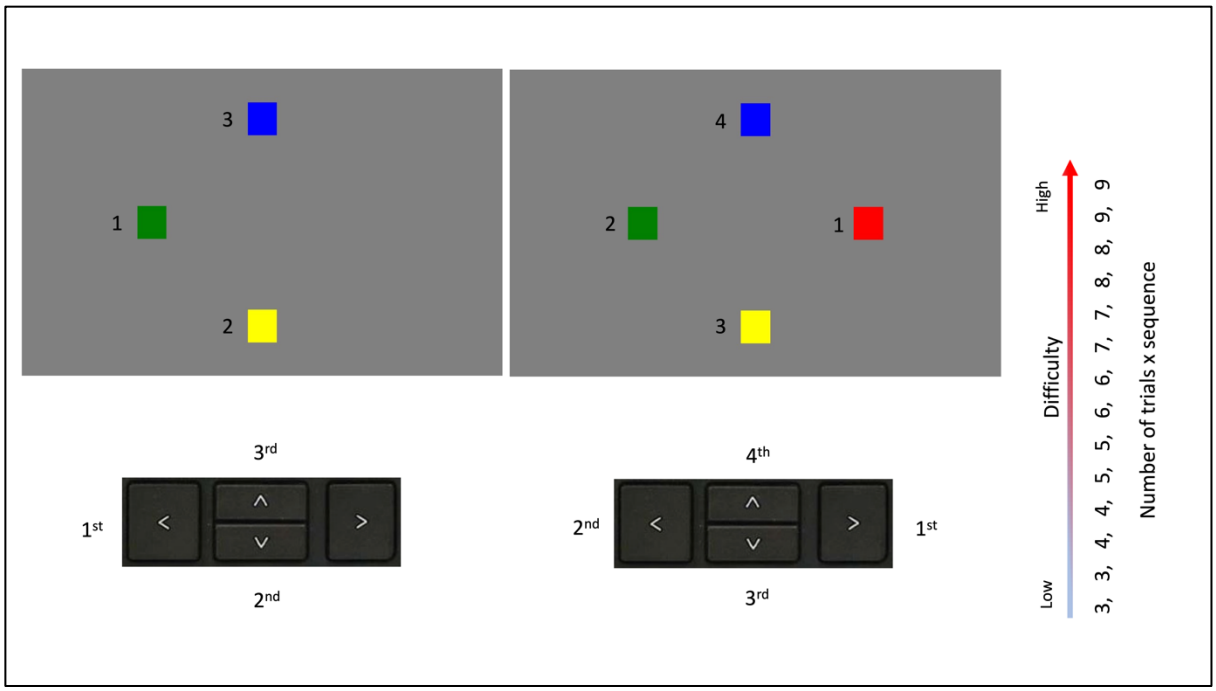
Participants were instructed to respond by pressing the keyboard letter “Z” whenever they saw an arrow pointing left and “M” when the arrow was pointing right. During congruent trials, direction and location appear on the same side of their associate response (e.g., an arrow pointing to the left will appear on the left side of the screen). During incongruent trials, the arrow will appear on the opposite side (e.g., an arrow pointing right will show on the left side of the screen). The three types of trials (congruent, incongruent and neutral) were randomly presented.

VISUOSPATIAL MEMORY

In order to investigate inflammation-induced spatial memory disturbance, I designed a visuospatial memory task where participants had to remember a randomly generated sequence of squares in space. The task consisted of 14 trials that increased in difficulty, beginning with 3 squares, and increasing by one square every two trials until a sequence of 9. Participants were instructed to remember the sequence and use the keyboard 'arrow keys' to recreate it (e.g., if the order of appearance of a sequence of 3 squares was left, bottom, up, participants when prompted, had to press the left, bottom, and up arrow keys respectively). The squares appeared on the screen for 2 seconds and trials were considered correct only when the whole sequence had been recreated correctly (all key presses were correct) **(Figure 17).** A practice run was completed prior to the main task and there were 2 different versions of the task which were randomly administered for the placebo and IFN-β conditions. Trials from each sequence were averaged (percentage of recall: 2 correct trials = 100%, 1 correct trial= 50%, no correct trials = 0%). Due to observed ceiling effects for sequences 3 and 4 and floor effects for sequences 8 and 9, and in order to avoid reduced variability associated with these measures, I opted to focus the analysis on sequences of intermediate difficulty (5, 6 and 7).

Repeated measures ANOVA (within factors: condition (Placebo/IFN-β) and sequence (5 to 7); between factor (Age category)) were used to investigate the main effects of IFN-β as well as condition x age interactions and age-associated differences. Pearson's correlation analysis was used to look at associations between recall ability and plasma concentration levels of cytokines IFN-β, IL-6 and TNF-α.

Figure 17. Visuospatial memory task



Participants were presented with squares that appeared and disappeared on the screen one at a time in each sequence. Squares appeared for 2 seconds. Once the sequence was finished participants were prompted to recreate it using the arrow keys. The figure shows an example of a 3 (left image) and 4 (right image) square sequence. For the sequence on the left, the squares appeared in the following order 1: left 2: bottom, and 3: up, In order to correctly recreate the sequence, participants had to press the left, followed by the bottom and up arrow keys. The task consisted of a total of 14 trials, 2 x sequence that increased in difficulty.

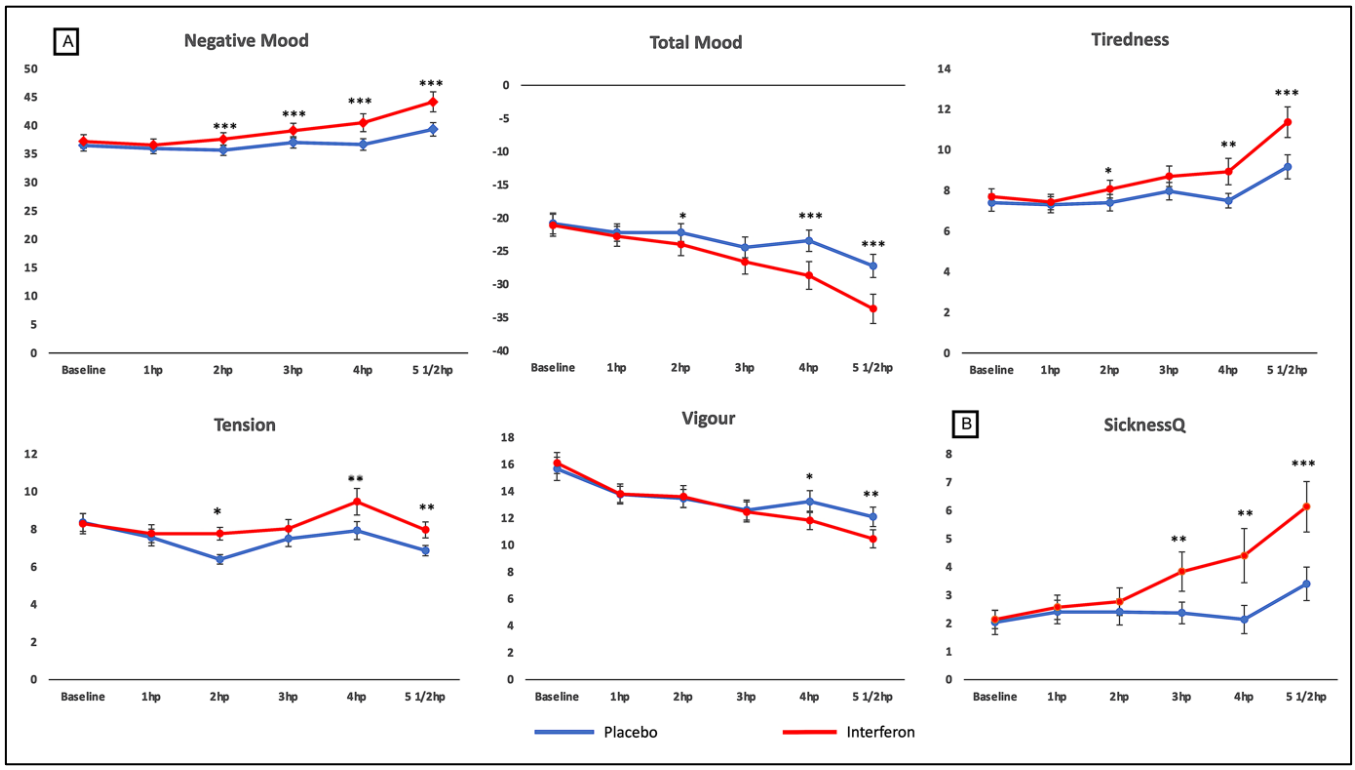
RESULTS

SUBJECTIVE RESPONSE

Interferon-beta induced significant shifts in mood that were observed in 5 of the POMS subscales. These shifts followed a consistent pattern, with the most pronounced changes seen at 5 ½ h hr post-injection. IFN-β significantly decreased total mood score (Condition x (Placebo/IFN-β) x Time $F_{(3.13,87.82)}$= 5.93, p<0.001) and Vigour (Condition x (Placebo/IFN-β) x Time $F_{(3.5,98.58)}$= 3.19, p= 0.021) and increased negative mood (Condition x (Placebo/IFN-β) x Time $F_{(3.28,91,98)}$= 5.61, p<0.001), Tiredness (Condition x (Placebo/IFN-β) x Time $F_{(2.44,68.5)}$= 4.97, p = 0.006) and Tension (Condition x (Placebo/IFN-β) x Time $F_{(3.89,109.13)}$= 2.73, p<0.034). A significant main effect of condition was also observed in total and negative mood, tiredness and tension subscales.

I also observed an increase in sickness symptoms as shown by the SicknessQ with significant condition ($F_{(1,28)}$= 7.64, p= 0.01) and condition x time interaction ($F_{(2.88,80,87)}$= 7.39, p= 0.001) and a peak change at 5 ½ hr post-injection **(Figure 18).** No significant effects were observed in the fatigue scale (fVAS) and no condition x age interactions or other age-related effects were found for any of the scales.

Figure 18. Subjective response.



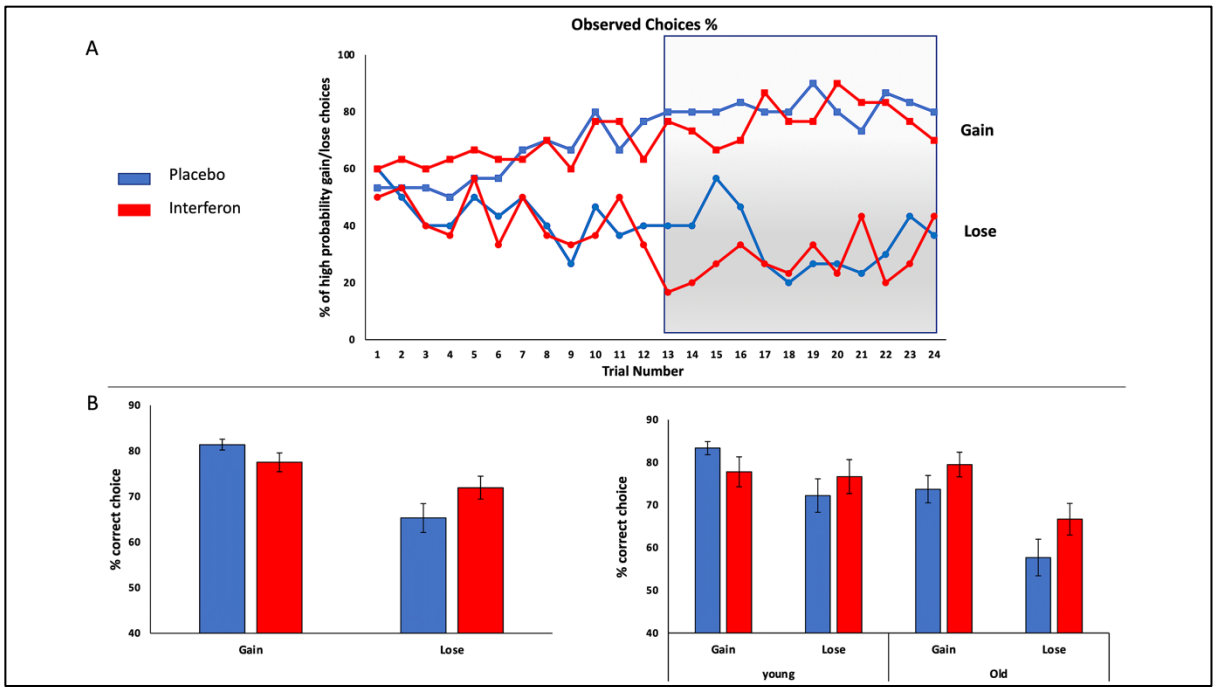
(A) Significant POMS subscales; (B) Sickness Questionnaire (SicknessQ). Blue represents placebo, red interferon. Error bars denote SEM. Significant values show the main effect of IFN-β relative to placebo (* p< 0.05, ** p< 0.01, *** p< 0.001).

REWARD VERSUS PUNISHMENT

The repeated measures ANOVA did not reveal a significant condition (Placebo/INF-b) x valence (Gain/Lose) interaction (p=0.139), yet, in line with other inflammation models like LPS and Typhoid vaccination (De Marco et al., 2023; Harrison et al., 2016), the findings indicated a pattern where IFN-β was associated with a shift in sensitivity to reward versus punishment. Specifically, the trend showed a decreased selection of high-probability reward (mean ± SEM -2.97 ± 3.3) but an *increased* tendency to avoid high-probability punishment stimuli (mean ± SEM 6.54 ± 5.1) in the last 50% of trials **(Figure 19).** To investigate age-related effects, I performed a condition (Placebo/IFN-β) x Valence (Gain/Lose) x age (as a between-subjects factor) repeated measures ANOVA, however no age interactions or age-associated effects were observed.

The analysis of the go response revealed a significant effect of condition ($F_{(1,26)} = 10.10$, $p = 0.004$) and valence ($F_{(1,26)} = 11.81$, $p = 0.002$). Participants performed more go responses after IFN-β compared to placebo and in gain compared to the lose trials. However, the condition did not differentially affect go responses in gain and lose trials as no condition x valence interaction was observed ($F_{(1,26)} = 0.56$, $p = 0.816$). Furthermore, the results did not reveal any age-related effects or interactions ($p>0.1$) confirming equal task engagement across age groups.

Figure 19. Reward vs. punishment task plots



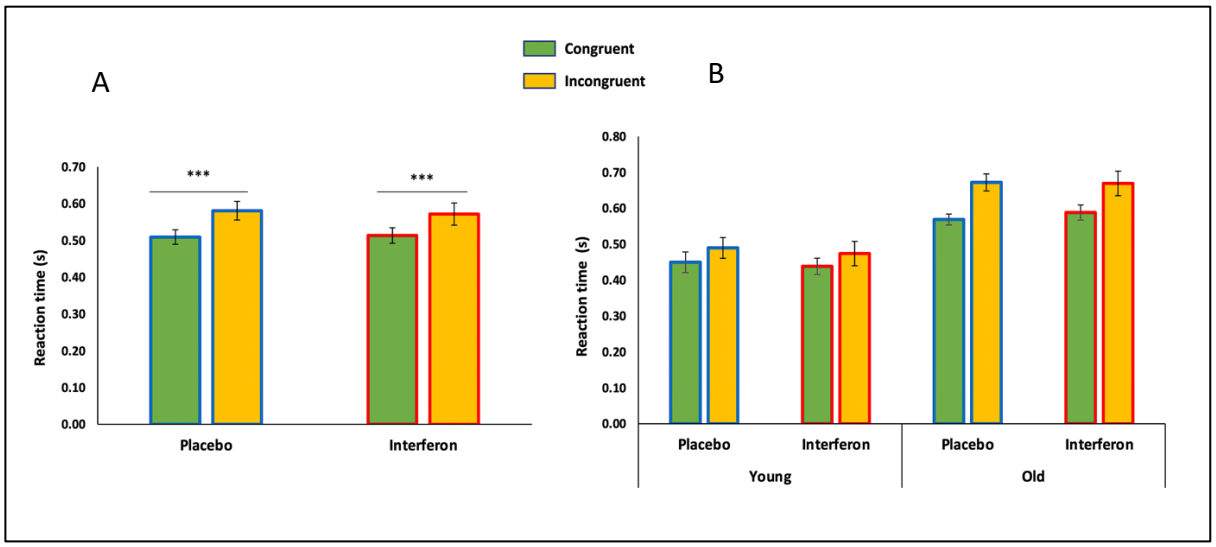
(A) Observed behavioural choices in response to gain and lose conditions following placebo (blue) and IFN-β (red) for the whole sample. The learning curves illustrate the percentage of participants who chose the correct stimulus (high probability of winning £1 -upper graph) and incorrect stimulus (high probability of losing £1-lower graph). The last 50% of the trials, averaged across participants and conditions (shaded area) was used for the analysis. The learning curves start at a chance level due to the lack of knowledge. Once participants start learning they keep selecting a high probability of winning in the win/nothing trials (increasing the curve in the Gain condition) and avoiding the high probability of losing in the lose/nothing condition (decreasing the curve in the Lose condition). **(B)** Mean and SEM of the last 50% of trials where participants chose the high probability of winning and avoided the high probability of losing (figure on the left represents the whole sample, on the right, participants split by age group).

PSYCHOMOTOR SLOWING

Repeated measures ANOVA showed a significant effect of trial (congruent/incongruent) ($F_{(1,26)}$= 54.29, p<0.001) confirming good task engagement. Participants showed significantly higher response time on the incongruent compared to the congruent trials in both placebo and IFN-β conditions (Placebo [$t_{(28)}$=6.46, p<0.01] IFN-β [$t_{(28)}$=4.75, p<0.01]). However, IFN-β did not affect the Simon task behavioural response and no differences in reaction time were associated with condition ($F_{(1,26)}$= 0.006, p=0.938) or condition x trial interaction ($F_{(1,26)}$= 1.52, p=0.228). No condition x age interactions were observed either. As expected, the between-subjects effects showed a significant main effect of age ($F_{(1,28)}$= 20.52, p<0.001). Older individuals were significantly slower than their younger counterparts regardless of condition and trial **(Figure 20).** Repeated measures ANOVAs were also performed for accuracy; however, no significant effects were observed.

To explore whether participants with a larger inflammatory response had increased reaction times, we ran Pearson's correlation analysis between plasma concentration levels of cytokines IFN-β, IL-6 and TNF-α (peak minus baseline) and reaction time (Incongruent minus congruent trials) for the IFN-β condition. The correlation analysis did not reveal any significant associations between changes in cytokines plasma concentrations and changes in the Simon task behavioural response.

Figure 20. Simon Task, congruent and Incongruent trials plots



(A) Reaction times were increased for incongruent compared to congruent trials for all participants in both conditions (***p<0.001). (B) Age-associated differences: older participants were slower than their younger counterparts for congruent and incongruent trials in both conditions. Green represents congruent, and yellow incongruent trials.

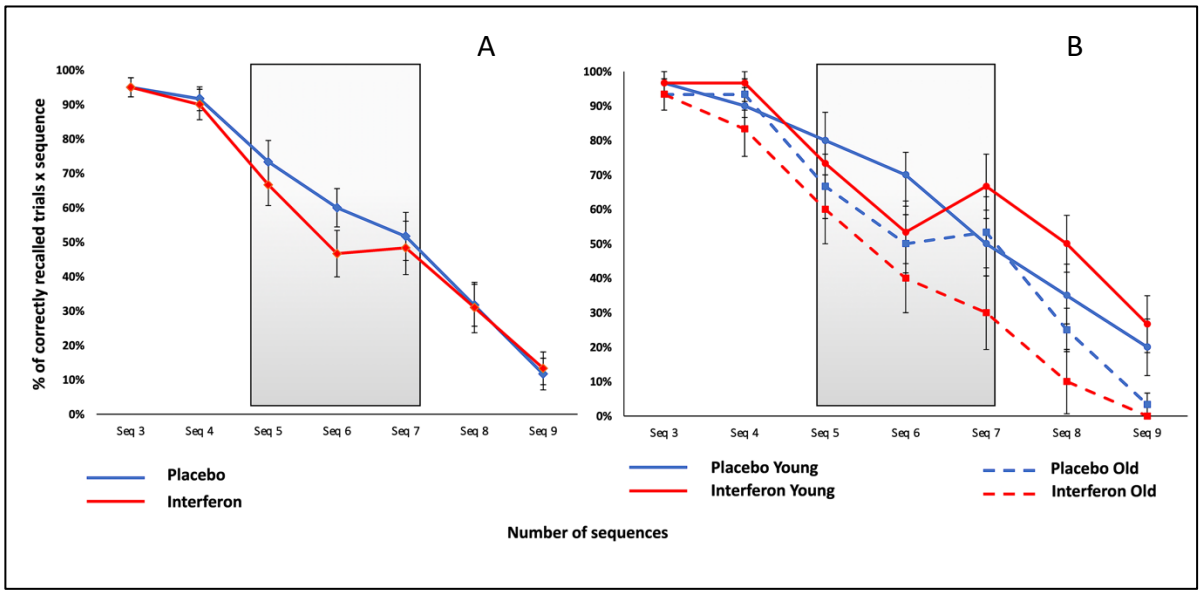
VISUOSPATIAL MEMORY

Performance on the memory task revealed the expected significant effect of sequence ($F_{(1.61,45.12)}$= 8.84, p<0.001). As anticipated sequences with an increased number of squares were more challenging to recall accurately. IFN-β did not influence performance and no differences in the percentage of recall were associated with the condition. No condition x sequence or age interactions were observed.

The results from the between-subjects analysis revealed a statistically significant effect of age ($F_{(1,28)}$= 4.82, p=0.037). Irrespective of the condition and sequence, the performance of older individuals was inferior compared to their younger counterparts. **(Figure 21).**

A Pearson's correlation analysis was conducted to examine the relationship between changes in plasma concentration levels of cytokines IFN-β, IL-6 and TNF-α (peak minus baseline) and accuracy (sequences 5, 6 and 7 grouped) within the IFN-β condition to determine if a larger inflammatory response influenced recall capacity. No significant associations were observed.

Figure 21. Visuospatial task plots



(A) Percentage of correctly recalled trials x sequence. Accuracy was computed by averaging the trials per sequence cross participants for the placebo and IFN-β conditions (100% 2 trials correct, 50% 1 trial, 0% no trials correct). For instance, sequence 3 showed near-perfect recall by most participants, resulting in approximately 100% recall. In contrast, sequence 9 had negligible recall, leading to a percentage score close to 0%. (B) Percentage of correctly recalled trials x sequence split by age. Sequences of intermediate difficulty

(shaded region) were selected for analysis. Blue denotes placebo, red interferon. Dashed lines represent older group.

DISCUSSION

In this chapter, I assess the effects of experimental IFN-β administration on behaviour and cognition. I administered self-rating questionnaires that have been commonly used to assess the impact of systemic inflammation on mood and fatigue. Consistent with previously reported findings using LPS, and typhoid vaccine immune challenges (De Marco et al., 2022, 2023; Eisenberger et al., 2010; Harrison, Cooper, et al., 2015), IFN-β induced similar transient changes in mood, fatigue and sickness symptoms, providing further support for employing IFN-β as a novel acute inflammatory challenge (refer to **Table 5** for a comparison of the model's mood changes and those from other immune challenges).

Approximately 6 hours post-administration of either IFN-β or saline injection, participants completed a series of cognitive tasks. In the reward task, the results did not reach significance, however they indicated a pattern where IFN-β was associated with a shift in sensitivity to reward versus punishment, which amplified participants' sensitivity to punishment versus reward. Furthermore, it is worth noting that while the results were not significant, the observed effect size of the interaction was medium to large suggesting that the observed pattern is of practical significance (condition x valence Partial Eta Squared ($\eta p2$) = 0.082). This trend was consistent with my hypothesis and previous literature on LPS, Typhoid vaccination and IFN-α immune challenges further supporting the notion that Inflammation leads to changes in reward-based behaviours as part of the motivational reorientation that occurs during sickness (Capuron et al., 2012; De Marco et al., 2023; Eisenberger et al., 2010; Harrison et al., 2016).

Alterations in dopaminergic neurotransmission are believed to play a causal role in inflammation-induced changes in reward sensitivity. Findings from human research have shown a decrease in dopamine uptake and reduced response to reward in the dopamine-rich ventral striatum of Hepatitis C patients following chronic IFN-α treatment (Capuron et al., 2012), Similarly, a decrease in reward sensitivity in the ventral striatum has been documented following endotoxemia studies (Eisenberger et al., 2010). Additional insights emerge from studies involving milder immune challenges, such as typhoid vaccine and show decreased ventral striatal reward outcomes and concurrently an increase in

punishment sensitivity associated with increased right insula activation in reaction to punishment cues (Harrison et al., 2016).

While my findings, along with earlier data from the same reinforcement learning task add to the evidence suggesting that inflammation can modify reward learning signals and sensitivity to rewards, other evidence based on endotoxemia studies indicates that the effects of inflammation may be driven by its influence on effort sensitivity rather than directly on reward sensitivity itself (Draper et al., 2018; Lasselin et al., 2017). Differences in task design could explain these differences. The task used in this study included a learning component during the trial sequence and only a single type of reward (win or nothing). Task designs exploring the effects on effort sensitivity do not include a learning component. For instance, Lasselin et al. (2017) used two different levels of reward and incentive motivation (high-effort/high-reward trials vs. low-effort/low-reward trials) while Draper et al. (2018) study design included 25 conditions with a combination of 5 efforts and 5 stake levels. Moreover, the somatic symptoms commonly Induced by some immune challenges, such as joint aches or muscular discomfort, could potentially account for the reduced engagement in high-effort trials rather than primarily reflecting a shift in motivational reorientation. However, IFN-β is a milder immune challenge, and while the task was administered when physical symptoms were at their peak (e.g., raised body temperature and fatigue), IFN-β did not have a significant effect on somatic symptoms (POMS scale $p=0.115$). In addition, the task reinforcement learning task used in our study required minimal effort (press/no press button response), the condition did not differentially affect go responses in gain and lose trials (condition x valence interaction $p = 0.825$) and was in fact associated with more go responses.

I also postulated that, following the IFN-β challenge, older individuals would exhibit a greater decrease in reward sensitivity coupled with an increase in sensitivity to punishment, relative to their younger counterparts. This hypothesis was based on clinical studies where elderly patients display a predisposition to developing behavioural and cognitive impairments in response to infections or chronic inflammation (Iwashyna et al., 2010; Jorge-Ripper et al., 2017; Naughton et al., 1995).

Interestingly, however contrary to my expectations, older participants exhibited an increased sensitivity to both reward and punishment after IFN-β. While not statistically significant, this increased sensitivity contrasts with some evidence from murine models where elderly mice showed pronounced decreases in behaviours such as sucrose intake and sexual activity and an increase in depression-like behaviour (Frenois et al., 2007; Godbout et al., 2008; Yirmiya, 1996). In healthy humans, the picture may be more complicated than that; one of the ways acute Inflammation might influence reward processing is based on its perceived advantages, and the way older and younger groups value monetary rewards (or punishments) may differ due to their distinct needs, therefore leading to different adaptative responses. Additional research with larger sample sizes could help detect smaller effect sizes and potentially identify age-specific associations that underlie the observed differences in the directionality of reward processing between groups. Given the potential for the effects of age to be nuanced or subtle, we would need a minimum sample size of at least 36 participants to achieve 95% power for detecting medium-sized effects (f = 0.25).

Following up on this, one potential explanation for the lack of condition x valence interaction in the overall results could be potential masking effects. Some of the contrasting responses between the young and old groups may have effectively "cancelled each other out", resulting in non-significant overall results. For instance, the sub-group analysis (young group, n=15), while not significant, showed a large effect size (condition x valence interaction: $\eta p2 = 0.142$); therefore, a larger sample would increase the power, thereby enhancing the likelihood of detecting a present effect and achieving and interaction. This notion aligns with findings observed in other mild models of inflammation that employed larger sample sizes, such as the study involving the typhoid vaccine (n=24) conducted by Harrison et al. (2016).

I also aimed to investigate whether IFN-β may trigger sickness-related psychomotor alterations and for that purpose, participants underwent a task that asses attentional and executive cognitive processes by examining how stimulus conflict impacts psychomotor reactions. Contrary to my hypothesis, inflammation did not have an impact on reaction time or performance on the Simon task. These results differ from prior studies that have

demonstrated a link between psychomotor dysfunction and increased cytokines in both animals and humans. In rodents, the administration of LPS or inflammatory cytokines (especially IL-1) routinely decreases both locomotor and motivational activities. This leads to prolonged periods of immobility, reduced responses to food incentives and a tendency for social isolation (Dantzer, 2009). Furthermore, IFN-α treatment in rodents suggests that neurovegetative symptoms like psychomotor disturbances, fatigue and changes in sleep patterns are linked with changes in basal ganglia circuitry and associated dopamine pathways (Capuron & Miller, 2004). Clinically, patients treated with low doses of IFN-α for chronic hepatitis C have exhibited Parkinson-like motor disturbances and levodopa has proven effective in significantly improving IFN-α-triggered psychomotor restlessness (Sunami et al., 2000). Further evidence from models of experimental inflammation indicates that individuals showing a stronger IL-6 response to the typhoid vaccine had prolonged reaction times and increased activity in the substantia nigra when completing an inhibitory control task (Brydon et al., 2008).

A potential explanation for the lack of significant findings could be attributed to the task itself. Firstly, I observed ceiling effects in accuracy where performance neared the highest possible level (94%), therefore leaving minimal room for variability and differentiation between conditions. The task may have not been challenging enough to evoke discernible differences between the conditions. Secondly, although the task was sensitive to the intrinsic contrasts of congruent vs. incongruent trials, this sensitivity may not extend to external conditions such as the effects of interferon or placebo. The similarity in reaction times between conditions further corroborates the ceiling effect observed in accuracy.
While the current version of the Simon task did not show condition effects, it was sensitive enough to detect the effects of age on performance. Older individuals consistently demonstrated slower reaction times across both trial types (congruent and incongruent), in line with findings from existing literature (Germain & Collette, 2008; van der Lubbe & Verleger, 2002). This emphasises the task's capability to discern performance variations linked to age, even if it did not reveal differences between conditions.

A more challenging task variant or with more graded levels of difficulty or a type of relevant dimension (in this case was the position of the arrow) could potentially tease

apart differences in reaction times and accuracy between conditions further allowing the exploration of age interactions as well as potential associations between changes in cytokine plasma concentrations and the behavioural response.

The final cognitive domain I examined was that of visuospatial memory. I hypothesised that IFN-β would be associated with deficits in spatial memory, however, my findings did not support this assumption. The most compelling data on inflammation-induced memory impairment comes from rodent models. Administration of inflammatory cytokines in hippocampal areas has been associated with spatial and contextual memory alterations in the Morris Water maze (Barrientos et al., 2002; Oitzl et al., 1993; Yirmiya & Goshen, 2011). In humans, performance on memory tasks homologues of the Morris water maze have been associated with object-location memory in parahippocampal areas (Bohbot & Corkin, 2007; Ploner et al., 2000). Additional evidence from clinical samples (i.e. Alzheimer's' disease) (Bird et al., 2009; Gabrieli et al., 1994; van Halteren-van Tilborg et al., 2007) and experimental inflammation models (Typhoid vaccine) (Harrison et al., 2014) has linked systemic inflammation to specific deficits in spatial but not MTL-independent procedural memory, suggesting that this region is especially sensitive to the impairing effects of inflammation.

Task sensitivity could be one of the reasons our results do not align with previous literature. My task may not be sensitive enough to detect subtle changes in spatial memory. For instance, remembering the location of coloured squares might not be as challenging as remembering the spatial location of objects in a virtual reality environment (like the human analogous of the Morris Water maze). Furthermore, The Morris water maze requires the integration of multiple environmental cues, whereas the study task relied on discrete, colour-based stimuli located in fixed screen locations. This difference in environmental complexity might influence the memory processes engaged.

Finally, it could be related to cognitive load. For instance, navigating space and remembering orientation cues to locate objects in space require more cognitive resources than recalling square locations, which could make the former more susceptible to impairment.

The study task effectively differentiated between levels of complexity in sequence recall (e.g., harder sequences resulted in lower percentages of recall) and detected age-associated differences. Consistent with previous findings, older individuals' recall ability was significantly lower than their younger counterparts (Heo et al., 2010; León et al., 2016). Its sensitivity to detect subtle changes associated with inflammation could potentially be improved with a bigger participant sample. A larger cohort would provide increased power potentially revealing nuanced effects that the current sample size might have missed.

In conclusion, to the best of my knowledge, this is the first study employing IFN-β as an experimental model of inflammation to investigate inflammation-induced behavioural and cognitive disturbances in both older and younger individuals. I provide two primary findings: Firstly, I demonstrate that IFN-β induces transient changes in mood, fatigue and sickness symptoms similar to those observed in other experimental immune challenges, providing further support for the employment of IFN-β as a novel acute inflammatory challenge.

Secondly, the trend results from the reward learning task add more evidence to the observation that systemic inflammation can reorient motivation, impairing sensitivity to rewards vs. punishments. The age-related associations behind the interesting results regarding the observed differences in the directionality of reward processing between groups warrant further investigation preferably with a larger sample and/or by comparing healthy individuals to clinical populations.

Table 5. Comparison of the IFN-β model with typhoid, IFN-α and Endotoxin challenges for the sickness response

Human systemic inflammation models

	Typhoid Vaccination (Typhim)	IFN-α2a (3MU)	Our model: IFN-b-1b (EXTAVIA® 3MU)	Endotoxin (1ng/kg NIH reference)
Sickness/Cognitive	Peripheral symptoms: None Sickness symptoms: + (2pts SicknessQ) Negative Mood: + (~2pts POMS) Fatigue: + (8pts fVAS)	Peripheral symptoms: Mild Sickness symptoms: + (3pts SicknessQ) Negative Mood: + (~3pts POMS) Fatigue: + (11pts fVAS)	Peripheral symptoms: None Sickness symptoms: + (2.5pts SicknessQ) Negative Mood: + (~4pts POMS) Fatigue: + (~2pts POMS) Fatigue fVAS: +/-	Peripheral symptoms: ++ Sickness symptoms: + (10pts SicknessQ) Negative Mood: + (~18pts POMS) Fatigue: + (30pts fVAS)

CHAPTER 5. DIFFUSION-WEIGHTED MAGNETIC RESONANCE SPECTROSCOPY (DW-MRS)

INTRODUCTION

Glial cells are the most widely distributed and abundant cells in the CNS, among these astrocytes and particularly microglia act as the first line of defence against different insults such as tissue damage, infection, or injury (Nimmerjahn et al., 2005).

In addition to their immune functions, microglia and astrocytes play crucial homeostatic roles in the healthy brain. Microglia are the resident brain macrophages and their phagocytic function is critical for the removal of dead cells, cell debris and other inflammatory stimuli (Damisah et al., 2020; Nimmerjahn et al., 2005). Furthermore, It has been shown that they are instrumental in maintaining and modulating neuronal plasticity (Weinhard et al., 2018) and preserving blood-brain barrier integrity (Joost et al., 2019; Mondo et al., 2020). Astrocytes also provide important homeostatic support, among others regulating synaptogenesis, maintaining ion levels and supplying metabolic support to neurons (Kucukdereli et al., 2011; Pellerin & Magistretti, 1994). Microglia can adopt different states based on environmental cues. In response to disturbances or threats in the CNS, they transition from a surveillant to a reactive state. This reactive state is characterised by morphological changes, upregulation of surface markers, release of pro-inflammatory mediators, or changes in phagocytic activity. Furthermore, they have been found to enter this 'reactive' state earlier than other glial cell types. (Davalos et al., 2005; Kreutzberg, 1996).

In line with this, evidence from murine models of systemic inflammation using a lipopolysaccharide challenge (LPS) (dose ranging between 0.33 and 200 mg/kg) have shown evidence of a shift between surveillant to a reactive phenotype in microglial cells, within a few hours of systemic immune challenge, which is concomitant with inflammatory parameters such as IL-1b, TNF-α, or TLR-2 and TLR-4 (Hoogland et al., 2015) and increased blood-brain barrier permeability (Varatharaj & Galea, 2017). As with many other cellular

and molecular processes, microglia and astrocytes' normal physiological support roles are vulnerable to ageing, with homeostatic function and phenotypic changes observed as common features in the ageing brain. For instance, in aging, microglia have been shown to have a decreased injury response, reduced cellular complexity, increased mitochondrial activity, and increased release of pro-inflammatory cytokines. Astrocytes, display increased mitochondrial activity, decreased cell complexity and glutamate clearance (Hanslik et al., 2021).

In animal studies, age-dependent changes in microglial responses to an intraperitoneal injection of LPS have been reported in middle-aged mice (Keane et al., 2021; Nikodemova et al., 2016; Sierra et al., 2007). Non-interventional studies show that the brains of aged rodents display region-specific microglial morphological changes such as reduced process length, increased soma volume or less ramified morphology (Hefendehl et al., 2014; Tremblay et al., 2012). Similarly, astrocytes in aged mice undergo morphological changes, with reduced size and short chubby processes, decreased astrocyte coupling through gap junction and variable changes in cell complexity (Bondi et al., 2021; Popov et al., 2021; Rodríguez et al., 2014).

In humans, in vivo methods to image microglia are mainly limited to Positron Emission Tomography (PET) using Translocation protein tracers (TSPO). TSPO is a protein found on the outer mitochondrial membrane. Following activation of microglia, levels increase, and it has therefore been considered to be a marker of microglial activation. Evidence from these studies has shown increased grey matter TSPO binding 3-5 hours after LPS immune challenge in humans (Sandiego et al., 2015) and after 4-6 hours in non-human primates (Hannestad et al., 2012). However, there are several challenges to using PET imaging: it is an invasive procedure involving exposure to radioactive tracers, and it lacks cell-specificity as TSPO is expressed in other glial cells and the endothelium. Furthermore, Increased expression of TSPO have been found increased in neurons after different types of neuronal stimulation (e.g., physiological or pharmacological) (Notter et al., 2021). A very recent study has also suggested that in contrast to what is seen in rodents, disease-associated increases in TSPO observed in humans relate almost exclusively to increases in microglial cell number (density) than changes in activation (Nutma et al., 2023)

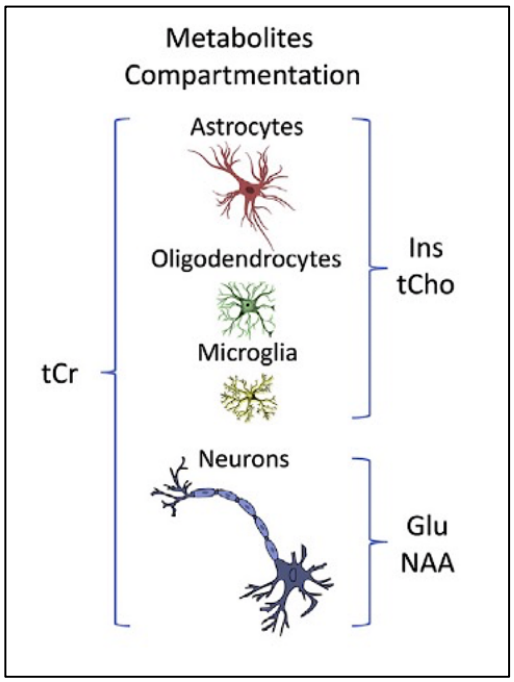
In addition, the redistribution of TSPO tracers across compartments makes the quantification of the PET signal in inflammation studies challenging and adds another level of complexity to the data acquisition and modelling processes (Yoder et al., 2015). Overall, these challenges restrict its wider application to research as well as its viability as a practical clinical tool (Schubert et al., 2021; L. Zhang et al., 2021).

Other types of imaging studies, such as MRI-based techniques have already been used to attempt to quantify the effects of systemic inflammation on the brain. The most commonly utilised have been fMRI and resting-state fMRI to explore functional reactivity and global connectivity (Dipasquale et al., 2016; Kitzbichler et al., 2021), quantitative magnetization transfer (qMT) and diffusion-weighted MRI to assess brain microstructure, and magnetic resonance spectroscopy (MRS) for brain neurochemistry (Critchley & Harrison, 2013; Garcia-Hernandez et al., 2020; Haroon et al., 2014, 2015, 2018; Kraynak et al., 2018). However, though sensitive to systemic inflammation, none of these techniques are able to provide the cell-specific information associated with the different underlying pathological and inflammatory mechanisms. MRI techniques such as diffusion MRI (DW-MRI) have proven to be powerful methods for non-invasively measuring brain microstructure, as water diffusion is restricted by the cellular membranes (Alexander et al., 2019). However, as water diffuses in a similar way across all cell types and the intra and extra-cellular spaces this method has limited cellular specificity.

The combination of DW-MRI with magnetic resonance spectroscopy (MRS) has the potential to overcome some of these limitations. Different brain cell types (e.g., astrocytes, neurons and microglia) differ in their neurochemical content (e.g., N-Acetylaspartate (NAA), choline, myoinositol, glutamate). Thus, as the signal obtained from brain neurochemicals resonates at different frequencies to water, DW-MRS is able to provide cell specific morphological information by estimating the apparent diffusion component (ADC) which measures the rate and direction of movement within the tissue. Furthermore, this signal comes predominately from the intra-cellular compartment (as opposite to water) and from particular cell types.

Using ADC as a metric, DW-MRS offers the ability to quantitatively map how different neurochemicals move within the brain, highlighting its potential for cell-specific imaging (Palombo et al., 2018). Evidence mostly obtained from cell culture studies shows that NAA and glutamate are found in higher concentration in neurons, total creatine (tCr= creatine + phosphocreatine) in all brain cells (Griffin et al., 2002; Urenjak et al., 1993) while total Choline (tcho= choline, glycerophosphocholine and phosphocholine) and myo-inositol (Ins) are more predominant in glial cells (Gill et al., 1989). (**Figure 22)**.

Figure 22. Metabolite compartmentation



The image shows the cell-specific compartmentalization of brain metabolites (tCr=creatine, Ins=myo-inositol, tCho=choline, Glu=glutamante, NAA= N-acetyl aspartate. (Image adapted from Palombo et al., 2018)

Consistent with this work, DW-MRS has been shown to be sensitive to metabolite diffusion changes during inflammation. In mice, the Culprizone mouse model of demyelination has shown elevated choline and myo-inositol ADCs in microglia and astrocytes respectively, compared with control mice after 6 weeks of treatment. Furthermore, these results correlated with histological measures of microglial and astrocytic alteration (Genovese et al., 2021). In humans, increased creatine and choline diffusivity have been found in patients with amyotrophic lateral sclerosis (ALS) and systemic lupus erythematosus (SLS), together suggesting a pattern of glial activation (Ercan et al., 2016; Reischauer et al., 2018).

Furthermore, increased choline diffusivity (but not NAA) has been observed in healthy controls after LPS peripheral administration (De Marco et al., 2022) and data from Multiple sclerosis (MS) patients have shown reduced thalamic NAA, which would be consistent with the neuronal damage and cell loss that are characteristic of the progression of autoimmune diseases (Bodini et al., 2018).

In this study, I use DW-MRS to evaluate the effect of IFN-β on the ADCs of total NAA, choline and creatine in the grey (thalamus) and white matter (corona radiata) of young and old healthy participants. These VOIs were chosen because (i) the thalamus and corona radiata are homogeneous regions of grey and white matter respectively, (ii) Research using TSPO PET that indicates that the thalamus exhibits amongst the highest TSPO uptake under normal conditions (Schubert et al., 2021). Additionally, it has been shown to be sensitive to LPS-induced inflammation (Buttini et al., 1996), (iii) recent evidence on DW-MRS showing changes in choline diffusivity in the left thalamus of healthy young individuals following LPS administration (De Marco et al., 2022).

I hypothesized that INF-b would (i) induce changes in glial but not neuronal ADCs, (ii) that there would be a significant association between IFN-β induced alterations in IL-6 levels and changes in choline ADC. Furthermore, I wanted to explore whether age may modulate changes in INF-β-induced metabolite diffusivity. I predicted a (i) greater effect of IFN-β on choline ADC with age (ii) age would have an effect on metabolites relative concentrations. Additionally, I conducted exploratory analysis to investigate potential associations between the ADC's and the behavioural and cellular immune responses.

Evidence looking at age-associated effects on the diffusivity of brain metabolites is scarce. A DW-MRS study with young and old healthy adults and ischemic patients did find differences in the ADCs of NAA, Choline and Creatine. Healthy older adults presented significantly reduced diffusivity compared to their younger counterparts. Furthermore, these metabolites decreased in patients with acute cerebral ischemia when compared with age-matched controls (Zheng et al., 2012). To the best of my knowledge, this study will be the first report of a non-invasive assessment of the intracellular diffusion changes

of tNAA, tCho and tCr using a mild model of inflammation, as well as considering the age effects in humans, in-vivo.

MATERIALS AND METHODS

PARTICIPANTS AND STUDY DESIGN

The same cohort of participants, a total of 30 (following the testing protocol described in chapter 2) underwent a DW-MRS scanning session 4½-5 hr after each injection of IFN-β or saline. This timing was informed by (i) previous TSPO PET studies in both humans and nonhuman primates that have indicated an increase in TSPO between 3-5 hr and 4-6 hr after an LPS challenge respectively (Hannestad et al., 2012; Sandiego et al., 2015) (ii) prior human Type I interferon studies that report sickness and systemic inflammatory responses as well as changes in mood, motivation and fatigue that can be observed within 4 hours of IFN-α administration, confirming an ongoing peripheral and central inflammatory response (Davies et al., 2020; Dowell et al., 2016).

DW-MRS ACQUISITION AND ANALYSIS

For this study, MRI and MRS data were obtained at 3T (Siemens Magnetom Prisma, Siemens Healthineers, Erlanger, Germany). After 3-plane localizer, a T1-weighted MPRAGE sequence (magnetization prepared rapid acquisition gradient echo) was acquired in sagittal orientation and reconstructed in 3 orthogonal planes (TR = 2100 ms, TE = 3.24 ms, T1 = 850 ms, flip angle = 8 degrees, FoV 256 x 256 mm^2). These scans were used to position two 4.5 cm^3 DW-MRS volumes of interest (VOIs) on the left thalamus and left corona radiata (20 x 15 x 15 mm^3 voxel size). These two regions were selected based on (i) the need to capture signal from homogeneous grey and white matter brain areas (ii) that the left hemisphere is more commonly implicated in inflammation-induced cognitive disturbance (Haroon et al., 2014; Harrison, 2017).

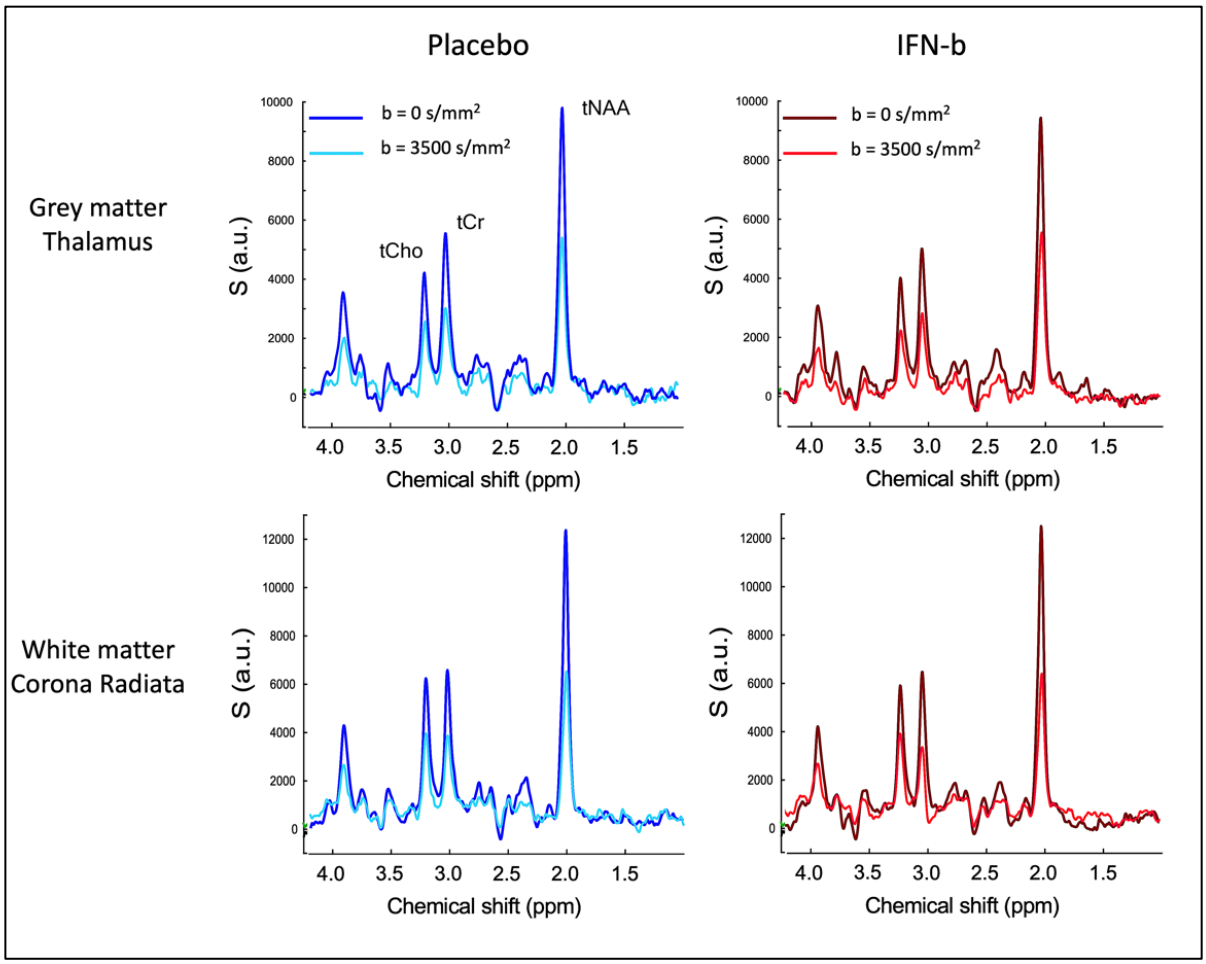
The DW-MRS sequence used was a bipolar sequence based on a semi-Localization by Adiabatic SElective Refocusing sequence (semi-LASER) (Genovese et al., 2021) with TE = 100 ms, TR = 3 s, spectral width = 2500 Hz, number of complex points = 1024. Four diffusion weighting conditions were applied: one at b = 0 s/mm^2 and three at b = 3500 s/mm^2 and diffusion gradients applied in three orthogonal directions [1, 1, -0.5], [1, -0.5, 1], [-0.5, 1, 1] in the VOI coordinate system.

For each condition, the number of signals averages (NSA) was 32 and a short scan without water suppression (NSA = 4) was performed for eddy current correction. B_0 homogeneity was achieved by employing a rapid automated shimming method that utilised echo-planar signals sequences and incorporated mapping along projections, referred to as FAST (EST) MAP (Gruetter & Tkáč, 2000).

Spectra data were transferred and analysed with customised software to pre-process DW-MRS data implemented in Matlab R2021b (Mathworks, Natick MA, USA). Data pre-processing consisted of three main steps: (i) generation of eddy current correction files based on water data acquisition and (ii) pre-processing of DWS and generation of output spectra files ready for analysis. For the last step, (iii) the analysis and quantification of spectral data, linear prediction singular value decomposition (LPSVD) was performed. The peak amplitude area estimates for the three orthogonal directions (at high b values) were averaged and used to compute the ADC for the three metabolites of interest (tCho, tNAA and tCr). **Figure 23** illustrates spectral data acquired in the grey and white matter regions of the same participant under the two conditions (placebo and IFN-β).

Amplitude values at b = 0 s/mm^2 were used to estimate relative tCho and tNAA (tNAA = NAA + NAAglutamate) concentrations (calculated as the ratio between their peak area and the tCr peak area). DW-MRS data were analysed completely blind to condition. Data from one participant was excluded due to CRLB above the 10% threshold (CRLB estimates the error associated to the ADCs, a lower CRLB indicates higher reliability of the estimate). Consequently, the results section will present findings from 29 participants

Figure 23. Example of MR spectra.



Data was acquired at b = 0 s/mm^2 and b = 3500 s/mm^2 in the Grey and White matter of one participant for the Placebo (blue) and IFN-β (red) conditions.

STATISTICAL ANALYSIS

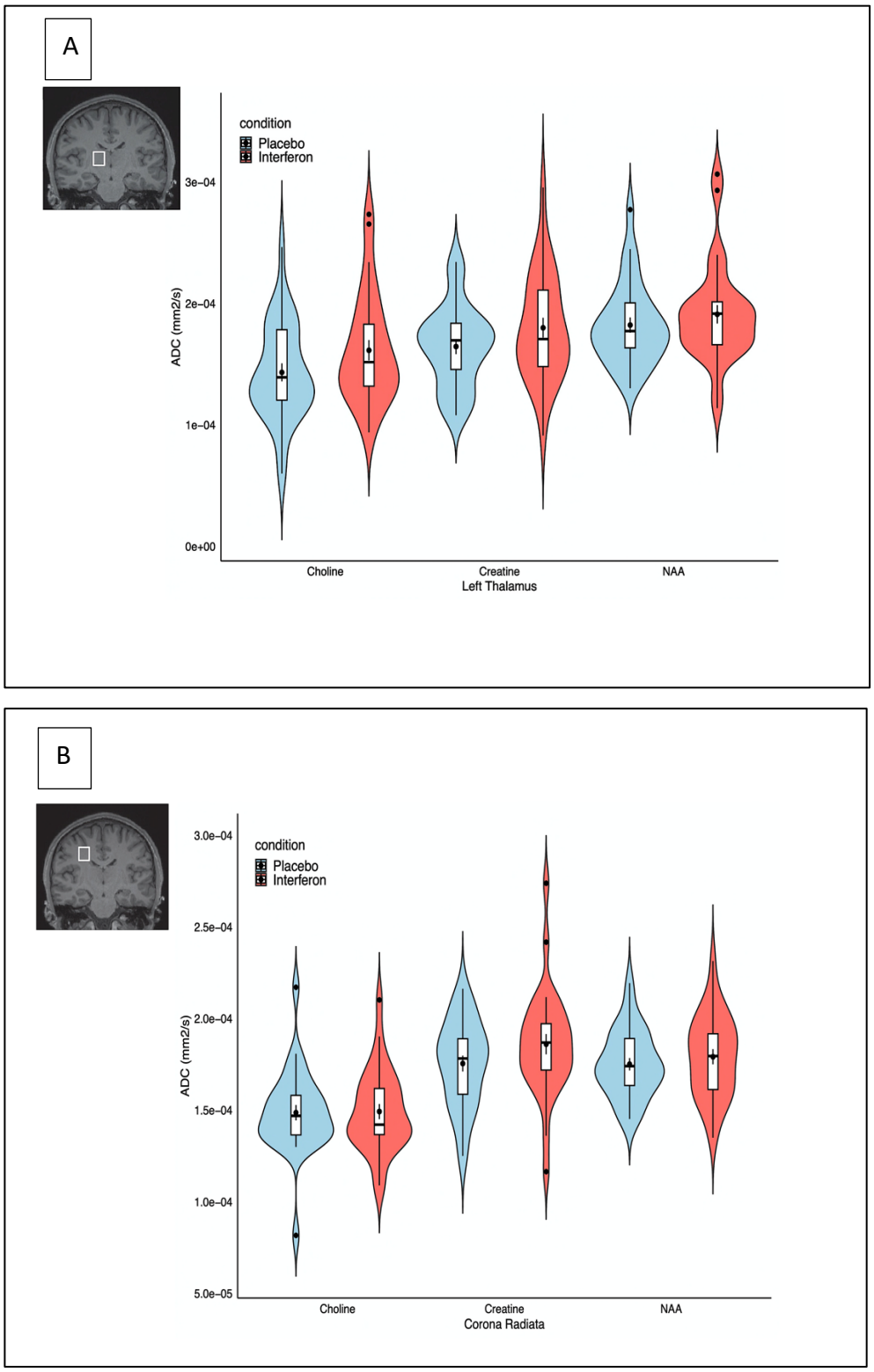
Statistical analysis was performed using IBM SPSS statistics version 27. The main effect of IFN on the ADC of the three metabolites as well as the tCho and tNAA relative concentrations ([tCho]/[tCr] and [tNAA]/[tCr]) were assessed between sessions (IFN vs placebo) using paired-samples t-tests. Mixed factorial ANOVA (within-subject factor: condition (IFN, placebo) between-subject factor: age category (young, old)) was used to look at condition x age interaction effects on the three ADCs and relative concentrations. Pearson's correlation coefficient was computed to assess associations between changes in ADCs and physiological, behavioural and cytokine measures. Greenhouse-Geisser adjusted repeated measures ANOVAs were used when appropriate to control for sphericity violations. Age-associated differences (young and old participants for the placebo condition) were assessed using independent-samples t-tests.

RESULTS

DW-MRS MAIN EFFECTS OF IFN-B AND AGE-ASSOCIATED INTERACTION

Metabolite dilfussion (ADCs): Paired t-test of the 29 participants showed a significant increase in tCho in the grey matter area of the thalamus for the INF-β condition compared to placebo ($t_{(28)}$= 2.15, p = 0.04) (ADC(tCho) -Thalamus: placebo (M = 1.43172e-04 mm^2/s, SD = 3.9643e-05), IFN-β (M = 1.61356e-04 mm^2/s, SD = 4.5196e-05)). No significant differences were observed between conditions for tCho in the WM region. tNAA and tCr ADCs also did not significantly differ between conditions in either grey or white matter regions. Repeated measures ANOVA did not reveal any condition x age interactions or age-associated effects for any of the ADCs. Data distribution for the placebo and IFN-β conditions in the thalamus and white matter are shown in **Figure 24A-B.**

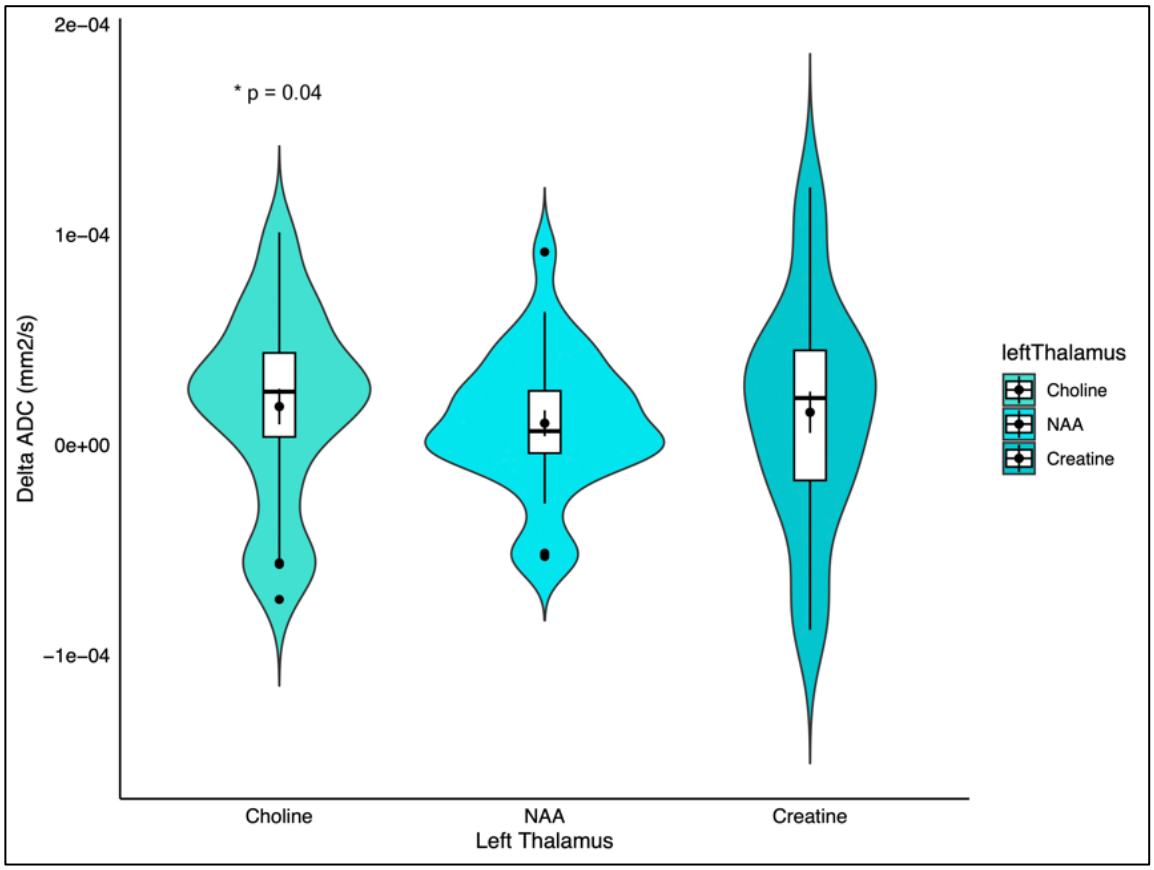
Figure 24. Grey and white matter ADC distribution



Violin plot illustrating, data distribution, mean, median and interquartile ranges for the ADCs of (A) the grey matter (thalamus) and (B) white matter area (corona radiata) of the brain. Blue denotes placebo, red denotes interferon.

The between-condition difference in the ACDs (IFN-β minus placebo) for the three metabolites in the left thalamus is shown in **Figure 25**. The average ADCs of tNAA, tCr and tCho are shown in **Table 6.**

Figure 25. Grey matter metabolite ADC response to IFN-β.



Differences between IFN-β and placebo sessions in the grey matter area of the brain. Mean, median and interquartile ranges are reported. P value shows between condition differences (IFN- β minus placebo) for choline ADC.

Table 6. Effects of IFN-β on metabolites ADC

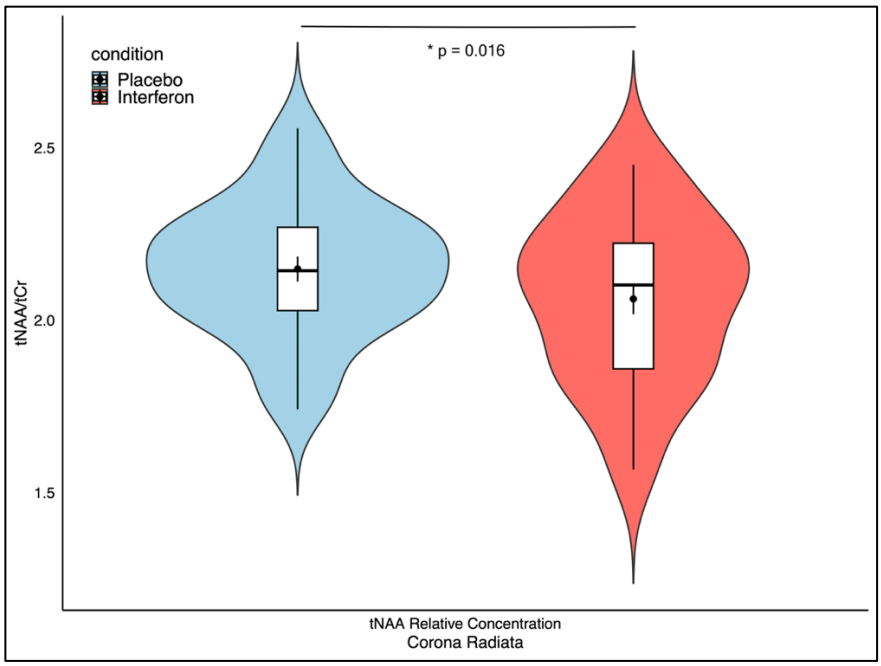
Mean ADC mm^2/s	Volume of Interest	Condition			
		Saline	IFN-β	Δ%	p-values (Saline vs. IFN- β)
tCho	**Thalamus**	**1.432e-04 (7.362e-06)**	**1.614e-04 (8.393e-06)**	**19.53 (7.85)**	**0.04**
	White Matter	1.486e-04 (4.208e-06)	1.492e-04 (4.113e-06)	2.19 (3.68)	0.894
tNAA	Thalamus	1.821e-04 (6.231e-06)	1.910e-04 (7.563e-06)	5.71 (3.2)	0.143
	White Matter	1.754e-04 (4.344e-06)	1.791e-04 (4.129e-06)	2.94 (2.39)	0.366
tCr	Thalamus	1.644e-04 (6.244e-06)	1.799e-04 (8.145e-06)	13.61 (6.39)	0.127
	White Matter	1.754e-04 (4.344e-06)	1.859e-04 (5.492e-06)	7.124 (3.37)	0.069

Data represents mean ± SEM.

Metabolite Concentrations: No significant differences in the relative concentration of Cho (computed as [tCho/tCr]) were found in either region between conditions or for thalamic tNAA relative concentration ([tNAA/tCr]). Repeated measures ANOVA did not reveal any age effects.

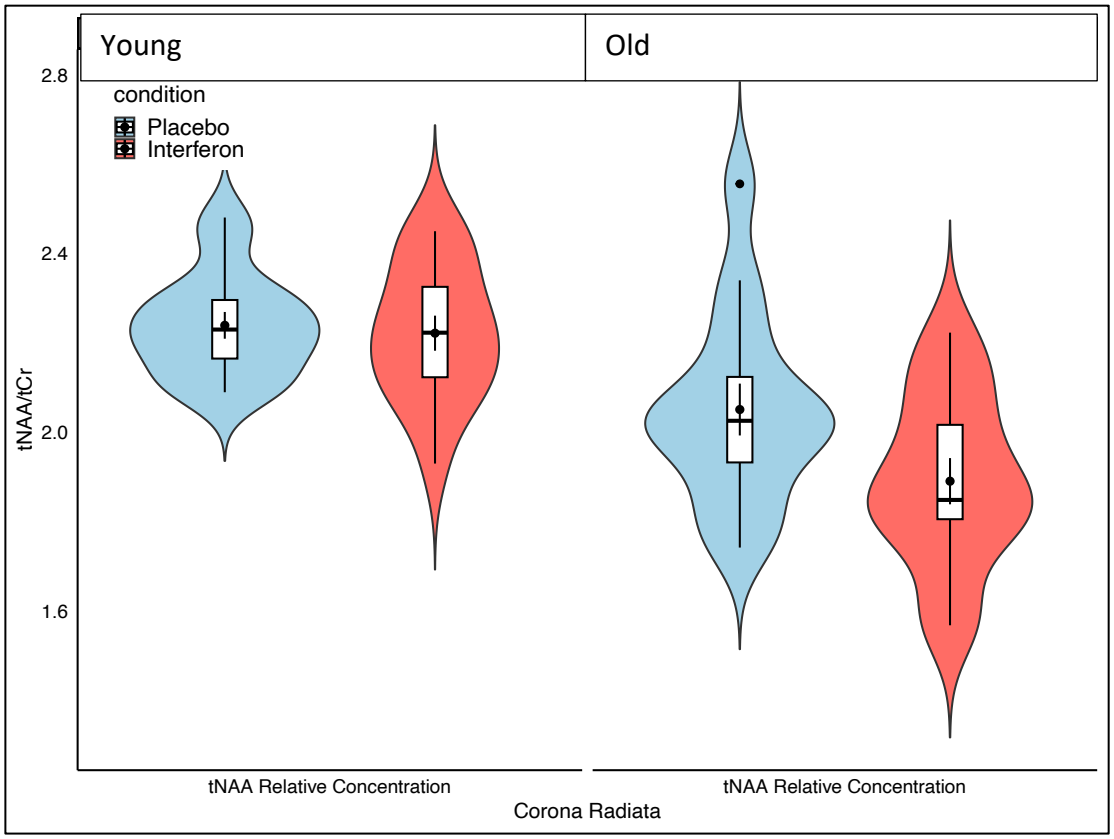
However, we observed a main effect of IFN-β in the tNAA relative concentration of the white matter region. NAA relative concentration was found significantly decreased for the IFN-β condition when compared to placebo ($t_{(28)}$ = -2.5, p = 0.016) (tNAA relative concentration – Corona Radiata: placebo (M = 2.148, SD = 0.194), IFN-β (M = 2.061, SD = 0.239)) (**Figure 26**). The repeated measures ANOVA also revealed a condition x age interaction ($F_{(1,27)}$ = 5.18, p = 0.031) as well as a between-subject age effect (($F_{(1,27)}$ = 21.52, p<0.001)) (**Figure 27**).

Figure 26. Distribution plot of tNAA relative concentration



tNAA relative concentration ([tNAA/Cr) for the IFN-β and Placebo conditions in the white matter area of the brain. Significant values show main paired t-test results. Blue denotes placebo, red interferon.

Figure 27. Distribution tNAA relative concentration split by age



Distribution plot of tNAA relative concentration ([tNAA/Cr)] split by age for the IFN- β (red) and placebo (blue) conditions.

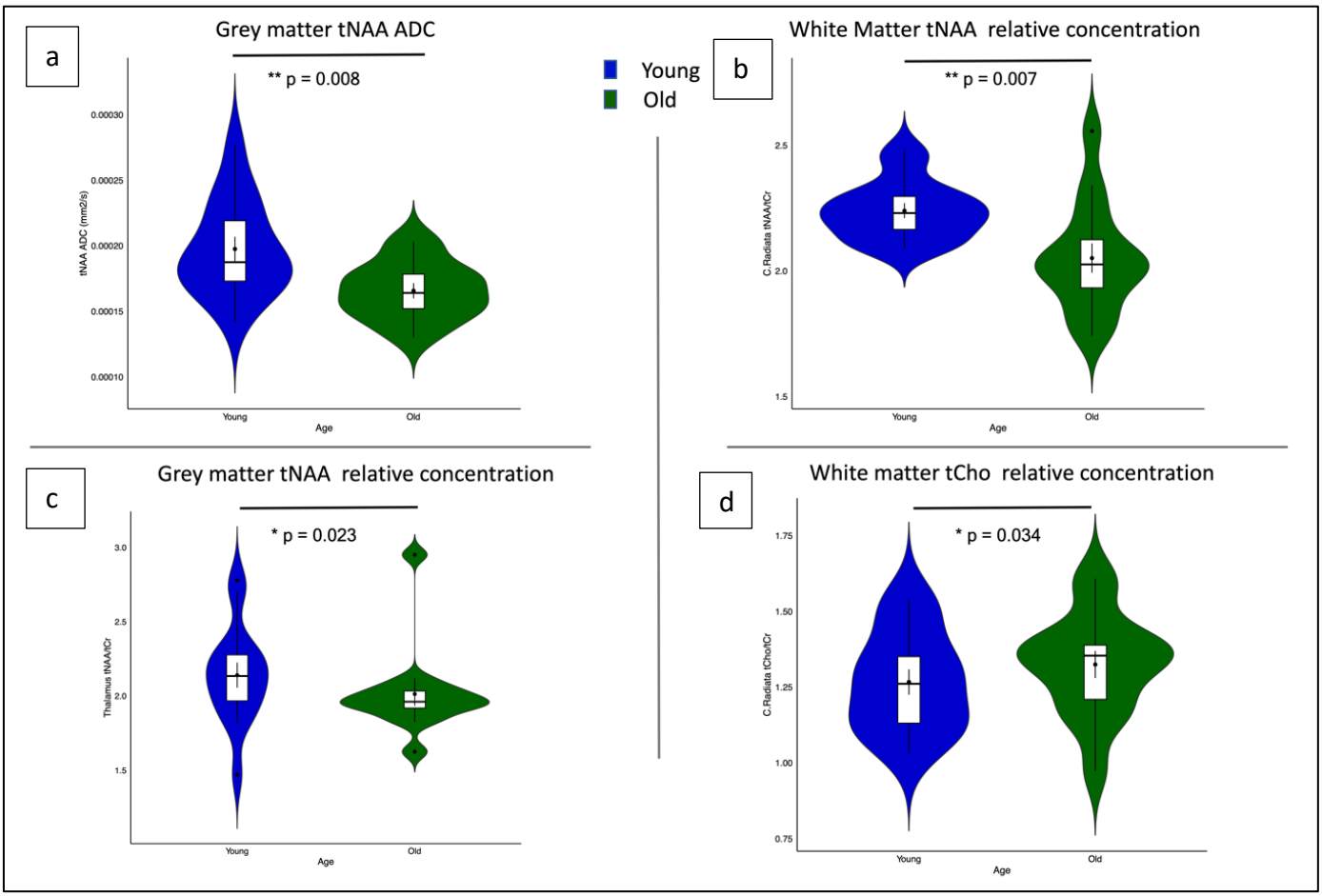
We found no significant correlation between changes in body temperature induced by IFN-β and ADC's for any of the metabolites (all p> 0.1).

Exploratory analysis investigating associations between the ADCs (computed as IFN minus baseline) and cellular immune responses (expressed as the peak change [6½ hours post-injection] minus baseline in the two sessions) found no significant associations (all p>0.1)

AGE DIFFERENCES IN ADCS AND RELATIVE CONCENTRATIONS UNDER PLACEBLO CONDITIONS

In examining differences related to age, independent samples t-test for the placebo condition showed a significantly lower thalamic tNAA ADC in the older group compared to younger adults ($t_{(27)} = 2.86$, p = 0.008) **(Figure 28a).** Also, the tNAA relative concentration (tNAA/tCr) was significantly lower in the white ($t_{(27)} = 2.94$, p = 0.007) **(Figure 28b)** and grey matter ($t_{(27)} = 2.42$, p = 0.023) **(Figure 28c)** in the old group compared to the young. Finally, the Choline relative concentration (tCho/tCr) in white matter was significantly higher in the older compared to the young group for the placebo condition ($t_{(24)} = -2.23$, p = 0.034)**(Figure28d).**

Figure 28. Age-associated differences in ADC and relative concentrations between young and old groups for the placebo condition.



(a) tNAA ADC, (b) tNAA relative concentration in the Corona Radiata, (c) tNAA relative concentration in the Thalamus, (d) tCho relative concentration in the Corona Radiata. Significant values show main paired t-test results. The young group is shown in blue, old group in green.

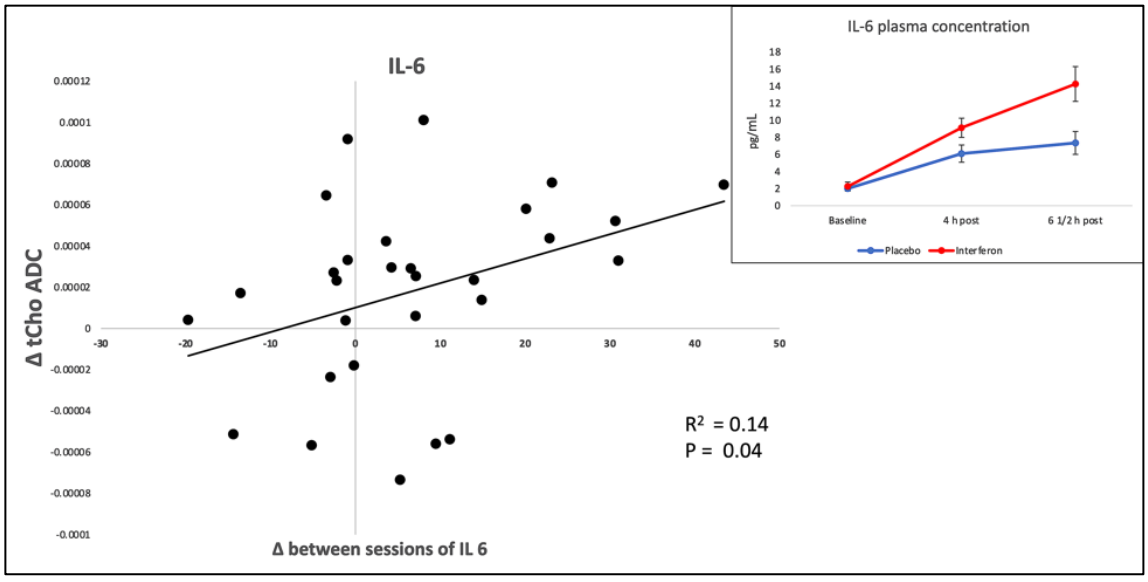
CYTOKINE LEVELS

Significant condition (IFN-β/Placebo) x time (baseline, 4 hr and 6½ hr post-injection) interactions were found for TNF-α ($F_{(1.58,42.81)} = 20.65$, $p<0.001$) and IL-6 ($F_{(1.4,37.81)} = 4.35$, $p = 0.032$) in the sample of 29 participants.

CORRELATIONS BETWEEN ADC (TCHO) AND CYTOKINE PLASMA CONCENTRATIONS.

In order to investigate potential associations between IL-6 levels and changes in the metabolite tCho, I calculated Δ values of IL-6 plasma concentration (6½ hours minus baseline) for IFN-β compared to placebo and correlated them with Δ tCho ADC (IFN minus placebo). IFN-β-associated changes in IL-6 were significantly correlated with changes in the ADC (tCho) of the thalamus ($R^2 = 0.14$, $p = 0.04$) (**Figure 29).**

Figure 29. Correlation between tCho ADC and IL-6



Correlation between tCho ADC change between sessions and the difference between IL-6 plasma concentration levels (6 ½ post-injection minus baseline) in the two sessions. The inset shows IL-6 changes throughout the 2 sessions (blue denotes placebo, red interferon).

BEHAVIOURAL RESPONSE

IFN-β induced significant transient changes in mood and sickness symptoms as shown by the POMS and SicknessQ questionnaires. IFN-β was associated with an increase in negative mood: main effect of condition ($F_{(1,27)}$ = 21.6, p<0.001) and condition x time interaction ($F_{(3.2,88.76)}$ = 5.57, p<0.001), and a reduction in total mood score: condition ($F_{(1,27)}$ = 13.8, p<0.001) and condition x time interaction ($F_{(3.1,84.76)}$ = 5.97, p<0.001) as well as in the tiredness scale: condition ($F_{(1,27)}$ = 11.6, p = 0.002) and condition x time interaction ($F_{(2.4,64.99)}$ = 4.72, p= 0.008). Moreover, IFN-β induced a significant increase in sickness symptoms: condition ($F_{(1,27)}$ = 7.42, p= 0.011) and condition x time interaction ($F_{(2.89,78.26)}$ = 7.3, p<0.001). Potential associations between mood and sickness behaviour and changes in tCho ADC were explored, however, all correlations were non-significant (p>0.1)

DISCUSSION

In this study, I used DW-MRS in a sample of 15 young and 14 older adults and showed evidence of changes in brain intracellular metabolite diffusion in the context of experimentally induced inflammation in healthy humans. As I hypothesized, our primary finding showed an increase in thalamic tCho ADC after IFN-β administration compared to placebo and suggests that DW-MRS is a tool sensitive to detecting morphometric changes in glial cells *in-vivo* in humans using a mild experimental model of inflammation. My results also add knowledge to age-associated differences in neurochemical concentrations and cell morphometry.

The primary finding is in line with evidence from animal models of systemic inflammation showing that reactive glia undergoes cytomorphological changes during activation, consistent with an increase in concentration of intraglial metabolites (Verkhratsky et al., 2014). For instance, studies using DW-MRS in myo-inositol (Ins) and Culprizone models of inflammation in mice have reported elevated Ins and tCho ADCs which also correlated with histological measures of severe inflammation and changes in astrocytic and microglial area fractions respectively (Genovese et al., 2021).

Furthermore, in humans, studies on neurological diseases such as systemic lupus erythematosus and ALS have also found elevated diffusivities in choline and creatine (Ercan et al., 2016; Reischauer et al., 2018). Other models of experimental inflammation such as LPS have reported microglial and astrocytic neuroinflammatory responses in mice (Ryu et al., 2019) with findings showing microglial activation after LPS administration measured with TSPO PET in humans (Sandiego et al., 2015). A recent preliminary study in healthy humans has also shown evidence of changes (increased ADC) in the diffusion properties of the metabolite choline after LPS injection (De Marco et al., 2022).

My findings further support the notion that changes in diffusivity during systemic inflammation are associated with cell-type-specific microstructural alterations that can be detected with DW-MRS and extend it to a milder, more ecologically valid model of inflammation. As recent evidence has indicated, in contrast to rodent models, the increase in TSPO observed in humans seems to be more closely linked to a rise in the number of microglial cells rather than shifts in their activation status (Nutma et al., 2023). Given this context, it can be argued that DW-MRS with its ability to discern changes in diffusivity associated with microstructural alterations, might be a more reliable method for studying inflammatory processes compared to the traditionally accepted 'gold standard' TSPO method.

Both microglia and astrocytes undergo metabolic, morphological and functional changes during neuroinflammation (Heneka et al., 2014). Microglia cells, the principal mediators of the immune response in the CNS, are highly dynamic and equipped with multiple finger-like processes that constantly monitor their local environment (Nimmerjahn et al., 2005). In response to any kind of injury, infection or brain damage microglia become reactive, displaying a phenotype characterised by a thickening and retraction of their processes, increasing in their cell body size and changing to a more amoeboid spherical shape (E. J. Davis et al., 1994; Hoogland et al., 2015). Upon activation, astrocytes are similarly characterised by hypertrophy of soma and processes (Escartin et al., 2019; K. Li et al., 2019) and therefore, I cannot definitively determine whether the changes in tCho diffusion we detected in the study were a result of microglia or astrocyte activation. However, Genovese et al. (2021) findings in their Culprizone mouse model of inflammation showed

an association between tCho and myo-inositol and histological changes in microglia and astrocytes, respectively. Furthermore, no changes in tCho diffusivity were found in a mouse model of reactive astrocytes where myo-inositol diffusion changes scaled with astrocytic hypertrophy while tCho remained unaltered (Ligneul et al., 2019). Findings on LPS mouse models seem to support the notion of a time-dependent neuroimmune process where reactive microglia appear first then seem to induce a later astrocyte activation (Liddelow et al., 2017). In line with these results, evidence from a DW-MRI study showed changes in microglia appearing at 8 hours post-injection while astrocytic reaction was observed 24 hours after the LPS injection (Garcia-Hernandez et al., 2020). Taking all these findings into consideration, we suggest that the most parsimonious interpretation of the changes in tCho observed in our data is of activation of microglia rather than astrocytes.

Glial activation plays a critical role in the development, progression, and resolution of a wide range of inflammatory responses in the central nervous system (CNS). Microglia and Astrocyte activation is a common feature of diverse neurological conditions such as Alzheimer's, Parkinson's Disease, stroke, and injury-induced neuropathic pain (Colburn et al., 1997; Giulian et al., 1993; McGeer et al., 1988). Moreover, glial pro-inflammatory immune phenotypes have also been found in autoimmune neuroinflammatory disorders such as MS or Neuro-SLE (McGeer & McGeer, 2002; Oh et al., 2011; Perry & Holmes, 2014). Further, studies focussing on heightened systemic inflammation and the neuropathology of psychiatric disorders such as MDD or Schizophrenia have also shown evidence of glial activation and excessive production of pro-inflammatory cytokines (Almeida et al., 2020; Dantzer et al., 2008; Lanquillon et al., 2000; Najjar et al., 2013). It is not clear yet what are the exact mechanistic factors underlying the response to inflammation-associated changes on the brain as in vivo methods for the assessment of glial activation and response to systemic inflammation are not fully developed. Nevertheless, DW-MRS sensitivity to cell-specific microstructural changes could potentially emerge as a valuable clinical tool applicable across a spectrum of disease.

In this study, I did not find a significant change in tNAA ADC. tNAA is generally considered a marker for neurons as it reaches detectable concentration in neuronal tissue only. Reductions in tNAA diffusivity have been previously reported in MS (Escartin et al., 2019;

Li et al., 2019) and cerebral ischemia (Zheng et al., 2012). In line with our data, no tNAA diffusivity changes have been found in other models of experimental inflammation such as LPS (De Marco et al., 2022). Altered tNAA ADC may reflect the neuronal damage and cell loss that characterize some neurological and autoimmune diseases, however, that is not a feature of our model of inflammation and indicates that IFN-β has no effects on neuronal morphology.

We observed an age effect in thalamic tNAA when we compared the ADCs for the placebo condition between our young and older cohorts. The older adult group showed significantly reduced tNAA diffusivity compared to their younger counterparts. Evidence from the literature shows that the main morphometric measures of neurons (e.g. total dendritic surface area, total volume, total dendritic length, dendritic spine numbers and densities and dendritic diameter) significantly decrease in healthy ageing, which may lead to decreased neuronal space for metabolite diffusion, and potentially reduce the ADCs (Bishop et al., 2010; Morrison & Hof, 2003; Raz et al., 2004). Moreover, the presence of neurofibrillary tangles and senile plaques in the neurons may as well be another factor that affects diffusion and reduces ADCs in older individuals (Kabaso et al., 2009). The incorporation of refined DW-MRS sequences might present a promising route to investigate intracellular compartments potentially offering avenues for further exploration of these phenomenon.

I also observed differences between young and old groups in the tNAA relative concentration (tNAA/tCre) for both VOIs (grey and white matter) in the placebo condition (the older group tNAA relative concentration was significantly lower than their younger counterparts). These results would also be consistent with data from human brain tissue showing significant age-associated specific variations in neuronal density. A decrease in neuronal density in the frontal cortex and hippocampal areas CA3 and CA4 was observed in healthy older subjects compared with the youngest group. Furthermore, astrocytes density increased with age in the entorhinal cortex and CA2, CA3, and CA4 hippocampal areas (Martínez-Pinilla et al., 2016).

Contrary to my second hypothesis (greater effect of IFN-β on metabolite diffusivity with age), I did not observe any condition x age interactions in Cho ADC or any of the other metabolites. This could be attributed to several factors. (i) The study might not have enough participants to detect a significant interaction effect, especially if those potential effects are subtle. (ii) young and old participants were healthy and without significant variations in health status, therefore the potential expected effects might not manifest clearly. (iii) Furthermore, it is possible that microglial activation as indicated by ADC Cho might not vary significantly between healthy young and healthy older adults with the effect being more pronounced and detectable only in individuals with a heightened inflammatory status or neurodegenerative conditions. (iv) The dosage of IFN-β administered in the study may have been a determining factor in the observed results. Given that the chosen dosage represents a mild inflammatory challenge, it is arguable that a more robust dosage might unveil distinct effects and possible interactions. (v) Additionally, it is worth considering the temporal dynamics of ADC Cho changes. This temporal trajectory might vary between age groups. Potentially, older participants could exhibit a different timeline for such responses compared to their younger counterparts, which might not have been captured within the assessment window of this study. Finally, (vi) The selection of VOIs in the study was informed by literature using LPS as an experimental model of inflammation. Given the distinct characteristics and effects of LPS vs. other inflammatory challenges, it is plausible that alternative VOIs, not captured in the current investigation, might be more sensitive or relevant.

To the best of my knowledge, this study is the first report of a non-invasive assessment of the intracellular diffusion changes of tNAA, tCho and tCr using a mild model of inflammation, as well as considering the age effects in humans, in-vivo. These considerations provide avenues for future research and could yield more nuanced insights into how age might increase susceptibility to inflammation.

In line with my third hypothesis (an association between IFN-β induced alterations in IL-6 levels and changes in choline ADC), the correlation analysis revealed a significant positive correlation between thalamic tCho and IL-6 plasma concentration. Research from TSPO PET and immunocytochemistry studies indicate that the thalamus is a grey matter area

known to have high microglial density and that is sensitive to inflammation (Buttini et al., 1996; Schubert et al., 2021). The thalamus has several vital functions such as relaying sensory and motor signals to the cerebral cortex and regulating functions such as consciousness, sleep and alertness with dense microglial presence potentially being an evolutionary adaptation to ensure rapid immune responses to potential threats. Participants with a larger inflammatory response may also exhibit a more pronounced central effect.

While we observed a main effect of IFN-β in the behavioural response (mood and sickness behaviour), no association between IFN-β-induced changes in thalamic Cho and mood changes was found. The thalamus has not been previously considered a brain structure linked to the psychological and behavioural changes associated with inflammation (Harrison, 2017). In line with this, previous literature has reported associations between the severity of mood symptoms and functional changes in the subgenual cingulate, amygdala and ventral striatum as well as decreased global functional connectivity in the ventral striatum following immune challenges (Capuron et al., 2012; Davies et al., 2020; Dipasquale et al., 2016; Harrison et al., 2009). De Marco's et al. recent DW-MRS preliminary study, however, showed a tight correlation between LPS induced changes in thalamic Cho and mood symptoms, which I did not replicate in this study.

Due to time implications, I restricted data acquisition only to the two mentioned VOIs which were chosen based on the available literature where LPS is the most common experimental model of inflammation. Microglia responses to pro-inflammatory stimuli such as LPS and Interferons have been found notably different, with LPS evoking higher pro and anti-inflammatory gene expression (Lively & Schlichter, 2018). Furthermore, and as mentioned before, the thalamus exhibits significant TSPO uptake under typical conditions (Schubert et al., 2021) and it has been shown to be sensitive to LPS-induced inflammation (Buttini et al., 1996). Diffusivity changes in this brain area may well reflect more general glial morphological changes in grey matter rather than a specific role in mood. Nevertheless, data acquisition considering a greater range of VOIs would allow more specificity when exploring regional changes and their association with behavioural features.

A potential bias in the study was the effect of IFN-β on body temperature and therefore metabolite diffusion. However, I did not find an effect of IFN-β on tNAA in either VOI or significant changes of tCho in the white matter region. Furthermore, I did not find any association between peak change in temperature and any of the metabolite's ADCs, which suggests that a causal relationship between temperature and our findings is improbable.
One limitation of this study is that the data were acquired at one time point (4½ -5 hours) after placebo/ IFN-β injection which did not allow us to address the question of how the temporal evolution of some brain changes is associated with the progression of specific behavioural and cellular phenotypes.

Physiological changes (e.g., body temperature, heart rate) tend to peak early following LPS administration (2-3 hours post-injection) with TSPO PET findings showing glial activation 3-5 hours after LPS in humans and from 4-6 hours in baboons (Hannestad et al., 2012; Sandiego et al., 2015). In our study, changes in the physiological, behavioural and cellular (WBC, lymphocytes and neutrophils) response peaked at 6½ hours after IFN-β administration which may give us an indication of the temporal evolution of glial activation after IFN-β. Future research could address questions such as how and when pro-inflammatory patterns may eventually resolve and how they associate with acute and more persistent symptoms in some participants.

DW-MRS data acquisition is challenging both at the acquisition and pre-processing level. Motion artifacts such as simple linear translational motion during the acquisition of the diffusion-weighted conditions affect the DW-MRS signal which leads to single phase shift. However, this can be corrected in the post-acquisition phase and has no significant effect in the metabolite ADC. Compressive motion, such as cardiac pulsation produces a non-constant phase shift that results in a drop in the intensity of the signal. Individual acquisitions with signal drop included in the signal average create a source of variance that overestimates the diffusion coefficients. In order to avoid that, the post-processing pipeline utilises a single criterion based on the strongest peak in the spectrum (NAA peak) which takes into account the variance of the peak amplitude across different acquisitions based on the variance of the error (Genovese et al., 2021).

Another challenge of DW-MRS acquisition relates to signal-to-noise ratio (SNR). The need to include the diffusion gradients within the sequence leads to longer echo times than standard MRS sequences. Furthermore, as three diffusion-weighted conditions are included, there is a limitation on the number of averages that are possible to acquire. SNR also affects the variance of the ADCs which can be estimated by calculating the propagation error. This type of error can amplify the variance of the ADCs a few times above that of the non-diffusion weighted condition signal. However, optimized acquisition and processing DW-MRS protocols, (such as the one applied in this study) have shown to be robust enough to provide statistical power to detect ADC differences (Wood et al., 2015).

In summary, my findings show that DW-MRS was able to detect changes in tCho diffusion associated to IFN-β and emerges as an MR imaging paradigm able to quantify glial morphological changes associated to mild inflammatory challenges in vivo in humans. I also observed differences in the ADC values between old and young participants suggesting that DW-MRS could be helpful to evaluate age-related changes in the intracellular environment in humans in vivo. The study did not demonstrate any significant interactions between condition and age. While the current findings provide a valuable foundation, future investigations can benefit from a number of methodological considerations. This could include, for instance, expanding the sample size, incorporating a wider range of VOIs, comparing outcomes between healthy and clinical populations and evaluating the impacts of varying dosage levels. Such refinements in the experimental design might elucidate more subtle effects and offer a deeper mechanistic understanding of how age may modulate the effects of inflammation.

CHAPTER 6. RESTING STATE FUNCTIONAL MAGNETIC RESONANCE IMAGING (RSFMRI)

INTRODUCTION

fMRI studies of emotional or cognitive task-related activation in the context of either acute experimental pro/anti-inflammatory challenges or chronic systemic inflammation have provided some of the most convincing evidence that peripheral inflammation can lead to alterations in the human brain. They have clarified which brain structures are the most sensitive to changes in systemic inflammation and have successfully identified cortical and subcortical structures that appear to play distinct roles in discrete elements of inflammation-related behavioural change. For instance, actions on the ventral striatum associated with impaired reward sensitivity have been reported in experimental studies after controlled administration of inflammatory challenges (i.e. typhoid vaccine and endotoxin) (Eisenberger et al., 2010; Harrison et al., 2016) as well as in patients undergoing IFN-α treatment for Hepatitis C (Capuron et al., 2012). Further evidence demonstrates significant effects of inflammation on hippocampal/parahippocampal areas and in spatial memory impairment (Harrison et al., 2014; Yirmiya & Goshen, 2011) with other regions such as the amygdala, insula, anterior and subgenual cingulate cortex also found to be sensitive to the impairing effects of inflammation (Dowell et al., 2016; Harrison et al., 2009; Kraynak et al., 2018). With more or less circumscribed roles, most of these brain areas are part of the extended limbic circuitry which is involved in the production of complex motivated behaviours and higher cognitive functions (e.g., memory or learning), as well as the integration of physiological and behavioural responses to inflammation (Critchley & Harrison, 2013; McEwen & Gianaros, 2010)

There are multiple competing conceptualisations of how the brain functions at a global level. One approach, based on mathematical concepts of graph theory, conceptualises the brain as an intricate network comprising interconnected grey matter areas referred to as nodes, which are interconnected by white matter fibre pathways. Whole brain resting-state fMRI data can be collected to investigate the functional connectivity (FC) between

these nodes enabling assessment of how recorded activity at each node is associated with the activity of all other nodes. rsfMRI data analysed using conventional or graph theoretic approaches has also been utilised to examine the relationship between peripheral inflammation and FC. For example, research employing whole-brain connectomic analysis has shown alterations in the connectivity within the Default mode network -DMN- (subgenual anterior cingulate cortex and medial prefrontal cortex) associated with higher IL-6 (Marsland et al., 2017), increased cortico-subcortical connectivity following LPS administration (Labrenz et al., 2016), increased subcortical connectivity as well as decreased cortical connectivity associated with TNF-α in adolescents (Swartz et al., 2021), decrease global network connectivity induced by IFN-α (Dipasquale et al., 2016), reduced network connectivity within the DMN of depressed patients (Kitzbichler et al., 2021). Further evidence has shown correlations between peripheral inflammation markers (using a composite measure of several cytokines) and decreased FC within emotion regulation and central executive networks (Nusslock et al., 2019) and dorsal attention and DMN in older individuals (Walker et al., 2020) as well as higher levels of CRP associated with decreased frontotemporal FC in older adults (Bang et al., 2019)

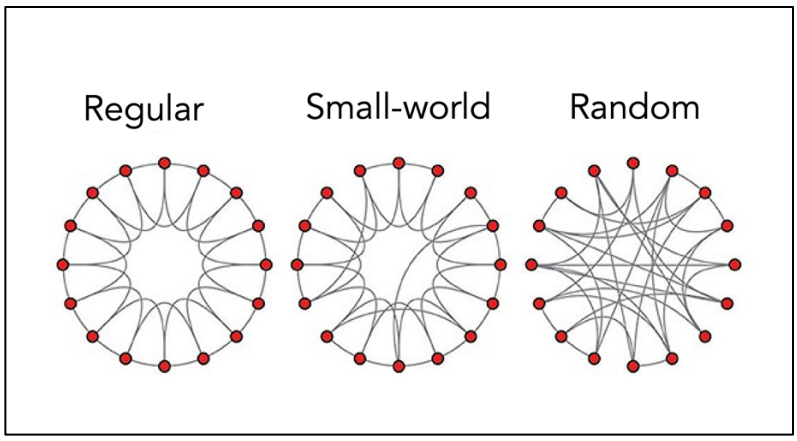
The conceptualization of the brain in this way (as a complex network of nodes) enables the utilisation of sophisticated mathematical network analysis, such as graph theory, which can provide a quantitative assessment of the essential characteristics of complex networks. Some examples of the graph metrics employed include (i) Node Strength which is defined as the sum of the weights (how strong a connection is) of the edges (connections) to and from a node. For instance, in a simple network with three nodes: A, B and C, the connection (edge) between A and B has a weight of 3 and the connection between A and C has a weight of 2, in this example the Node Strength of A is 5 and this is calculated by summing the weights of all edges connected to node A. High-degree nodes (also called 'hubs') will have stronger connections and it is assumed they make a greater contribution to global network efficiency compared to less well-connected nodes.
(ii) Betweenness centrality aims to capture the role of nodes as bridge between other groups of nodes. For instance, a node with high centrality would lie on the shortest path between other nodes or (iii) Network efficiency which is considered a measure of the network's ability for parallel information transfer (the brain's ability to process and

transmit multiple streams of information simultaneously across various regions or nodes). Higher network efficiency is associated to the brain's ability to rapidity integrate information from diverse sources and execute complex cognitive tasks.

Utilization of graph theory methodologies in the study of the human brain has revealed that the human brain follows an effective "small-world" functional structure (Achard et al., 2006). Specifically, this implies that the individual elements within the network (nodes) possess a higher degree of localized interconnections (edges) than would be anticipated in a random network, while also exhibiting shorter minimum distances between pairs of nodes compared to regular or lattice-type networks (Watts & Strogatz, 1998) **(Figure 30).**

This type of 'small world" organisational structure offers several notable advantages: reduced wiring cost and increased robustness, characterised by the network's ability to maintain its integrity even in the face of random node or edge damage (van den Heuvel et al., 2008). These networks also exhibit a smaller number of 'hubs' (nodes with high centrality and highly connected) and provide the shortest connection path for numerous node pairs, therefore playing a vital role in facilitating efficient communication. Although critical for efficient communication they can also be susceptible to brain insults which in turn can result in a rapid decline in network efficiency and overall connectivity within the brain.

Figure 30. The small-world network model



Regular networks (left) show high clustering and long path lengths, Random networks (right) exhibit low clustering and short path lengths and small-world networks (centre) combine elements of both. Small-world networks have a balance of short-range and long-range connections, resulting in a high clustering coefficient and a short characteristic path length (image adapted from Farahani et al., 2019).

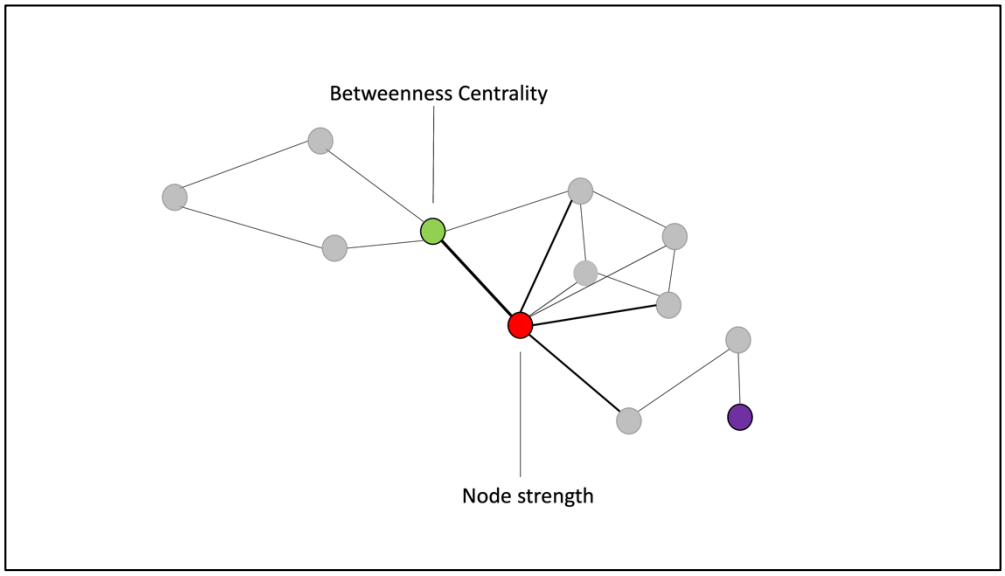
In this chapter, I use rsfMRI to investigate the acute effects of IFN-β on FC with a particular focus on the efficiency of information transfer. rsfMRI from 30 participants (15 young and 15 old) was parcellated into 410 cortical and subcortical regions and, weighted undirected (i.e. if there is a connection between node A and node B, it is the same as a connection between node B and node A as edges as considered not have a specific direction) graph theory metrics were applied to explore the effects of IFN-β on: (i) Node Strength (number of weighted connections -edges- of each node to the other nodes), (ii) betweenness centrality (number of shortest paths that traverse a specific node, connecting all other pairs of nodes within the network) **(Figure 31)** and (iii) global and Local Efficiency (minimum path length between nodes; for instance, whether there is a direct transmission of information between node A and node B (shorter path length), or the information has to traverse through one or more intermediate nodes (resulting in a longer path length). Global Efficiency provides information about whole-brain network efficiency of information exchange in a parallel system while Local Efficiency provides an estimation of the significance of individual nodes in facilitating the exchange of information within the network (Fornito et al., 2016a). In order to identify networks sensitive to the acute effects of IFN-β, graph measures were also estimated for eight parcellated resting state networks.

My prediction was that IFN-β would acutely impair global network connectivity, specifically a reduction in mean nodal strength, betweenness centrality and Global Efficiency.

Hubs or highly-connected nodes, are distinguished by their topological centrality and associated biological costs, such as increased metabolic demands. This makes them especially prone to various pathogenic influences. When these hubs are affected by pathological factors, it can disrupt Global Efficiency and information processing (Crossley et al., 2014). Therefore, I also hypothesised that nodes with high connectivity (hubs) would be particularly susceptible to the impairing effects of IFN-β. Furthermore, I predicted that overall FC and graph measures of Node Strength and Global Efficiency would be more affected by IFN-β in older individuals when compared to their younger counterparts. Finally, I investigated global and local changes in brain network function and their

association with plasma concentrations of cytokines affected by IFN-β (TNF-α and IL-6) as well as acute changes in mood and sickness behaviours scales impacted by IFN-β

Figure 31. Graphical representation of Node Strength and betweenness centrality



In this network of twelve nodes, the red dot represents a high-degree node as it is directly connected to six other nodes. Conversely, the purple dot represents a low-degree node as it is only directly connected to one node. The green dot represents high betweenness centrality as it lies on a high number of shortest paths connecting disparate parts of the network.

METHODS

STUDY DESIGN AND PROCEDURE

The same cohort of participants, a total of 30 (following the testing protocol already described in previous chapters) underwent a resting state fMRI session 4-4 ½ hr after each injection of IFN-β or saline. This timing was informed by (i) previous human Type I interferon studies that report sickness and systemic inflammatory responses as well as changes in mood, motivation and fatigue that can be observed within 4 hours of IFN-α administration, confirming an ongoing peripheral and central inflammatory response (Davies et al., 2020; Dowell et al., 2016). (ii) rsFMRI studies reporting inflammation-induced alterations in functional connectivity of resting state networks as well as impaired Global Efficiency and parallel information exchange (Dipasquale et al., 2016; Kitzbichler et al., 2021; Labrenz et al., 2016).

In the human connectomics literature, it is common practice to apply a threshold to a connectivity matrix in order to reduce noise and spurious links while emphasising the network's topological qualities. One common approach is to use proportional, also called density-based, thresholding where a fixed proportion of the strongest connections are retained (e.g., a density value *k* that keeps the top 20%, 30% of connections or any other desired percentage of connections based on their strength). This approach allows for comparisons across different datasets or groups by using a consistent proportion (Fornito et al., 2016b). There is not a clear consensus regarding the most appropriate threshold. A range of costs have been reported in the literature, overall going from 5 to 40%. A very low cost of 5% may involve excess network fragmentation while a more liberal cost of 40% or higher may give rise to network randomness which would not conform with knowledge that the human brain functions as a small world network not a random one (Fornito et al., 2010).

Because brain functional networks have been shown to exhibit economical small-world properties (Achard et al., 2006; Latora & Marchiori, 2001; Watts & Strogatz, 1998), I

applied graph metrics on a functional connectivity matrix thresholded with a low *K* value of 0.25 (25% density), which both preserves only the strongest functional connections and enables highly efficient parallel information processing at a low wiring cost.

IMAGE ACQUISITION AND DATA PRE-PROCESSING

MR imaging was performed on a 3T (Siemens Prisma, Siemens Healthineers, Erlanger, Germany). After a 3-plane localizer, a T1-weighted anatomical scan was acquired for each participant using an MPRAGE sequence (TR = 2100 ms, TE = 3.24 ms, T1 = 850 ms, flip angle = 8 degrees, FoV 256 x 256 mm^2). Functional MRI data were obtained during rest using a T2*-weighted multi-echo EPI sequence (TR= 2691 ms; TE_1= 12.6, TE_2=30.25, TE_3=47.9 ms; total acquisition time: 11.21 min; flip angle= 80 degrees; voxel size: 3.4 x 3.4 x 3.4 mm; 250 volumes).

Data pre-processing was performed using the fMRIPrep pipeline (Esteban et al., 2019) which performs standard processing steps (co-registration, normalization, field unwarping, motion correction and brain extraction) and TE-Dependent Analysis (TEDANA), a robust tool for denoising multi-echo fMRI data (DuPre et al., 2021).

TEDANA decomposes multi-echo BOLD data using principal component analysis (PCA) and multi-echo independent component analysis (ICA). The extracted components are then subjected to analysis to determine whether they are TE-dependent (classified as BOLD) or TE-independent (classified as non-BOLD). TE-independent components are discarded as part of data cleaning. Non-bold components are discarded because they often capture non-neuronal fluctuations that can arise from a variety of sources, including motion artefacts, physiological processes (e.g., cardiac and respiratory cycles) and potential scanner instabilities. By removing these components, the focus is maintained on BOLD signal changes related to neuronal activity, minimizing the influence of noise signals.
TEDANA first step is to combine the signal across echoes, and this is done by calculating a weighted average and producing an optimally combined time series that will be later decomposed and used to identify BOLD and non-BOLD components. The next step TE-

dependent principal component analysis (TEDPCA) involves removing thermal noise and applying PCA to decompose the optimally combined data into component maps and time series that will facilitate the later ICA decomposition to converge. Finally, TEDANA applies TE- dependent independent component analysis (TEDICA) in order to identify "non-BOLD" components. A number of independent time series and estimate maps are generated to visualise the spatial distribution of these components within the brain. This will yield an ICA classification of TE-dependent (BOLD signal), TE-independent (non-BOLD noise) or neither (ignored). Components identified as noise are removed from the optimally combined data which results in a denoised time series ready for post-processing.

The eXtensible Connectivity Pipeline (XCP) (Ciric et al., 2018; Satterthwaite et al., 2013) a robust postprocessing pipeline of fMRI data was then used to process the TE-dependent outputs identified by TEDANA. The retained components provided a denoised fMRI time series at each voxel that were then band-pass filtered to retain signals within the 0.01-0.1 Hz frequency band (wavelet scales 1-2, where resting state BOLD signal frequencies are predominantly located). Processed BOLD was smoothed with a Gaussian Kernel of 6mm (FWHM). In order to identify high-motion outlier volumes, FD (framewise displacement) was calculated and volumes with an FD>0.5 mm were flagged as outliers and excluded from nuisance regression. Scans from 3 participants were discarded due to imaging artefacts.

The analysis packages and protocols used in this study, are free source and accessible through the following links: https://github.com/nipreps/fmriprep, https://github.com/ME-ICA/tedana, and https://github.com/PennLINC/xcp_d

FUNCTIONAL CONNECTIVITY

Processed functional time series were parcellated into 360 cortical and 50 subcortical areas using the Glasser and Tian scale III atlases respectively (Glasser et al., 2016; Tian et al., 2020). The Yeo map was used for parcellation into 7 resting-state networks (Yeo et al., 2011). The regional mean fMRI time series were estimated, producing a 410 x 250 regional

time series matrix for each participant for the placebo and Interferon conditions. The functional connectivity between each fMRI time series was obtained by Pearson's correlation for each pair of regions, resulting in a 410 x 410 functional connectivity matrix. Correlation matrices were converted into z-values and averaged across participants for the placebo and interferon conditions.

GRAPH ANALYSIS

As discussed above, and informed by graph theory and its applications to brain networks, four high-level graph metrics: Node Strength, betweenness centrality, Global and Local Efficiency were calculated using the Brain Connectivity Toolbox (Rubinov & Sporns, 2010) applying a density threshold of 25%

My prediction was that IFN-β would acutely impair global network functional connectivity resulting in a reduction of mean Node Strength, betweenness centrality as well as global and Local Efficiency. The four graph measures were estimated using weighted (i.e., non-binarized and therefore incorporating information on the strength of the connection) and undirected graphs (i.e., edges can be traversed in both directions).

Repeated measures ANOVA with two within factors: Condition (IFN/Placebo) and network (Ventral Attention (VA), Dorsal Attention (DA), Default mode, Visual, Frontoparietal, Somatosensory, Limbic and Subcortical) and one between factor (age category) were used to investigate the effects of IFN-β on the graph measures. Greenhouse-Geisser adjusted repeated measures ANOVAs were used when appropriate to account for any deviations. False discovery rate (FDR) was used to correct for multiple comparisons.

Additionally, I aimed to determine if the effects of IFN-β were specific to nodes with high or low Node Strength/Betweenness Centrality as well as potential age effects. To achieve this, I split the data into quartiles and applied a repeated measures linear mixed model.

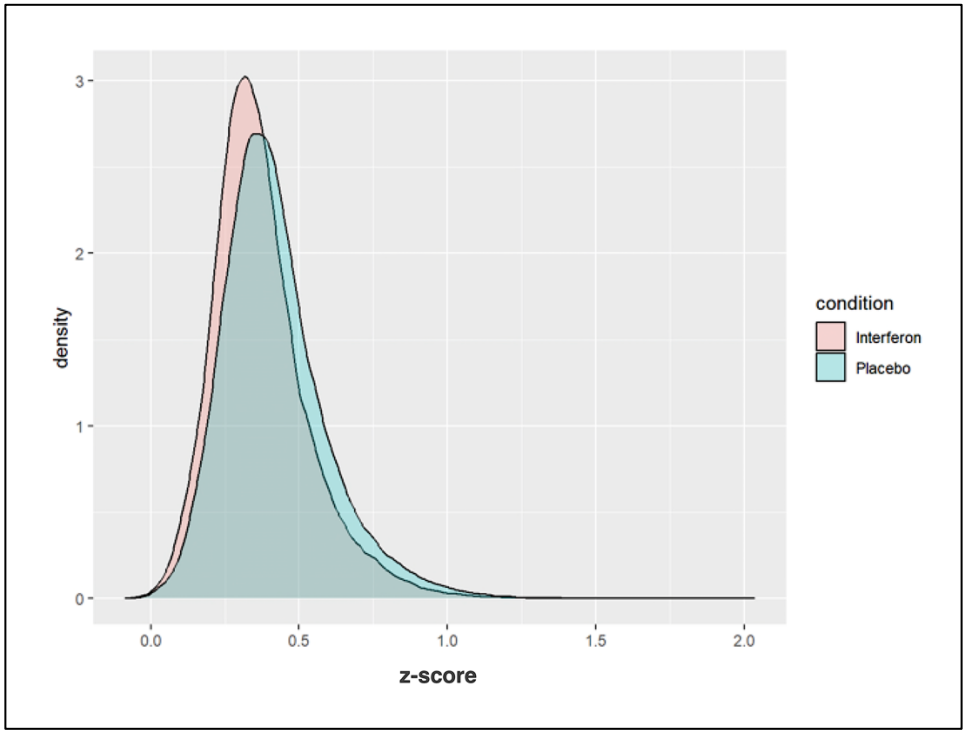
CORRELATIONS BETWEEN GRAPH MEASURES AND BEHAVIOURAL AND CYTOKINE DATA

I explored the relationship between the graph measures sensitive to IFN-β and the individuals' susceptibility to the behaviourally impairing effects of IFN-β. I examined the changes in POMS subscales that were significantly affected by Interferon (tension, fatigue, vigour, and negative and total mood scores) and the SicknessQ scale scores. Finally, I investigated whether any of the graph measures were associated with plasma concentration of cytokines affected by IFN-β (IL-6, TNF-α). Correlation analysis was performed using Pearson's correlation coefficients and FDR was used to control for multiple comparisons.

FUNCTIONAL CONNECTIVITY

I first plotted the distribution of all pairwise correlations (84,050) over all participants for the placebo and interferon conditions. Distributions were mostly positive but significantly different between conditions (Wilcoxon test, V = 1.5123e+12, p<2.2e-16). Thus, compared to placebo, the interferon distribution showed a significantly left-shifted pattern indicating a greater proportion of weaker functional connections **(Figure 32).**

Figure 32. Group mean distribution of pair-wise correlations

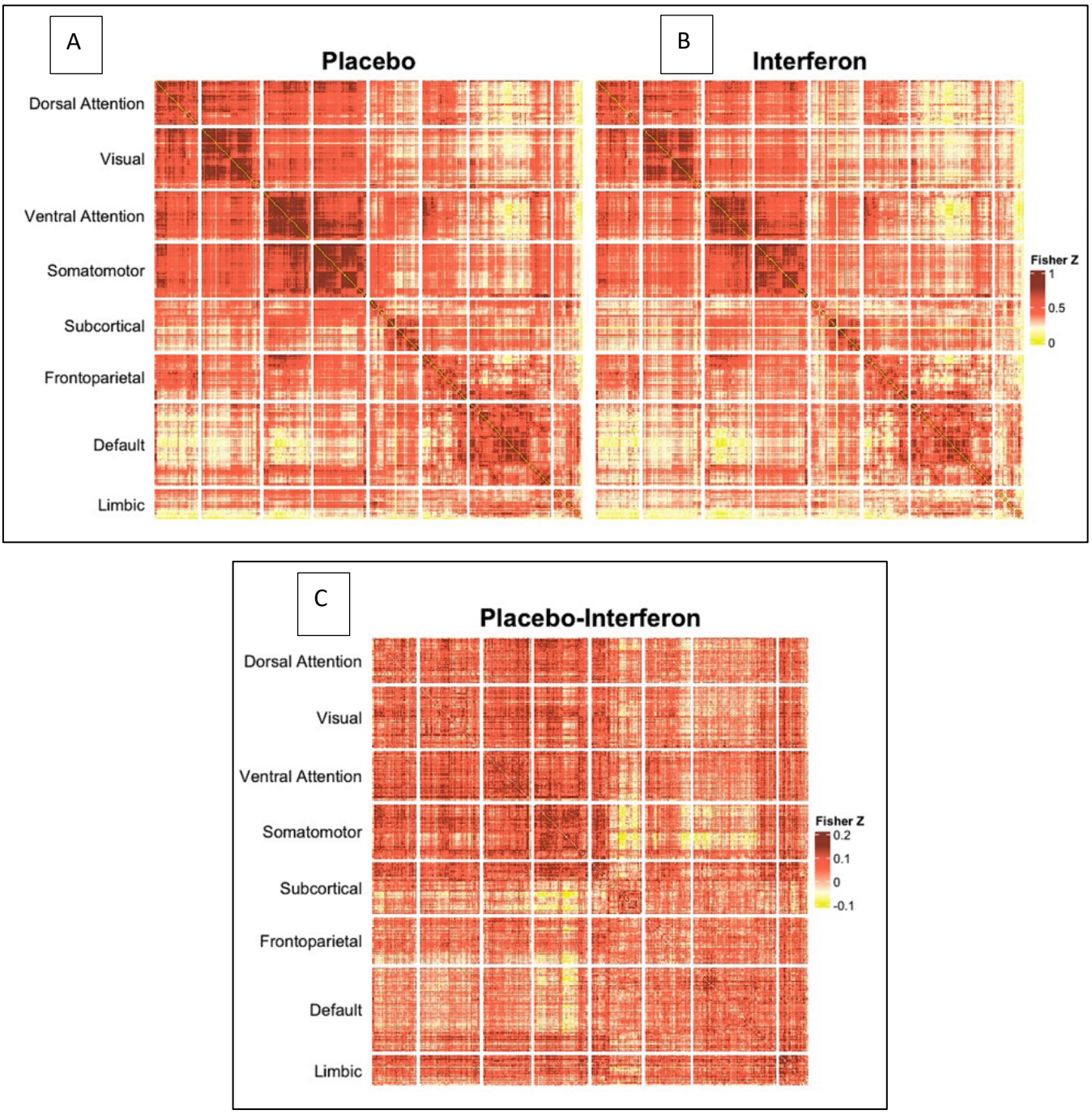


Group mean distribution of pair-wise correlations for placebo (green) and interferon (red) conditions.

To investigate this further, I next averaged, mean inter-areal FC correlation matrices across all participants for both conditions (Placebo/Interferon) based on the pre-defined Yeo and subcortical networks. As anticipated, nodes within the same network generally positively correlated with each other, while more negatively weighted functional connections were observed between some networks, for instance between the Default and VA and DA areas.

As already observed by comparing distributions of connectivity strengths, the difference between conditions (delta: Placebo minus IFN-β) revealed an overall reduction in functional connectivity in the IFN-β condition, though a contrary increase in mean functional connectivity was observed for the IFN condition between subcortical and somatosensory and between default and somatosensory networks **(Figure 33).** None of these effects were significant (no main effect of condition or age or condition x network interaction was observed ($p>0.05$)). However post-hoc analyses revealed a significant effect of condition for the Limbic network with the Limbic area showing a significantly reduced mean functional connectivity to the rest of the brain during IFN-β ($t_{(26)}=3.74$, $p_{FDR}=0.03$).

Figure 33. Functional connectivity matrices



FC matrices for placebo (33A) and IFN-β (33B) averaged across all participants. Figure 33C shows the difference matrix (Placebo-IFN-β) with red and yellow colours respectively representing higher or lower FC at the placebo than at the IFN-β condition. Colour denotes z-scores. The differences between Placebo and Interferon are subtle but observable as indicated by more "yellow "colours (lower values) in the Interferon condition (B) and by the red colours in the difference matrix (C).

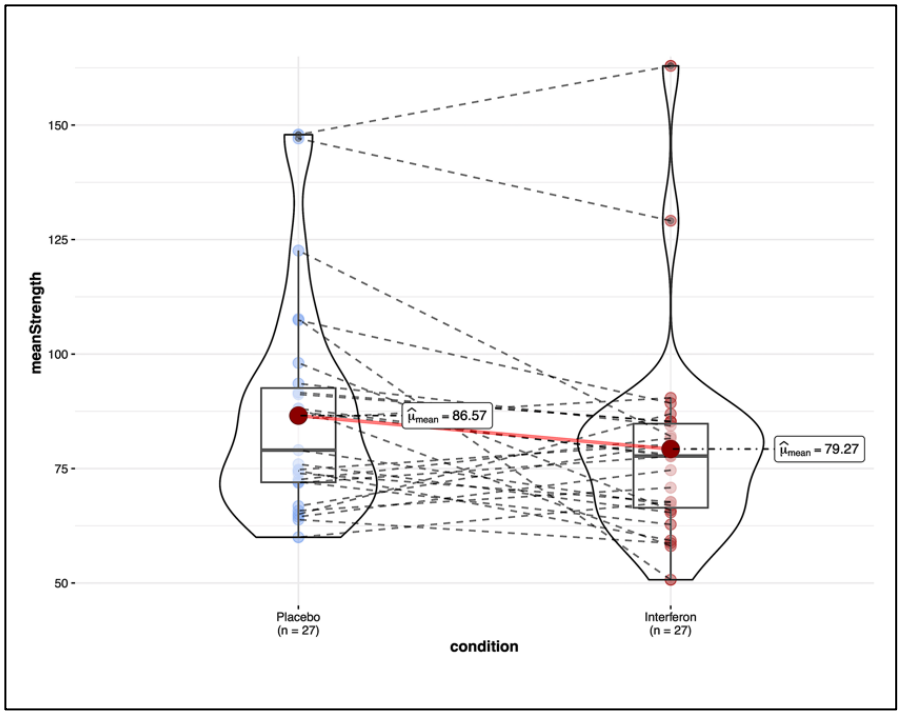
GRAPH MEASURES

To investigate this further, I then applied graph metrics on a functional connectivity matrix with a density threshold of 25%

NODE STRENGTH

Node Strength (number of weighted connections -edges- of each node to the other nodes) repeated measures ANOVA (within factors: condition (plac/IFN-β) and network; between factors: age category) revealed a main effect of condition for Node Strength ($F_{(1,26)}$= 4.76, p=0.038). A significant reduction in Node Strenght was observed for the IFN-β condition **(Figure 34).**

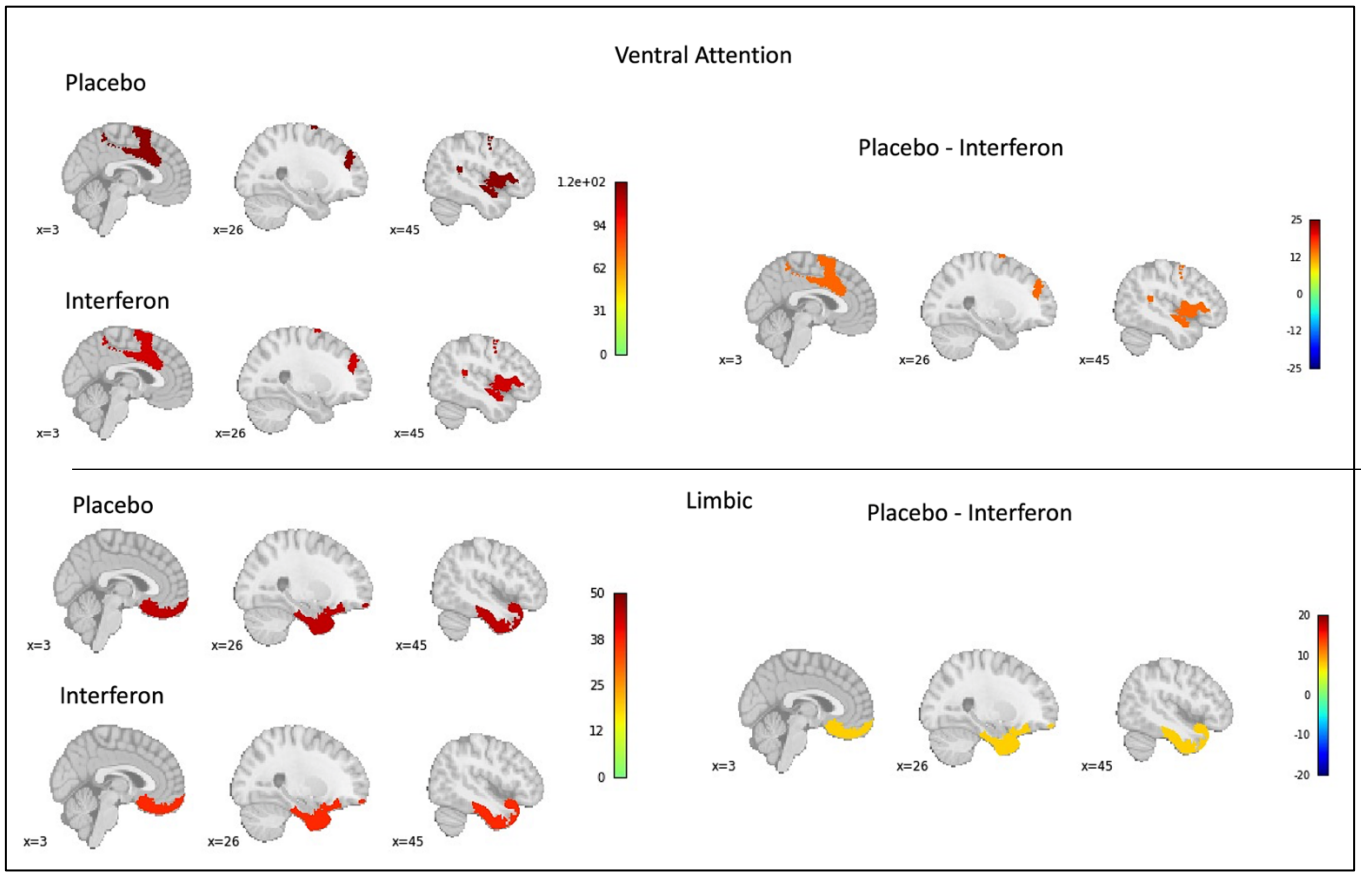
Figure 34. Distribution of Node Strength



Distribution of Node Strength for the Placebo and Interferon conditions. Blue represents placebo, red IFN-β. (meanStrength: mean Node Strength).

No condition x network interactions were found. However, post-hoc analysis revealed a significant reduction in Node Strength during IFN-β within the Limbic ($t_{(26)}$=2.88 p_{FDR}<0.05) and Ventral Attention areas ($t_{(26)}$=2.65, p_{FDR}<0.05) **(Figure 35).** No other interactions were observed.

Figure 35. Group mean Node Strength for Ventral Attention and Limbic networks



Ventral attention (Top) and Limbic (bottom) network maps of group mean Node Strength for placebo and interferon (left) and the map of significant Placebo-Interferon differences (right).

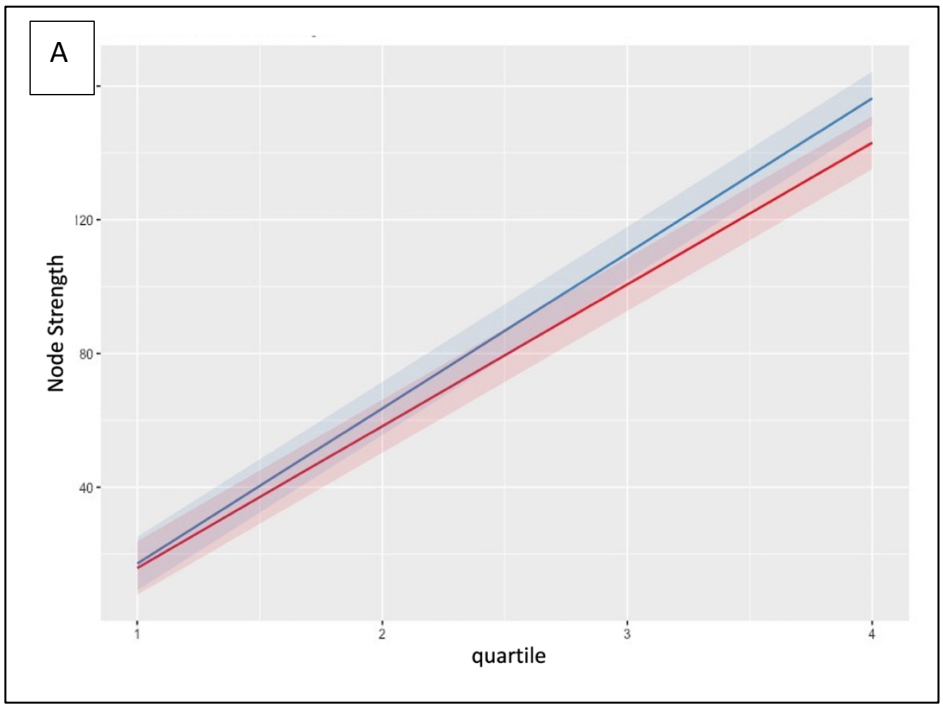
In order to investigate whether the effects of IFN-β were greater for the 'hubs' (highly connected nodes), I split Node Strength distribution into quartiles (1st quartile: low connected nodes, 4thquartile: highly connected nodes) and ran a linear mixed model analysis which included condition, quartile, age and their interactions as predictors for the models.

I observed a significant condition x quartile interaction (Estimate= [2.24], 95% CI= [1.36-3.11], p<0.001). The positive estimate (2.24) shows that as we move from lower to higher quartiles, the effect of condition increases, confirming that the influence of IFN-β significantly varied between nodes of high and low degree and also demonstrated a more pronounced impairing effect of IFN-β on highly connected nodes **(Figure 36A-B).**

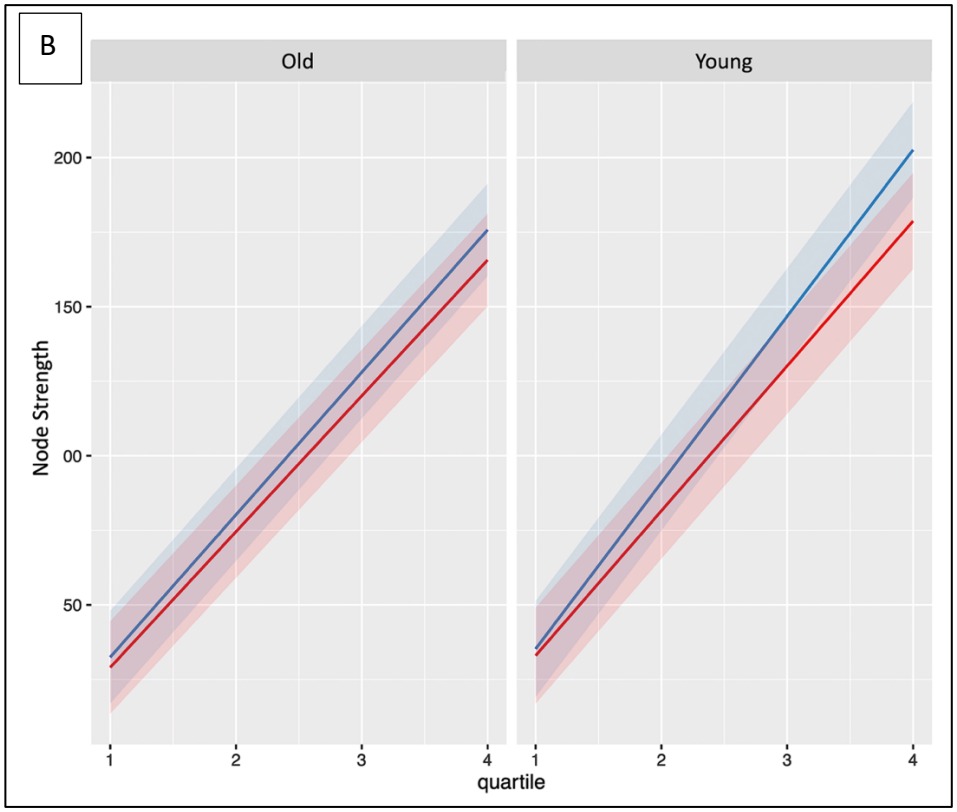
After exploring the effects of IFN-β on highly connected nodes, I further extended the analysis to examine potential interactions and influences of age. I observed a quartile x

age (Estimate= [1.84], 95% CI= [0.94-2.73], p<0.001), a condition x age (Estimate= [-4.9], 95% CI= [-8.36-1.44], p=0.006), and a condition x quartile x age interaction (Estimate= [3.63], 95% CI= [2.37-4.9], p<0.001). Interestingly, the negative estimate (-4.9) for the condition x age interaction suggests that as age increases, the effects of condition tend to decrease rather than increase as we hypothesised **(Figure 36C).** Finally, and also contrary to my hypothesis, the three-way interaction between condition x quartile x age implies the effect of condition on quartile varies depending on age, in other words the effect between condition and quartile changes as age increases. As age increases the effect of condition on highly connected nodes is less pronounced. A detailed description of the linear model fixed and random effects results can be found in **Table 1.**

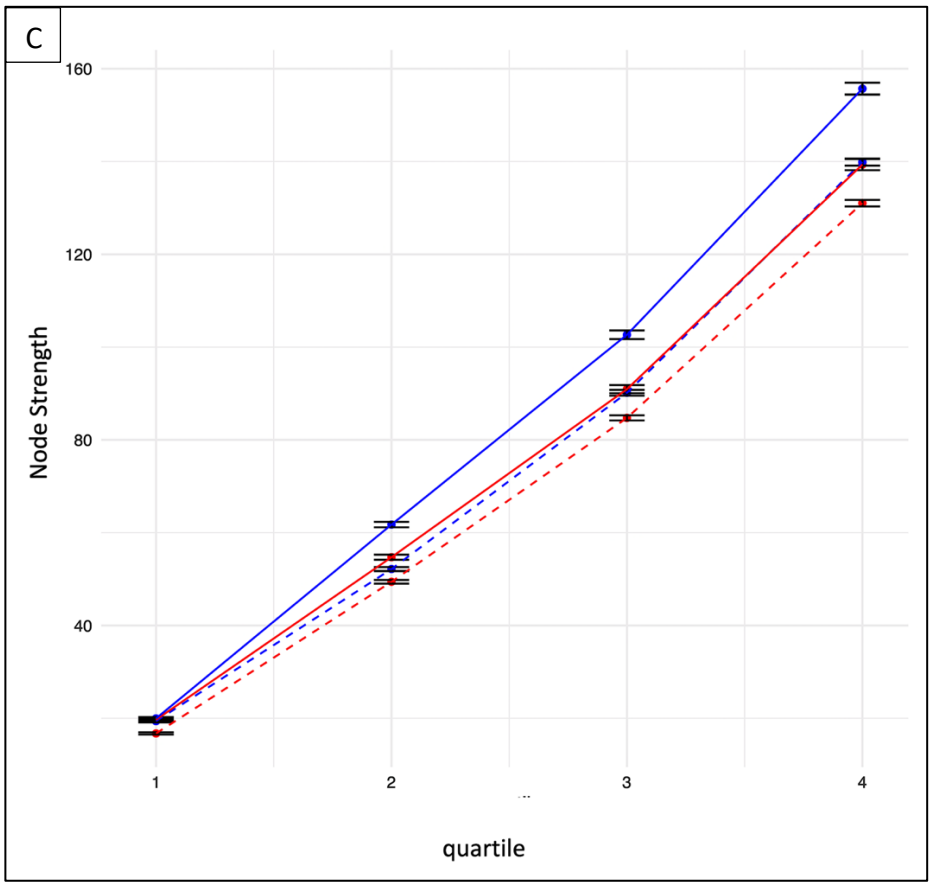
Figure 36. Graphical representation of interaction effects for Node Strength



Node Strength. (A) condition x quartile interaction: The plot demonstrates an increasing trend of the condition impact as we move from lower to higher quartiles ($p<0.001$) (the impairing effect of IFN-β is higher in hubs). Blue line denotes placebo, red interferon.



Node Strength. (B) plot of condition x quartile effects, differentiated by age groups. The graph illustrates the varying intensity of the condition effect across quartiles, showing a clear variation with respect to age ($p=0.006$). Blue denotes placebo, red interferon



Node Strength (C) Plot visualising the condition x quartile x age interaction ($p<0.001$), blue lines denote placebo, red interferon. Dashed lines show older group. Error bars denote SEM.

Table 7. Summary of Linear mixed-effects model for Node Strength

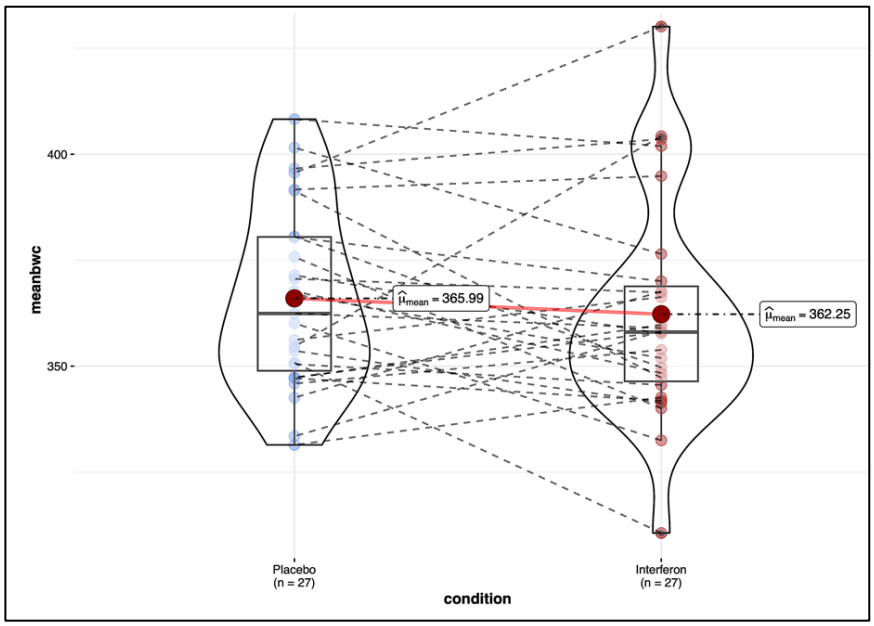
	Node Strength		
Predictors	*Estimates*	*CI*	*p*
Intercept	-27.20	-38.35 – -16.05	**<0.001**
condition	-0.28	-2.68 – 2.11	0.816
quartile	41.51	40.89 – 42.13	**<0.001**
age	1.43	-14.64 – 17.50	0.861
condition x quartile	2.24	1.36 – 3.11	**<0.001**
condition x age	-4.90	-8.36 – -1.44	**0.006**
quartile x age	1.84	0.94 – 2.73	**<0.001**
condition x quartile x age	3.63	2.37 – 4.90	**<0.001**
Random Effects			
σ^2	719.12		
τ_{00}	442.55		
ICC	0.38		
N	27		
Observations	22140		
Marginal R^2 / Conditional R^2	0.683 / 0.804		

The table shows the estimates, CI (confidence intervals) and p values for the fixed effects and the variance components for the random effects as well as intraclass correlation coefficients (ICC). The marginal and conditional R-squared values, indicating the proportion of variance explained by the fixed effects alone and by the entire model, respectively are also presented.

BETWEENNESS CENTRALITY

Betweenness centrality aims to capture the role of nodes as bridge between other groups of nodes **(Figure 37).** The analysis from the repeated measures ANOVA revealed no significant effect of IFN-β on Betweenness Centrality ($F_{(1,26)}$= 1.86, p=0.18) and no interaction effects were detected.

Figure 37. Distribution of Betweenness Centrality

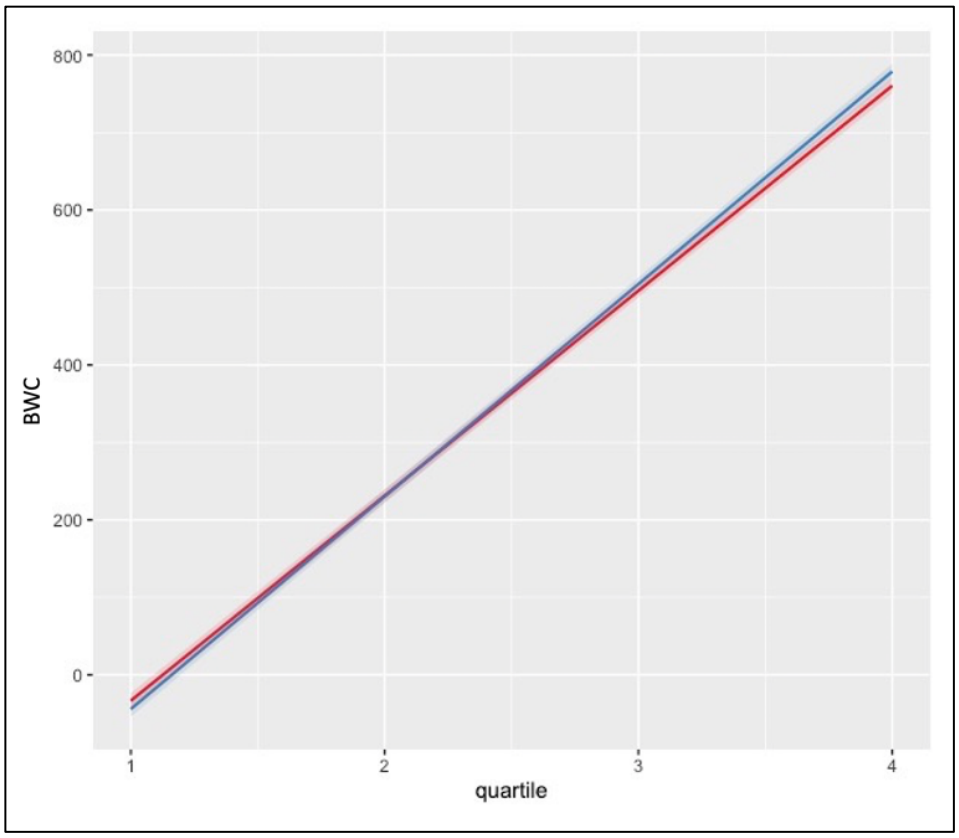


Betweenness Centrality for the Placebo and Interferon conditions. Blue represents placebo, red IFN-β. (meanbwc: mean Betweenness Centrality).

I then conducted an analysis similar to that for Node Strength and ran a linear mixed model after splitting the betweenness centrality scores into quartiles. Here, the 1st quartile corresponds to low Betweenness Centrality while the 4th quartile indicates a higher Betweenness Centrality.

The results showed a significant effect of condition (Estimate= [-25.28], 95% CI= [-45.04- -5.52], p=0.012), and condition x quartile interaction (Estimate= [13.87], 95% CI= [6.64-21.1], p<0.001). The effect of condition varied across quartiles indicating that IFN-β had a greater impairing effect in nodes displaying higher Betweenness Centrality **(Figure 38).** No other interactions were observed. Full linear model is described in **Table 8**.

Figure 38. Condition x quartile interaction for Betweenness Centrality



The plot shows an increased effect of condition in 3rd and 4th quartiles, suggesting a stronger influence as Betweenness Centrality scores rise ($p<0.001$). (BWC: Betweenness Centrality). Blue denotes placebo, red interferon.

Table 8. Summary of the Linear mixed-effects model for Betweenness Centrality

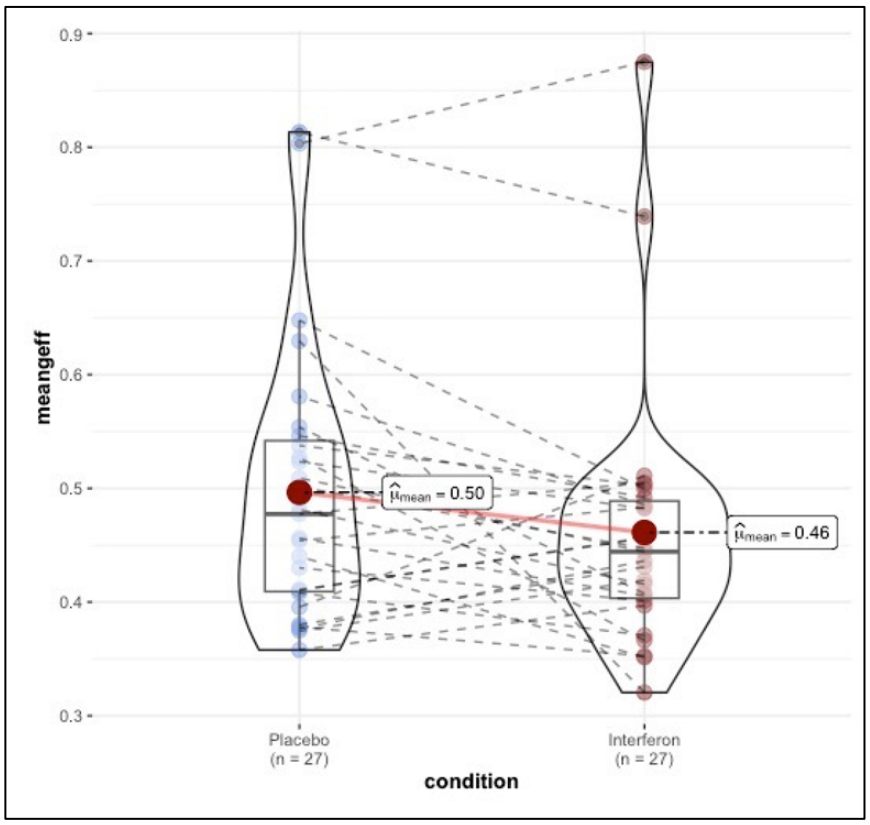
	BWC		
Predictors	*Estimates*	*CI*	*p*
Intercept	-289.48	-306.69 – -272.26	**<0.001**
condition	-25.28	-45.04 – -5.52	**0.012**
quartile	261.89	256.78 – 267.00	**<0.001**
Age	-17.64	-42.45 – 7.17	0.163
condition x quartile	13.87	6.64 – 21.10	**<0.001**
condition x Age	9.88	-18.59 – 38.36	0.496
quartile × Age	5.64	-1.73 – 13.00	0.133
condition x quartile x Age	-8.62	-19.03 – 1.80	0.105
Random Effects			
σ^2	48775.86		
τ_{00}	368.36		
ICC	0.01		
N	27		
Observations	22140		
Marginal R^2 / Conditional R^2	0.649 / 0.652		

The table shows the estimates, CI (confidence intervals) and p values for the fixed effects and the variance components for the random effects as well as intraclass correlation coefficients (ICC). The marginal and conditional R-squared values, indicating the proportion of variance explained by the fixed effects alone and by the entire model, respectively are also presented.

GLOBAL EFFICIENCY

Global Efficiency provides information about whole-brain network efficiency of information exchange in a parallel system. The Repeated measures ANOVA (within factors: condition (plac/IFN-β) and network; between factors: age category) revealed a main effect of condition for Global Efficiency ($F_{(1,26)}$= 8.94, p=0.006). IFN-β significantly reduced Global Efficiency **(Figure 39).**

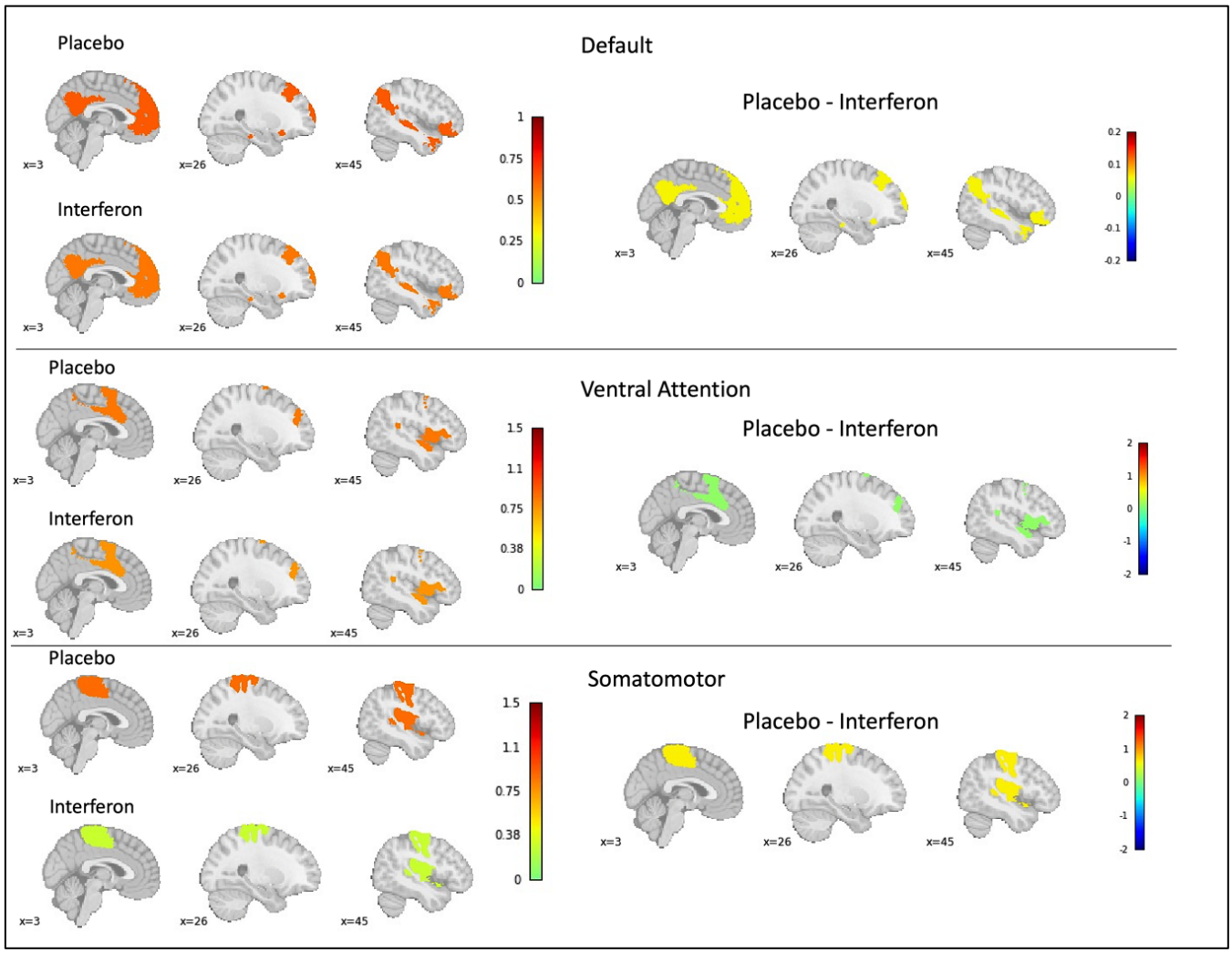
Figure 39. Distribution of Global Efficiency



Global Efficiency for the Placebo and interferon conditions. Blue represents placebo, red IFN-β. (meangeff: mean Global Efficiency).

No condition x network interactions were found. However, post-hoc analysis revealed a significant reduction in Global Efficiency during IFN-β within the Default ($t_{(26)}$=2.57 p_{FDR}<0.05) Ventral Attention ($t_{(26)}$=2.98, p_{FDR}<0.05) and somatomotor areas ($t_{(26)}$=2.7 p_{FDR}<0.05) **(Figure 40).** No other interactions were observed.

Figure 40. Global Efficiency for Default, Ventral Attention, Limbic and Somatosensory networks



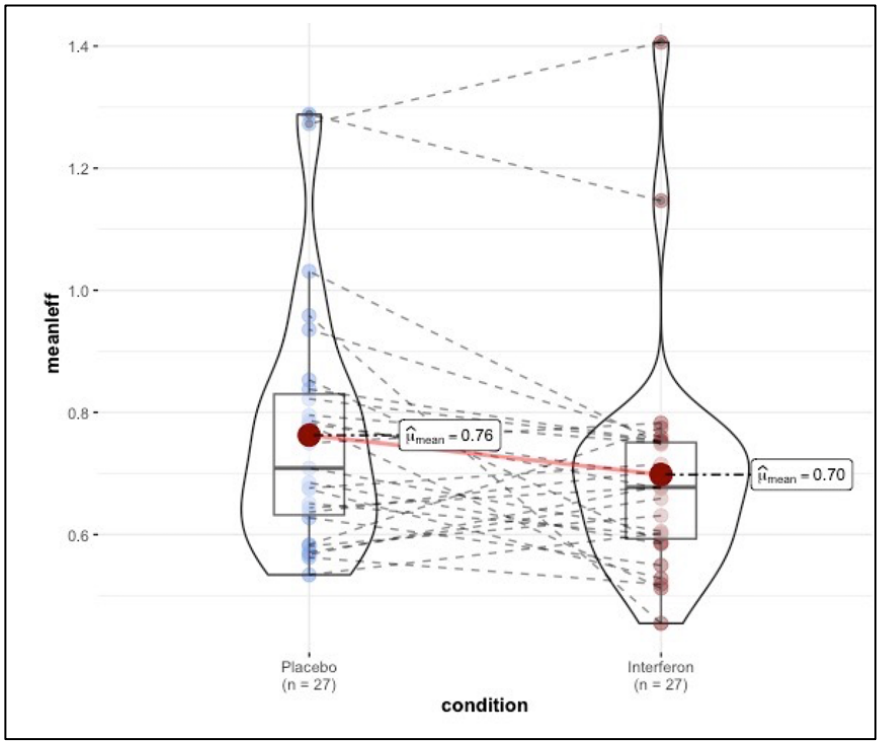
Default (Top), Ventral Attention (middle) and Somatomotor (bottom) network maps of group mean Global Efficiency for placebo and interferon (left) and the map of significant Placebo-Interferon differences (right).

CHAPTER 1 LOCAL EFFICIENCY

Local Efficiency provides an estimation of the significance of individual nodes in facilitating the exchange of information within the network. Similar to Global Efficiency the repeated measures ANOVA indicated a significant main effect of condition ($F_{(1,26)}$= 5.88, p=0.023) pointing to a decrease in Local Efficiency under the IFN-β condition **(Figure 41).** I did not find any condition x network interaction. Local Efficiency was significantly reduced during the IFN-β condition in the Default ($t_{(26)}$=2.23 $p_{(uncorr)}$=0.03), Somatomotor ($t_{(26)}$=2.3 $p_{(uncorr)}$=0.02), Limbic ($t_{(26)}$=2.18 $p_{(uncorr)}$=0.03) and Ventral attention ($t_{(26)}$=2.15

$p_{(uncorr)}$=0.04) networks, however, they did not survive FDR correction for multiple comparisons (p_{FDR}>0.05). No other interactions were observed.

Figure 41. Distribution of Local Efficiency



Local Efficiency for the Placebo and interferon conditions. Blue represents placebo, red IFN-β. (meanleff: mean Local Efficiency)

MAIN EFFECT OF IFN-B ON BEHAVIOUR

The behavioural analysis of the 27 participants revealed significant IFN-β induced changes in the following POMS subscales: total mood ($F_{(1,25)}$=11.05, p=0.003), negative mood ($F_{(1,25)}$=18.29, p<0.001), fatigue ($F_{(1,25)}$=10.08, p=0.004) and tension ($F_{(1,25)}$=8.38, p=0.008). IFN-β significantly reduced global mood and increased negative mood, fatigue and tension. The results from the SicknessQ scale also showed a significant increase in sickness symptoms for the IFN-β condition ($F_{(1,25)}$=7.46, p=0.011).

CORRELATION WITH BEHAVIOURAL MEASURES

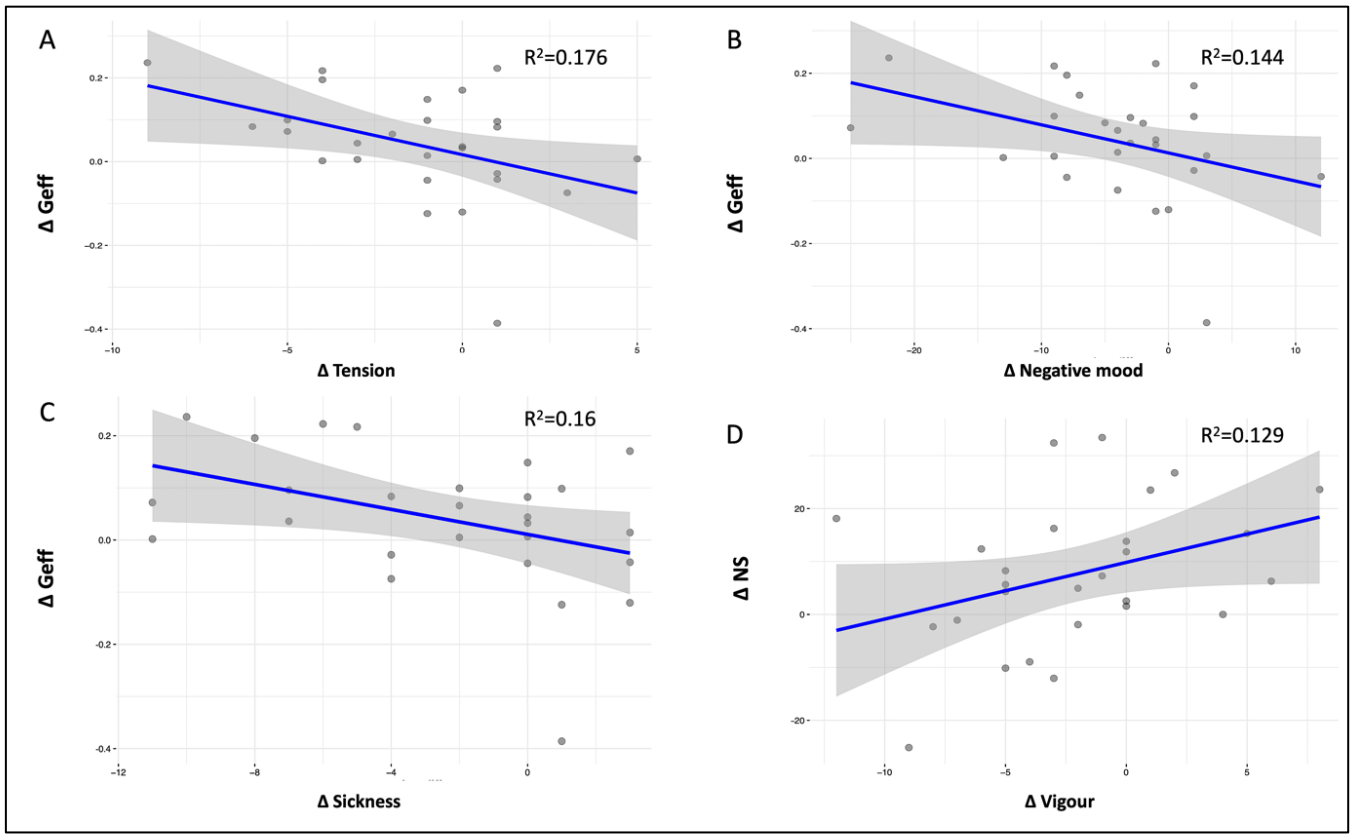
I next conducted an analysis to determine if interferon-induced changes in Node Strength, Global and Local Efficiency were also associated with individual differences in sensitivity to the behaviourally impairing effects of IFN-β. In this investigation, I examined the changes in the scales affected by IFN-β: POMS subscales (vigour, tension, fatigue, confusion, negative and total mood scores) and the SicknessQ scale scores. Additionally, i explored whether these graph metrics correlated with plasma concentration levels of cytokines impacted by IFN-β: IL-6, and TNF-α.

Changes in Global (whole brain) Efficiency did not show any significant correlations with any of the behavioural measures; however, I observed significant negative correlations between Global Efficiency within the limbic network and 2 of the POMS scales: tension (r=-0.42, p=0.02) and negative mood (r=-0.38, p=0.04) as well as for the SicknessQ scale (r=-0.4, p=0.03) **(Figure 42 A-C).** This suggests that an increase in negative mood, induced by IFN-β is concomitant with a reduction in Global Efficiency.

A marginal positive correlation between Node Strength and the POMS subscale vigour was observed as well within the limbic network (r=0.36, p=0.06) (Figure 13D). Increased Global Efficiency was associated with increased levels of vigour

No correlations survived FDR correction for multiple comparisons, however, these results suggest that alterations associated with IFN-β in the limbic network may be one of the underlying mechanisms for the acute shift in motivation induced by IFN-β.

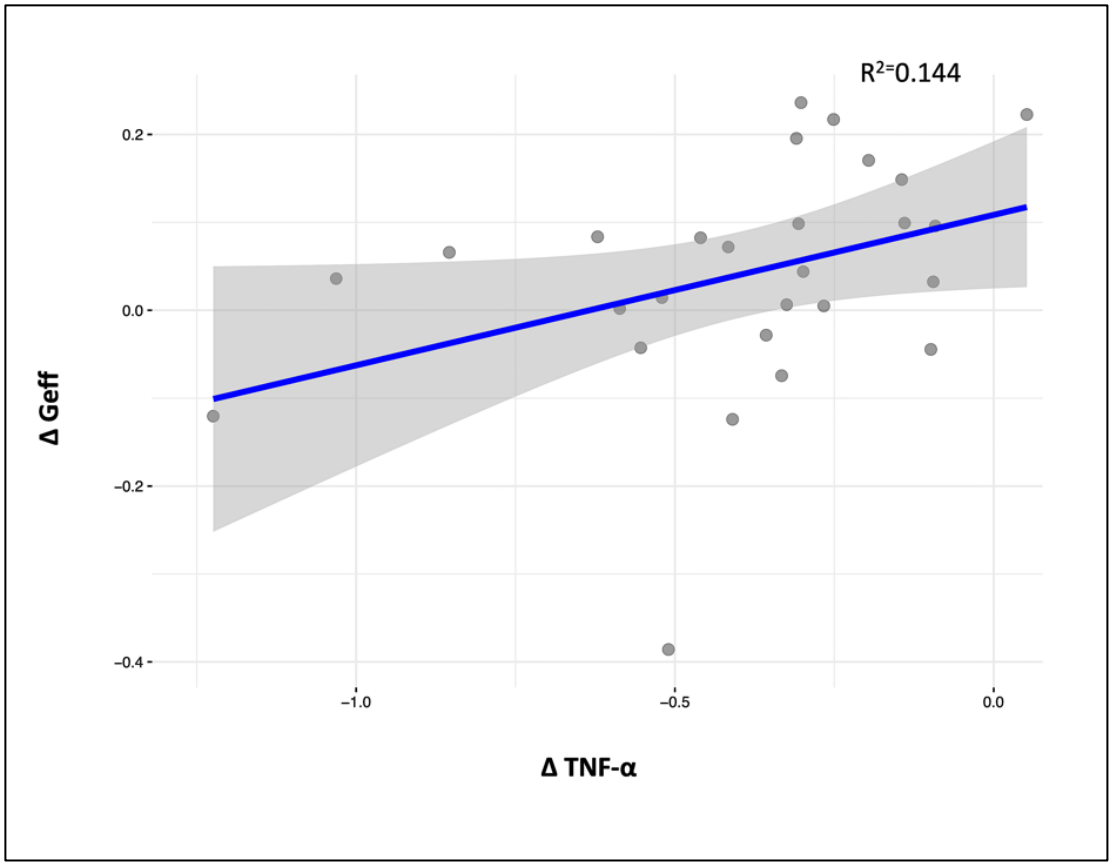
Figure 42. Correlations between graph metrics and behavioural measures for the Limbic network



Between session Δ correlations of graph metrics and behavioural measures for the Limbic Network. (A-B) Global Efficiency correlations with POMS scales: tension and negative mood. (C) SicknessQ scale. (D) Correlation between Node Strength and POMS scale vigour. (Geff: Global Efficiency, NS: Node Strength).

IFN-β significantly increased plasma concentrations of IL-6 ($F_{(1,25)}$=7.76, p=0.01) and TNF-α ($F_{(1,25)}$=17.15, p<0.001), nevertheless no significant correlations between IL-6 and any of the graph measures were found. However, I observed a marginally significant correlation between TNF-α and Global Efficiency also within the limbic network (r=0.38, p=0.051) **(Figure 43).**

Figure 43. Between session Δ correlations of Global Efficiency and TNF-α



DISCUSSION

In this chapter, I used graph-theoretic analysis of rsfMRI to investigate the acute effects of IFN-β on functional network architecture, with a particular focus on efficiency of information transfer. I showed that peripherally administered IFN-β altered the overall whole brain functional architecture (i.e. distribution of connections strength). This resulted in a reduction in mean nodal strength connections, a diminished global capacity for parallel information exchange (Global Efficiency) and a decrease in the efficiency and strength connections of a number of specific subnetworks. Interestingly, I observed that the effects of IFN-β were greater for more highly connected nodes (hubs), and furthermore the intensity of the condition effect in those hubs also varied with respect of age. Additionally, changes at a specific cortical-subcortical resting state network (Limbic network) correlated with concomitant mood and behavioural alterations, suggesting that IFN-β could quickly trigger coordinated behavioural shifts by influencing the organization of these brain areas.

I applied graph metrics on a functional connectivity matrix thresholded with a low *K* value of 0.25 and, in line with our prior hypothesis and evidence (Dipasquale et al., 2016), IFN-β led to a rapid reduction in global network efficiency (an index of parallel information transfer) and in mean Node Strength (number of weighted connections of each node to the other nodes). Importantly and consistent with my prediction, this effect was stronger for highly connected nodes (hubs). Hubs possess substantial topological value; however, this comes at an additional biological expense such as elevated blood flow and glucose metabolic rates. These metabolic demands may increase their vulnerability to pathological factors and disease processes (Crossley et al., 2014). Moreover, studies in neurodegenerative diseases like Alzheimer's demonstrate that highly connected nodes that bridge functionally specialized areas appear to be more selectively impacted in Alzheimer's patients (Buckner et al., 2009).

Contrary to our hypothesis, no main effect of IFN-β was found in betweenness centrality (an index of how central a node is within the whole-brain network). Interestingly, when I split the betweenness centrality scores into quartiles, we did observe a condition x quartile

interaction. Nodes displaying high betweenness centrality seemed to be more affected by the impairing effects of IFN-β. These findings are consistent with the study findings regarding Node Strength and the greater impact IFN-β has on high-degree nodes. Hubs or high-degree nodes are characterized by their numerous connections with other nodes and due to their multiple connections, they also frequently display high betweenness centrality as they serve as crucial links or bridges between different parts of the network. While hubs are central in brain communication and integration, centrality may make them vulnerable and susceptible to disconnection and dysfunction under conditions such as inflammation (van den Heuvel & Sporns, 2013).

The results of this study are the first to show that the acute administration of IFN-β to healthy individuals quickly compromises global network efficiency and weighted node degree, with age influencing some of these outcomes.

I observed that age modulated the effect of IFN-β in high and low-connected nodes however, contrary to my hypothesis the effect of IFN-β in highly connected nodes was less pronounced in older participants compared to their younger counterparts. While high connectivity hub regions appeared to be more selectively targeted in the young, IFN-β showed a more global impact on network connectivity in the older adult group. Two potential explanations for these findings: (i) Reduced metabolic activity associated with age (Cunnane et al., 2011) could potentially lead to less pronounced differences between low and highly connected nodes, in terms of metabolic demands which might subsequently manifest as a more widespread impact. (ii) the structural and functional changes that occur throughout the brain as individuals age which may lead to a decrease in network specialization and an increase in network integration (with overall brain networks becoming less segregated and more interconnected globally) (Crowell et al., 2020; Deery et al., 2023; T. Zhao et al., 2015). As a result of these topological and metabolic changes with age, the main effect of inflammation might be more distributed across the whole network rather than focused on specific hubs, potentially leading to a more global impact on network connectivity in healthy older individuals.

Additionally, the study highlights particular networks vulnerable to the impairing effects of inflammation and their potential link to related disruptions in mood and behaviour. To identify specific networks sensitive to the acute effects of IFN-β, I computed graph metrics for eight parcellated resting state networks.

Following IFN-β several networks showed a statistically significant reduction (FDR corrected): Default and Somatomotor networks displayed reduced parallel information transfer (Global Efficiency). The limbic network showed a decrease in the number of connections (Node Strength), while the ventral attention network was particularly affected as showed by both Node Strength and Global Efficiency reduction after IFN-β administration.

Altered connectivity in the ventral attention as well as limbic networks has been already found associated with inflammation (Felger et al., 2016; Lekander et al., 2016; Nusslock et al., 2019). Evidence from a meta-analysis study has also shown that changes in the ventral attention network have been observed following LPS administration (Kraynak et al., 2018). The ventral attention network also known as the 'salience network', is mainly comprised of the Temporo-parietal junction, middle and inferior frontal Gyrus and the insula and is characterised by abundant connections between cortical and subcortical regions (Ongür & Price, 2000). It may play a significant role in identifying survival-relevant environmental cues as well as predictive regulation of internal physiological functions (Barrett & Simmons, 2015; Ginty et al., 2017; Hermans et al., 2011). In this context, peripheral inflammation could serve as a crucial physiological mediator, transferring visceral signals from and to the ventral attention network and potentially modulating its activity (Barrett & Satpute, 2013; Critchley & Harrison, 2013).

The limbic network is also known as 'the emotion regulation network' and mainly comprises the amygdala, hippocampus, ventromedial prefrontal cortex, orbitofrontal cortex and anterior and posterior cingulated cortex. Animal studies have already suggested the involvement of the limbic network in the interplay between the brain and inflammation (Haas & Schauenstein, 1997). In humans, associations between higher levels of peripheral inflammatory markers in the Limbic network have been found in young

adults (Nusslock et al., 2019) whereas further evidence has reported a correlation between increased IL-6 levels and reduced Limbic network connectivity in healthy older adults (Walker et al, 2020).

In general, changes in the functional viscerosensory activity in these limbic areas are believed to modulate the effects of inflammation on sickness behaviour and associated motivational and mood alterations (Capuron & Miller, 2011; Dantzer, 2014; Harrison et al., 2009). In line with this and supporting the functional significance of the results on the effects of IFN-β in the Limbic network, I observed significant correlations (at uncorrected p-values) between changes in Global Efficiency and IFN-β-induced sickness behaviour and mood alterations. Participants who experienced the most severe mood disturbances following IFN-β administration also showed the most significant decrease in limbic network connectivity. Remarkably the most impact was observed in Global Efficiency, which evaluates the information processing capacity of the network. Changes in Global Efficiency showed significant associations with two POMS subscales including negative mood, and tension as well as with the SicknessQ scale, a global measure of sickness behaviour. Altogether, these results underly and further support the significance of changes in limbic activity in association with the behaviourally impairing effects of IFN-β.

There are a number of limitations to this study. (i) The model focuses solely on the acute effects of IFN-β on functional brain network architecture, therefore I cannot ascertain whether the acute changes observed may evolve or resolve following chronic exposure to IFN-β. (ii) I adopted a robust repeated-measure within-subject study design, however, there is a risk this approach was not powerful enough to detect smaller-size interaction effects. The study was powered to detect medium interaction effects (f=0.3). Following up on these results in a larger sample in future research could increase statistical power and the capacity to potentially detect more nuanced effects. (iii) Head motion during the scanning process is an important source of bias in fMRI connectivity analysis. I employed a robust and well-validated data processing pipeline for movement correction and all data underwent standard quality control for head motion prior to statistical analysis. (iv) The criteria applied for brain parcellation, which determines the number of nodes and thresholds set to identify connections (edges) can significantly impact the results. In order

to minimise that, I used the Glasser (Glasser et al., 2016) and Yeo (Yeo et al., 2011) atlases, two commonly used and well-implemented parcellation maps that have been designed to provide neuroanatomical precision for studies of the structural and functional human brain and can allow data comparison with other studies using graph theory approaches. (v) Furthermore, I applied graph metrics on a functional connectivity matrix thresholded with a low *K* value of 0.25 (25% density), which both preserved only the strongest functional connections and enabled highly efficient parallel information processing at a low wiring cost. In this manner, I was able to prevent the possibility of excessive fragmentation of the network that could happen at extremely low costs and at the same time, I also managed to regulate potential network randomness that could occur at higher k values.

In summary, this study is the first to show that peripherally administrated IFN-β rapidly induced alterations in the overall structure of the brain network, impairing global functional connectivity and parallel information exchange efficiency as well as particularly affecting efficiency within specific subnetworks.

The findings presented here align with evidence from previous research exploring topological features estimated by graph theory within the domain of inflammation. For instance, Dipasquale et al. (2016) reported a decreased node degree and Global Efficiency associated with IFN-α treatment in patients with Hepatitis C while Kitzbichler et al. (2021) identified reduced hubness within the DMN in the context of peripheral inflammation and its association with depressive symptoms. The results from this study, along with previous evidence presented in this thesis, further validate IFN-β as a model of acute experimental inflammation and demonstrate its capacity to induce temporary alterations in the brain's functional architecture.

Additionally, A novel finding was that the impact of IFN-β was greater on highly connected and central nodes and the intensity of the condition effect in these hubs also varied based on age. Moreover, changes observed within the limbic network were linked to concomitant mood and behavioural alterations suggesting that IFN-β could quickly trigger

coordinated behavioural shifts by affecting the organization of the cortical-subcortical connections within the limbic network.

Future work could include coupling the present protocol with for instance magnetization transfer measurements of microstructure in an attempt to elucidate how differences in local, biophysical properties of the brain tissue associated with inflammation, may correlate with broader alterations in functional connectivity between cortical and subcortical nodes and within specific brain networks. Furthermore, future designs could be complemented by integrating data from the Allen Human Brain Atlas (AHBA). Incorporating AHBA would allow for a multimodal analysis approach, combining gene expression, and functional and anatomical data. The integration of genetic and connectivity data may enhance the development of predictive models regarding the actions of inflammation on the brain, potentially identifying age or condition-specific vulnerabilities or resilience factors.

CHAPTER 7. ACUTE IMMUNE-RESPONSE SIGNATURES TO IFN-A THAT PREDICT THE DEVELOPMENT OF FATIGUE

INTRODUCTION

Interferon-α (IFN-α) is a key mediator of antiviral immune responses used to treat Hepatitis-C virus (HCV) infection. Though clinically effective, chronic IFN-α administration frequently induces severe sickness responses, including functionally impairing fatigue and major depressive episodes (Capuron et al., 2002; Capuron & Miller, 2004; Maddock et al., 2005). To date, many studies have focused on mechanisms of depression, which typically emerges 4 to 8 weeks after IFN-α treatment onset and affects approximately a third of patients (Whale et al., 2019). However, fewer studies have focused on fatigue which emerges far more quickly, usually within hours of the first injection and affects almost all treated patients (Capuron et al., 2002; Dowell et al., 2016). Furthermore, fatigue is frequently cited as one of the most functionally impairing features of IFN-α-based therapy and can persist even after treatment completion (Russell et al., 2019)

Traditionally, the study of fatigue has been split into central (brain) and peripheral (neuromuscular) mechanisms (Chaudhuri & Behan, 2004; Greenhouse-Tucknott et al., 2022). IFN-α has been implicated in both processes. Regarding central mechanisms, type-I interferons have been shown to enter the brain by saturable transport systems at the blood brain barrier (BBB) (Banks, 2005; Banks & Erickson, 2010) and IFN-α is readily detected in the cerebrospinal fluid (CSF) of humans undergoing systemic IFN-α treatment for Hepatitis-C infection (Raison et al., 2009). In rodents, intra-peritoneal administration of IFN increases neuronal expression of STAT1 (signal transducer and activator of transcription 1), a common pathway in IFN signalling, within hours of administration (Wang, 2009; Wang & Campbell, 2005), and has been shown to modulate neuronal firing rates within the hypothalamus and other deep brain grey matter structures (Dafny et al., 1996). Supporting this, human neuroimaging studies of patients receiving IFN-α have demonstrated changes in basal ganglia glucose metabolism (FDG-PET), spectroscopic indices of neuronal activity (glutamate/glutamine concentrations) and tissue

microstructure, each of which correlates with IFN-induced changes in fatigue or motivational state (Capuron et al., 2007, 2012; Dowell et al., 2016, 2017; Haroon et al., 2014). Taken together, these findings suggest that one of the mechanisms through which IFN-α may alter brain function is by crossing the BBB and inducing transcriptional changes, particularly within deep grey matter structures, such as the basal ganglia. However, how these IFN-mediated changes in neuronal gene expression and function relate to fatigue remain to be determined.

Regarding peripheral mechanisms, interferon-induced transcriptional changes in muscular tissue have also been implicated in peripheral fatigue, particularly in the context of cancer or cancer-treatment-associated fatigue. A molecular pathway particularly implicated in cancer fatigue is the 'mammalian target of rapamycin' (mTOR) pathway, which plays an important role in cell growth, survival and proliferation, protein synthesis, transcription and translation and ribosomal biogenesis (Dowling et al., 2010) and has been shown to translationally regulate Type-I interferon responses (Ivashkiv & Donlin, 2014; Livingstone et al., 2015). mTOR inhibitors show potent anticancer properties, however, they are also commonly associated with severe cancer treatment-related fatigue (Peng et al., 2015). In rodents, the mTOR pathway has been linked to healthy muscle structure, mass and composition (Maiole et al., 2019; Risson et al., 2009) and its ability to modulate ubiquitin proteins, particularly Ubiquitin-40S Ribosomal protein S27a (RPS27A) which has been specifically linked to cancer treatment-induced skeletal muscle weakness and atrophy (Sakai et al., 2020). IFN-α can also induce a rare, non-inflammatory myopathy that is also thought to relate to its catabolic effects (Stübgen, 2009). Together, these studies suggest a potential association between IFN-α-induced transcriptomic changes, particularly activation of mTOR and the experience of fatigue.

Ascertaining the molecular mechanisms by which IFN-α induces fatigue in both brain and muscle tissue in humans is challenging due to lack of non-invasive methods. Consequently, our understanding of IFN-α induced fatigue remains limited. Whole blood transcriptomics may provide a minimally invasive approach for inferring brain transcriptional effects based on changes in peripheral blood gene expression, given that some genes have shown correlated gene expression between blood and brain (Basu et al., 2021; Hess et al., 2016;

Qi et al., 2018; Sullivan et al., 2006; Tylee et al., 2013). However, the gene expression of most peripheral blood transcripts is not correlated with brain gene expression, and thus there is a pressing need to discover peripheral blood transcriptomic biomarkers that are associated with fatigue and provide insights into the underlying molecular mechanisms.

In this exploratory prospective cohort study 27 HCV patients initiating IFN-α based therapy completed whole blood transcriptomic analyses at baseline and again 4½ hours after their first IFN-α injection. Patients were followed up with regular assessment of fatigue and sickness response to determine whether we could identify acute blood-based response signatures to IFN-α that could be associated with the development of severe fatigue 4 weeks after initiation of treatment.

MATERIALS AND METHODS

PARTICIPANTS

Twenty-seven patients (21 male, mean 50.4 +/- 11.1 years) with Hepatitis-C due to commence combination antiviral therapy with IFN-α and ribavirin were recruited for this study. Participants were aged 30-68 years, fluent in English and fulfilled NICE guidelines for initiating IFN-α based therapy. Participants' current mental state and previous psychiatric history were evaluated at baseline using the Mini-International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998). Exclusion criteria included history of psychotic illness or autoimmune disease, treatment for depression at study enrolment, if they had not abstained from substance abuse for at least 6 months, were co-infected with human immunodeficiency virus (HIV) or had any cause for liver disease other than Hepatitis-C. Ethical approval was granted by the Cambridge Central National Research Ethics Committee (12/EE/0491) and all individuals provided written informed consent.

STUDY DESIGN

In this exploratory prospective cohort design, participants were evaluated at baseline (mean 7 days before treatment), 4½ hours after their first IFN-α injection and 4, 8 and 12 weeks into IFN-α based treatment. Blood samples for whole-blood mRNA analysis were collected in PAXgene Blood RNA tubes (PreAnalytiX, Switzerland) at baseline and 4½ hours after the first IFN-α injection (but before the first dose of ribavirin) following standard protocols. Fatigue symptoms were assessed at each visit using a fatigue Visual Analogue Scale (fVAS) (Gift, 1989). The Hamilton Depression Rating Scale (HAMD) (Hamilton, 1960), State and Trait Anxiety Inventory (STAI) (Spielberger, et al., 1983) and the Epworth Sleepiness Scale (ESS) were also completed to describe the broader psychological response to IFN-α based therapy. Demographic data are summarized in **Table 9.**

Table 9. Hepatitis C patients demographic data.

	Baseline	4-Wks	*P* value
Age (years)	50.4±11.3		
Sex			
Male	21		
Female	6		
f-VAS	29.9±26.3	61.2±29.4	<0.001
HAM-D	5.96±7.00	13.6±7.59	<0.001
STAI	30.4±7.40	38.4±13.7	<0.005
ESS	5.85±3.37	9.18±4.48	<0.005

Data represent mean ± standard deviation. fVAS: Fatigue Visual Analogue score; HAM-D: Hamilton Depression Rating Scale; STAI: State and Trait Anxiety Inventory; ESS: Epworth Sleepiness Scale.

BEHAVIOURAL ANALYSIS

Effects of IFN-α on fatigue symptoms, defined as the difference in fVAS between baseline and 4 weeks after starting treatment, were used as the primary outcome variable. The fVAS was designed as a 100-mm-long horizontal line with two vertical anchoring lines labelled 'no fatigue' and 'extremely fatigued' at the left (0 mm) and right ends (100 mm) respectively. Participants were asked to rate their fatigue level by marking the point on the line that best represented their current perception of fatigue.

Effects of IFN-α on fatigue symptoms were analyzed in R (v4.0.2) using repeated measures ANOVA with Holm correction for multiple comparisons. Pearson's correlation analysis of IFN-α induced gene expression and fatigue symptoms was performed in R (v4.0.2) and used to predict IFN-α induced fatigue 4 weeks after initiation of treatment.

RNA ISOLATION AND TRANSCRIPTOMICS ANALYSIS.

Isolation of total RNA was performed using the PAXgene blood mRNA kit following the manufacturer's protocol (PReAnalytix, Hombrechtikon, CHE). RNA quantity and quality were assessed using the A260/280 and A260/230 ratios and evaluated using a Nanodrop spectrophotometer (NanoDrop Technologies, Delaware, USA). Samples were stored at -80 °C. RNA integrity number (RIN), assessed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), was in the range of 7–10.

Microarray assays were performed following the protocol described in the Affymetrix GeneChip Expression Analysis technical manual (Affymetrix, California, USA). Briefly, 250 ng RNA were used to synthesize cDNA with the Ambion WT Expression Kit (ThermoFisher Scientific), which was then purified, fragmented, labelled, and hybridized onto Human Gene 1.1 ST Array Strips (Affymetrix). Gene expression data visualization and quality control were assessed using Partek Genomics Suite V6.6. Probe set normalization and summarization were computed using Robust Multi-Array (RMA) algorithm. Quality control and batch effects were assessed using the Principal Component Analyses (PCA). No outliers or batch effects were observed. Genes modulated by IFN-α (expression profile of the sample at 4½ hours after the first injection minus the profile at baseline) were

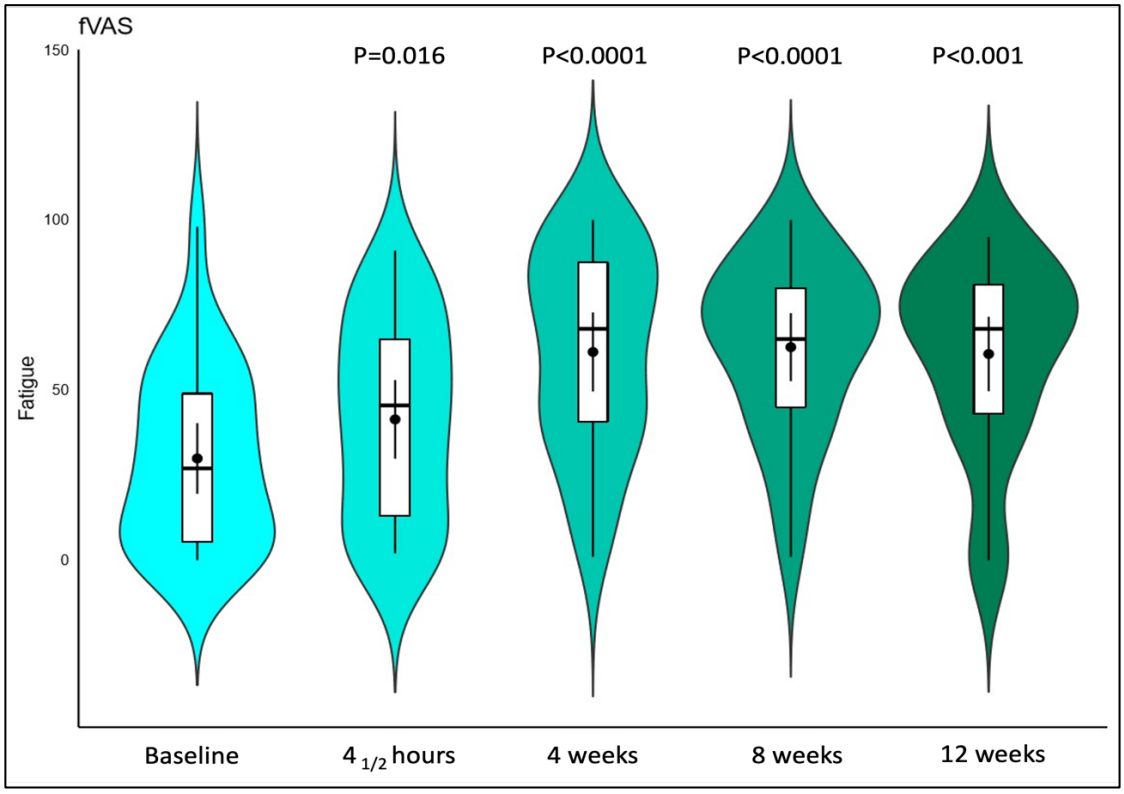
identified with fold changes (FC) of >1.2 and <-1.2 and p<0.05 (uncorrected). This less stringent cut-off was used in order to prioritize genes modulated by INF-α and to conduct protein-protein interactions and pathway analyses by uploading gene lists that encompasses a sufficient quantity of genes.

Genes identified as differentially expressed (FC >1.2 and <-1.2 and uncorrected p<0.05) and genes correlating with an increase in fatigue at 4 weeks (uncorrected p<0.05) were then imported into STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins, https://string-db.org/) and used to identify protein-to-protein interaction (PPI) networks that showed (1) a significant main effect of IFN-α or (2) were significantly associated with an increase in fatigue at 4 weeks respectively. Functional enrichment and network analysis in STRING were restricted to data from experimental, co-expression and protein homology studies (i.e., weaker data from text-mining were excluded) and the highest confidence rating was used (i.e. >0.9). STRING uses a graph-theoretic approach to provide statistical control (i.e., whether the complexity (number of edges) of observed networks of functionally-interconnected transcripts are likely to arise through chance alone). Finally, Ingenuity Pathway analysis (IPA) was used to identify which upstream regulators may underpin the set of transcripts found to significantly predict IFN-alpha induced-fatigue (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA). STRING and IPA p-values were computed at an uncorrected level.

FATIGUE SYMPTOMS

IFN-α significantly increased fatigue symptoms (fVAS: $F_{(4,104)}$=15.96, p<0.001) from a mean of 29.9±26.25 at baseline to a peak mean of 62.62±25.24 at 8 weeks. The increase in fatigue was rapid with a significant effect already observed at $4_{1/2}$ hours (t_{26}=11.46, p=0.016) and significant increases also observed at 4 (t_{26}=31.27, p<.0001), 8 (t_{26}=32.70, p<0.001) and 12 weeks (t_{26}=30.68, p<0.001) (**Figure 44**).

Figure 44. Fatigue response to IFN-α



Violin plot illustrating data distribution, mean, median, interquartile ranges and standard deviation of the fatigue Visual Analogue Scale (fVAS) at baseline, 4 ½ hours, 4, 8 and 12 weeks after initiation of IFN- α based therapy. P values relate to comparison with baseline values.

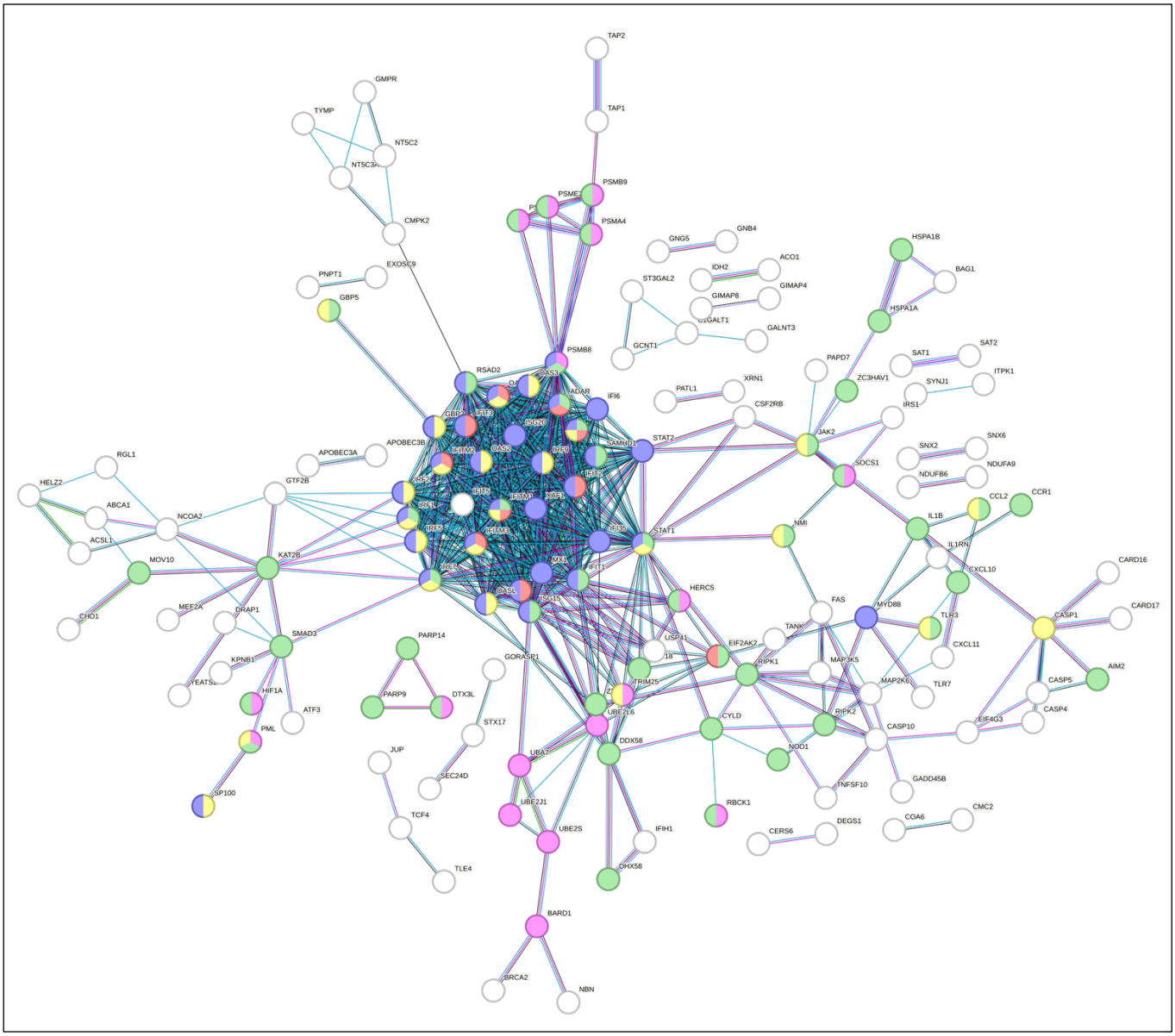
MAIN EFFECT OF INTERFERON-A ON GENE EXPRESSION

Genes modulated by IFN-α were identified by comparing the expression profile of the sample at 4½ hours after the first injection with the profile at baseline. IFN-α modulated 754 genes, 526 were upregulated (at an uncorrected $p<0.05$; FC >1.2) and 228 were downregulated (at uncorrected $p<0.05$; FC< -1.2).

STRING was then used to explore protein-to-protein interactions (PPI) and perform functional enrichment analysis of the upregulated genes. The PPI network generated by STRING consisted of 515 nodes and 654 edges with an average node degree of 2.54 (average number of connections per node) and average clustering coefficient of 0.213 (the degree to which nodes in the graph tend to cluster together) which are higher than expected by chance alone. The number of edges was significantly larger than expected for a random network of the same size ($p<1x10^{-16}$) (**Figure 45**).

The GO biological processes that were most enriched among genes coding proteins in this network were: type-I interferon signalling (ID: 0060337; FDR $p=7.00 \times 10^{-24}$), response to Interferon-α (ID: 0035455; FDR $p=8.17 \times 10^{-8}$), response to Interferon-gamma (ID: 0034341; FDR $p=7.22 \times 10^{-20}$), protein ubiquitination (ID: 0016567; FDR $p=3.3 \times 10^{-5}$) and regulation of the immune system process (ID: 0002682; FDR $p=8.02 \times 10^{-15}$).

Figure 45. Main effect of IFN-α



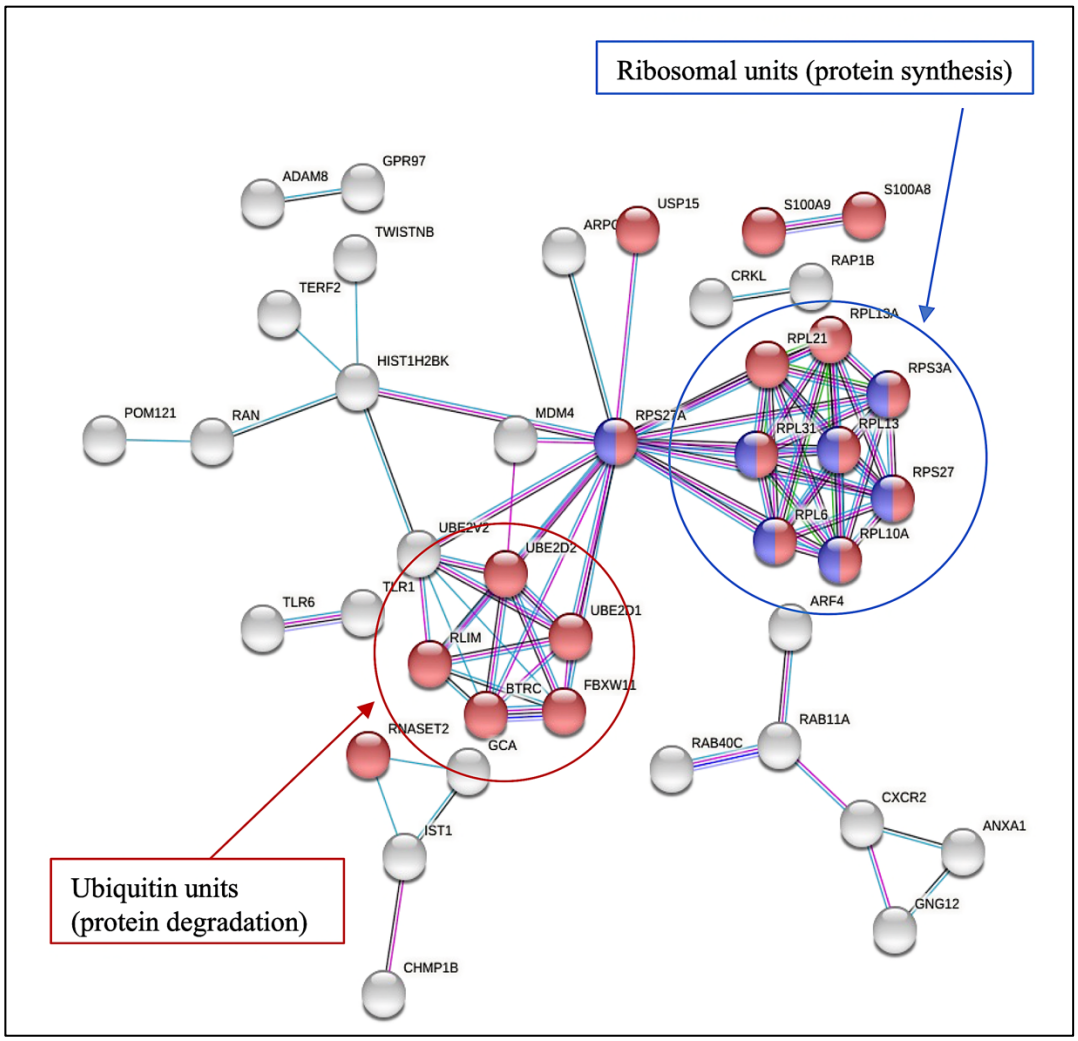
The PPI network generated 515 nodes and 654 edges with significantly more interactions than expected by chance alone ($p < 10^{-16}$). Line color indicates the type of interaction evidence (light blue: from curated databases, purple: experimentally determined, green: gene neighbourhood, red: gene fusions, dark blue: gene co-occurrence, black: co-expression, lilac: protein homology). Node colors indicate the biological processes that best illustrate the clustering (purple: Type-I interferon signalling ($p=7.00 \times 10^{-26}$), green: regulation of immune system process ($p=1.89 \times 10^{-14}$), pink: protein ubiquitination, (p=0.0205), yellow: response to interferon-gamma (5.68×10^{-21}), red: response to IFN-α ($p=5.09 \times 10^{-08}$).

CHANGES IN GENE EXPRESSION MODULATED BY IFN-A AND THE DEVELOPMENT OF IFN-A-INDUCED FATIGUE

Early changes in gene expression induced by IFN-α were correlated with an increase in fatigue symptoms at 4 weeks (minus baseline). Correlation coefficients at 4 weeks were computed in R (cor function at index cut-off $r>0.5$, $p<0.05$). A total of 178 genes correlated with fatigue symptoms at 4 weeks.

Among those 178 genes, 93 positively correlated with fatigue symptoms. PPI analysis of these 93 transcripts produced a network that was significantly more complex than expected for a random network of the same size ($p <0.00783$) consisting of 84 nodes and 81 edges (average node degree: 1.93; cluster coefficient: 0.424) centred on two highly functionally interconnected main nodes (**Figure 46**). The pathways that were most enriched among genes coding proteins in this network involved cellular catabolic (ubiquitin transcripts) (ID: 0044248; FDR $p= 2.4 \times 10^{-4}$) and cytosolic ribosomal processes (ID: 0022626; FDR $p=2.23 \times 10^{-5}$) that have been implicated in modulated mTOR signalling.

Figure 46. Prediction of IFN-α induced fatigue



STRING analysis of IFN-induced genes that positively correlated with fatigue. STRING produced a network (84 nodes and 81 edges) (p-value=0.00783) Line color indicates the type of interaction evidence (light blue: from curated databases, purple: experimentally determined, green: gene neighborhood, red: gene fusions, dark blue: gene co-occurrence, black: co-expression, lilac: protein homology). Node colours indicate the biological processes that best illustrate the clustering (red: cellular catabolic processes, 21 genes (p=0.00024), blue: cytosolic ribosome cellular components, 7 genes ($p=2.21 \times 10^{-06}$)).

Among the upstream regulators of genes correlated with fatigue that were identified by IPA, 3 of the top 4 regulators were associated with the mTOR pathway (p-value of overlap = 1.04e-11, 8.95e-10 and 1.91e-07) (**Table 10**)

Table 10, IFN-α upstream regulators

Upstream Regulator	Target Molecules
LARP1	**RPL10A,RPL13,RPL13A,RPL21,RPL31,RPL6,RPS27, RPS27A,RPS3A**
Torin1	**ATP6V1E1,RPL10A,RPL13,RPL13A,RPL21,RPL31,RPL6,RPS27, RPS27A,RPS3A**
Acyline	BCL6,RPL10A,RPL13,RPL13A,RPL21,RPL31,RPL6
Sirolimus	**IL16,RAN,RPL10A,RPL13,RPL13A,RPL21,RPL6,RPS27,RPS27A, RPS3A,USP15,VMP1,ZNF845**
MLXIPL	RPL10A,RPL13,RPL13A,RPL21,RPL31, RPL6,RPS27A
IL17RA	CSF3R,CXCR2,S100A8,S100A9
Mir-802	ARRDC3,GNG12,OAZ2,PPP1CB,RAN,RAP1B
MRGPRX3	CSF3R,CXCR2,S100A8,S100A9
MYCN	RPL13,RPL13A,RPL21,RPL31,RPL6,RPS27,RPS3A,UBE2V2
IFN-β-1a	IL16,RAN,RPS27,S100A9,TLR1

List of the first 10 upstream regulators identified by IPA. In bold: upstream regulators associated with the mTOR pathway: LARP1, torin1 and sirolimus, p-value of overlap = 1.04e-11, 8.95e-10 and 1.91e-07 respectively.

DISCUSSION

In this study, we employed microarrays to explore acute interferon-induced changes in the whole transcriptome and to ascertain their relationship with the subsequent development of fatigue symptoms in a sample of 27 patients undergoing IFN-α treatment for Hepatitis-C. Firstly, IFN-α administration was associated with a rapid transcriptomic response in whole blood, as differential gene expression analysis revealed 546 upregulated and 228 downregulated transcripts, within 4 ½ hours of injection; which coincided with the onset of IFN-α-induced fatigue. This finding is consistent with previous studies which report a rapid stimulation of Interferon sensitive genes in blood following peripheral administration of IFN in humans (Hepgul et al., 2016; Ji et al., 2003; MacParland et al., 2015). The second finding was that network of transcripts, which comprised predominantly ubiquitin and ribosomal genes, was significantly associated with the subsequent development of fatigue 4 weeks after the first IFN injection.

This connected network consisted of two tightly connected clusters of proteins which are interconnected by the ubiquitination ribosomal gene RPS27A, a stress sensor that is translationally regulated by the mTOR pathway and interestingly previously associated with cancer treatment-induced muscle atrophy in murine models (Sakai et al., 2020). The first cluster in our network was composed of genes expressing protein degradation units belonging to the E2 (UBE2D1, UBE2D2, UBEV2) and E3 (FBXW11, BTRC, RLIM) ubiquitin ligase family. The UBE2D family are E2-Ub conjugating enzymes, that interact highly with E3s providing them with catalytic activity. Interestingly, these proteins have previously been shown to play a role in muscle wasting across a range of different physiological situations (Polge et al., 2015). Furthermore, E3 protein ligases such as F8XW11 and BTRC are also involved in the degradation of DEPTOR, an endogenous regulator of the mTOR pathway, whereby inhibition of DEPTOR by E3 ligases promotes mTOR expression (Jiang et al., 2019; Y. Zhao et al., 2011). The second cluster of genes identified in our network were RPL and RP ribosomal units. The mTOR pathway regulates gene expression of ribosomal biosynthesis by promoting the translation of RP mRNAs (Mayer & Grummt, 2006) and activation of mTOR signaling can result in both acute and long-term

upregulation of protein synthesis (Bolster et al., 2004). mTOR is a dual regulator of anabolism and catabolism in muscle mass (Yoon, 2017). Disruption of the fine balance between protein synthesis and protein degradation, likely disrupts muscle function, which suggests a plausible mechanism through which IFN-α may induce fatigue.

Type-I IFN-α/β (IFNAR) receptors are ubiquitously expressed across almost all tissue types. Furthermore, peripherally administrated IFN-α readily crosses the vascular endothelium and blood-brain barrier (BBB) to gain access to the CNS and peripheral neuromuscular tissue (Banks, 2005). It has been shown that intraperitoneal IFN injection is followed by a marked increase in the expression of Interferon sensitive genes in the brain parenchyma of murine models (Wang et al., 2008; Wang & Campbell, 2005).

Within the brain, IFN has a particular predilection for subcortical structures such as the basal ganglia (GoutièRes et al., 1998) and IFN-induced fatigue/ motivational impairment has been associated with disruption of basal ganglia glucose metabolism, dopamine turnover (Capuron et al., 2007; Nakagawa et al., 2016), glutamate/glutamine concentrations (Haroon et al., 2014) and tissue microstructure (Harrison, Cooper, et al., 2015). Interestingly, there is also a small literature suggesting that mTOR expression in the basal ganglia may play a role in motor learning skills and motor memory (Bergeron et al., 2014).

Though offering potential new insights into the pathophysiology of IFN-induced fatigue the study has a number of limitations. Firstly, although certain gene transcripts exhibit a correlated expression pattern in both blood and brain (Basu et al., 2021; Hess et al., 2016; Qi et al., 2018; Sullivan et al., 2006; Tylee et al., 2013) and peripheral blood transcriptomic approaches have been successfully applied to identify molecular signatures and changes in gene expression during chronic IFN-α treatment (Felger et al., 2012; Hepgul et al., 2016), it is important to note that transcripts detected in peripheral blood may be substantially different and not align with gene expression in the brain. Furthermore, this study is unable to capture IFN-mediated tissue-specific changes in gene expression. In addition, whole blood encompasses multiple cell types and this study accessed how IFN mediates changes

at the global level and therefore cannot provide information about which specific cells mediate the observed effect. A more in-depth characterization of cell-specific mRNA profile should be considered for further studies. Secondly, patients underwent combination therapy with IFN-α and the antiviral drug ribavirin. Arguably, this pharmacological agent may have had an influence in some of the behavioral changes observed though it is worth noting that it will not have influenced the transcriptional changes we observed as the first dose of Ribavirin was administered after both blood samples were taken. Both STRING and IPA, the tools I used in the analysis, do not inherently control for correlations among genes, treating each gene as an independent entity. In reality, genes are interconnected, with their expression often being correlated. This limitation might have led to higher false positive rates, overrepresentation of certain pathways, or a failure to detect complex gene-gene interactions. However, the fact that STRING considers several types of evidence for interaction, and not just co-expression, somewhat mitigates the potential effects of correlation among genes. Muscle mass and function were not measured as part of this study. Inclusion of this in future studies would further evaluate evidence linking IFN-α induced fatigue to mTOR.

Finally, though the repeated measures design adds power, the sample size was relatively modest and these findings must be viewed as hypothesis generating and requiring confirmation in future larger studies.

To conclude, this exploratory study provides evidence of an acute immune-response signature to the administration of IFN-α that is associated with the subsequent development of fatigue. Identification of this novel network of predominantly cytosolic ribosomal and ubiquitin transcripts implicated in modulating mTOR signalling may provide new insights into how interferons induce fatigue and highlight a potential novel therapeutic target for virus-induced fatigue.

CHAPTER 8. SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

The overarching aim of this project was to (i) develop and validate a novel experimental inflammatory challenge (subcutaneous interferon beta injection) and then (ii) use this together with a series of neuroimaging techniques to investigate why older individuals appear to be more susceptible to the behaviourally and cognitively impairing effects of inflammation. More specifically, I aimed to validate IFN-β as a new experimental model of acute inflammation by comparing its effects on physiological, immunological and behavioural responses with those of other immune challenges previously reported in the literature. Another objective was to quantify the effects of IFN-β on the brain using two non-invasive neuroimaging modalities, diffusion-weighted Magnetic resonance spectroscopy (DW-MRS) and resting-state fMRI (rsfMRI). Finally, I aimed to explore whether age might modulate the effects of IFN-β induced inflammation. Data from a third neuroimaging modality, Dynamic Contrast Enhancement (DCE), as well as heart rate variability and mRNA data for transcriptomic analysis, were also collected, however, due to time constraints linked to the pandemic, analysis completion was not possible within the timeframe of this PhD, and therefore, has not been presented as part of this thesis.

For years, studies of patients receiving IFN-α based therapies for the treatment of Hepatitis C and malignant melanoma have provided empirical support for inflammation in the aetiology of depression. They have also shown that IFN-α readily induces sickness symptoms (usually within a couple of hours of the first injection). At the outset of this thesis, I therefore planned to use a single injection of IFN-α as the selected immune challenge for my project. However, due to market-driven factors, the commercial availability of the unpegylated (i.e., short-acting form) of IFN-α was discontinued during the COVID-19 pandemic. While this scenario presented challenges, it paved the way for the introduction of a new model of experimental inflammation utilizing another closely related type I interferon: IFN-β. Type I interferons, alpha and beta, share structural similarities and modulate inflammatory reactions by signalling to the IFNAR1 and IFNAR2 receptors and the JAK-STAT family of signal transducers. Notably, many cells secrete IFN-α, whereas fibroblasts are the primary producers of IFN- β.

The aim in developing this model was to produce a challenge that induced robust sickness responses in a relatively short timeframe but had fewer cardiovascular responses than low dose (i.e., 0.8-1ng/Kg) endotoxin meaning that it could be used without the need for continuous cardiac monitoring. Moreover, it was intended to be suitable for a broader range of experimental uses and suitable for various age groups, from the young to the elderly.

IFN-β therapies were the first major therapeutic class of medications to be developed for the treatment of MS and have been used therapeutically for more than twenty years and given to millions of patients word-wide (Jacobs et al., 1981, 1982; Jacobs et al., 1996). However, to the best of my knowledge, this study is pioneering in its application of IFN-β as an experimental model of mild acute systemic inflammation. Additionally, it also represents the first investigation using an experimental inflammatory challenge to investigate age-related differences in the effects of inflammation.

In Chapter 3, I illustrated the effect of 100 µg of IFN-β-1b subcutaneous injection on physiological parameters, white blood cell count and circulating cytokines plasma concentration. The dosage selection was guided by the escalation regime used in MS starting from 0.65 µg, in weeks 1-2, 125 µg in weeks 3-4, 185 µg in weeks 5-6 and to 250 µg, from week 7 and thereafter). We used 40% of the recommended dose with the objective to induce mild sickness symptoms.

Plasma concentration of IFN-β dramatically increased and peaked at 4 hr and showed a decrease at 6½ hr post-challenge, which would be consistent with its known half-life. Pro-inflammatory cytokines IL-6 and TNF-α displayed a robust increase that peaked at 6½ hr post-challenge. Differential white cell counts showed significant changes at 4 hr which peaked at 6½ hr for Lymphocytes and NLR (Neutrophil/Lymphocyte ratio). Significant changes in Monocytes and Neutrophils were observed at 4 hr and 6½ post respectively. Physiological measures, temperature and heart rate showed significant changes from 4 hr reaching a peak at 6½ hr post-challenge. The time-course progression of the physiological data confirmed that inflammation was present at the time of the MRI scanning session (~4-4 ½ hr) and during the completion of the cognitive tasks (6 hr).

In Chapter 4, I reported the central effects of the IFN-β challenge on subjective sickness symptoms and mood. Self-report questionnaires for mood, fatigue and sickness symptoms were completed at baseline and 5-time points after the IFN-β/saline injection. The IFN-β injection was associated with an acute decline in mood and an increase in the subjective experience of fatigue as recorded by the POMS scales, as well as an increase in classical sickness symptoms as documented by the SicknessQ questionnaire. Consistent with previously reported findings using other immune challenges (e.g., LPS, typhoid vaccine, IFN-α), IFN-β induced similar transient changes in the physiological and behavioural responses (Capuron & Miller, 2004; De Marco et al., 2022, 2023; Harrison, Cooper, et al., 2015). The effects observed in these responses demonstrate that this new model of inflammation can serve as a minimally invasive and efficacious experimental design, capable of inducing temporary alterations in systemic inflammation in healthy subjects. Given that IFN-β triggers a mild yet robust response, it is suitable for broader experimental applications and can be utilized in more challenging and intricate populations (i.e., older individuals). IFN-β did not induce any peripheral effects (i.e., aching muscles or joint pain) which in the case of other models may indirectly and non-specifically change behaviour. Furthermore, it did not produce any adverse side effects. There was only one participant who experienced a minor injection side reaction which resolved on its own. After further consultation, I established that this likely occurred due to the reconstituted dose being stored in the fridge prior to administration, as there was a delay in getting the participant ready. We can therefore view IFN-b as a more refined, ecologically valid model similar to the mild inflammation typically reported in psychiatric disorders like depression or cognitive impairment.

While I did not observe any condition x age interactions, the study revealed some trends (i.e., lymphocytes (condition x age) IL-6 (condition x time x age) and IFN-β (time x age). This suggests that the model can be sensitive to detect age-related changes, however, the capacity to detect subtle effects may have been limited due to the statistical power.

Additionally, in Chapter 4 I reported the effects of IFN-β on 3 different cognitive measures. Firstly, I explored the effects of IFN-β on reward vs punishment sensitivity using a reinforcement learning task. While the results in this study did not reach significance, they showed the same pattern of shift in motivational reorientation observed in other findings. Previous evidence using the same task have shown that inflammation (i.e., LPS and typhoid vaccine challenges in healthy individuals) is linked to increased sensitivity to punishments versus rewards i.e. after inflammation participants show a decrease in the selection of stimuli associated with a high probably of reward but an increased selection of stimuli associated with avoiding a (financial) punishment (De Marco et al., 2023; Harrison et al., 2016). Furthermore, administration of this task during fMRI revealed that inflammation *impaired* ventral striatal encoding of reward prediction errors but *enhanced* encoding of punishment prediction errors in the anterior insula, circuits that are known to play a central role in learning to rewards and punishments respectively (Harrison et al., 2016; Pessiglione et al., 2006).

I also postulated that following inflammation older individuals would exhibit the same pattern of motivational change but with a greater decrease in reward sensitivity coupled with increased sensitivity to punishment. However, interestingly the older individuals displayed an increased sensitivity to both reward and punishment following inflammation. These differences in the directionality of effects of inflammation on reward processing between the two groups could have produced masking effects, thereby diminishing the likelihood of identifying non-significant overall results. Indeed, when I looked at the young group alone (which showed a similar mean age to earlier studies) I did observe the same effect previously reported in the literature. In order to further investigate this intriguing difference in the reward processing between groups a larger sample would be recommended. This would help uncover subtle age-specific associations that may underlie the observed differences.

Another area that has been actively investigated is the effect of inflammation on psychomotor response times. I therefore also investigated the impact of IFN-β on psychomotor slowing using an adapted version of the Simon task. Contrary to my hypothesis, inflammation did not influence either the reaction time or performance of the

task. My findings contrasted with those from animal and human models where associations between neurovegetative symptoms such as psychomotor disturbances and increased cytokine levels have been documented (Brydon et al., 2008; Capuron & Miller, 2004; Dantzer, 2009). The Simon task was chosen as is widely used to explore how stimulus conflict may impact psychomotor reactions. Furthermore, it presents similarities to other paradigms (i.e., Stroop task) previously used in other experimental challenges (Brydon et al., 2008). The contrasting results might be attributable to task sensitivity since ceiling effects were observed in performance; the task version used may not have been challenging enough to elicit discernible differences between conditions and to explore subtle age interactions further.

The last cognitive domain I examined was that of visuospatial memory. The aim was to design a task that could be seamlessly integrated into the rest of the cognitive assessments. It also incorporated varying degrees of difficulties but was mindful of not being overly long (e.g., the human version of the Morris water maze was too lengthy to be implemented as part of the study's experimental design) or cognitively too strenuous for either of the age groups. Finally, it had similitudes to other paradigms that investigate visuospatial processing (e.g., paired-associate learning tasks). I hypothesised that inflammation would be associated with deficits in MTL-dependent memory in support of evidence previously described in rodent and human models (Barrientos et al., 2002; Bird et al., 2009; Bohbot & Corkin, 2007; Harrison et al., 2014). The task effectively differentiated between levels of complexity in sequence recall and also detected age-associated differences (harder sequences resulted in lower percentages of recall, and older individuals' recall ability was lower when compared to the young). However, my results did not align with early findings as no condition effects or condition x age interactions were observed. One of the potential explanations for these results is the marked differences between the task used for this study (recalling a sequence of coloured squares that increased in difficulty) and the classical task used in animal and human models (the Morris water maze and its analogous human version). Various factors such as discrete colour-base stimuli versus the integration of multiple environmental cues or the dissimilarities in cognitive load between tasks, might plausibly serve as the foundational causes for these observed differences.

In Chapters 5 and 6, I presented the findings from two neuroimaging studies that employed non-invasive techniques to index neuroinflammation. In the first, I used DW-MRS, a novel MRI method that is able to provide specific information about cell metabolism by quantifying the diffusion properties of intracellular metabolites. The results showed an increase in choline diffusion (predominantly considered a glial metabolite) but not a selective effect on NAA diffusion (predominantly found in neurons) after IFN-β. This finding replicates the results of a recent proof of concept study using the more potent inflammatory challenge LPS (1ng/Kg) (De Marco et al., 2022) and contributes additional evidence to the notion that the morphological changes in glial cells associated with inflammation can be sensed using MRI, additionally extending the previous findings to a milder, more ecologically valid model of inflammation. This study also represents the first exploration into the effects of age on metabolite diffusion, using an experimental model of inflammation. Although the results did not unveil any significant interactions between condition and age, differences in diffusivity values between older and younger participants were observed (e.g., reduced tNAA diffusivity in the older group compared with the young in the placebo condition) which aligns with prior evidence detailing changes in morphometric measures of neurons related to ageing (Bishop et al., 2010; Morrison & Hof, 2003; Raz et al., 2004). This suggests that DW-MRS could be instrumental in evaluating changes in neurochemical concentrations and cell morphometry related to ageing.

The accessibility of affordable and non-invasive MRI methods, which are sensitive to glial activation, might enable studies aiming to evaluate the temporal dynamics of neuroinflammation in both disease and experimental models. The practical benefits become especially apparent in comparison to studies utilizing the gold standard TSPO PET, where the study population is frequently limited due to factors such as cost, invasiveness, or intricate procedures. The capability to apply DW-MRS in extensive and more varied populations and in longitudinal studies could enhance sensitivity, facilitating the detection of potential immunomodulant agents.

While the findings from this study are encouraging, they would need to be complemented by future studies. It would be crucial to evaluate the DW-MRS signal across a greater

number of volumes of interest, encompassing both cortical and subcortical areas. Furthermore, comparing outcomes between healthy and clinical populations, evaluating the impact of varying dosage levels of IFN-β and expanding the sample size and age groups would potentially allow the detection of more subtle interaction and correlation effects.

A key consideration is the interpretation of the choline signal, given that the precise relative contribution of choline to each glial cell type remains partially undefined. I hypothesised that the changes observed in choline diffusion were more linked to microglial than astrocyte reactivity. This presumption stemmed from prior studies using mouse models of multiple sclerosis and post-mortem histological analysis, which found associations between choline and myo-inositol and histological alterations in microglia and astrocytes, respectively (Genovese et al., 2021). It is worth noting that I could not determine myo-inositol levels with the DW-MRS sequence employed, therefore additional studies using animal models would be necessary to validate this assumption, for instance examining the cell-specificity of choline and myo-inositol diffusivity at various stages following microglial depletion. This would enable the evaluation of whether myo-inositol concentration is exclusively related to astrocytes and whether choline levels would rise as microglia repopulate the brain.

In Chapter 6, I used rsfMRI to explore the acute effects of IFN-β on functional connectivity. Based on the assumption that the brain functions as a sophisticated network of nodes, I employed graph theory to explore functional network architecture, with a particular focus on the efficiency of information transfer. The findings from the functional connectivity maps revealed an overall reduction in functional connectivity in the IFN-β condition. Furthermore, I also observed a reduction in mean nodal strength connections and a diminished global capacity for parallel information exchange which aligns with prior studies that have looked at topological features estimated by graph theory in the context of inflammation (e.g., patients undergoing IFN-α therapy and suffering from major depressive disorder) (Dipasquale et al., 2016; Kitzbichler et al., 2021).

Two more interesting and novel findings in this study. Firstly, I hypothesised that highly connected nodes or hubs (i.e., nodes with high mean nodal strength) would be particularly

susceptible to the impairing effects of IFN-β. My results corroborated this hypothesis which aligns with the notion that hubs that have higher topological centrality also display higher metabolic activity which would make them more vulnerable to pathological factors. This vulnerability stems from their higher demand for blood flow, increased production of metabolic waste and greater susceptibility to oxidative stress, factors frequently associated with inflammatory processes (Crossley et al., 2014). Furthermore, research in neurodegenerative conditions has shown that nodes with numerous connections that link areas with specialized functions tend to be more selectively affected in individuals with Alzheimer's disease (Buckner et al., 2009).

Secondly, I observed that the intensity of the condition effect varied with respect to age. Intriguingly, and contrary to my hypothesis the effects of IFN-β tended to decrease rather than increase with age. Additionally, in contrast to my prediction, I observed that as age increased, the effects of IFN-β on highly connected nodes were also less pronounced.

At present there is no preceding evidence regarding how age might affect the impact of an immune challenge on the efficiency of information transfer in healthy participants. Potential explanations for these findings might be rooted in neural network dynamics and compensatory mechanisms observed in ageing (i.e., decrease in network specialization and increase in integration) (Crowell et al., 2020; Deery et al., 2023; T. Zhao et al., 2015). Furthermore, the reduced metabolic activity associated with age (Cunnane et al., 2011) could potentially result in less pronounced differences between various brain nodes in terms of their metabolic demands.

In summary, this study provides further as well as novel evidence and shows how rsfMRI and graph theory analysis can inform about inflammation-induced alterations in global network architecture. While the results of this study are promising, they warrant further exploration and confirmation through subsequent studies. For instance, longitudinal designs following chronic exposure to IFN-β would help ascertain the progression of the acute changes observed, and whether they may evolve or resolve. A larger cohort of participants may help detect subtle interaction effects that might have been missed due to statistical power.

Future designs may couple the present protocol with for instance magnetization transfer measurements of microstructure in an attempt to elucidate how differences in local, biophysical properties of the brain tissue, associated with inflammation may correlate with broader alterations in functional connectivity between cortical and subcortical nodes and within specific brain networks. Moreover, other designs could benefit from the inclusion of data from the Allen Human Brain Atlas (AHBA). By incorporating AHBA data, a multi-faceted analysis methodology could be employed combining functional, gene expression and anatomical data with the potential of improving the development of models predicting the actions of inflammation on the brain.

In my last chapter, I analysed secondary data using whole blood transcriptomics and protein-to-protein interaction network analysis from a longitudinal sample of 27 Hepatitis C patients initiating IFN-α and Ribavirin therapy. Though clinically effective, IFN-α frequently induces functionally impairing mood and motivation symptoms, particularly fatigue (Capuron & Miller, 2004; Maddock et al., 2005). Unlike depressive symptoms, which typically emerge after weeks of treatment, fatigue tends to emerge and evolve rapidly, typically within hours of the first IFN-α injection (Capuron et al., 2002; Dowell et al., 2016). Despite being a major source of functional impairment during IFN-α and other immune-based therapies, the biological mechanisms underlying fatigue remain poorly understood. Here, I aimed to identify acute bold-based response signatures to IFN-α that could be associated with the experience of fatigue 4 weeks after initiation of treatment.

I showed that IFN-α was associated with a rapid transcriptomic response in whole blood, as the differential gene expression analysis showed 546 upregulated and 228 downregulated transcripts, within 4 ½ hours of injection which coincided with the onset of IFN-α induced fatigue. Furthermore, protein-to-protein interaction analysis revealed a network of transcripts, primarily composed of ubiquitin and cytosolic ribosomal genes that was significantly correlated with the subsequent development of fatigue 4 weeks after the first IFN-α injection. Interestingly, this network of transcripts is implicated in the modulation of mTOR signalling, a pleiotropic pathway that has already been associated with cancer-treatment-related fatigue in humans (Peng et al., 2015) and cancer treatment-induced muscle atrophy in murine models (Sakai et al., 2020).

Findings from this exploratory study provide potentially new insights into the pathophysiology of IFN-α induced fatigue, however, several elements warrant additional investigation and consideration. It is worth noting that while peripheral blood transcriptomics methods have effectively identified molecular signatures during IFN-α (Felger et al., 2012; Hepgul et al., 2016), transcripts in peripheral blood may substantially diverge and not correspond with gene expression in the brain. Additionally, further investigations should consider using a larger sample and a more detailed characterization of cell-specific mRNA profiles, as whole blood analysis cannot provide information about the specific cells that might mediate the observed effects.

In conclusion, my study innovatively employs IFN-β and demonstrates it is an effective and valid experimental model of inflammation. It also validates the use of non-invasive imaging techniques coupled with a series of cognitive tasks as a tool to explore the effects of experimentally induced neuroinflammation in young and old individuals. The understanding that influences on the immune system on the brain can significantly modify behaviour has motivated the development of new immune-targeted drugs. The findings from this project hold relevance in guiding the design and application of more ecologically sound experimental paradigms for evaluating new therapeutic approaches, which may ultimately benefit individuals suffering from some of the most prevalent and serious neurodegenerative and neuropsychiatric conditions.

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APPENDIX A: SELF-REPORTED QUESTIONNAIRES

PROFILE OF MOOD STATES (POMS)

Directions: Read each statement and then circle the appropriate number to the right of the statement to indicate **HOW YOU FEEL RIGHT NOW**:

	Not at all	**A little**	**Moderately**	**Quite a bit**	**Extremely**
1. Tense	1	2	3	4	5
2. Feverish	1	2	3	4	5
3. Worn out	1	2	3	4	5
4. Angry	1	2	3	4	5
5. Lively	1	2	3	4	5
6. Confused	1	2	3	4	5
7. Shaky	1	2	3	4	5
8. Aching joints	1	2	3	4	5
9. Sad	1	2	3	4	5
10. Grouchy	1	2	3	4	5
11. Active	1	2	3	4	5
12. On edge	1	2	3	4	5
13. Annoyed	1	2	3	4	5
14. Energetic	1	2	3	4	5
15. Hopeless	1	2	3	4	5
16. Relaxed	1	2	3	4	5
17. Resentful	1	2	3	4	5
18. Unworthy	1	2	3	4	5

19. Uneasy	1	2	3	4	5
20. Can’t concentrate	1	2	3	4	5
21. Fatigued	1	2	3	4	5
22. Nauseated	1	2	3	4	5
23. Listless	1	2	3	4	5
24. Nervous	1	2	3	4	5
25. Lonely	1	2	3	4	5
26. Muddled	1	2	3	4	5
27. Furious	1	2	3	4	5
28. Cheerful	1	2	3	4	5
29. Exhausted	1	2	3	4	5
30. Gloomy	1	2	3	4	5
31. Sluggish	1	2	3	4	5
32. Headache	1	2	3	4	5
33. Weary	1	2	3	4	5
34. Bewildered	1	2	3	4	5
35. Alert	1	2	3	4	5
36. Bitter	1	2	3	4	5
37. Efficient	1	2	3	4	5
38. Hungry	1	2	3	4	5
39. Forgetful	1	2	3	4	5
40. Guilty	1	2	3	4	5
41. Vigorous	1	2	3	4	5
42. Thirsty	1	2	3	4	5

Visual Analogue Scale (VAS) of Fatigue

Please score below how fatigued you currently feel

SICKNESSQ

Read the statements below and then circle the number that best corresponds to ***how you currently feel in this very moment.*** There are no right or wrong answers. Don't use too much time at each statement, just pick the answer you think best describes how you fell right now.

		Disagree	**Agree Somewhat**	**Mostly Agree**	**Agree**
1	I want to keep still	0	1	2	3
2	My body feels sore	0	1	2	3
3	I wish to be alone	0	1	2	3
4	I don't wish to do anything at all	0	1	2	3
5	I feel depressed	0	1	2	3
6	I feel drained	0	1	2	3
7	I feel nauseous	0	1	2	3
8	I feel shaky	0	1	2	3
9	I feel tired	0	1	2	3
10	I have a headache	0	1	2	3