THE UNIVERSITY of ADELAIDE

From the Bottom Up: Chemotherapy-Induced Gut Toxicity, Glial Reactivity and Cognitive Impairment

A thesis submitted in fulfillment for the degree of

Doctor of Philosophy

in

The Department of Physiology

Adelaide Medical School

The University of Adelaide

by

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April, 2019

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‘I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.’

Juliana Esma Bajic

April, 2019

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Acknowledgments

I would like to sincerely give thanks to my supervisors, Professor Mark Hutchinson and Professor Gordon Howarth for their encouragement and support as I've journeyed through this PhD. I am grateful for the opportunity you have both given me to undertake my PhD and appreciate your patience, guidance, reassurance and the time you have dedicated in assisting me to this point.

I'd like to thank the the Australian Government Research Training Program Scholarship, the Freemason's Society Supplementary Scholarship and the various in-house travel awards I was awarded, as well the Walter and Dorothy Duncan Trust. These scholarships and awards enabled me to share my research with the rest of the world, at the local, national and international levels. I give thanks to these awards and my family for enabling me to present my research in Miami, Montana, Vienna and around much of Australia.

My PhD journey wouldn't have been possible without the support and friendship of two amazing women, Dr. Kelsi Dodds and Krystal Iacopetta. Thank you from the bottom of my heart, for being there for me, in whatever capacity I needed you over the years. I wouldn't have been able to get through the ups and downs of this roller coaster adventure without you and will treasure our friendships forever. I will forever be in debt to you Kelsi, for your keen eyes and attention to detail in helping me throughout the final stages of my thesis writing. I will always be indebted to the **amazing** Vicky Staikopoulos (she truly is a superwoman with superpowers!) for her tireless hours in assisting me over the years… from general lab work and knowledge, to fine tuning my western blot technique and offering guidance, reassurance and even just listening to me. I thank you wholeheartedly – you are and will always be a true friend and an integral part of the Neuroimmunopharmacology lab. Speaking of the lab, special thanks must be given to the one-and-only superstar, Dr. Jaijay Liu for her quirky sense of humour, guidance and

support. A big thanks to the rest of the lab for the many ways in which they have supported me, made me laugh, taken me for coffee or brought in some cake (thanks Dr. Sanam Mustafa) over the years – I thank you!

I'd also like to thank Professor Howarth and his gastro group for enabling me to isolate and examine the many brains and spinal cords of mice and rats with various gut inflammatory disorders since my Honours exploration. Special thanks must be given to Dr. Suzanne Mashtoub, for whom I enjoyed working with so much in my 3rd year physiology practical, that if it weren't for this incredible experience, I probably wouldn't have continued an Honours project at all.

I would further like to show my appreciation to some people or groups who have positively impacted my research experience and who are still within the scientific community. This includes Dr. Elizabeth Beckett (thank you so much for your support and friendship), Dr. Ian Johnston (for your valuable guidance in my literature reviews and for hosting me during the chemotherapy-related cognitive impairment workshop at Sydney University), the Cancer Treatment Toxicities Group, Dr. Amanda Hutchinson, Dr. Larisa Bobrovskaya and Mr. Jim Manavis.

To my network outside of the confines of the PhD, thank you for being so understanding and supportive of me over the years, especially in the last few months of putting together the thesis. Thank you for always trying to understand and acknowledge the big elephant that has existed in the room since I started my PhD. Your encouragement in helping me believe in myself, that I could complete my PhD, has not gone unnoticed and will never be forgotten.

I feel so grateful to have such an incredible family and fiancé. Their unconditional and unwavering support and love have enabled me to realise my dreams and reach my full potential. I appreciate absolutely everything they have done for me, particularly during my PhD journey… from the simple things (like phone calls, hugs, flowers and meals together), to the massive things (like the emotional and financial support – thank you in helping make some of my overseas dreams come true!) … I thank you wholeheartedly and love you so much. In addition to learning a lot about the neuroimmune system and the central comorbidities associated with gut inflammatory disorders, I have also learnt a lot about what is truly important in life. My family and fiancé are owed this credit, as I have learnt what it means to be unconditionally loved and supported, and also how to do this in return. Dad, you have taught me many lessons throughout this process and I wouldn't be here now, without your unwavering support – I love you. Mum, you have supported me in more ways than you realise – I love you. I'm so, so grateful to have such an incredible, inspirational, loving brother, Nick and I'm so pleased to have Jess as an integral part of our family – I love and value you both wholeheartedly.

A special mention must be given to the best study buddy in the world, Ludo the dog; who doesn't talk but makes me laugh, reminds me to take breaks, always releases endorphins in my brain and who reminds me of how to be a good human. You are truly the best dog.

Last, but certainly not least, a special heartfelt thanks goes out to my one and only, my amazing fiancé, Andy. You have been my rock throughout this sometimes-tumultuous PhD journey. You have supported me in more ways than imaginable and I feel so blessed with your unfaltering friendship, support, patience, dedication and love. Thank you for always looking after me, believing in me, bringing out the best in me and reminding me of how important the simple things in life are. I'm excited about what the future holds for us.

Publications arising from PhD research program

Bajic JE, Howarth GS, Johnston IN and Hutchinson MR (2016). Neuroimmunological Manifestations of Chemotherapy Exposure: Implications for Mucositis, Glia and Cognition. *Journal of Cancer Science and Research,* 1(2):1-9.

Bajic JE, Johnston IN, Howarth GS and Hutchinson MR (2017). From the Bottom Up: Chemotherapy and Gut-Brain Axis Dysregulation. *Frontiers in Behavioural Neuroscience*, 12:104.

Publications currently under submission to journals

Bajic JE, Howarth GS, Mashtoub S, Whittaker AL, Bobrovskaya L and Hutchinson MR. Neuroimmunological Complications Arising from Chemotherapy-Induced Gut Toxicity and Opioid Exposure in Rats. *British Journal of Cancer*.

Bajic JE, Howarth GS, Selway CA, Weyrich LS, Bobrovskaya L and Hutchinson MR. "Big Brain" and "Little Brain" Consequences in 5-Fluorouracil-Induced Gut Toxicity and Indomethacin-Induced Enteropathy; Neuroimmune and Microbiome Implications. *Journal of Molecular Cancer Therapeutics.*

Bajic JE, Howarth GS, Mashtoub S, Chartier LC and Hutchinson MR. Comparing Neuroimmunological Consequences in the Brain and Spinal Cord of mice with Experimentally-Induced Chronic Ulcerative Colitis and Colitis-Associated Colorectal Cancer. *European Journal of Cancer*.

Bajic JE, Howarth GS, Hutchinson AD and Hutchinson MR. Understanding the Side-Effects of Chemotherapy: A Cross Sectional Correlational Study in Australian Breast Cancer Survivors. *Breast Cancer Research and Treatment Journal.*

Supporting publications and co-authored contributions

During my candidature, I was involved in studies investigating the effects of naturally-sourced products on CIGT. This involvement resulted in me being invited to be the primary investigator and co-author of the following manuscripts. Although these publications are not presented in my thesis, they are listed below.

Bajic JE, Eden GE, Eden GL, Lampton LS, Cheah KY, Lymn KS, Pei J, Yool A and Howarth GS (2016). Aqueous Rhubarb Extract Partially Improves Mucosal Integrity in a Rat Model of Chemotherapy-Induced Mucositis. *World Journal of Gastroenterology,* 22(37):8322-8333.

Mashtoub S, Lampton LS, Eden GL, Cheah KY, Lymn KA, **Bajic JE** and Howarth GS (2016). Emu Oil Combined with Lyprinol™ Reduces Small Intestinal Damage in a Rat Model of Chemotherapy-Induced Mucositis. *Nutritional Cancer*, 68(7):1171-80.

Conference presentations arising from PhD research program

Presented in 2018:

Abstract: "**Gut-Brain Axis Dysregulation in Chemotherapy: Comparing Gut Toxicity, Microbial Shifts and Neuroimmune Implications in Rats with Chemotherapy-Induced Intestinal Mucositis and NSAID-Induced Enteropathy**"

Bajic JE, Howarth GS, Selway C, Bobrovskaya L and Hutchinson MR.

Multinational Association for Supportive Care in Cancer, Vienna, Austria, 28-30 June 2018

Abstract: "**Comparing Neuroimmunological Consequences in the Brain and Spinal Cord of Mice with Experimentally-Induced Chronic Ulcerative Colitis or Colitis-Associated Colorectal Cancer**"

Bajic JE, Howarth GS, Bobrovskaya L and Hutchinson MR.

Multinational Association for Supportive Care in Cancer, Vienna, Austria, 28-30 June 2018

Presented in 2016:

Abstract: "Intestinal Mucositis Induced by 5-Fluorouracil results in Glial Changes Modified by Analgesics via Neuro-Immune Mechanisms"

Bajic JE, Whittaker AL, Bobrovskaya L, Hutchinson MR and Howarth GS.

Multinational Association for Supportive Care in Cancer, Adelaide Conv Centre, 23-25 June

Australian Society for Medical Research, Adelaide Convention Centre, 9 June

Digestive Diseases Week, San Diego Convention Centre, California, 21-24 May

Presented in 2015:

Abstract: "Intestinal Mucositis Induced by 5-Fluorouracil Results in Spinal Astrocyte Expression Changes in Rats"

Bajic JE, Eden GL, Lampton LS, Abimosleh SM, Howarth GS and Hutchinson MR.

Australian Society for Medical Research, Adelaide Convention Centre, 4 June

International Society for Neurochemistry and Australasian Neuroscience Society, Cairns Convention Centre, Queensland 23-27 August

Presented in 2014:

Abstract: "Intestinal mucositis induced by 5-fluorouracil results in spinal astrocyte expression changes in rats"

Bajic JE, Eden GL, Lampton LS, Abimosleh SM, Howarth GS and Hutchinson MR.

Australian Gastroenterology Week, Gold Coast Convention Centre, 22-24 October 2014

Translational Neuroimmunology, Big Sky Ski Resort, Montana, 13-18 July 2014

Multinational Association Supportive Care in Cancer, Miami Conv Centre, 25-27 June 2014

In 2016, I was invited to attend the Cognition and Cancer Workshop held at Sydney University. It was truly a privilege to address the many challenges faced by researchers in this area. It was a humbling experience to be amongst, and discuss these global health issues with some of the leading researchers in this field from Australia and over the world.

In 2018, I was invited by the Uniting Care Group to present a talk on 'Caring for the Aging Brain' to a group of 60+ year old Australian citizens who were either carers, or cared for their partners. I was again, humbled by this experience in a different way, as it enabled me to discuss current research in the field and advise the aging community on evidence-based strategies that will ultimately, improve their cognitive abilities. I was honoured to be in the presence of such an incredible community to openly discuss their individual cases.

List of abbreviations

Analysis of variance; ANOVA

Autonomic nervous system; ANS

Azoxymethane; AOM

Blood brain barrier; BBB

Buprenorphine; BUP

Breast Cancer Network Australia; BCNA

Breast cancer survivors; BCS

Central nervous system; CNS

Carprofen; CAR

Chemotherapy-induced; CI

Chemotherapy-induced cognitive impairment; CICI

Chemotherapy-induced gut toxicity; CIGT

Chemotherapy-induced peripheral neuropathy; CIPN

Chronic ulcerative colitis; UC

Cluster of differentiation molecule 11b; CD11b

Comments from others; OTH

Colitis-associated colorectal cancer; CA-CRC

Crohn's disease; CD

Crypt depth; CD

Cyclophosphamide, methotrexate and 5-FU; CMF

Danger-associated molecular patterns; DAMPs

Dendritic cells; DCs

Depression, anxiety and stress scale; DASS Dextran sulphate sodium; DSS

Dihydropyrimidine dehydrogenase; DPD

Disease activity index; DAI

Dorsal root ganglia; DRG

Early breast cancer; EBC

Electrochemiluminescence; ECL

Enteric glial cells; EGC

Enteric nervous system; ENS

Extraction blank controls; EBC

Fluorodeoxyuridine monophosphate; FdUMP

Fluorodeoxyuridine triphosphate; FUTP

Fluorouridine triphosphate; FUTP

Functional assessment for cancer therapy; FACT

FACT-Cognitive; FACT-Cog

FACT-Fatigue; FACT-Fat

Functional assessment for chronic illnesses therapy; FACIT

FACIT-Diarrhoea and abdominal disturbances; FACIT-D/AD

Gastrointestinal (GI) tract; GIT

Glial fibrillary acidic protein; GFAP

Haematoxylin and eosin; H&E

Hypothalamic pituitary adrenal; HPA

Indomethacin; INDO

Inflammatory bowel diseases; IBDs

Interleukin-1 beta; IL-1β

Ionized calcium binding adaptor molecule 1; Iba-1

Irritable bowel syndrome; IBS

Lipopolysaccharide; LPS

Medial prefrontal cortex; mPFC

Microbe-associated molecular patterns; MAMPs

Mu; μ

Myeloperoxidase; MPO

No-template control; NTC

Non-steroidal anti-inflammatory drug; NSAID

Perceived cognitive abilities; PCA

Operational taxonomic units; OTU

Perceived cognitive impairment; PCI

Perceived learning and memory difficulties; PLMD

Pathogen-associated molecular patterns; PAMPs

Pattern recognition receptors; PRRs

Polymerase chain reactions; PCR

Quality of life; QOL

Serotonin; 5-hydroxytryptamine, 5-HT

Serotonin re-uptake inhibitors; SSRIs

Tumour necrosis factor-alpha; TNF-α

Toll-like receptors; TLRs

Tramadol; TRAM

Ulcerative colitis; UC

Vehicle; VEH

Villus height; VH

Western blot; WB

World Health Organisation; WHO

Xenobiotic-associated molecular patterns; XAMPs

2,4,6-trinitrobenzenesulfonic acid; TNBS

5-Fluorouracil; 5-FU

5-FU-induced gut toxicity; 5IGT

General introduction

Abstract

Patients with inflammatory disorders of the gut often experience central comorbidities, which include depression, anxiety and cognitive deficits. This is not surprising considering the gastrointestinal tract (GIT; gut) and the central nervous system (CNS, brain and spinal cord) are connected by a myriad of bidirectional pathways. The dialogue between the peripheral immune system and the brain (neuroimmune interactions) has been implicated in the development of sickness behaviours, which interestingly mimic the central comorbidities reported by patients with gut inflammatory disorders, particularly those with inflammatory bowel diseases and cancer. The neuroimmune system lies at the heart of this thesis and its overarching hypotheses. This stems from evidence implicating microglial and astrocyte cell reactivity/priming in contributing to central changes via peripheral inflammatory events. Once primed, glial cells undergo morphological and functional changes which often result in neurotoxicity and neuroinflammation due to their intimate relationship with the synapse. In their quiescent state, however, microglial and astrocytes are critical in central homeostasis, health and development. Reactive glia induce a destructive microenvironment via tissue damage and neuronal loss, contributing to pathological pain pathways and neurodegeneration. Whilst glial reactivity is initiated as an innate response aimed at assisting the host in healing, often microglia and astrocytes can remain primed after the peripheral inflammatory event subsides. Consequently, this thesis examined microglial and astrocyte dysregulation in the brain and spinal cord of rodents across a range of inflammatory conditions of the gut. This work compared acute and chronic models involving a commonly used chemotherapy drug and non-steroidal anti-inflammatory drug. The chronic models assessed ulcerative colitis and colitis-associated colorectal cancer. Finally, this thesis explored the relationship between gut disturbances

and cognitive impairment in an Australian breast cancer cohort. The findings from this thesis suggest that neuroimmunological manifestations occur in the spinal cord and brain of rodents across a range of acute and chronic inflammatory disorders of the gut. Importantly, this indicates that current investigations into the central side-effects associated with gut inflammatory conditions may be critically missing the direct and indirect influence of simultaneously occurring side-effects. Rather than focussing on an individual mechanism relating to a specific side-effect of chemotherapy treatment, perhaps we should be more accurately reflecting a clinical setting and develop our understanding of how multiple side-effects work in unison. The findings from the clinical trial assist us in further understanding the relationships between the perceived severity of side-effects associated with chemotherapy treatment and identify relationships between allergies, pain, gut disturbances and cognition. The mechanistic implications of these findings should be further explored to elucidate whether the gut-inflammatory-induced glial dysregulation described in this thesis has the potential to modulate brain function and behaviour. Ultimately, such studies may critically lead to the development of novel treatment approaches that target multiple side-effects and positively influence the central comorbidities associated with inflammatory disorders of the gut.

Thesis Explanation

The format of my thesis is as follows: a general introduction which highlights important background literature to the themes presented. This is followed by two published literature reviews, whereby the hypotheses and aims are presented. Therein follows three *in vivo* research chapters, a clinical research chapter, a general discussion, references and three appendices. I made significant efforts to publish these chapters throughout my candidature, chapters one and two have been published, whilst chapters three-six have been submitted to journals. Whilst all the chapters are presented in manuscript format, repetition of abbreviation definitions, theoretical concepts and methodological approaches/techniques occur throughout these works, particularly in the B*ackground* and *Methods* sections of the primary research chapters. The first two appendices contains PDF versions of my publications arising from Chapters One and Two, whereas Appendix Three includes a copy of the questionnaire designed for Chapter Six (some minor formatting issues remain). Each research chapter is presented in its original format, apart from some *Background* and *Discussion* sections due to word limit restrictions for journal specifications. In my thesis I have included further discussion points in the *Background* and *Discussion* sections of my research chapters.

Framing the Thesis

At its core, this thesis stems from my interest in the interactions between body systems under inflammatory conditions of the gut, with a particular focus on the neuroimmune system, peripheral-to-central immune signalling and gut-brain axis dysregulation. Three overarching themes explore the neuroimmunological mechanisms contributing to central pathological manifestations associated with chemotherapy-induced gut toxicity (CIGT), chronic ulcerative colitis (UC) and colitis-associated colorectal cancer (CA-CRC). First, I sought to determine whether acute administration of the commonly utilised chemotherapy

drug, 5-Fluorouracil (5-FU), was unique in causing spinal cord glial dysregulation, and to clarify whether such changes occur in the hippocampus of rats with experimentally-induced gut toxicity. Second, I examined whether glial dysregulation would occur in chronic inflammatory conditions of the gut lacking chemotherapy exposure, comparing chronic UC (non-malignant inflammation) with CA-CRC (inflammation-associated malignancy). Using the three gut inflammatory models described above, I covered acute versus chronic models, inflammation versus malignancy, and chemotherapy alone whilst examining different regions of the alimentary tract, from the small intestine to the colon. Finally, I sought to identify whether breast cancer survivors subjectively reported chemotherapy-induced gastrointestinal (GI) symptoms that could be correlated with the severity of their cognitive changes during their treatment. I also wanted to identify whether a relationship between allergies, perceived GI and cognitive disturbances were present.

In order to address these themes, I characterised the time-point and specific CNS regions expressing glial dysregulation in an acute 5-FU-induced gut toxicity (5IGT) rat model and intervened with three mechanistically different analgesic interventions. The clinically relevant interventions chosen in this study further influenced glial dysregulation as well as having effects on small intestinal inflammatory parameters (Research Chapter One). Following on from this, the second research chapter compared the glial dysregulation observed in the high-dose inflammatory response of the 5IGT rat model with a low-dose of indomethacin (non-steroidal anti-inflammatory drug; NSAID), characterising gut toxicity and analysing microbiota changes in both models (Research Chapter Three). I then wanted to assess glial dysregulation in chronic inflammatory models further along the alimentary tract, in the colon of mice. Utilising experimentally-induced chronic UC and CA-CRC models, I examined additional higher order brain regions in these studies (Research Chapter Four). Findings from the clinical trial in my thesis sought to understand the impact

of perceived gut- and cognitive-related side-effects associated with chemotherapy treatment in an Australian breast cancer cohort. This study aimed to identify whether the perceived severity of chemotherapy-induced GI disturbances positively correlated with the perceived severity of cognitive issues reported by these patients. This study also used exploratory questions to identify whether allergy susceptibility could be related with the perceived gut or cognitive side-effects associated with chemotherapy treatment.

Nomenclature

Please note that variations in terminology relating to GI toxicity associated with chemotherapy occur throughout this thesis. These inconsistencies paradoxically reflect advancements as well as failures in the field to adequately define this pathology. During the first few years of my research, mucositis, including oral and/or intestinal mucositis was the most commonly used term. However, advancements in the field suggested that the previously defined characterisation of mucositis by Sonis [1] perhaps more accurately reflected the terminology CIGT. As a result of this, and requests made by reviewers during the publication stages, I have not changed the terminology from what was used in the original publications to avoid altering content.

Please review the following nomenclature.

Alimentary tract: any region from the mouth to anus.

Mucositis: chemotherapy-induced inflammation/ulceration of the mucosal layers of the GIT which include the mouth or intestines.

Oral mucositis: chemotherapy-induced mucosal inflammation/ulceration of the mouth.

Intestinal mucositis/gut toxicity: chemotherapy-induced mucosal inflammation/ulceration of the intestines.

Please note: unless otherwise stated, the terms glia/glial cells throughout this thesis are inclusive of microglial and astrocyte cell types only.

From the Bottom Up: Pathways and Signalling in the Gut and the Brain

Multiple bidirectional, parallel pathways control signalling between the CNS and GIT, form the gut-brain axis [for extensive reviews 2, 3-5]. The complexity of this network is best appreciated in its ability to integrate information from a variety of systems encompassing the central, autonomic and enteric nervous systems, whilst simultaneously considering neuroendocrine, enteroendocrine and neuroimmune input (Please review Figure 2.1 in Chapter Two) [5]. Nonetheless, a brief analysis of the top down, Bottom Up and enteric nervous system (ENS) literature is necessary to appreciate the integration of these pathways. In doing so, this will highlight mechanisms by which these pathways influence behaviour and impact central comorbidities in disorders of the gut. Firstly, the top down (brain-to-gut) signalling will be examined.

Top Down Signalling

The branches of the autonomic nervous system (ANS) responsible for brain communication to the viscera are the sympathetic and parasympathetic limbs [5]. Descending monoaminergic pathways modulate the intensity of spinal reflexes and modify dorsal horn excitability. The subcortical regions that generate these outputs are the hypothalamus and amygdala which receive inputs from a number of cortical areas, such as the anterior cingulate cortex and medial prefrontal cortex (mPFC) [6]. The mPFC receives input from the lateral PFC and orbitofrontal cortex containing integrated multisensory information about homeostatic gut states, such as food intake, pain and immune insults. Output from these networks are projected to the periaqueductal grey [7] where the information is relayed to the dorsal vagal complex, locus coeruleus and raphe nuclei in the pons and medulla [4].

This cortico-limbic-pontine network is referred to as the emotional motor system as it integrates motor autonomic, neuroendocrine and pain modulatory mechanisms [7]. The

medial component regulates spinal reflexes (e.g. serotonergic, noradrenergic and opioidergic descending spinal pathways) directly related to GIT function, whilst the lateral component integrates and modulates motor autonomic, neuroendocrine and pain behaviours [8]. Modulation of descending opioid-dependent pain pathways may occur via behavioural and motivational states involving, for example, food intake and fear [9, 10]. Top down influences may override local reflexes in response to certain cognitive or emotional states that include stress [8], anger [11] and memories, as well as environmental stimuli (threat or safety) [4]. Signals are projected from the medullary pontine nuclei to sympathetic preganglionic fibres in the spinal cord which modulate function-specific vagal and sacral parasympathetic outflows [12]. The hypothalamic pituitary adrenal (HPA) axis coordinates adaptive responses to any stressor and forms a part of the limbic system which is heavily involved in memory and emotional responses [13]. Cortisol is the key stress hormone which has functional effects on many cells in various organs of the body, including the gut and the brain. Several lines of neural and hormonal communication combine and enable the brain to influence a variety of intestinal cells, such as immune, epithelial and enteric neurons. Recently, emerging evidence has revealed that these same cells are constantly under the influence of the intestinal microbiota [14]. From this, the concept of gut-brain-microbiome axis has been developed and strong evidence has accrued supporting its involvement in various pathologies in, not only the GIT but centrally as well [5, 14-23]. This fascinating and complex phenomenon extends beyond the scope of these works. However, several important findings that specifically support the influence of the microbiome in oncology are presented in Chapter Two of this thesis (Table 2.1). Some of the key results emphasised in this chapter include the ability of specific microbial strains showing anti-tumour potential, mechanisms by which chemotherapy-induced dysbiosis influences CIGT, drug efficacy and the complex interplay it has in cancer immunotherapy.

The top down effects of sympathetic and parasympathetic interactions have been well established [3, 24, 25] but overall their actions suppress secretion, motility and GI transit via inhibitory cholinergic transmission including immune- and emotion-related alterations [4]. Mucosa and microflora interactions may also be modified by sympathetic innervation [26]. Importantly, emotional states such as depression and anxiety have been associated with tonic ANS dysfunction involving altered top down signalling. Sympathetic and parasympathetic innervation facilitates emotion-related changes in motor, secretory and immune activity in the GIT. In turn, distinct facial expressions and postures (facilitated by the somatic motor system) reflecting different moods have direct effects on the GIT and indirectly influence feedback to the brain [4]. Reduced expression of Toll-like receptors (TLRs; discussed later) on epithelial cells is an example of peripheral target cells negatively affected by prolonged alterations in ANS output [27]. In a chronic setting, such as functional gut disorders, increased efferent signalling has been associated with regional brain remodelling [28]. The above literature briefly describes the integration of top down signalling pathways which have effects on various gut functions. Simultaneously, the gut and ENS are constantly relaying signals up to the brain in a continual feedback loop.

From the Bottom Up

The GIT elicits a myriad of functions ultimately resulting in absorption of nutrients and expulsion of noxious and pathogenic chemicals, including undigested content. This is primarily achieved by muscular contractions mixing contents and propelling them from the mouth toward the colon for defecation. The GIT contains an extensive intrinsic nervous system (ENS), unique in its ability to control certain functions of the small and large intestines even when it is not connected to the CNS [2]. However, the ENS should not be considered fully autonomous. The ENS is the largest and most complex division of the peripheral nervous system comprising 400-600 million neurons and an extensive network

of enteric glial cells (EGC) [24]. EGCs share similarities with astrocytes (discussed later), their CNS counterparts, in the mechanisms they adopt to support enteric neurons, including their morphology, function and molecular capabilities [29]. Importantly, EGCs play key roles in mounting an immune response, particularly during intestinal inflammation.

Local environmental factors of the gut are signalled to the brain via endocrine, immune and neuronal afferent pathways which have been extensively reviewed [3, 4, 24, 25]. Signalling molecules communicate gut sensory information to the CNS via functional effector cells, such as epithelial cells, smooth muscle cells, interstitial cells of Cajal, enterochromaffin cells, intrinsic and extrinsic primary afferent neurons, immune and enteroendocrine cells [5]. To further complicate gut-brain crosstalk, various neurotransmitters commonly produced centrally, are also expressed by these cells in the GIT [25]. One of the most abundantly produced neurotransmitters in the gut is serotonin (5-hydroxytryptamine; 5-HT) which is released by epithelial enterochromaffin cells. 5-HT activates intrinsic and extrinsic primary afferent neurons and induces peristaltic and secretory effects [30]. In the brain, 5-HT regulates many brain functions which result in behavioural effects, having implications in cognition, reward pathways and learning and memory, to mention a few [31]. Some neuropsychological and gut disorders have a serotonergic component, such as Alzheimer's and Parkinson's disease, experimental colitis and chronic constipation [32-34]. 5-HT has multiple action sites which enable selective serotonin re-uptake inhibitors (SSRIs) therapeutic promise in treating various disorders of the brain. However, its non-selective nature can also result in less desirable effects in the GIT. Consequently, further research on these neurotransmitters expressed in both the gut and the brain, such as 5-HT is warranted.

Recent advances in our understanding of the impact microflora has on the gut-brain axis has led to common use of the term *microbiota-gut-brain axis* [35, 36]. Communication

along the microbiota-gut-brain axis has been shown to alter certain aspects of brain development, function, mood and cognitive processes [17]. In the gut, disruption of the intestinal microbiota can heavily influence pathological intestinal conditions, such as inflammatory bowel diseases (IBD) or gut disorders induced by chemotherapy [37, 38]. Evidence linking microbial changes to a range of disorders, both in the gut and centrally highlights the need for continued research in this area.

Numerous afferent and efferent pathways connect the gut and brain, presenting the host with a multitude of possible platforms for malfunction, dysregulation and disease, both in the periphery and centrally. Accumulating evidence has identified the pivotal role gut-brain axis dysfunction plays in our mechanistic understanding of various gut disorders and their effects on cognition. Disorders of the gut and chronic inflammation often occur simultaneously with psychological abnormalities, including anxiety and depression [39]. Additionally, physiological responses can be induced by stress, for instance triggering relapse in experimental colitis [40]. Recently, great interest has been paid to the importance of gut health on mental health and vice versa. This has become particularly apparent in anecdotal evidence on the central comorbidities associated with various gut disorders, particularly in irritable bowel syndrome (IBS) and IBD [41, 42]. The following sections will briefly introduce the central comorbidities associated with these gut disorders and identify similarities in oncology. This will lead into an exploration of the complexities surrounding cancer and chemotherapy treatment, from the Bottom Up, with an emphasis on the gut and central changes associated with chemotherapy treatment.

Functional Gut Disorders and Central Comorbidities

A range of gut disorders have been associated with central comorbidities. Specifically, this thesis will discuss the central morbidities associated with IBD, IBS, cancer and chemotherapy treatment. It is unclear as to whether a cause and effect relationship occurs

between the brain and gut in these disorders, nonetheless it is widely accepted that stress and psychological challenges contribute to the pathogenesis of functional gut disorders and that gut disturbances can contribute to central comorbidities.

IBS and IBD

Gut disorders are commonly associated with poorer mental health. For instance, 54-94% of IBS patients actively seeking treatment also present with emotional, psychological and cognitive comorbidities [42]. Changes in cognitive performance have been identified in IBS patients, particularly stress-related impairment in visuospatial memory [43]. Similarly, mild forms of memory impairment have been observed in IBD patients, including verbal IQ [44, 45]. IBS attacks present episodically and are often triggered by stressors of the psychological, physical or environmental kind [46, 47]. Consequently, psychiatric conditions, in particular depression and anxiety, play a pivotal role in the development as well as the outcome and prognosis of both disorders [48, 49].

It is for this reason that gut disorders, such as IBS and IBD, involve clinical input from a range of health care providers, including general practitioners, gastroenterologists, radiologists and psychologists or psychiatrists. The emergence of multidisciplinary research and theories in these gut disorders, have resulted in a *biopsychosocial model* which considers a lifetime perspective from the patient's childhood through to their adult life [50]. The model integrates different aspects of the body and the mind to include the ENS and intestinal microenvironment, diet, the immune system and the microbiota. In addition, this model acknowledges and involves different aspects of the individual's life, to include their genetic makeup, environment, cognitive status, stress, emotions, spirituality and traumatic events. Perhaps this kind of multidisciplinary biopsychosocial model should be further explored in other gut disorders which also have a central component, such as in

oncology where patients often similarly experience physical, mental, immune and gut disturbances whilst undergoing anti-cancer treatment.

IBS and IBD aetiology are complex and multifactorial. Several factors associated with gut-brain axis dysregulation play a role in the pathological development of IBS and IBD. Some of these factors include stress, chronic pain and immune activation, as well as CNS modulation, microbiota alterations, genetic susceptibility, HPA dysregulation and visceral hypersensitivity [51-53]. Brain imaging studies have reported structural alterations in patients with both disorders when compared to healthy controls. Changes in grey matter density and volumes have been reported in various cortical regions of IBS and IBD patients [28, 54]. Importantly, these cortical regions play a role in cognitive pain modulation and hence, alterations may be indicative of impaired inhibitory descending nociception.

Although extensive evidence supports the hypothesis for the involvement of central changes in the aetiology of IBS and IBD, a certain level of caution should be employed, as it remains unclear whether these changes are specific to both disorders, or if they overlap with other disease activity, for example with depression or anxiety. The literature above provides clear evidence that IBS and IBD occur simultaneously with central comorbidities. Functional gut disorders have been strongly associated with a range of psychological comorbidities; yet our understanding of the central consequences of other gut disorders, such as in the oncology arena remains unclear. Patients with various cancer types often report a myriad of peripheral and central changes which negatively affect quality of life. These symptom clusters can include GI disturbances, lack of appetite, malaise, depression, anxiety, cognitive deficits, lethargy and sleep disturbances and fatigue, to name a few. The following sections will briefly introduce cancer, focussing on breast cancer and CRC and CIGT, CICI and immune dysregulation in the context of this thesis.

Cancer and chemotherapy

Cancer

Since the year 2000, estimates on cancer incidence have increased by approximately 80% and yet estimates on deaths resulting from cancer have increased by only 60% [55, 56]. Importantly, this highlights that anti-cancer treatment advancements have significantly improved the survival rates of cancer survivors. In doing so, increased attention has been given to not only the acute but also the delayed side-effects associated with treatment. The increasing incidence of cancer diagnoses and use of anti-cancer treatments will result in a future with a significant proportion of cancer survivors who will be cancer free, yet may still endure some of the side-effects from their anti-cancer treatment.

Breast cancer and colorectal cancer (CRC) incidence are amongst the top three most reported cancers in the world [55]. In women, breast cancer accounts for approximately one in every four cancer cases. Overall, CRC ranks third in the context of incidence, but second in terms of mortality due to its often-late diagnosis and aggressive nature. These stark statistics associated with both cancer types as well as the high incidence of cognitive impairment reported in breast cancer patients justified exploration of the central themes presented in this thesis.

Patients with both cancer types often present with simultaneous side-effects in the gut and centrally, yet it is unclear as to whether these side-effects are induced by the malignancy or by anti-cancer treatments [57-59]. Separate investigations into anti-cancer treatment associated gut and cognitive changes have implied various immunological mechanisms contributing to the pathogenesis of each disorder [1, 60-65]. Unsurprisingly, at the crux of cancer lies immune dysregulation as host immune processes attempt to eliminate malignancies, yet paradoxically encourage its progression. The complexities of these

treatment-induced disorders will be further discussed after briefly describing immune dysregulation within the context of cancer.

Cancer and Immune Dysregulation

Cancer and anti-cancer treatments have been associated with complex immune maladaptation's involving inflammatory processes, both surrounding the site of the malignancy, within the malignancy itself and as a result of anti-cancer treatments. This is further explored in Chapter One of this thesis under the subtitle, "The contradiction: host immunity, dysregulation and cancer". Briefly, in oncology, immune processes become compromised in a myriad of contexts and can be considered somewhat paradoxical. For example, whilst endogenous immune processes aim to eliminate malignant cells, within the malignancy, similar immune processes are initiated which encourage cancer establishment and progression [66, 67]. Accordingly, inflammation is now considered one the hallmarks of cancer establishment and progression [68]. A strong association exists between chronic inflammatory responses and cancer, for example IBD is strongly associated with the development of colon cancer [69]. Additionally, allergy (immune dysregulation) has been linked to an increased risk for developing certain cancer types [70, 71]. Infectious organisms (e.g. lipopolysaccharide (LPS); a cell wall component of gram negative bacteria) and exogenous pro-inflammatory cytokine administration triggers inflammatory responses, activating innate immune pattern recognition receptors (PRRs), such as TLRs which recognise pathogen associated molecular patterns (PAMPs) [72]. The interaction between PAMPs and PRRs initiates inflammatory responses which can become dysregulated and lead to chronic inflammation, having beneficial effects on tumour development [73]. In particular, TLR-4 signalling enhances tumour development yet, is capable of initiating the production of cytokines, such as tumour necrosis factor-alpha (TNF-α), which can have an anti-tumour effect [74, 75]. Tumour cells also express TLRs

and specific ligands, such as TLR-1, -7 and -9, which stimulate tumour growth and protect tumour cells from apoptosis [76].

Immune dysregulation is further complicated upon commencement of anti-cancer treatment. Most chemotherapy drugs, regardless of the mechanism of action, elevate circulating pro-inflammatory cytokines and have been linked to chemotherapy-induced side-effects, such as pain, fatigue and cognitive impairment [77, 78]. In addition, localised inflammatory responses induced by chemotherapy drugs, result in gut toxicity and further increased expression of pro-inflammatory cytokines in the circulatory system. From this, and evidence confirming the role of pro-inflammatory cytokines in cytokine-induced sickness responses (briefly discussed in the following section and in more detail throughout this thesis), the complexities surrounding the aetiology of chemotherapy-induced gut and cognitive impairment may be related to the symptom clusters experienced by cancer patients. Although a cytokine-based neuroimmunological mechanism has been proposed to contribute to cancer and anti-cancer treatment-related symptom clusters, little research has been undertaken in this area [62]. Accordingly, these concepts formed the basis of the hypotheses outlined in this thesis.

Chemotherapy-Induced Symptom Clusters

Chemotherapy treatment comes with a cost as recipients often experience a cluster of symptom side-effects. Chemotherapy recipients may report acute and delayed side-effects which include gastrointestinal disturbances, learning and memory difficulties, fatigue, and depression [79]. The cytotoxic and non-selective nature of chemotherapy drugs results in them targeting non-malignant and healthy rapidly dividing cells. The GIT constantly replenishes its cells, having a high regenerative capacity. In the CNS, cellular (neuronal and glial) death is a natural part of the aging process and accordingly, like the GIT, the CNS must replenish these cells. This primarily occurs in the hippocampal region,

specifically the dentate gyrus. It is for this reason that the two organs most susceptible to chemotherapy-induced toxicity are the GIT and the CNS.

Interestingly, chemotherapy-induced symptom clusters mimic cytokine-induced sickness responses; a coordinated set of behavioural adaptations which develop in unwell individuals during the course of an infection [80]. During infection, the behavioural repertoire of individuals changes dramatically as they express little motivation to achieve/perform normal daily functions which include eating and drinking, socialising, and they are often fatigued and have trouble sleeping [81]. The individual may also experience increased sensitivity to pain, trouble concentrating, and are unable to experience pleasure. This state has been replicated experimentally in laboratory animals following both central and peripheral administration of LPS or recombinant pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β) [80-82]. Whilst the sickness response produces negative symptoms for the individual, this innate central motivational state actually promotes recovery and is mediated by pro-inflammatory cytokines, such as IL-1β, acting directly or indirectly in the brain [81, 82]. Whilst the altered immune profile of cancer patients has been attributed to cancer progression and some treatment-induced symptom toxicities, the role of the neuroimmune system and glial dysregulation in cancer and treatment-induced toxicities in the gut and the brain remain elusive.

Chemotherapy-Induced Toxicities: From the Bottom Up

Briefly, *From the Bottom Up*, Sonis (the 'godfather' of mucositis) [1] characterised chemotherapy-induced mucositis by inflammation, ulceration, mucosal damage and malabsorption. Mucositis was defined into four phases including the Initiation Phase, the Signalling Phase, the Ulceration Phase and the Recovery Phase [1]. Mucositis may occur anywhere along the alimentary tract and usually resolves post treatment cessation. During the Ulceration Phase, mucosal integrity is compromised and the clinical symptoms may

include diarrhoea, constipation, significant pain, abdominal bloating, nausea and vomiting. Although Sonis provided a solid basis for the characterisation of mucositis, research groups have more recently reported additional mechanisms directly contributing to gut toxicity caused by chemotherapy treatment and accordingly renamed the disorder, CIGT. Some mechanisms are discussed further in this thesis within Chapter Two, 'From the Bottom Up: chemotherapy and gut-brain axis dysregulation' but involve alterations in intestinal permeability and microbiome implications.

Although doctors initially ignored cognitive complaints from chemotherapy recipients, it soon became evident that many chemotherapy drugs readily crossed the blood brain barrier (BBB). Breast cancer patients called their difficulties with learning and memory, 'chemobrain', yet later this was clinically referred to as CICI [83]. The cognitive deficits reported by chemotherapy recipients are typically subtle but in severe cases lead to employment issues and relationship breakdowns [57, 58]. The main cognitive domains affected in CICI include working memory, executive function and processing speed [57, 84, 85]. Studying cognitive decline in chemotherapy recipients presents researchers with an array of methodological challenges. For example, subjective versus objective findings, cognitive reserve, education and other external factors have the potential to influence an individual's susceptibility to CICI. Consequently, investigations utilising rodent models have been critical in developing our understanding of mechanisms specifically resulting from chemotherapy exposure, in the absence of malignant cells and removing other potential individual confounds.

Systematic rodent studies confirmed that single doses of various chemotherapy drugs with different mechanisms of action were having negative behavioural effects, directly related to learning and memory deficits [86-88]. Furthermore, the findings from these experimental models suggested that the behavioural and memory deficits were

hippocampal and PFC dependent. Hippocampal neurogenesis is one of the most commonly reported changes following systemic chemotherapy treatment [86, 87, 89]. Whilst it is widely accepted that neurogenesis in the hippocampus is involved in learning and memory processes [90], the precise role of neurogenesis is unclear and debated amongst the literature [91, 92]. From this it seems that other neurobiological processes, such as cellular apoptosis and neuronal death may contribute to the pathogenesis of CICI. Commonly used chemotherapy drugs, such as 5-Fluorouracil (5-FU; discussed below), methotrexate and cyclophosphamide have induced hippocampal changes and subsequent cognitive deficits in malignancy-absent rodent models [86, 87, 93].

Mechanisms relating to the pathogenesis of CICI are complex, and the sheer existence of the phenomenon has been challenged by some groups in both pre-clinical and clinical studies [94, 95]. Nonetheless, a significant proportion of CICI patients report a reduced quality of life and continued cognitive changes for many years post chemotherapy cessation [96-98]. It is for this reason that I wanted to examine potential mechanisms contributing to CICI pathogenesis throughout this thesis. Moreover, studies examining either CIGT or CICI focus on single mechanisms relating specifically to each disorder. Although these disorders appear separate, it is plausible that inflammatory events in the gut may be contributing to central changes and this may be occurring by direct or indirect mechanisms. One of the most commonly utilised chemotherapy drugs for breast cancer and CRC is 5-FU, which may be used alone or in conjunction with other chemotherapy drugs. In addition to this, 5-FU is frequently observed throughout both the CIGT and CICI literature as it induces negative physiological changes from the Bottom Up. As a result of this, 5-FU was the chemotherapy drug examined in this thesis.

The Chemotherapy Drug, 5-FU

5-FU has been used as a frontline chemotherapy drug improving the prognosis and survival rate of cancer patients worldwide [99, 100]. This invaluable chemotherapy drug has successfully treated various cancer types, including breast [101] and CRC [102]. It formed part of a frontline breast cancer regime used with cyclophosphamide and methotrexate in the CMF (cyclophosphamide, methotrexate and 5-FU) regime [103]. 5-FU is an anti-metabolite that misincorporates into RNA and results in DNA mutations, ultimately initiating apoptotic pathways and causing cellular death (Figure 2).

5-FU utilises the same facilitated transport mechanisms as the nucleic acid, uracil, to enter each cell and is converted into several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FUTP) and fluorouridine triphosphate (FUTP) [104]. The metabolites of 5-FU disrupt RNA and DNA synthesis, by inhibiting the production of thymidylate synthase. The rate limiting enzyme in 5-FU metabolism is dihydropyrimidine dehydrogenase (DPD) which converts the metabolites of 5-FU into dihydrofluorouracil [105]. 5-FU is mostly catabolised in the liver (80%) and the activities of DPD in laboratory rats are within the same range as humans [106].

This widely utilised chemotherapy drug was chosen for studies in this thesis as it induces toxicities from the Bottom Up, in both the gut and the brain. GI dysfunction following 5-FU administration often results in significant challenges surrounding the clinical application of this chemotherapeutic drug due to symptom severities and, at times, the need to prematurely cease treatment. GI symptoms associated with 5-FU treatment are derived from Sonis' characterisation of mucositis and may include visceral pain, diarrhoea, constipation, nausea and vomiting [1].

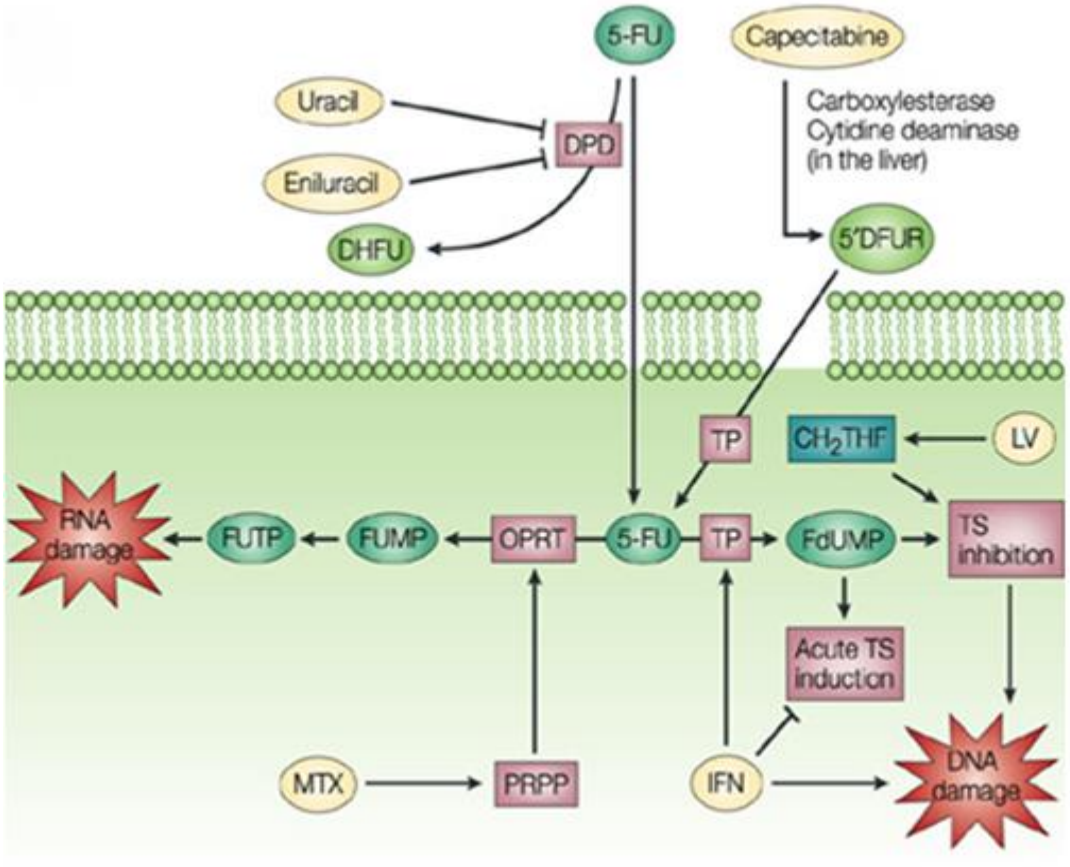

Figure 2. The mechanism of action of 5-FU. 5-FU enters the cell mimicking uracil where it is metabolised into FUTP, FUMP and FdUMP. The 5-FU metabolites inhibit the synthesis of thymidylate synthase and misincorporate into RNA and DNA. Misincorporation of 5-FU metabolises into RNA and DNA cause mutations, strand breaks and ultimately induces apoptotic pathways, leading to cell death (Figure from Longley, *et al*., 2003). Fluorodeoxyuridine monophosphate; FdUMP, fluorodeoxyuridine triphosphate; FUTP, fluorouridine triphosphate; FUTP, dihydropyrimidine dehydrogenase; DPD, dihydrofluorouracil; DHFU.

Although mucositis symptoms were originally believed to resolve upon treatment cessation, recently long-term GI dysmotility has been reported to outlast the acute intestinal inflammatory response in mice treated with 5-FU [65]. Further studies revealed this delayed dysmotility to be in part mediated by 5-FU-induced ENS dysfunction, such as myenteric neuronal loss [107]. 5-FU induces intestinal dysfunction in various experimental models across a wide range of doses and combined treatment regimens [107, 108]. A single high-dose of 5-FU induces a significant reduction in bodyweight, shortened intestinal villi and crypt depths and an acute intestinal inflammatory response, as routinely

shown by increased myeloperoxidase (MPO) expression [108-110]. MPO is an enzyme expressed by neutrophils and elevated expression indicates neutrophil infiltration and thus, acute inflammation [111]. MPO has been routinely used as an indicator of acute intestinal inflammation and was thus used in the acute studies in this thesis.

In the CNS, 5-FU rapidly penetrates the brain and cerebrospinal fluid, crossing the BBB via simple diffusion [112]. Many breast cancer patients report significant problems with concentration and memory, which have negative effects in many facets of their lives [83, 113]. Neuropsychological testing has confirmed that a certain subset of breast cancer patients on the CMF regime showed cognitive impairment in attention, mental flexibility, speed of information processing, visual memory and motor function [114]. Experimental models have supported these findings, reporting that 5-FU alone and when used in combination with other chemotherapy drugs negatively affects spatial working memory and executive function [115, 116]. These cognitive changes were linked to 5-FU-induced alterations in neurotrophin levels and neurogenesis in the hippocampus of rats, as identified by reduced cellular proliferation in the dentate gyrus of 5-FU treated animals. *In vitro* and *in vivo* evidence has linked systemic 5-FU treatment to a syndrome of delayed myelin destruction which importantly highlights that 5-FU also targets non-dividing cells within the CNS [115]. This study reported increased apoptosis and prolonged cell proliferation in the sub-ventricular zone, dentate gyrus and corpus callosum of the hippocampus for up to six months post short-term systemic administration of 5-FU in mice. Neuronal and oligodendrocyte precursors (another glial cell type) were particularly susceptible to 5-FU toxicity and these findings confirm imaging studies of the prevalence of white matter tract pathologies developing from systemic chemotherapy administration [61, 117]. Taken together, the above evidence indicates mechanisms by which 5-FU induces toxicity to the gut and the brain and consequently justifies further examination of

the neuroimmunological manifestations of this drug. As proposed, current investigations into the mechanisms contributing to CICI may be critically missing the direct and indirect influence of other simultaneously occurring treatment-induced side-effects, such as gut toxicity. Further, it is highly likely that in many of the CICI studies presented above, a significant proportion of those laboratory animals would have been experiencing CIGT, though this region was not examined. The neuroimmune interface will now be presented as a potential mechanism contributing to the central comorbidities associated with gut inflammatory conditions, such as in the case of chronic UC, CRC and CIGT.

Tying it All Together: the Neuroimmune Interface

Multiple lines of evidence have demonstrated that bidirectional communication occurs between neurons and glial cells [118-122]. This is now widely accepted as the neuroimmune interface [123]. Whilst oligodendrocytes are responsible for myelination, microglia and astrocytes are the focus of this thesis and are largely considered the immune-like cells of the CNS. They play a pivotal role in brain development, function and plasticity in both health and various neurodegenerative disease states [121, 122]. Microglia and astrocytes are discussed further in Chapter Two of this thesis, in '"Little brain" to "big brain" inflammation and signalling pathways'. Briefly, when primed from either direct (central; e.g. traumatic brain injury) or indirect (peripheral; e.g. bone cancer) insults, microglia and astrocytes change their morphology and functional output from a quiescent resting state into a ramified state [124]. Reactive glial cells trigger a neuroinflammatory response, augmenting the production of pro-inflammatory cytokines, chemokines, mediators and prostaglandins all aimed at assisting the host in healing [125]. Unfortunately, these functional adaptations often become dysregulated and may persist after the initial trigger has resolved. Central to this matter is the synergistic nature of microglia and astrocytes activating one another; microglia release substances that activate astrocytes and vice versa [126].

My fascination with the neuroimmune system stems from the widely accepted mechanism by which peripheral inflammatory events induce glial cell reactivity and neuroinflammation, having the potential to influence brain functions and behavioural outcomes. In the spinal cord microglia and astrocytes become reactive in various models of peripheral inflammation, including bone cancer and in chemotherapy-induced peripheral neuropathies [127, 128]. In higher order brain regions, they contribute to the pathogenesis of central disorders, such as Alzheimer's and Parkinson's disease [124, 129-131]. Glial

reactivity and neuroinflammation dysregulates synaptic transmission via increased release of neuroexcitatory mediators, which are known to contribute to pathological pain states, such as allodynia (painful response from non-harmful stimuli), hyperalgesia (heightened sensitivity to pain) and in pain associated with CIGT and peripheral neuropathy [123, 132-135]. The effects of glial dysregulation in the spinal cord and higher order brain regions continue to be heavily researched.

Glial dysregulation has been shown to contribute to pathological pain states at various sites involved in pain pathways, including the spinal cord, dorsal root ganglia and peripheral nerves. Peripheral nerve injury results in spinal glial activation, which can then amplify pain signalling and induce central sensitisation [126, 136]. Considering the influence glial cells have on pain pathways, it is not surprising that they are also reactive to analgesics, specifically opioids. Chronic morphine administration activates glia and increases pro-inflammatory cytokine expression in rodents [137, 138]. Further studies have revealed that opioid-induced glial activation compromises opioid efficacy as it contributes to opioid reward, dependence and withdrawal [139]. The anti-analgesic effects induced by opioids can be ameliorated by coadministration of glial attenuators, such as minocycline [140]. Opioids also exhibit actions in the GIT affecting motility and fluid secretion [141, 142]. The aforementioned studies confirm the complex nature of the background literature surrounding this thesis. In particular, these factors can complicate analgesic intervention in the oncology arena where pain and anti-cancer treatment toxicities already coexist. It is for this reason that the first research chapter of this thesis included the influence of three analgesics, including a NSAID, a partial opioid and an atypical opioid in exacerbating 5IGT-induced glial dysregulation in the brain and spinal cord of rats.

Few studies have examined the neuroimmunological manifestations of the gut inflammatory disorders described in this thesis, which is surprising considering the

bidirectional nature of the gut-brain axis and the sensitivity of glial cell to peripheral inflammatory events. Taken into consideration, we will now briefly review the proposed peripheral-to-central immune signalling pathways proposed to be responsible for the glial dysregulation observed in these models.

Peripheral-to-Central Immune Signalling and Gut Inflammation

Immune signals and mediators expressed by an inflamed gut are able to signal the brain via three main immune signalling pathways: neural, humoral and cellular routes (for an extensive review of all pathways, see [143]). For the purpose of this thesis, the neural and humoral pathways will only be outlined as they formed a significant element of the hypotheses. The neural route is considered the fastest passageway, largely characterised via the vagus nerve [144, 145]. Immune signals released from localised peripheral inflammation, such as macrophages and epithelial cells in CIGT, are detected by primary afferent neurons. This information is transduced into a neural message, which can be relayed to higher order brain regions. In the brain parenchyma, the neural message is re-transduced into an immune message, which produces cytokines that act on nearby neurons and glia to evoke behavioural changes. Some of these behaviours can be related to cytokine-induced sickness responses, which also include cognitive deficits.

The humoral route is considered a slower route of transmission which involves molecular intermediates at circumventricular organs (devoid of the BBB), such as the choroid plexus [144]. At these organ sites, pro-inflammatory cytokines from the periphery directly access the brain via a range of mechanisms involving active transport via cytokine-specific transport molecules on brain endothelium, endothelial cell activation and subsequent release of secondary messengers, such as prostaglandins. In turn, these secondary messengers may act on specific brain targets and induce behavioural changes. The concept of a direct vs indirect pathway is introduced here. This thesis proposes that pro-

inflammatory cytokines released from macrophages and monocytes in CIGT will result in glial dysregulation in the spinal cord, which is indicative of the neural route being activated. In this sense, the *indirect* actions of the drug (e.g. the gut toxicity) will result in spinal cord changes. Whereas, glial dysregulation observed in higher order brain regions may be mediated by the humoral immune signalling pathway. It is proposed that systemic pro-inflammatory cytokines/mediators or the drug itself will *directly* cross the BBB via the humoral route and induced higher order brain adaptations.

Concluding Remarks

The general introduction highlighted the intimate and bidirectional communication pathways shared between the gut and the brain. Additionally, it identified that the central comorbidities associated across a range of gut inflammatory conditions, including those outside of the oncology area (chronic UC) and those within (malignancy-related; CRC and chemotherapy-related; CIGT) remains under-investigated. The increasing incidence of gut inflammatory disorders and subsequent central comorbidities is ample evidence to encourage continued research in this area. In saying so, current research focuses on specific mechanisms relating to either the GI or central disorders. Critically, this angle of research may be missing the potential influence that other side-effects may be having on each disorder. One research area that has not received much attention is the neuroimmune interface and, accordingly, this is proposed throughout this thesis.

During inflammatory disorders of the gut, such as in the case of chronic UC, CRC and CIGT, the immune dialogue between the gut and the brain becomes significantly compromised, resulting in immune dysregulation. Peripheral cytokines from the gut inflammatory disorders described above, have the potential to access the brain via direct and indirect peripheral-to-central immune signalling pathways. It is proposed that in the chronic models both humoral and neural routes mediate glial dysregulation and

neuroinflammation, yet the acute models implicate only the neural pathway. In specific higher order brain regions, such as the hippocampus, it is anticipated that glial reactivity and neuroinflammation may contribute to the central comorbidities associated with such chronic gut inflammatory disorders. Both the higher order brain region and spinal cord ramifications warrant further investigation.

CHAPTER ONE: Literature Review

Neuroimmunological Manifestations of Chemotherapy Exposure: Implications for Mucositis, Glia and Cognition

Context statement

This work has been published in the *Journal of Cancer Science and Research.*

Bajic JE, Howarth GS, Johnston IN and Hutchinson MR (2016). Neuroimmunological Manifestations of Chemotherapy Exposure: Implications for Mucositis, Glia and Cognition. *Journal of Cancer Science and Research,* 1(2):1-9.

NOTE: This publication is included in Appendix One and will also be available to authorised users at: DOI: 10.4172/2576-1447.1000105

Chapter One provides an overview of chemotherapy-induced side-effects, focusing on CICI and CIGT. Briefly describing the complex immune dysregulation which occurs in chemotherapy recipients, this review introduces inflammation in the gut ("the little brain") and the brain ("the big brain") and presents pathways in which "little brain" inflammation results in "big brain" inflammation, neuroinflammation. It proposes that the neuroimmune system and glial cell dysregulation may be contributing to or exacerbating CICI via CIGT and presents peripheral-to-central immune signalling pathways as potential mechanisms.

Statement of authorship

Statement of Authorship

Title of Paper	Neuroimmunological manifestations of chemotherapy exposure: implications for mucositis, glia and cognition
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	**Bajic JE, Howarth GS, Johnston IN and Hutchinson MR (2016). Neuroimmunological Manifestations of Chemotherapy Exposure: Implications for Mucositis, Glia and Cognition.** ***Journal of Cancer Science and Research,*** **1(2):1-9.**

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Contribution to the Paper	Manuscript conception, reviewed all papers cited in manuscript, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/04/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);

II. permission is granted for the candidate in include the publication in the thesis; and

III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Chemotherapy drugs reduce quality of life often causing acute and delayed central side-effects, termed chemotherapy-induced cognitive impairment (CICI). Another dose-limiting chemotherapy-induced side-effect is oral and intestinal mucositis which results in significant gastrointestinal (GIT) damage and intestinal inflammation. Recent interest has been paid to neurological complications arising in patients with gut disorders, yet little attention has been paid to the role GIT damage plays in CICI. Our current understanding of neuronal adaptations and behavioural consequences resulting from immune system dysregulation has paved the way for investigation into the neuroimmunological manifestations associated with chemotherapy. In a clinical setting cancer patients experience a cluster of symptoms, similar to that manifested in cytokine-induced sickness responses. Accordingly, it is suggested that peripheral inflammatory events, such as chemotherapy-induced mucositis, may cause glial dysregulation and potentiate cognitive changes in CICI. Perhaps it is time to examine the cancer experience in a multidisciplinary manner, in order to encapsulate the direct and indirect mechanisms underlying treatment-related side-effects. Specifically, understanding the neuroimmunological implications of chemotherapy-induced mucositis will provide further insight into the direct and indirect mechanisms underlying CICI pathogenesis.

Background

Chemotherapy drugs have proven invaluable in treating many cancers and improved the outcome for millions of cancer patients worldwide. Whilst the ultimate goal of chemotherapy is to prevent malignant cells from metastasizing, chemotherapy drugs are generally non-specific as they also target healthy, non-malignant cells. Chemotherapy induces a range of acute and delayed side-effects. In the central nervous system (CNS) the phenomenon is clinically recognised as chemotherapy-induced cognitive impairment (CICI) [1]. Peripherally, chemotherapy drugs negatively affect the gastrointestinal tract (GIT) lining causing oral and/or intestinal mucositis. Although mucositis is an acute disorder which usually resolves upon treatment cessation, it is often a dose limiting side-effect due to the painful nature of the disorder [2, 3]. Traditionally, these chemotherapy-induced side-effects have been considered to be separate disorders. However, recent evidence suggests that bidirectional communication pathways connecting the GIT and CNS may be implicated in the pathogenesis of both disorders. These pathways regulate a myriad of physiological and immune functions in health and various disease states manifesting from the periphery or centrally [4].

Peripheral inflammatory events or immune insults trigger a characteristic cluster of behavioural, cognitive, and affective changes, which are commonly referred to as cytokine-induced sickness responses [5, 6]. Interestingly, many symptoms associated with cytokine-mediated sickness responses mimic the cognitive and behavioural changes commonly reported by chemotherapy recipients, including learning and memory dysfunction, fatigue and depression [7]. Cancer and chemotherapy exposure are associated with substantial immune dysregulation, involving inflammation [8], changes in cytokine levels [9] and mucositis which may be contributing to cognitive changes. Nonetheless, previous studies have failed to determine whether a link exists between these already established, yet

disparate side-effects of chemotherapy. This review proposes that neuroimmune mechanisms and glial dysregulation may contribute to CICI symptoms both directly and indirectly via a peripherally driven inflammatory event: chemotherapy-induced mucositis.

As we unravel the complex aetiology of CICI, it soon becomes clear that the challenge in examining CICI lies within the cluster of symptoms cancer patients' experience. In a clinical setting, cancer patients reporting cognitive dysfunction often concurrently experience depression, anxiety, sleep deprivation, fatigue and pain [10-13]. Accordingly, Lee *et al.* [14] proposed a biological basis for cancer (and cancer treatment) related symptom clusters; a *cytokine-based neuroimmunological mechanism*. This concept stems from well-established studies which indicate that cytokine-induced sickness behaviours can be evoked by exposing animals to either infectious, inflammatory or certain pro-inflammatory cytokines [5, 6]. Additionally, various gut disorders have been associated with psychological and cognitive comorbidities (reviewed below). From this it can be concluded that direct and indirect mechanisms may be contributing to cognitive changes observed in the chemotherapy setting. Neuroimmunological approaches in managing cancer and treatment-related side-effects may pave the way for novel and effective therapeutic and preventative approaches, ultimately improving the quality of life of cancer patients and survivors worldwide.

Gut Disorders and Cognition

CNS dysfunction has been recognised as a prominent feature in functional gut disorders, including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) [15, 16]. The idiopathic diseases comprising IBD include ulcerative colitis (UC) and Crohn's disease (CD). UC usually involves the colon and ileum whereas CD mainly involves the rectum and colon. IBS, however, is currently viewed as being caused by dysregulation of the gut-brain axis. Whilst the symptomatology of both disorders includes pain, altered

bowel movements and a host of physical, emotional, psychological and cognitive responses, IBD is strongly associated with intestinal inflammation unlike IBS.

The aetiology of functional gut disorders is complex and multifactorial, however it has been suggested and somewhat accepted that IBD and IBS may be triggered by psychological, environmental or physical stressors [17, 18]. Consequently, several pathophysiological factors negatively affecting the gut-brain axis are pivotal to the disorders and include stress, chronic pain and immune activation [19, 20]. Although a substantial body of literature exists linking stress, chronic pain and immune activation to cognitive deficits, this area in the context of functional gut disorders has received little attention. Nonetheless, primary studies assessing these pathological factors in this patient population have shown deficits in specific aspects of cognition, such as verbal IQ [21]. In CD, regional morphological differences in cortical and subcortical structures have been critically linked to abdominal pain [22].

Psychopathological factors, such as depression and anxiety, are frequently observed to have effects in patients with functional gut disorders, and have been shown to play a role in cognitive deficits [23]. Approximately 70% of patients with functional gut disorders experience some psychological comorbidity [24]. It is unclear as to which disease develops first, however it is well accepted that stress and anxiety is associated with IBS/IBD. Regardless of this, the impact of functional gut disorders on psychological processes is undeniable as the stress associated with symptom progression severely affects patients' quality of life. Taken into account, a biopsychosocial model has been proposed to clinically approach and conceptualise IBS pathophysiology [25]. This model encompasses a lifetime perspective from the patient's childhood through to their adult life integrating genetic, environmental, learning, stress and traumatic events. Fundamentally, it takes into account the interaction of the mind and emotions, the brain, the enteric nervous system

(discussed later) and the intestinal microenvironment, including food, the immune system and microbiota. Literature demonstrates that functional gut disorders are strongly associated with a range of psychological comorbidities, specifically cognitive impairment; yet our understanding of the central consequences of other gut disorders, such as chemotherapy-related mucositis remains undetermined. Many chemotherapy drugs, such as oxaliplatin and 5-Fluororuacil (5-FU) are responsible for inducing both gut disorders and cognitive impairment, yet whether these comorbidities interact remains to be elucidated.

Gut Disorders Caused by Chemotherapy: Mucositis

The pathogenesis of mucositis was defined in five phases by Sonis [2]. Mucosal barrier injury may occur throughout the entire GIT and result in oral and/or intestinal mucositis. The rapidly dividing epithelial layer lining the GIT is particularly prone to tissue injury from different chemotherapy drugs including 5-FU, methotrexate and cyclophosphamide. Consequently, apoptotic pathways are initiated in healthy mucosal tissue causing reduced cellular proliferation in the small intestine. Some hallmark characteristics of intestinal mucositis include villus atrophy, shallow crypts, inflammation and ulceration. Mucositis results in a heightened inflammatory response via the up-regulation and activation of various transcription factors, ultimately resulting in elevated circulating pro-inflammatory cytokines, in particular interleukin-1 beta (IL-1β) and tumour necrosis factor-alpha (TNF-α).

The most significant phase of mucositis for patients is during the ulceration phase as this involves loss of mucosal integrity. Painful ulcerating lesions in the GIT become susceptible to microbial infiltration and in severe cases can lead to bacteraemia and sepsis [2]. The clinical symptoms of mucositis generally begin five to ten days after chemotherapy treatment and include significant pain, abdominal bloating, nausea and vomiting, diarrhoea and constipation [26]. Although mucositis is an acute phenomenon

which usually resolves once chemotherapy treatment has ceased, treatment may be prematurely ceased as a result of progressively worsening symptoms. Current guidelines for the prevention and management of mucositis fail to reveal effective treatment options [27]. Whilst mucositis pathogenesis is well understood, the indirect central effects of mucositis remain unknown. The complex neuroimmune axis has been suggested to be implicated in depression, a comorbidity of cancer diagnosis, and chemotherapy exposure [14]. Neurological manifestations from elevations in cytokine levels imply that neuroimmunological mechanisms underlying the pathogenesis of these chemotherapy-induced side-effects may be at play.

Cognitive Changes Following Chemotherapy Exposure

Although reports of cognitive decline in chemotherapy patients pre-date the 1980's, systematic research only commenced in the 1990's. Patients collectively termed the cognitive disturbances 'chemobrain' or 'chemofog' which heavily impacted daily functioning and quality of life, yet initial complaints were dismissed by doctors and the scientific community [7]. Previously, it was assumed that the brain was protected from systemically administered chemotherapy drugs by the blood-brain barrier (BBB) and additionally, cognitive symptoms could be explained by the stress, anxiety and depression associated with cancer diagnosis. Extensive research in the recent years clearly indicate that many systemically administered chemotherapy drugs readily cross the BBB inducing structural, molecular and cellular changes that impact upon cognitive function [1]. Mechanisms underlying the pathogenesis of CICI remain to be elucidated although suggestions include hippocampal damage and immune dysregulation (discussed below). In order to better understand the suggested mechanisms, it is important to review the clinical evidence and understand the negative impact imposed upon patients.

Clinical Evidence of CICI

The main cognitive domains affected by CICI are executive functioning, attention and concentration, processing speed, reaction time, motor speed and dexterity [1]. Whilst current estimates of CICI prevalence differ greatly (14-85%), the worldwide prediction of cancer incidence reaching 70 million in 2020 highlights the need for continued research [28]. Consequently, the estimate of high survivability rates for many cancers results in increased survivor numbers and in turn, we will see an increase in the incidence of post-treatment issues [29]. Those affected by CICI experience stressors in many facets of their lives, including relationships (familial, friends and colleagues), employment, self-esteem/worth and finances; leading to a reduced quality of life. CICI patients commonly expressed frustration in having difficulty with simple tasks, such as remembering names, misplacing everyday items and trouble finding common words [30, 31]. Emotions regularly described by CICI patients included distress, anxiety, frustration, irritability, depression and embarrassment [30, 32, 33]. Many summed up their feelings by describing as if they "felt stupid" or were "going crazy" and sometimes related their memory disturbances to the fear of being at risk for early dementia or Alzheimer's disease [30, 34]. This evidence collectively supports the negative impact of CICI on patients and emphasises the increased amount of effort and time required to complete everyday tasks.

Breast Cancer Cohorts and Duration of Cognitive Effects

Whilst the majority of CICI studies focus on breast cancer populations, cognitive deficits in a range of cancer types have been investigated, including myeloma, testicular and ovarian cancer [35-37]. Nonetheless, breast cancer populations offer researchers completion of extensive retrospective studies due to their typically good prognosis, allowing for more thorough evaluations of parallel short- and long-term sequelae [38-40]. The duration of cognitive changes is of particular interest to patients and for this reason,

continues to be an area of much research. There is considerable variability surrounding the duration of chemotherapy-induced cognitive deficits or even existence of the phenomenon. Majority of the studies report improvement in cognitive symptoms after a period of time, yet some studies have indicated the presence of symptoms for ten-twenty years after treatment cessation [41-43]. Functional magnetic resonance imaging and neuropsychological testing was observed in a group of breast cancer survivors who had received adjuvant chemotherapy treatment and was compared with a breast cancer control group who were not treated with chemotherapy [42]. The chemotherapy group demonstrated hypo-responsiveness in executive functioning tasks performed 10 years post treatment, indicating significant long-term cognitive impairments when compared to the non-chemotherapy control group.

Neuroimaging studies have identified structural and molecular changes associated with chemotherapy treatment. Reductions in specific brain regions, such as frontal cortex, temporal lobes and cerebellar grey matter regions have been reported in breast cancer patients [44]. These reductions were evident for twelve months post-chemotherapy cessation yet improvements were reported in most regions four years later. Global brain networks become re-organised under chemotherapy treatment and thus, indicate a reduced ability for information processing [45]. Additionally, chemotherapy-induced white matter tract alterations may be interpreted as demyelination or axonal damage [46].

Animal Models of CICI

Animal studies have confirmed that central structural and molecular changes may be accountable for the cognitive domains affected by common chemotherapy drugs. Several studies in rodents report declines in abilities to perform behavioural tasks following single drug administration of many chemotherapy drugs, including 5-FU, methotrexate and oxaliplatin [47-49]. Rodent behavioural tests have been adopted to understand the central

pathological changes following systemic chemotherapy exposure, such as fear conditioning, novel object recognition and the Morris Water maze. These behavioural adaptations may be interpreted as hippocampal and frontal cortex region alterations which importantly, overlap with the brain structures implicated in CICI [1].

Specific CNS cell populations are sensitive to a range of chemotherapy drugs. One of the most widely reported central changes following chemotherapy exposure is reduced hippocampal cellular proliferation. This has been documented to occur with cyclophosphamide, methotrexate, thioTEPA and 5-FU [50-52]. These CICI animal models suggested that the hippocampal changes were associated with the hippocampal-dependent behavioural changes and memory deficits. Although cyclophosphamide most frequently reports cognitive changes and cellular alterations, negative findings on long-term hippocampal changes have also been reported [53]. Nonetheless, stem cells of the dentate gyrus are particularly susceptible to chemotherapy toxicity [54-56]. This is important to note as neurogenesis within the dentate gyrus is responsible for the proliferation and division of neural stem cells that form into new neurons or astrocytes, playing a pivotal role in hippocampal circuit plasticity and memory consolidation [57, 58]. Consistent with patient observations of leukoencephalopathies and white matter tract lesions, animal and *in vitro* studies have shown that both mature oligodendrocytes and their precursors may be susceptible to the actions of chemotherapy drugs [1]. Whilst there is clear evidence that specific central cell populations are susceptible to reductions in cellular proliferation following chemotherapy exposure, some studies have reported no changes [59, 60]. This evidence reflects the complex aetiology of CICI, indicating various structural, molecular and cellular changes contributing to cognitive impairment following chemotherapy exposure. The aforementioned studies fail to take into account neuroimmune mechanisms that may be at play, whether directly or indirectly. Perhaps it is time to consider the impact

other chemotherapy-induced peripheral inflammatory events may be having on CICI, such as immune challenges in the context of malignant tissues, more specifically gut toxicities, such as mucositis.

The Contradiction: Host Immunity, Dysregulation and Cancer

The ultimate goal of the immune system is to protect and defend the host from infection and insults by recognising, repelling and eliminating pathogens and foreign molecules. Further, inflammation is an essential defensive response resulting in physiological processes critical in host healing. The toll that both malignancies and chemotherapy treatments have on the host is particularly enigmatic in the context of the immune system, whereby complex inflammatory processes contradict and manipulate responses; a dynamic network that primarily ensures protection against foreign pathogens whilst remaining tolerant of self-antigens. This somewhat contradictory phenomenon results in immune dysregulation which in turn, may result in central effects via the neuroimmune interface and signalling pathways.

Inflammatory processes become dysregulated in cancer and anti-cancer treatments. On one hand, endogenous immune processes and inflammatory cascades attempt to eliminate malignant cells from the host. Yet, simultaneously within malignant cells, similar pathways are initiated and inflammatory signalling molecules contribute to cancer establishment and progression. Several lines of evidence have suggested inflammatory processes are the *seventh hallmark* for cancer establishment and progression [8, 61, 62]. To further complicate matters, chemotherapy treatments are associated with increased circulating inflammatory markers, yet suppression of immune activity is commonly reported (discussed below). It is well established that immune dysregulation occurs in several

disorders negatively affecting the CNS and in some cases, the gut. To illustrate this point, a few disorders, such as neuropathic pain will now be further discussed.

Immune Dysregulation in Animal Models

Several convergent lines of experimental and clinical evidence have supported the hypothesis, that pro-inflammatory cytokines are pivotal in the pathophysiology of not only, cancer-related and anti-cancer treatment-induced symptoms, but other disorders, including chronic fatigue syndrome, neuropathic pain and major depression. Elevated circulating pro-inflammatory cytokines, such as IL-1 and TNF-α have been reported in clinical studies examining chronic fatigue syndrome, major depression and various pain states [63, 64]. IL-1 action is regulated by a complex network of molecules and is a potent stimulus of corticotrophin-releasing hormone, activating the hypothalamic-pituitary-adrenal axis, an important stress hormone which has been well documented in major depression [63]. Additionally, TNF-α is widely recognised as an important factor in the mediation of major depression, chronic fatigue syndrome and neuropathic pain [63-65]. Rodent models have reported that intraperitoneal administration of TNF-α resulted in a dose dependent pain responsivity, indicative of hyperalgesia (heightened sensitivity to pain) [66]. The hippocampus is associated with pain perception and cognition [67] and accordingly, a rat model of chronic constriction injury of the sciatic nerve reported increased hippocampal TNF-α levels [65]. These studies indeed demonstrate a pivotal role for the aforementioned pro-inflammatory cytokines in the pathogenesis of a range of disorders and disease states. It should be noted that the disorders mentioned in this section also often occur simultaneously in cancer patients undergoing chemotherapy treatment.

Immune Dysregulation in Cancer and Chemotherapy

There is growing consensus on two recognised interactions between cancer and the immune system. Firstly, host immunity has the ability to recognise and reject malignant

cells and immuno-surveillance can prevent tumour development and control recurrence. Consequently, activation of the innate immune system leads to the production of highly immuno-stimulatory cytokines, systemic inflammation and T- and B-cell activation, with the goal of eliminating malignant cells. Secondly, many inflammatory mediators and cells involved in detecting and eliminating malignancies also play a key role in the migration, invasion and metastasis of malignant cells, thus promoting tumour expansion [68, 69]. This double-edged sword results in a plethora of intertwined and complex interactions, in which the immune system recognises and tries to reject tumour formations, whilst inflammatory processes simultaneously enable tumour progression and development.

Additionally, chemotherapy drugs also induce inflammatory responses which may be either local, around the site of administration or systemic in nature resulting in mucositis. Several chemotherapy drugs including 5-FU (anti-metabolite), etoposide (topoisomerase II inhibitor) and doxorubicin (anthracycline) elevate pro-inflammatory cytokine production *in vitro* [70]. Importantly, this demonstrates that most cytotoxic anti-cancer drugs, regardless of their mechanism of action, increase circulating cytokines. Such findings have been translated into clinical studies linking circulating pro-inflammatory cytokine elevations with common chemotherapy-induced side-effects, such as fatigue, depression, pain and cognitive impairment [71, 72]. Extensive studies revealed the importance of elevated circulating pro-inflammatory cytokines in sickness responses which often result in cognitive changes and interestingly, mimic CICI reports. Finding therapeutic approaches that target the immune system has the potential to improve multiple chemotherapy-related side-effects which all have an immune component to their aetiology.

The intimate bidirectional relationship shared between the CNS and the GIT presents as a potential mechanism that may contribute to CICI symptom severities. As such, it is plausible that chemotherapy-induced peripheral inflammatory events, such as mucositis,

may trigger central cell population changes. Peripheral-to-central changes occurring via neuroimmunological pathways may result in behavioural (cognitive) changes, similar to those apparent in cytokine-induced sickness responses [5, 73, 74]. Although a *cytokine-based neuroimmunological mechanism of cancer-related symptoms* has been suggested [14], CICI researchers are yet to examine the indirect central effects of chemotherapy-induced peripheral inflammatory events, such as mucositis.

"Little Brain" to "Big Brain" Inflammation and Signalling Pathways

The ability of the enteric nervous system (ENS) to self-regulate (hence "*little brain*") and act similarly to the CNS ("*big brain*") makes it the largest and most complex division of the peripheral nervous system [75]. Previous literature has suggested that the GIT is a vulnerable passageway through which pathogens may influence the CNS and lead to abnormalities, for example, neuroinflammation contributing to autism [76] and multiple sclerosis [77]. A well-established link exists between various neurodegenerative diseases and the role neuroinflammation plays in their pathogenesis [78, 79]. However, few studies have examined the influence of peripheral-to-central immune signalling and neuroinflammation in the context of chemotherapy-induced mucositis and CICI.

Inflammation in the "Little Brain": ENS Inflammation

The ENS contains more than 400-600 million neurons [80] and an extensive network of enteric glial cells (EGC). Although EGCs support enteric neurons, the precise mechanisms by which EGCs support enteric neurons remains to be fully elucidated. EGCs share similarities with their CNS counterparts, astrocytes in morphological, functional and even molecular capabilities [81]. As well as exerting protective functions, EGCs are key players of the ENS during intestinal inflammation and immune responses. Their intimate relationship with enteric neurons and their responsiveness to local inflammation makes

them a prime target for therapeutic intervention as has been investigated in the CNS with targeting glial cells.

From our understanding of the intimate bidirectional relationship shared between the GIT and the CNS, it is not surprising that a diverse range of neurodegenerative diseases arise from systemic infections and inflammation, such as multiple sclerosis and Alzheimer's disease [76, 82]. We have all experienced the change in mood, emotion and cognition when one is faced with systemic infection, a cold or influenza. Numerous reports indicate that this immune response is driven by a dialogue between the peripheral systemic infection and our brain [73]. The gastrointestinal immune system is considered the primary immune organ of the body as it induces and maintains peripheral immune tolerance. This is achieved via complex cellular networks with specialised immuno-regulatory functions, including interactions between the microbiota and host. Impaired host immune defences and mutations in pattern recognition receptors leads to GIT dysfunction and enables invasion of pathogens [83]. The downstream effect of such events results in chronic GIT inflammation and/or a loss of control of local immune responses resulting in an unbalanced enteric microbiota having substantial implications in the pathogenesis of rheumatoid arthritis, IBD and asthma [84-86]. From this evidence it is clear that GIT inflammatory events may modify central processes controlling behaviour and aligns with our central hypothesis that chemotherapy-induced mucositis may result in central changes via neuroimmune mechanisms involving glia, discussed in more detail below.

Glia; the "Other Brain"

Glial cells are critical in brain development, function and plasticity in both health and disease and fall into three cell types; astrocytes, microglia and oligodendrocytes. Neurons, astrocytes and oligodendrocytes arise from neural progenitor cells whilst microglial cells originate from peripheral macrophage cell lines [87]. Glia perform a host of regulatory

functions within the CNS, from supporting neurons and regulating synaptic neurotransmission, to maintaining calcium homeostasis and clearing intracellular ions and neurotransmitters [88]. A bidirectional communication occurs between neurons and glia (astrocytes and microglia) which is now widely accepted as the neuroimmune interface; the tripartite (pre- and post-synapse and astrocyte relationship) and tetrapartite (pre- and post-synapse, astrocyte and microglia relationship) synapses describes these complex intertwined relationships in health and disease [89, 90].

Glia play a vital role in various aspects of brain function. The ambiguities of glial cells in health go far beyond our current understanding and deserve much more attention. An area of particular interest is the mechanism by which these central immune cells are involved in the pathogenesis of CNS disease states. Several researchers have gained valuable insight to this question and begun to unravel the mechanisms by which glia contribute to the pathogenesis of neurological and neurodegenerative diseases, such as Alzheimer's disease, neuropathic pain, ischaemia and migraine. The common thread linking these diseases is glial priming and subsequent neuroinflammation.

"Big Brain" Inflammation

Microglia and astrocytes may become reactive or primed either from direct-central insults or indirect-peripheral inflammatory events triggering neuroinflammatory responses. Microglia are highly sensitive to insults so are the first to react, unlike astrocytes which respond more slowly and in a more controlled manner [88]. In their reactive states, both glial cell types undergo morphological changes, augmenting a cascade of detrimental functional outcomes leading to tissue damage and neuronal death [91]. In particular, reactive glia overproduce prostaglandins, pro-inflammatory cytokines, chemokines, mediators and reactive oxygen and nitrogen species, having detrimental effects on neuronal function and survival via oxidative stress [92]. Primed glial cells reduce output of anti-

inflammatory molecules, decrease neurotrophic support, dysregulate calcium, glutamate and brain derived neurotrophic factor, resulting in excitotoxicity and neuroinflammation [93]. Interestingly, both cell types may remain in a primed state whereby they continue to be sensitised after the initial stimulus has resolved. Although primed glial cells appear active due to their morphological form, they do not overproduce inflammatory mediators until challenged, whereby they react quickly and elicit an exaggerated immune response [94]. In particular brain regions this may influence behaviours involving cognition [89, 95].

Glia modulate neurotransmission and cause neuronal injury via various mechanisms including a reduced ability to produce neurotrophic support, excitotoxic glutamate-receptor mediated damage and oxidative stress [96]. Glutamate is the primary excitatory neurotransmitter instrumental in neuronal plasticity and thus, key in learning and memory consolidation [97]. The glutamate transporters GLAST and GLT-1 are localised on astrocyte membranes [98]. Reactive astrocytes undergo reduced expression of glutamate transporters and lose their ability to re-uptake glutamate, yet continue to release glutamate into the synapse [99, 100]. Additionally, reactive astrocytes inhibit production of glutamine synthetase, an enzyme that converts extracellular glutamate to glutamine, vital in neuroprotection [101]. From this, it is not difficult to see that a significant feature of many neurodegenerative disorders is reactive or primed glia, and subsequent neuroinflammation. In the context of chemotherapy exposure, inflammation (central or peripheral) occurring via either direct or indirect mechanisms may trigger glial dysregulation and neuronal consequences, impacting negatively on cognition.

The host immune system utilises innate immune signalling to recognise microorganisms, detected by molecular structures shared by a large number of pathogens; exogenous microbe-associated molecular patterns (MAMPs) and endogenous molecules (danger-

associated molecular patterns; DAMPs). Toll-like receptors (TLRs) represent a class of innate immune receptors belonging to the IL-1/TLR superfamily and act as pattern recognition receptors capable of responding to MAMPs, DAMPs and more recently, xenobiotics (XAMPs) [102]. XAMPs represent foreign chemicals that include alcohol, methamphetamine and cocaine [103]. The mechanism by which XAMPs modify glial expression levels and morphology via TLRs may then present as a plausible mechanism contributing to CICI.

Although reactive glia might start as a beneficial process responding to an insult (disease, trauma, infection or drug exposure), it may, depending on the nature, duration and intensity of the insult, turn to a detrimental neuroinflammatory state. Defining neuroinflammation is by no means a simple task; however, it is generally accepted to include microglial and astrocyte reactivity and increased expression of pro-inflammatory cytokines and chemokines [104]. Chronic neuroinflammatory states are known to contribute to neuronal loss and central homeostatic disturbances. It is widely accepted that systemic inflammation influences brain function and behaviours. The last two decades have revealed the pivotal roles microglia, astrocytes and neuroinflammation play in various neurodegenerative diseases. In addition to neurodegenerative diseases and central injuries, neuroinflammation has also been implicated in neuropathic pain, schizophrenia, epilepsy and perhaps most recently, cancer and cognitive decline following chemotherapy exposure [105-109]. Of particular interest to this review, is the potential for chemotherapy drugs to influence glial cell populations in both the brain and the spinal cord, having implications in cognition and pain pathways. Various chemotherapy drugs appear to be causing a generalised glial response which is not limited to specific drug classes [108-110]. These studies primarily focussed on the direct-central effects of chemotherapy exposure, not accounting for the potential of GIT damage to indirectly exacerbate central changes via neuroimmune

pathways. Peripheral-to-central immune signalling pathways offer a potential way in which peripheral inflammatory events, such as mucositis may be implicated in CICI.

"Little Brain" to "Big Brain" Signalling

Histories of abuse, life stressors and other psychological factors have been shown to play an important role in the onset of various functional bowel disorders [111, 112]. As information is relayed in a bidirectional manner between the gut and the brain, it makes sense that the CNS may be modified by gut dysregulation. Information from the "*little brain*" to the "*big brain*" may be relayed via afferent neurons connecting the gut to the CNS. Pathways responsible for the transmission of various endocrine, neuronal, paracrine and humoral signals are vagal, humoral or neural. The vagus nerve provides a cytokine responsive neural pathway indirectly triggering the brain via afferent vagal input or leaky circumventricular organs [113].

Peripheral immune messages, such as focally produced pro-inflammatory cytokines may travel indirectly to the CNS via neural signalling pathways, including but not exclusive to the vagus nerve [74]. Information detected by primary afferent neurons is transduced into a neural message which is then relayed to higher order brain regions. In the brain parenchyma this message is then re-transduced back into an immune message where locally produced cytokines alter brain function by acting either directly or indirectly on neurons or glia. In specific brain regions, this may result in behavioural adaptations, involving cognition and mood. Alternatively, the slower and more direct humoral pathway occurring at leaky circumventricular organs involves molecular intermediates, such as prostaglandins. Local inflammation activates peripheral tissue macrophages to increase release of pro-inflammatory cytokines, such as IL-1β and TNF-α. Consequently, macrophages and endothelial cells release chemokines and adhesion molecules that attract leukocytes [74]. As well as their essential roles in peripheral inflammation, circulating IL-

1β and TNF-α are also key initiators of neuroinflammation. From this knowledge, we present these immune-to-brain signalling pathways as potential mechanisms by which chemotherapy-induced intestinal inflammation may directly and indirectly lead to neuroinflammation and glial dysregulation. Pro-inflammatory cytokines and mediators expressed during the pathogenesis of chemotherapy-induced mucositis may access the CNS via leaky circumventricular organs, resulting in a neuroinflammatory response.

What the Future Holds

In the year 2020 it is estimated that 70 million cancer survivors will be disease free [7, 28]. Nonetheless, a substantial proportion of survivors will have experienced either acute or delayed cognitive deficits during or post treatment cessation. Therefore, it is of paramount importance to consider the direct and indirect mechanisms underlying CICI to develop new strategies and treatments that will improve the quality of life of cancer survivors. To date, CICI animal models have failed to consider the impact of peripheral inflammatory responses on cognitive deficits. In fact, in most CICI animal studies, it is almost unquestionable that mucositis tissue damage would have certainly been present, yet these organs were not analysed. This limited angle of analysis may be missing incidental, yet crucial mechanisms in the aetiology of CICI. Irrespective of this, we acknowledge the many challenges faced by researchers undertaking CICI studies and teasing apart both the direct and indirect mechanisms presents with its own myriad of complications. Perhaps now is the time to examine chemotherapy-induced side-effects which more accurately reflect a clinical setting; elucidating how multiple chemotherapy side-effects work in unison.

One might argue that in general, the two major areas of the human body which become dysregulated following chemotherapy exposure are the gut and CNS; the "*little*" and "*big*" brains. The above sections clearly illustrate the recent substantial increase in literature

implying that brain function is somewhat dependent upon gut function and vice versa. Many questions still remain and research should continue to clarify how the neuroimmune interface and signalling pathways may be implicated in CICI. The literature reviewed presents our theory that chemotherapy-induced intestinal inflammation may drive glial dysregulation via direct and indirect neuroimmune signalling pathways which may ultimately, potentiate cognitive impairment. Harnessing our understanding of these mechanisms and outlining ways in which the gut can modulate brain function and behaviours via neuroimmune signalling pathways may guide us to novel treatment approaches that encapsulate more targeted therapies aimed at treating multiple side-effects of chemotherapy treatment.

CHAPTER TWO: Literature Review

From the Bottom Up: Chemotherapy and Gut-Brain Axis Dysregulation

Context statement

This work was published in a special edition of the Journal for *Frontiers of Behavioural Neuroscience: Clinical Relevance of the Immune-to-Brain and Brain-to-Immune Communications.*

Bajic JE, Johnston IN, Howarth GS and Hutchinson MR (2018). From the Bottom Up: Chemotherapy and Gut-Brain Axis Dysregulation. *Frontiers in Behavioural Neuroscience*, 12:104.

NOTE: This publication is included in Appendix One and it is also available to authorised users at: DOI: 10.3389/fnbeh.2018.00104

Chapter two provides an overview of chemotherapy-induced side-effects from the Bottom Up (the GIT to the CNS), briefly describing CIGT and CICI and presents several stages of the gut-brain axis which become dysregulated under these conditions. It highlights the influence of functional gut disorders (such as IBD and IBS) in the development of central comorbidities and links this to CIGT and CICI. It provides a brief overview of communication pathways making up the gut-brain axis with a focus on humoral and neural mechanisms. Critically, this chapter emphasises the complex nature of gut-brain axis dysregulation in the chemotherapy setting. It also examines the pivotal and interchangeable role the microbiota plays in intestinal permeability and vice versa, as well as identifying mechanisms relating to peripheral nerve and ENS dysfunction.

Statement of authorship

Statement of Authorship

Title of Paper	From the bottom up: chemotherapy and gut-brain axis dysregulation
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	**Bajic JE, Johnston IN, Howarth GS and Hutchinson MR (2018). From the bottom up: chemotherapy and gut-brain axis dysregulation.** ***Frontiers in Behavioural Neuroscience,*** **12:104.**

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Contribution to the Paper	Manuscript conception, reviewed all papers cited in manuscript, wrote manuscript, designed figures and acted as corresponding author.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/04/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);

II. permission is granted for the candidate in include the publication in the thesis; and

III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

The central nervous system (CNS) and gastrointestinal tract (GIT) form the primary targets of chemotherapy-induced toxicities. Symptoms associated with damage to these regions have been clinically termed chemotherapy-induced cognitive impairment (CICI) and mucositis. Whilst extensive literature outlines the complex aetiology of each pathology, to date neither chemotherapy-induced side-effect has considered the potential impact of one on the pathogenesis of the other disorder. This is surprising considering the close bidirectional relationship shared between each organ; the gut-brain axis. There are complex multiple pathways linking the gut to the brain and vice versa in both normal physiological function and disease. For instance, psychological and social factors influence motility and digestive function, symptom perception, and behaviours associated with illness and pathological outcomes. On the other hand, visceral pain affects central nociception pathways, mood and behaviour. Recent interest highlights the influence of functional gut disorders, such as inflammatory bowel diseases and irritable bowel syndrome in the development of central comorbidities. Gut-brain axis dysfunction and microbiota changes have served as key portals in understanding the potential mechanisms associated with these functional gut disorders and their effects on cognition. In this review we will present the role gut-brain axis dysregulation plays in the chemotherapy setting, highlighting peripheral-to-central immune signalling mechanisms and their contribution to neuroimmunological changes associated with chemotherapy exposure. Here, we hypothesise that dysregulation of the gut-brain axis plays a major role in the intestinal, psychological and neurological complications following chemotherapy. We pay particular attention to evidence surrounding microbiota dysbiosis, the role of intestinal permeability, damage to nerves of the enteric and peripheral nervous systems and vagal and humoral mediated changes.

Background

The chemotherapy experience is associated with powerful psychological, neurological and somatic side-effects. Cancer diagnosis and the complications arising from treatment induce anxiety and depression, fatigue, pain, and cognitive impairments while patients struggle to maintain hope for recovery and continue normal daily functions, routines and roles [57, 146, 147]. Due to the non-selective and systemic nature of most chemotherapy drugs, they also target healthy, rapidly-dividing non-malignant cells. The regions of the body most susceptible to the unwanted toxicities of chemotherapy exposure are the gastrointestinal tract (GIT) and the central nervous system (CNS) – the gut and brain. Many chemotherapy drugs are small enough to readily cross the blood-brain barrier (BBB) and result in molecular, structural and functional changes within the CNS, manifesting as cognitive changes in a subset of patients [148]. Outside of the CNS, the cells of the GIT are particularly vulnerable to damage following chemotherapy exposure. In particular, epithelial cells within the mucosal layer lining the alimentary tract form prime targets due to chemotherapy drugs targeting proliferating enterocytes [1]. Although the gut and the brain appear disparate, they are intimately connected. The complex network of pathways linking the gut to the brain will be discussed in more detail below as we present mechanisms by which chemotherapy results in gut-brain axis dysregulation.

This network has a bidirectional relationship. For instance, psychological and social factors have the ability to influence motility and digestive function, symptom perception, behaviours associated with illness and the pathological outcome [149]. On the other hand, visceral pain affects central pain perception and pathways, mood and behaviour [150]. Importantly, systemic and gut immunity is tightly regulated by the inflammatory reflex and cholinergic anti-inflammatory pathway [151, 152]. Integral components of the inflammatory reflex include innate immune cell activation, release of inflammatory

mediators, such as cytokines, vagal innervation and responses from higher order brain regions, such as the nucleus tractus solitarius. Vagal innervation is of particular importance in the chemotherapy setting as it is pivotal in the transmission of chemo and mechanosensory information from the gut to the brain (Figure 2.1) [145, 151]. In this sense, pro-inflammatory mediators and cytokines, such as interleukin-1 beta (IL-1β) and tumour necrosis factor-alpha (TNF-α) activate primary afferent nerve fibres within the vagal sensory ganglia. Vagal ganglia signal several nuclei of the dorsal vagal complex responsible for the integration of visceral sensory input. This information is relayed to higher order brain regions like the hypothalamus, hippocampus and forebrain. Coordinated autonomic and behavioural responses are initiated to assist in restoration of homeostasis. Importantly, efferent vagal motor activity inhibits cytokine synthesis, creating the inflammatory reflex effect. Humoral anti-inflammatory pathways can be activated, stimulating the release of adrenocorticotropin hormone. Sympathetic outflow can also increase localised adrenaline and noradrenaline expression and further suppress inflammation. The activation of these innate components of the inflammatory reflex, including the vagally-mediated cholinergic efferent output, ultimately results in the regulation of systemic and localised inflammation, having important implications in gut immunity (Figure 2.1). A more comprehensive outline of the inflammatory reflex has been reviewed elsewhere [151, 152].

Additionally, activation of the neuroimmune system via glial priming and neurogenic inflammation further complicates immune to brain signalling. Although glial cells are non-neuronal cell types which can be found in the CNS and periphery, such as oligodendrocytes and Schwann cells, for the remainder of this manuscript we specifically refer to microglia and astrocytes. For an in-depth analysis of glial priming and neuroinflammation several excellent reviews exist [121, 122, 124, 143, 153-155].

Nonetheless, to illustrate this point in the context of cancer and chemotherapy, inflammation (either centrally or locally derived from either the malignancy or chemotherapy) and the release of pro-inflammatory cytokines signals the brain and activates neuroimmunological cells, glia (Figure 2.2). Pro-inflammatory cytokines access the brain either directly via leaky circumventricular organs or indirectly via a neural route (e.g. vagal transmission). Microglia and astrocytes form an integral part of the tri- and tetra-partite synapses and maintain a close bidirectional relationship with neurons; the neuroimmune interface, which has wide implications in central health and disease [118, 121, 123, 155]. Reactive glial cells undergo morphological changes and overproduce pro-inflammatory mediators whilst reducing anti-inflammatory output [125, 156]. Ultimately, glial reactivity results in a neuroinflammatory environment whereby neurotoxicity causes damage to surrounding tissues and neurons [129, 157, 158]. Centrally derived neurogenic inflammation and signalling also contributes to the exacerbation of peripheral inflammatory conditions. Although glial reactivity may begin with beneficial intentions by responding to insults (disease, trauma, infection or drug exposure), glia may remain in a primed state and be sensitised even after the initial insult has resolved, eliciting an exaggerated immune response (Figure 2.2). Critically, in particular brain regions primed glia and neuroinflammation influence behaviours involving cognition and are involved in the pathogenesis of various neurodegenerative diseases and pathological pain states [129, 159]. Due to the altered immune profile of cancer and chemotherapy patients, it has been suggested that neuroinflammatory processes may be contributing to the cognitive deficits often experienced by this patient group [61, 160]. This form of innate immune (peripheral-to-central) signalling represents a plausible mechanism meditating mucositis and neurological changes (Figure 2.2).

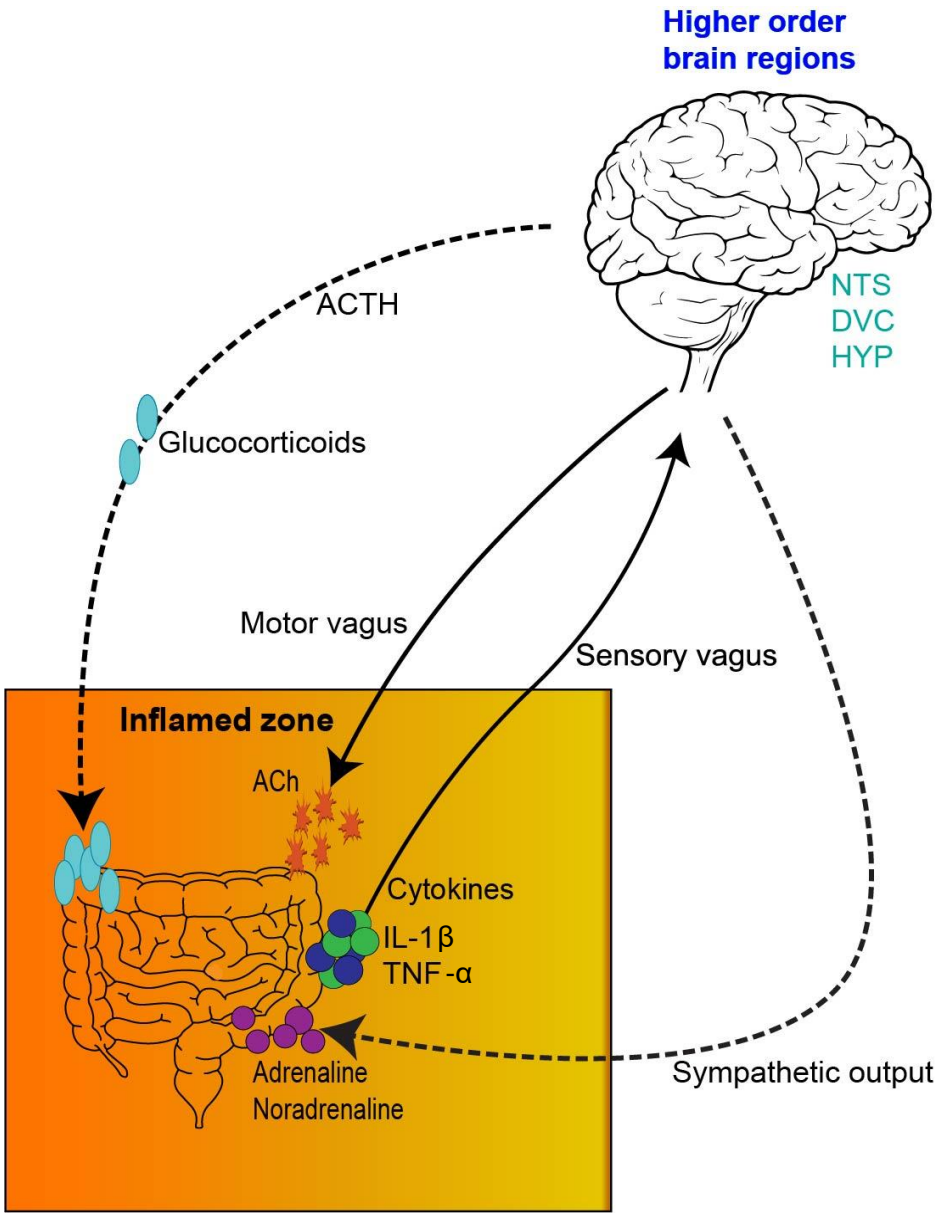

Figure 2.1. The inflammatory reflex. 1) The inflamed zone represents tissue damage, infection and ischemia; 2) Increased expression of inflammatory mediators and cytokines, such as Il-1β and TNF-α are released by cells in the inflamed zone; 3) Cytokines activate primary afferent neurons within the vagal sensory ganglia; 4) Afferent visceral signals are relayed to nuclei in the DVC, such as the NTS; 5) Visceral information is further relayed from the DVC to higher order brain regions, such as the hypothalamus, hippocampus and forebrain; 6) Activation of efferent vagal motor activity inhibits cytokine synthesis; 7) Hypothalamus activation stimulates the release of adrenocorticotrophic hormone from the pituitary gland, initiating a humoral anti-inflammatory pathway; 8) Sympathetic outflow can increase localised adrenaline and noradrenaline expression further suppressing inflammation. IL-1β; interleukin-1 beta, TNF-α; tumour necrosis factor – alpha, DVC; dorsal vagal complex, NTS; nucleus tractus solitarius, HYP; hypothalamus, ACTH; adrenocorticotrophic hormone.

Following on from this, it is not surprising that interactions between the immune system and the brain become dysregulated under cancer and chemotherapy conditions. Further, recent evidence has highlighted the impact gut commensal bacteria has in both central and peripheral development and health [161]. Importantly, dysbiosis (microbial imbalance/maladaptation) and gut-brain axis dysfunction have been associated with functional gut disorders having negative effects on cognition [37, 46]. Previously, research has focussed on a single pathological manifestation of chemotherapy exposure, for example gut toxicity or regional structural brain changes [93, 162]. Such studies have failed to consider the *indirect* effects of simultaneously occurring treatment-induced toxicities, which may be contributing to the primary pathology under investigation. Consequently, we hypothesise that chemotherapy treatment causes severe and prolonged psychosocial impacts on the survivor. Furthermore, we suggest that the gut-brain axis is an important mediator of a diverse range of cognitive and emotional disorders similar to those experienced by cancer survivors. Here, we will determine whether chemotherapy affects the gut-brain axis and present several key stages. Following on from this, we suggest that the psychosocial side-effects of chemotherapy treatment could be caused by the effects of chemotherapy on the gut-brain axis.

Following a brief analysis of gut-brain communication, we will review some key studies linking gut-brain axis dysregulation to specific psychiatric disorders, highlighting similarities between these conditions and the chemotherapy setting. From the Bottom Up (GIT to the brain) we will examine chemotherapy-induced gut and central changes and present several mechanisms meditating gut-brain axis dysregulation in the chemotherapy setting; focussing on the microbiome, intestinal integrity, peripheral neuropathies and enteric nervous system (ENS) dysfunction. Finally, we will address the role vagal-, neural- and humoral-mediated responses may play in these complex chemotherapy-induced

pathological conditions. Overall, we aim to illustrate the complex role gut-brain axis dysregulation plays in shaping neurological changes associated with chemotherapy exposure.

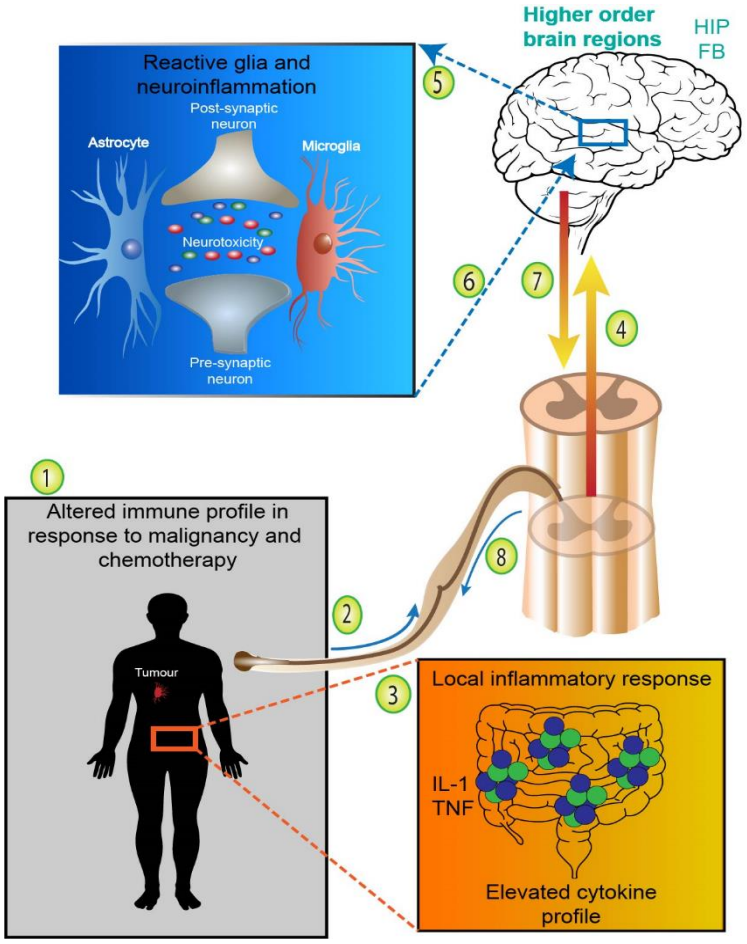

Figure 2.2. Adapted from Dodds *et al.*, 2016. Neuroimmunological complications arising from cancer and chemotherapy treatment. 1) Cancer patients undergoing chemotherapy treatment express an altered immune profile with increases in pro-inflammatory cytokines. 2) Systemic pro-inflammatory cytokines and mediators, such as IL-1 and TNF released either from the malignancy or treatment-associated toxicities access the brain directly via leaky circumventricular organs or (3) indirectly via neural transmission. (4) Systemic or localised pro-inflammatory mediators and cytokines signal higher order brain regions and (5) activate microglia and astrocytes. Reactive glia undergo morphological changes and overproduce pro-inflammatory mediators whilst reducing anti-inflammatory output – resulting in neurotoxicity and neuroinflammation. 6) In particular brain regions primed glia and neuroinflammation influence behaviours involving cognition and contribute to various neurodegenerative diseases. 7) Centrally derived neurogenic inflammation and descending signalling in the spinal cord (8) contributes to the exacerbation of peripheral inflammatory conditions and exaggerated pain states. Therefore, peripheral-to-central innate immune signalling represents a plausible mechanism meditating chemotherapy-induced gut toxicity and neurological changes. FB; forebrain, HIP; hippocampus, IL-1; interleukin-1, TNF; tumour necrosis factor.

Gut-Brain Cross Talk

Since Pavlov's Nobel Prize-winning discovery on the role neural innervation plays in gastric secretion – the first functional evidence connecting the gut and brain – our understanding of the pathways connecting the CNS and the GIT have significantly advanced [163]. The multiple bidirectional pathways responsible for controlling signalling from the brain to the gut and vice versa have been extensively reviewed and is outside the scope of this manuscript [2-5]. The complexity of this network is best appreciated in its ability to integrate information from a variety of systems encompassing the central, autonomic and enteric nervous systems (including the influence of the intestinal microbiota), whilst simultaneously considering neuroendocrine, enteroendocrine and neuroimmune input (summarised in Figure 2.3) [5]. A brief analysis of Bottom Up and ENS mechanisms is necessary to appreciate the systems by which the integration of these pathways influence behaviour and impact central comorbidities in disorders of the gut. We begin this section from the Bottom Up; presenting key pathways, cell types and signalling mechanisms involved in communication from the gut to the brain. We also illustrate mechanistic evidence relating to disorders of the gut which often have a central comorbidity component, such as in the case of inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS). Whilst research covering the central comorbidities associated with IBD and IBS continues to expand, the potential mechanisms linking neurological and gut changes following chemotherapy exposure remains under investigated.

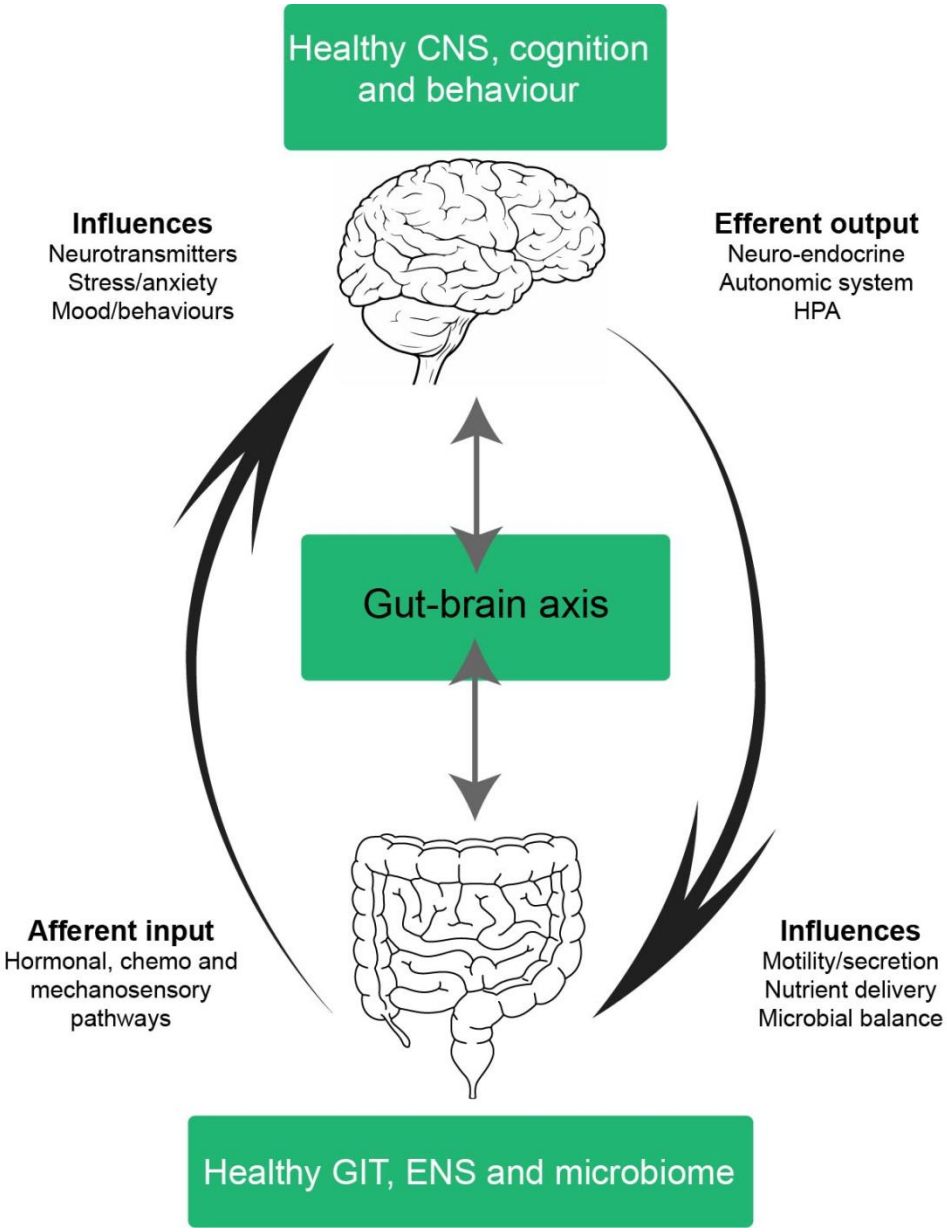

Figure 2.3. Schematic of a healthy gut-brain axis. In a healthy system, the gut-brain axis integrates information from many systems; the CNS, ANS, ENS, neuroendocrine, enteroendochrine, neuroimmune and HPA. The complex bidirectional communication pathways and systems shared between the gut and the brain maintain health and homeostasis in the CNS, GIT and microbiota. Efferent signals from the brain involving neuro-endocrine, autonomic and HPA outputs influence motility, secretion, nutrient delivery and microbial balance in the GIT. Whilst afferent inputs from the GIT, such as intestinal hormones, cytokines and sensory perceptions influence neurotransmitter expression, stress, anxiety, mood and behaviour. CNS; central nervous system, chemo; chemotherapy, ANS; autonomic nervous system, ENS; enteric nervous system, HPA; hypothalamic-pituitary axis, GIT; gastrointestinal tract.

From the Bottom Up

The GIT elicits a myriad of functions ultimately resulting in absorption of nutrients and expulsion of noxious chemicals and pathogens via muscular contractions, cellular, endocrine and immune mechanisms. Critically, the gut harbours a diverse microbial community (e.g. bacteria, fungi, archaea, viruses, and protozoa) and has prolific central effects mediating a healthy host [161]. Consequently, changes in gut-microbial composition disrupts physiological homeostasis, often contributing to central maladaptations [164, 165]. Recent advances in our understanding of the impact the microbiota has on the gut-brain axis has led to common use of the term *microbiota-gut-brain axis* [35, 36]. Microbiota-gut-brain axis communication alters certain aspects of brain development, function, mood and cognitive processes from both the Bottom Up and vice versa [5, 14, 15, 17, 36, 166]. Evidence specifically related to chemotherapy-induced microbiota changes will be discussed further below (see reviews on microbiota-gut-brain axis [14, 17, 35, 36]).

The GIT maintains an extensive intrinsic nervous system, the ENS which is unique in its ability to control certain functions of the small and large intestines even when it is disconnected from the CNS [2]. However, the ENS should not be considered fully autonomous due to the constant top down input it receives. The ENS is the largest and most complex division of the peripheral nervous system comprising 400-600 million neurons and an extensive network of enteric glial cells (EGC) [24]. EGCs share similarities with astrocytes, their CNS counterparts in the mechanisms they adopt to support enteric neurons, including their morphology, function and molecular capabilities [29]. Importantly, EGCs play key roles in mounting an immune response, particularly during intestinal inflammation.

Luminal environmental factors, such as mechanical and chemical changes are signalled from the gut to the brain via endocrine, immune and neuronal afferent pathways [3, 4, 24, 25]. Information regarding the level of distension, concentrations of specific nutrients, electrolytes, pH, and the presence of danger and immune signals is transmitted from the gut to the brain via a wide variety of neural and systemic communication pathways. Visceral changes are detected by a variety of sensory cell types including enterocytes, intrinsic and extrinsic primary afferent neurons, immune and enteroendocrine cells [5].

Hence, a wide variety of hormones and metabolites from the gut communicate homeostatic information to the brain via functional effector cells (enterocytes, smooth muscle cells, interstitial cells of Cajal, enterochromaffin cells, intrinsic and extrinsic primary afferent neurons, immune and enteroendocrine cells) [5]. Examples of homeostatic information relayed from the functional effector cells include but are not exclusive to sensory, pH, water metabolism, chemical, danger and immune signals. Each cell type responds to luminal environmental changes and secretes specific signalling molecules which may include but are not exclusive to ghrelin, cholecystokinin, glucagon-like peptide-1, corticotrophin releasing hormone, proteases and cytokines, etc. [25]. To further complicate gut-brain crosstalk, various neurotransmitters commonly produced centrally are also expressed in the GIT [25]. Gut derived neurotransmitters, such as dopamine, serotonin and neuropeptide Y influence many aspects of central homeostasis, yet in the gut are responsible for appetite, satiety, hunger, pain and are implicated in the activation of reward pathways relating to food and beverage intake [2].

Numerous afferent and efferent pathways connect the gut and brain, presenting the host with a multitude of platforms for malfunction, dysregulation and disease, both in the periphery and centrally. Whilst the basic principles outlining top down signalling have been extensively reviewed [2, 3, 25] and is outside the scope of this review, it is crucial to

acknowledge that these effects occur simultaneously with those described From the Bottom Up. Importantly, top down sympathetic and parasympathetic interactions suppress secretion, motility and GI transit, having direct effects on immune-, emotion-, mucosa- and microflora-related alterations [4, 26]. Gut-brain axis dysfunction has played a pivotal role in our mechanistic understanding of various gut disorders and their effects on cognition. Indeed, experimentally induced gut disorders have critically developed our understanding of the mechanisms underlying central changes induced by disruptions in gut homeostasis. Disorders of the gut and chronic inflammation often result in psychological abnormalities, such as anxiety and depression [39]. Additionally, physiological responses can be induced by stress, for instance triggering relapse in experimental colitis [40].

Great interest has recently been paid to the importance of gut health on mental health and vice versa. This has become particularly evident in the continual expansion of anecdotal evidence on the central comorbidities associated with various gut disorders, particularly in IBS and IBD [41, 42]. Disorders of the gut are commonly associated with poorer mental health. For instance, 54-94% of IBS patients actively seeking treatment also present with emotional, psychological and cognitive comorbidities [42] as do chemotherapy recipients. The literature presented above provides clear evidence that gut disorders often occur simultaneously with central comorbidities, aligning with our hypothesis that gut-brain axis dysregulation may be mediating both chemotherapy-induced mucositis and neurological changes. Therefore, it is pivotal that we determine the *direct* and *indirect* central consequences of drug-induced gut disorders, such as chemotherapy-induced mucositis. Chemotherapy induces a range of peripheral and central side-effects, significantly reducing quality of life. In the gut this has been termed chemotherapy-induced mucositis and in the CNS, chemotherapy-induced cognitive impairment (CICI). The current review will now explore whether mucositis and CICI are linked and whether they exacerbate other

symptoms, such as pain associated with mucositis, or cognitive impairment which are often experienced simultaneously in the chemotherapy setting.

Chemotherapy From the Bottom Up: the Gut and Cognition

Chemotherapy drugs can be considered paradoxical at the most basic level. Primarily, they offer recipients' survivorship as they target malignant cells in an attempt to rid the host of cancer. On the other hand, due to their non-selective nature, they also target healthy cells and induce a range of side-effects reducing patient quality of life. The organ where their actions are perhaps often first noticed is the GIT due to its high regenerative capacity. Mucositis occurs in up to 70% of chemotherapy recipients and may manifest anywhere along the alimentary tract, termed oral or intestinal mucositis (Figure 2.4) [167]. It is one of the most significant dose-limiting side-effects of intensive anti-cancer therapy due to the painful nature of the disorder.

Sonis classified the pathogenesis of mucositis into five stages [1]. Hallmark characteristics of mucositis include villus atrophy, shallow crypts, inflammation and ulceration. Mucositis results in a high inflammatory response via the up-regulation and activation of various transcription factors, ultimately causing elevations in circulating pro-inflammatory cytokines (Figure 2.4), in particular IL-1β and TNF-α [1]. Whilst mucositis is an acute phenomenon which usually resolves upon cessation of chemotherapy treatment, clinical symptoms generally begin five to ten days post chemotherapy exposure and include significant pain, abdominal bloating, nausea and vomiting, diarrhoea and/or constipation [168]. Although guidelines for the prevention and treatment of mucositis exist, they fail to include effective treatment options [169]. Novel complementary treatment approaches are showing positive results utilising naturally sourced products, such as Emu Oil and Rhubarb extract [108, 110]. Although these treatment strategies show promise, to date they are still in the pre-clinical stages. Our understanding of the central consequences of drug-induced

gut disorders, such as mucositis remains elusive, yet evidence on CICI is expanding as various mechanisms underlying its pathogenesis are becoming clearer.

CICI occurs in 15-45% of patients undergoing anti-cancer therapy [170]. Subjective (self-reported) report rates are considerably higher than objective measures with some studies reporting 95% of patients experiencing changes in cognitive performance [57]. Subjective measures are nonetheless important as they identify the impact of cognitive impairment and the strain it places on patients' lives and daily functioning [94]. The breast cancer population forms the majority of the CICI literature as they offer researchers completion of extensive retrospective studies due to their typically good prognosis [113]. Regardless, CICI has been investigated in a range of other cancer types including myeloma and testicular cancer [171, 172].

The cognitive domains most commonly reported in CICI are executive functioning, attention and concentration, processing speed, reaction time and motor speed and dexterity [79]. Perceived cognitive impairment affects various facets of the patient's life, including relationships, employment, self-esteem/worth, finances and independence. The CICI experience leaves patients feeling distressed, anxious, frustrated, irritable, depressed and embarrassed, often reducing confidence [173, 174]. Current estimates on the duration of CICI are varied with some studies identifying deficits up to twenty years post chemotherapy cessation, yet most indicate improvements up to twelve months later [96, 98]. Neuroimaging studies have confirmed molecular and structural changes in the grey matter of the frontal and temporal lobes and the cerebellum of breast cancer patients following chemotherapy exposure [175, 176]. Additionally, chemotherapy induces white matter tract changes and the reorganisation of global brain networks, which have undoubtable associative if not causal impacts on cognitive performance [117, 177].

Animal studies have begun to unravel various mechanisms underlying the pathogenesis of CICI and involve structural and behavioural changes. Hippocampal and frontal cortical alterations have correlated with behavioural memory changes in various rodent models [89, 148, 178]. Neurogenesis occurs in the dentate gyrus and cellular proliferation is critical in hippocampal circuit plasticity and memory consolidation [179, 180]. CICI models have reported on the vulnerability of stem cells to proliferate in the dentate gyrus irrespective of chemotherapy drug class [87, 181]. Considering the pivotal role neural stem cells in this region have to divide into new neurons or astrocytes, disruptions in this process present as a direct mechanism which may be contributing to CICI. More recently, neuroimmunological manifestations, such as glial dysregulation and neuroinflammation, have been reported to contribute to CICI [182, 183].

Currently, effective prevention strategies or treatment approaches for CICI remain undetermined although two evidence-based guidelines are available to assist oncologists in addressing cognitive deficits [184]. Other interventions for CICI are broadly categorised into cognitive training, compensatory strategies, pharmacological, and complementary and integrative medicines [185]. Recently, Toll-like receptors (TLRs) have been suggested as a common component in the pathology of neuropathy/pain and gut toxicity resulting from chemotherapy, presenting a novel and much needed therapeutic approach in the treatment of chemotherapy-induced toxicities [186]. TLRs have profound homeostatic effects, tightly regulating innate immune and gut functions, modulating pain behaviours [187-192]. Wardill *et al*. [186], hypothesised that TLR-4 mediated glial activation and neuropathy driven by the molecular signals released from gut toxicity caused by chemotherapy. Primary studies have indicated that an altered TLR expression profile may contribute to chemotherapy-induced pain and diarrhoea [189]. This study importantly highlights the need for further research examining both peripheral and central toxicities associated with

chemotherapy treatment. Interestingly, the selective serotonin reuptake inhibitor, fluoxetine, has shown promising results in a rat model of CICI. Fluoxetine co-administration with the chemotherapy drug improved cognitive performance in rats assessed by object location recognition [193]. Whilst cellular proliferation in the dentate gyrus significantly reduced in the chemotherapy group, co-administration with fluoxetine reversed this reduction. Regardless of the evidence presented here indicating CNS changes following chemotherapy exposure, it is important to note that some studies have reported no structural or cognitive changes [95, 194]. Various cytotoxic insults have revealed no morphological changes to neurons located in the CNS [195, 196]. These negative findings could result from a range of factors, including differences in species, drug, dose, type of administration and cognitive parameters examined; but importantly, suggests that more complex mechanisms are likely to play a role in CICI.

Whilst the direct mechanisms presented here reflect the complex aetiology of CICI, they fail to acknowledge the influence other simultaneously occurring side-effects may be having on CICI symptoms. Many of the CICI models described above utilised chemotherapy drugs that are also often used to examine mucositis, for example 5-Fluorouracil (5-FU), methotrexate and oxaliplatin. Although mucositis would have most likely been present in these models, the gut tissue would not have been examined and thus, the potential for *indirect* mechanisms relating to gut-brain axis dysregulation would have been ignored. In doing so, we may be missing critical mechanisms contributing to or exacerbating CICI pathogenesis. In order to explore this theory, we will now consider the influence chemotherapy exposure has on the gut-brain axis, opening novel hypotheses surrounding how mucositis may contribute to the aetiology of CICI.

Chemotherapy Interrupts Several Stages of the Gut-Brain Axis

As presented above, the two organs most vulnerable to the toxicities of chemotherapy treatment are the gut and the brain. Therefore, it is plausible that several stages of the gut-brain axis may become dysregulated in the chemotherapy setting (Figure 2.4). Here, we propose that chemotherapy exposure influences the gut-brain axis via several mechanisms which include: altering intestinal microbiota composition and function; upsetting the balance of "beneficial" and "detrimental" bacteria in the lumen, deleteriously affecting the gut lining, impairing the ENS and activating neuroimmune and pain signalling pathways (Figure 2.4). The interaction of the gut-brain axis and the neuropsychological comorbidities associated with specific gut disorders have been extensively reviewed, for example depression/cognitive deficits and IBS/IBD [42, 45, 47-49]. However, this angle of research is yet to be reviewed in the context of chemotherapy exposure and cognitive impairment. Research in this area will continue to develop as we begin to appreciate that chemotherapy-induced side-effects involving the gut-brain axis may continue to linger for some time after treatment cessation, placing significant strain on health care systems and importantly, patient quality of life.

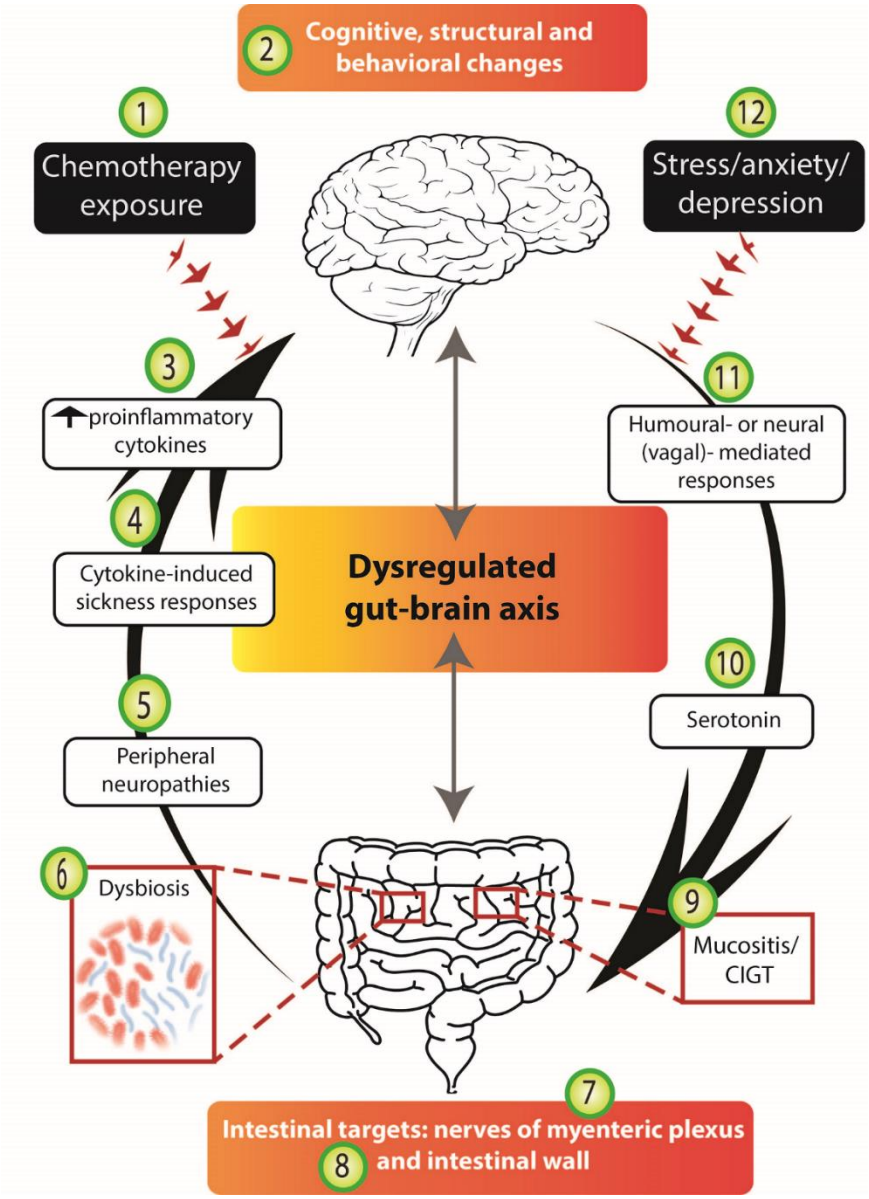

Figure 2.4. Chemotherapy disrupts several stages of the gut-brain axis. We suggest that chemotherapy-induced gut-brain axis dysregulation plays a major role in the intestinal, psychological and neurological complications experienced by many cancer patients. Chemotherapy often results in molecular and structural changes in the brain, e.g. hippocampal changes as identified in rodent models. Chemotherapy exposure causes cognitive and behavioural changes to a subset of patients and these findings have been supported by some experimental models. The altered immune profile of chemotherapy recipients results in increased circulating pro-inflammatory cytokines which have been reported to cause cytokine-induced sickness-like responses (which mimic chemotherapy-induced side-effects). Damage to peripheral nerves resulting in peripheral neuropathies are experienced by some chemotherapy recipients. Chemotherapy targets the intestines by impairing the nerves of the myenteric plexus, damaging intestinal wall parameters and resulting in mucositis and causing microbiota dysbiosis. Serotonin dysregulation under chemotherapy conditions may play a role in chemotherapy-induced intestinal and neurological changes. Finally, peripheral-to-central immune signalling pathways (humoral or neural/vagal) may mediate chemotherapy-induced gut-brain axis dysregulation.

The Microbiome

It has been estimated that our gut contains 100-fold more genes than the human genome and approximately 1000 bacterial species [197, 198]. Our gut microbiome coevolves with us [199] and changes may be either beneficial or detrimental to human health. In healthy individuals, the gut microbiota is responsible for a number of health benefits, such as pathogen protection, nutrition, host metabolism and immune modulation [200]. Although a core microbial population has been established in individuals, changes can be caused by many factors including age, diet, antibiotic and analgesic use and environmental factors [201]. The microbiome facilitates intestinal homeostasis and more specifically, has the capacity to influence inflammation and immunity, both at the local (mucosal) and systemic levels [202]. Commensal bacteria play important roles in anti-viral immunity, regulating systemic immune activation [203]. Signals released by commensal bacteria assist in immune development and thereby, have important implications for infectious and inflammatory disease susceptibility [203, 204], such as in the case of chemotherapy-induced mucositis. Consequently, dysbiosis can heavily influence pathological intestinal conditions with an inflammatory component, for example in experimentally-induced IBD [205-208]. Critically, IBD patients reported microbial composition changes with major shifts in genomic landscape and functional outcomes [209]. Undoubtedly, the implications of such IBD studies have heavily impacted oncology, raising many questions specifically relating to the intestinal microbiota, immune, malignancy and anti-cancer treatment interactions.

Whilst Sonis' five-phase model of mucositis [1] lacked any potential influence on the microbiota, unequivocal research has indeed confirmed that intestinal inflammation modulates microbiome composition and function [208, 209]. As intestinal inflammation is a common characteristic of mucositis, it makes sense that chemotherapy induces functional

and compositional changes to the microbiome. It has been suggested that mucositis development is influenced by commensal microbiota in multiple pathways involving inflammation and oxidative stress, intestinal permeability (discussed below), mucus layer composition , epithelial repair mechanisms and via the release of immune effector molecules [38]. Indeed, research has begun to unravel the complexities surrounding the interactions between the host and the intestinal microbiota following chemotherapy exposure and consequently, several excellent reviews exist [38, 208, 210-212]. Commonly used chemotherapy drugs, such as 5-FU and irinotecan report drastic shifts in intestinal microflora, from commensal bacteria which maintain a symbiotic relationship with the host, to elevated levels of *Escherichia* spp., *Clostridium* spp., and *Enterococcus* spp. which can be associated with several pathologies involving inflammation and infection [213-216] (Table 2.1). Several clinical studies have supported pre-clinical findings describing alterations in faecal microbial composition following chemotherapy treatment. Literature reveals a general decrease in the overall diversity of bacteria in the microbiota of cancer patients undergoing anti-cancer treatment when compared to healthy individuals, irrespective of cancer type or chemotherapy regime [217-219] (Table 2.1).

In addition to the direct effects microorganisms and their enzymes have on cancer initiation and progression [16, 220], the microbiota also modifies drug absorption and metabolism via gene expression changes [221, 222]. This has become a pivotal research angle in oncology as chemotherapy-microbiota-immune interactions have identified microbial-mediated innate and adaptive immune responses and their effect on the efficacy of cancer immunotherapy and chemotherapy drugs [223-226]. Two crucial studies presented in *Science* (2013) [223, 224] reported that microbiota disruption by antibiotic treatment impaired chemotherapy drug efficacy on tumours, utilising cyclophosphamide and oxaliplatin. More recent studies have illustrated the important role certain microbial

strains (*Bifidobacterium*) play in anti-tumour immunity [225, 226]. Although these studies were performed in mice, their findings indicate the potential risks associated with the use of antibiotics during chemotherapy treatment.

Table 2.1. Summary of key papers highlighting chemotherapy-microbiota-immune interactions.

Study	Subjects	Treatment	Commentary
Lin, *et al.*	Tumour bearing rats	Irinotecan alone Irinotecan/5-FU	Increased abundance *clostridial clusters I*, *XI*, and *Enterobacteriaceae*.
Von Bültzingslöwen, *et al.*	Rats	5-FU	Increased facultative and anaerobic bacteria from the oral cavity. Increased facultative anaerobes in large intestine. Proportion of facultative gram-negative rods increased in both oral cavity and intestine.
Stringer, *et al.*	Rats	Irinotecan	Increased jejunal samples of *Escherichia spp*., *Clostridium spp*., *Staphylococcus spp*., Increased colonic samples of *Escherichia spp*., *Clostridium spp*., *Enterococcus spp*., *Serratia spp*., *Staphylococcus spp*. No changes in faecal flora except *E. coli*.
Stringer, *et al.*	Rats	Irinotecan	Extensive changes were evident in stomach, jejunum, colon and faeces. Most significant changes were in colon, indicating a relationship between colon bacteria modification and diarrhoea incidence.
Montassier, *et al.*	Patients with non-Hodgkin's lymphoma	Bone marrow transplantation with chemotherapy conditioning	Steep reduction in alpha diversity during chemotherapy. Decreases in *Firmicutes bacteria* and *Bifidobacterium* whilst *Bacteriodes* and *Esterichia* were increased
Manichanh, *et al.*	Patients with abdominal tumours	Pelvic radiotherapy	Faecal samples reported significant microbiota profile changes in patients with postradiotherapy diarrhoea. Not all patients reported diarrhoea. Importantly, this study suggests initial microbial colonisation may be linked to susceptibility or protection against diarrhoea following radiotherapy treatment.
Zwielehner, *et al.*	Patients with various malignancies	Chemotherapy and antibiotic treatment	Chemotherapy decreased *Clostridium cluster IV* and *XIVa*. *C. difficile* was present in three out of seventeen patients and was accompanied by a decrease in the genera

			Bifidobacterium, *Lactobacillus*, *Veillonella* and *Faecalibacterium prausnitzii*. *Enterococcus faecium* increased following chemotherapy.
Iida, *et al*.	Tumour bearing mice	Oxaliplatin and cisplatin	Chemotherapy-induced dysbiosis impairs response to immunotherapy and chemotherapy.
Viaud, *et al*.	Tumour bearing mice	Cyclophosphamide	Jejunal and faecal samples reported dysbiosis and induces translocation of specific Gram-positive bacteria to secondary lymphoid organs whereby they stimulate subsets of T cells. These results suggest that the gut microbiota may affect anticancer immune response.
Sivan, *et al*.	Tumour bearing mice	Co-housing, faecal transfer, programmed cell death protein 1 ligand 1 (PD-L1)–specific antibody therapy (checkpoint blockade), oral *Bifidobacterium*	Changes to anti-tumour immunity were eliminated by co-housing and faecal transfer. Oral *Bifidobacterium* administration improved tumour control to same degree as PDL-1 therapy; combination treatment nearly abolished tumor outgrowth.
Vetizou, *et al*.	Tumour bearing mice and metatstatic melanoma patients	Ipilimumab (CTLA-4 blocker) regulates T cell activation and improves survivability of metastatic melanoma patients.	CTLA-4 blockade is influenced by the microbiota. Changes in *B. fragilis* and/or *B. thetaiotaomicron* and *Burkholderiales* affects immune response facilitating tumour control in mice and humans.

The growing field of microbiome research has raised a lot of questions and comments on the complex interplay and interwoven relationships between microbes and cancer, including anti-cancer treatments [227-231]. Further, some of the above studies [225, 226] have implications for microbial therapy in cancer immunotherapy. As our understanding of these interactions continues to progress, new knowledge in this area will open up possibilities of novel paradigm shifts in treatment approaches which may improve anti-cancer efficacy and even prevent toxicity. The studies presented in this section suggest a role for chemotherapy-induced microbiota changes in intestinal disease pathogenesis and chemotherapy-induced gut-brain axis dysregulation (Figure 2.4). As mentioned,

commensal bacteria are critical in regulating intestinal homeostasis and more specifically, intestinal integrity. In fact, the effects commensal bacteria have on intestinal integrity and vice versa, go hand-in-hand. Accordingly, the reciprocal relationship shared between commensal bacteria and the intestinal wall will be presented together in the following section. Chemotherapy compromises intestinal integrity and leads to profound effects on the gut lining, eventually leading to a dysbiotic microbial community and consequently risking microbial invasion into the systemic circulation.

Chemotherapy Impairs Intestinal Barrier-Microbiota Interactions

The even comprehensively described pathogenesis of mucositis [1] is unable to fully encapsulate the mechanisms underlying the pathogenesis of chemotherapy-induced gut damage. It does however, cover many essential aspects of the pathological processes underlying mucositis, such as epithelial barrier damage. More recently, some research groups have re-defined gut damage caused by chemotherapy as chemotherapy-induced gut toxicity (CIGT). The proposed term includes additional pathological manifestations caused by chemotherapy treatment, such as abnormalities in tight junctions, immune dysfunction and microbiota influences [208, 217, 232].

Nonetheless, the epithelial barrier lining of the GIT is fundamental in ensuring the maintenance of intestinal integrity. As well as forming a mechanical barrier to separate the inside of the body from the outside world, it is heavily involved in the communication shared between the body and the intestinal contents [233]. Tight junctions are intertwined throughout the epithelial barrier and regulate diffusion of solutes according to strict size and charge limitations [234]. Chemotherapy exposure increases intestinal permeability and the most widely studied mechanisms to date have included apoptosis of intestinal crypts and villous atrophy [64, 235]. Early clinical studies assessing the severity of intestinal damage in high-dose regimes reported abnormalities in intestinal permeability and defects

in tight-junction integrity (Figure 2.4) [162]. Convincing rodent evidence has implicated mucosal barrier injury and tight junction deficits with gut toxicity induced by various chemotherapy drugs, including irinotecan and methotrexate [232, 236]. However, it should be noted that rodent model application in gut immunity and microbiome research has serious limitations and pitfalls due to compositional microbiota differences between species. Whilst the rodent microbiome shares some common features with human commensal bacteria, unique commensals in rodents have differential effects in immune responses and disease pathogenesis [237]. Consequently, animal models of inflammation are different to human models of inflammation in terms of microbial colonisation, morphology of lesions and clinical manifestations. Nonetheless, research in experimental models continues to provide critical insight into complex interactions between the host, microbiota and immune responses. More recently, it has been becoming more evident the impact intestinal integrity has on the microbiota, especially under chemotherapy conditions. The health and stability of the intestinal wall influences the microbiota and vice versa.

Commensal bacteria in the microbiota have a protective effect on intestinal integrity, interacting with TLR and Nuclear Factor kappa B pathways, ensuring the development of an innate immune response [188]. These components of innate immunity in the gut and the activation of these pathways are pivotal in maintaining barrier function, promoting mucosal repair and protecting the gut against injury [192, 238]. Chemotherapy exposure alters commensal microbial composition in the microbiota, thus negatively affecting barrier function, repair pathways and compromising intestinal integrity [215]. Accordingly, further investigations are required to fully appreciate the role chemotherapy-induced intestinal permeability changes play in gut-brain axis dysregulation. As intestinal integrity becomes compromised under chemotherapy conditions, it is not surprising that nerves of

the myenteric plexus and peripheral nerve endings become damaged as these neural components also reside outside of the BBB as will be discussed below.

Chemotherapy Results in Peripheral and Enteric Neuropathy

The PNS is particularly vulnerable to the cytotoxic nature of different chemotherapy drug classes, including platinum analogues, antitubulins, proteasome inhibitors, immunomodulatory agents and some newer biologics, such as brentuximab [239]. Chemotherapy-induced peripheral neuropathy (CIPN) is experienced by 30-40% of chemotherapy recipients and is often responsible for early cessation of treatment, decreasing chemotherapeutic efficacy and causing higher relapse [128, 240]. Typically, sensorimotor symptoms are more common than motor involvement, presenting in a bilateral "glove-and-stocking" distribution in the hands and feet to include paraesthesia, numbness, burning pain, allodynia (pain following non-painful stimuli; central pain sensitisation) and hyperalgesia (heightened sensitivity to pain) [241]. However, the development of motor and autonomic neuropathic symptoms may also occur, such as sensory ataxia, pain, weakness of distal muscles, reduced deep tendon reflexes and severe numbness that can severely affect the patient's ability to function and their quality of life [242]. Often symptoms fail to improve after cessation of treatment, referred to as a "coasting" phenomenon [241].

The pathogenesis of CIPN is primarily related to axonopathy and neuronopathy in which dorsal root ganglia (DRG) are involved. Peripheral nerves and their ganglia are particularly susceptible to chemotherapy-induced damage due to their location as they lack the protective defences associated with the BBB [25]. For example, chemotherapy interrupts the cell cycle, inducing structural and functional changes in DRG which partly explain the development of sensory symptoms in CIPN [243, 244]. Many pathophysiological mechanisms mediating chemotherapy-induced peripheral nerve damage have been

identified. Some examples include, but are not exclusive to dysregulated axonal transport and trophic factor support via microtubule structural changes [245], mitochondrial stress [246, 247] and reduced blood supply to nerves [245, 248]. Further changes contributing to CIPN pathogenesis include dysregulated ion channels, neurotransmitter release and receptor sensitivity [249-251]. The evidence presented here clearly describes mechanisms by which the PNS is damaged following chemotherapy exposure, forming an important element of the proposed central hypothesis (Figure 2.4).

In addition to peripheral neuropathies, neurons residing within the ENS are also susceptible to the deleterious effects of various chemotherapy drugs, including cisplatin, oxaliplatin and more recently, 5-FU [107, 252, 253]. Systemic administration of these chemotherapy drugs induces structural and functional changes to myenteric neurons (Figure 2.4), consequently resulting in downstream negative effects on GI motility. Interestingly, acute exposure of 5-FU increases intestinal transit whilst prolonged treatment decreases transit time [107]. These findings outline the complex nature of the impact chemotherapy drugs have on enteric neurons and altered motility patterns. Here, we highlight that chemotherapy results in damage to neurons and ganglia residing outside of the BBB, exerting functional maladaptations in both the PNS and ENS. So far, we have described several mechanisms relating to chemotherapy-induced gut-brain axis dysregulation, yet we have not identified how immune signals from the intestinal cavity may communicate to the brain and potentially contribute to the pathogenesis of CICI. In the following section we present peripheral-to-central immune pathways as being critical in the transmission of signals from the gut to the brain following chemotherapy exposure.

Peripheral-to-Central Immune Signalling Pathways Mediating Chemotherapy-Induced Gut-Brain Axis Dysregulation

Historically there has been controversy surrounding the theory that a communication system existed between the immune system and the CNS [82]. Traditionally it was assumed that pro-inflammatory cytokines were unable to pass through the BBB due to their size. However, the humoral route explained that cytokines expressed in the periphery could in fact, cross the BBB at leaky circumventricular organs through fenestrated capillaries. At these sites blood-borne cytokines act on parenchymal astrocytes that express secondary mediators, such as nitric oxide and prostaglandins which freely diffuse to nearby brain regions, such as the hypothalamus to mediate the effects of pyrogenic and corticotropic cytokines [254]. Whilst this hypothesis points towards the existence of a communication system between the immune system and the CNS, it was unable to fully account for other contributing pathways that may be mediating physiological responses. Consequently, it is now widely accepted that peripheral cytokines signal the brain and in turn, this triggers sickness behaviour responses [255].

Central or peripheral immune challenges trigger a range of physiological, behavioural and motivational changes to assist the host in healing (Figures 2.2 and 2.4). Non-specific symptoms which accompany sickness behaviours include, but are not exclusive to fever, depressed activity, a loss of interest in regular activities (e.g. appetite, sexual, cleaning, hygiene), weakness, malaise, listlessness and cognitive changes [110]. As demonstrated by Dantzer and Kelley [82], the last two decades of research on this phenomenon have confirmed that local or systemic pro-inflammatory cytokines expressed at physiological levels, during both acute and chronic inflammatory responses, serve as true communication molecules between the immune system and brain. For example, direct administration of IL-1β or TNF-α to the lateral ventricle decreased social exploration and feeding behaviour in

rats [110]. In the chemotherapy setting, this phenomenon may be related to either the systemic nature of the drugs themselves or localised inflammatory responses occurring as a result of the toxicities associated with their use, such as in the case of mucositis (Figure 2.2).

Interestingly, whilst IL-1β and TNF-α are key pro-inflammatory cytokines instigating sickness behaviours, they also play a pivotal role in the pathogenesis of mucositis. Since both pro-inflammatory cytokines play an important role in the pathogenesis of mucositis and sickness behaviours which involve cognitive deficits, it is plausible that these cytokines and the pathways mediating their activation may present as key mechanisms underlying the central hypothesis in this review (Figure 2.4). Various animal models have identified that sickness behaviour responses may be induced by a range of clinical conditions, such as systemic or central administration of lipopolysaccharide (active fragment of gram negative bacteria) or recombinant pro-inflammatory cytokines [82, 134, 256, 257]. Furthermore, many symptoms associated with cytokine-induced sickness responses mimic the cluster of chemotherapy-induced side-effects, including fatigue, depression, reduced appetite, heightened sensitivity to pain and cognitive impairment (Figure 2.4). As previously mentioned, up to 70% of chemotherapy recipients experience mucositis [167] and an altered immune profile due to the systemic nature of anti-cancer treatments, yet whether these side-effects may be contributing to CICI remains elusive. Accordingly, we present pathways which may be enabling the communication of peripheral immune signals to the brain, more specifically defining how mucositis-driven inflammation may signal the brain via vagal- and neural-mediated mechanisms and contribute to the pathogenesis of CICI.

Information from pro-inflammatory cytokines and mediators expressed under chemotherapy-induced mucositis conditions may signal the brain via a vagal

communication pathway (Figure 2.4). Dendritic cells (DCs) are a specialised subset of immune cells located within the vagus nerve and surrounding paraganglia [256]. The signals (pro-inflammatory cytokines, chemokines and mediators) expressed by DCs are capable of communicating to the brain [258, 259]. Vagal immunosensation requires primary afferent neuron activation as the initial interface triggering the brain. Following chemotherapy exposure, pro-inflammatory cytokines and mediators, such as those from the IL-1 family arise from mucositis-induced inflammation. IL-1 binds to receptors on the paraganglia surrounding vagal afferents and release neurotransmitters onto the vagus, consequently activating vagal fibres. A vagal-mediated neural signal is then carried to the nucleus tractus solitarius which projects the message to higher order brain regions, such as the hypothalamus and hippocampus, whereby, IL-1 production is increased and other neural cascading events are initiated to produce sickness behaviour responses [82, 186]. Whilst this evidence clearly demonstrates the role vagal afferent nerves play in peripheral-to-central transmission of immune messages from the abdominal cavity, to date these pathways have not been examined under chemotherapy conditions and are therefore presented as potential mechanisms contributing to gut-brain axis dysregulation.

DCs play a role in immunomodulation and neuroimmune regulation, crucially bridging innate and immune adaptive processes. Importantly, DCs express pattern recognition receptors for a range of chemicals (e.g. TLRs), chemokines, microorganisms and neurotransmitters, such as serotonin [258, 259]. Damage to surrounding GIT tissues and increased levels of pro-inflammatory mediators, such as cytokines and chemokines induce maturation of DCs [260]. Matured DCs migrate to secondary lymphoid organs to initiate a localised immune response via interacting with naïve T cells [258]. Recent data implies an emerging role for DC-expressed serotonin and receptor activation in regulating innate and immune responses associated with gut inflammatory conditions [261, 262]. Mechanisms

underlying DC-mediated serotonin and receptor-sub types affect various levels of localised inflammation, even having anti-inflammatory effects preventing excess inflammation and tissue damage [261]. These findings coupled with the aforementioned positive cognitive effects of fluoxetine in a rat model of CICI [193], serotonin presents as a new therapeutic approach for inflammatory disorders, having effects in both the gut and the brain. Accordingly, serotonin is presented as an underestimated contributing factor potentially implicated in chemotherapy mediated gut-brain axis dysregulation (Figure 2.4).

From the Bottom Up, the gut and the brain are the two primary organs most susceptible to toxicity associated with the non-selective nature of chemotherapy drugs. As chemotherapy exposure induces cognitive decline and mucositis in a subset of recipients, it makes sense that several stages of the gut-brain axis are prone to negative effects in this setting. The gut-brain axis is largely responsible for the maintenance of homeostasis and achieves this delicate balance by integrating a vast array of signals and information from many systems, as described above and shown in the figures. In this regard, upsetting the balance of any stage in the gut-brain axis following chemotherapy treatment has the potential to exacerbate side-effects, such as in the case of mucositis and CICI. The findings from our review support our main hypotheses that chemotherapy treatment causes severe and prolonged psychosocial impacts on the survivor. Secondly, the gut-brain axis is an important mediator of a diverse range of cognitive and emotional disorders similar to those experienced by cancer survivors. Evidently, chemotherapy affects the gut-brain axis at several key stages which are outlined above. Collectively, this review concludes that the psychosocial side-effects of chemotherapy treatment may be caused by the effects of chemotherapy on the gut-brain axis.

Apart from chemotherapy treatments crossing the BBB and *directly* causing damage to specific regions, peripheral inflammatory responses from either the malignancy or

systemic treatment also *indirectly* cause cellular changes in the spinal cord. Preliminary findings from our group recently demonstrated glial reactivity in the thoracic spinal region of rats with 5-FU-induced intestinal mucositis, indicating an indirect regional-specific neuroimmune response to CIGT [263]. These data provide evidence that experimentally-induced jejunal toxicity *indirectly* dysregulates thoracic astrocytic protein expression. In addition to this recent finding, the evidence presented here suggests a role for chemotherapy-induced microbiota changes in intestinal inflammation. This further complicates intestinal inflammation and ulceration induced by chemotherapy exposure which may potentially influence CICI. Neurons in both the ENS and the PNS are also vulnerable to the cytotoxic nature of chemotherapy treatments. The implications of co-administration of pharmacological interventions (e.g. fluoxetine) with chemotherapy drugs remains undetermined, although preliminary studies showing improvements in cognitive performance warrants further investigation. In view of the aforementioned data, we conclude that several stages of the gut-brain axis become dysregulated following chemotherapy exposure and may be implicated in the pathogenesis of CICI. Harnessing our understanding of the role gut-brain axis dysregulation plays in modulating brain function may offer clues for more targeted therapeutic strategies to prevent CICI and warrants further investigation.

Hypotheses and Aims

Overarching thesis hypotheses derived from General Introduction and Chapters One and Two:

- 5-FU is unique in causing peripheral inflammatory events, such as gut toxicity and glial dysregulation, in the brain and spinal cord of rats;
- Opioid exposure further exacerbates 5IGT glial dysregulation in the brain and spinal cord of rats;
- Chronic inflammatory conditions of the gut (malignant and non-malignant) result in glial cell activation in the brain and spinal cord of mice;
- Acute low inflammation induced in the small intestine of rats with NSAID-enteropathy is sufficient to induce spinal cord glial reactivity;
- 5-FU-induced glial dysregulation directly (direct effects of drug on brain) or indirectly (from gut toxicity) may be associated with or potentiate learning and memory deficits, in the context of CICI; *and*
- Breast cancer patients undergoing chemotherapy treatment experience perceived GI symptom severities that may be associated with perceived cognitive disturbances.

Specific hypotheses and aims relating to each research chapter:

Research Chapter Three

Hypothesis 1. 5IGT induces microglial and astrocyte dysregulation in the brain and spinal cord of rats via humoral and/or neural pathways.

Aims

- Quantify glial and pro-inflammatory cytokine protein expression via glial fibrillary acidic protein (GFAP) for astrocytes, cluster of differentiation molecule 11b (CD11b)

for microglia, and IL-1β expression changes in the hippocampus and spinal cord of rats with 5IGT.

- Determine whether glial changes are occurring via peripheral-to-central immune signalling pathways:
    - *Humoral pathway:* examine the hippocampal region (indicative that glial changes are occurring as a result of 5-FU directly crossing the BBB),
    - *Neuronal pathway:* examine the thoracic (T6-T9) region (indicative that glial changes are occurring as a result of the indirect drug actions of 5-FU in the jejunum).
- Determine whether spinal cord changes are general or region specific:
    - Examine cervical (C2-C5) region for glial expression changes.

Hypothesis 2. 5-FU-induced microglia and astrocyte reactivity marker expression changes persist beyond the Recovery Phase of intestinal mucositis in rats.

Aims

- Assess 5-FU-induced glial reactivity (GFAP and CD11b) at **day 3** (72 h; Peak Injury Phase of mucositis) and **day 5** (120 h; Recovery Phase of mucositis) via western blot (WB) analysis.

Hypothesis 3. Analgesia in the form of opioid/NSAID exposure will further impact regional glial changes in rats with 5IGT.

Aim

- Quantify glial reactivity (GFAP and CD11b) and IL-1β expression via WB in hippocampus and spinal cord of rats with 5IGT and compare to analgesic intervention.

Research Chapter Four

Hypothesis 1. The commonly used NSAID, indomethacin (INDO), induces mild enteropathy in rats and increases hippocampal and thoracic microglial and astrocytic expression.

Aims

- Determine whether spinal glial expression changes occurred as a result of a *generalised* (lower) inflammatory response in the small intestine utilising INDO, or whether the glial changes were specifically related to chemotherapy drug exposure (5IGT).
    - Compare GFAP and ionized calcium binding adaptor molecule-1 (Iba-1) changes in the hippocampus, hypothalamus, pre-frontal cortex and cervical, thoracic and lumbar spinal regions of rats with 5IGT (high inflammatory response) to INDO-induced enteropathy (low inflammatory response).

Hypothesis 2. The microbial diversity of rats with 5IGT decreases when compared to INDO-induced enteropathy.

Aim

- Examine caecally-derived microbial changes using 16S-PCR sequence profiling.

Research Chapter Five

Hypothesis 1. Chronic inflammation resulting from dextran sulphate sodium (DSS)-induced chronic UC would increase glial expression in higher order brain regions and the lumbar region of mice.

Aims

- Assess glial (GFAP and Iba-1) expression changes in the brain and spinal cord (6 regions in total) of mice with DSS-induced UC via WB analysis.
    - In the brain, the hippocampus, PFC and hypothalamus regions.

        - The hippocampus and PFC are implicated in cognitive processes and glial dysregulation in these regions may directly be mediated by activation of the humoral pathway, for example, systemic pro-inflammatory cytokines or 5-FU crossing the BBB and directly exerting effects.
        - The hypothalamus receives input from the GIT, thus gut inflammation may indirectly induce glial dysregulation in this region via neurally-mediated immune signalling mechanisms.
    - Examination of the cervical, thoracic and lumbar regions of the spinal cord to determine whether glial changes are region specific. If regional changes occur in the spinal cord region that is innervated by the inflamed GIT region, for example colonic changes infer lumbar regional changes, then this will indicate neural pathway activation.

Hypothesis 2. Chronic inflammation and malignancy resulting from colitis-associated CRC (CA-CRC) would increase glial expression in higher order brain regions and the lumbar region of mice.

Aims

- As described in first hypothesis aims, assess glial (GFAP and Iba-1) expression changes in the brain and spinal cord of mice with DSS/azoxymethane (AOM)-induced CRC via WB.

Research Chapter Six

Hypothesis 1. Breast cancer patients undergoing chemotherapy treatment experience perceived GI symptom severities that may be associated with perceived cognitive disturbances.

Hypothesis 2. Allergy susceptibility in breast cancer survivors correlates with susceptibility of developing perceived GI and cognitive disturbances during treatment.

Aims

- Identify relationships between allergies, pain, cognition and gut disturbances using exploratory questions on participants perceptions following chemotherapy treatment.
- Quantify chemotherapy recipient's perceived severity of mucositis symptoms and cognitive deficits using Functional Assessment for Cancer Therapy (FACT) or Chronic Illnesses Therapy (FACIT) cognitive and gastrointestinal survey measures, controlling for related variables (depression, anxiety, fatigue, age).
- Approach utilized the following measurement systems:
    - Self-report (subjective) online questionnaire (exploratory investigator led questions in addition to validated scale systems),
    - Measured scores using the FACT and FACIT scale systems:
        - Perceived cognitive measurements (FACT-Cog)
        - Perceived intestinal disturbances (FACIT-D/AD)
        - Perceived fatigue symptoms (FACT-Fat)
    - Control for variables such as depression and anxiety using the depression, anxiety and stress scale (DASS)

CHAPTER THREE, *in vivo*

Neuroimmunological Complications arising from 5-Fluorouracil-Induced Gut Toxicity and Opioid Exposure in Rats

Statement of context

This work has currently been submitted for review with the *British Journal of Cancer*.

Bajic, JE, Howarth GS, Mashtoub S, Whittaker AL, Bobrovskaya L and Hutchinson MR.

Chapter Three examines glial and IL-1β dysregulation in the spinal cord and hippocampus of rats with 5IGT. It was proposed that peripheral-to-central immune signalling pathways mediate these changes and that spinal cord changes would indicate a neural route, whereas hippocampal changes would occur via the humoral pathway. It also determined whether regional glial dysregulation would continue following resolution of the acute gut inflammation, comparing the Peak Injury to Recovery Phases of 5IGT. In addition, the neuroimmune complications arising from 5IGT were further explored with three analgesic options used to treat pain associated with CIGT, of particular interest was the opioid-based interventions, as they are also known for their neuroimmune-modulating effects.

Statement of authorship

Statement of Authorship

Title of Paper	**Neuroimmunological Complications arising from 5-Fluorouracil-Induced Gut Toxicity and Opioid Exposure in Rats**
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Juliana Esma Balic, Gordon Stanley Howarth, Suzanne Mashtoub, Alexandra Louise Whittaker, Larisa Bobrovskaya & Mark Rowland Hutchinson

Principal Author

Name of Principal Author (Candidate)	Juliana Bajic		
Contribution to the Paper	I was responsible for the compilation of all bodyweight and MPO data, assisted with animal experimental work, performed all western blot experimental work, reviewed all papers cited in manuscript, performed statistical analyses and interpreted data, designed figures and acted as corresponding author.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/04/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);
II. permission is granted for the candidate in include the publication in the thesis; and
III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon Howarth		
Contribution to the Paper	Reviewed manuscript.		
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Contribution to the Paper	Performed animal experimentation for experiment one.		

Signature		Date	10/04/19

Name of Co-Author	Alexandra Whittaker		
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Signature		Date	10/04/19

Name of Co-Author	Larisa Bobrovskaya		
Contribution to the Paper	Assisted with training and equipment use for western blot analyses.		
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Contribution to the Paper	Reviewed manuscript and assisted with data interpretation.		
Signature		Date	10/04/19

Abstract

Background: Cancer patients experience chemotherapy-induced (CI) gut toxicity (CIGT) and cognitive impairment (CICI). Analgesic selection for CIGT is difficult as opioids induce glial cell activation (neuroinflammation) and unwanted side-effects. Therefore, we examined whether peripheral-to-central immune pathways would directly-humorally or indirectly-neurally induce neuroinflammation in rats with CIGT being treated with three mechanistically different analgesics.

Methods: Utilising a 5-Fluorouracil-induced GT (5IGT) rat model and analgesic intervention (carprofen; CAR, buprenorphine; BUP and tramadol; TRAM), neuroimmune consequences were examined via microglial, astrocyte and pro-inflammatory protein expression changes (cluster of differentiation molecule 11b; CD11b; glial fibrillary acidic protein; GFAP, and interleukin-1 beta; IL-1β). Hippocampal and thoracic spinal cord investigation determined the direct and indirect actions of 5IGT and analgesia.

Results: BUP and TRAM with 5IGT synergistically increased hippocampal GFAP expression. 5IGT significantly increased thoracic GFAP ($p<0.05$) and IL-1β ($p<0.0001$) expression, CAR and BUP ameliorated these effects. CAR administered with 5IGT significantly increased hippocampal and thoracic CD11b expression levels ($p<0.05$).

Discussion: Peripheral-to-central immune pathways are implicated in the neuroimmune adaptations observed here. Opioid-induced hippocampal changes inferred a humorally-mediated mechanism, whereas thoracic neuroinflammation indicated activation of an indirect neural route. Although TRAM ameliorated 5IGT-intestinal inflammation, this opioid presents complications relating to bodyweight and regional glial dysregulation (neuroinflammation) and may not be optimal to alleviate pain associated with 5IGT treatment.

Background

Mucositis is a painful side-effect of cancer treatment, often resulting in reduced or premature cessation of treatment and thus, compromising the efficacy of cancer therapy. Mucositis incidence occurs in approximately 40% of patients undergoing a standard-dose chemotherapy regime and up to 100% of patients undergoing a high-dose regime and stem cell or bone marrow transplantation [264, 265]. The condition may occur anywhere along the alimentary tract and results in significant pain, ulcerating lesions, abdominal bloating, nausea, vomiting, diarrhea and/or constipation [168]. Intestinal mucositis commonly occurs in the small intestine and is characterised by inflammation, ulceration, mucosal damage and malnutrition, and in severe cases, may lead to bacteraemia and sepsis [1]. In addition to the significant impact on patient quality of life, further downstream effects of mucositis become evident in longer hospital stays, increased economic burden and increased use of analgesic treatment for pain management. Although the pathogenesis of intestinal mucositis is gradually becoming more clearly defined, management interventions that either prevent or treat the symptoms associated with the disorder remain sub-optimal and largely ineffective. Research has identified additional mechanisms contributing to gut disturbances induced by chemotherapy drugs, and in doing so have re-named mucositis to chemotherapy-induced gut toxicity (CIGT) which is more inclusive of other factors contributing to the disorder. For example, defects in tight junctions have been implicated in irinotecan-induced mucosal barrier dysfunction [232] and Toll-like receptor (TLR)-4-mediated mechanisms contributing to CIGT associated pain [134].

Despite this, pain management remains an important factor in the treatment of CIGT. The World Health Organisation (WHO) analgesic ladder is a therapeutic guideline for pain associated with cancer [266]. The first line of treatment proposed in the guidelines is a non-opioid analgesic, such as a non-steroidal anti-inflammatory drug (NSAID). If the pain

persists, a weak opioid should replace the first medication option. Finally, if this treatment is insufficient in providing analgesia, a more powerful opioid should be used. The challenge with administering opioids to this patient population falls onto the neuroimmunological consequences associated with their use as they contribute to the development of drug tolerance, addiction and enhanced pain states [126].

Glia represent the non-neuronal immune-like cells of the central nervous system (CNS) and are key players in the modulation of pain pathways, including the pharmacodynamics of opioids [136]. They play a pivotal role in various central homeostatic mechanisms due to their close relationship with the synapse (reviews [121, 122, 153]); forming the tetrapartite synapse [122] and contribute to neuronal consequences involving neurodegeneration (reviews [118, 121, 124, 131, 157, 267]). More specifically, opioids activate brain and spinal glia as demonstrated by up-regulation of glial fibrillary acidic protein (GFAP) expressed on astrocytes and cluster of differentiation 11b (CD11b) for microglia, morphologically changing from a ramified (resting) to amoeboid (activated) state [136, 139]. This central immune response driven by opioid exposure closely mimics the typical immunogen response to lipopolysaccharide (LPS), an endotoxin expressed by Gram negative bacteria [240]. LPS relies upon peripheral immune signals to activate glia and initiate a central pro-inflammatory immune response; neuroinflammation [268]. Alternatively, opioids readily permeate the brain, having direct effects on glia in neuroanatomical areas implicated in addiction and acting in a similar manner to neurotransmission, now commonly referred to as central immune signalling [269].

TLRs are innate immune pattern-recognition receptors expressed primarily on glia and become up-regulated following immune insults, resulting in downstream activation of inflammatory transcription factors (e.g. NF-κB) and up-regulation of chemokines and pro-inflammatory cytokines (e.g. interleukin-1 beta; IL-1β) [187]. Specifically, opioid-induced

glial activation and neuroinflammation are mediated by TLR-4 signalling [190, 191]. Certain behavioural adaptations may be induced by immune signals and result in cytokine-induced sickness responses [80]. Hart (1988) [270] characterised sickness responses as anhedonia, malaise, anxiety, social withdrawal, fever, pain, and depression and proposed that these adaptive and functional changes were critical for survival.

In oncology, chemotherapy patients are also prone to experiencing a cluster of physical and central symptoms, like cytokine-induced sickness responses [82]. In addition to the initial stress, depression and anxiety caused by cancer diagnosis, patients undergoing anti-cancer treatment often experience changes in their cognitive performance, clinically termed chemotherapy-induced cognitive-impairment (CICI) [271]. Rodent models of CICI have widely reported hippocampal-dependent behavioural adaptations following chemotherapy exposure, which importantly overlap with brain regions implicated in CICI [86, 116, 272-274]. Molecular studies have confirmed that the hippocampus is particularly susceptible to damage across a range of chemotherapy drug exposures [87, 116, 181, 275]. Various chemotherapy drugs reduce hippocampal cellular proliferation, specifically targeting stem cells of the dentate gyrus [87, 89, 178, 275]. Critically, these rodent studies support many of the subjective and objective reports on CICI indicating that higher order brain processing from this region is implicated in CICI [79, 276]. Whilst CIGT would almost certainly be present in these CICI models, no intestinal data were reported. Peripheral-to-central immune pathways offer a mechanism by which gut inflammation can result in behavioural changes [143, 144, 277]. Information from peripheral pro-inflammatory cytokines and mediators signal the brain via humoral-, neural- and cellular-mediated pathways, triggering physiological, behavioural and motivational changes [144]. The slower humoral route enables passage of cytokines or drugs (via diffusion) directly to the brain at circumventricular organs containing fenestrated capillaries and molecular

intermediates, such as prostaglandins and TLRs [268, 278]. Another system transporting blood-borne cytokines to the brain utilises specific, saturable carrier systems, for example binding to brain endothelial cells [279, 280]. Neural signalling is a more rapid route whereby primary afferent neurons detect peripheral immune messages, including pathogen associated molecular patterns, transducing them into translatable messages which are relayed to higher order brain regions, (e.g. vagal innervation to the nucleus tractus solitarius) [281]. The message is then re-transduced into an immune message in the brain parenchyma, where the production of cytokines impacts brain function acting either directly or indirectly on neurons and/or glia. Although the time course engaging these immune-to-brain communication pathways may differ, they all result in glial activation and neuroinflammation.

The mechanisms associated with CIGT continue to be heavily researched, yet the potential indirect neuroimmune consequences of CIGT remain elusive. The biology of the system and the wider implications associated with opioid exposure and neuroimmunological complications during the Peak Injury Phase of CIGT also remain unknown. Thus, we hypothesised that glial dysregulation would occur in the brain and spinal cord of rats via humoral and neural immune-to-central communication pathways during the Peak Injury Phase of CIGT in an acute setting. We also determined whether chemotherapy-induced glial changes would persist into the Recovery Phase of CIGT. Finally, we assessed whether analgesic intervention, following the WHO analgesia ladder guidelines, would further impact regional glial changes in rats with CIGT. The first experiment aimed to characterise the time-course of glial dysregulation following chemotherapy exposure using the antimetabolite chemotherapy drug, 5-Fluorouracil (5-FU), comparing the Peak Injury to Recovery Phase of GT. Secondly, we examined the effects of three analgesics during the Peak Injury Phase of 5-FU-induced GT (5IGT), replicating the time-points at which

analgesia would be administered in a clinical setting. The analgesics selected were the selective COX-2 inhibitor NSAID, carprofen [282]; a partial mu (μ) opioid agonist, buprenorphine [283]; and an 'atypical' opioid analgesic, tramadol [284]. Ultimately, this study determined the wider implications of analgesics on certain aspects of the gut-brain axis, specifically their effects on GT and then assessed neuroimmunological adaptations in the hippocampus and spinal cord of rats with 5IGT.

Materials and Methods

Female Dark Agouti rats (110-140g, *n*=80) were sourced from Laboratory Animal Services, The University of Adelaide, Adelaide, SA, Australia (barrier maintained Specific-Pathogen Free production facility). Female rats were selected for this study as the majority of clinical investigations come from the breast cancer population [58, 285]. Animal experimentation was approved by the Animal Ethics Committee of the University of Adelaide. The animal protocol described in this study minimised pain and discomfort to the animals by complying with the National Health and Medical Research Council Code of Practice for Animal Care in Research and Teaching (8th Edition, 2013). Rats were acclimatised and group housed in a room maintained at 21-23°C with a 12 h reversed light-dark cycle. All rats were given *ad libitum* access to water and standard rat chow (Specialty Feeds, Glen Forrest, WA, Australia). Depending on group allocation, following acclimatisation period rats were either individually housed in metabolic cages (Tecniplast, Exton, PA, United States) for a further 2 days [110, 257] or remained in group housing. Bodyweight, disease activity index (DAI), feed and water intake, and faecal and urine output were recorded daily.

Experiment One: Determining the Time-Point of 5IGT Glial Changes

The first experimental design determined the time-point with most significant glial changes according to intestinal disease progression (Figure 3.1; timeline). In order to achieve this, two end-points were examined based on findings from our lab [108]: end-point one (72 h post 5-FU administration, Peak Injury Phase); and end-point two (120 h post 5-FU exposure, Recovery Phase). Following acclimatisation period (-48 h to 0 h), rats were randomly assigned to one of four groups (Figure 3.1): 1. water + saline (SAL; 0.9% NaCl w/v); 2. water + 5-FU; 3. water + SAL; and 4. water + 5-FU. Groups 1 and 2 assessed end-point 1 and groups 3 and 4 assessed end-point 2. SAL or 5-FU (Mayne Pharma Pty, Ltd,

Mulgrave, VIC, Australia) were administered via single intraperitoneal injection after the acclimatisation period (150mg/kg at 0 h).

Experiment Two: Direct and Indirect Analgesic-Induced Glial Adaptations in 5IGT

Glial changes were identified during the Peak Injury Phase of 5IGT in Experiment One and thus, the remainder of the study design was focused at 72 h post 5-FU injection (Figure 3.1). Rats were randomly allocated to one of six groups (group housed): 5. Carprofen (CAR; 15mg/kg, Zoetis, US) + SAL; 6. Buprenorphine (BUP; 0.05mg/kg, Reckitt Benckiser, NSW) + SAL; 7. Tramadol (TRAM; 12.5mg/kg, CSL Ltd. SA) + SAL; 8. CAR (15mg/kg) + 5-FU; 9. BUP (0.05mg/kg) + 5-FU; and 10. TRAM (12.5mg/kg) + 5-FU (Figure 3.1). SAL or 5-FU (150mg/kg) was intraperitoneally injected at 0 h and analgesic treatments were also commenced depending on group allocation. Subcutaneous injections of each analgesic were performed into the scapular region, dosed as per clinical practice, at 12 hourly intervals for the remainder of the study (6 doses in total).

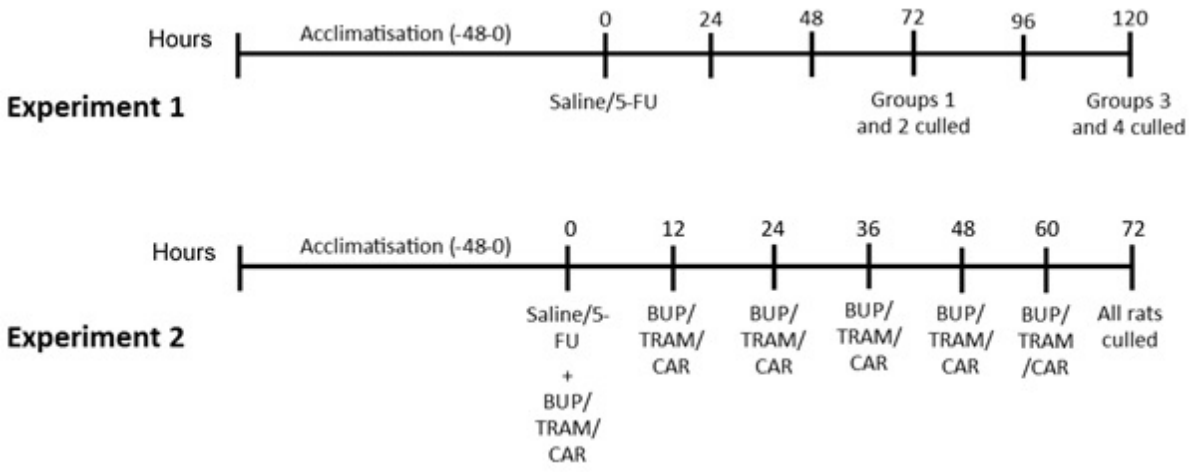

Figure 3.1. Timeline of experimental procedures. During Experiment One and Two, 5-FU or SAL was administered intraperitoneally at 0 h. During the first experiment euthanasia occurred at 72 h for groups 1 and 2 (Peak Injury Phase of 5IGT) and 120 h (Recovery Phase) for groups 3 and 4. During Experiment Two, all rats underwent the same experimental procedure as groups 1 and 2 in the first experiment. The rats in Experiment Two also commenced one of the analgesic interventions subcutaneously administered at 0 h and every 12 h for the remainder of the study (6 doses in total). 5-Fluororuacil; 5-FU, saline; SAL, carprofen; CAR, buprenorphine; BUP, tramadol; TRAM.

Tissue Collection

Metabolic data, including indicators of disease activity, behavioural (pain scores via facial grimace) and intestinal measures from 192 animals in 24 groups have been previously published and have been reported here, as these data were collected at the same time and served as the key controls across multiple studies [108, 110, 286, 287]. The rats in Experiment One formed part of larger studies examining the metabolic and intestinal efficacy of naturally sourced plant and animal extracts (e.g. Emu Oil, Lyprinol and Rhubarb Extract) in 5IGT [108, 110, 286]. The remaining animals in the current study formed part of a larger study investigating the analgesic efficacy and welfare of rats with 5IGT [287]. Importantly, these studies focused on intestinal parameters and behavioural outcomes without a multiple injection control group, not considering the direct and indirect neuroimmunological implications of 5IGT or analgesic intervention.

Following CO_2 asphyxiation and cervical dislocation, the gastrointestinal contents of each animal were emptied and intestines weighed. Biochemical analysis of intestinal samples was achieved via myeloperoxidase (MPO) assay [108, 110, 286, 287]. MPO assay was used an indicator of intestinal acute inflammation and the method has been extensively covered [109, 288]. All remaining visceral organs (thymus, spleen, lungs, heart, liver and kidneys) were weighed and discarded. Brain and spinal cord were dissected and snap frozen for further processing in western blot analysis.

Western Blot Protocol for CNS Tissue

Hippocampal, cervical (C2-C5) and thoracic (T6-T9) regions were dissected and homogenised in cell lysis buffer (Tris-base saline; pH 8.0; 50mM, 1% Triton-x100, 1% protease inhibitor cocktail; #P8340, Sigma-Aldrich, Castle Hill, NSW, Australia); 300µl (spinal cord) and 500µl (hippocampus) aliquots. Samples were sonicated (10 sec followed by at least 1 min on ice) until they were homogenised then centrifuged (15,000 x g) for 40

min. Total protein concentration in the supernatant was determined via BCA-Protein Assay (Pierce® BCA Protein Assay Kit; #23225, Thermo Fisher Scientific Inc., Victoria, Australia). Homogenised proteins of known concentrations were suspended in 2 x sample buffer (SDS reducing sample buffer: dH^2O, 0.5M Tris-HCl pH 6.8, glycerol, 10% SDS solution and 0.5% bromophenol blue) and heated at 70˚C for 10 min. Equal amounts of protein from each sample (25µg) were loaded and separated by gel electrophoresis in acrylamide gradient gels. Gels were homemade (SDS page 8-10%; 40% Acrylamide/Bis, gel Buffer (Resolving gel: 1.5M Tris-HCl, pH 8.8 & Stacking gel: 0.5M Tris-HCl pH 6.8); 10% w/v SDS; 10% ammonium persulfate; TEMED). Protein transfer onto nitrocellulose filter paper (Sigma-Aldrich, Castle Hill, NSW, Australia) used boric acid (pH 8.9; boric acid, EDTA for 2 h @ 600mA) transfer buffer and ponceau stains were performed. Membranes were blocked with either 5% skim milk (lower kDa proteins) or 5% BSA (for higher kDa proteins) at room temperature for 1.5 h. Protein expression utilised antibodies for glial fibrillary acidic protein (GFAP; 1:2000, #ab7260, Abcam, Cambridge, UK), cluster of differentiation molecule 11b (CD11b; Integrin aM (M19); 1:2000, #sc6614, Santa Cruz biotechnology, Dallas, Texas, US) and interleukin-1 beta (IL-1β; 1:1000, #ab9722, Abcam, Cambridge, UK); indicators of astrocyte, microglia and pro-inflammatory changes, respectively. Overnight incubation at 4˚C achieved primary antibody staining. β-actin (1:2000, #a2066, Sigma Aldrich) standardised sample loading (control) and accounted for protein loading variability during gel electrophoresis. An additional internal sample control (2µl from each sample) determined changes between gel integrity and normalised band detection. Electrochemiluminescence (ECL) enabled detection of all experimental analyses using the following secondary antibodies: Anti-rabbit/sheep/mouse antibody (peroxidase-conjugated Affinipure Donkey Anti-rabbit/sheep/mouse IgG; 1:20,000; #711-035-152, #713-035-003, #715-035-150, Jackson Immuno Research, West Grove, PA, USA).

Immunoblots were developed using an in-house ECL reagent (10mL of 100mM Tris HCl (pH 8.5), 22µL of 90mM coumaric acid, 50µL of 250mM luminol and 3µL of H_20_2) and bands were visualised using the ImageQuant 4100 imaging system (GE Health Care, Buckinghamshire, UK) [289]. Ratio-metric analysis quantified band densities and were measured as a fold change relative to control group, accounting for normalisation as described earlier.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 6.02 for Windows (GraphPad Software Inc., San Diego, CA, USA). Data were assessed for normality using the Shapiro-Wilks analysis. All parametric data (bodyweight, MPO and western blots) were analysed using a two-way analysis of variance (ANOVA) with Tukey's *post hoc* comparison tests. Whilst some main effects were not observed using an ANOVA, significant interactions at different levels were reported utilising *post hoc* analyses. Consequently, it may be anticipated that the main effect would not translate to a *post hoc* and thus, it is acknowledged that this approach of statistical reporting represents a potential limitation of this study. Data expressed as mean ± SEM with values of $p<0.05$ deemed significant.

Results

Bodyweight

Rats administered 5-FU significantly reduced bodyweight during the Peak Injury Phase (72 h) of 5IGT when compared to saline controls ($p<0.0001$; Figure 3.2A). During the Recovery Phase of 5IGT, rats returned to similar baseline bodyweight for the control group ($p<0.05$; Figure 3.2A). During Experiment Two, at the Peak Injury Phase of 5IGT, rats co-administered CAR and BUP significantly reduced bodyweight compared to the SAL control group ($p<0.0001$; Figure 3.2B). TRAM potentiated the bodyweight reduction in rats with 5IGT ($p<0.0001$; Figure 3.2B).

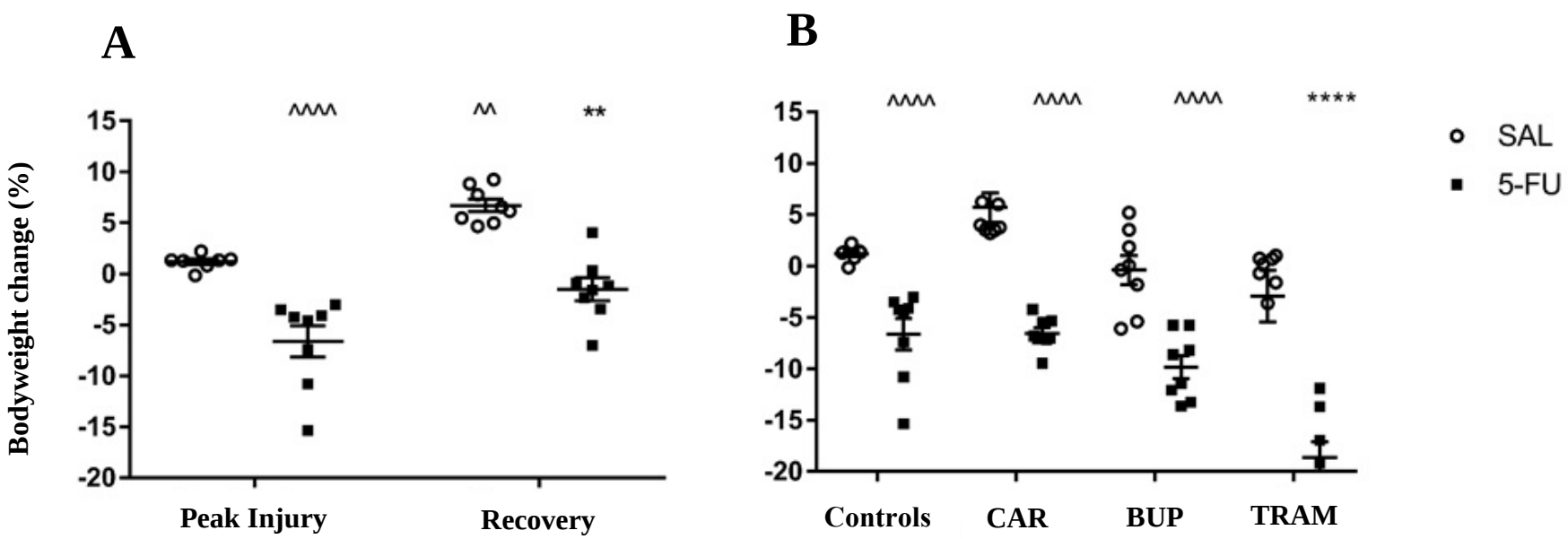

Figure 3.2A and B. Effects of 5-FU during the Peak Injury and Recovery Phase of 5IGT on bodyweight change as represented from baseline (A). 5-FU significantly reduced bodyweight compared to SAL control group during the Peak Injury Phase but this reduction returned to baseline levels in the Recovery Phase of 5IGT. Effects of 5-FU and analgesics on bodyweight change as represented from baseline (B). 5-FU significantly reduced bodyweight compared to SAL control group. CAR and BUP also substantially reduced bodyweight in the presence of 5-FU compared to SAL controls. Co-administration of TRAM potentiated weight loss when compared to the 5-FU control group. Data represented as mean bodyweight (% change from baseline) ± SEM. ^ indicates significance compared to SAL control group. * indicates significance compared to 5-FU control group. ^^/** indicates $p<0.01$, ^^^^/**** indicates $p<0.0001$. 5-Fluorouracil; 5-FU, saline; SAL, carprofen; CAR, buprenorphine; BUP, tramadol; TRAM.

Meloperoxidase

During Experiment One, jejunal and ileal MPO expression significantly increased in rats during the Peak Injury Phase of 5IGT compared to SAL controls ($p<0.0001$; Figure 3.3A). In the Recovery Phase of 5IGT, MPO expression returned to baseline SAL expression levels ($p>0.05$; data not shown) [108]. In Experiment Two, jejunal MPO activity was attenuated by co-administration of both BUP and TRAM during the Peak Injury Phase of 5IGT ($p<0.01$; Figure 3.3B). Whilst there was a downward trend of MPO expression in the ileum with all analgesics, this failed to reach significance when compared to the 5-FU control group.

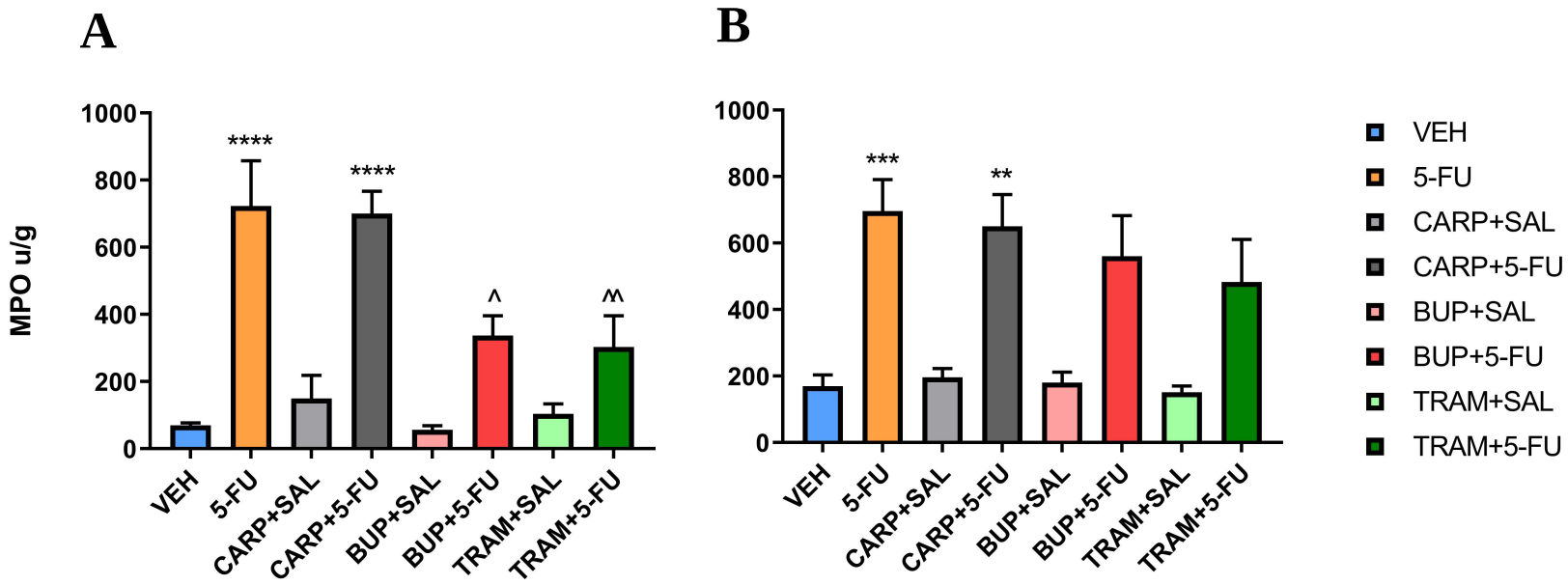

Figure 3.3A and B. Effects of 5-FU and analgesics on myeloperoxidase (MPO) in the jejunum (A) and ileum (B) of rats during the Peak Injury Phase (72 h) of 5IGT. 5-FU significantly elevated jejunal and ileal MPO expression compared to SAL group during the Peak Injury Phase of 5IGT. BUP and TRAM attenuated this response as shown by a reduction in jejunal MPO expression compared to rats with 5IGT with no analgesic intervention. In the ileum, all analgesics reduced MPO expression compared to 5IGT control group. Data represented as mean (MPO units/g tissue) ± SEM. ^ indicates significance compared to SAL control group. * indicates significance compared to 5-FU control group. ^ indicates 0.05, ^^/** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$. 5-Fluorouracil; 5-FU, 5-FU-induced gut toxicity; 5IGT, saline; SAL, carprofen; CAR, buprenorphine; BUP, tramadol; TRAM.

CD11b expression changes

In Experiment One, no significant CD11b changes were observed during the peak or Recovery Phases of 5IGT in the hippocampus, thoracic or cervical regions ($p>0.05$; Recovery Phase data not shown; peak injury hippocampal and thoracic data shown on Figures 3.4A and 3.4B). Hippocampal CD11b expression was significantly reduced by co-administration of CAR during the Peak Injury Phase of 5IGT compared to 5-FU controls ($p<0.05$; Figure 3.4A). Gut toxicity induced by 5-FU had a subtle downward effect on thoracic CD11b expression compared to controls although no statistical significance was determined ($p>0.05$; Figure 3.4B). Thoracic CD11b expression was significantly elevated by co-administration of CAR during the Peak Injury Phase of 5IGT ($p<0.01$; Figure 3.4B). BUP and TRAM alone significantly elevated thoracic CD11b expression only when compared to 5-FU treated animals ($p<0.05$; Figure 3.4B), although no statistical significance was observed when compared to the SAL control group.

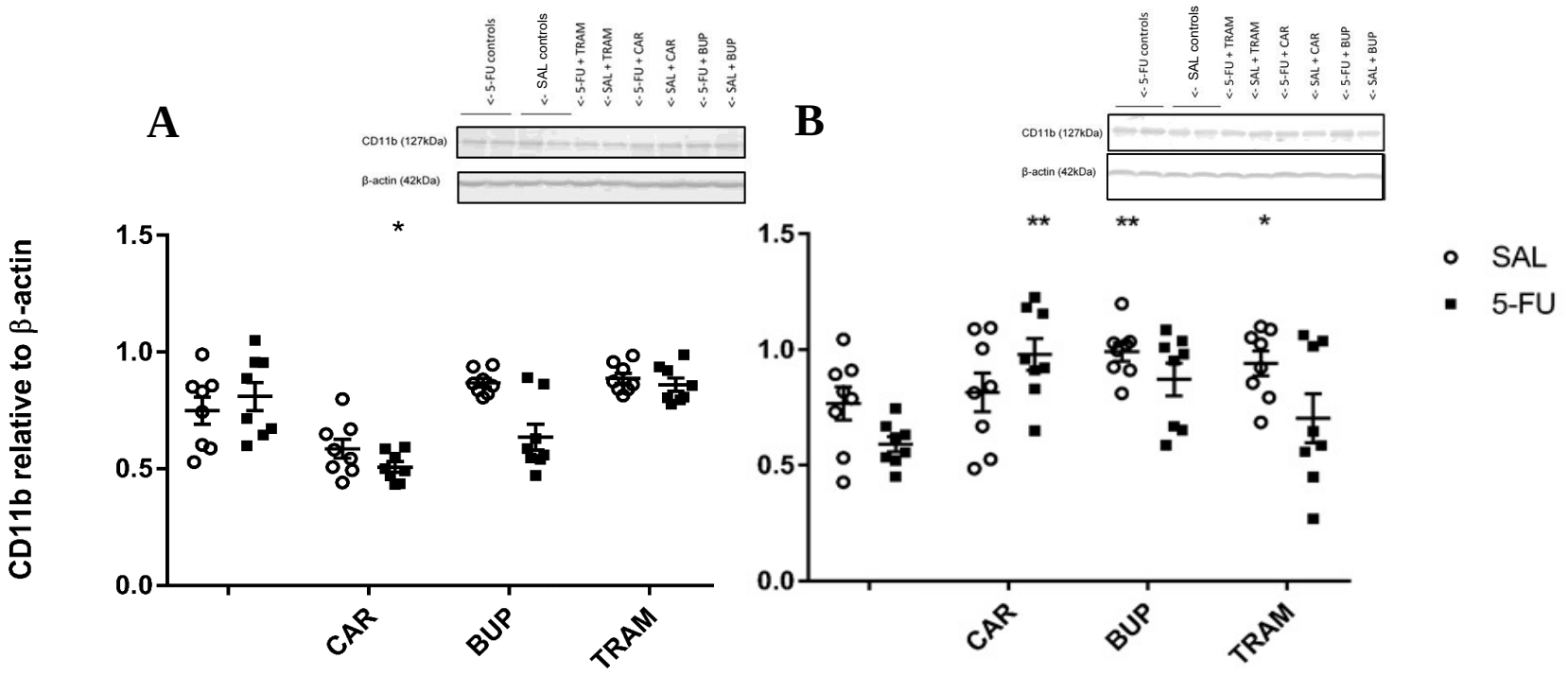

Figure 3.4A and B. Effects of analgesics on hippocampal (A) and thoracic (B) CD11b expression during the Peak Injury Phase (72 h) of 5IGT. 5-FU alone had no effect on hippocampal CD11b expression, yet CAR in the presence of 5-FU significantly reduced CD11b expression compared to 5-FU control group. Although 5-FU alone had no statistically significant effect on CD11b expression, a trend in down-regulation is evident. CAR in the presence of 5-FU significantly elevated CD11b expression compared to the 5-FU control group. Data represented as mean (CD11b expression relative to β-actin) ± SEM. * indicates $p<0.05$ and ** indicates $p<0.01$ significance compared to 5-FU control group. 5-Fluorouracil; 5-FU, saline; SAL, carprofen; CAR, buprenorphine; BUP, tramadol; TRAM.

GFAP expression changes

5IGT had no effect on hippocampal GFAP expression when compared with SAL controls ($p>0.05$; Figure 3.5A). Thoracic GFAP expression was significantly increased in rats with 5IGT compared to SAL controls ($p<0.01$; Figure 3.5B). GFAP expression returned to baseline levels during the Recovery Phase of 5IGT ($p>0.05$; data not shown). CAR and BUP ameliorated this main effect, significantly reducing GFAP expression ($p<0.01$ and $p<0.001$, respectively; Figure 3.5B). However, co-administration with BUP and TRAM during the Peak Injury Phase of 5IGT significantly elevated hippocampal GFAP expression when compared to 5-FU controls ($p<0.0001$ and $p<0.01$, respectively; Figure 3.5A). Cervical GFAP expression was unchanged by 5-FU administration compared to SAL controls, yet CAR alone significantly decreased whilst TRAM alone significantly increased GFAP expression when compared to SAL controls ($p<0.01$ and $p<0.0001$, respectively; Figure 3.5C).

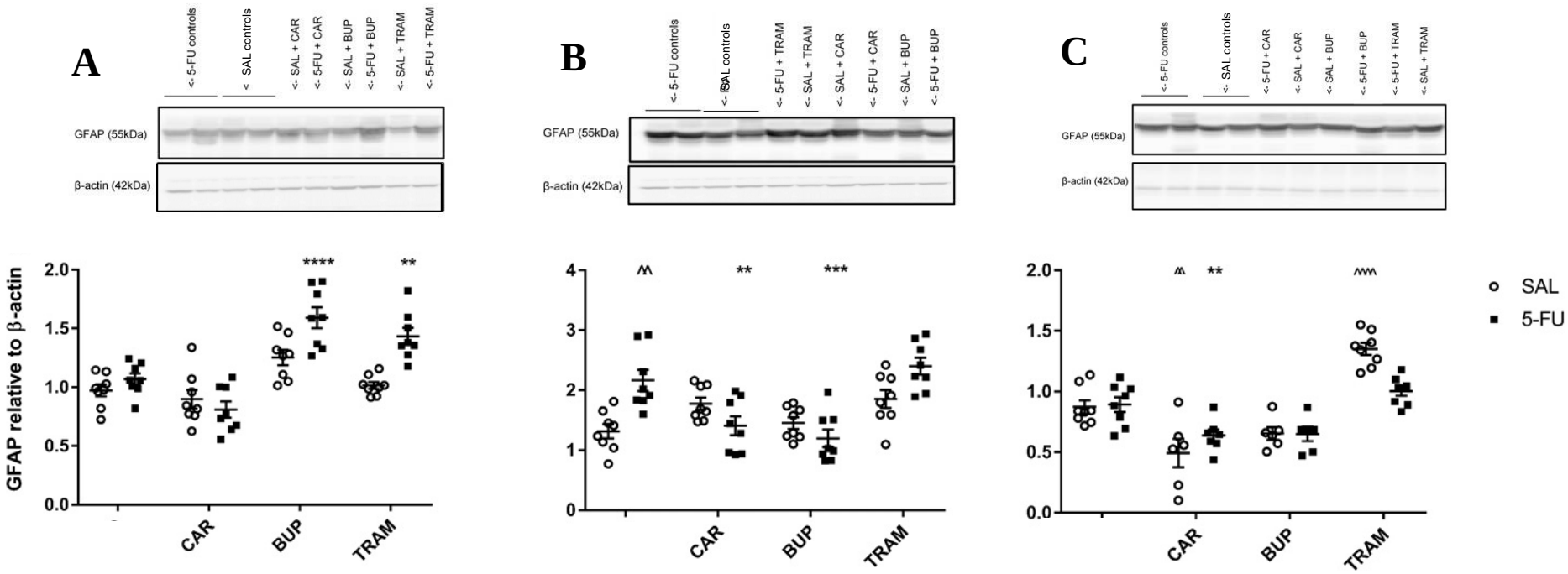

Figure 3.5A, B and C. Effects of 5-FU and analgesics on hippocampal (A), thoracic (B) and cervical (C) GFAP expression during the Peak Injury Phase (72 h) of 5IGT. Thoracic GFAP expression significantly increased following 5-FU administration compared to SAL controls, CAR and BUP attenuated this response (A). BUP and TRAM in the presence of 5-FU significantly increased hippocampal GFAP expression compared to the 5-FU control group (B). CAR in the presence of 5-FU significantly reduced cervical GFAP expression compared to the 5-FU control group (C). Cervical GFAP expression was also significantly reduced in CAR alone groups, whereas TRAM alone was increased when compared to the SAL control group (C). Data represented as mean (GFAP expression relative to β-actin) ± SEM. ^ indicates significance compared to SAL control group. * indicates significance compared to 5-FU control group. **/^^ indicates $p<0.01$, *** indicates $p<0.001$, ^^^^/**** indicates $p<0.0001$. Glial fibrillary-acidic protein; GFAP, 5-Fluorouracil; 5-FU, 5-FU-induced gut toxicity; 5IGT, saline; SAL, carprofen; CAR, buprenorphine; BUP, tramadol; TRAM.

IL-1β expression changes

Although a subtle up-regulation of hippocampal IL-1β was observed, this failed to reach statistical significance during 5IGT compared to vehicle controls ($p>0.05$; Figure 3.6A). BUP in the presence of 5-FU significantly decreased hippocampal IL-1β expression when compared to 5-FU controls ($p<0.05$; Figure 3.6A). BUP alone also significantly reduced hippocampal IL-1β expression compared to SAL controls ($p<0.05$; Figure 3.6A). Conversely, hippocampal IL-1β expression significantly increased in rats co-administered TRAM with 5-FU and in the TRAM control group when compared to SAL controls ($p<0.001$; Figure 3.6A). In the thoracic region, IL-1β expression was significantly up-regulated during the Peak Injury Phase of 5IGT compared to SAL controls ($p<0.0001$; Figure 3.6B). Co-administration of all analgesics attenuated thoracic IL-1β expression changes when compared to 5-FU controls ($p<0.001$; Figure 3.6B).

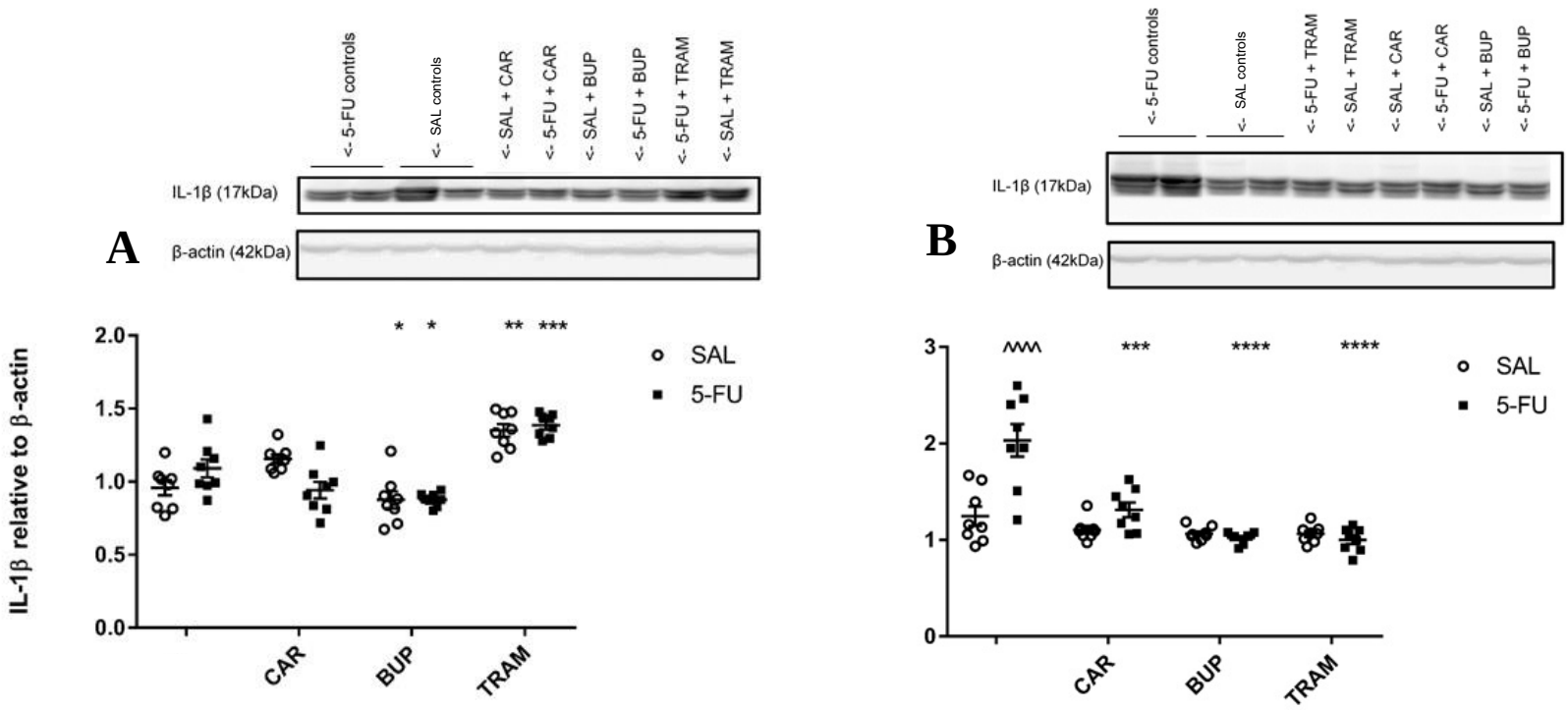

Figure 3.6A and B. Effects of 5-FU and analgesics on hippocampal (A) and thoracic (B) IL-1β expression during the Peak Injury Phase (72 h) of 5IGT. BUP in the presence of 5-FU significantly reduced hippocampal IL-1β expression compared to the 5-FU control group yet, TRAM in the presence of 5-FU increased IL-1β expression (A). GFAP expression was significantly increased in the thoracic region following 5-FU administration compared to the SAL control group (B). Co-administration of all analgesics attenuated this response when compared to the 5-FU control group (B). Data represented as mean (IL-1β expression relative to β-actin) ± SEM. ^ indicates significance compared to SAL control group. * indicates significance compared to 5-FU control group. * indicates $p<0.05$ and *** indicates $p<0.001$. 5-Fluorouracil; 5-FU, 5-FU-induced gut toxicity; 5IGT, interleukin-1 beta; IL-1β, saline; SAL, carprofen; CAR, buprenorphine; BUP, tramadol; TRAM.

Discussion

Whilst research assessing the mechanisms relating specifically to CIGT continues to expand, the indirect neuroimmune consequences and wider implications of opioid treatment during the Peak Injury Phase of CIGT remain elusive. This is the first study characterising glial complications arising in the brain and spinal cord of rats treated with analgesics under 5IGT conditions. Most investigations on chemotherapy side-effects have focused on direct mechanisms relating to a specific side-effect, such as gut toxicity or cognitive impairment. Whilst these studies have importantly shaped our understanding of such chemotherapy-induced toxicities, they have failed to account for the potential influence other side-effects may have on simultaneously occurring symptoms (cluster symptoms). Here, our data highlights the need for research into simultaneous side-effects occurring as a result of chemotherapy treatment, focusing on the gut and CNS, in particular examining neuroimmune consequences resulting from 5IGT and opioid treatment.

The significant weight loss and increased intestinal MPO data following 5-FU administration confirmed that this model induced gut toxicity in rats. Rats co-administered with CAR and BUP maintained a similar reduction in bodyweight as the 5-FU control group when compared to the SAL control group. The potentiated bodyweight reduction following TRAM co-administration was anticipated due to anecdotal evidence on the effects of opioids on feeding behaviour and bodyweight [290, 291]. Although conflicting results on opioid-induced behavioural and bodyweight changes report increased or decreased bodyweight depending on opioid selectivity and dosage, the feeding behaviour and bodyweight changes following exogenous opioid administration have been unequivocally linked to changes in the endogenous opioid system [291].

The MPO enzyme found in the intracellular granules of neutrophils was used as an indicator of acute intestinal inflammation [292]. As anticipated in the small intestine, acute intestinal inflammation was identified by a significant increase in jejunal and ileal MPO expression following 5-FU administration. This finding was in accordance with previous studies [108, 109, 288]. It was anticipated that acute intestinal inflammation during the Peak Injury Phase of 5IGT would be attenuated following co-administration of CAR, considering the selective COX-2 inhibitory nature of CAR and its anti-inflammatory action yet, MPO levels remained similar to the 5-FU control group. Interestingly, jejunal MPO expression during the Peak Injury Phase of 5IGT was ameliorated by both opioids.

Opioid receptors are not only widely distributed throughout the central and peripheral nervous systems, but also in the gastrointestinal tract [293, 294]. Opioids have long been used in clinical gastroenterology due to their effects on gut motility and visceral pain [141]. Visceral pain is often managed with opioids as they exert their effects on peripheral (spinal afferents) and central (spinocerebral pathways and anterior cingulate cortex) sites, which reduce gut sensitivity and modify pain perception [142, 295, 296]. In fact, some opioids protect the gastric mucosa under stress, having anti-inflammatory effects in experimental colitis [297]. The analogue of buprenorphine (BU08070) used in their study reported a dual activity at the nociceptive and μ opioid receptor levels, showing efficacy in reducing the severity of experimental colitis. Furthermore, delta and μ opioid receptor agonists, as well as typical opioids like tramadol, exert protective effects in the rat mucosa under gastric stress [298, 299]. Importantly, Gyires and Ronai (2001) [299] discovered that vagal innervation played an integral role in the gastroprotective effects of opioids. Further, they identified that nitric oxide and prostaglandin synthesis in the mucosa may also be involved in opioid-induced gastric protection. From this, it was not surprising that BUP and TRAM attenuated MPO levels during the Peak Injury Phase of 5IGT and we

suggest this anti-inflammatory effect may at least be partially due to opioid-induced gastroprotective effects. Further investigations are warranted to clarify the mechanistic implications for the BUP- and TRAM-related MPO effects under the 5IGT conditions described in the current study.

Neuroinflammation includes increased microglial and astrocyte expression and the subsequent synthesis of various pro-inflammatory cytokines and mediators, such as IL-1β [118, 120, 121]. Many chemotherapy drugs readily cross the blood brain barrier, including 5-FU, resulting in direct cellular and molecular changes [87, 115]. Nonetheless, the acute high 5-FU dose used in the current study failed to modulate hippocampal GFAP, CD11b or IL-1β expression levels. This was surprising considering short-term systemic 5-FU administration at a lower dosage induced apoptosis in multiple CNS regions, including the hippocampus of mice [115]. It is plausible that the peak injury time-point selected for the current study may have been suboptimal for hippocampal changes or that hippocampal changes require multiple dosages, more representative of a clinical setting. Neural progenitor cells, glial precursors and non-dividing oligodendrocytes are particularly vulnerable to toxicities associated with chemotherapy drugs and regimes, such as cisplatin and cyclophosphamide-methotrexate-5-FU [182, 300]. Although a hippocampal neuroinflammatory response was not present in the current study under 5IGT conditions, neuroinflammation has been previously suggested as a possible mechanism contributing to CICI utilising 5-FU as part of a regime [182]. This study did not present any spinal cord or gastrointestinal data, although gut toxicity would have most likely been present in their model.

Thoracic GFAP expression was significantly increased during the Peak Injury Phase of 5IGT, which supports our hypothesis that gut toxicity would induce thoracic astrogliosis. It was predicted this change would occur as a result of gastric innervation; that acute

intestinal inflammatory changes would activate a neurally-driven peripheral-to-central communication pathway mediating thoracic GFAP expression changes. Enteric astrogliosis (abnormal increase in the number of astrocytes) has been reported in a model of irinotecan-induced intestinal mucositis [301, 302]. Whilst this study importantly indicated that irinotecan-induced intestinal inflammation modified enteric glial cell expression, once again this study focused on intestinal neuronal and astrocyte segments, potentially missing critical data in other CNS regions. To our knowledge, one other study has examined spinal cord gliosis under CIGT conditions [134]. Wardill *et al.* (2016) [134] reported TLR-4 dependent mechanisms mediating irinotecan-induced gut toxicity and pain as identified by lumbar astrocyte activation and facial grimace changes. Facial grimace scores are regularly used as an indicator of pain associated with various disorders, but in this case CIGT. Although Wardill *et al.* (2016) indicated significant changes in this pain measure, Whittaker *et al.* (2016) was unable to detect any significant changes in a 5IGT model using the same measure [134, 287]. Whilst both studies utilised a single dose chemotherapy model, 270mg/kg of irinotecan was a significantly higher dose compared to the 150mg/kg of 5-FU and may have accounted for the lack of pain scores reported by Whittaker *et al.* (2016). It is astounding that so few studies exist considering the role spinal glia play in pain and the incidence of pain associated with both cancer and CIGT [1, 169, 264]. Nonetheless, some data exists on the role spinal astrogliosis plays in various models of cancer-induced pain [303-305]. In other peripheral inflammatory models, such as multiple sclerosis, neuropathic pain, arthritis and more recently, endometriosis, the role spinal glia play in pain continues to be intensively studied [304, 306-308].

Considering the steep elevation in thoracic GFAP expression, it was predicted that a similar increase in CD11b expression would occur, however this was not the case in the current model. Microglia have long been considered the major resident immunocompetent

cells in the CNS [118, 119, 121, 309]. Due to their innate surveillance role in their quiescent state, microglia are in a favourable position to be the first in-line to respond to stressors [119]. Substantial evidence suggests an interaction between microglia and astrocytes; that both cells work in unison to modulate the microenvironment, playing pivotal roles in CNS development, maintenance and pathology [124, 125]. As microglia have classically been referred to as the initial neuroimmunological defence cells, it was surprising that 5IGT induced astrocyte activation, yet had no effect on microglial CD11b expression. However, the lack of CD11b expression changes in the current study was in accordance with a previous study whereby, lumbar region glial activation was immunohistochemically identified using GFAP and Iba-1 expression markers in rats with irinotecan-induced gut toxicity [6]. At 72 h, GFAP expression was significantly increased compared to the control group, yet Iba-1 changes were not reported. GFAP expression was significantly increased at 6 h and 72 h following irinotecan administration. Other compelling evidence linking central astrogliosis to CIGT has been identified in models using oxaliplatin and vincristine [132, 133, 310]. These findings coupled with the lack of microglial marker changes in the current study implicate astrocytes in playing a preponderant role in response to peripherally driven inflammatory events, such as CIGT. It is possible that the current study missed the peak time-point of microglial activation, as this may have occurred prior to the 72 h end-point. Future studies should fully characterise the time course of glial activation. Whilst it is widely accepted that microglia are the first to respond to central insults, our data suggests that astrocytes take on this role in response to peripheral inflammatory events, such as 5IGT.

Experimentally-induced glial activation models have also reported the simultaneous up-regulation of various pro-inflammatory cytokines, such as TNF-α and IL-1β [303, 307]. Thus, it was anticipated that 5IGT-induced GFAP up-regulation would also result in

elevated IL-1β expression levels. This was indeed the case in the thoracic region of rats with 5IGT. Regardless, the current study was unable to clarify the mechanistic significance of the thoracic astrocyte and IL-1β modulation observed in 5IGT and hence further investigation is warranted. Nonetheless, the findings presented here suggest that 5IGT resulted in thoracic neuroimmunological changes mediated by a neural immune-to-central signalling pathway. Characterising the pro-inflammatory and anti-inflammatory cytokine profiles as well as behavioural changes in future studies of 5IGT-induced glial dysregulation would identify potential mechanistic adaptations. Importantly, behavioural and pain assessment would identify whether CIGT has negative effects on cognition, as this has been previously suggested [61, 302].

Centrally, it was expected that the selected opioids would modify glial and IL-1β expression levels due to the relationship between opioids and spinal cord immune signalling [126]. CAR also has central effects indicating that analgesia may be partially mediated by serotonergic mechanisms [311]. CAR significantly altered hippocampal and thoracic CD11b expression in rats with 5IGT. However, it is important to note that in the hippocampus, CD11b and GFAP expression was not altered by 5IGT when compared to controls. As there was no modulation identified in the 5IGT group, the question remains as to why CAR reduced CD11b from normal expression levels. This could be explained by the anti-inflammatory effect CAR had in a mouse model of traumatic brain injury [312]. More specifically, CAR reduced microglial numbers in the post-traumatic brain via inhibition of COX-2 [312]. From this it is plausible that CAR in the presence of 5IGT resulted in a synergistic effect on microglia in the hippocampus. Future behavioural data on hippocampal-dependent cognitive changes utilising this analgesic would clarify potential functional adaptations and the significance of these findings.

In the thoracic region, CAR significantly increased CD11b expression during the Peak Injury Phase of 5IGT. Here, it should also be noted that CD11b in the 5IGT control group was not statistically different from the SAL control group, although a trend in down-regulation was evident. Whilst the BUP and TRAM control groups elevated thoracic CD11b expression when compared to 5-FU treated rats, the 5-FU-induced CD11b changes were not statistically significant. In this instance, although the thoracic CD11b results are somewhat ambiguous, we can conclude that CAR in 5IGT and BUP and TRAM alone result in microglial activation as indicated by increased CD11b expression. Whether these changes in expression level occurred as a result of a reduction in microglial cell number or morphological changes remains to be elucidated and further studies will elucidate the changes seen in this model.

Neuroinflammation was present in the thoracic region of rats with 5IGT as determined by increased GFAP expression and elevated IL-1β expression, yet CAR and BUP attenuated this response. TRAM failed to have any direct effect on GFAP expression during the Peak Injury Phase of 5IGT. Accordingly, CAR and BUP administration had an anti-neuroinflammatory effect during the Peak Injury Phase of 5IGT; central immune signalling could explain this finding. Clinically, the findings of the current study suggest that CAR and BUP would be the optimal analgesic if the goal were to reduce thoracic neuroinflammation in 5IGT. The anti-neuroinflammatory effect of CAR and BUP in 5IGT could be at least partially mediated by the reduced IL-1β expression at this time-point. Interestingly, whilst TRAM failed to attenuate GFAP expression changes, it effectively reduced IL-1β expression to normal levels, like CAR and BUP. The mechanism underlying the reduction in thoracic IL-1β in the TRAM group is therefore unable to be explained by central immune signalling or mediated by the neuroimmune system in 5IGT.

Here we identified a main neuroimmunological effect on thoracic astrocyte activation and increased IL-1β expression during the Peak Injury Phase of 5IGT. We suggest that this regional neuroinflammatory state was indirectly driven by 5IGT thoracic innervation and mediated by a neural peripheral-to-central immune signalling pathway. From a neuroimmunological perspective, these findings indicate that TRAM had the least ameliorating effect on GFAP expression changes in either region where neuroinflammation was present. Taken into consideration, this may have significant implications clinically as TRAM may not be optimal at reducing pain associated with CIGT. However, we acknowledge that making such clinical suggestions warrants more detailed characterisation of the neuroimmunological consequences of 5IGT and importantly, studies more replicative of a clinical oncology setting are critical. Similarly, the up-regulation of hippocampal IL-1β expression following TRAM co-administration in 5IGT indicated a pathological synergistic adaptation, which could potentially contribute to a neurotoxic environment with detrimental outcomes for surrounding neurons. Nonetheless in conclusion, BUP co-administration during 5IGT was the most efficacious analgesic option in this study. Not only did BUP offer some anti-neuroinflammatory and potentially neuroprotective effect as determined by thoracic GFAP expression attenuation and reduced pro-inflammatory parameters, but it also had a positive effect on intestinal acute inflammation as it reduced MPO expression during the Peak Injury Phase of 5IGT.

Despite recent advancements in our understanding of pain associated with CIGT, pain management for cancer and CIGT continues to be an issue. Choosing the optimal analgesic for pain associated with CIGT is challenging due to the risks associated with opioid tolerance, dependence and enhanced pain states. It is important to understand the wider implications of opioid use in the oncology arena and this study has helped to fill the gap on the neuroimmune consequences of 5IGT and opioid treatment. Microglia and astrocytes

are critical in CNS homeostasis forming a close relationship with the synapse. Glial dysregulation plays a role in a range of neurodegenerative diseases and pathological pain syndromes and it is plausible that under chemotherapy conditions, they may contribute to cognitive changes and pain states. Future studies should critically assess the morphological glial adaptations described in the current study via immunohistochemistry and this is acknowledged as a limitation of the current study. Chemotherapy-induced glial dysregulation and the consequences arising from opioid treatment should be further investigated as the benefit of using this form of analgesic may outweigh the risks and complications associated with opioid use.

CHAPTER FOUR, *in vivo*

“Big Brain” and “Little Brain” Consequences in 5-Fluorouracil-Induced Gut Toxicity and Indomethacin-Induced Enteropathy; Neuroimmune and Microbiome Implications

Statement of context

This manuscript has been submitted to the Journal of Molecular Cancer Therapeutics.

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Chapter Four provides a detailed characterisation of intestinal damage in rats with acute 5IGT and INDO-enteropathy. It describes and quantifies astrocyte protein expression changes in the thoracic region of rats with 5IGT and suggests this was mediated by a neural peripheral-to-central immune signalling mechanism. Additionally, this chapter provides histological data which supports the reactive astrocyte phenotype suggested by the up-regulation of thoracic GFAP expression. Finally, this chapter reports on the caecal microbiota changes observed in the chemotherapy group and discusses the potential functional significance of these findings.

Statement of Authorship

Title of Paper	"Big brain" and "little brain" consequences in 5-Fluorouracil-induced gut toxicity and indomethacin-induced enteropathy; neuroimmune and microbiome implications
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Juliana Esma Ballc, Gordon Stanley Howarth, Caitlin Selway, Laura Weyrich, Larisa Bobrovskaya & Mark Rowland Hutchinson

Principal Author

Name of Principal Author (Candidate)	Juliana Ballc		
Contribution to the Paper	I was responsible for the conceptual framework and execution of all elements of this study; conducted all experimental work, reviewed all papers cited in manuscript, wrote manuscript, performed statistical analyses and interpreted data, designed tables and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);
II. permission is granted for the candidate in include the publication in the thesis; and
III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Chemotherapy-induced (CI) gut toxicity (CIGT) and cognitive impairment (CICI) are a significant burden at the individual, clinical and global levels. The need to clarify mechanisms contributing to CIGT and CICI are critical, and the neuroimmune system and gut microbiota are ideally positioned to influence both toxicities. Using a single high-dose rat model of 5-Fluorouracil (5-FU)-induced gut toxicity (5IGT) we sought to determine whether intestinal inflammation would induce caecal microbiota changes and glial reactivity in the brain and spinal cord. Effects were compared to a low-dose, non-chemotherapy inflammatory model of non-steroidal anti-inflammatory drug (NSAID)-enteropathy. It was proposed that glial reactivity may be mediated by peripheral-to-central immune signalling pathways (humoral and neural). 5IGT reduced bodyweight, elevated myeloperoxidase activity and significantly altered the mucosal architecture in the small intestine of rats during the Peak Injury Phase (72 h). 5IGT resulted in changes to the caecal microbiota, significantly reducing alpha phylogenetic diversity, decreasing the relative abundance of commensal bacterial taxa and elevated some non-commensals, such as *Bacteroides*. Spinal thoracic astrocyte reactivity was observed in rats with 5IGT via increased protein marker expression changes and histological assessment. The mucosa of NSAID-treated rats was maladapted compared to vehicle controls (reduced villus height and crypt depth), and bodyweight, acute intestinal inflammatory parameters, caecal microbial and astrocyte marker changes were not affected at 72 h. The findings demonstrate that inflammation in the "little brain" (5IGT) results in "big brain" inflammation (spinal thoracic astrocyte reactivity) via a neurally-mediated immune signalling mechanism. The microbiota perturbations and neuroimmune findings indicate that these two disparate, yet connected mechanisms, are not only playing a role in the pathogenesis of regimen-related gut and central toxicities, but suggest that they are uniquely positioned to influence both CIGT and CICI symptoms.

Background

The central nervous system (CNS; "big brain") and gastrointestinal tract (GIT; "little brain") form the primary organs susceptible to toxicities associated with chemotherapy treatment. Clinically, CNS changes referred to as chemotherapy-induced cognitive impairment (CICI), whilst GIT perturbations are termed chemotherapy-induced gut toxicity (CIGT) [1, 272, 313]. Both disorders often occur simultaneously and cause a significant strain on patient quality of life. Extensive research has provided mechanistic explanations for each toxicity, yet they have not considered the direct or indirect influence each disorder has on the other. This is surprising considering the multiple mechanisms and pathways involved in gut-brain signalling and vice versa – the integration of information from many systems. The central, autonomic and enteric nervous systems, the microbiota-gut-brain axis, neuroendocrine, enteroendocrine and neuroimmune input make up some of these systems and have been extensively reviewed elsewhere [3-5, 8, 14, 15, 36, 314, 315].

CIGT is comprised of five constantly overlying pathophysiological phases described by Sonis (2004) [1]. Whilst chemotherapy drugs have differing mechanisms of action, the model proposed by Sonis can be applied to most treatment modes. Recently, the unique roles of additional pathological features to specific drugs have been highlighted. For instance, the widely utilised chemotherapy drug, 5-Fluorouracil (5-FU) induced enteric neurotoxicity and colonic dysmotility [107]. Particular interest has been given to the role of an unbalanced intestinal microbiota in 5-FU-induced gut toxicity (5IGT) [213, 316]. Specifically, a shift in the bacterial composition from Gram-positive to Gram-negative organisms was observed in 5-FU-treated rats [214]. Development of the research term "oncomicrobiome" purely focuses on the role of the microbiota in cancer aetiology, including cancer risk, as well as identifying mechanisms that influence the toxicity and efficacy of various chemotherapy drugs [38, 208]. This is important as commensal bacteria

play pivotal roles in host innate and adaptive immune responses [317]; thus, changes in microbiota can influence CNS disorders, such as Alzheimer's disease, depression and Parkinson's disease [18-20]. The influence of CIGT-induced microbiota changes has yet to be examined in the context of CICI, regardless of the influential and bidirectional nature of the microbiota-gut-brain axis.

Intestinal epithelial homeostasis is maintained by an interaction between commensal bacteria and Toll-like receptors (TLRs), innate pattern recognition receptors [192]. TLRs belong to a family of receptors which are sensitive to immune insults and critically initiate innate immune responses [318]. Release of immune messages, such as pro-inflammatory cytokines, from the intestine are able to reach higher order brain regions via humoral and neural communication routes [144, 277]. This triggers a neuroimmunological response involving glial cell reactivity, increased synthesis of immune cytokines, mediators and neurotrophic factors, which include glutamate, interleukins (ILs) and bone-derived neurotrophic factor (BDNF), ultimately assisting the host in healing [121, 122, 143]. The immune-like cells of the CNS, primarily glial cells (microglia and astrocytes), form an intimate relationship with the synapse and are pivotal in CNS homeostasis [118, 120]. Increased glial cell expression and neuroinflammation contributes to many central and peripheral disorders, ranging from neuropathic pain and peripheral neuropathies, to neurodegenerative disorders, such as Parkinson's disease and depression [124, 131, 133, 157, 267, 319]. Nevertheless, few studies have focused on the role of the neuroimmune system (glial cells) in CIGT [134, 320]. Due to the intimate relationship glial cells have with the synapse, they are ideally positioned to influence, contribute to or potentiate learning and memory deficits in the context of CICI.

CICI affects a substantial subset of cancer patients [321-323] in memory, attention, executive function and processing speed [321]. Various biological and molecular factors

predispose cancer patients to CICI, such as cognitive reserve, immune function and genetics [84, 113]. Direct chemotherapy drug interactions in higher order brain regions, regardless of drug mechanism of action, reduce hippocampal neurogenesis in CICI experimental models [86, 87]. These studies link chemotherapy-induced hippocampal changes to learning and memory deficits in rodents. Drugs widely utilised in cancer and CICI studies, such as 5-FU, are also selected in CIGT studies. Whilst all CICI investigations will have most likely resulted in gut toxicity, the influence of intestinal inflammation on CICI has yet to be examined. A neuroimmunological mechanism for CICI has been proposed [62, 302], although few studies have reported findings [134, 320] and this highlights the need for further examination. Regional spinal astrocyte activation and pain were mediated by TLR-4 following systemic administration of irinotecan in mice [134], whereas methotrexate activated hippocampal microglia but was unable to elevate central cytokine expression [320]. Importantly, the findings from these studies indicate a potential role for the neuroimmune system in mediating the pathogenesis of both regimen-related toxicities, in the "little brain" and "big brain".

In light of the increasing incidence of cancer and use of chemotherapy treatments, there is a clear need to better understand the pathophysiological mechanisms underpinning both disorders. The current study sought to determine whether 5IGT induced acute intestinal inflammation and caecal microbiota changes in rats. Secondly, it determined whether 5-FU would *directly* induce glial cell reactivity in higher order brain regions, by directly crossing the blood brain barrier (BBB), indicating a humorally-mediated immune signalling pathway. It was also postulated that systemic 5-FU would induce spinal cord glial reactivity, *indirectly* resulting from 5-FU-induced intestinal inflammation, indicative of a neurally-mediated signalling pathway. Thirdly, utilising a single high-dose model of 5IGT,

the current study aimed to determine whether the effects were unique to 5-FU by comparing microbiota and regional glial changes in a rat model of reduced intestinal inflammation, induced by a low-dose of the non-steroidal anti-inflammatory drug (NSAID), indomethacin.

Materials and methods

Female Dark Agouti rats (110-140g, *n*=36) were sourced from the Animal Resources Centre in Western Australia (barrier maintained Specific-Pathogen Free production facility). Animal experimentation was approved by the Animal Ethics Committee of the University of Adelaide. The animal protocol described in this study minimised pain and discomfort to the animals by complying with the Australian National Health and Medical Research Council Code of Practice for Animal Care in Research and Teaching (8th Edition, 2013). Rats were randomly grouped (*n*=12/group; 8/group for molecular and 4/group for immunohistochemical analysis) and group-housed (with a combination of rats from each group) in a room maintained at 21-23°C with a 12 h reversed light-dark cycle. All rats were provided *ad libitum* access to water and standard rat chow. Following an acclimatisation period (>2 weeks), at 0 h, rats were intraperitoneally administered a single dose of saline (SAL), 5-FU (150mg/kg; Mayne Pharma Pty Ltd, Mulgrave, Victoria, Australia) or indomethacin (INDO, 20mg/kg; Sigma-Aldrich, Missouri, United States of America, #17378-10G).

Daily Metabolic and Disease Activity Index

Bodyweight and severity of gut toxicity were measured daily using a disease activity index (DAI) [324]. To assess the severity of gut toxicity, overall condition, bodyweight and stool consistency (evidence of rectal bleeding) were measured using a scale (0-3) with increasing severity for each parameter. Scores were totalled to achieve an overall DAI, described previously [324, 325]. Overall condition was determined by, 1) mobility/agility: healthy rats are active and alert, whereas rats treated with 5-FU or INDO characteristically become weakened, often sitting hunched with little movement, and (2) fur: healthy rats are well groomed with flat, shiny hair that sits close to their body, whereas unhealthy rats appear scruffy, with a dull, ruffled coat.

Tissue Collection and Preparation

Gastrointestinal tract. Following CO_2 asphyxiation, the gastrointestinal contents of each animal were removed, measured, emptied of contents and the intestine weighed. Small intestinal segments were collected at approximately 10% (jejunum) and 90% (ileum) of the total small intestinal length and snap frozen for biochemical analysis. Further segments of the small intestine (jejunum, jejunum-ileum (JI) junction and ileum; 2 cm) were dissected and placed in 10% neutral-buffered formalin for histological analysis. The remaining visceral organs (thymus, lungs, heart, liver, kidneys and spleen) were weighed and discarded. Caecal contents were isolated and frozen at -20ºC for bacterial diversity profiling.

CNS. Following removal of organs, rats were decapitated and the vertebral column dissected. The entire spinal cord was isolated and the brain was carefully removed whole from the skull and snap frozen in liquid nitrogen for biochemical analysis. An additional 4 animals from each group were perfused with 4% PFA and decapitated. The spinal cord was isolated and sectioned into cervical (C3-C5), thoracic (T7-T9) and lumbar (L2-4) regions. Coronal sections of the hippocampus were isolated between bregma -5.00 to -5.50 following a conventional rat brain atlas (Paxinos and Watson, 2006).

Intestinal Biochemical Analyses

Myeloperoxidase (MPO) is an enzyme in the intracellular granules of neutrophils, thus providing a quantitative analysis of acute inflammation [292]. Jejunal and ileal segments were thawed and prepared for MPO assay via homogenisation in 10mM phosphate buffer (pH 6.1). Homogenised samples were centrifuged at 13,000 rpm for 12 min and the supernatant discarded. The remaining pellet was resuspended with 0.5% hexadecyltrimethyl ammonium bromide buffer and vortexed prior to a final centrifuge (13,000 rpm for 2 min). Supernatant from each sample (50µL aliquot) was dispensed into a

96-well plate and the MPO reaction was initiated with an O-dianisidine dihydrochloride solution (200µL/well; 4.2mg O-dianisidine dihydrochloride, 12.5µL hydrogen peroxide (30%) in 2.5mL potassium phosphate buffer (50mM, pH 6.1) and 22.5mL distilled H_2O). A spectrometer (Victor X4 Multilabel Reader, Perkin Elmer, Singapore, South East Asia) measured absorbance (450nm) at 1 min intervals over a 15 min period. The change in absorbance was used to calculate MPO activity within a tissue sample (MPO units/g of intestinal tissue).

Intestinal Histological Analyses

Following removal from formalin, intestinal samples were transferred to 70% ethanol 24 h post-collection. Samples were embedded in paraffin wax and sections (4µm) were routinely processed and stained with haematoxylin and eosin (H&E). Nanozoomer™ and Digital Pathology Software (Hamamatsu Photonics, Japan and Histalim, Montpellier, France) was utilised to visualise histological intestinal changes at 3.5 x and 10 x magnification. Villus height (VH) and crypt depth (CD) were measured in the small intestine and determined for 20 well-orientated villi and crypts per small intestinal tissue section, per rat and a mean value was obtained for statistical analysis [326]. All intestinal histological analyses were performed in a blinded-fashion using ImageJ 1.49 software (NIH Image, Maryland, USA).

Immunohistochemical assessment of Astrocyte Reactivity Expression Markers

Thoracic spinal cord (T7-9) sections (5µm) were sliced on a rotary microtome and mounted on to Superfrost® microscope slides (Menzel-Glăser, Braunschweig, Germany). Astrocytic reactivity was performed using Glial Fibrillary Acidic Protein (GFAP; Clone 6F2, DakoCytomation, Dako, Denmark; #M0761. Briefly, sections were dewaxed in xylene and dehydrated in 100% ethanol before being quenched for endogenous peroxidase activity in methanol with 0.5% hydrogen peroxide for 30 min. Slides were washed in 0.1%

M PBS (pH 7.4, 3 x 2min) before undergoing heat-mediated antigen retrieval using 0.1 M citrate buffer (pH 6.0). Non-specific binding was blocked by 3% normal horse serum (NHS; Sigma-Aldrich, NSW, Australia) at room temperature for 30 min. GFAP (diluted in 3% NHS) was applied overnight at room temperature in a humid chamber (GFAP 2mg/µL). Following removal of GFAP stain, a secondary goat biotinylated anti-rabbit IgG antibody (Vector Laboratories, California, USA, anti-rabbit #BA1000, at 6mg/µL) was applied to the sections at room temperature for 30 min. After washing with PBS (3 x 2 min) slides were incubated with Pierce™ streptavidin peroxidase conjugate (ThermoFisher Scientific, VIC, Australia, #21130, at 2mg/µL) at room temperature for 30 min followed by a PBS rinse. Immunocomplex was visualised with DAB (Sigma-Aldrich, NSW, Australia, #D5637) precipitation in the presence of hydrogen peroxide (3%). Slides were washed to remove excess DAB and lightly counter-stained with haematoxylin, dehydrated and mounted with DePeX from histolene. Slides were scanned and assessed using the Nanozoomer™ and Digital Pathology Software (Hamamatsu Photonics, Japan and Histalim, Montpellier, France). Staining was assessed in the dorsal column of the spinal cord using ImageJ 1.49 software and the previously validated colour deconvolution method [327].

Western Blot Protocol for CNS Tissue

Hippocampal, pre-frontal cortex and hypothalamus brain regions, and cervical (C2-C5) and thoracic (T7-T9) (spinal) regions were dissected and immersed in cell lysis buffer (Tris-base saline; pH 8.0, 50mM, 1% Triton X-100; 1% protease inhibitor cocktail, #P8340, Sigma-Aldrich, Castle Hill, NSW, Australia); 300µl (spinal cord) and 500µl (hippocampus) aliquots. Samples were sonicated (10 sec followed by at least 1 min on ice) then centrifuged (15,000 x g) for 40 min. Protein concentration of the supernatants were determined via BCA-Protein Assay (Pierce® BCA Protein Assay Kit; #23225, Thermo

Fisher Scientific Inc., Victoria, Australia.). Homogenised proteins of known concentrations were suspended in 2 x sample buffer (SDS reducing sample buffer: deionised H_2O, 0.5M Tris-HCl pH 6.8, glycerol, 10% SDS solution and 0.5% bromophenol blue) and heated at 70˚C for 10 min. Equal amounts of protein from each sample (25µg) were loaded and separated by gel electrophoresis in acrylamide gradient gels. Gels were homemade (SDS page 8-10%; 40% Acrylamide/Bis; gel buffer (Resolving gel: 1.5M Tris-HCl, pH 8.8, and Stacking gel: 0.5M Tris-HCl pH 6.8); 10% w/v SDS; 10% ammonium persulfate; TEMED) and optimised to suit antibody specifications. Protein transfer onto nitrocellulose filter paper (Sigma-Aldrich) used a boric acid transfer buffer (pH 8.9; boric acid, EDTA for 2 h at 600 mA) and ponceau stain performed. Membranes were blocked with 5% skim milk (lower kDa proteins) at room temperature for 1.5 h. Protein expression utilised antibodies for GFAP (1:2000; #ab7260, Abcam, Cambridge, UK) and Ionized calcium binding adaptor molecule-1 (Iba-1; 1:1000 WAKO; #019-19741, Osaka, Japan) indicators of astrocyte and microglia expression changes, respectively. Overnight incubation at 4˚C achieved primary antibody staining. β-actin (1:2000; #a2066, Sigma Aldrich, St. Louis, MO, USA) standardised sample loading (control) and accounted for protein loading variability during gel electrophoresis. An additional internal sample control (2µL from each sample) determined changes between gel integrity and normalised band detection. Electrochemiluminescence (ECL) enabled visualisation of the protein bands using secondary antibodies: anti-rabbit/sheep/mouse antibody (peroxidase-conjugated Affinipure Donkey Anti-rabbit/sheep/mouse IgG; 1:20,000; #711-035-152, #713-035-003, #715-035-150; Jackson ImmunoResearch Laboratories, Inc., PA, USA). Immunoblots were developed using an in-house ECL reagent (10mL of 100mM Tris HCl (pH 8.5), 22µL of 90mM coumaric acid, 50µL of 250mM luminol and 3µL of H_2O_2) and were visualised using the ImageQuant 4100 imaging system (GE Health Care, Buckinghamshire, UK)

[289]. Ratio-metric analysis quantified band densities and were measured as a fold-change relative to control group, accounting for normalisation as described earlier.

Bacterial Diversity Profiling

Bacterial DNA was extracted from 500mg of rat caecal material using the DNeasy PowerSoil Kit (Qiagen, MO Bio Laboratories, Carlsbad, CA, USA, #12888-100), following manufacturer's instructions. Extraction blank controls (EBC) were performed in parallel to monitor DNA present in the laboratory and within reagents. Using Illumina Inc. (San Diego, CA, USA) primers, the V4 region of the 16S rRNA was targeted and amplified in triplicate. Individual polymerase chain reactions (PCR) (25µL) contained 18.05µL of DNA-free water, 2.5µL 10x HiFi Taq buffer (ThermoFisher), 1.0µL $MgSO_4$ (25mM), 0.2µL dNTPS (25mM) (ThermoFisher), 0.25µL HiFi Platinum Taq DNA polymerase (ThermoFisher), 1µL of each V4 primer (10mM; barcoded reverse primer) and 1µL of DNA extract. Cycling conditions were as follows: 95°C for 6 min; 38 cycles of 95° C for 30 sec, 50°C for 30 sec, 72°C for 90 sec; and a final extension at 60°C for 10 min. A no-template control (NTC) monitored laboratory and reagent contamination for each PCR group. Once amplified, triplicate PCR products were pooled and the presence of a 16S rRNA V4 gene product was verified by gel electrophoresis, using a 2.5% agarose gel. Amplifiable products from EBCs and NTCs were prepared for sequencing and investigated laboratory and reagent contamination in order to avoid the presence of false positives as true biological signal. 16S rRNA libraries were quantified using a Qubit dsDNA HS assay kit (ThermoFisher) and pooled in equimolar concentrations (30 samples per group). Pooled libraries were then size selected and cleaned using AxyPrep to remove excess primers and primer dimers. The pooled, cleaned libraries were then quantified using a broad sensitivity D1000 Screentape on an Agilent 2200 Tapestation. Quantitative PCR (qPCR; KAPA Illumina Primer mix) was carried out to accurately determine the

concentration of this final library (LightCycler 96 System, Roche Life Science), before diluting to a 2nM concentration for sequencing. Libraries were sequenced on the Illumina Miseq using 2 x 150 bp kits with custom sequencing primers. DNA sequences were demultiplexed, quality filtered and operational taxonomic units (OTU) were identified from fastq sequences in QIIME (v 1.8.). After demultiplexing and quality filtering sequences based on their unique barcode identifier, a file containing all sequences assigned to each sample (seq.fna) was produced and combined with a seqs.fna from all 16S rRNA libraries created at the Australian Centre for Ancient DNA (ACAD; n=1692). Finally, open and closed reference OTU strategies were applied to identify sequences according to the Greengenes reference database (v13.8). The open reference OTU picking provides all the closed OTUs (those identified in the Greengenes (v13.8) database), as well as any unique OTUs that cluster separately (*de novo*). A filtering process was performed to ensure contaminant sequences were removed before proceeding with diversity analyses and taxonomic profiling.

Statistical Analysis

Bodyweight and Metabolic Parameters: Statistical analyses were conducted using IBM SPSS Statistics Program (v 25). These data were tested for normality using the Shapiro-Wilk analysis. All data were analysed using a one-way analysis of variance (ANOVA) with Tukey's *post hoc* comparison tests. Bodyweight was analysed using a repeated measures ANOVA. Whilst some main effects were not observed using an ANOVA, significant interactions at different levels were reported utilising *post hoc* analyses. Consequently, it may be anticipated that the main effect would not translate to a *post hoc* and thus, it is acknowledged that this approach of statistical reporting presents as a potential limitation of this study. All data expressed as mean (unless otherwise stated in Figure legends) ± SEM with values of $p<0.05$ deemed significant.

Microbial Diversity Analyses

Alpha and beta diversity (diversity within and between samples, respectively) was calculated, and the taxonomic composition of samples was summarised. For alpha diversity, two metrics were used: (1) observed species; bacterial species richness, and (2) phylogenetic diversity; phylogenetic distance of bacteria within samples. Samples were rarefied to 8000 sequences and an alpha diversity statistic (two-way t-test) was applied. Beta diversity measured the diversity between samples using a distance matrix. Due to the abundance of various taxa within 16S data sets being biased, only the unweighted UniFrac (a phylogenetic metric not weighted on the abundance of taxa) metric was used. OTUs were summarised into their taxonomic classification via analysing the genera driving the alpha- and beta-diversity. Category significance (beta diversity statistics) was determined via adonis and ANOSIM analyses which investigated the variation between categories within each metadata field. The effect size (R^2) and dissimilarity between groups (R value) determined the *p*-value.

Results

INDO and 5-FU Treatment Decreased Bodyweight

Following administration, bodyweight percentages decreased on day 1 in the majority of rats compared to experimental day -2, regardless of treatment type, although this failed to reach significance between treatment groups ($p>0.05$). When compared to VEH controls, INDO and 5-FU treatment significantly decreased bodyweight on day 2 ($p<0.05$ and $p<0.01$, respectively; Figure 4.1). The average reduction in bodyweight percentage for 5-FU treated rats on this day was -2.6g, compared to VEH controls averaging an increase of 1.9g. Whilst the INDO group bodyweight percentage returned to baseline levels on day 3, 5-FU rats maintained a significantly reduced bodyweight (average -1.29g) on day 3 compared to VEH controls ($p<0.001$; Figure 4.1).

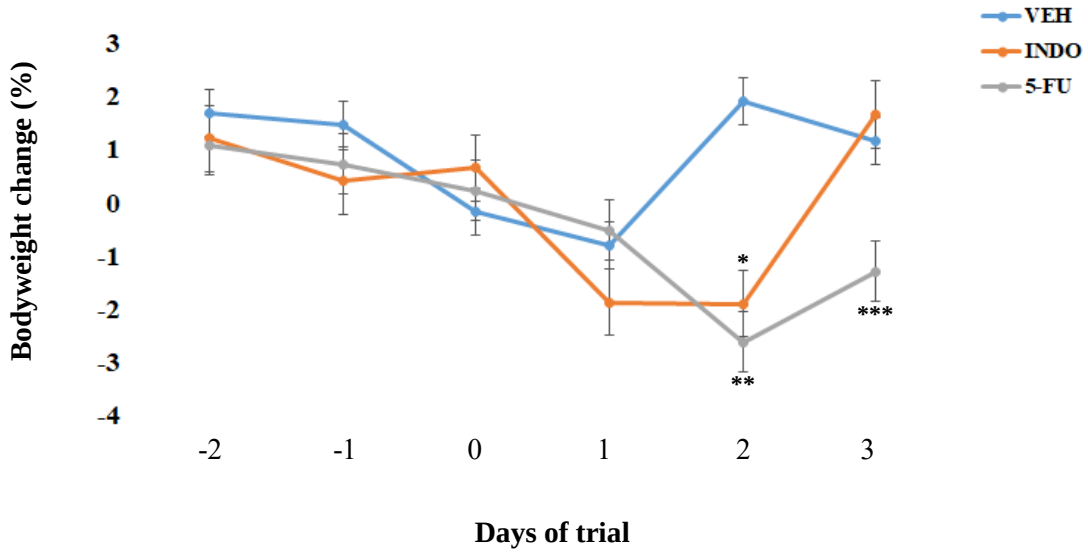

Figure 4.1. Bodyweight represented as a percentage from baseline (day -2). All data expressed as mean % of each treatment group from baseline (day -2) ± SEM. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ compared to vehicle controls. 5-Fluorouracil; 5-FU, indomethacin; INDO, vehicle; VEH.

INDO and 5-FU Reduced Small Intestinal Length and Differentially Affected Visceral Organ Weights

The total length of the small intestine (SI; jejunum and ileum) of rats treated with INDO and 5-FU significantly reduced compared to VEH controls ($p<0.01$ and $p<0.001$, respectively; Table 4.1A). Additionally, SI weight was significantly increased in INDO treated rats compared to 5-FU treated animals, which decreased compared to VEH controls ($p<0.05$ and $p<0.001$, respectively). Colon and duodenum lengths were unaffected by either drug ($p>0.05$). Other significant INDO- and 5-FU-induced organ weight changes were evident in the thymus, liver and spleen compared to vehicle controls ($p<0.05$; Table 4.1B). Colonic weight was significantly increased in 5-FU treated rats compared to VEH controls ($p<0.01$).

A

Length (mm)	VEH	INDO	5-FU
SI	69.9 ± 1.5	63 ± 1.4**	58.1 ± 1.4***
Colon	12.8 ± 0.4	12.1 ± 0.5	12.3 + 0.2
Duodenum	4.4 ± 0.6	4.5 ± 0.4	4.4 ± 0.7

B

Weight (%)	VEH	INDO	5-FU
Stomach	65.6 ± 2.9	68.7 ± 2.1	70.7 ± 1.2
Duodenum	35 ± 2.1	33.8 ± 0.8	34.9 ± 3.3
SI	282.4 ± 5.2	314.8 ± 7.3*	222.1 ± 9.1***
JI	18.7 ± 1.7	24.2 ± 1.2	18.1 ± 1.3
Caecum	74.5 ± 8	53.7 ± 3.9	74.5 ± 8
Colon	70 ± 4.5	74.1 ± 4.2	91.6 ±3.8**
Thymus	24.4 ± 1	17.7 ± 2**	7.5 ± 0.3***
Heart	42.4 ± 1.6	43.6 ± 1.4	42.5 ± 0.8
Lungs	69.2 ± 4.7	67 ± 2.3	61.1 ± 1.3
L kidney	42.5 ± 1.4	44.4 ± 1.2	44.2 ± 1
R kidney	43.8 ± 1.8	44.5 ± 0.7	44.7 ± 0.9
Liver	362.9 ± 13.9	420.1 ± 8.4**	409.7 ± 14.2*
Spleen	20.2 ± 1.1	25.2 ± 1**	13.7 ± 0.3***

Table 4.1. GI and organ lengths (A) and weights (B). Weight data were calculated according to bodyweight adjustments for each animal in each group. Data expressed as mean (% relative to bodyweight) ± standard error of the mean. * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$ compared to VEH control. Gastrointestinal; GI, vehicle; VEH, indomethacin; INDO, 5-Fluororuacil; 5-FU, small intestine; SI and jejuno-ileum junction; JI.

5-FU Administration Increased DAI on Day 2

Although a slight increase in DAI scores was evident on day 1 following VEH, INDO and 5-FU administration (mean increasing from an average of 0.3-0.5 on day 0 to 0.6-0.9 on day 1), this was not statistically significant (p>0.05; Table 4.2). On day 2, DAI scores increased in rats with INDO-enteropathy and 5IGT, though this only reached statistical significance with the 5-FU group when compared with VEH controls (p=0.002; Table 4.2). Whilst rats with 5IGT maintained an elevated DAI mean on day 3, this was insignificant compared with VEH controls (p=0.06).

	DAY 0	DAY 1	DAY 2	DAY 3
VEH	0.5(0-1)	0.6(0-1)	0	0.4(0-2)
INDO	0.3(0-1)	0.9(0-3)	0.7(0-3)	0.3(0-2)
5-FU	0.3(0-1)	0.8(0-3)	1.6(1-4)**	1.3(0-3)

Table 4.2. DAI scores of rats with 5IGT and INDO-enteropathy at 72 h. Data expressed as mean score (range). ** indicates p<0.01 compared to VEH controls. Disease activity index; DAI, 5-Fluorouracil-induced gut toxicity; 5IGT, indomethacin; INDO, vehicle; VEH.

5-FU Administration Significantly Elevated MPO Activity in the Small Intestine

MPO expression in the jejunum and ileum was significantly elevated in rats with 5IGT compared to VEH controls (>435% increase and $p<0.001$ in both regions, Figure 4.2A and B). Whilst the MPO expression of rats with INDO-induced enteropathy failed to reach significance compared to VEH controls, there was a trend for elevated levels in both regions (average of 44% in the jejunum and 55% in ileum, $p>0.05$, Figure 4.2A and B).

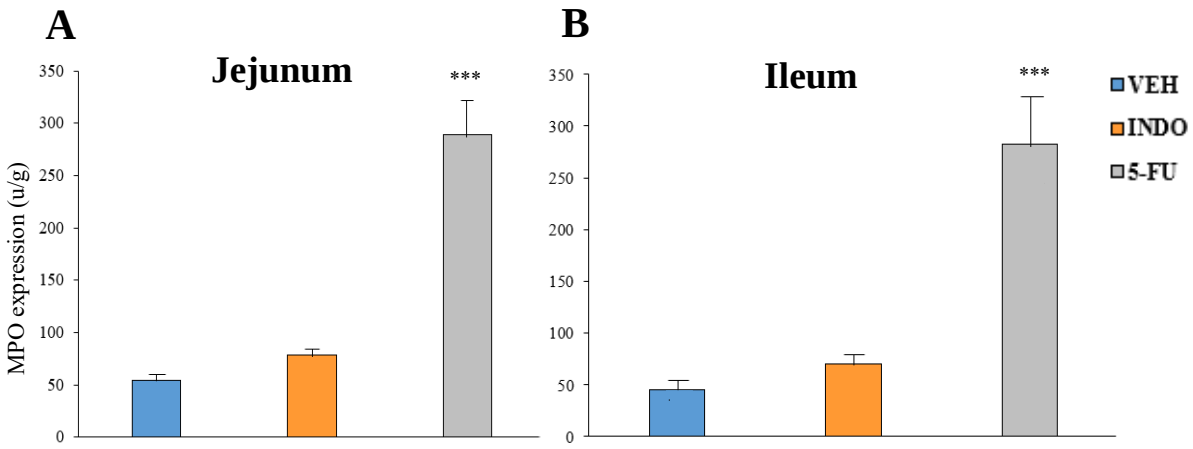

Figure 4.2. MPO activity in the jejunum (A) and ileum (B) of rats with 5IGT and INDO-enteropathy. Data expressed as units of MPO per gram of tissue ± SEM. *** indicates $p<0.001$ compared to VEH controls. Myeloperoxidase; MPO, vehicle; VEH, indomethacin; INDO, 5-Fluorouracil; 5-FU.

5-FU and INDO administration significantly altered jejunal mucosal structural architecture

5-FU and INDO administration significantly reduced VH and CD compared to VEH controls ($p<0.0001$ for both groups; Table 4.3). Representative H&E images of jejunal sections indicate drug-induced gut toxicity compared to VEH group via morphological changes in 5IGT and INDO-enteropathy groups (Figure 4.3; panels A, B and C respectively). INDO revealed moderate signs of tissue injury and toxicity in the jejunum as characterised by crypt ablation and villous shedding and fusion (Figure 4.3B). 5-FU treated rats showed severe signs of mucosal injury with compromised villus and crypt integrity, decreased muscularis layer, areas of luminal surface showing little to no evidence of villi structures, inflammatory infiltrate and epithelial shedding from muscularis wall (Figure 3C).

	Villus height	Crypt depth
VEH	516 ± 7.9	197 ± 6.8
INDO	324 ± 15.7****	135 ± 6.3****
5-FU	169 ± 4.6****	99 ± 3.36****

Table 4.3. Jejunal villus height (VH) and crypt depth (CD) of rats with 5IGT and INDO-enteropathy at 72 h. Data expressed as mean (length; μm) ± SEM. **** indicates $p<0.0001$ compared to VEH controls. 5-Fluorouracil-induced gut toxicity; 5IGT, indomethacin; INDO, vehicle; VEH.

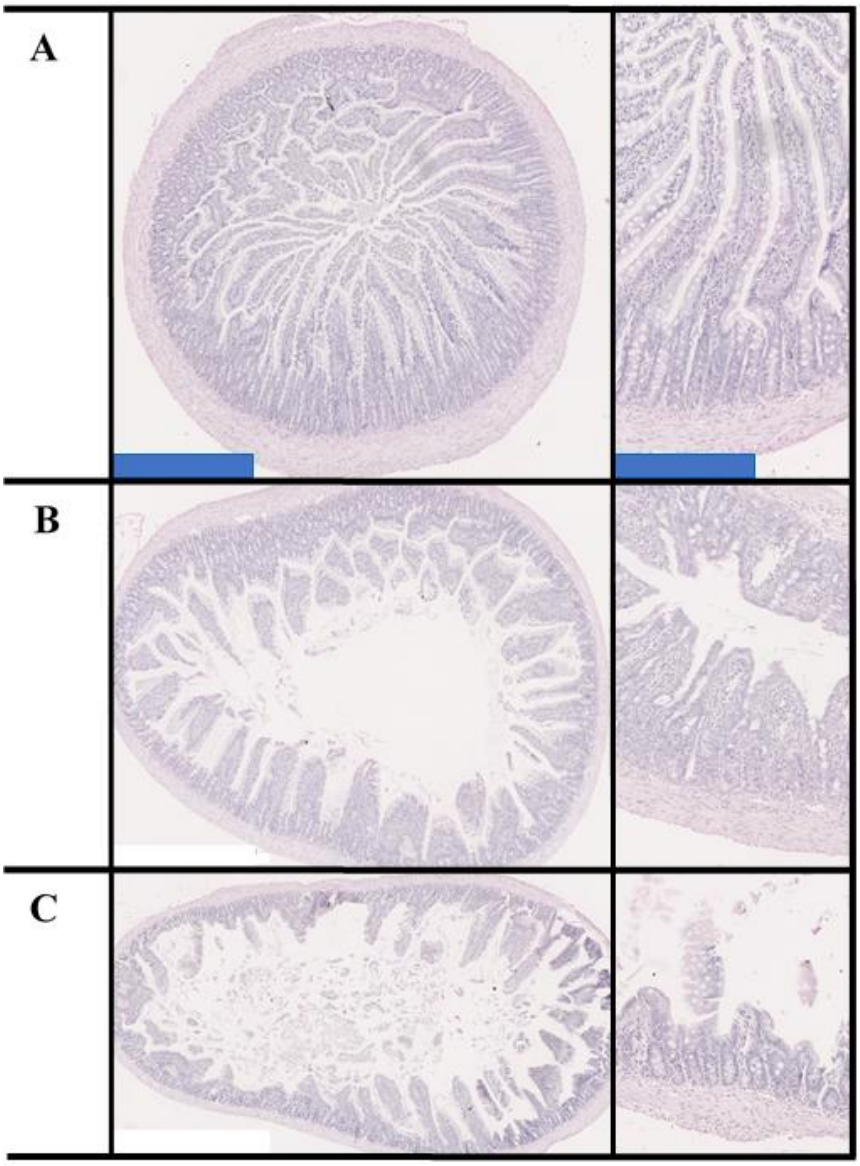

Figure 4.3A, B and C. Representative histological H&E images of rat jejunal sections (4µm); VEH treated (panel A), INDO-enteropathy (panel B) and 5IGT (panel C) rats. INDO-enteropathy rats show moderate jejunal injury and intestinal toxicity by reduced VH and CD. Panel C shows severe villus and crypt injury and toxicity. Blue scale bars show 500µm (left) and 250µm (right). Hematoxylin and eosin; H&E, vehicle; VEH, indomethacin; INDO, 5-Fluorouracil-induced gut toxicity; 5IGT, villus height; VH, crypt depth; CD.

Evidence for Thoracic Spinal Region Astrocyte Reactivity in Rats Following 5-FU Injection

During the Peak Injury Phase of 5IGT, thoracic spinal cord GFAP expression was significantly increased compared to the VEH group, as shown by WB analysis (p=0.003; Figure 4.4A.1). A marginal down-regulation in the INDO-enteropathy group was observed compared to the control group, though this was insignificant. IL-1β expression was also elevated in the thoracic spinal cord region of rats following 5-FU injection, compared to VEH controls (p=0.0004; Figure 4.4A.2). GFAP DAB staining was significantly elevated in the thoracic region (T7 and T8) of rats with 5IGT compared with VEH controls (p<0.01 and p<0.001, respectively; Figure 4.4B.1). Qualitative images of the thoracic spinal dorsal horn of VEH controls (Figure 4.4B.2) and rats with 5IGT (Figure 4.4B.3) support the increased GFAP molecular and staining findings. Astrocyte reactivity (astrogliosis) is evident through changes in cellular phenotype; somatic hypertrophy (yellow box), elongated (blue box) and thickened (black box) processes. No other molecular regional GFAP, IL-1β or Iba-1 protein expression changes were observed in the other brain or spinal cord regions of rats with 5IGT or INDO-enteropathy (data not shown).

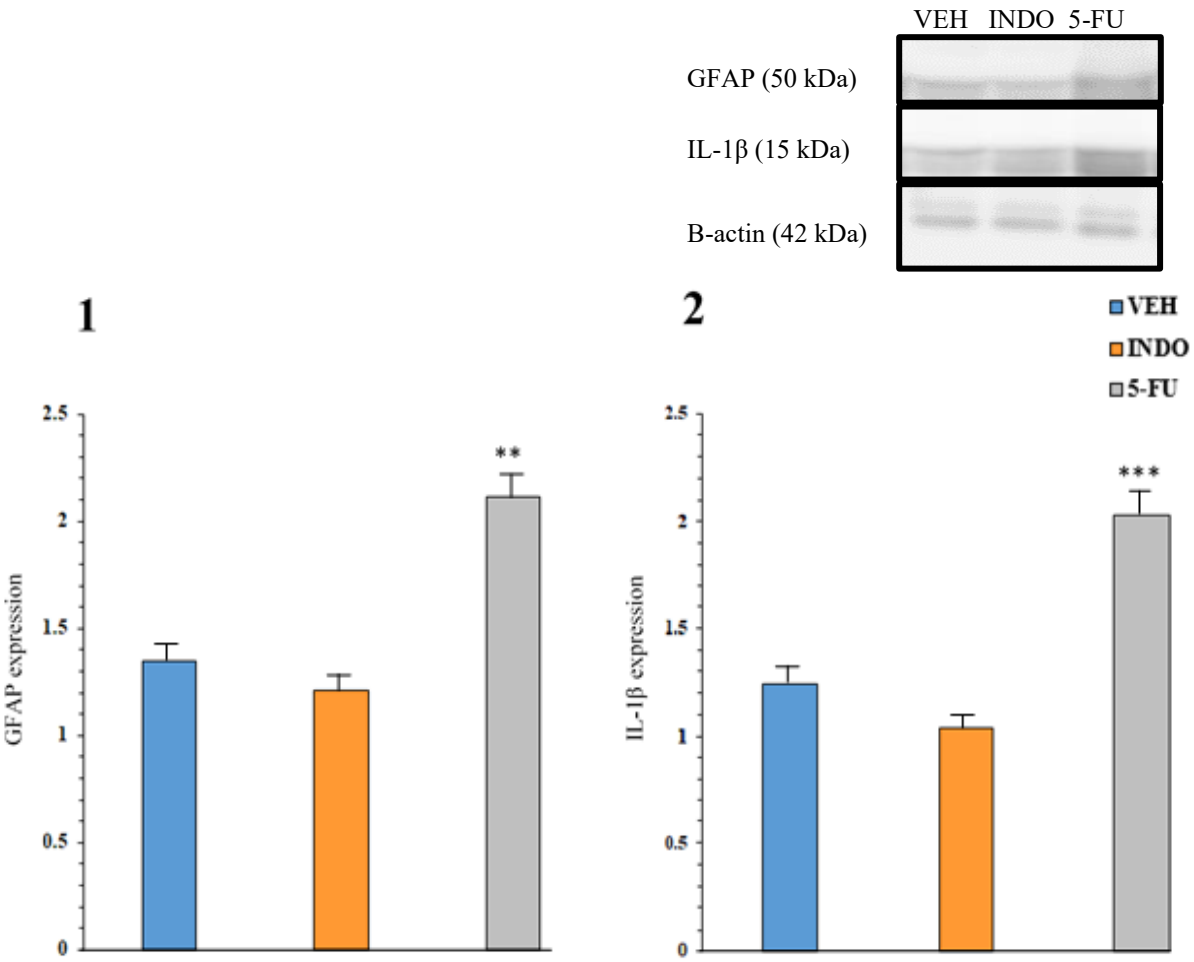

Figure 4.4A. Thoracic spinal cord GFAP (1) and IL-1β (2) expression in VEH, INDO and 5-FU treated rats, as assessed by WB. Data expressed as GFAP/IL-1β expression relative to β-actin (% change) ± SEM. ** indicates $p<0.01$ and *** indicates $p<0.001$ compared to VEH controls. Glial fibrillary acidic protein; GFAP, western blot; WB, vehicle; VEH, indomethacin; INDO, 5-Fluorouracil; 5-FU.

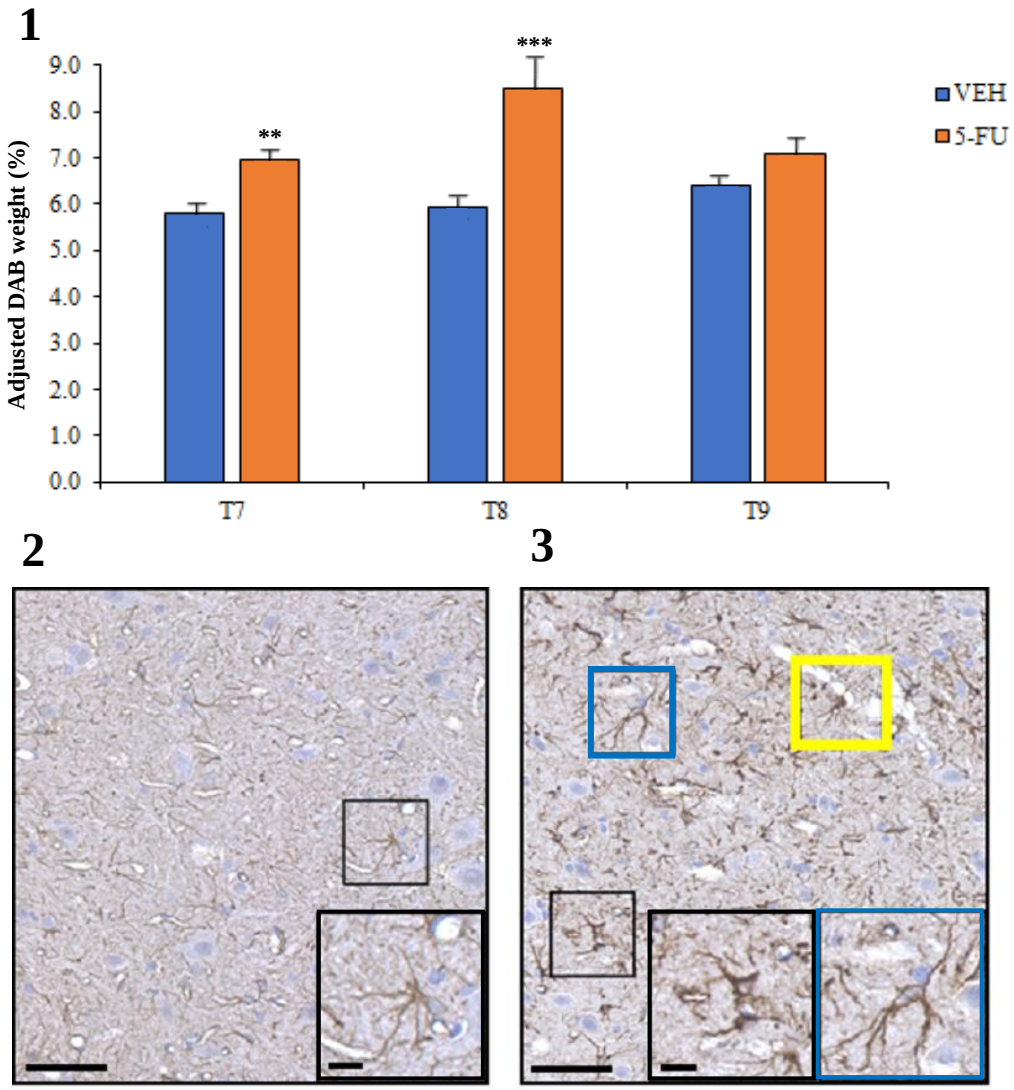

Figure 4B. GFAP immunostaining was assessed in the left dorsal column of the thoracic spinal cord (1). Data expressed as adjusted DAB weight (%) group mean ± SEM. ** indicates $p<0.01$ and *** indicates $p<0.001$, compared to VEH controls. Representative images of VEH (2) and 5-FU (3) T8 spinal cord sections (left dorsal horn). Rats with 5IGT displayed morphological changes in astrocyte phenotype indicative of astrocyte activation (C); somatic hypertrophy (yellow box), elongated (blue box) and thickened (black box) processes. Scale bars represent 50µm and 10µm for representative images and subset images, respectively. Glial fibrillary acidic protein; GFAP, 5-Fluorouracil-induced gut toxicity; 5IGT, vehicle; VEH.

5IGT Results in a Change in Microbiota Composition in the Rat Caecum

The caecal contents of rats with 5IGT resulted in significantly decreased phylogenetic alpha diversity compared to VEH and INDO rats (approximately -40% reduction; Figure 4.5A). The diversity of the 5IGT caecal contents were significantly reduced compared to the VEH group using X metric (p=0.036; Figure 4.5B). Similarly, beta diversity was also significantly different in the 5-FU treated rats than those from the VEH and INDO groups (adonis: R^2= 0.228, p=0.001; Figure 4.6). Changes in alpha and beta diversity were demarcated by significant changes in the relative abundance of different bacterial genera (Figure 4.7). *Bacteroides, Phascolarctobacterium* and *Desulfovibrio* increased in rats treated with 5-FU compared to VEH controls (Figure 4.8). Noticeable reductions were also observed in the relative abundance of commensal taxa in 5IGT compared to VEH controls, and included *Oscillospira*, *Ruminococcus*, *Clostridiales*, *S24-7*, *Prevotella* and *Lachnospiraceae* taxa. Interestingly, the commensal bacterial taxa *Oscillospira* and *S24-7* taxa were elevated in INDO treated rats (Figure 4.8).

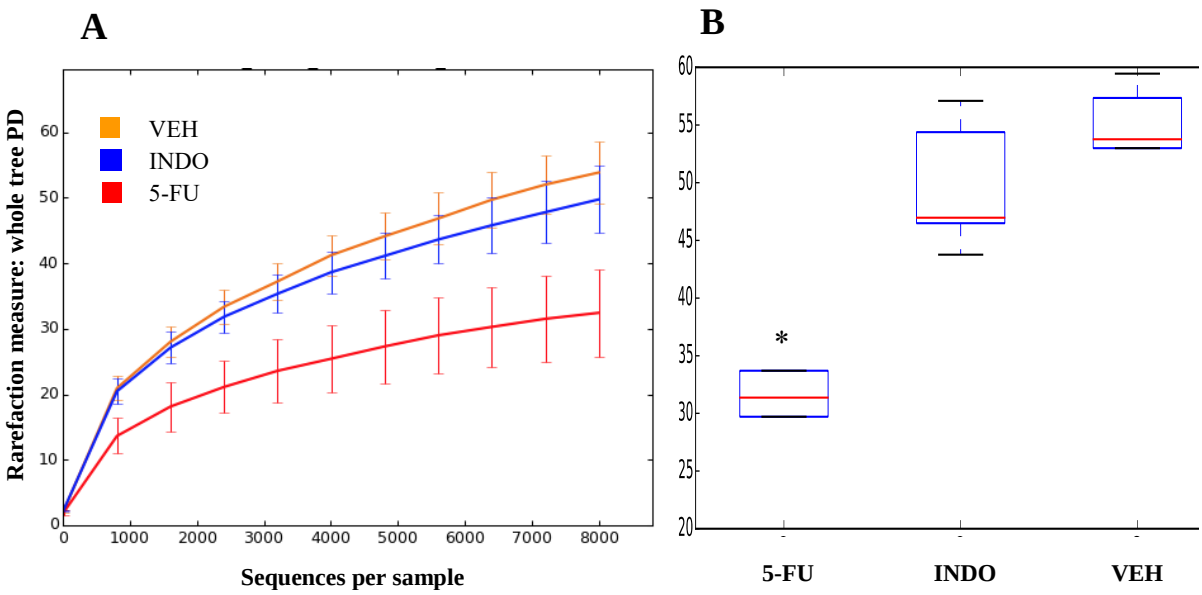

Figure 4.5. Phylogenetic alpha diversity as represented by rarefaction measure (A) and box plot (B). The caecal contents of VEH and INDO groups display more phylogenetic diversity than the 5-FU group (p<0.05). * indicates p<0.05 compared to vehicle controls. Vehicle; VEH, indomethacin; INDO, 5-Fluorouracil; 5-FU.

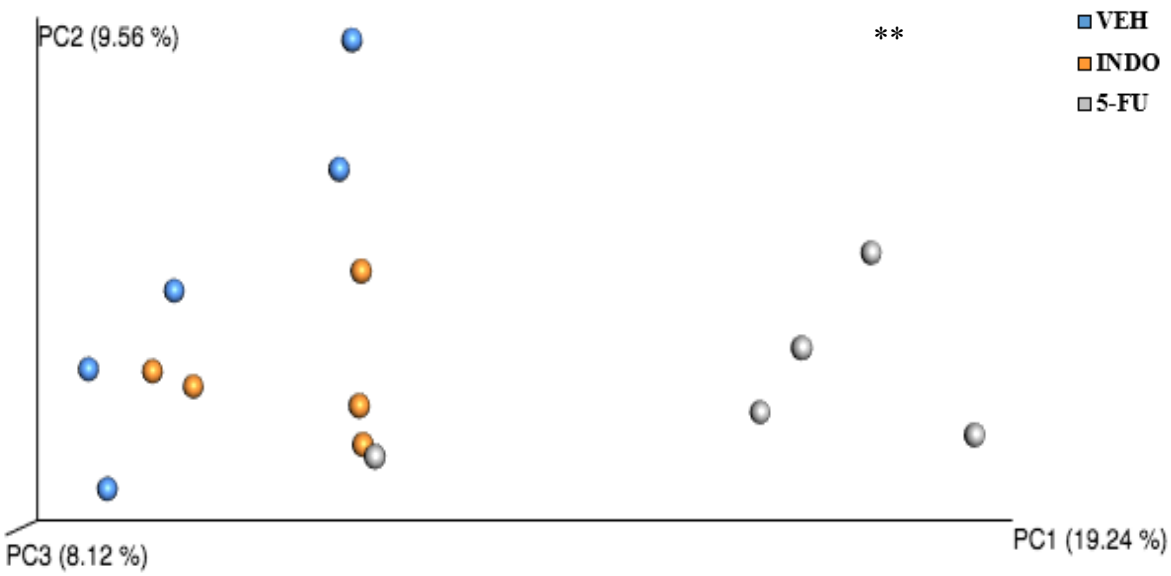

Figure 4.6. Beta diversity as represented by a principal component plot using a distance matrix based on the unweighted UniFrac metric. Caecal samples are represented by a sphere and the distance between samples identifies variance (similar or dissimilar) in the microbial communities. ** indicates $p<0.01$ compared to vehicle controls. Vehicle; VEH, indomethacin; INDO, 5-Fluorouracil; 5-FU.

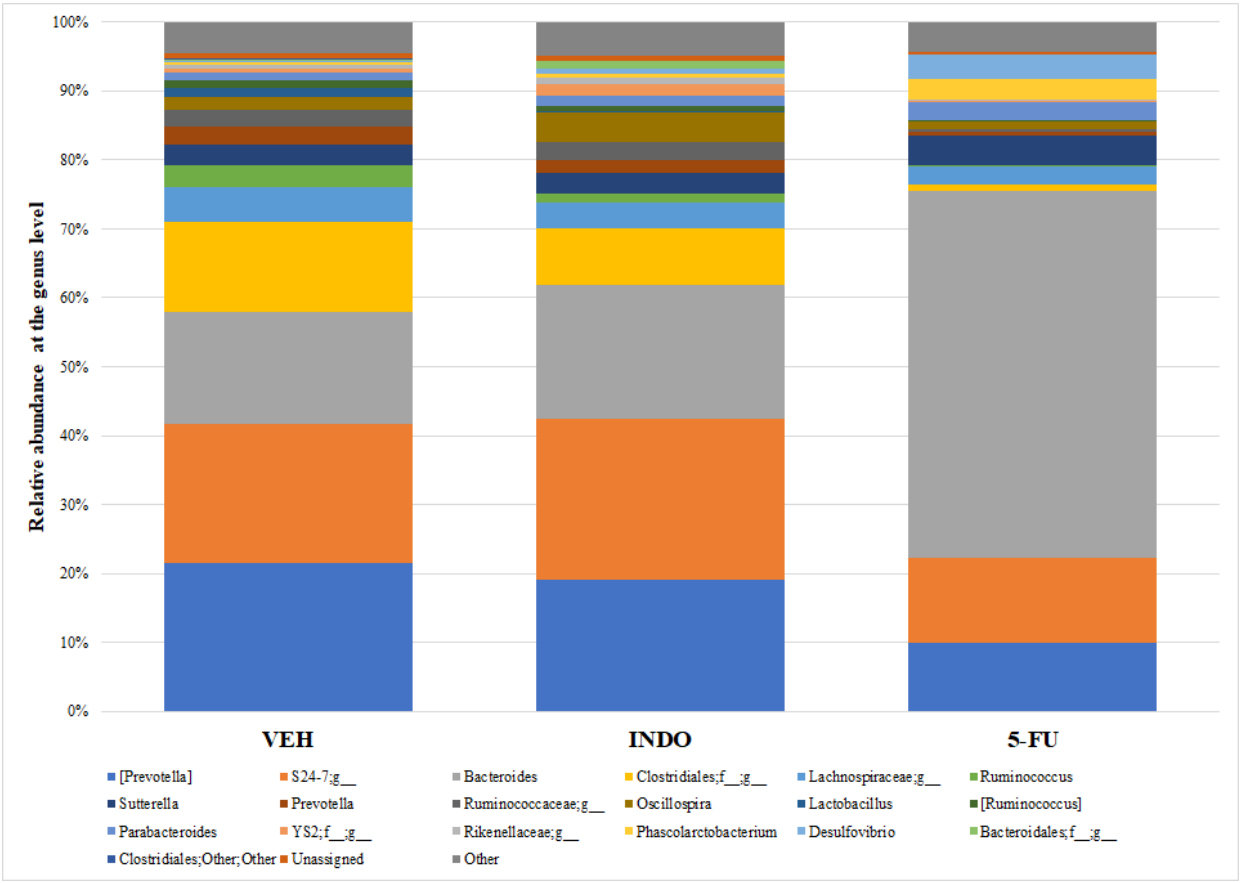

Figure 4.7. Taxa summary of the caecal samples in relative abundance at the genus level. Each colour is representative of a specific genus as identified in the legend. Vehicle; VEH, indomethacin; INDO, 5-Fluorouracil; 5-FU and phylogenetic diversity; PD. Data presented as relative abundance (%).

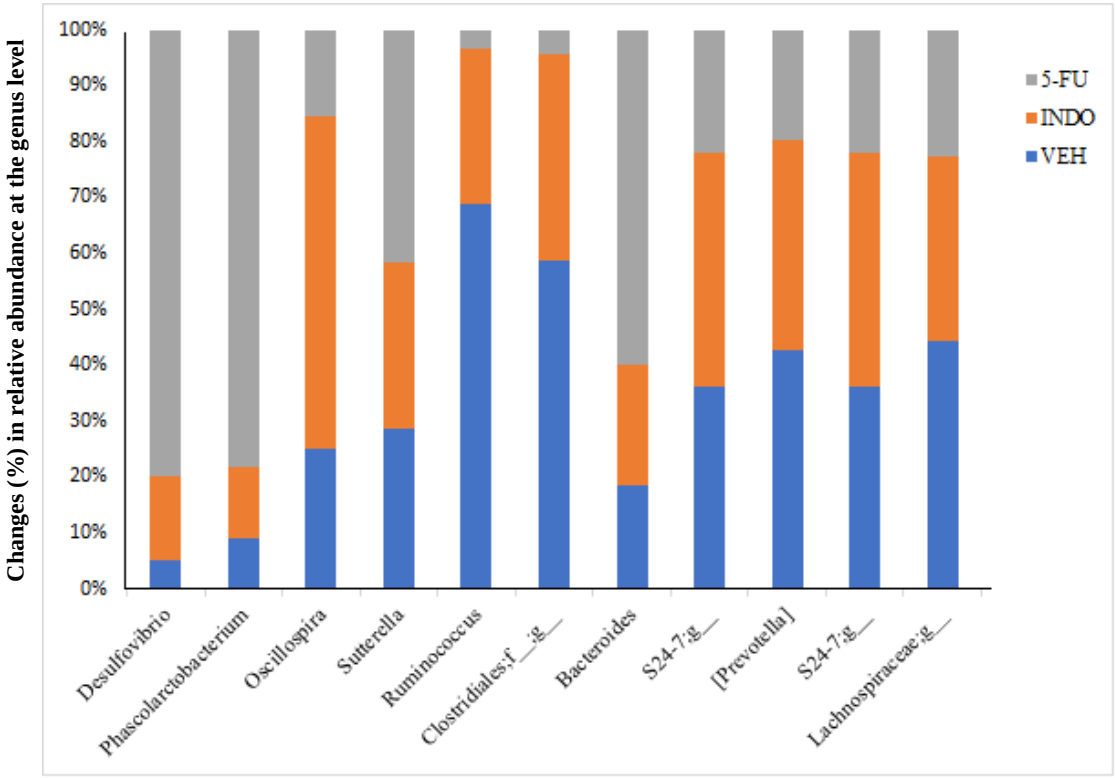

Figure 4.8. Caecal microbial changes as represented by % of relative abundance at the genus level: taxa of interest taken from Figure 4.7. Data presented as relative abundance (%) and expressed as 10^{-2}. Vehicle; VEH, indomethacin; INDO and 5-Fluorouracil; 5-FU.

Discussion

GI and cognitive disturbances associated with chemotherapy treatment remain a significant burden to cancer patients, and mechanisms are still unclear. Recent research has suggested involvement of the innate immune system, including TLRs, glial reactivity and neuroinflammation, in the aetiology of both disorders as well as pain associated with CIGT [134, 182, 189, 302, 328, 329]. The present study implicates 5-FU-induced microbiota changes in the pathogenesis of CIGT. In addition, the thoracic astrogliosis response to 5IGT suggests an indirect activation of a neural-driven immune signalling pathway.

5IGT resulted in significant weight loss, increased DAI, reduced thymus and spleen weight, severe SI injury and acute intestinal inflammation in accordance with previous studies [108, 110, 286, 330]. Whilst the bodyweight of rats with 5IGT remained significantly reduced until 72 h, weight loss in rats administered INDO only occurred on experimental days 1 and 2, yet returned to baseline at 72 h. INDO also resulted in decreased SI length and weight, decreased thymus weight and increased liver and spleen weight, as well as elevated DAI scores on day 1 and 2 in accordance with previous studies [331]. VH and CD were significantly decreased in the jejunum of both 5-FU and INDO treated rats compared to VEH controls. Upon closer examination of jejunal histological H&E staining, mild-to-moderate structural changes were evident in the mucosa of the INDO group, whereas severe mucosal injury and toxicity was evident in rats administered 5-FU. The metabolic and intestinal findings in the current study confirmed that the selected INDO dose was sufficient to cause mild-to-moderate gut toxicity when compared to the severity of intestinal damage observed in the 5-FU treated rats.

The current study sought to determine whether the chemotherapy drug, 5-FU was unique at inducing gut toxicity and spinal astrocyte activation, by comparing its effects to a lower intestinal inflammatory response, induced using a non-chemotherapy drug, INDO. In order

to achieve this, a high-dose of 5-FU and lower dose of INDO was utilised which resulted in a high versus low intestinal response, as indicated by the MPO data. Whilst some evidence for INDO-induced intestinal injury was present (e.g. days 1-2 weight loss and increased DAI; mild to moderate histological jejunal injury at 72 h), the absence of MPO changes at 72 h implied that the peak mucosal injury occurred earlier. As MPO is released by neutrophils upon intestinal injury [292] and neutrophil infiltration has been demonstrated to play a role in the primary events leading up to mucosal damage in both 5IGT and INDO-enteropathy [65, 332], it was anticipated that MPO would be elevated in both experimental groups at 72 h. The lack of INDO-MPO expression changes at 72 h may be partially explained by its differing mechanistic action to 5-FU.

NSAID-enteropathy develops following a direct intestinal insult coupled with enterohepatic and systemic effects [333]. The downstream effects result in increased mucosal permeability and activation of the inflammatory cascade, elevating pro-inflammatory cytokines, such as IL-1β [332]. Neutrophils are recruited to the ulcerated area to facilitate the recovery and healing process [332, 334]. Previously, INDO treated rats showed significant increased MPO expression in the small intestine at 24h post-injection [335]. Whilst the previous study used the same dosage as the current study, the route of administration differed (intragastric injection). 5-FU on the other hand enters the cell by mimicking the nucleic acid, uracil, and misincorporating into RNA and DNA, leading to mutations in strands and inducing cellular apoptosis [1, 336]. In the present study, the timing for the assessment of intestinal injury may have not reflected optimal alignment of the peak effects of the two models. In future, pilot studies should be employed to determine the precise characterisation of 5-FU and INDO gut toxicity, with differing dosages and time-points which can adequately assess the similarity of varying levels of intestinal inflammation in each group. Alternatively, the use of other methods,

such as ELISA, effectively determining the cytokine profile of these animals, would verify the inflammatory condition of the intestine.

As well as inducing severe GI disturbances, 5-FU (mostly in combination with other chemotherapy drugs) is commonly reported to induce acute and delayed central toxicities. Chemotherapy recipients undergoing 5-FU treatment often report trouble in several cognitive domains, involving executive function, attention and verbal memory [322, 337]. Imaging studies and experimental models have confirmed that 5-FU induces structural and cellular brain alterations, such as reductions in frontal cortex size and the restructuring of brain networks, leukoencephalopathy and delayed cerebral demyelinating syndrome [175, 177, 338]. One of the most common findings in experimental models of CICI is hippocampal cellular reductions, frequently associated with learning and memory deficits [87, 115, 116]. Considering previous short-term systemic administration of 5-FU using a lower dose than that used in the present study, resulted in apoptosis in multiple brain regions, including the hippocampus, it was surprising that the acute high-dose used resulted in no sign of inflammation or glial activation in the higher order brain regions examined at the selected time-point. Differences in dosing regimens and species strain may have accounted for some of the regional apoptosis observed in the previous models. Not all studies have reported cognitive deficits following 5-FU administration. Some models have even reported improvements in mice [95]. The complex and sometimes contradictory nature of CICI mechanisms highlight the need for continued detailed investigations.

In the CNS, the selected time-point for optimal intestinal injury assessment may have also critically missed glial reactivity and may partially explain the lack of either glial cell changes in the INDO group and microglial changes in the 5-FU group. Microglia are highly mobile, dynamic brain surveillance cells (even in their quiescent 'resting' state) [339], which typically become activated upon acute immune insults, whether the

inflammatory source is central or peripheral [340, 341]. Astrogliosis generally occurs more slowly at later stages of immune responses [121, 124, 342]. As microglia express mediators which recruit astrocytes, it is plausible that in the current study, microglial reactivity occurred at an earlier time-point and future studies would benefit from a thorough time course of 5-FU-induced glial changes. Astrocyte and IL-1β activity were up-regulated in the thoracic spinal region of rats with 5IGT, indicating that the drug indirectly induced neuroinflammation via a neurally-driven immune signalling pathway [302].

Spinal astrocytes are particularly reactive to a variety of peripheral immune insults, such as bone cancer and endometriosis and have been suggested to play a role in pain associated with these disorders [127, 303, 305, 343]. In the GIT, astrogliosis is evident in the lumbar region of mice during the chronic phase of experimental colitis, whereas microglia play an active role in the acute stage [344]. In this model of chronic colonic inflammation, hippocampal neurogenesis was also reduced and importantly this indicates that inflammatory events in the "little brain" resulted in a neuromodulatory response in the "big brain" [344]. Thus, providing mechanistic evidence supporting the hypotheses outlined in the current study. Further, enteric astrogliosis occurred in rats following administration of the chemotherapy drug, oxaliplatin [301]. From this it is plausible that enteric astrocytes may actively communicate with their spinal counterparts and contribute to this maladaptation. Astrocytes are capable of communicating with each other, propagating information over long distances through calcium ion waves [121]. The present study can only speculate reasons for the 5-FU-induced thoracic astrocyte up-regulation. Nonetheless, the spinal astrogliosis findings presented here are consistent with other chemotherapy models investigating pain associated with gut toxicity, neuropathic pain and peripheral neuropathy [133, 134, 310, 345]. Accordingly, it could be assumed that astrogliosis in the present model may have contributed to pain associated with 5IGT. Importantly, the

aforementioned studies highlight the primary role of spinal astrogliosis as opposed to microglial activation in the pathogenesis of these chemotherapy-induced disorders.

The importance of a balanced microbial environment is pivotal in host development and immunity as it assists in the maintenance of multiple aspects of central and gut homeostasis, and modulates adaptive and innate immune responses [5, 346]. Disruptions in microbial composition can be caused by various factors, including age, diet, environmental changes and drugs, such as analgesics, antibiotics and chemotherapy treatment [201, 217]. It is now widely recognised that a bidirectional relationship exists between the microbiota, CNS and gut; recognised as the microbiota-gut-brain axis [5, 17, 36]. From this, changes in microbial composition have been linked to the pathogenesis of many gut- and central-related disorders, such as inflammatory bowel and coeliac disease, Alzheimer's disease and autism spectrum disorder [14, 15, 18, 36, 166, 347, 348]. Changes in microbiota composition also play a critical role in the development of CIGT [38, 134, 208, 213, 215]. Microbiota-induced mechanisms influencing the pathogenesis of CIGT involve inflammatory and oxidative stress pathways, changes in mucous layer composition, intestinal permeability and hence, epithelial repair mechanisms and release of immune effector molecules [38]. Healthy mice transplanted with the faeces of mice with 5IGT show reduced bodyweight and colon length, which infers the direct impact of the microbiota on gut toxicity and metabolic parameters [349].

In accordance with previous studies investigating 5-FU-induced microbiome effects, the present study showed a reduction in caecal microbial diversity in 5IGT rats [214, 316]. Specifically, an increase in the abundance of gram-negative bacterial taxa were evident and has been reported previously [214, 350]. Whilst *Bacteroides* and *Desulfovibrio* generally have symbiotic relationships with the host, they also can transition into pathogenic bacterium involved in the development of chronic GI disorders and lethal infections [351,

352]. *Phascolarctobacterium* is found in relative abundance in the healthy human gut [353]. Interestingly, although the present study revealed an elevated abundance of *Phascolarctobacterium* compared to controls, reductions have been linked with colonic inflammation in patients with ulcerative colitis [354]. Importantly, this implied that elevated levels of this bacterium may be implicated in the pathogenesis of 5-FU-induced intestinal inflammation. Recent studies have highlighted gender-specific prevalence for this bacterium in metabolic syndromes [355]. From this, caution should be taken with translating the significance of changes in the relative abundance of some intestinal bacterium from experimental to clinical studies. For instance, the reductions in the relative abundance of several commensal species (*Oscillospira*, *Ruminococcus*, *Clostridiales*, *S24-7*, *Prevotella* and *Lachnospiraceae*) in the rats with 5IGT. The precise mechanistic implications of these changes require further investigation, though relative changes in some of these strains mentioned here, have been suggested to contribute to inflammatory processes [356, 357]. Interestingly, whilst a reduced abundance of *Prevotella* was present in rats with 5IGT and is also observed in lung conditions, such as asthma and chronic obstructive pulmonary disease, increased abundance is commonly reported in systemic and localised inflammatory conditions [357]. Whist the microbial changes noted here implicate 5-FU-induced alterations, it is acknowledged that these changes may result from reductions in bodyweight and food/water intake. As food/water intake was not recorded, this represents a limitation of the current study.

Mounting evidence of the roles the microbiome plays in carcinogenesis [358] and interactions on the efficacy and toxicity of anti-cancer treatments [359], coupled with the literature presented here, highlight the diverse and complex nature of the microbiome-gut-CNS relationships. Recent findings supporting the concept of the intestinal microbiota as a key regulator priming neuroinflammation in response to brain injury [21], aligns with the

concepts presented in the present study. Further, the emerging roles of probiotics [316, 360] and faecal microbiota therapy [361] in treating not only gastrointestinal, but central disorders also shows promise for alleviating symptoms associated with CIGT and CICI.

Until recently, the GI and central toxicities associated with chemotherapy treatment have been studied independently. Importantly, the present study provides evidence of an indirectly (neurally)-mediated central neuroinflammatory response in rats with 5IGT; that inflammation in the “little brain” results in intestinal microbiota changes and “big brain” inflammation, as indicated by spinal thoracic GFAP and IL-1β up-regulation. In conclusion, given the ubiquitous involvement of the microbiota, innate immunity, signalling and glia in central and gut homeostasis, glia and the microbiota may be uniquely positioned to influence both CIGT and CICI.

CHAPTER FIVE, *in vivo*

Comparing and Characterising Glial Consequences in the Brain and Spinal Cord of Experimentally-Induced Chronic Ulcerative Colitis and Colorectal Cancer

Statement of context

This manuscript has been submitted to the *European Journal of Cancer*.

Bajic JE, Howarth GS, Mashtoub S, Chartier LC, Bobrovskaya L and Hutchinson MR.

Chapter Five examines neuroimmunological responses in the brain and spinal cord of mice with chronic UC and CA-CRC. As I identified that thoracic spinal GFAP expression changes occurred in rats with 5IGT, it was time to determine whether spinal glial cells were reactive to chronic inflammatory conditions of the gut. Importantly, these studies included no chemotherapy treatment and included the addition of a malignancy in the CA-CRC group. This study aimed to confirm whether chronic conditions of the gut were able to induce glial cell reactivity in higher order brain regions, as this did not occur in the acute-chemotherapy models used in the preceding chapters. Due to unforeseen circumstances, I was unable to collect caecal contents to examine the microbiota changes in these groups.

Statement of authorship

Statement of Authorship

Title of Paper	**Comparing and characterising glial consequences in the brain and spinal cord of experimentally-induced chronic ulcerative colitis and colorectal cancer**
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Juliana Esma Balic, Gordon Stanley Howarth, Suzanne Mashtoub, Lauren Claire Chartier, Larisa Bobrovskaya & Mark Rowland Hutchinson

Principal Author

Name of Principal Author (Candidate)	Juliana Bajic		
Contribution to the Paper	I was responsible for the compilation of all bodyweight and MPO data, assisted with animal experimental work, performed all western blot experimental work, reviewed all papers cited in manuscript, performed statistical analyses and interpreted data, designed figures and acted as corresponding author.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11.04.19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);
II. permission is granted for the candidate in include the publication in the thesis; and
III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon Howarth		
Contribution to the Paper	Reviewed manuscript.		
Signature		Date	12/04/19
Name of Co-Author	Suzanne Mashtoub		
Contribution to the Paper	Performed animal experimentation and reviewed manuscript.		

Signature		Date	08.04.19

Name of Co-Author	Lauren Chartier		
Contribution to the Paper	Performed animal experimentation.		
Signature		Date	08.04.19

Name of Co-Author	Larisa Bobrovskaya		
Contribution to the Paper	Assisted with equipment use for western blots.		
Signature		Date	09.04.19

Name of Co-Author	Mark Hutchinson		
Contribution to the Paper	Reviewed manuscript.		
Signature		Date	11.04.19

Abstract

The central comorbidities associated with chronic inflammatory disorders of the colon remain under-investigated. Chronic ulcerative colitis (UC) patients are at higher risk of developing colitis-associated colorectal cancer (CA-CRC). We investigated the role of the neuroimmune system (glial cell reactivity and neuroinflammation) and peripheral-to-central immune pathways mediating the pathogenesis of central comorbidities associated with CA-CRC. Therefore, we sought to determine whether experimentally-induced chronic UC and CA-CRC resulted in regional higher order brain region and spinal cord changes in mice using glial cell markers in western blot: ionised calcium binding adaptor molecule 1 (Iba-1) for microglia; and glial fibrillary acidic protein (GFAP) for astrocytes. Chronic UC was induced via dextran sulphate sodium (DSS) water cycling and azoxymethane (AOM)/DSS was used to induce CA-CRC. GFAP and Iba-1 expression was up-regulated in the hippocampus of both treatment groups ($p<0.05$). Hypothalamic GFAP expression was also elevated in the CA-CRC group when compared to saline controls ($p<0.01$). In the lumbar spinal region, GFAP expression was downregulated in the chronic UC group ($p<0.05$) in contrast to GFAP up-regulation in the CA-CRC group when compared to controls ($p<0.01$). Iba-1 expression in the lumbar region was increased in both treatment groups compared to saline controls ($p<0.0001$). Our data implicates glial dysregulation in the pathogenesis of chronic UC and CA-CRC and suggests these changes occur via neural- and humoral-mediated peripheral-to-central immune signalling pathways.

Background

The incidence and reported prevalence rates of inflammatory bowel disease (IBD) and colorectal cancer (CRC) have been increasing each year [362, 363]. Ulcerative colitis (UC) and Crohn's disease are the two major forms of IBD and both prominently feature chronic intestinal inflammation. The risk of developing CRC is higher in patients with IBD and approximately twelve percent of patients develop colitis-associated CRC (CA-CRC) in ten years following IBD symptom onset [364]. During episodes, histopathological manifestations include ulceration, mucosal damage, breakdown of the intestinal epithelial lining and consequently, a marked increase of infiltrating cells from the innate and adaptive immune systems into the lamina propria [365]. Local invasion of these immune cells results in the synthesis and release of a battery of pro- and anti-inflammatory cytokines and mediators, such as interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α.

Genetic and environmental factors are key players in IBD development, yet accumulating evidence has indicated the critical role the unbalanced or dysfunctional gut microbiota plays in intestinal barrier function [366, 367]. Classical IBD symptoms include increased or decreased stool consistency, abdominal pain (visceral hypersensitivity), cramping and in severe cases, malnutrition and the presence of blood and mucus in stools [368]. Extraintestinal manifestations of IBD may include fever, weight loss, delayed growth and arthritis [369]. Other common but less reported comorbidities associated with IBD are neurological, such as seizure disorders and peripheral neuropathy [370]. More recently however, behavioural and cognitive alterations have been associated with IBD, although these comorbidities are often overlooked and poorly understood with little to no treatment options available [371, 372]. Depression, anxiety, fatigue, low self-esteem and cognitive impairment, particularly in the executive function domain affect a significant proportion of

IBD patients and these complications, combined with managing clinical symptoms, puts enormous strain on the patients' occupational, financial, social and relationship well-being [373]. In addition, it is common for IBD patients to present to doctors with a recent stressful event and accordingly, it is thought that stress may exacerbate IBD symptoms or episodes [40]. Suggested mechanisms in the development of central disorders stemming from IBD include malabsorption, metabolic agents, infections arising from immunosuppressive complications, side-effects of medication and immunological abnormalities [370]. More recently however, a growing body of literature suggests that immune dysregulation associated with various chronic disease states, including cancer and peripheral inflammatory disorders results in an innate central-immune response which can modify behaviours. Whilst chronic UC and CA-CRC have been associated with central comorbidities [49, 59], the potential mechanisms remain under investigated.

Neuroinflammatory processes initiate as a host defence mechanism, resolving insults by returning normal structure and function to injured tissue [374]. The resultant neuroinflammatory microenvironment may become neurotoxic, unregulated and self-propagate with deleterious effects contributing to the pathogenesis of various neurodegenerative diseases, such as Alzheimer's disease, multiple sclerosis and neuropathic pain [267, 319, 375]. Neuroimmunological cells (glia), particularly microglia and astrocytes, are not only responsible for maintaining neuronal and central nervous system (CNS) homeostasis in their resting/quiescent state, but also initiate neuroinflammatory responses to a range of central (direct; e.g. traumatic brain injury) and peripheral (indirect; e.g. bone cancer) insults [127, 153, 374]. Due to the close bidirectional relationship glia share with neurons at the synapse (tetrapartite synapse) they have the potential to influence brain function, modify behaviours and contribute to neuropathic pain [122]. Glia may become primed via peripheral-to-central immune pathways (humoral or

neural immune signalling) whereby circulating pro-inflammatory cytokines either directly or indirectly access the brain [82, 144]. Glial priming and chronic neuroinflammation have been associated with cognitive impairment following systemic exposure to noxious, infectious or even chemical exposure, such as chemotherapy [182, 277]. Importantly, chronic intestinal inflammation induces microglial activation in the hippocampus and spinal cord which has been attributed to visceral hypersensitivity [376, 377].

The current study aimed to characterise neuroimmunological complications arising from chronic intestinal inflammatory conditions, focussing on chronic UC and CA-CRC, *in vivo*. We sought to identify whether neuroimmune modifications (glial activation) occurred in the lumbar spinal cord region as a result of the inflamed/malignant colon, and if changes occurred in higher order brain regions of mice with experimentally-induced chronic UC and CA-CRC. To date, the potential role glial dysregulation plays in the central comorbidities of both disorders remain elusive. In the present study we examined microglia and astrocyte markers (ionized calcium binding adaptor molecule 1, Iba-1; and glial fibrillary acidic protein, GFAP; respectively) in mouse models of dextran-sulphate sodium (DSS)-induced chronic UC and CA-CRC utilising azoxymethane (AOM)/DSS.

Material and Methods

Female C57BL/6 mice (8 wks) were group-housed (10 per cage) in a temperature-controlled room with a 12 h light-dark cycle. All animals were sourced from the Animal Resource Centre (Perth, Western Australia) and were acclimatised for one week before the trial commencement. All animal studies complied with the Australian National Health and Medical Research Council Code of Practice for Animal Care in Research and Teaching (8^{th} Edition, 2013) and were approved by the Children, Youth and Women's Health Service and The University of Adelaide Animal Ethics Committees. The animals used in this study formed the controls for larger studies examining naturally sourced treatments in the treatment of chronic UC and CA-CRC [378, 379]. Mice (n=9-12) were randomly allocated into either control (saline), chronic UC (DSS) or CA-CRC (AOM/DSS) groups. Chronic UC was induced via *ad libitum* drinking water (2% w/v DSS dissolved in water bottles) every three weeks for a period of seven weeks (Figure 5.1). During weeks one, three and six, DSS bottles replaced standard water drinking bottles in the chronic UC group. CA-CRC mice followed the same DSS water cycling schedule, however at day zero an AOM injection was administered (7.4mg/kg bodyweight; Sigma-Aldrich, Castle Hill NSW). The CA-CRC groups had an additional 12 days with standard water to allow for tumour development following the third cycle of DSS water before euthanasia (Figure 5.1). Chronic UC mice were euthanised on day 51 and CA-CRC mice on day 63 via CO_2 asphyxiation. Metabolic data, including indicators of disease activity and intestinal measures from 192 animals in 24 groups have been previously published and have been reported here, as these data were collected at the same time and served as the key controls across multiple studies [378, 379]. UC and CA-CRC was determined by clinical and histological means already published [378, 379]. Diseased animals showed reductions in bodyweight, increased DAI and crypt depth in the colon. Colitis progression was monitored

by colonoscopy at regular intervals and colonic tumour size and numbers were recorded. The brain and spinal cord of each animal were snap frozen for future molecular analysis.

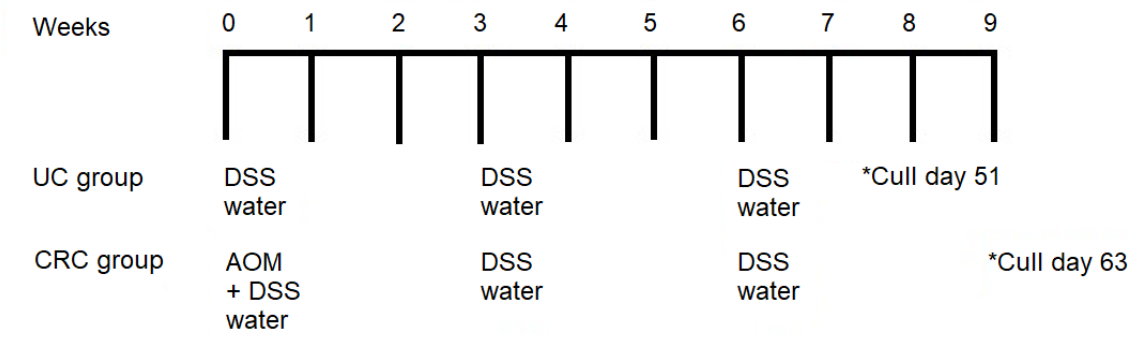

Figure 5.1. Timeline of mice with experimentally-induced chronic UC and CA-CRC. UC was induced via ad libitum drinking water containing DSS (2%) during weeks 1, 3 and 6. UC mice were euthanised 2 days after the third DSS cycle at day 51. The CA-CRC group followed the same DSS cycles except this group received a single AOM injection at day 0 and were euthanised on day 63. UC; ulcerative colitis, CA-CRC; colitis-associated colorectal cancer, DSS; dextran-sulphate sodium, AOM; azoxymethane.

Western Blot Analysis

Following thawing, mice brains were isolated into hippocampal, pre-frontal cortex and hypothalamus sections whilst the spinal cords were sectioned into cervical (C2-C5), thoracic (T7-T9) and lumbar (L3-L6) regions. CNS sections were homogenised in cell lysis buffer (Tris-base saline; pH 8.0; 50mM, 1% Triton-x100, 1% protease inhibitor cocktail; Sigma Aldrich, #P8340); 300µl (spinal cord, pre-frontal cortex and hypothalamus) and 500µl (hippocampus) aliquots. Samples were sonicated (10 sec bursts, followed by a minimum of 1 min on ice) and centrifuged at 15,000 x g for 40 min. Protein was determined using BCA-Protein Assay (Pierce® BCA Protein Assay Kit; #23225, Thermo Fisher Scientific Inc., Victoria, Australia). Homogenised proteins of known concentrations were suspended in 2X sample buffer (SDS reducing sample buffer: dH_2O, 0.5M Tris-HCl pH 6.8, glycerol, 10% SDS solution and 0.5% bromophenol blue) and heated at 70˚C for 10 min. Protein

samples (25µg) were loaded and separated by gel electrophoresis in acrylamide homemade gels (SDS page 8-10%; 40% Acrylamide/Bis, gel Buffer (Resolving gel: 1.5M Tris-HCl pH 8.8 & Stacking gel: 0.5M Tris-HCl pH 6.8); 10% w/v SDS; 10% ammonium persulfate (APS); TEMED). Protein transfer occurred onto nitrocellulose filter paper (Sigma-Aldrich) using boric acid pH 8.9 (boric acid, EDTA for 2 h @ 600mA) transfer buffer. Membranes were blocked with 5% skim milk at room temperature for 1.5 h. Protein expression utilised antibodies for GFAP (1:2000 Abcam; #Ab7620, Cambridge, UK) and Iba-1 (1:1000 WAKO; #019-19741, Osaka, Japan); indicators of astrocyte and microglial activation, respectively, with an overnight incubation (4˚C). The total protein content standardised sample loading and was used as the relative control. An additional internal sample control (2µl from each sample) determined changes between gel integrity and was used to normalise band detection. Secondary antibodies allowed visualisation of the protein bands (peroxidase-conjugated Affinipure Donkey Anti-rabbit IgG; 1:20,000; #711-035-152; Jackson ImmunoResearch Laboratories, Inc., PA, USA). Enhanced chemiluminescence (ECL) enabled detection of bands using an in-house ECL reagent (10mL of 100mM Tris HCl; pH 8.5, 22µL of 90mM coumaric acid, 50µL of 250mM luminol and 3µL of H_20_2) and bands were visualised using the ImageQuant 4100 imaging system (GE Health Care, United Kingdom) described previously [289]. Band densities were measured as a fold change relative to control group, accounting for normalisation as described earlier.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 6.02 for Windows (GraphPad Software Inc., San Diego, CA, USA). Data were tested for normality using the Shapiro-Wilk analysis. All western blot data were analysed using unpaired t-tests. All data were expressed as mean ± SEM with values of $p<0.05$ deemed significant.

Results

Chronic UC and CA-CRC Disrupts Glial Marker Expression in the Hippocampus and Hypothalamus

Hippocampal GFAP expression was up-regulated in both chronic UC and CA-CRC groups when compared to vehicle controls (p=0.0275 and p=0.0002, respectively; Figure 5.2A and B). Iba-1 expression was also elevated in both chronic UC and CA-CRC groups (p=0.0016 and p=0.0007, respectively; Figure 5.3A and B). GFAP expression was significantly increased in the hypothalamus of mice with CA-CRC (p=0.004; Figure 5.4). Although hypothalamic Iba-1 expression resulted in no significant changes, the range of Iba-1 expression in this group was considerably modified in the CA-CRC group when compared to the data range of Iba-1 expression in the vehicle controls (Figure 5.4). No significant changes were evident in the pre-frontal cortex in either disease group (p>0.05; data not shown).

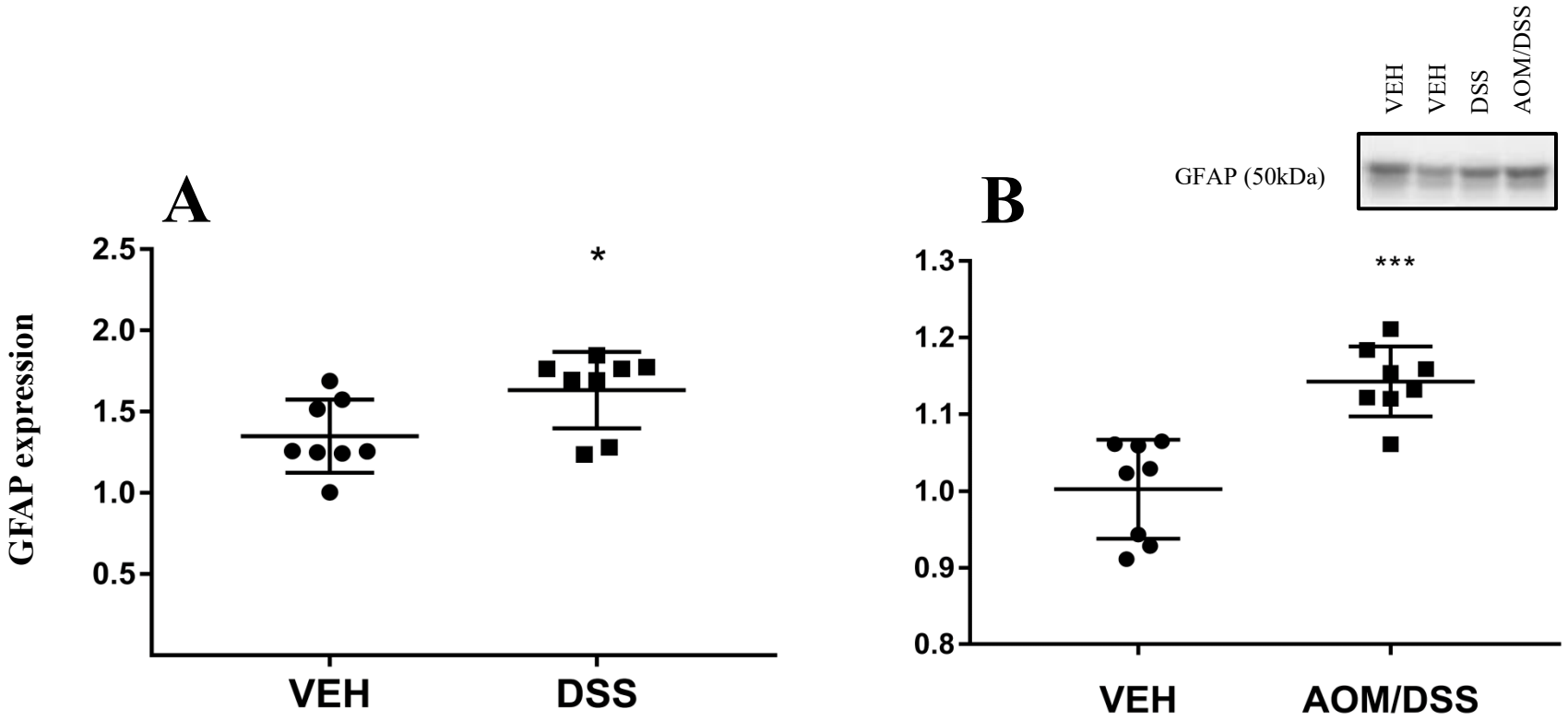

Figure 5.2A and B. Effects of DSS and AOM/DSS on hippocampal GFAP expression in mice with experimentally-induced chronic UC (A) and CA-CRC (B). GFAP expression is represented as a percentage of the total protein content. Both chronic UC and CRC groups resulted in a significant increase in hippocampal GFAP expression when compared to VEH control groups. *p<0.05, ***p<0.001 when compared to VEH controls. VEH; vehicle treated mice, DSS; dextran-sulphate sodium, AOM; azoxymethane, GFAP; glial fibrillary acidic protein, UC; ulcerative colitis, CA-CRC; colitis-associated colorectal cancer.

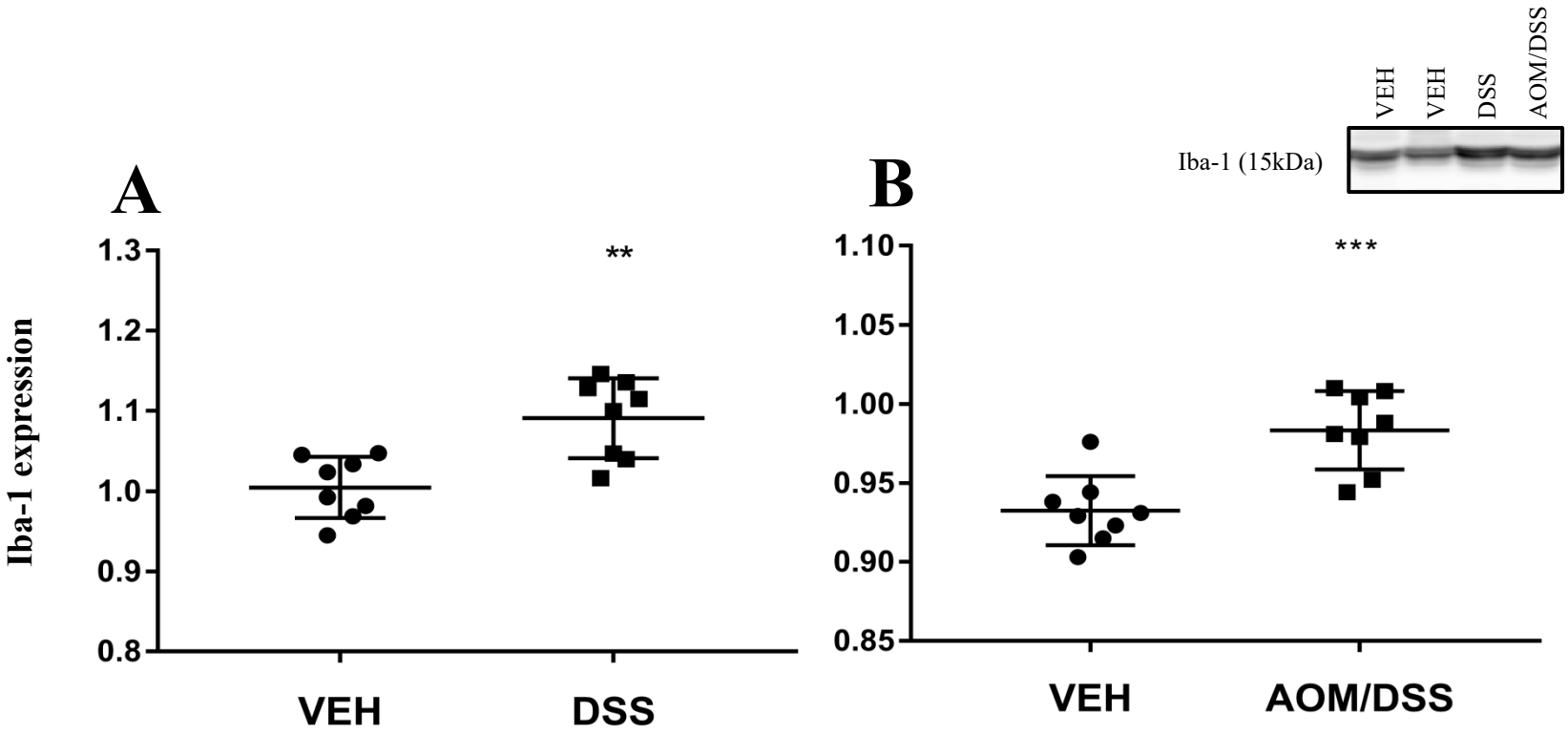

Figure 5.3A and B. Effects of DSS and AOM/DSS on hippocampal Iba-1 expression in mice with experimentally-induced chronic UC (A) and CA-CRC (B). Iba-1 expression is represented as a percentage of the total protein content. Both chronic UC and CRC resulted in significant increases in hippocampal Iba-1 expression when compared to VEH control groups. $^{**}p<0.01$, $^{***}p<0.001$ when compared to VEH controls. VEH; vehicle treated mice, DSS; dextran sulphate sodium, AOM; azoxymethane, IBA-1; ionized calcium binding adaptor molecule 1, UC; ulcerative colitis, CA-CRC; colitis-associated colorectal cancer.

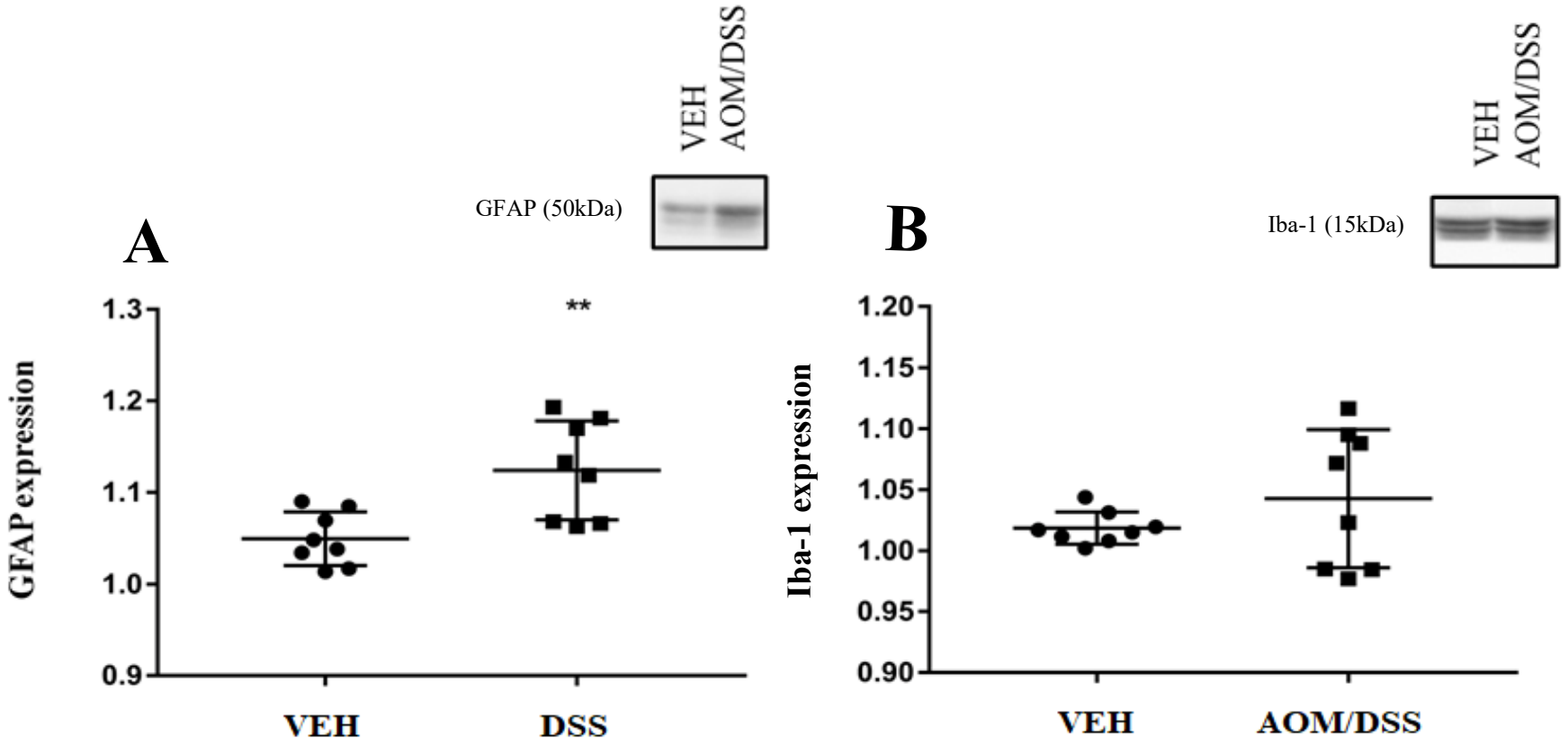

Figure 5.4A and B. Effects of AOM/DSS on hypothalamic GFAP (A) and Iba-1 (B) expression in mice with experimentally-induced CA-CRC. GFAP and Iba-1 expressions are represented as a percentage of the total protein content. AOM/DSS resulted in a significant increase in hypothalamic GFAP expression when compared to VEH control groups. Iba-1 expression was disrupted by AOM/DSS when compared to the VEH control group. $^{**}p<0.01$ when compared to vehicle controls. VEH; vehicle treated mice, DSS; dextran sulphate sodium, AOM; azoxymethane, Iba-1; ionized calcium binding adaptor molecule 1, GFAP; glial fibrillary acidic protein, CA-CRC; colitis-associated colorectal cancer.

Glial Marker Expression Changes in the Lumbar Region of Mice with Chronic UC and CA-CRC

GFAP expression was significantly reduced in the lumbar spinal region of mice with chronic UC when compared to vehicle controls (p=0.0293; Figure 5.5A). However, GFAP expression was increased in the lumbar region of CA-CRC mice when compared to vehicle controls (p=0.0014; Figure 5.5B). Both chronic UC and CA-CRC mice showed elevations in Iba-1 expression in the lumbar region when compared to vehicle controls (both groups p<0.0001; Figure 5.6A and B). Cervical and thoracic spinal cord regions yielded no significant changes in the expression of either glial marker in each disease group (p>0.05; data not shown).

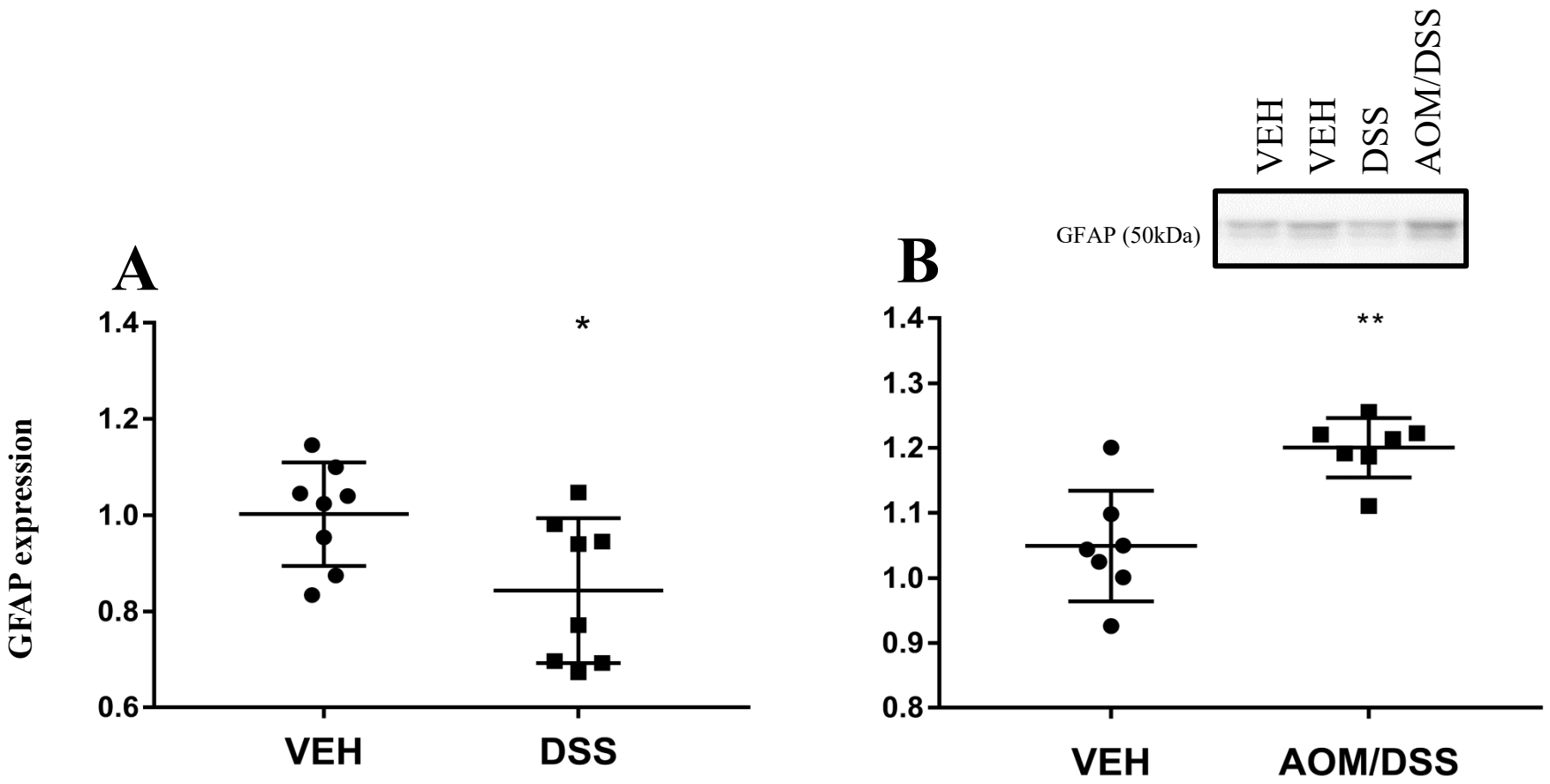

Figure 5.5A and B. Effects of DSS and AOM/DSS on lumbar GFAP expression in mice with experimentally-induced chronic UC (A) and CA-CRC (B). GFAP expression is represented as a percentage of the total protein content. Chronic UC caused a significant reduction in GFAP expression in the lumbar region, compared with CA-CRC mice which resulted in significant increase in lumbar GFAP expression when compared to VEH control groups. *p<0.05, **p<0.01 when compared to VEH controls. VEH; vehicle treated mice, DSS; dextran sulphate sodium, AOM; azoxymethane, GFAP; glial fibrillary acidic protein, UC; ulcerative colitis, CA-CRC; colitis-associated colorectal cancer.

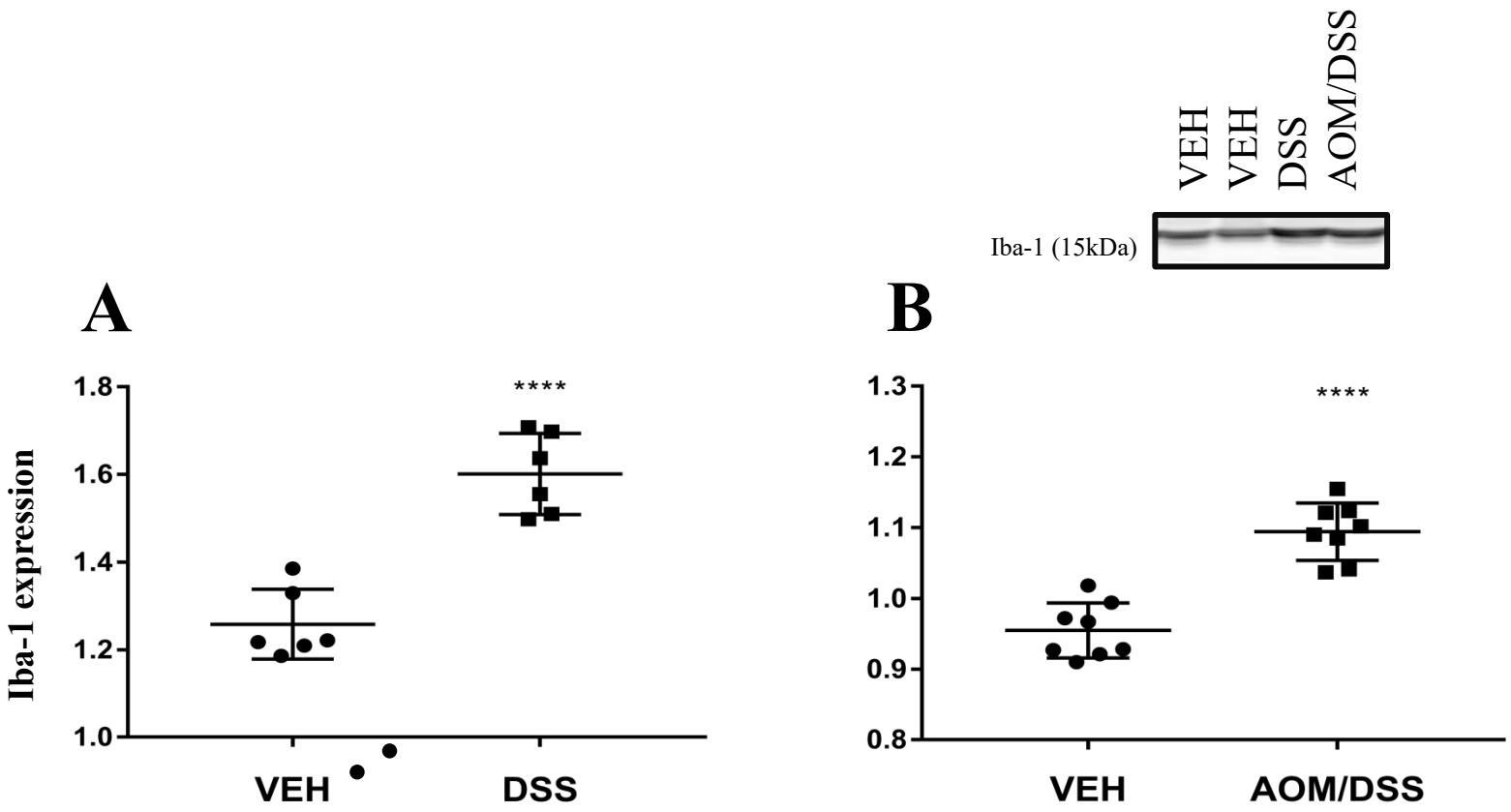

Figure 5.6A and B. Effects of DSS and AOM/DSS on lumbar Iba-1 expression in mice with experimentally-induced chronic UC (A) and CRC (B). Iba-1 expression is represented as a percentage of the total protein content. Chronic UC caused a significant increase in Iba-1 expression in the lumbar region, compared with the CRC group which resulted in substantial increase in lumbar Iba-1 expression when compared to vehicle control groups. ****p<0.0001 when compared to vehicle controls. VEH; vehicle treated mice, DSS; dextran sulphate sodium, AOM; azoxymethane, Iba-1; ionized calcium binding adaptor molecule 1, UC; ulcerative colitis, CA-CRC; colitis-associated colorectal cancer.

Discussion

The central comorbidities associated with chronic inflammatory disorders of the bowel remain under-investigated. This is surprising for two reasons: firstly, the central comorbidities associated with IBD place enormous strain on many aspects of patient quality of life. The emotional toll central comorbidities place on IBD patients affects the individual's self-value and confidence, thought processing, social functioning and relationship capabilities, which in turn has negative effects on education, employment opportunities and often results in financial burden [49]. Secondly, the gut and the brain are intimately connected via a multitude of pathways and mechanisms which opens many doorways for experimental investigation. The findings from our study add to the growing literature on the neuroimmunological manifestations associated with chronic gut inflammatory disorders. Importantly, we have described neuroimmunological changes in the lumbar spinal region and higher order brain regions of mice with experimentally-induced chronic UC and CRC. Elevated GFAP and Iba-1 expression indicated astrocyte and microglial reactivity, respectively, which are prominent features of neuroinflammation [118, 120, 121]. We anticipated this central response in our models, as preliminary data by our group has shown glial activation and neuroinflammation in the thoracic region of rats with 5-FU-induced gut toxicity in an acute setting [263]. Primarily, the current study determined the presence of regional neuroimmunological responses in experimentally-induced chronic UC and CRC, and also that spinal cord glial changes were representative of the inflamed/malignant gut section which innervated the spinal cord, e.g. inflamed colon innervated the lumbar spinal region. Importantly, we identified that higher order brain regions may also be susceptible to neuroinflammation under experimentally-induced chronic UC or CRC conditions, as determined by hippocampal increases in GFAP and Iba-1 expression. Previously, we reported no changes in higher order brain regions utilising

markers for microglia and astrocytes in rats with 5-FU-induced gut toxicity, and we suggest that this may have resulted from the acute setting used in this model [263].

In the current study, both chronic UC and CRC resulted in a stark increase in hippocampal GFAP and Iba-1 expression. Furthermore, increased hypothalamic GFAP expression was present in the CRC group when compared to the control group, yet this was not evident in the chronic UC group. Hypothalamic changes were anticipated in response to the generally recognised role of stress and hypothalamic-pituitary-adrenal axis in the pathogenesis of IBD [13, 380]. Whilst hypothalamic Iba-1 expression in the CRC group in the current study was not statistically different to the control group, the distribution of individual Iba-1 scores when compared to the control group suggested that CRC induced dysregulation of Iba-1 expression in the hypothalamus. Nonetheless, as we were unable to collect histological data in our study, we were unable to determine morphological changes in the treatment groups.

Irrespective of this, the hippocampal findings in our study raises three important points/questions. Firstly, are immune messages from the inflamed colon directly accessing the brain via a humorally-mediated peripheral-to-central immune communication pathway? Although the humoral route is slower than the neural route, it enables passage of cytokines and pro-inflammatory mediators from the periphery to enter the brain via circumventricular organs [144, 268]. In this capacity, glial cells may have been directly activated by peripheral immune messages accessing the brain at circumventricular organs. Or were the intestinal immune signals indirectly altered and transduced by primary afferent neurons? In this sense, highly reactive spinal glia could have stimulated neurogenic inflammatory pathways and promoted peripheral inflammatory processes which could have conducted the immune messages to higher order brain regions, such as the hippocampus. In attempt to answer these questions, we examined all spinal cord regions

and reported no significant changes in GFAP or Iba-1 expression in the thoracic or cervical regions of mice with chronic UC or CRC. This suggested that the hippocampal GFAP and Iba-1 expression changes occurred via humorally-mediated mechanisms. Further investigations are warranted to clarify this finding, in particular performing a vagatomy on mice under the same experimentally-induced UC and CRC conditions would validate this finding if the same hippocampal findings were reported.

Neuroinflammation is a widely accepted central immune response classically characterised by glial activation and subsequent inflammatory cytokine profile changes; reduced anti-inflammatory cytokine output; and increased release of pro-inflammatory cytokines [125]. Neuroinflammation contributes to the pathogenesis of various central disease states, involving neurodegeneration and pain syndromes, such as in the case of Alzheimer's disease and neuropathic pain, as well as in the periphery, for example in bone cancer-induced pain [127, 129, 319]. This innate immune response may be driven by lipopolysaccharide (LPS; the active fragment of endotoxin from Gram negative bacteria) and cytokines, such as IL-1β, which can be detected by the specific immunosurveillance Toll-like receptor (TLR), TLR-4 [381, 382]. The physiological and behavioural responses triggered by LPS and exogenous cytokines are referred to as sickness behaviours which include, but are not exclusive to, fever, malaise, social withdrawal and cognitive changes [80, 382, 383]. Interestingly, cytokine-induced sickness behaviours mimic the symptom cluster experienced by cancer and chemotherapy recipients [62].

TLRs belong to a transmembrane protein family responsible for recognising common structural patterns (for example, pathogen associated molecular patterns) and a diverse range of molecules (pro-inflammatory cytokines) which mediate appropriate innate immune responses to assist with host survival [381, 384]. At the mucosal level, TLR signalling plays an integral part in intestinal integrity and in inflammatory processes. More

specifically, TLR-4 activation in enterocytes promotes intestinal injury and inhibits intestinal repair [385]. Recently it has been shown that TLR-4 plays an important role in mediating chemotherapy-induced gut toxicity and pain [134]. As well as being key players in intestinal inflammation, TLRs mediate astrocyte and microglial cell activation displaying exaggerated expression levels and initiate neuroinflammation via the activation of signalling cascades, such as NF-κB and My-D88 [386]. Expanding our understanding of the multiple organ sites of inflammation in gut inflammatory disorders may lead to the exploration and discovery of treatment approaches that target inflammation in both the gut and the brain. It has already been proposed that TLR-4 mediates pain associated with chemotherapy-induced gut toxicity [387] and evidence has indeed confirmed the validity of this hypothesis [134]. This evidence combined with the findings from our study suggest that further investigations are warranted into the role of TLR, as it may mediate intestinal inflammation and neuroinflammation in experimentally-induced chronic UC and CRC.

One study has attempted to elucidate our understanding of the simultaneous and regional inflammatory mechanisms at play in a rat model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis utilising positron emission tomography (PET) imaging [388]. In this study, a tracer ([^{11}C]PBR28) was used to target a translocator protein which becomes up-regulated when microglia and macrophages are activated. Although the PET imaging resulted in no significant changes of the tracer between the diseased and controls groups at day 4, *ex vivo* biodistribution studies contrastingly revealed significant changes in the gut and the brain of rats with TNBS-colitis when compared to the control group at day 4. At day 11, PET imaging resulted in higher tracer uptake in the cerebellum of rats with TNBS-colitis. The lack of PET imaged inflammatory changes could be partially explained by the acute nature of this model and that the selected time-points may have not been optimal to

detect/identify the acute and chronic inflammatory responses occurring in the higher order brain regions.

Future studies involving the collection of cognitive data will clarify the mechanistic implications of the neuroimmune findings in the current study. Abundant evidence implicating the development of central comorbidities with chronic gut inflammatory disorders warrants further exploration of the potential cognitive behavioural changes in the models described in the current study. It is important to note that experimentally-induced colitis-like colonic inflammation studies have reported impaired spatial recognition memory and depressive- and anxiety-like behaviours in mice [389, 390]. Both of the aforementioned studies did not examine glial changes, yet importantly confirmed the link between cognitive and mood fluctuations in experimentally-induced colitis.

Further insights into the cognitive and mood changes induced by chronic gut inflammatory-induced glial activation may result in the exploration of targeted therapeutic approaches which are readily available, for example utilising the glial attenuators, Ibudilast (AV-411) or minocycline [140, 390, 391]. When administered prior to chemotherapy, Ibudilast has shown therapeutic promise in supressing tactile allodynia (pain from stimuli that does not usually induce pain) and improving cognitive impairment associated with the chemotherapy drug, oxaliplatin [392]. Minocycline suppresses microglial activation and plays a pivotal role in morphine-induced reward and pain modulation [140]. In experimentally-induced colitis, minocycline has effectively attenuated TNBS-induced visceral hypersensitivity, a hallmark feature of IBS [376]. In colitis patients, grey matter changes appear to be mediated by intestinal inflammation, supporting the gut-brain involvement in IBD [393]. Further investigation into the therapeutic potential of such glial attenuators in chronic inflammatory conditions of the gut, including CRC may lead to the

discovery of not only positive central molecular changes, such as reduced glial activation, but also behavioural improvements.

In addition to the hippocampal findings, we reported glial dysregulation via expression changes in GFAP and Iba-1 in the lumbar region of rats with chronic UC and CRC. Interestingly, we report a reduction in GFAP expression in the chronic UC group when compared to controls which was contrastingly different to the increase in GFAP expression in the CRC group. It is difficult to determine whether the reduction in GFAP expression resulted from a reduced number of astrocyte cells or that the cells themselves retracted their processes (which occurs prior to astrocyte activation). It is plausible that the intestinal inflammatory response was greater in the CRC group when compared to the chronic UC group due to the addition of the malignancy. Inflammation has been acknowledged as the seventh hallmark of tumour progression and development [68]. We anticipated the neuroimmunological manifestations in this region would occur via the neurally-mediated peripheral-to-central immune signalling mechanism. Previous studies have revealed the sensitivity of spinal glia in reacting to various models of peripheral inflammation, including cancer-induced pain, multiple sclerosis, neuropathic pain models and chemotherapy-induced gut toxicity [134, 303, 306, 307].

Studies using TNBS-induced colitis mouse models have reported similar microglial reactivity in the hippocampus and spinal cord [377]. We postulate that spinal glia were activated by the neural (vagal) pathway in which primary afferent neurons detect immune messages from the colon. Colon-projecting afferent neurons undergo exacerbated spontaneous activity during experimentally-induced colonic inflammation in mice [389]. As a result of this, at the spinal level glia may indirectly become dysregulated due to the inflammatory innervation of the colon in both diseased groups in our study. These immune messages then become transduced into a neural message which is then able to be relayed

by higher order brain regions [144]. Within the brain parenchyma, the neural messages are re-transduced back into an immune signal whereby local expression of cytokines increases, this in turn, has direct effects on neurons and glia. This immune-to-central signalling pathway may also, partially account for the hippocampal changes evident in both diseased groups as well as the hypothalamic GFAP expression increase and Iba-1 dysregulation evident in the CRC group. Due to the GFAP and Iba-1 expression changes identified in the hippocampus, hypothalamus and lumbar regions, we suggest that both humoral and neural pathways were activated and at least, in part, explain the glial marker expression changes evident in the models used in our study.

Previously, experimentally-induced colitis has reported elevated pro-inflammatory cytokines and reduced neurogenesis in the hippocampus [344]. Hippocampal neurogenesis is responsible for the proliferation and division of neural stem cells into new neurons or astrocytes, whilst critically contributing to hippocampal circuit plasticity and memory consolidation [179, 180]. In a study by Zonis, *et al*. (2015) a similar DSS water cycling model was used; 3% wt/vol DSS for five days with two days break reverting back to normal drinking water before the next DSS cycle begun for a total of 26 days [344]. During the acute inflammatory phase, hippocampal neuroinflammation was present as assessed by microglial marker activation and increased expression of pro-inflammatory markers. The neuroinflammatory response continued into the chronic phase of DSS-induced colitis whereby evidence was provided of reduced hippocampal neurogenesis, yet astrocyte activation was present. Molecular and cellular changes in the hippocampus are of particular significance as this region is fundamental in multiple aspects of cognition including neurogenesis, functioning processes, mood control and memory formation [179, 394]. This is of primary importance considering the cognitive and mood comorbidities associated with UC and CRC, warranting further investigation.

In view of the aforementioned data, we conclude that regional neuroimmunological manifestations of experimentally-induced chronic UC and CRC contribute to the complex pathophysiology of both disorders. To our knowledge, this is the first study showing an increase in GFAP expression in the hypothalamus of mice with CA-CRC. The mechanistic understanding of the spinal and higher order brain region neuroimmunological findings requires further investigation. Future exploration into the behavioural and histological parameters, including cytokine profile changes and microbiota analysis in chronic UC and CRC will assist in our understanding of the central comorbidities associated with chronic UC and CRC. Nonetheless, our findings support existing literature on the neuroimmune gut-brain interactions of chronic gut inflammatory disorders and suggests humoral and neural peripheral-to-central pathways mediate these responses.

CHAPTER SIX, clinical trial

Understanding the Side-Effects of Chemotherapy: A Cross-Sectional Correlational Study of Gastrointestinal Symptoms, Allergies and Cognitive Impairment in Australian Breast Cancer Survivors

Statement of Context

This manuscript has been submitted to the *Breast Cancer Research and Treatment Journal.*

Bajic JE, Hutchinson AD, Howarth GS and Hutchinson MR.

Chapter Six examines various perceptions of BCSs on their cognition, gut disturbances and other confounding factors since their chemotherapy treatment. It uses exploratory investigator-led questions to determine whether allergies are related to perceptions on cognition, gut disturbances and pain. It also used validated measures to assess mood-disorders and fatigue to identify the impact these have on patient quality of life. To my knowledge, this is the first study of its kind to investigate the relationship of allergies to cognitive, gut and pain disturbances. This novel approach opens up a pathway for future studies that may predict gut, pain and cognitive outcomes in chemotherapy recipients following allergy-related measures.

Statement of Authorship

Title of Paper	Understanding the side-effects of chemotherapy: A cross-sectional correlational study of gastrointestinal symptoms, allergies and cognitive impairment in Australian breast cancer survivors
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Juliana Esma Bajic, Gordon Stanley Howarth, Amanda Hutchinson & Mark Rowland Hutchinson

Principal Author

Name of Principal Author (Candidate)	Juliana Bajic		
Contribution to the Paper	I was responsible for the conceptual framework and execution of all elements of this study; developed the questionnaire, reviewed all papers cited in manuscript, wrote manuscript, performed statistical analyses and interpreted data, designed tables and acted as corresponding author.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	12/04/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);
II. permission is granted for the candidate to include the publication in the thesis; and
III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Gordon Howarth		
Contribution to the Paper	Reviewed manuscript.		
Signature		Date	12/04/19

Name of Co-Author	Amanda Hutchinson		
Contribution to the Paper	Assisted with initial conceptual framework, advising on analyses and reviewed/edited the manuscript.		
Signature		Date	4/4/19

Name of Co-Author	Mark Hutchinson		
Contribution to the Paper	Assisted with initial conceptual framework, advising on analyses and reviewed manuscript.		
Signature		Date	11/04/19

Abstract

The breast cancer (BC) and chemotherapy experience results in a myriad of cellular and molecular responses throughout the body. The two primary organs most susceptible to the toxicities of chemotherapy are the brain and gastrointestinal tract. Cognitive deficits and gut-related disturbances have been clinically termed chemotherapy-induced cognitive impairment (CICI) and gut toxicity (CIGT), respectively. The gut and brain are intrinsically connected in a bidirectional manner in both health and disease. Whether symptoms associated with CIGT contribute to, or exacerbate, cognitive symptoms remains undetermined. We hypothesised that CIGT would be positively correlated with CICI symptoms via neuroimmune and peripheral-to-central immune signalling; and secondly, that allergies, as a marker of general immune reactivity, may predispose chemotherapy recipients to gut or cognitive changes and increased sensitivity to pain. Here, in this retrospective correlational pilot study we examined the subjective responses of Australian BC survivors (BCS) experiencing cognitive changes after commencing chemotherapy treatment. We explored perceived cognitive impairment and its relationships to other symptoms (fatigue, depression, anxiety, stress, abdominal symptoms and allergies). Using Pearson's coefficients and Chi-square analyses, the current study identified positive relationships between perceived increased sensitivity to pain and perceived learning and memory difficulties (PLMD) following chemotherapy treatment (r=2.77; p<0.05). Allergy susceptibility in this patient population was also related to the incidence of PLMD (r=3.22; p<0.05) and the presence of gastrointestinal disturbances during treatment (p<0.01). Perceived cognitive issues positively correlated with fatigue (p<0.01), yet negatively correlated to depression, anxiety, and stress. When controlling for the effects of fatigue, depression, anxiety and stress, perceived cognitive ability positively correlated with perceived comments from others (p<0.05). From this preliminary study we conclude

that relationships occur between allergy susceptibility and cognitive changes, and allergy gastrointestinal disturbances during chemotherapy treatment. Although greater power is required in future studies to confirm the clinical significance of these findings, mechanistically we propose that neuroimmunological manifestations may be mediating the perceived gut and cognitive induced changes in a subset of BCS. Further, our findings imply that patient allergy susceptibility may be used as a predictive tool in the identification of individuals susceptible to unwanted gut and cognitive changes following chemotherapy treatment.

Background

Central- and gut-induced toxicities associated with chemotherapy treatment place significant strains on patient quality of life. In the central nervous system (CNS), reports of learning and memory difficulties have been clinically termed chemotherapy-induced cognitive impairment (CICI) and incidence estimates differ substantially, ranging from 15-85% of cancer patients [93, 172, 321, 322, 395]. The most commonly reported cognitive domains affected by CICI are executive functioning, attention, memory, decision making and speed of processing [174].

Other systemic unwanted side-effects of chemotherapy exposure are also common. In the gastrointestinal (GI) tract (GIT), mucositis is the umbrella term used to describe regimen-related mucosal damage and its aetiology has been comprehensively characterised by Sonis [1]. Since then, additional mechanisms contributing to the pathogenesis of mucositis have been identified, and the term mucositis has been superseded by chemotherapy-induced gut toxicity (CIGT). CIGT incorporates the influence of the microbiome and damage to enteric neurons [107, 208]. CIGT occurs in up to 40% of patients undergoing a standard dose regimen and up to 80% with higher dose regimens [396, 397].

The symptoms associated with both CICI and CIGT affect patients' quality of life in many domains, such as social, emotional, financial and physical, with symptom severity negatively impacting the ability to withstand treatment at effective doses [113, 322, 398]. The clinical and economic implications of both disorders warrant further investigation of the mechanisms related to the pathologies.

Whilst the gut and the brain are regionally disparate, they are intimately connected via various mechanisms and pathways. Information is signalled bidirectionally to both regions encompassing the central, autonomic and enteric nervous systems, with input from the gut-

brain-microbiota axis, neuroendocrine, enteroendocrine and neuroimmune systems [4, 5, 15, 314, 315]. The immune system becomes particularly compromised during cancer and its treatment. Many immunological processes become dysregulated due to the complex, and at times contradictory, nature of endogenous inflammatory events occurring at different sites [66, 68]. For example, inflammatory signalling molecules initiate pathways which attempt to rid the host of the malignancy, yet can simultaneously assist in the establishment and progression of cancer [67]. Accordingly, the immune profiles of patients undergoing anti-cancer treatments often become dysregulated; whilst increased systemic pro-inflammatory markers are commonly reported, suppressed immune activity may also be promoted by immunomodulatory antibodies [78, 399]. Finally, anti-cancer treatments target rapidly regenerating healthy cells. The gut lining and hippocampal brain region are particularly susceptible to toxicities associated with treatments, resulting in symptoms which have been attributed to CIGT and CICI, independently [64, 86, 116, 235]. Nonetheless, investigations into whether the two disorders contribute to, or potentiate, the pathogenesis of one another are lacking.

Further complicating immune dysregulation in the context of cancer is the association of allergies. Several systematic reviews have highlighted lines of evidence linking allergies either increased or decreased risk of developing specific cancer types [70, 71, 400, 401]. Whilst there is strong evidence for allergies having a protective role in cancer development (glioma, cancers of the GIT; oral, oesophageal, stomach and colorectal cancer), a history of allergies has also been linked to increased risk for lung, bladder, prostate and lymphoma cancer [71]. The hypothesis that allergies can increase risk for certain cancer types has been challenged and disputed amongst the literature. Accordingly, three major mechanisms mediating the relationship between allergies and cancer have been postulated, which involve: (1) chronic inflammation; (2) immune-surveillance (enhanced immune

responsiveness); and (3) prophylaxis (allergic reactions expel mutagenic triggers); all of which have yet to be elucidated [70].

The allergy status of an individual may demonstrate differing set-points of the system which promote pro-inflammatory processes. Accordingly, a challenge such as chemotherapy exposure may result in worse outcomes for gut toxicity and cognition, hence allergy status may serve as a predictive tool for patients at risk of severe toxicities to these regions, including visceral pain, severe mouth ulcers, diarrhoea and cognitive dysfunction that affect daily functioning. The current study thus proposed an association between allergies and risk of developing chemotherapy-induced toxicities. We hypothesised that if an individual already has an established heightened immune response to certain triggers, they may be predisposed to developing more severe gut and cognitive symptoms associated with chemotherapy treatment. Therefore, allergies may predispose a subset of chemotherapy recipients to more severe perceived CIGT and CICI symptoms.

The neuroimmune system and peripheral-to-central immune signalling pathways present as a plausible mechanism linking both disorders. Intestinal inflammation arising from CIGT may act as the catalyst, whereby peripheral inflammatory signals and mediators, such as interleukin-1 beta (IL-1β) or lipopolysaccharide (Gram-negative bacteria) signal the brain to orchestrate a series of physiological and behavioural responses [256, 382, 383]. Peripheral-to-central immune signalling pathways (humoral, neural and cellular routes) activate what is now commonly recognised as cytokine-induced sickness behaviours [382, 394]. Profound CNS activation initiates a host of "sickness responses" which include fever, fatigue, heightened sensitivity to pain, anorexia, and social isolation [382]. This constellation of behaviours ultimately aims to return the host to a homeostatic state. Interestingly, cytokine-induced sickness behaviours mimic many of the symptoms experienced by chemotherapy recipients, such as cognitive dysfunction, gastrointestinal

disturbances, depression, pain, anxiety and fatigue [79, 80]. Therefore, it has been postulated that interactions between CIGT and CICI may be mediated by peripheral-to-central immune signalling pathways which implicate the neuroimmune system in the pathogenesis of cognitive symptoms [302].

Neuroimmune cells, glia, form an intimate relationship with the synapse and are critical in health and disease [121, 122]. In particular, microglia and astrocytes are sensitive to increases in systemic pro-inflammatory cytokines and mediators released by peripheral inflammatory events. Once activated, glia release a host of pro-inflammatory cytokines and mediators, inducing neuroinflammation, and the subsequent neurotoxic microenvironment can contributes to various peripheral and central disorders, such as neuropathic pain and Parkinson's disease [131, 319]. In higher order brain regions involved in learning in memory, such as the hippocampus, glial activation and neuroinflammation have been proposed to contribute to CICI which warrants further investigation [84, 302]. In preliminary studies, our group has shown that glial activation occurs in the hippocampus of mice with colitis-associated colorectal cancer and in the spinal cord of rats in a non-malignant CIGT model (unpublished). Whilst the acute chemotherapy model failed to observe higher order brain glial dysregulation, further exploration utilising a more clinically representative approach (chronic model) may yield support for this hypothesis. Increased expression of microglial and astrocyte markers in the hippocampus of mice with colitis-associated colorectal cancer suggested that glial-induced chronic inflammatory gut conditions were mediated by peripheral-to-central immune signalling pathways, such as the humoral (hippocampal changes) and neural (spinal changes) routes.

Our current understanding of mechanisms relating to CICI and CIGT fail to acknowledge the impact CIGT may have on CICI. Therefore, the current study additionally sought to bridge this gap and identify the potential impact these chemotherapy-induced side-effects

have on the quality of life in breast cancer survivors (BCS) in Australia. Through an exploration of subjective CIGT and CICI in BCS, the current study hypothesised that the severity of CIGT and CICI symptoms would be correlated. To further explore the relationship between symptoms and quality of life in BCS, various factors such as, depression, anxiety, stress, fatigue, allergy susceptibility and pain were also measured. This was a cross-sectional correlational pilot study which used exploratory statistical analyses to identify associations between a range of peripheral and central side-effects induced by chemotherapy treatments. The current study was separated into two arms whereby the initial analyses (*Phase One: partial dataset*) directed a more guided, detailed and appropriate statistical approach for the second arm of analyses (*Phase Two: fulll dataset*). The first arm of the study focused on subjective exploratory questions surrounding perceived cognitive impairment (PCI), identifying specific time-points for perceived changes, as well as other side-effects of treatment; for example relating to pain, stressors and allergy susceptibility. The Phase One analysis aimed to determine whether individual susceptibility to allergies could serve as a predictive sign for CIGT development in BCS, owing to their hypothesised shared immune basis. The Phase One questionnaire was developed by the lead investigators and answers were converted into categorical variables for statistical analyses. Phase Two utilised already validated measures, such as the Functional Assessment for Cancer Therapies (FACT), Functional Assessment of Chronic Illnesses Therapy (FACIT) (FAC(I)T) and the Depression, Anxiety and Stress Scale (DASS). The Phase Two analysis aimed to quantify relationships between perceived symptoms and quality of life, offering insight into the impact these side-effects have on BCS perceived quality of life.

Materials and Methods

Procedure

Approval was granted by The University of Adelaide's Human Research Ethics Committee (# H-2015-167; Appendix Five) and all elements of the survey were guided by the National Statement on Ethical Conduct in Human Research (2007). BCS (total *n*=72) were recruited through the Breast Cancer Network Australia (BCNA) Review and Survey Group. Initial power analysis was based on an effect size of 0.3 with 90% power; sample size n=88, however, the recruited sample size did not reach this number. As a result of this, the current study was separated into two arms to ensure sufficient statistical power in identifying potential links between allergies and perceived GI or learning and memory difficulties. Fewer participants than anticipated completed the questionnaire in full (Phase One and Two) or met the inclusion criteria. However, their responses informed the question of the relationship between allergy, PLMD and GI disturbances. Therefore, they were considered separately in Phase One of the analyses. It is for these reasons that Phase One (*n*=58; effect size of 0.5 with 95% power); focused on exploratory questions, whereas Phase Two (*n*=31; effect size of 0.5 with 80% power) were a subset of the original (*n*=58) population and adopted more stringent inclusion and exclusion criteria, including specific validated measures (see Figure 6.1: Venn diagram). Basic inclusion criteria for all consenting participants included in both phases of this study were that they: 1) had all been diagnosed with breast cancer; 2) were 30+ years old; and 3) had all undergone and completed chemotherapy treatment. BCS were only included in Phase Two of the study if they were non-smokers, non-diabetic, free from psychiatric (including major depression) or neurological disorders and had no pre-existing gut disorders (for example, inflammatory bowel diseases) prior to chemotherapy treatment. Participants not meeting these criteria were excluded because smoking, diabetes and gut disorders may predispose BCS to CIGT due to altered cytokine profiles and increased risks

of infection and disease [364, 402, 403]. As some pharmaceuticals alter cognition, participants were also free from using any medications, such as opioids, which can impact cognitive outcomes [404]. BCS included in the Phase Two analysis reported experiencing difficulties with their cognition and GI disturbances since commencement of chemotherapy treatment. Finally, the Phase Two participants were only included if they had received their last anti-cancer treatment within two years of the current study.

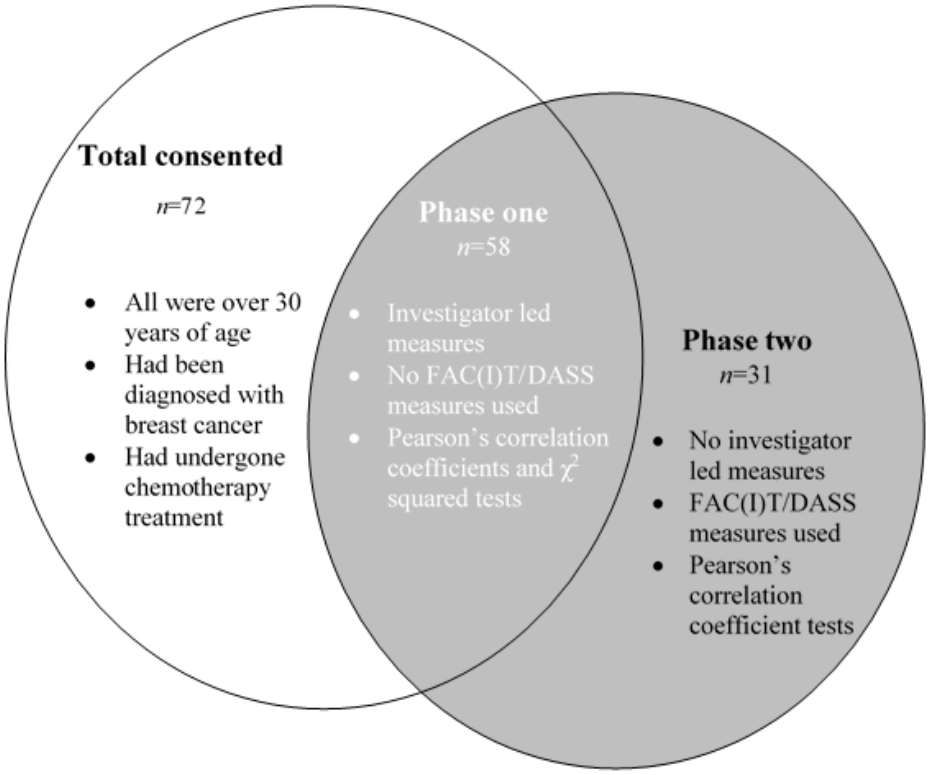

Figure 6.1. Venn diagram depicting Phase One and Two breakdown. Of the 72 consented participants, 58 were eligible for Phase One analysis involving exploratory questions/measures only, not using the FAC(I)T/DASS measures. Inclusion and exclusion criteria were applied to the Phase One arm, resulting in 31 eligible participants to be included in the Phase Two arm of the study focusing on the FAC(I)T/DASS measures.

Participants

All initial correspondence with participants was via the BCNA by email (Appendix Four), which contained a participant information sheet, inclusion and exclusion criteria checklist, and the participant's contact project and complaints form. Willing participants were given the option to complete the study online (SurveyMonkey), or alternatively by requesting a hardcopy from the primary investigator by email. Participant responses were anonymous.

Measures

Participants completed an online self-report questionnaire which were separated into two phases (four participants completed hard-copy version; see Appendix Three for full questionnaire).

The first phase of the questionnaire (see Appendix Three) was primarily designed by investigators to determine demographic, medical and personal lifestyle information, as well as identifying specific exploratory questions relating to the time-course of perceived learning and memory difficulties (PLMD) and perceived GI disturbances after chemotherapy treatment. For example, participants were asked whether PLMD or GI issues were noticed before treatment but after diagnosis, during treatment or after treatment ceased. This initial phase also identified the importance of exercise to each individual by rating it on a scale (1: extremely important to 5: not at all important) and identified how often exercise was undertaken in a typical week. Specific questions relating to allergies, pain and factors associated with pain were examined. BCS were asked to report on how often they experienced pain prior to diagnosis (1: daily to 7: rarely) and commented on the type of pain most commonly experienced. Following treatment cessation, they were also asked whether they noticed an increase in their sensitivity and frequency of pain (yes/no) and to indicate their previous and current use of various 'habits' associated with pain including dieting, consumption of caffeine and alcohol, illicit drug use, and vitamin supplementation. Participants responded currently, yes; currently, no; previously, yes; or previously, no. Participant stressors were assessed by categories focusing on the following areas: familial or employment, relationships in both areas, financial and health stress. All stressors were rated in terms of the level of stress in these areas of their life (1: no stress; 2: intermittent stress; 3: persistent stress and 4: overwhelming stress).

The second phase of the questionnaire (see Appendix Three) followed a directed statistical approach by quantifying perceived cognitive and GI disturbances using valid and reliable surveys: FACT-Cognitive (Cog) Scale, FACIT-Abdominal Disturbances (AD), FACIT-Diarrhoea (D) and FACIT-Fatigue (F). Depressive and anxiety symptoms were assessed using the DASS to control for these as confounding factors potentially associated with both cognition and GI disturbances. The Phase One analysis of this study exclusively relates to data outlined in the first phase of the questionnaire, which included demographic and exploratory questions regarding personal, lifestyle and medical issues designed specifically for this study by the lead investigators (see Appendix Three). Phase Two followed a directed statistical approach utilising the data obtained from the FAC(I)T and DASS measures.

Phase One: Demographic and medical information. All participants were asked to report their age, sex, education, employment status, exercise level, allergy susceptibility, stress levels in various aspects of life, marital status and medical information. Medical information included time post-treatment, types of adjuvant therapies, and a range of investigator-led exploratory questions surrounding PLMD and GI issues, as well as reporting on their use of additional therapies, such as anti-hormonal medications. Following the initial results obtained from the global Phase One analysis, stringent screening of the inclusion and exclusion criteria were applied to the Phase One participants. Ultimately, this resulted in a subsample which enabled more targeted statistical analyses in Phase Two.

Phase Two: Perceived cognitive function. Perceptions on cognitive changes were measured using the FACT-Cog (v 3) [405]. This version of FACT-Cog has 37 questions rated on a five point Likert scale (0: never or not at all – 4: several times a day/very much) [406]. Four subscales cover PCI (20 items), perceived cognitive abilities (PCA; 9 items),

perceptions on comments from others (OTH; 4 items) about their cognitive function and quality of life (QOL; 4 items). Negatively worded items were reverse scored. Overall, higher scores reflect fewer cognitive problems. FACT-Cog is a self-report measure which has demonstrated good validity and reliability in previous BCS studies examining CICI [276, 406, 407].

Fatigue. The FACIT-Fat (v 4) was used to measure the severity of fatigue symptoms over a seven-day period on a five point-scale with response options ranging from 0 (not at all) to 4 (very much so). This tool has been specifically designed for cancer patients [408] and has shown good validity and reliability in BCS populations [409-411]. Responses are summed to calculate a total score with high scores reflecting low levels of fatigue and the FACIT-F scale has strong internal consistency, including test-re-test reliability [406].

Depressive and anxiety symptoms. The DASS is a 42 item questionnaire [412]. Participants indicate the extent to which symptoms applied to them over the past week on a four-point Likert scale ranging from 0 (Did not apply to me at all) to 3 (Applied to me very much, or most of the time). DASS measures participants' emotional states of depression, anxiety and stress during the past week. DASS has high internal consistency with Cronbach alphas of 0.91, 0.84 and 0.90 for depression, anxiety and stress subscales respectively [413, 414]. Although this instrument was not created specifically for cancer patients, it has previously demonstrated reliability when used in breast cancer studies [413, 414].

Gastrointestinal discomfort. The FACIT-AD and FACIT-D scales assessed participants' perceived well-being and gastrointestinal disturbances during their treatment. Subscales were classified into physical well-being (7 items), social/family well-being (6 items), emotional well-being (6 items) and functional well-being (7 items). Responses were scored using a five-point Likert scale ranging from 0 (not at all) to 4 (very much). Both

instruments produced three subscales: physical well-being, social/family well-being, emotional and functional well-being. To avoid repetition of the same questions, the primary investigator received permission from the FACT/FACIT organisation to orchestrate a unique set of questions which were tailored specifically for the participants in this study and in turn, relabelled it the FACIT-D/AD. The 'additional concerns' sub-sections from both instruments (3:AD + 11:D) were combined to make a specific 14 item section which avoided repetition of questions presented in both surveys. Higher scores indicated that participants had a better perceived QOL and reduced GI disturbances.

Statistical Analysis

All statistics were performed using IBM SPSS Statistics program (v 25). Frequencies were used to describe the samples in both phases of analysis and were also used to report on the exploratory questions in Phase One analysis. In Phase One, Chi-squared tests were employed to explore relationships between PLMD and GI symptom severity during treatment, including allergy susceptibility and increased sensitivity to pain. A Pearson's correlation matrix was also performed as an overall guide to identify potential links in Phase One of the analysis. The Phase Two analyses used further Pearson's correlation coefficients to explore relationships between PCI, FACT-Cog total scores and the subscale scores of other symptoms associated with perceived QOL, such as emotional well-being and abdominal disturbances whilst controlling for the effects of fatigue, depression, anxiety and stress. This nested statistical approach was developed due to the preliminary nature of this study.

Results

Demographics

Table 6.1 contains participant demographic and medical information. Of the 72 participants, 58 were eligible for inclusion in the Phase One analysis with all demographic and exploratory questions fully completed. Demographic frequencies remained similar for participants in Phase One and Phase Two of the analysis. The majority of participants were 50-69 years of age, post-menopausal and married. Participants most commonly reported they were working full time (32-34.4% in both phases) and the majority had completed some form of tertiary education. Early breast cancer (EBC) was the primary diagnosis for the majority of BCS (60.3-65% in both phases). Six participants reported being diagnosed with at least one or more additional diagnoses, such as ductal or lobular carcinoma in situ. One EBC survivor disclosed multiple diagnoses which included Paget's disease of the nipple, ductal carcinoma in situ and locally advanced breast cancer. In addition to chemotherapy treatment, most BCSs had surgery (partial or full mastectomy) and/or radiation. Participants most commonly reported undergoing their anti-cancer treatment regimens for less than one year and were in remission for less than 24 months.

In Phase Two the number of participants reached 31 after the inclusion and exclusion criteria were applied. The most common reasons for exclusion were that participants had exceeded 24 months since their last chemotherapy treatment, had not experienced any gastrointestinal disturbances during their chemotherapy treatment, or had pre-existing mental health issues. Although some participants reported no pre-existing mental health issues, they reported that they reported anti-depressant medication. These participants were included in the Phase Two analysis under the assumption it was for another use, such as in the treatment for hot flushes during menopause. For a breakdown of the Phase One and Phase Two demographic, medical and exploratory data, refer to Table 6.1.

Internal consistency of the subscales in all FACT and DASS questionnaires were evaluated using Cronbach's coefficient alpha. Internal consistency is considered acceptable for group comparisons if $\alpha > 0.7$. Internal consistency was high for all DASS subscales (depression, anxiety and stress scores; >0.9), PCI and PCA reached >0.9, yet comments from others and QOL scales were <0.5, FACIT-F was 0.8, whereas FACIT:D-AD subscales were weak (<0.4).

Table 6.1: Sample descriptive statistics personal demographics, medical and treatment

Parameters	**n (%)**	**n (%)**
Consented participants	72 (100)	58 (100)
	Phase One	***Phase Two***
Eligible particpants	58 (80.5)	31 (53.4)
Age		
30-39	2 (3.4)	2 (6.5)
40-49	14 (24.1)	5 (16.1)
50-59	24 (41.4)	15 (48.4)
60-69	14 (24.1)	8 (25.9)
70-79	3 (5.2)	1 (3.2)
Post-menopausal	51 (87.9)	26 (83.9)
Relationship status		
Single, not married	9 (15.5)	1 (3.2)
Single, cohabiting significant other/domestic ı	6 (10.4)	4 (12.9)
Married	33 (56.9)	17 (54.8)
Separated	2 (3.4)	2 (6.5)
Divorced	7 (12.1)	6 (19.4)
Widowed	1 (1.7)	1 (3.2)
Employment		
Unemployed, not looking	6 (10.4)	4 (12.9)
Unemployed, looking	4 (6.9)	3 (9.7)
Employed, part-time	16 (27.6)	8 (25.8)
Employed, full-time	20 (34.4)	10 (32.3)
Retired	11 (18.9)	5 (16.1)
Disabled	1 (1.7)	1 (3.2)
Education		
PhD/Masters/higher education	14 (24.1)	8 (25.8)
Honours degree	5 (8.6)	4 (12.9)
Undergrad degree	10 (17.2)	5 (16.1)
Tafe/Diploma	13 (22.4)	8 (25.8)
Completed year 12	3 (5.2)	3 (9.7)
Did not complete year 12	8 (13.8)	3 (9.7)
Primary BC diagnosis		
Ductal carcinoma in situ	13 (22.4)	9 (29)
Lobular carcinoma in situ	6 (10.3)	3 (9.7)
Paget's disease of the nipple	2 (5.7)	2 (6.5)
Locally advanced BC	8 (13.8)	4 (12.9)
Early BC	35 (60.3)	20 (64.5)
Secondary BC	2 (5.7)	0 (0)
Unsure	1 (1.7)	1 (3.2)
Treatment type		
Surgery (full mastectomy)	19 (54.3)	15 (48.4)
Surgery (partial mastecomy)	11 (31.4)	9 (29)
Chemotherapy	32 (91.4)	**31 (100)**
Radiotherapy	12 (34.3)	22 (71)
Other	19 (35.4)	19 (61.3)
Treatment length		
Less than 6 months	8 (13.8)	4 (12.9)
Less than 1 year	27 (46.6)	16 (51.6)
More than 1 year	15 (25.9)	9 (29)
More than 2 years	8 (13.8)	2 (6.5)
Less than 24 months in remission	38 (65.5)	27 (87.1)
Last cancer tmt less than 24 months	45 (77.6)	**31 (100)**

Lifestyle and Reported Changes Since Treatment

In Phase One, exercise was important to approximately 70% of participants with 43.1% susceptible to allergies. The most commonly reported allergies in the current study related to medication (e.g. penicillin) and plants/pollen, followed by food and insects/animals, with only a few individuals reporting allergies to dust, alcohol and tapes/latex. Hormone therapy was the most commonly reported regular medication (58.6%). Persistent or overwhelming stressors were reported by 29 of the participants and were mainly related to health, family, parents, children, finances, work and co-workers. Of those, 13 reported multiple areas of stress in their life, as an example one participant expressed persistent or overwhelming stress in relation to their health, family, parents and children, as well as in their workplace.

When asked whether their cancer diagnosis or anti-cancer treatment prevented them from working, 67.2% participants agreed. The main reasons for not being able to continue working related to treatment side-effects (71.4%), with cognitive changes (33.3%) and pain (23.8%) most commonly reported. Aside from work duties, other normal daily activities were negatively affected by cancer diagnosis and anti-cancer treatment (81%). Prior to receiving a cancer diagnosis and anti-cancer treatment, the majority of participants reported rarely experiencing pain (56.9%), though 70.7% reported noticing an increase in their sensitivity to pain (and frequency) following commencement of anti-cancer treatments.

All participants in Phase One reported experiencing problems with cognition since their cancer diagnosis but the majority specifically reported noticing issues following commencement of their anti-cancer treatment (70.7%). Of those, 86.2% continued to experience PLMD. Cognitive changes were reported to be more noticeable post-chemotherapy treatment sessions (62.1%) and 87.9% said that their PLMD had not

returned to their capacity prior to anti-cancer treatment. Although 84.5% of participants reported GI disturbances throughout their anti-cancer treatment, only 69% reported that they consulted their doctor. Most doctors provided participants with analgesic intervention or coping strategies to help manage GI issues (56%). Of those reporting GI disturbances, only 10.3% of the participants were clinically diagnosed with mucositis (oral and/or intestinal) although 56.9% reported that their GI complaints were more severe post chemotherapy treatment.

Phase One: *Categorical Relationships Between Perceived Increased Sensitivity to Pain, PLMD, Perceived Severity of GI Disturbances and Allergies*

Initial Pearson's correlation analysis was performed to identify potential links between perceived allergy, pain, PLMD and GI issues in Phase One BCS (n=58). A significant interaction between BCSs reporting increased frequency and sensitivity to pain with PLMD was identified (Pearson's r=0.277, p<0.05; Table 6.2). When comparing the frequency of self-reported susceptibility to allergies and PLMD since undergoing treatment, a significant interaction was also found (Pearson's r=0.322, p<0.05; Table 6.2). Following the initial analysis, multiple chi-squared tests of independence were performed to further explore these relationships. The majority (85.5%) of participants who reported PLMD since undergoing treatment also reported GI disturbances during treatment ($\chi^2(1)=0.766$, p=0.403; Table 6.3). There was a significant interaction between the exploratory pain and allergy data with 51% of participants reporting increased sensitivity and pain frequency, also reporting allergy susceptibility ($\chi^2(1)=3.575$, p>0.048; Table 6.3). 45.5% of participants who were susceptible to allergies also reported experiencing cognitive changes since undergoing treatment ($\chi^2(1)=2.397$, p=0.255; Table 6.3).

Table 6.2. Pearson's correlations for perceived pain, learning and memory difficulties, GI disturvances and allergy susceptibility

Parameters	Increased pain	PLMD	GI disturbances	Allergies
Increased pain	1	.277*	0.128	0.255
PLMD		1	0.181	.322*
GI disturbances			1	0.125
Allergies				1

*. Correlation is significant at the 0.05 level (2-tailed).

Table 6.3. Pearson's Chi-squared tests on PLMD, GI disturbances, allergy and pain data

Parameters	GI issues during treatment	Allergies	Increased pain
Cognitive issues during treatment	0.766	2.397	2.131
GI issues during treatment		0.008	0.083
Allergies			3.757*

*. Correlation is significant at the 0.05 level (2-tailed).

Perceived learning and memory difficulties (PLMD), gastrointestinal (GI).

Phase Two*: *Relationships Amongst PCI, QOL and Other Symptoms (Fatigue, Depression, Anxiety and Stress)

Phase Two included only those participants who met more stringent inclusion criteria to control for the impact of potential confounders. In this analysis, PCI was significantly related to QOL and fatigue (r=0.55, p<0.05 and r=0.69, p<0.01; respectively; Table 6.4). PCI was negatively associated with depression, anxiety and stress scores (r<-0.5 and p<0.01 for all measures; Table 6.4). There were also negative correlations with fatigue and depression, anxiety and stress scores (r<-0.5 and p<0.01 for all measures; Table 6.4).

Relationships Between PCI, Well-being and GI Disturbances, Controlling for Confounding Factors

In Phase Two, the subscale scores generated from the FACT-Cog, well-being and GI disturbances data were examined, whilst controlling for DASS and fatigue measures. This analysis identified whether positive relationships occurred between PCI and well-being or GI disturbances; higher scores for PCI (worse perceived cognitive impairment) would positively correlate with higher scores for well-being or GI disturbances (better perceived well-being or worse perceived GI symptoms). Following application of the stringent eligibility criteria to participants, an n=31 was reached. PCA was related to perceived comments from others (r=0.388; p=0.04; Table 6.5). Whilst a negative trending association was observed between PCI and social/family well-being, and PCI and diarrhoea, these failed to reach statistical significance (r=-0.33, p=0.095 and r=0.37, p=0.057, respectively; Table 6.5). Perceived comments from others was also trending towards a negative association with emotional or physical well-being (r=-0.320 and r=-0.336, respectively; Table 6.5), as well as with abdominal disturbances (r=-0.353; Table 6.5), though these also failed to reach statistical significance (p=0.10, p=0.09 and p=0.07, respectively; Table 7.5). Perceived diarrhoea symptoms were positively related to perceived social and family well-

being (r=0.530; p=0.004; Table 6.5), as well as showing a positive trend toward emotional well-being, though this just failed to reach statistical significance (r=0.361; p=0.06; Table 6.5).

Table 6.4. Relationships between PCI, QOL and other symptoms.

Measures	QOL	Fatigue	Abdominal	Diarrhoea	Depression	Anxiety	Stress
PCI	.561**	.695**	0.106	-0.151	-.701**	-.617**	-.573**
QOL		.621**	0.172	0.321	-.468**	-.462**	-.360*
Fatigue			0.059	-0.028	-.579**	-.721**	-.550**
Abdominal				0.155	-0.081	-0.099	-0.062
Diarrhoea					-0.087	0.028	0.097
Depression						.868**	.837**
Anxiety							.885**

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

Table 6.5. Partial correlations of subscale scores in FACT-Cog, well-being and FACIT:D-AD, controlling for confounding factors.

Control variables		PCA	OTH	Physical WB	Family/Social WB	Emotinal WB	Functional WB	Abdominal	Diarrhoea
DASS	PCI	0.540	0.206	-0.092	-0.328	-0.086	-0.197	0.103	-0.370
Fatigue	PCA		0.388*	0.038	-0.087	-0.254	0.251	0.136	-0.055
	OTH			-0.320	-0.027	-0.336	0.069	-0.353	-0.162
	Physical WB				0.262	-0.009	0.168	0.278	0.148
	Family/Social WB					-0.001	0.122	0.251	0.530*
	Emotional WB						-0.169	0.256	0.361
	Abdominal							-0.291	0.057

*Correlation is significant at the 0.05 level (2-tailed).

Perceived cognitive impairment (PCI), quality of life (QOL), functional assessment of cancer therapy-cognition (FACT-Cog), well-being (WB), functional assessment of chronic illnesses therapy-diarrhoea/abdominal disturbances (FACTI:D-AD), others (OTH).

Discussion

The cognitive and GI disturbances associated with cancer and anti-cancer treatments place significant burdens on the individual and on health services at a national level [264, 415]. Regimen-related GI and cognitive symptoms negatively impact cancer patients in many ways, placing significant strains on their ability to function (from eating and speaking, to functioning in normal roles, such as parenting or working), to financial hardships. Cancer patients with moderate-to-severe GI and cognitive deficits experience reductions in income due to maintaining oncology appointments, feeling unwell or unfit for work, and even hospitalisations [264]. This, combined with increased financial strain associated with cancer diagnosis, treatment and hospitalisations, results in a significant burden on cancer survivorship. At the national level, these regimen-related toxicities often become so severe that infection results in hospitalisations, which can have significant impacts on national healthcare systems in an aging population.

Whilst regimen-related GI symptoms are generally acute and resolve upon treatment cessation, cognitive deficits are often delayed, persistent and distressing to survivors as they are difficult to manage [321]. At the heart of oncology, is the complex nature of the simultaneous symptom clusters that patients experience. In an effort to elucidate global mechanisms contributing to concurrent central and GI symptoms in BCS, the neuroimmune system is ideally positioned as it incorporates immune dialogue between the periphery and the CNS. Therefore, the purpose of the present study was to determine whether relationships between the perceived severity of CIGT symptoms were correlated with perceived cognitive difficulties in BCS. Additional side-effects associated with treatment (fatigue and depression) were also accounted for, as well other individual factors which may affect immunological outcomes, such as, allergy susceptibility and lifestyle choices, such as exercise.

One of the prominent findings from Phase One was that frequency and sensitivity to pain following chemotherapy was positively related to PLMD after treatment. Pain and cognition are intrinsically and reciprocally intertwined, yet mechanisms contributing to their interaction are unclear [416, 417]. The concept of pain perception factors in not only the sensory modality, but the conscious experience which involves a myriad of cognitive processes, including emotional and psychological factors [417]. The integration of these processes results in a cognitive-evaluative dimension of pain, which encompasses memory retrieval, adaptive learning and active decision making [418]. Chronic pain sufferers appear to be particularly susceptible to reporting learning and memory deficits in attention and working and verbal memory domains [419, 420]. Studies utilising placebo analgesia and cognitive tasks (distraction) to alleviate pain in chronic pain syndromes provide strong evidence that the two paradigms are intimately linked [421, 422]. Pain is amongst one of the most commonly reported symptoms associated with cancer and anti-cancer treatments [423]. Approximately 16-73% of BCS report pain as a significant emotional, financial and physical burden, yet the ability to appropriately evaluate pain is difficult due to the complexity of the disease itself and the subjective experience of pain [424]. Chronic pain occurs in 43% of BCS for up to 3 years post mastectomy surgery [425]. Accordingly, pain management guidelines for cancer and its treatment have been developed by the World Health Organisation which advise using (in increasing intensity) mild, moderate and more powerful analgesic options, such as non-steroidal anti-inflammatory drugs, partial or weak opioids, followed by typical/classic opioids [266].

Growing evidence has highlighted the role innate immune signalling and the neuroimmune system has in modulating general and neuropathic pain, opioid analgesia, cognitive changes and symptom clusters (alike to sickness behaviours) in the cancer population [123, 136, 191, 319, 426]. Due to the intimate relationship glia have at the synapse, microglia

and astrocytes are uniquely positioned to influence neuronal excitability, which in particular brain regions may result in central sensitisation, increased nociceptive signalling and behavioural and cognitive adaptations [123, 127, 132, 134, 136, 329, 427]. Importantly, recent data has implicated genetic polymorphisms in neuroimmune activation of the innate pathway, My-D88, in cognitive dysfunction amongst cancer patients [329]. My-D88 (adaptor protein for many Toll-like receptors; TLRs) is pivotal in innate immune activation involving opioids, danger-associated molecular patterns and interleukin [IL]-1 receptors [428]. Combined with molecular studies identifying a role for neuroinflammation in CICI [182] and glial activation in mediating pain-associated with CIGT [134], this may partially explain the cognitive-pain interaction observed in the present study. Pain-cognition interactions can be two-way (effects of pain on cognition and cognitive load effects on pain) and are observed in acute and chronic pain settings [429-431].

There are three theories that could, at least partially explain, the association between perceived pain and PLMD found in the current study [416]. Firstly, it is plausible that the same (or at least some) cognitive processes involved in the perception of pain are competing with the negatively-affected cognitive domains; the limited resource theory. Persistent nociceptive inputs from pain have been suggested to compete with other sensory inputs, resulting in a negative effect on cognitive performance [432]. Alternatively, the neuroplasticity theory involves neural rewiring or reorganisation in chronic pain which interferes with normal cognitive functioning. For example, morphological adaptations, such as volume changes or cellular changes (e.g. oligodendrocytes, white matter) in specific brain regions, such as the pre-frontal cortex, or deficits in hippocampal neurogenesis or long-term potentiation modifications, have been proposed to have deleterious effects on cognition in patients experiencing chronic pain [417]. Finally, pain may result in the dysregulated release of intermediate neuromediators, such as pro-

inflammatory cytokines, monoamines and neurotrophic factors; some of which are released by glial cells. Increased or decreased release of these intermediate neuromediators may negatively affect cognition in various brain regions which can be explained by the neuromediator theory. It is unlikely that one mechanism or theory is responsible for the perceived pain-cognitive relationship observed in the BCS in the current study, rather all these theories could work in unison. Whilst a mild pain-cognition association was observed in Phase One, it was unable to be determined whether the pain perception BCSs influenced cognitive dysfunction, or vice versa, due to the cross-sectional approach adopted. In addition, it is also important to consider depression, anxiety and stress as confounding factors as they have all been associated with learning and memory deficits [419]. Nonetheless, these emotional components were not measured or accounted for in the Phase One analysis. Teasing apart these factors would require further detailed longitudinal examination of both objective and subjective cognitive, emotional and pain measures taken prior to chemotherapy, during and after treatment cessation.

Allergy susceptibility in the current BCS population was associated with PLMD, pain and GI disturbances during treatment in Phase One. Whilst the link between allergies and cancer development is well established [71], evidence connecting interactions between allergies and regimen-related toxicities is lacking. To our knowledge, this is the first study that identifies a relationship between allergies and PLMD, pain and GI disturbances in BCS. Certain chemotherapy drug types, including platinum compounds and taxanes, have high potential to cause allergic reactions (hypersensitivity) in some cancer patients [433], though none were reported in the current study. Evidence suggests that risk factors associated with hypersensitivity to chemotherapy drugs include prior chemotherapy exposure and a history of allergies [434, 435]. In a similar light, it is plausible that BCS with a prior history of allergies may have a primed immunological response that can be

triggered by chemotherapy treatment and potentially contribute to perceived severity of CICI, CIGT and pain symptoms. The aetiology of immunologic responses, such as atopic dermatitis, food allergies and hypersensitivity to drugs (e.g. penicillin) are multifactorial and, aside from genetic susceptibility, other influences, such as early-life and environmental exposures, infection and autoreactivity, have been identified [436-439]. Recently, it has been suggested that food allergies and asthma may be associated with changes in cognitive status [440, 441]. From this, one can assume that the allergy-cognition relationship identified in the current study may somewhat contribute to these findings. Although a link has been suggested to occur between food allergies and other GI disorders, such as IBD and IBS [442, 443], whether this is purely a coincidental or causal relationship, remains elusive. The potential involvement of an allergy-related primed or heightened immune (innate and adaptive) response and its effect on cognition in BCS in the current study warrants further investigation.

Innate and adaptive immune responses create a cascade of non-specific and specific defence mechanisms to protect the host from invading pathogens [238, 444]. Immune cells constantly circulating the lamina propria and submucosal areas of the GIT are pivotal to the mucosal immune system [346]. Consequently, the most noticeable manifestations of primary or acquired immunodeficiency disorders are infections of the soft tissue, in particular the oral cavity whereby recurrent viral, fungal and parasitic infections are commonly reported [445]. Patients undergoing radiotherapy or chemotherapy are included in this category as they are usually also immunocompromised, and like patients with autoimmune disorders, they frequently report inflammation and ulceration in the oral cavity (oral mucositis) or gut-related disturbances (visceral pain, bloating, diarrhoea, constipation, rectal bleeding, nausea and vomiting) [264]. Recently, the role of the neuroimmune system in the development of allergic reactions has been recognised [446].

Advancements in our understanding of the neuroimmune interactions mediating food, skin and lung allergic reactions have provided us with insights into mechanisms that have superseded the classical role of the immune system and nervous system in allergies [447-449]. This exciting avenue of research may offer novel targeted therapeutic approaches that dampen allergic responses via inhibiting the neuroimmune system, though detailed studies are required to elucidate these mechanisms. In the same sense, the neuroimmune system could be targeted in chemotherapy recipients, in an attempt to globally reduce regimen-related toxicities with an inflammatory component, such as CICI or CIGT, and even cancer-induced pain.

Taken together, these data presented here, coupled with the involvement of the neuroimmune system in allergic reactions [447], provide some mechanistic evidence for the allergy-related findings in Phase One of the present study. The translatable potential for the preliminary allergy-related findings in this study, suggest that chemotherapy recipients who are susceptible to allergies, may in fact have a heightened immune response and therefore, may be prone to developing more severe cognitive, pain or gut disturbances during and/or after treatment. This predisposition may allow clinicians to encourage patients who will be undergoing chemotherapy regimens to use preventative strategies that may alleviate or reduce the severity of these symptoms associated with chemotherapy-induced toxicities. Larger studies, with greater statistical power, are required to clarify the mechanisms contributing to the weak allergy-related relationships with PLMD, pain and gut disturbances reported in the current study.

In Phase Two, PCI positively correlated with QOL and fatigue, yet, in this small BCS population, PCI was negatively associated with depression, anxiety and stress. Strong positive associations between PCI and depression, anxiety and fatigue have been frequently reported in both BCS and other cancer patient populations undergoing

chemotherapy treatment [59, 337, 450-453]. Breast cancer diagnosis alone often results in feelings of depression and anxiety which can have negative effects on cognition and QOL [146]. Interpretation of this strong association should be treated with caution as the direction of the relationship is unclear, and may be cyclical; depression and anxiety may have substantial effects on PCI, yet PCI may also affect depression and anxiety [190]. Although it has been previously argued that depression and anxiety may in fact potentiate CICI [415, 454], in the general population negative associations between these parameters have been reported [454]. Some studies report mild-to-moderate negative depressive and anxiety effects on objective neuropsychological functioning [455]. Higher recruitment numbers and more statistical power, including the addition of effective control groups (healthy and cancer, no chemotherapy) will assist in future non-biased comparisons. Furthermore, as recommended by the International Cognition and Cancer Task Force, pre-chemotherapy cognitive scores are critical to address potential cognitive issues and/or reserve prior to treatment commencement as a baseline.

In the Phase Two analysis, FACT-Cog total scores strongly correlated with fatigue scores which was in accordance with previous studies [456]. Nonetheless, other studies using different fatigue scales have yielded negative associations between perceived cognitive changes and fatigue [451]. Various factors contributing to the prevalence of fatigue in cancer survivors have been attributed to physiological (pain, anaemia), psychological (depression, anxiety), chronobiological (circadian sleep rhythms) and social/cultural (education, socio-economic status) factors [457]. The discrepancies in the positive and negative associations between perceived cognitive changes and fatigue in these BC populations may be related to methodological differences and individual patient variances, such as, the use of different tools and measures, to testing cognitive reserve and emotional status prior to treatment and time-point of data collection, as examples.

Web-based technologies are advantageous as they offer researchers an economically viable and readily accessible tool to examine various factors of regimen-related toxicities. Online questionnaires are less invasive for participants, as they can complete tasks in the comfort of their home (or even a café). This methodological approach was utilised in the current pilot study to take a quick snap-shot of links, albeit some of the correlations were weak in nature, which may support further neuroimmunological examination on the perceived side-effects associated with chemotherapy treatment in this BCS population. Several pitfalls were identified with this approach and mostly related to difficulties surrounding the recruitment of BCS and incompletion or only partial completion of the questionnaire. The challenges with recruitment and uncompleted questionnaires resulted in a lack of statistical power which required the current study to consider participants who did not meet all criteria for Phase One of the study. This was the primary reason for dividing the analysis into two phases; whereby one focussed on specific questions relating to the investigator-led questions, and Phase Two utilised the fully completed questionnaire containing responses from the FAC(I)T and DASS measures only. A more detailed methodological approach containing subjective versus objective measures, and qualitative versus quantitative data, pre- and post-chemotherapy time-points, is recommended for future analyses to overcome these limitations.

Whilst objective neuropsychological testing has been considered the gold standard for cognitive impairment assessment, it is not always feasible for several reasons. For instance, neuropsychological test batteries often demand qualified and trained professionals making them not only unaffordable, but time intensive [458]. The time associated with neuropsychological testing limits their use in practice and more importantly may lead to conflicting test results due to fatigue, loss of concentration and decreased motivation [459]. Paradoxically, this complicates neuropsychological testing as fatigue, lapses in

concentration and decreased motivation compound cognitive abilities, negatively affecting the cognitive domains of which the tests are initially intended to determine.

Further complicating research is the subtle nature of cognitive changes that have been objectively assessed and reported in BCS [83, 94, 170]. Moreover, objectively assessed cognitive changes may not appropriately reflect the day-to-day cognitive struggles BCS experience. Perceived cognitive changes in this patient population are equally important as they offer researchers critical insights to the impact cognitive changes have on QOL. Due to the cost, time and possibility of conflicting test results, it is not surprising that subjective cognitive changes in BCS are more commonly reported than objective measures [460]. Some researchers have attempted to overcome these inconsistencies by conducting both objective and subjective measures [85, 113, 170, 461], yet inconsistencies in the prevalence of CICI still remain. To highlight this variance, Hutchinson, *et al*. (2012) examined the relationship between objective and subjective measures of cognitive impairment in BCS [460]. This extensive systematic review identified that only 8 out of the 24 studies included in the study, found a relationship between objective and subjective measures. In addition, the prevalence of objectively-reported cognitive impairment in these studies varied from 1-63% compared with 8-95% for subjective assessments. Importantly, this study suggested that discrepancies between objective and subjective measures may be attributed to inconsistencies in the definition of cognitive impairment, study design (longitudinal versus cross-sectional), actual time measured (objective tests measure specific points in time, whereas subjective tests measure performance over a period of time), the effect of mood and/or psychological distress and the awareness of research purpose. Given these limitations, both forms of assessment are useful tools in understanding the cognitive side-effects associated with chemotherapy exposure in BCS.

Irrespective, subjective measures importantly provide us with valuable insights to perceptions on daily functioning and quality of life.

Research in this area is compounded by a number of factors. The majority of BCS are over 40 years old and age alone may influence findings and must be considered. The natural process of aging involves biological processes which promote the gradual loss of homeostatic maintenance, including the structure and function of the brain [462-464]. Cognitive decline naturally occurs with age and has been associated with reduced hippocampal size [465]. Additionally, BCS in this age bracket may be undergoing hormonal changes as a result of menopause which can also affect cognition [466]. Natural age-induced cognitive and hormonal changes occur at different rates for everyone across an individual's life. This presents researchers with considerable challenges, due to difficulties surrounding measuring natural age-related changes on an individual basis. However, several studies have reported that age was not a confounding factor accounting for CICI symptoms [467]. In addition, the time course of cognitive changes in this patient population may be difficult to reconcile, as some studies report acute deficits that resolve within 12 months of cessation of treatment [322, 323, 453], whilst others indicate delayed onset and continued difficulties up to 20 years post treatment cessation [98]. Discrepancies also exist with the interaction of PCI and changes in the cytokine profile of BCS. Whilst PCI has been associated with higher plasma concentrations of pro-inflammatory cytokines, such as IL-1β and IL-6, some studies have reported no significant correlations [60, 468]. The discrepancies in these findings may result from different treatment regimens and methodological variances.

The complex nature of CICI is multifactorial and variances in findings are frequently reported in the BC population. Research in this field has significantly advanced since the 1990s, when cognitive complaints were initially dismissed by doctors as a result of the

stress, depression and anxiety associated with the initial diagnosis of cancer [272]. The realisation of methodological problems and continued expansion of more detailed longitudinal studies have resulted in studies yielding more clinical validity and translatable evidence. Experimental models have supported clinical evidence and begun to unravel the complex nature of the mechanisms associated with CICI [116, 272, 392]. Of particular interest to the present study is recent data implicating the neuroimmune system in CICI and pain associated with CIGT [134, 182, 392].

In summary, the implication of the neuroimmune system in allergic reactions, and the findings presented in the current study related to the links between allergies, perceived learning and memory difficulties and pain, highlight the need for continued research in this area. Indeed, the neuroimmune system is uniquely positioned to have beneficial effects on chemotherapy-induced peripheral and central toxicities. Larger longitudinal studies with greater power will assist in elucidating the functional implications of the somewhat weak correlations observed in the current study, assisting in our understanding of the impact the simultaneous side-effects have on daily life. To understand the mechanistic implications, allergy-cancer animal models determining pain, GI and cognitive outcomes should be adopted to assess cellular and molecular neuroimmunological complications in the brain and spinal cord. This will critically identify the influence glial cells have on the relationships observed in the current study. Ultimately, harnessing the neuroimmune system may enable us to intervene with potential novel treatments, which target both chemotherapy-induced toxicities and improve the quality of life for cancer survivors.

General Discussion

The deleterious GI and CNS effects produced by cancer and chemotherapy are well established, yet our understanding of the mechanisms contributing to their development are incomplete. The impact both cognitive and GI treatment-related toxicities have on daily functioning, coupled with the financial burden they place on the individual, as well as national health systems, makes them a considerable problem. Pain, ulceration, bleeding and gut motility issues associated with chemotherapy-induced oral and gut toxicity renders patients unable to perform normal daily tasks such as eating, drinking or speaking [1]. Mild-to-moderate oral and gut toxicities can often be managed with analgesic interventions, antibiotics, histamines, mouth creams/lozenges, certain probiotics and gut motility modifying agents (e.g. laxatives for constipation or anti-diarrhoea medication). However, severe cases can often result in infections requiring hospitalisations and additional therapies, involving liquid diets, fluid replacements, anti-fungal and anti-viral medications [264].

The average estimated costs of cancer patients with chemotherapy-induced oral/gut toxicity hospitalisations is US$6277 with oral toxicity, and US$9132 with gut toxicity, per chemotherapy cycle [264]. Predictions on the financial impact of cognitive complications arising from cancer and anti-cancer treatments are limited as differences in employment status and pay amongst patients prior to diagnosis are so broad. Nonetheless, reductions in work hours, unpaid leave and, in some cases, unemployment, have been linked to treatment-induced cognitive changes [83]. These down-stream work-related financial effects, combined with the need to commence treatment and seek assistance from health care services, therapies, and medication associated with cancer diagnosis, results in significant hardship on patient survivorship [469]. The financial impact that chemotherapy-induced oral/gut toxicity has on the patient is short-term compared with the potential for

cognitive issues to continue for many years following completion of cancer treatment therapies.

Involvement of the neuroimmune system in the pathological manifestations associated with chemotherapy treatment is becoming more evident. The neuroimmune interface is uniquely positioned to influence both the gut and the brain in mediating the development of both comorbidities and also has the potential to play a key role in treatment approaches and preventative strategies. Collectively, the gut inflammatory-mediated neuroimmune findings described in this thesis offer novel hypotheses relating to the activity of glial cells and the activation of peripheral-to-central immune signalling pathways under these conditions. The allergy-cognition and allergy-pain relationships identified in the BCS study (Chapter Six) are the first of its kind and will shape further investigations. A discussion of the overall findings from this thesis now follows.

This body of work aimed to explore the neuroimmunology of 5-FU and to identify whether altered central glial reactivity was unique to this widely utilised chemotherapy drug, or whether changes occurred due to a generalised inflammatory response in the GIT. In order to investigate this, several experimental gut inflammatory models were adopted. The acute models included a high dose of chemotherapy (5-FU) (Chapters Three and Four), a low dose non-chemotherapy (INDO) drug (Chapter Four) and the influence of analgesics on regional glial reactivity and IL-1β expression (Chapter Three). The chronic models compared glial reactivity in non-chemotherapy treated mice with chronic UC (non-malignancy) to CA-CRC (inflammatory-induced malignancy) (Chapter Five). The preliminary studies forming these Chapters revealed that spinal cord astrocytes displayed heightened reactivity in disorders of the gut which involve a high inflammatory component, irrespective of GI region; whether the disorder was acute or chronic; and whether the inflammation was malignancy or chemotherapy-related. Interestingly, changes

in spinal cord microglial reactivity only occurred simultaneously with altered astrocyte reactivity in the chronic models employed in this thesis (Chapter Five).

The collection of works presented here highlights the need to address current limitations in the assessment of gut and central chemotherapy-related toxicities. Critically, 5IGT, chronic UC and CA-CRC resulted in neuroimmunological manifestations which have the potential to contribute to central comorbidities associated with each disorder. Specifically, the findings generated from these works and the overarching hypotheses determined that:

- 5-FU was not unique in causing gut toxicity and altered glial reactivity in the brain and spinal cord of rats (Chapters Three and Four). Thoracic GFAP expression adaptations occurred at the spinal level corresponding with small intestinal toxicity, yet this single high dose did not result in hippocampal changes. Alterations to glial reactivity were also present in the lumbar spinal cord of both chronic UC and CA-CRC models, as well as the hippocampus (Chapter Five). Although INDO-enteropathy was unable to induce any central glial changes, gut toxicity was confirmed by reduced bodyweight and histological assessment (Chapter Four). Therefore, the glial changes reported here are indicative of a generalised inflammatory response in the gut, rather than systemic 5-FU alone. This is an important discovery which adds to the field, as the incidence of inflammatory conditions of the gut continues to rise, so do the central comorbidities associated with their pathogenesis;
- Opioid exposure attenuated 5-FU-induced thoracic GFAP expression, rather than exacerbating the response as postulated (Chapter Three). The analgesics selected for these experiments resulted in regional glial cell expression adaptations which highlight the complex and conflicting nature of glial cell reactivity to pain and their functional implications; *and*

- Positive relationships between allergies and PLMD, allergies and pain, pain and PLMD, PLMD and GI disturbances were observed in the BCS population (Chapter Six).

Whilst one of the primary overarching hypotheses proposed that 5-FU-induced glial dysregulation would be associated with, or potentiate, learning and memory deficits in the context of CICI, this was unable to be ascertained as behavioural data were lacking. The spinal and hippocampal glial ramifications of these works indicate a potential neuroimmunological role in the pathogenesis of CICI which will be discussed below. Additional key results from the studies presented which can be specifically related to the aims presented in this thesis are:

- 5IGT resulted in altered thoracic spinal cord astrocyte reactivity during the Peak Injury Phase of mucositis and resolved during the Recovery Phase (Chapter Three);
- 5-FU-induced thoracic astrocyte reactivity was proposed to be mediated by a neural peripheral-to-central immune signalling pathway (Chapter Three);
- 5IGT resulted in caecal microbiota changes, yet the low-dose INDO-enteropathy model failed to induce glial reactivity or microbiota changes (Chapter Four);
- The findings presented above support the hypotheses outlined in the first two chapters, that CIGT results in neuroimmunological manifestations (Chapter One) and several stages of the gut-brain-axis become dysregulated under chemotherapy conditions (Chapter Two);
- Chronic inflammation resulting from the chronic UC and CA-CRC models induced astrocyte and microglial marker expression changes in the lumbar spinal cord and hippocampal region (Chapter Five);
- Similar to the thoracic results in Chapters Three and Four, the spinal lumbar region glial marker expression changes in Chapter Five indicated a neurally-mediated

peripheral-to-central immune signalling pathway. However, the concurrent hippocampal glial changes indicated that the humoral route was mediating this response;

- The findings from Phase One of the BCS trial identified an association between patient allergy susceptibility and PLMD and GI disturbances, independently. Additionally, increased pain perception correlated with PLMD since chemotherapy treatment cessation. Phase Two analysis revealed that PCI correlated with QOL and fatigue, yet a negative relationship occurred between PCI and depression, anxiety and stress.
- The findings in relation to allergies and its relationship with PLMD, GI disturbances and pain warrant further investigation.

The causal and directional nature of these distinct, yet connected pathological findings, opens up a pathway for further investigation into gut-mediated neuroimmune manifestations, specifically in relation to cancer, chemotherapy and IBD. The findings summarised above leave many new questions which will be addressed below. For example, is 5-FU-induced gut toxicity driving microbiota changes, or vice versa? Also, are the 5-FU-induced microbiota changes important in mediating changes in spinal astrocyte reactivity?

Glial Adaptations in the Spinal Cord: Implications for 5-FU, Chronic UC and CA-CRC

The spinal glial adaptations identified throughout this thesis demonstrated a neuroimmunological role in the pathogenesis of 5IGT, chronic UC and CA-CRC. Nonetheless, we can only speculate on the mechanistic significance of these findings and, as such, future prospective studies should focus on specific functional relationships between gut toxicity and spinal glial reactivity. Astrocytes and microglia are classically

recognised as playing key roles in pathological and chronic pain conditions [123]. Both neuroimmune cell classes are uniquely positioned around the synapse (forming the tetrapartite synapse) to enhance neuronal transmission and amplify nociceptive signalling under pathological conditions. Intense, repeated and sustained nociceptive signalling results in central sensitisation in the spinal dorsal horn. Neuronal and biochemical processing at the central synapses at these sites are transitioned into a "pain-facilitatory" state, involving the increased expression of pro-inflammatory receptors and mediators [470, 471]. These adaptations have been well-characterised and extensively reviewed [123, 471, 472], but briefly involve the phosphorylation of many receptors, which can increase synaptic efficacy by promoting receptor trafficking to the membrane. These conditions enable low-threshold sensory fibres, which are usually activated by innocuous stimuli, to activate high-threshold sensory neurons [472]. Critically, glial and immune cell-derived mediators modulate excitatory and inhibitory synaptic transmission by binding to receptors on pre- and post-synaptic terminals in the spinal dorsal horn [123]. This results in nociceptive hypersensitisation, which is characteristic of central sensitisation [123].

TLRs are expressed by glial cells and their involvement in central immune signalling plays a pivotal role in the initiation and maintenance of heightened pain states [473]. However, the reactive phenotype of some glia is always not synonymously connected with pro-inflammatory and pro-nociceptive phenomena. Interestingly, some reactive glial cells have been shown to exhibit anti-inflammatory properties, which contribute to the resolution of nociceptive hypersensitivity [319, 474]. Recently, it has been argued that gliosis may be considered a beneficial process which assists the host in healing, yet when glial processes become dysfunctional, a condition referred to as a "gliopathy", they are key players in pathological pain states [475, 476]. From this, it is plausible that the reactive phenotype of astrocytes observed in the acute 5-FU model in this thesis, were in fact exerting a

protective effect. Although the simultaneous increased expression of IL-1β was indicative of a regional pro-inflammatory response, no anti-inflammatory cytokines were assessed in this model. We can only presume that the reactive astrocytes and the increase in IL-1β were linked. We are unable to determine whether the increase in spinal thoracic IL-1β expression was mediated by immune signals from the inflamed intestine or whether the pro-inflammatory change was driven by reactive astrocytes. Future studies clarifying IL-1β receptor changes on astrocyte membranes during the astrocyte response to 5-FU, would mechanistically elucidate the findings from these works. Identifying a complete cytokine profile would enable detection of changes in pro- and anti-inflammatory cytokines. Additionally, the assessment of pain behaviours would further identify whether the spinal cord glial adaptations were associated with pain in rats with 5IGT.

Although it is widely recognised that microglia are the "first responders" in various models of pain [477-481], astrocytes are increasingly becoming recognised for having a primary role in pain conditions, such as acute pain associated with arthritis [482]. Recently it was shown that microglial signalling was actively involved in the transformation of astrocytes from a quiescent to reactive state [483]. Whilst the acute 5-FU model presented in this thesis observed adaptations in thoracic spinal astrocyte reactivity marker expression with no change in microglial reactivity marker expression, it is possible that microglial changes would have presented at the earlier stage of intestinal inflammation (day one or two) [480]. Such changes in microglial expression/phenotype may have subsided by day three, and accordingly played a role in the initiation of the reactive astrocyte phenotype present at this time-point.

The increased expression of microglial markers in the lumbar spinal region of mice with chronic UC and CA-CRC may have occurred via the amalgamation of numerous "hits" of acute inflammation. In these models, a series of acute inflammatory states were induced by

the DSS water cycles, however over the course of the experimental period, a secondary immune response develops. In the CA-CRC model, the administration of AOM and the repeated “hits” of acute inflammation accelerate the development of tumours in the colon [378]. Accordingly, repeated intestinal inflammatory “hits” may prime (sensitise) lumbar spinal cord microglia and explain the increased expression of microglial reactivity markers observed in these models. The “two-hit” paradigm explains this phenomenon, whereby an initial immune challenge primes microglia to over-respond to subsequent challenges, enhancing pain intensity and duration [484]. It is plausible that the multiple inflammatory “hits” used in both models primed microglia and astrocytes and hence, resulted in the significant increased expression of CD11b/Iba-1 and GFAP reactivity markers, respectively.

Spinal Glial Involvement in Pain Associated with Cancer and CIGT

Although astrocytic marker expression changes are commonly reported in experimental models of cancer-induced pain, the functional and mechanistic adaptations are still unclear. Bone cancer pain is the most widely used model and a commonly reported feature is spinal microglial and astrocyte reactivity [127, 303, 305, 485, 486]. Rodent models have implicated various signalling pathways and receptors in the development of gliosis-dependent bone cancer pain, such as TNF-α, TLR4, metabotropic glutamate receptors and chemokine signalling [127, 303, 305, 486]. Increased expression of the chemokines, CXCL12 and CXCR4, mediate neuronal sensitisation and spinal glial reactivity and have been linked to the development and maintenance of bone cancer pain in rats [486]. Some studies have however, suggested that cancer pain is not necessarily correlated with overexpression of spinal reactive glial markers [487]. This importantly highlights the complex nature of cancer pain with the potential involvement of multiple pain syndromes,

which have inflammatory, neuropathic, compressive and ischaemic mechanisms [488]. It is plausible that in the chronic UC and CA-CRC models, multiple pathways that drive hyper-nociception were occurring simultaneously. Consequently, teasing apart these mechanisms is problematic.

Although recent data has implicated a role for spinal gliosis in the development of pain associated with CIGT, few studies have identified the mechanistic origins for these findings. Innate immune receptors, such as TLR4, are expressed on microglia and astrocytes [489, 490]. Wardill *et al*. (2016), reported that TLR4 mediated irinotecan-induced gut toxicity and pain in mice, which was attributed to increased spinal lumbar astrocyte reactivity and heightened facial grimace scores [134]. TLR signalling has also been shown to impair host-microbial communication mediating gut commensal bacteria and critically, contribute to the severity of intestinal damage followed by chemotherapy [491]. Coller *et al.* (2015), identified a diagnostic marker capable of predicting CIGT in certain cancer types via TLR2 and TNF-α genetic variability [492]. Whilst the studies presented here highlight the involvement of innate immune signalling in pain associated with CIGT, mechanisms underpinning the spinal gliosis presented in this thesis remain undetermined. However, it is plausible that the acute or chronic intestinal inflammation induced by 5-FU, DSS or AOM/DSS, led to reactive gliosis in the spinal cord, which in turn may contribute to pain signalling. To clarify the mechanistic pain-related implications, further studies examining the role of innate immune signalling, such as the TLR4 pathway, as well as utilising rodent pain measures, such as Von Frey or hot plate testing, should be explored.

Further complicating innate immune signalling in CIGT are enteric immune cells, namely EGC which are prone to becoming reactive following systemic chemotherapy administration. EGC reactivity has been reported to contribute to intestinal damage in two

CIGT models using chemotherapy drugs with different mechanisms of action [301, 493]. In Swiss mice, irinotecan enhanced S100β marker (specifically released by EGC in the intestine) and GFAP expression, increasing TNF-α and IL-6 expression, yet reduced enteric neuronal (HUC/D marker) expression in the small intestine [301]. In another 5IGT model which utilised the same markers, a similar trend of intestinal EGC up-regulation (increased co-expression for GFAP and S100β) and reduced neuronal (HUC/D) expression were attributed to the S100B/RAGE/NFκB pathway [493]. In this model, the S100β inhibitor, pentamidine, reduced intestinal glial reactivity (attenuating GFAP and S100β expression) and improved neuronal loss, as shown by an increase in HUC/D expression. Whether EGCs communicate with their central counterparts remains undetermined and should continue to be investigated. This knowledge may lead the way in novel preventative or treatment approaches which target EGC pathologies, and in turn, they may have a positive effect on astrocyte reactivity in the CNS. From this, the mechanisms of signalling between EGC and central glial cells present as a possible pathway mediating pain in not only CIGT, but other gut disorders, such as IBD and cancer. Future gut inflammatory models which examine the central and enteric glial components (GFAP and S100β), as well as the role of innate immune signalling (TLR-4) and pro-inflammatory cytokines, will assist in unravelling the complex nature of these findings, offering insight to further peripheral-to-central mechanisms which may influence CIGT and CICI.

Implications for Chronic UC- and CA-CRC-Induced Reactive Glia in the Hippocampus

It is increasingly becoming evident that central comorbidities occur in gut disorders with an inflammatory component. These are heavily researched in IBD and in oncology, where malignancy and systemic chemotherapy often induce multiple toxicities in the periphery (gut) and centrally (cognitive) in a subset of patients [44, 494]. The concept that intestinal

inflammation results in altered hippocampal neurogenesis [39, 93, 344] was the foundation for exploring whether acute and chronic intestinal inflammation results in central GFAP and CD11b/Iba-1 expression changes. Astrocytes form an integral part of the synapse and contribute to the molecular signalling of the tripartite synapse [154] which has been extensively reviewed elsewhere [154, 495-499]. Accordingly, the concept of glia-neuron bidirectional signalling is widely accepted. Given the role glia play in healthy brain function, it is not surprising that glia may play a role in the early stages of various diseases of the CNS, involving neurodegeneration. The widely recognised perspective on microglia being the "first responders" has indeed, been challenged as emerging data has revealed the dynamic exchange that occurs between hippocampal astrocytes and synapses [500]. A study using hippocampal slices containing the simultaneous manipulation of neurons and astrocytes, provided evidence for astrocytes changing morphologically and behaviourally within minutes of an immune challenge [500]. In order to determine which glial cell was the "first responder" in the gut inflammatory conditions presented in this thesis, a full characterisation of the time-course for the acute- and chronic-induced glial dysregulation is required.

The essential role microglia have in central homeostasis enables them to contribute to many central pathologies, and potentially influence cognition. Microglia release cytokines, neurotransmitters and extracellular matrices which, regulate neuronal and synaptic activity involved in functional plasticity [501-503]. In early development, unchallenged microglia regulate neurogenesis in the sub-granular zone of the hippocampus by eliminating excess newborn neurons which are processed by apoptotic pathways [504]. It has been suggested that microglia have a dual role in neurogenesis [505]. Acute LPS exposure induces microglial phenotype changes which have detrimental effects on the survival and differentiation of neural stem cells in the adult brain [506, 507]. The morphology, phenotype and cytokine expression changes observed in microglia when they transition

from an acute to chronic insult, mean that they may be detrimental in the early stages of acute injury, yet offer a supportive or even protective role in the later stages of injury. Taken together, the functional role for the elevated Iba-1 protein expression in the hippocampus of rats with chronic UC and CA-CRC may have been a beneficial adaptation. Importantly, determining the morphology, phenotype and cytokine profile of glia under the disease states presented in this thesis, will offer insights into the mechanistic (detrimental or beneficial) alteration of their expression. For example, ramified glia may be contributing to neuroinflammation, yet in this state they may also be releasing neurotransmitters (glutamate and dopamine) into the synapse, which are critical for functional plasticity. To tease apart these potential beneficial or detrimental outcomes, it is suggested that the simultaneous release of neurotransmitters be examined alongside the microglial-mediated cytokine profiles.

A summary of the findings from this thesis and new questions relating to central glial changes in the various gut inflammatory models are presented in Figure 7.1. The chronic models reported suggest a role of peripherally-mediated hippocampal glial reactivity and we propose that this may contribute to or exacerbate CICI symptoms. Further studies utilising behavioural models to elucidate learning and memory consequences, immunohistochemistry for morphological cell changes, and cytokine immune profiling, will clarify mechanisms directly relating to microglial and astrocyte responses under these experimentally-induced conditions. Furthermore, the concept of an “astrocyte activation spectrum” [508] and debate surrounding the beneficial and detrimental heterogenous phenotype of microglia [509-511], highlight the need for continued research into the varied cellular profiles and functional significance of glial cells in both acute and chronic inflammatory states. From the bottom up, spinal cord glial reactivity has the potential to mediate intestinal microbiota changes and contribute to nociceptive signalling (Figure 7.1).

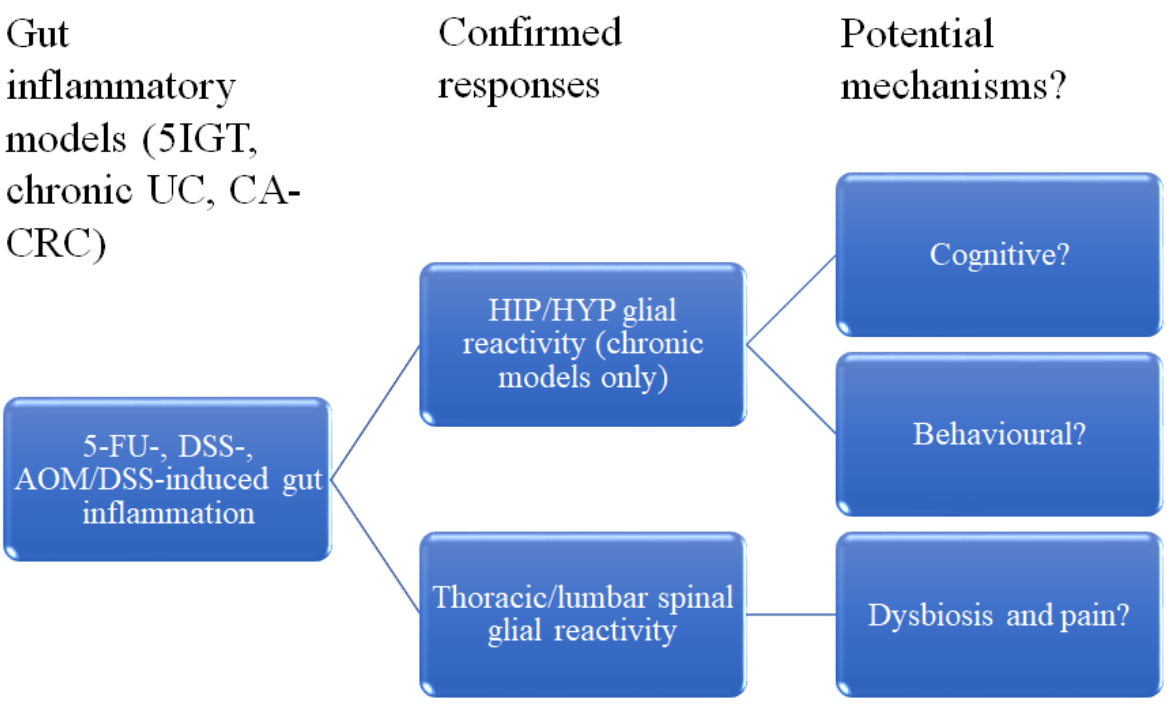

Figure 7.1. Proposed mechanisms from the neuroimmune findings presented in this thesis. The gut inflammatory models (5-FU, DSS and AOM/DSS) confirmed that spinal cord glial reactivity occurred in both acute and chronic settings. Additionally, the chronic gut models confirmed that some higher order brain regions resulted in altered glial reactivity. We propose that the changes in glial reactivity in the higher order brain regions may contribute to or exacerbate CICI (cognitive and behavioural). Finally, the changes in spinal cord glial reactivity is postulated to contribute to intestinal microbiota changes and pain signalling. 5-Fluorouracil; 5-FU, 5-FU-induced gut toxicity; 5IGT, ulcerative colitis; UC, colitis-associated colorectal cancer; CA-CRC, dextran-sulphate sodium; DSS, Azoxymethane; AOM, hippocampus; HIP, hypothalamus; HYP.

Implications for Hypothalamic Glial dysregulation

The hypothalamus (and HPA axis; stress system) is responsible for the maintenance of basal and stress-related homeostasis [512]. The hypothalamus senses a wide array of inputs from the periphery, such as blood-borne cytokines. Acute activation of the stress system results in various physiological and behavioural responses, such as accelerated motor reflexes, increased tolerance of pain, and inhibition of immune-mediated inflammation [513]. The increased expression of the circulating pro-inflammatory cytokines, such as IL-1β and TNF-α, plays a major role in activating the HPA axis [514, 515]. As IL-1β and TNF-α are elevated in patients with CIGT, IBD and colorectal cancer [516], it was

postulated that hypothalamic glial reactivity marker expression changes would occur in these experimentally-induced disease states. The increased expression of GFAP and the dysregulated distribution of Iba-1 in the hypothalamus of CA-CRC mice implied that, in this region, glia were sensitive to immune-mediated input from the gut. As the hypothalamus integrates immune signals and responds accordingly, it is plausible that in chronic gut inflammatory conditions, regional glial cells in close proximity to the hypothalamus, respond by becoming dysregulated or reactive to assist the host in healing.

Although GFAP and Iba-1 changes in the hypothalamus of CA-CRC rats demonstrated glial dysregulation, the absence of glial changes in the chronic UC (and acute 5IGT) animals implies that the addition of the malignancy in this model, may at least partially explain the glial discrepancies. Here, in these preliminary findings we report gross protein expression changes. Future studies should clarify the functional significance by undertaking immunohistochemistry for cellular morphological adaptations and visualisation of cell positioning via immunofluorescence and confocal imaging. Due to the significant impact the hypothalamus has on the immune system and vice versa, emphasis on mechanisms involving the neuroimmune (glial) system and the HPA axis warrant further exploration. This may lead the way for new therapeutic approaches that target HPA-neuroimmune signalling, which may alleviate symptoms associated with chronic inflammatory disorders, such as CA-CRC.

IL-1β and Neuroinflammation

The pro-inflammatory function of IL-1β is to respond to tissue injury via recognising PAMP and DAMP signals released by the injured tissue [517]. IL-1β mediates tightly regulated innate immune responses which rapidly induce the transcription of multiple genes in many cell types, from monocytes to endothelial cells [518, 519]. Simultaneously,

these genes are capable of causing a positive-feedback loop which amplifies the IL-1β response by inducing its own expression [520]. IL-1β is a key cytokine associated with CIGT and CICI aetiology [1, 468]. It is for this reason that Chapters Three and Four sought to explore the potential for IL-1β expression changes to occur in the brain and spinal cord of rodents with experimentally-induced CIGT. Additionally, astrocytes and microglia express IL-1β and in their heightened reactive states increase synthesis and receptor availability for this cytokine [521]. In particular, morphological changes in microglia, together with the up-regulation of central IL-1β, has accrued formidable experimental support for the role they play in amplifying neuroinflammation in various CNS disorders, such as Alzheimer's disease [375, 522-524]. In specific brain regions, increased IL-1β expression is also a potent mediator of cytokine-induced sickness responses, which closely mimic chemotherapy-related toxicities [80, 82]. Recently it has been shown that the rapid increase in expression of microglial IL-1β (mRNA) preceded elevations in Iba-1 immunoreactivity following systemic LPS administration in mice [525]. This study also showed that IL-1β mRNA in astrocytes was significantly increased and correlated with sickness responses in these animals. As previously mentioned, the cognitive and gastrointestinal symptoms associated with chemotherapy treatment mimic some of the physiological and behavioural adaptations in cytokine-mediated sickness responses.

Further studies examining the inflammatory profile of the experimental models used in this thesis will determine whether the brain IL-1β expression changes can be linked to glial dysregulation, though chronic chemotherapy models should be used as they are more clinically representative. The thoracic spinal IL-1β findings in the rats treated with 5-FU may be directly related to the altered astrocyte reactivity observed in this model. However, it is difficult to ascertain whether the observed IL-1β findings were a consequence of

astrocyte reactivity, or whether astrocyte reactivity mediated this pro-inflammatory response.

As well as being involved in CNS diseases, IL-1β is also critical at initiating astrocyte reactivity [526, 527] and plays an active role in central regenerative processes [528]. Specifically, IL-1β-mediated astrocyte reactivity is paradoxically influential in its effects on the synapse and the microenvironment: it is able to release cytokines with pro- and anti-inflammatory properties, trigger the expression of chemokines, provide trophic factors and alter gene expression, which can result in interrupted astrocyte-to-astrocyte communication [517, 529-531]. Nonetheless, in the 5IGT model presented in this thesis, we propose that the thoracic spinal IL-1β expression changes were contributing to a neuroinflammatory microenvironment, due to the simultaneous up-regulation of GFAP and thus, astrocyte reactivity observed in this region.

The Use of Probiotics to Improve Symptoms Associated with CIGT: Implications for 5IGT-Induced Microbial Changes

The recent expansion of evidence linking microbiota changes to a range of gut- and centrally-mediated disorders warrants investigation of these mechanisms. We have only just begun to unravel the complex gut microbial genome in terms of the mechanistic implications of dysregulated microbial populations under chemotherapy conditions. The well-characterised mucositis model by Sonis [1] made no mention of the intestinal microbiota, yet investigations since the early 2000's have indeed reported that chemotherapy exposure can have a profound effect on intestinal microbial composition [38, 215, 532]. Furthermore, the microbiota has been implicated in carcinogenesis and is reported to influence the efficacy and toxicity of some anti-cancer therapies [358, 359]. The changes in caecal microbial composition of rats with 5IGT support evidence of

microbiota changes in CIGT, owing to the reduced abundance of bacterial diversity and increased abundance of *Bacteroides.* The individual changes in species abundance requires further exploration as the mechanisms relating specifically to the pathogenesis of 5IGT are not clear. As the microbiome plays an integral role in maintaining intestinal integrity, it appears that microbiota changes may contribute to alterations in intestinal permeability. Under chemotherapy conditions, it should also be clarified which event occurs first: do microbial changes precede impaired intestinal permeability or vice versa?

Given the microbial perturbations identified in this thesis, treatments with probiotics may have broader implications for CICI. The use of probiotics in alleviating symptoms associated with CIGT have been generally supported by clinicians, yet studies have only recently identified mechanisms supporting this consensus. Pre-treatment with probiotics prior to chemotherapy ameliorated intestinal mucosal injury and intestinal permeability induced by 5-FU in rats [316]. The probiotic mixture used in this study, improved mucosal architecture, ameliorated MPO expression and reduced intestinal pro-inflammatory cytokine profiles. Critically, this study also reported that the probiotic treatment was able to restore caecal microbiome homeostasis, as well as reversing the 5-FU-induced TLR-2 and TLR-4 expression changes in the ileal mucosa. Although the precise mechanisms relating to microbial-immune and microbial-intestinal barrier interactions are largely unknown, gut microbial-host interactions are mediated through TLRs and have been extensively reviewed elsewhere [22, 23, 238, 491, 533-535]. In this sense, chemotherapy-induced microbial perturbations act as a catalyst in the initiation of innate immune responses and may have played a role in the astrocyte changes evident in the rats with 5IGT. As astrocytes express TLRs, it is plausible that the 5IGT-microbial changes triggered an innate neuroimmune response via TLR signalling, although thorough investigations will be needed to confirm this suggestion.

Recent findings which implicate reduced species diversity and bacterial overgrowth of *Bacteroides* in intestinal barrier dysfunction and priming the neuroinflammatory response to brain injury (stroke) support the overarching hypotheses presented in this thesis [21]. The reduced diversity of species and the increased abundance of *Bacteroides* identified in the caecal microbiota of rats with 5IGT may, in this same manner, partially explain the spinal neuroimmune adaptations observed via thoracic GFAP up-regulation. Research specifically relating to these findings within the context of oncology, whereby chemotherapy induces intestinal barrier dysfunction, microbiota changes, neuroimmune dysregulation and cognitive impairment, are lacking. This opens a path for discovery into the potential microbiome-neuroimmune links mediating CIGT and CICI responses (Figure 7.2). Importantly, this highlights the potential for targeting the microbiome to protect or enhance central processes in inflammatory conditions of the gut.

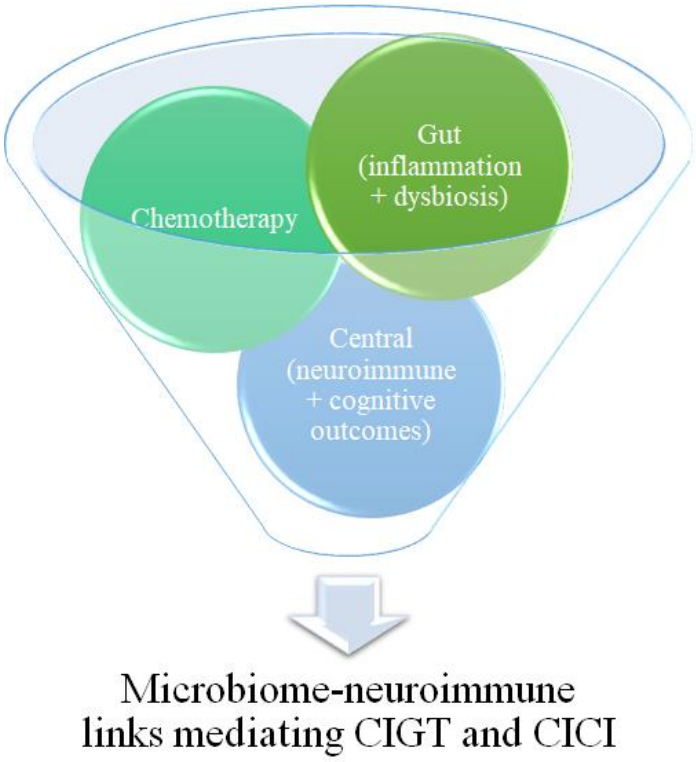

Figure 7.2. Potential microbiome-neuroimmune link mediating CIGT and CICI. Chemotherapy induces gut responses (intestinal inflammation and microbiota changes; CIGT). Chemotherapy also results in central responses (neuroimmune dysregulation and cognitive impairment). It is suggested that chemotherapy-induced gut inflammation and microbiota changes contribute to or potentiates central responses, such as glial dysregulation and cognitive impairment. Highlighting a potential microbiome-neuroimmune mechanism linking CIGT and CICI .

Allergy, GI and Cognitive Disturbances in BCS: A Novel Paradigm Warranting Further Exploration

In the final research chapter of this thesis, the proposed concept that an innate immune system primed by allergy susceptibility may be linked to, or exacerbate, CICI and CIGT symptoms, was unchartered territory. The preliminary findings in this chapter indicated an association between allergies and treatment-induced GI disturbances and PLMD. Whilst more rigorous investigations with greater power are necessary to validate these findings, to our knowledge this was the first study identifying an association between allergies and PLMD and GI issues in BCS. Importantly, this implied that allergies may serve as a predictive tool for patient susceptibility to cognitive and GI chemotherapy-induced toxicities. If these preliminary findings are indeed confirmed, the potential for clinicians to offer/advise on taking preventative measures, such as increasing physical activity and taking probiotics prior to commencing anti-cancer treatment, may reduce patients' risk of developing, or the severity of, GI and cognitive disturbances. Additionally, the relatively simple and blunt nature of the allergy question presented here presents further queries. For example, are 'better' immune-based predictive biomarkers available that can be identified prior to treatment, which predict outcomes based on the immune reactive phenotype? Future investigations should employ pre-treatment, during treatment and post-treatment subjective measures as well as effective control groups to generate reliable group comparisons.

Targeting the Neuroimmune System to Alleviate Symptoms Associated with CICI and CIGT - Attenuating Glial Reactivity and IL-1β Expression as Potential Therapies

Although the gut-induced neuroimmunological complications presented in this thesis require further exploration to identify symptom-related associations with CICI and CIGT,

several therapeutic approaches are presented for consideration. Some glial attenuators, such as minocycline, have provided neuroprotection in stroke and alleviated depressive symptoms in bipolar disorders and multiple sclerosis [536-538]. However, blocking the inflammatory activities of reactive microglia can exacerbate certain CNS diseases [539, 540]. Emerging evidence targeting the neuroimmune system has resulted in positive effects on some psychiatric and cognitive symptoms across a range of central disorders, such as LPS-induced cognitive impairment, Alzheimer's disease and addiction [541-544]. Minocycline may improve some cognitive parameters, yet differences in its effect on hippocampal neurogenesis in adult compared with aged mice, imply the potential variance in microglial functions across the lifespan of an animal, implicating limitations in its use for different age groups [545]. Considering the elevated hippocampal GFAP and Iba-1 expression observed in both chronic models in Chapter Five of this thesis, we can assume that reactive gliosis was occurring. It was unclear whether this regional glial reactivity was having a negative or positive effect on cognition, or even whether it had an effect at all. LPS induces cognitive dysfunction in rats via prolonged activation of hippocampal microglia [546, 547].

Further, the increased expression of immune molecules associated with hippocampal microglial and astrocyte reactivity in neurodegenerative disorders, such as Alzheimer's disease, provides evidence of the strong association between hippocampal glial reactivity and cognitive outcomes [129, 498]. Thus, it was most likely that under the chronic conditions presented in this thesis, microglia and astrocytes were contributing to neuroinflammation in this region.

Morphological examination of glial cell states, including detailed cytokine profiling and assessment of pain and behavioural outcomes, involving hippocampal-dependent learning and memory tests, will clarify the mechanistic implications of these findings. In order to

tease apart the distinction between whether gut inflammatory disorders have the ability to potentiate cognitive deficits, particularly in oncology, it is recommended that a full characterisation of both the short-term and delayed (time-course) central- and gut-related consequences of CIGT and CICI be assessed in the same animals. This would be achieved by comparing the aforementioned parameters in a healthy group, a non-malignant/non-chemotherapy but inflammation (acute versus chronic UC) group, a non-malignant/chemotherapy (5-FU alone or in combination with other chemotherapies – acute and chronic dosages) group, a malignant (comparing breast to colorectal cancer)-non-chemotherapy group and a malignant/chemotherapy group. This kind of thorough examination has the potential to clarify the synergistic and functional effects of gut-mediated glial dysregulation in the spinal cord and higher order brain regions. Ultimately, it will offer insight into the influence gut-mediated neuroimmunological manifestations may have on cognitive outcomes in pre-clinical studies, having important implications for cancer patients in the longer term.

Following on from this, targeted neuroimmune therapeutic approaches may indeed improve symptoms associated with both distinct regional toxicities.

In models where neuroinflammation plays a pathogenic role in the development of central disorders, focussing attention on the inflammatory profile of reactive glial cells may offer a deeper understanding of the cognitive adaptations. Slight increases in IL-1 have been shown to improve hippocampal-dependent memory in mice, whereas substantial deviations from normal physiological levels impair cognition, including ablation of IL-1 signalling [548]. Several lines of evidence have linked increased expression of IL-1 to treatment-related symptom clusters in cancer patients [549] which, as previously mentioned, mimic cytokine-induced sickness responses. This response has been specifically related to cognitive deficits in breast cancer patients [60, 468]. Although the involvement of IL-1β in

the pathogenesis of CICI and CIGT has been strongly supported, therapies targeting this signalling molecule may have detrimental downstream effects as its central and peripheral inflammatory functions are critical in host homeostasis.

Opioids have been a commonly prescribed analgesic to alleviate pain associated with cancer and treatment-related toxicities [550], yet the burden associated with opioid-induced adverse events may significantly influence patients' symptomatology [551]. Adverse side-effects associated with opioids have the potential to exacerbate GI and cognitive symptoms in cancer patients. For example, dry mouth, nausea and vomiting are regularly reported in patients undergoing chemotherapy (whether diagnosed with CIGT or not), yet are also adverse events following opioid exposure. Centrally, opioids cause drowsiness and confusion, which can further complicate CICI symptoms in cancer patients. The myriad of confounding opioid findings presented in Chapter Three, support the suggestion that caution should be taken by clinicians when prescribing opioids to cancer patients. The analgesic benefit for opioid use may be negatively outweighed by not only the adverse effects on patient symptomatology, but also that spinal glial and IL-1β changes have the ability to influence pain signalling and activity in higher order brain regions that contribute to normal cognitive function. The mechanistic implications of the central neuroimmune adaptations in 5IGT and analgesic interventions therefore warrant further exploration. Identifying behavioural, learning and memory deficits, as well as pain and histological assessment of glial morphology, will assist in better understanding the influences these effects have on CIGT and CICI.

The multifaceted nature of the neuroimmune system in the outcomes of various central physiological processes makes glial- and IL-1β-induced manipulation challenging. As mentioned, the risks for complications may outweigh the benefits observed, and a one size-fits-all approach may be unfeasible. For instance, long-term treatment of cognitive or

psychiatric deficits by pharmacologically blocking microglia, may prevent microglial cells from exhibiting their neuroprotective functions later in life. One of the major limitations in pharmacologically attenuating neuroimmune activity resides within the need to preserve the neuroprotective function of glia and IL-1β yet reduce the deleterious outcomes; requiring careful consideration. Accordingly, continued research on the short-term and delayed consequences of glial attenuation is of paramount importance.

Exploring Preventative Treatment Strategies that have Potential in Improving Cognitive Outcomes or Reversing Neuroinflammation in CICI

Anti-inflammatory paradigms have been examined in the search for therapies that improve cognitive outcomes in central disorders. For example, the protective effect of NSAIDs in Alzheimer's disease emphasised that rather 'simple' and well-established therapies have the potential to improve cognitive outcomes [552]. However, this treatment approach may be unsuitable in the context of CICI, whereby chemotherapy-cancer-host interactions are complex, including the involvement of drug-drug interactions. Interestingly, the findings in Chapter Three showed that CAR (NSAID) ameliorated spinal thoracic GFAP and IL-1β expression in rats with 5IGT. Although we were unable to conclude whether the increased expression of GFAP and IL-1β was beneficial or negative, CAR displayed a robust ability to reverse this effect, whereas CAR elevated spinal thoracic CD11b in rats with 5IGT. Comparatively, CAR reduced CD11b expression in the hippocampus of rats with 5IGT, yet 5-FU alone was unable to alter CD11b expression. CAR also attenuated MPO expression in the ileum of rats with 5IGT, which confirmed that this drug has both gut- and central-mediated effects. Considering CAR co-administration elevated thoracic CD11b, yet attenuated GFAP expression in rats with 5IGT, regionally, this NSAID may induce differential effects on glial cell populations at this disease time-point. Accordingly, NSAID

therapies may present as an effective mediator of both spinal and hippocampal neuroinflammation in such settings. Future studies should explore the potential for pharmacological intervention using NSAIDs to improve neuroinflammation in central disorders, including CICI.

Current evidence indicates that the pharmacological management of cognitive alterations in CICI is generally unfavourable [553]. Turning the attention away from reversing cognitive alterations to either enhancing cognitive function or preventing onset is a more practicable approach. Non-pharmacological approaches utilising cognitive behavioural therapy and computerised cognitive training have yielded conflicting results [554, 555, 556]. Nonetheless, the speed of processing training and memory training have produced positive findings, which supports the use of these tools to improve specific cognitive domains in CICI [554]. Although these strategies are promising, methodological challenges have prevented concrete recommendations for clinical practice. Accordingly, the formation of the International Cognition and Cancer Taskforce has recommended principles and guidelines to overcome these pitfalls in making future assessments standardised [557]. These recommendations, such as assessing cognitive parameters prior to commencing anti-cancer treatment, will improve the homogeneity of research designs and facilitate inter-study comparisons (including meta-analyses).

The rather “simple act” of exercise or increasing physical activity has been shown to be extremely efficacious in ameliorating cognitive deficits across a wide range of experimental models, from mild cognitive impairment to multiple sclerosis and CICI [274, 5558, 559]. Several mechanisms mediating the beneficial cognitive effects of physical activity include neurogenesis, synaptogenesis and angiogenesis [560]. As neuroinflammation is a common underlying feature of many neurodegenerative diseases, it has been hypothesised that physical activity could minimise brain diseases via modifying

glial-induced neuroinflammation [561]. Exercise has also shown promise in ameliorating pain and reversing glial hyperactivity in the dorsal horn of mice with nerve injury-induced neuropathic pain [562]. From these novel findings, it is proposed that exercise may improve cognitive symptoms in CICI patients, ameliorate altered central glial reactivity, and potentially offer pain relief to rodents with CIGT, CICI, chronic UC and CA-CRC. Whilst at a first glance this approach seems appealing on many levels (affordability, sustainability, global health benefits – improving perceived quality of life, practicality, etc.), patient cooperation to perform exercise or increase their level of activity may prove difficult. At the heart of this difficulty is that some of the very symptoms 'the act' may improve, may also contribute to patients' inability to make such lifestyle changes during this already tumultuous time in their lives. For example, fatigue, gastrointestinal disturbances, depression and anxiety, and difficulties with cognitive processes (including the sheer act of remembering to exercise) may inhibit CICI, UC and CRC patients' ability to exercise. Nonetheless, the importance of exercise in managing cognitive deficits should be fundamentally emphasised in the early stages of disease and diagnosis, regardless of disease type or treatment strategies, provided that patients are fit to do so.

Evidence for Neural- and Humoral-Mediated Peripheral-to-Central Immune Signalling Pathways in the Development of CIGT and CICI

Throughout this thesis, it was proposed that neural- and/or humoral-mediated peripheral-to-central immune signalling pathways would mediate the gut inflammatory-induced spinal and higher order brain glial and IL-1β dysregulation (Figure 7.3). It was anticipated that either the actions of the drugs or the immune signals released from the drug actions (e.g. damage to intestinal epithelial layer) would induce glial/IL-1β changes via the neural or humoral immune signalling routes. In view of the spinal-mediated glial and IL-1β changes

in the collection of these works, it was proposed that the effects of 5-FU, CAR, BUP, TRAM, DSS and AOM/DSS on the various models of gut inflammation, indirectly resulted in spinal cord changes which were innervated by the inflamed gut region. This was considered an indirect pathway as the action of the drug induced intestinal inflammation, which in turn, indirectly mediated the activation of the neural pathway through immune signals from the inflamed intestine (see Figure 7.3; yellow arrows). This neural route has been extensively researched and reviewed in its influence on sickness behaviours [25, 145, 256, 383]. In order to determine whether this route was indeed activated and responsible for the glial/IL-1β spinal changes evident in these models, surgical vagotomy interventions are suggested.

The glial changes observed in the hippocampal region implied that the humoral route was potentially mediated by two mechanisms (Figure 7.3; blue arrows): 1) drugs directly crossing the BBB and causing central damage, or 2) immune signals from systemic infiltration of mediators and cytokines released by inflamed gut crossing the BBB and causing neuroinflammation. Teasing apart the two mechanisms presented here is challenging in this setting, as the drugs induce gut toxicity which will always ultimately result in increased expression of pro-inflammatory cytokines in the systemic circulation. Assessing hippocampal glial changes in an experimental model which utilises a chemotherapy drug that does not result in severe gut toxicity may help to elucidate whether the humoral route is indeed responsible for these changes. Further detailed assessment of cytokine profiles (as mentioned above) will also assist in teasing apart this mechanism.

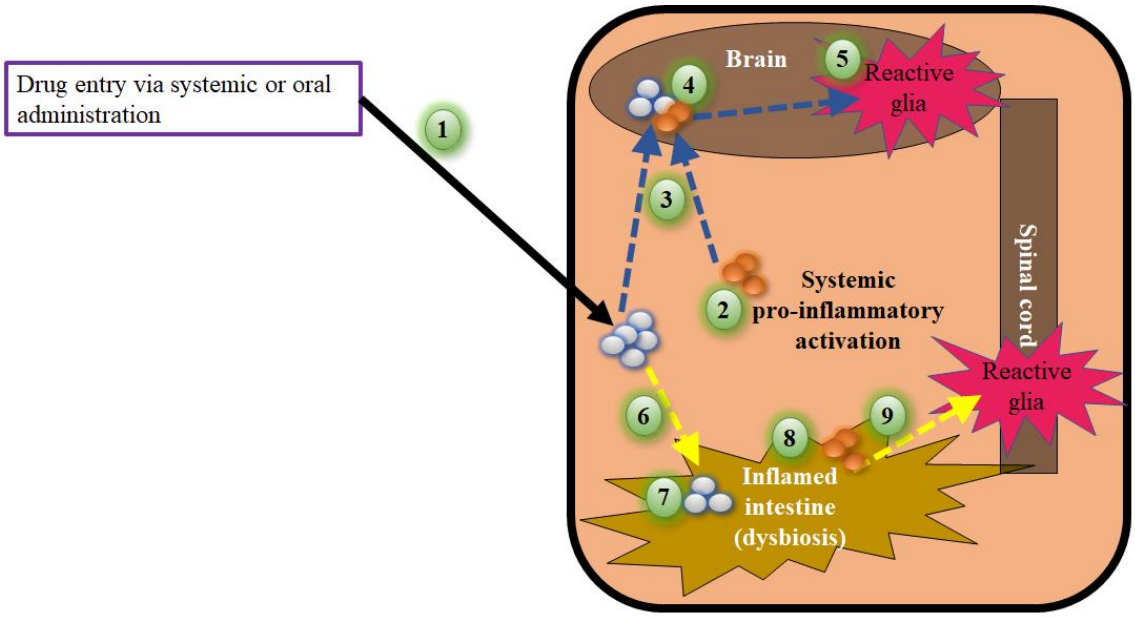

Figure 7.3. Neural vs humoral peripheral-to-central immune signalling pathways. HUMORAL PATHWAY (blue arrows): 1) Drug enters circulation via systemic (intraperitoneal administration of 5-FU/INDO/AOM or subcutaneous CAR/BUP/TRAM) or oral (DSS) administration; 2) Drug induces systemic pro-inflammatory activation; 3) Drugs or pro-inflammatory cytokines from the peripheral circulation access the brain, *directly* crossing the BBB via the humoral route; 4) Drugs or intermediate pro-inflammatory molecules at BBB travel to higher order brain regions; 5) Drugs or intermediate pro-inflammatory molecules activate glia in higher order brain regions. NEURAL PATHWAY (yellow arrows): 1) Drug enters circulation via systemic or oral administration; 2) Drug induces systemic pro-inflammatory activation; 6) Drugs target rapidly dividing intestinal cells; 7) Resulting in an inflamed intestine and microbiota changes; 8) Pro-inflammatory cytokines and mediators signal primary afferent neurons in the intestinal wall; 9) Pro-inflammatory cytokines and mediators are transduced into a neural message and relayed to higher order brain regions via afferent signalling pathways (dorsal horn of the spinal cord). 5-Fluorouracil; 5-FU, indomethacin; INDO, azoxymethane; AOM, carprofen; CAR, buprenorphine; BUP, tramadol; TRAM, dextran-suphate sodium; DSS.

Concluding Remarks

Cognitive and gut disturbances continue to be some of the most debilitating side-effects of chemotherapy treatment. The ever-increasing incidence of cancer, and hence the acute-gut and delayed-cognitive consequences of chemotherapy, pose a considerable burden on the health and well-being of millions of cancer patients, their families, clinicians and national health systems. A complete understanding of the molecular mechanisms of glial dysregulation in CICI and CIGT will aid greatly in the development of new strategies and treatment approaches to alleviate chemotherapy-related toxicities.

The central glial alterations presented throughout this thesis have provided evidence for neuroimmune adaptations to occur under acute chemotherapy and chronic inflammatory conditions of the gut. The significance of these findings requires further morphological and functional investigation, to determine whether the neuroimmune adaptations contribute to, or potentiate the pathogenesis of either disorder. As the dichotomous nature of glial cells and their reactivity orchestrates both beneficial and detrimental processes in health and disease, harnessing their neuroprotective mechanisms may enhance the beneficial outcomes in CNS disorders. It will be important to understand the functional implications of the varying states of glial reactivity, to define the regional differences in the models presented here. Moreover, in an effort to develop urgently needed therapies that improve the gastrointestinal and cognitive status of cancer patients, it is also necessary to probe the molecular and cellular targets of IL-1β, as an influential driver of many inflammatory processes.

Chemotherapy disrupts the delicate equilibrium of bacterial communities in the GIT and recent data on host-microbiota-neuroimmune interactions highlights the need for continued research. Understanding and normalising the microbial balance of commensal bacteria in the intestine could be an appropriate strategy to improve not only the intestinal

architecture, but also the cognitive status of patients receiving chemotherapy. In addition, the novel allergy-related findings in the BCS patient population may serve as a predictive tool for clinicians to identify patients, who may be at a higher risk for developing more severe gut or cognitive disturbances following chemotherapy treatment. Critically, further studies with higher statistical power may have the potential to introduce early intervention strategies that can improve the negative gut and cognitive outcomes of not only BCS, but also patients with other cancer-types. Allergy-cognitive relationships should be further explored in other gut inflammatory disorders which have a cognitive/mood component.

The increasing rates of cancer survivorship and negative impact CIGT and CICI have on patient quality of life, highlight the need for continued research into the common biological pathways and mechanisms linking the disparate disorders. From the bottom up, this thesis confirmed that several gut inflammatory conditions influence the neuroimmune system, as identified by glial adaptations in spinal and higher order brain regions. In conclusion, it is anticipated that the present findings will promote future research efforts that will ultimately improve our understanding and treatment of the central comorbidities associated with gut inflammatory disorders. In particular, the ultimate aim will be to significantly improve the livelihood of patients with gut disorders, involving cancer, chemotherapy and inflammatory bowel diseases – I know the patients, survivors, their families and friends will certainly look forward to a future like this.

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Appendix One

Cancer Medicine & Anti Cancer Drugs

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Research Article OMICS International

Neuroimmunological Manifestations of Chemotherapy Exposure: Implications for Mucositis, Glia and Cognition

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Received date: November 14, 2016**Accepted date:**December 15, 2016**Published date:**December 20, 2016

Abstract

Chemotherapy drugs reduce quality of life often causing acute and delayed central side-effects, termed chemotherapy-induced cognitive impairment (CICI). Another dose-limiting chemotherapy-induced side-effect is oral and intestinal mucositis which results in significant gastrointestinal (GIT) damage and intestinal inflammation. Recent interest has been paid to neurological complications arising in patients with gut disorders, yet little attention has been paid to the role GIT damage plays in CICI. Our current understanding of neuronal adaptations and behavioral consequences resulting from immune system dysregulation has paved the way for investigation into the neuroimmunological manifestations associated with chemotherapy. In a clinical setting cancer patients experience a cluster of symptoms, similar to that manifested in cytokine-induced sickness responses. Accordingly, it is suggested that peripheral inflammatory events, such as chemotherapy-induced mucositis, may indirectly cause glial dysregulation and potentiate cognitive changes in CICI. Perhaps it is time to examine the cancer experience in a multidisciplinary manner, in order to encapsulate the direct and indirect mechanisms underlying treatment-related side-effects. Specifically, understanding the neuroimmunological implications of chemotherapy-induced mucositis will provide further insight into the direct and indirect mechanisms underlying CICI pathogenesis.

Keywords: Chemotherapy-induced cognitive impairment; Chemotherapy-induced mucositis; Neuroimmune signalling pathways; Glial reactivity; Neuroinflammation; Microglia; Astrocytes

Introduction

Chemotherapy drugs have proven invaluable in treating many cancers and improved the outcome for millions of cancer patients worldwide. Whilst the ultimate goal of chemotherapy is to prevent malignant cells from metastasizing, chemotherapy drugs are generally non-specific as they also target healthy, non-malignant cells. Chemotherapy induces a range of acute and delayed side-effects. In the central nervous system (CNS) the phenomenon is clinically recognised as chemotherapy-induced cognitive impairment (CICI) [1]. Peripherally, chemotherapy drugs negatively affect the gastrointestinal tract (GIT) lining causing oral and/or intestinal mucositis. Although mucositis is an acute disorder which usually resolves upon treatment cessation, it is often a dose limiting side-effect due to the painful nature of the disorder [2,3]. Traditionally, these chemotherapy-induced side-effects have been considered to be separate disorders. However, recent evidence suggests that bidirectional communication pathways connecting the GIT and CNS may be implicated in the pathogenesis of both disorders. These pathways regulate a myriad of physiological and immune functions in health and various disease states manifesting from the periphery or centrally [4].

Peripheral inflammatory events or immune insults trigger a characteristic cluster of behavioural, cognitive, and affective changes, which are commonly referred to as cytokine-induced sickness responses [5,6]. Interestingly, many symptoms associated with cytokine-mediated sickness responses mimic the cognitive and behavioural changes commonly reported by chemotherapy recipients, including learning and memory dysfunction, fatigue and depression [7]. Cancer and chemotherapy exposure are associated with substantial immune dysregulation, involving inflammation [8], changes in cytokine levels [9] and mucositis which may be contributing to cognitive changes. Nonetheless, previous studies have failed to determine whether a link exists between these already established, yet disparate side-effects of chemotherapy. This review proposes that neuroimmune mechanisms and glial dysregulation may contribute to CICI symptoms both directly and indirectly via a peripherally driven inflammatory event: chemotherapy-induced mucositis.

As we unravel the complex aetiology of CICI, it soon becomes clear that the challenge in examining CICI lies within the cluster of symptoms cancer patients' experience. In a clinical setting, cancer patients reporting cognitive dysfunction often concurrently experience depression, anxiety, sleep deprivation, fatigue and pain [10-13]. Accordingly, Lee *et al.* proposed a biological basis for cancer (and cancer treatment) related symptom clusters; a cytokine-based neuroimmunological mechanism [14]. This concept stems from well-established studies which indicate that cytokine-induced sickness behaviours can be evoked by exposing animals to either infectious, inflammatory or certain pro-inflammatory cytokines [5,6]. Additionally, various gut disorders have been associated with psychological and cognitive comorbidities (reviewed below). From this

Citation: Bajic JE, Howarth GS, Johnston IN, Hutchinsona MR (2016) Neuroimmunological Manifestations of Chemotherapy Exposure: Implications for Mucositis, Glia and Cognition . Cancer Med Anticancer Drug 1: 105.

it can be concluded that direct and indirect mechanisms may be contributing to cognitive changes observed in the chemotherapy setting. Neuroimmunological approaches in managing cancer and treatment-related side-effects may pave the way for novel and effective therapeutic and preventative approaches, ultimately improving the quality of life of cancer patients and survivors worldwide.

Gut disorders and cognition

CNS dysfunction has been recognised as a prominent feature in functional gut disorders, including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) [15,16]. The idiopathic diseases comprising IBD include ulcerative colitis (UC) and Crohn's disease (CD). UC usually involves the colon and ileum whereas CD mainly involves the rectum and colon. IBS, however, is currently viewed as being caused by dysregulation of the gut-brain axis. Whilst the symptomatology of both disorders includes pain, altered bowel movements and a host of physical, emotional, psychological and cognitive responses, IBD is strongly associated with intestinal inflammation unlike IBS.

The aetiology of functional gut disorders is complex and multifactorial, however it has been suggested and somewhat accepted that IBD and IBS may be triggered by psychological, environmental or physical stressors [17,18]. Consequently, several pathophysiological factors negatively affecting the gut-brain axis are pivotal to the disorders and include stress, chronic pain and immune activation [19,20]. Although a substantial body of literature exists linking stress, chronic pain and immune activation to cognitive deficits, this area in the context of functional gut disorders has received little attention. Nonetheless, primary studies assessing these pathological factors in this patient population have shown deficits in specific aspects of cognition, such as verbal IQ [21]. In CD, regional morphological differences in cortical and subcortical structures have been critically linked to abdominal pain [22].

Psychopathological factors, such as depression and anxiety, are frequently observed to have effects in patients with functional gut disorders, and have been shown to play a role in cognitive deficits [23]. Approximately, 70% of patients with functional gut disorders experience some psychological comorbidity [24]. It is unclear as to which disease develops first, however it is well accepted that stress and anxiety are associated with IBS/IBD. Regardless of this, the impact of functional gut disorders on psychological processes is undeniable as the stress associated with symptom progression severely affects patients' quality of life. Taken into account, a biopsychosocial model has been proposed to clinically approach and conceptualise IBS pathophysiology [25]. This model encompasses a lifetime perspective from the patient's childhood through to their adult life integrating genetic, environmental, learning, stress and traumatic events. Fundamentally, it takes into account the interaction of the mind and emotions, the brain, the enteric nervous system (discussed later) and the intestinal microenvironment, including food, the immune system and microbiota. Literature evidence demonstrates that functional gut disorders are strongly associated with a range of psychological comorbidities, specifically cognitive impairment; yet our understanding of the central consequences of other gut disorders, such as chemotherapy-related mucositis remains undetermined. Many chemotherapy drugs, such as oxaliplatin and 5-Fluororuacil (5-FU) are responsible for inducing gut disorders and cognitive impairment, yet whether these comorbidities interact remains to be elucidated.

Gut disorders caused by chemotherapy: mucositis

The pathogenesis of mucositis was defined in five phases by Sonis [2]. Mucosal barrier injury may occur throughout the entire GIT and result in oral and/or intestinal mucositis. The rapidly dividing epithelial layer lining the GIT is particularly prone to tissue injury from different chemotherapy drugs including 5-FU, methotrexate and cyclophosphamide. Consequently, apoptotic pathways are initiated in healthy mucosal tissue causing reduced cellular proliferation in the small intestine. Some hallmark characteristics of intestinal mucositis include villus atrophy, shallow crypts, inflammation and ulceration. Mucositis results in a heightened inflammatory response via the up-regulation and activation of various transcription factors, ultimately resulting in elevated circulating pro-inflammatory cytokines, in particular interleukin-1 beta (IL-1β) and tumour necrosis factor-alpha (TNF-α).

The most significant phase of mucositis for patients is during the ulceration phase as this involves loss of mucosal integrity. Painful ulcerating lesions in the GIT become susceptible to microbial infiltration and in severe cases can lead to bacteraemia and sepsis [2]. The clinical symptoms of mucositis generally begin five to ten days after chemotherapy treatment and include significant pain, abdominal bloating, nausea and vomiting, diarrhoea and constipation [26]. Although mucositis is an acute phenomenon which usually resolves once chemotherapy treatment has ceased, treatment may be prematurely ceased as a result of progressively worsening symptoms. Current guidelines for the prevention and management of mucositis fail to reveal effective treatment options [27]. Whilst mucositis pathogenesis is well understood, the indirect central effects of mucositis remain unknown. The complex neuroimmune axis has been suggested to be implicated in depression, a comorbidity of cancer diagnosis, and chemotherapy exposure [14]. Neurological manifestations from elevations in cytokine levels imply that neuroimmunological mechanisms underlying the pathogenesis of these chemotherapy-induced side-effects may be at play.

Cognitive changes following chemotherapy exposure

Although reports of cognitive decline in chemotherapy patients pre-date the 1980's, systematic research only commenced in the 1990's. Patients collectively termed the cognitive disturbances "chemobrain" or "chemofog" which heavily impacted daily functioning and quality of life, yet initial complaints were dismissed by doctors and the scientific community [7]. Previously, it was assumed that the brain was protected from systemically administered chemotherapy drugs by the blood-brain barrier (BBB) and additionally, cognitive symptoms could be explained by the stress, anxiety and depression associated with cancer diagnosis. Extensive research in the recent years clearly indicated that many systemically administered chemotherapy drugs readily cross the BBB inducing structural, molecular and cellular changes that impact upon cognitive function (see review 1) [1]. Mechanisms underlying the pathogenesis of CICI remain to be elucidated although suggestions include hippocampal damage and immune dysregulation (discussed below). In order to better understand the suggested mechanisms, it is important to review the clinical evidence and understand the negative impact imposed upon patients.

Clinical evidence of CICI

The main cognitive domains affected by CICI are executive functioning, attention and concentration, processing speed, reaction

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time, motor speed and dexterity [1]. Whilst current estimates of CICI prevalence differ greatly (14% to 85%), the worldwide prediction of cancer incidence reaching 70 million in 2020 highlights the need for continued research [28]. Consequently, the estimate of high survivability rates for many cancers results in increased survivor numbers and in turn, we will see an increase in the incidence of post-treatment issues [29]. Those affected by CICI experience stressors in many facets of their lives, including relationships (familial, friends and colleagues), employment, self-esteem/worth and finances; leading to a reduced quality of life. CICI patients commonly expressed frustration in having difficulty with simple tasks, such as remembering names, misplacing everyday items and trouble finding common words [30,31]. Emotions regularly described by CICI patients included distress, anxiety, frustration, irritability, depression and embarrassment [30-33]. Many summed up their feelings by describing as if they "felt stupid" or were "going crazy" and sometimes related their memory disturbances to the fear of being at risk for early dementia or Alzheimer's disease [30,34]. This evidence collectively supports the negative impact of CICI on patients and emphasises the increased amount of effort and time required to complete everyday tasks.

Breast cancer cohorts and duration of cognitive effects

Whilst the majority of CICI studies focus on breast cancer populations, cognitive deficits in a range of cancer types have been investigated, including myeloma, testicular and ovarian cancer [35-37]. Nonetheless, breast cancer populations offer researchers completion of extensive retrospective studies due to their typically good prognosis, allowing for more thorough evaluations of parallel short- and long-term sequelae [38-40]. The duration of cognitive changes is of particular interest to patients and for this reason, continues to be an area of much research. There is considerable variability surrounding the duration of chemotherapy-induced cognitive deficits or even existence of the phenomenon. Majority of the studies report improvement in cognitive symptoms after a period of time, yet some studies have indicated the presence of symptoms for ten-twenty years after treatment cessation [41-43]. Functional magnetic resonance imaging and neuropsychological testing was observed in a group of breast cancer survivors who had received adjuvant chemotherapy treatment and was compared with a breast cancer control group who were not treated with chemotherapy [42]. The chemotherapy group demonstrated hyporesponsiveness in executive functioning tasks performed 10 years post-treatment, indicating significant long-term cognitive impairments when compared to the non-chemotherapy control group.

Neuroimaging studies have identified structural and molecular changes associated with chemotherapy treatment. Reductions in specific brain regions, such as frontal cortex, temporal lobes and cerebellar grey matter regions have been reported in breast cancer patients [44]. These reductions were evident for twelve months post-chemotherapy cessation yet improvements were reported in most regions four years later. Global brain networks become re-organised under chemotherapy treatment and thus, indicate a reduced ability for information processing [45]. Additionally, chemotherapy-induced white matter tract alterations may be interpreted as demyelination or axonal damage [46].

Animal models of CICI

Animal studies have confirmed that central structural and molecular changes may be accountable for the cognitive domains affected by common chemotherapy drugs. Several studies in rodents report declines in abilities to perform behavioural tasks following single drug administration of many chemotherapy drugs, including 5-FU, methotrexate and oxaliplatin [47-49]. Rodent behavioural tests have been adopted to understand the central pathological changes following systemic chemotherapy exposure, such as fear conditioning, novel object recognition and the Morris Water maze. These behavioural adaptations may be interpreted as hippocampal and frontal cortex region alterations which importantly, overlap with the brain structures implicated in CICI [1].

Specific CNS cell populations are sensitive to a range of chemotherapy drugs. One of the most widely reported central changes following chemotherapy exposure is reduced hippocampal cellular proliferation. This has been documented to occur with cyclophosphamide, methotrexate, thioTEPA and 5-FU [50-52]. These CICI animal models suggested that the hippocampal changes were associated with the hippocampal-dependent behavioural changes and memory deficits. Although cyclophosphamide most frequently reports cognitive changes and cellular alterations, negative findings on long-term hippocampal changes have also been reported [53]. Nonetheless, stem cells of the dentate gyrus are particularly susceptible to chemotherapy toxicity [54-56]. This is important to note as neurogenesis within the dentate gyrus is responsible for the proliferation and division of neural stem cells that form into new neurons or astrocytes, playing a pivotal role in hippocampal circuit plasticity and memory consolidation [57,58]. Consistent with patient observations of leukoencephalopathies and white matter tract lesions, animal and *in vitro* studies have shown that both mature oligodendrocytes and their precursors may be susceptible to the action of chemotherapy drugs [1]. Whilst there is clear evidence that specific central cell populations are susceptible to reductions in cellular proliferation following chemotherapy exposure, some studies have reported no changes [59,60]. This evidence reflects the complex aetiology of CICI, indicating various structural, molecular and cellular changes contributing to cognitive impairment following chemotherapy exposure. The aforementioned studies fail to take into account neuroimmune mechanisms that may be at play, whether directly or indirectly. Perhaps it is time to consider the impact other chemotherapy-induced peripheral inflammatory events may be having on CICI, such as immune challenges in the context of malignant tissues, more specifically gut toxicities, such as mucositis.

The contradiction: host immunity, dysregulation and cancer

The ultimate goal of the immune system is to protect and defend the host from infection and insults by recognising, repelling and eliminating pathogens and foreign molecules. Further, inflammation is an essential defensive response resulting in physiological processes critical in host healing. The toll that both malignancies and chemotherapy treatments have on the host is particularly enigmatic in the context of the immune system, whereby complex inflammatory processes contradict and manipulate responses; a dynamic network that primarily ensures protection against foreign pathogens whilst remaining tolerant of self-antigens. This somewhat contradictory phenomenon results in immune dysregulation which in turn, may result in central effects via the neuroimmune interface and signalling pathways.

Inflammatory processes become dysregulated in cancer and anti-cancer treatments. On one hand, endogenous immune processes and inflammatory cascades attempt to eliminate malignant cells from the

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host. Yet, simultaneously within malignant cells, similar pathways are initiated and inflammatory signalling molecules contribute to cancer establishment and progression. Several lines of evidence have suggested inflammatory processes are the seventh hallmark for cancer establishment and progression [8,61,62]. To further complicate matters, chemotherapy treatments are associated with increased circulating inflammatory markers, yet suppression of immune activity is commonly reported (discussed below). It is well established that immune dysregulation occurs in several disorders negatively affecting the CNS and in some cases, the gut. To illustrate this point, a few disorders, such as neuropathic pain will now be further discussed.

Immune dysregulation in animal models

Several convergent lines of experimental and clinical evidence have supported the hypothesis that pro-inflammatory cytokines are pivotal in the pathophysiology of not only cancer-related and anti-cancer treatment-induced symptoms, but other disorders, including chronic fatigue syndrome, neuropathic pain and major depression. Elevated circulating pro-inflammatory cytokines, such as (IL-1) and (TNF-α) have been reported in clinical studies examining chronic fatigue syndrome, major depression and various pain states [63,64]. IL-1 action is regulated by a complex network of molecules and is a potent stimulus of corticotrophin-releasing hormone, activating the hypothalamic-pituitary-adrenal axis, an important stress hormone which has been well documented in major depression [63]. Additionally, TNF-α is widely recognised as an important factor in the mediation of major depression, chronic fatigue syndrome and neuropathic pain [63-65]. Rodent models have reported that intraperitoneal administration of TNF-α results in dose dependent pain responsivity, indicative of hyperalgesia (heightened sensitivity to pain) [66]. The hippocampus is associated with pain perception and cognition [67] and accordingly, a rat model of chronic constriction injury of the sciatic nerve reported increased hippocampal TNF-α levels [65]. These studies indeed demonstrate a pivotal role for the aforementioned pro-inflammatory cytokines in the pathogenesis of a range of disorders and disease states. It should be noted that the disorders mentioned in this section also often occur simultaneously in cancer patients undergoing chemotherapy treatment.

Immune dysregulation in cancer and chemotherapy

There is growing consensus on two recognised interactions between cancer and the immune system. Firstly, host immunity has the ability to recognise and reject malignant cells and immuno-surveillance can prevent tumour development and control recurrence. Consequently, activation of the innate immune system leads to the production of highly immuno-stimulatory cytokines, systemic inflammation and T- and B-cell activation, with the goal of eliminating malignant cells. Secondly, many inflammatory mediators and cells involved in detecting and eliminating malignancies also play a key role in the migration, invasion and metastasis of malignant cells, thus promoting tumour expansion [68,69]. This double edged sword results in a plethora of intertwined and complex interactions in which the immune system recognises and tries to reject tumour formations whilst inflammatory processes simultaneously enable tumour progression and development.

Additionally, chemotherapy drugs also induce inflammatory responses which may be either local, around the site of administration or systemic in nature resulting in mucositis. Several chemotherapy drugs including 5-FU (anti-metabolite), etoposide (topoisomerase II inhibitor) and doxorubicin (anthracycline) elevate pro-inflammatory cytokine production *in vitro* [70]. Importantly, this demonstrates that most cytotoxic anti-cancer drugs, regardless of their mechanism of action, increase circulating cytokines. Such findings have been translated into clinical studies linking circulating pro-inflammatory cytokine elevations with common chemotherapy-induced side-effects, such as fatigue, depression, pain and cognitive impairment [71,72]. Extensive studies revealed the importance of elevated circulating pro-inflammatory cytokines in sickness responses which often result in cognitive changes and interestingly, mimic CICI reports. Finding therapeutic approaches that target the immune system has the potential to improve multiple chemotherapy-related side-effects which all have an immune component to their aetiology.

The intimate bidirectional relationship shared between the CNS and the GIT presents as a potential mechanism that may contribute to CICI symptom severities. As such, it is plausible that chemotherapy-induced peripheral inflammatory events, such as mucositis, may trigger central cell population changes. Peripheral-to-central changes occurring via neuroimmunological pathways may result in behavioural (cognitive) changes, similar to those apparent in cytokine-induced sickness responses [5,73,74]. Although a cytokine-based neuroimmunological mechanism of cancer-related symptoms has been suggested [14], CICI researchers are yet to examine the indirect central effects of chemotherapy-induced peripheral inflammatory events, such as mucositis.

"Little brain" to "big brain" inflammation and signalling pathways

The ability of the enteric nervous system (ENS) to self-regulate (hence "little brain") and act similarly to the CNS ("big brain") makes it the largest and most complex division of the peripheral nervous system [75]. Previous literature has suggested that the GIT is a vulnerable passageway through which pathogens may influence the CNS and lead to abnormalities, for example, neuroinflammation contributing to autism [76] and multiple sclerosis [77]. A well-established link exists between various neurodegenerative diseases and the role neuroinflammation plays in their pathogenesis [78,79]. However, few studies have examined the influence of peripheral-to-central immune signalling and neuroinflammation in the context of chemotherapy-induced mucositis and CICI.

Inflammation in the "little brain": enteric nervous system inflammation

The ENS contains more than 400-600 million neurons [80] and an extensive network of enteric glial cells (EGC). Although EGCs support enteric neurons, the precise mechanisms by which EGCs support enteric neurons remains to be fully elucidated. EGCs share similarities with their CNS counterparts, astrocytes in morphological, functional and even molecular capabilities [81]. As well as exerting protective functions, EGCs are key players of the ENS during intestinal inflammation and immune responses. Their intimate relationship with enteric neurons and their responsiveness to local inflammation makes them a prime target for therapeutic intervention as has been investigated in the CNS with targeting glial cells.

From our understanding of the intimate bidirectional relationship shared between the GIT and the CNS, it is not surprising that a diverse range of neurodegenerative diseases arise from systemic infections and inflammation, such as multiple sclerosis and Alzheimer's disease

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[76,82]. We have all experienced the change in mood, emotion and cognition when one is faced with systemic infection, a cold or influenza. Numerous reports indicate that this immune response is driven by a dialogue between the peripheral systemic infection and our brain [73]. The gastrointestinal immune system is considered the primary immune organ of the body as it induces and maintains peripheral immune tolerance. This is achieved via complex cellular networks with specialised immuno-regulatory functions, including interactions between the microbiota and host. Impaired host immune defences and mutations in pattern recognition receptors leads to GIT dysfunction and enables invasion of pathogens [83]. The downstream effect of such events results in chronic GIT inflammation and/or dysbiosis (a loss of control of local immune responses resulting in an unbalanced enteric microbiota) having substantial implications in the pathogenesis of rheumatoid arthritis, IBD and asthma [84-86]. From this evidence it is clear that GIT inflammatory events may modify central processes controlling behaviour and aligns with our central hypothesis that chemotherapy-induced mucositis may result in central changes via neuroimmune mechanisms involving glia, discussed in more detail below.

Glia: the "other brain"

Glial cells are critical in brain development, function and plasticity in both health and disease and fall into three cell types; astrocytes, microglia and oligodendrocytes. Neurons, astrocytes and oligodendrocytes arise from neural progenitor cells whilst microglial cells originate from peripheral macrophage cell lines [87]. Glia perform a host of regulatory functions within the CNS, from supporting neurons and regulating synaptic neurotransmission, to maintaining calcium homeostasis and clearing intracellular ions and neurotransmitters [88]. A bidirectional communication occurs between neurons and glia (astrocytes and microglia) which is now widely accepted as the neuroimmune interface; the tripartite and tetrapartite synapse describes this complex intertwined relationship in health and disease [89,90].

Glia plays a vital role in various aspects of brain function. The ambiguities of glial cells in health go far beyond our current understanding and deserve much more attention. An area of particular interest is the mechanism by which these central immune cells are involved in the pathogenesis of CNS disease states. Several researchers have gained valuable insight to this question and begun to unravel the mechanisms by which glia contributes to the pathogenesis of neurological and neurodegenerative diseases, such as Alzheimer's disease, neuropathic pain, ischaemia and migraine. The common thread linking these diseases is glial priming and subsequent neuroinflammation.

"Big brain" inflammation

Microglia and astrocytes may become reactive or primed either from direct-central insults or indirect-peripheral inflammatory events triggering neuroinflammatory responses. Microglia is highly sensitive to insults so are the first to react, unlike astrocytes which respond more slowly and in a more controlled manner [88]. In their reactive states, both glial cell types undergo morphological changes augmenting a cascade of detrimental functional outcomes leading to tissue damage and neuronal death [91]. In particular, reactive glia overproduce prostaglandins, pro-inflammatory cytokines, chemokines, mediators and reactive oxygen and nitrogen species having detrimental effects on neuronal function and survival via oxidative stress [92]. Primed glia reduces output of anti-inflammatory molecules, decrease neurotrophic support, dysregulate calcium, glutamate and brain derived neurotrophic factor resulting in excitotoxicity and neuroinflammation [93]. Interestingly, both cell types may remain in a primed state whereby they continue to be sensitised after the initial stimulus has resolved. Although primed glia appears active due to their morphological form, they do not overproduce inflammatory mediators until challenged, whereby they react quickly and elicit an exaggerated immune response [94]. In particular brain regions this may influence behaviours involving cognition [89,95].

Glia modulates neurotransmission and cause neuronal injury via various mechanisms including a reduced ability to produce neurotrophic support, excitotoxic glutamate-receptor mediated damage and oxidative stress [96]. Glutamate is the primary excitatory neurotransmitter instrumental in neuronal plasticity and thus, key in learning and memory consolidation [97]. The glutamate transporters GLAST and GLT-1 are localised on astrocyte membranes [98]. Reactive astrocytes undergo reduced expression of glutamate transporters and lose their ability to re-uptake glutamate, yet continue to release glutamate into the synapse [99,100]. Additionally, reactive astrocytes inhibit production of glutamine synthetase, an enzyme that converts extracellular glutamate to glutamine, vital in neuroprotection [101]. From this, it is not difficult to see that a significant feature of many neurodegenerative disorders is reactive or primed glia, and subsequent neuroinflammation. In the context of chemotherapy exposure, inflammation (central or peripheral) occurring via either direct or indirect mechanisms may trigger glial dysregulation and neuronal consequences, impacting negatively on cognition.

The host immune system utilises innate immune signalling to recognise microorganisms, detected by molecular structures shared by a large number of pathogens; exogenous microbe-associated molecular patterns (MAMPs) and endogenous molecules (danger-associated molecular patterns; DAMPs). Toll-like receptors (TLRs) represent a class of innate immune receptors belonging to the IL-1/TLR superfamily and act as pattern recognition receptors capable of responding to MAMPs, DAMPs and more recently, xenobiotics (XAMPs) [102]. XAMPs represent foreign chemicals that include alcohol, methamphetamine and cocaine [103]. The mechanism by which XAMPs modify glial expression levels and morphology via TLRs may then present as a plausible mechanism contributing to CICI.

Although reactive glia might start as a beneficial process responding to an insult (disease, trauma, infection or drug exposure), it may, depending on the nature, duration and intensity of the insult, turn to a detrimental neuroinflammatory state. Defining neuroinflammation is by no means a simple task; however, it is generally accepted to include microglial and astrocyte reactivity and increased expression of pro-inflammatory cytokines and chemokines [104]. Chronic neuroinflammatory states are known to contribute to neuronal loss and central homeostatic disturbances. It is widely accepted that systemic inflammation influences brain function and behaviours. The last two decades have revealed the pivotal roles microglia, astrocytes and neuroinflammation play in various neurodegenerative diseases. In addition to neurodegenerative diseases and central injuries, neuroinflammation has also been implicated in neuropathic pain, schizophrenia, epilepsy and perhaps most recently, cancer and cognitive decline following chemotherapy exposure [105-109]. Of particular interest to this review, is the potential for chemotherapy drugs to influence glial cell populations in both the brain and the spinal cord, having implications in cognition and pain pathways.

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Various chemotherapy drugs appear to be causing a generalised glial response which is not limited to specific drug classes [108-110]. These studies primarily focussed on the direct-central effects of chemotherapy exposure, not accounting for the potential of GIT damage to indirectly exacerbate central changes via neuroimmune pathways. Peripheral-to-central immune signalling pathways offer a potential way in which peripheral inflammatory events, such as mucositis may be implicated in CICI.

"Little brain" to "big brain" signalling

Histories of abuse, life stressors and other psychological factors have been shown to play an important role in the onset of various functional bowel disorders [111,112]. As information is relayed in a bidirectional manner between the gut and the brain, it makes sense that the CNS may be modified by gut dysregulation. Information from the "little brain" to the "big brain" may be relayed via afferent neurons connecting the gut to the CNS. Pathways responsible for the transmission of various endocrine, neuronal, paracrine and humoral signals are vagal, humoral or neural. The vagus nerve provides a cytokine responsive neural pathway indirectly triggering the brain via afferent vagal input or leaky circumventricular organs [113].

Peripheral immune messages, such as locally produced pro-inflammatory cytokines may travel indirectly to the CNS via neural signalling pathways, including but not exclusive to the vagus nerve [74]. Information detected by primary afferent neurons is transduced into a neural message which is then relayed to higher order brain regions. In the brain parenchyma this message is then re-transduced back into an immune message where locally produced cytokines alter brain function by acting either directly or indirectly on neurons or glia. In specific brain regions, this may result in behavioural adaptations, involving cognition and mood. Alternatively, the slower and more direct humoral pathway occurring at leaky circumventricular organs involves molecular intermediates, such as prostaglandins. Local inflammation activates peripheral tissue macrophages to increase release of pro-inflammatory cytokines, such as IL-1β and TNF-α. Consequently, macrophages and endothelial cells release chemokines and adhesion molecules that attract leukocytes [74]. As well as their essential roles in peripheral inflammation, circulating IL-1β and TNF-α are also key initiators of neuroinflammation. From this knowledge, we present these immune-to-brain signalling pathways as potential mechanisms by which chemotherapy-induced intestinal inflammation may directly and indirectly lead to neuroinflammation and glial dysregulation. Pro-inflammatory cytokines and mediators expressed during the pathogenesis of chemotherapy-induced mucositis may access the CNS via leaky circumventricular organs resulting in a neuroinflammatory response.

What the future holds

In the year 2020 it is estimated that 70 million cancer survivors will be disease free [7,28]. Nonetheless, a substantial proportion of survivors will have experienced either acute or delayed cognitive deficits during or post-treatment cessation. Therefore, it is of paramount importance to consider the direct and indirect mechanisms underlying CICI to develop new strategies and treatments that will improve the quality of life of cancer survivors. To date, CICI animal models have failed to consider the impact of peripheral inflammatory responses on cognitive deficits. In fact, in most CICI animal studies, it is almost unquestionable that mucositis tissue damage would have certainly been present, yet these organs were not analysed. This limited angle of analysis may be missing incidental, yet crucial mechanisms in the aetiology of CICI. Irrespective of this, we acknowledge the many challenges faced by researchers undertaking CICI studies and teasing apart both the direct and indirect mechanisms presents with its own myriad of complications. Perhaps now is the time to examine chemotherapy-induced side-effects which more accurately reflect a clinical setting; elucidating how multiple chemotherapy side-effects work in unison.

One might argue that in general, the two major areas of the human body which become dysregulated following chemotherapy exposure are the gut and CNS; the "little" and "big" brains. The above sections clearly illustrate the recent substantial increase in literature implying that brain function is somewhat dependent upon gut function and vice versa. Many questions still remain and research should continue to clarify how the neuroimmune interface and signalling pathways may be implicated in CICI. The literature reviewed presents our theory that chemotherapy-induced intestinal inflammation may drive glial dysregulation via direct and indirect neuroimmune signalling pathways which may ultimately, potentiate cognitive impairment. Harnessing our understanding of these mechanisms and outlining ways in which the gut can modulate brain function and behaviours via neuroimmune signalling pathways may guide us to novel treatment approaches that encapsulate more targeted therapies aimed at treating multiple side-effects of chemotherapy treatment.

Acknowledgements

JB would like to acknowledge the Australian Postgraduate Award and the Freemason's Society for their Supplementary Scholarship. MH is a fellow of the Australian Research Council. GH is a South Australian Cancer Council Senior Research Fellow.

Submission Declaration

The authors hereby declare that this work has not been previously published (except in the form of an abstract for conference presentation), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder. No funding sources were utilised to produce this manuscript.

Conflict of Interest Statement: The authors wish to acknowledge no conflict of interest.

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Appendix Two

frontiers in Behavioral Neuroscience

REVIEW
published: 22 May 2018
doi: 10.3389/fnbeh.2018.00104

From the Bottom-Up: Chemotherapy and Gut-Brain Axis Dysregulation

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The central nervous system and gastrointestinal tract form the primary targets of chemotherapy-induced toxicities. Symptoms associated with damage to these regions have been clinically termed chemotherapy-induced cognitive impairment and mucositis. Whilst extensive literature outlines the complex etiology of each pathology, to date neither chemotherapy-induced side-effect has considered the potential impact of one on the pathogenesis of the other disorder. This is surprising considering the close bidirectional relationship shared between each organ; the gut-brain axis. There are complex multiple pathways linking the gut to the brain and vice versa in both normal physiological function and disease. For instance, psychological and social factors influence motility and digestive function, symptom perception, and behaviors associated with illness and pathological outcomes. On the other hand, visceral pain affects central nociception pathways, mood and behavior. Recent interest highlights the influence of functional gut disorders, such as inflammatory bowel diseases and irritable bowel syndrome in the development of central comorbidities. Gut-brain axis dysfunction and microbiota dysbiosis have served as key portals in understanding the potential mechanisms associated with these functional gut disorders and their effects on cognition. In this review we will present the role gut-brain axis dysregulation plays in the chemotherapy setting, highlighting peripheral-to-central immune signaling mechanisms and their contribution to neuroimmunological changes associated with chemotherapy exposure. Here, we hypothesize that dysregulation of the gut-brain axis plays a major role in the intestinal, psychological and neurological complications following chemotherapy. We pay particular attention to evidence surrounding microbiota dysbiosis, the role of intestinal permeability, damage to nerves of the enteric and peripheral nervous systems and vagal and humoral mediated changes.

Keywords: chemotherapy-induced cognitive impairment, mucositis, chemotherapy-induced gut toxicity, gut-brain axis, microbiota

OPEN ACCESS

Edited by:
Julia Lassolin, Stockholm University, Sweden

Reviewed by:
Attila Szabo, University of Oslo, Norway
Yvonne Ritze, Universität Tübingen, Germany

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Received: *17 January 2018*
Accepted: *30 April 2018*
Published: *22 May 2018*

Citation:
Bajic JE, Johnston IN, Howarth GS and Hutchinson MR (2018) From the Bottom-Up: Chemotherapy and Gut-Brain Axis Dysregulation. Front. Behav. Neurosci. 12:104. doi: 10.3389/fnbeh.2018.00104

INTRODUCTION

The chemotherapy experience is associated with powerful psychological, neurological and somatic side-effects. Cancer diagnosis and the complications arising from treatment induce anxiety and depression, fatigue, pain, and cognitive impairments while patients struggle to maintain hope for recovery and continue normal daily functions, routines and roles (Kuzeyli Yildirim et al., 2005; Downie et al., 2006; Chan et al., 2014). Due to the non-selective and systemic nature of most chemotherapy drugs,

they also target healthy, rapidly-dividing non-malignant cells. The regions of the body most susceptible to the unwanted toxicities of chemotherapy exposure are the gastrointestinal tract (GIT) and the central nervous system (CNS)—the gut and brain. Many chemotherapy drugs are small enough to readily cross the blood-brain barrier and result in molecular, structural and functional changes within the CNS, manifesting as cognitive changes in a subset of patients (Wigmore, 2012). Outside of the CNS, the cells of the GIT are particularly vulnerable to damage following chemotherapy exposure. In particular, epithelial cells within the mucosal layer lining the alimentary tract form prime targets due to chemotherapy drugs targeting proliferating enterocytes (Sonis, 2004). Although the gut and the brain appear disparate, they are intimately connected. The complex network of pathways linking the gut to the brain will be discussed in more detail below as we present mechanisms by which chemotherapy results in gut-brain axis dysregulation.

This network has a bidirectional relationship. For instance, psychological and social factors have the ability to influence motility and digestive function, symptom perception, behaviors associated with illness and the pathological outcome (Bhatia and Tandon, 2005). On the other hand, visceral pain affects central pain perception and pathways, mood and behavior (Chakiath et al., 2015). Importantly, systemic and gut immunity is tightly regulated by the inflammatory reflex and cholinergic anti-inflammatory pathway (Tracey, 2002; Pavlov and Tracey, 2012). Integral components of the inflammatory reflex include innate immune cell activation, release of inflammatory mediators, such as cytokines, vagal innervation and responses from higher order brain regions, such as the nucleus tractus solitarius. Vagal innervation is of particular importance in the chemotherapy setting as it is pivotal in the transmission of chemo and mechanosensory information from the gut to the brain (**Figure 1**; Goehler et al., 2000; Tracey, 2002). In this sense, proinflammatory mediators and cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) activate primary afferent nerve fibers within the vagal sensory ganglia. Vagal ganglia signal several nuclei of the dorsal vagal complex responsible for the integration of visceral sensory input and relay information to higher order brain regions like the hypothalamus, hippocampus and forebrain. Coordinated autonomic and behavioral responses are initiated to assist in restoration of homeostasis. Importantly, efferent vagal motor activity inhibits cytokine synthesis, creating the inflammatory reflex effect. Humoral anti-inflammatory pathways can be activated, stimulating the release of adrenocorticotrophin hormone. Sympathetic outflow can also increase localized adrenaline and noradrenaline expression and further suppress inflammation. The activation of these innate components of the inflammatory reflex, including the vagally-mediated cholinergic

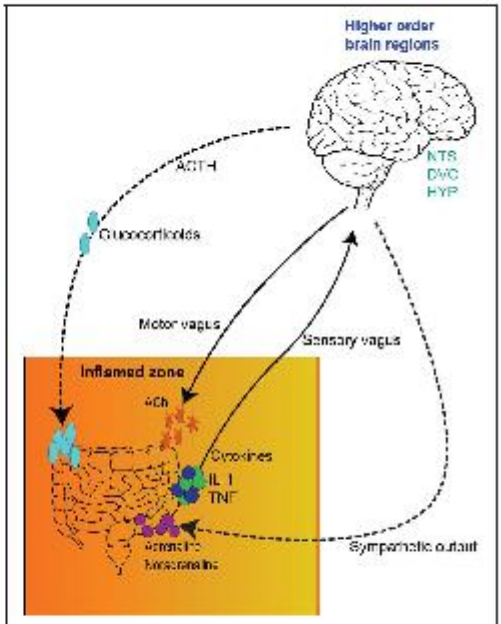

FIGURE 1 | The inflammatory reflex. (1) The inflamed zone represents tissue damage, infection and ischemia. (2) Increased expression of inflammatory mediators and cytokines, such as IL-1β and TNF-α are released by cells in the inflamed zone. (3) Cytokines activate primary afferent neurons within the vagal sensory ganglia. (4) Afferent visceral signals are relayed to nuclei in the dorsal vagal complex (DVC), such as the nucleus tractus solitarius. (5) Visceral information is further relayed from the DVC to higher order brain regions, such as the hypothalamus, hippocampus and forebrain. (6) Activation of efferent vagal motor activity inhibits cytokine synthesis. (7) Hypothalamus activation stimulates the release of adrenocorticotrophic hormone from the pituitary gland, initiating a humoral anti-inflammatory pathway. (8) Sympathetic outflow can increase localized adrenaline and noradrenaline expression further suppressing inflammation. IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor—alpha; DVC, dorsal vagal complex; NTS, nucleus tractus solitarius; HYP, hypothalamus; HIP, hippocampus; FB, forebrain; ACTH, adrenocorticotrophic hormone.

efferent output, ultimately results in the regulation of systemic and localized inflammation, having important implications in gut immunity (**Figure 1**). A more comprehensive outline of the inflammatory reflex has been reviewed elsewhere (Tracey, 2002; Pavlov and Tracey, 2012).

Additionally, activation of the neuroimmune system via glial priming and neurogenic inflammation further complicates immune to brain signaling. Although glial cells are non-neuronal cell types which can be found in the CNS and periphery, such as oligodendrocytes and Schwann cells, for the remainder of this manuscript we specifically refer to microglia and astrocytes. For an in depth analysis of glial priming and neuroinflammation several excellent reviews exist (Araque et al.,

Abbreviations: CICI, Chemotherapy-induced cognitive impairment; ENS, enteric nervous system; PNS, peripheral nervous system; EGC, enteric glial cells; IBS, irritable bowel syndrome; IBD, inflammatory bowel diseases; FGIDs, functional gastrointestinal disorders; BBB, blood-brain barrier; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumour necrosis factor alpha; 5-FU, 5-Fluorouracil; DSS, dextran sodium sulfphate; CIGT, chemotherapy-induced gut toxicity; TLRs, toll-like receptors; CIPN, chemotherapy-induced peripheral neuropathy; 5-HT, serotonin 5-hydroxytryptamine; DCs, dendritic cells.

1999; Bains and Oliet, 2007; He and Sun, 2007; Allen and Barres, 2009; Capuron and Miller, 2011; Parpura et al., 2012; Dodds et al., 2016). Nonetheless, to illustrate this point in the context of cancer and chemotherapy, inflammation (either centrally or locally derived from either the malignancy or chemotherapy) and the release of proinflammatory cytokines signals the brain and activates neuroimmunological cells, glia (**Figure 2**). Proinflammatory cytokines access the brain either directly via leaky circumventricular organs or indirectly via a neural route (e.g., vagal transmission). Microglia and astrocytes form an integral part of the tri- and tetra-partite synapses and form a close bidirectional relationship with neurons; the neuroimmune interface which has wide implications in central health and disease (Allen and Barres, 2009; Graeber and Streit, 2010; Grace et al., 2014; Dodds et al., 2016). Reactive glia undergo morphological changes and overproduce proinflammatory mediators whilst reducing anti-inflammatory output (O'Callaghan et al., 2008; Agrawal and Yong, 2011). Ultimately, glial reactivity results in a neuroinflammatory environment whereby neurotoxicity causes damage to surrounding tissues and neurons (Eikelenboom et al., 2006; Bilbo et al., 2012; Laskaris et al., 2015). Centrally derived neurogenic inflammation and signaling also contributes to the exacerbation of peripheral inflammatory conditions. Although glial reactivity may begin with beneficial intentions by responding to insults (disease, trauma, infection or drug exposure), glia may remain in a primed state and be sensitized even after the initial insult has resolved, eliciting an exaggerated immune responses (**Figure 2**). Critically, in particular brain regions primed glia and neuroinflammation influence behaviors involving cognition and are involved in the pathogenesis of various neurodegenerative diseases and pathological pain states (McGeer et al., 1988; Eikelenboom et al., 2006). Due to the altered immune profile of cancer and chemotherapy patients, it has been suggested that neuroinflammatory processes may be contributing to the cognitive deficits often experienced by this patient group (Myers, 2009; Johnston, 2014). This form of innate immune (peripheral-to-central) signaling represents a plausible mechanism meditating chemotherapy-induced gut toxicity and neurological changes (**Figure 2**).

Following on from this, it is not surprising that interactions between the immune system and the brain become dysregulated under cancer and chemotherapy conditions. Further, recent evidence has highlighted the impact gut commensal bacteria has in both central and peripheral development and health (Feng et al., 2018). Importantly, dysbiosis (microbial imbalance/maladaptation) and gut-brain axis dysfunction have been associated with functional gut disorders having negative effects on cognition (Jones et al., 2006; Frank et al., 2007). Previously, research has focussed on a single pathological manifestation of chemotherapy exposure, for example gut toxicity or regional structural brain changes (Keefe et al., 1997; Christie et al., 2012). Such studies have failed to consider the *indirect* effects of simultaneously occurring treatment-induced toxicities, which may be contributing to the primary pathology under investigation. Consequently, we hypothesize that chemotherapy treatment causes severe and prolonged psychosocial impacts on the survivor. Furthermore, we suggest that the gut-brain axis is an important mediator of a diverse range of cognitive and emotional disorders similar to those experienced by cancer survivors. Here, we will determine whether chemotherapy affects the gut-brain axis and present several key stages. Following on from this, we suggest that the psycho-social side effects of chemotherapy treatment could be caused by the effects of chemotherapy on the gut-brain axis.

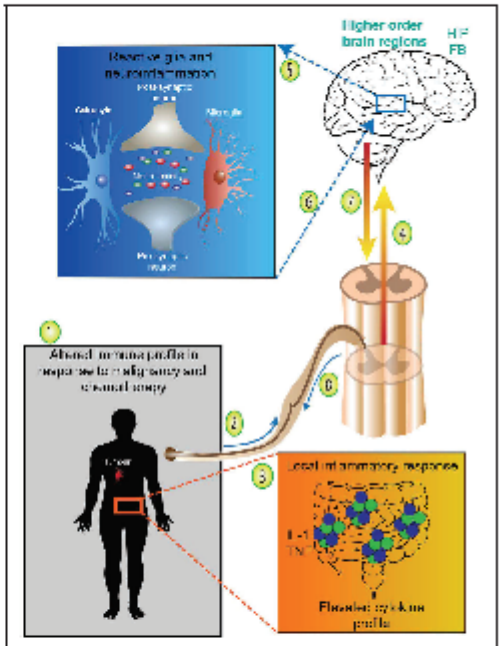

FIGURE 2 | Adapted from Dodds et al. (2016). Neuroimmunological complications arising from cancer and chemotherapy treatment. (1) Cancer patients undergoing chemotherapy treatment express an altered immune profile with increases in proinflammatory cytokines. (2) Systemic proinflammatory cytokines and mediators, such as IL-1 and TNF released either from the malignancy or treatment-associated toxicities access the brain directly via leaky circumventricular organs or (3) indirectly via neural transmission. (4) Systemic or localized proinflammatory mediators and cytokines signal higher order brain regions and (5) activate microglia and astrocytes. Reactive glia undergo morphological changes and overproduce proinflammatory mediators whilst reducing anti-inflammatory output—resulting in neurotoxicity and neuroinflammation. (6) In particular brain regions primed glia and neuroinflammation influence behaviors involving cognition and contribute to various neurodegenerative diseases. (7) Centrally derived neurogenic inflammation and descending signaling in the spinal cord (8) contributes to the exacerbation of peripheral inflammatory conditions and exaggerated pain states. Therefore, peripheral-to-central innate immune signaling represents a plausible mechanism meditating chemotherapy-induced gut toxicity and neurological changes. FB, forebrain; HIP, hippocampus; IL-1, interleukin-1; TNF, tumor necrosis factor.

Following a brief analysis of gut-brain communication, we will review some key studies linking gut-brain axis dysregulation to specific psychiatric disorders, highlighting similarities between these conditions and the chemotherapy setting. From the bottom-up (GIT to the brain) we will examine chemotherapy-induced gut and central changes and present several mechanisms meditating gut-brain axis dysregulation in the chemotherapy setting; focussing on the microbiome, intestinal integrity, peripheral neuron and enteric nervous system (ENS) dysfunction. Finally we will address the role vagal-, neural-, and humoral-mediated responses may play in these complex chemotherapy-induced pathological conditions. Overall, we aim to illustrate the complex role gut-brain axis dysregulation plays in shaping neurological changes associated with chemotherapy exposure.

GUT BRAIN CROSSTALK

Since Pavlov's Nobel Prize-winning discovery on the role neural innervation plays in gastric secretion—the first functional evidence connecting the gut and brain—our understanding of the pathways connecting the CNS and the GIT have significantly advanced (Keller and William, 1950). The multiple bidirectional pathways responsible for controlling signaling from the brain to the gut and vice versa have been extensively reviewed and is outside the scope of this manuscript (Mayer, 2011; Al Omran and Aziz, 2014; Carabotti et al., 2015; Furness, 2016). The complexity of this network is best appreciated in its ability to integrate information from a variety of systems encompassing the central, autonomic and enteric nervous systems (including the influence of the intestinal microbiota), whilst simultaneously considering neuroendocrine, enteroendocrine and neuroimmune input (summarized in **Figure 3**; Carabotti et al., 2015). A brief analysis of bottom-up and ENS mechanisms is necessary to appreciate the systems by which the integration of these pathways influence behavior and impact central comorbidities in disorders of the gut. We begin this section from the bottom-up; presenting key pathways, cell types and signaling mechanisms involved in communication from the gut to the brain. We also illustrate mechanistic evidence relating to disorders of the gut which often have a central comorbidity component, such as in the case of inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS). Whilst research covering the central comorbidities associated with IBD and IBS continues to expand, the potential mechanisms linking neurological and gut changes following chemotherapy exposure remains under investigated.

From the Bottom-Up

The GIT elicits a myriad of functions ultimately resulting in absorption of nutrients and expulsion of noxious chemicals and pathogens via muscular contractions, cellular, endocrine and immune mechanisms. Critically, the gut harbors a diverse microbial community (bacteria, fungi, archaea, viruses, and protozoa) and has prolific central effects mediating a healthy host (Feng et al., 2018). Consequently, changes in gut-microbial composition disrupts physiological homeostasis, often contributing to central maladaptations (Mu et al., 2016; Dinan and Cryan, 2017). Recent advances in our understanding of the impact the microbiota has on the gut-brain axis has led to common use of the term *microbiota-gut-brain axis* (Rhee et al., 2009; De Palma et al., 2014). Microbiota-gut-brain axis communication alters certain aspects of brain development, function, mood and cognitive processes from both the bottom-up and top-down (Catanzaro et al., 2014; De Palma et al., 2014; Mayer et al., 2014; Tillisch, 2014; Carabotti et al., 2015; Barbara et al., 2016). Evidence specifically related to chemotherapy-induced microbiota changes will be discussed further below (see reviews on microbiota-gut-brain axis; Rhee et al., 2009; De Palma et al., 2014; Mayer et al., 2014; Tillisch, 2014).

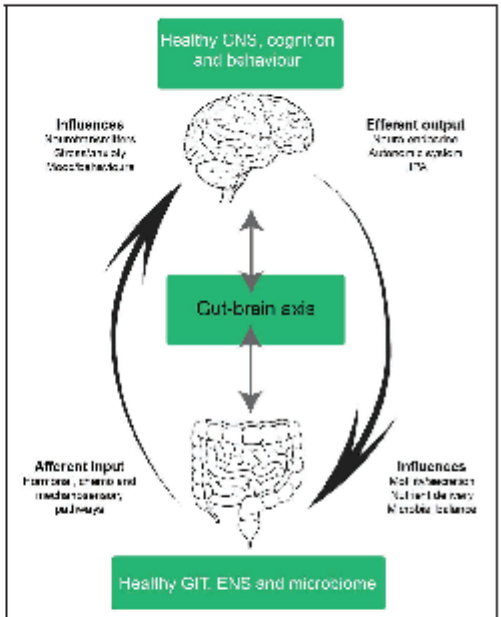

FIGURE 3 | Schematic of a healthy gut-brain axis. The arrows highlight the bidirectional nature of the gut-brain axis; in a balanced system mechanisms from the bottom-up (and vice versa) exist in cohesion. In a healthy system, the gut-brain axis integrates information from many systems; the central nervous system (CNS), autonomic nervous system (ANS), enteric nervous system (ENS), neuroendocrine, enteroendocrine, neuroimmune, and hypothalamic-pituitary axis (HPA). The complex bidirectional communication pathways and systems shared between the gut and the brain maintain health and homeostasis in the CNS, GIT, and microbiota. Efferent signals from the brain involving neuro-endocrine, autonomic and HPA outputs influence motility, secretion, nutrient delivery and microbial balance in the GIT. Whilst afferent inputs from the GIT, such as intestinal hormones, cytokines and sensory perceptions influence neurotransmitter expression, stress, anxiety, mood and behavior. CNS, central nervous system; ANS, autonomic nervous system; ENS, enteric nervous system; HPA, hypothalamic-pituitary axis; GIT, gastrointestinal tract.

The GIT maintains an extensive intrinsic nervous system, the ENS which is unique in its ability to control certain functions

of the small and large intestines even when it is disconnected from the CNS (Furness, 2016). However, the ENS should not be considered fully autonomous due to the constant top-down input it receives. The ENS is the largest and most complex division of the peripheral nervous system (PNS) comprising 400–600 million neurons and an extensive network of enteric glial cells (EGC) (Furness, 2012). EGCs share similarities with astrocytes, their CNS counterparts in the mechanisms they adopt to support enteric neurons, including their morphology, function and molecular capabilities (Gulbransen and Sharkey, 2009). Importantly, EGCs play key roles in mounting an immune response, particularly during intestinal inflammation.

Luminal environmental factors, such as mechanical and chemical changes are signaled from the gut to the brain via endocrine, immune and neuronal afferent pathways (Mayer, 2011; Furness, 2012; Al Omran and Aziz, 2014; Furness et al., 2014). Information regarding the level of distension, concentrations of specific nutrients, electrolytes, pH, and the presence of danger and immune signals is transmitted from the gut to the brain via a wide variety of neural and systemic communication pathways. Visceral changes are detected by a variety of sensory cell types including enterocytes, intrinsic and extrinsic primary afferent neurons, immune and enteroendocrine cells (Carabotti et al., 2015).

Hence, a wide variety of hormones and metabolites from the gut communicate homeostatic information to the brain via functional effector cells (enterocytes, smooth muscle cells, interstitial cells of Cajal, enterochromaffin cells, intrinsic and extrinsic primary afferent neurons, immune and enteroendocrine cells) (Carabotti et al., 2015). Examples of homeostatic information relayed from the functional effector cells include but are not exclusive to sensory, pH, water metabolism, chemical, danger and immune signals, etc.). Each cell type responds to luminal environmental changes and secretes specific signaling molecules which may include but are not exclusive to ghrelin, cholecystokinin, glucagon-like peptide-1, corticotrophin releasing hormone, proteases and cytokines, etc. (Furness et al., 2014). To further complicate gut-brain crosstalk, various neurotransmitters commonly produced centrally are also expressed in the GIT (Furness et al., 2014). Gut derived neurotransmitters, such as dopamine, serotonin and neuropeptide Y influence many aspects of central homeostasis, yet in the gut are responsible for appetite, satiety, hunger, pain and are implicated in the activation of reward pathways relating to food and beverage intake (Furness, 2016).

Numerous afferent and efferent pathways connect the gut and brain, presenting the host with a multitude of platforms for malfunction, dysregulation and disease, both in the periphery and centrally. Whilst the basic principles outlining top-down signaling have been extensively reviewed (Al Omran and Aziz, 2014; Furness et al., 2014; Furness, 2016) and is outside the scope of this review, it is crucial to acknowledge that these effects occur simultaneously with those described from the bottom-up. Importantly, top-down sympathetic and parasympathetic interactions suppress secretion, motility and GI transit, having direct effects on immune-, emotion-, mucosa-, and microflora-related alterations (Lyte et al., 2011; Mayer, 2011). Gut-brain axis dysfunction has played a pivotal role in our mechanistic understanding of various gut disorders and their effects on cognition. Indeed, experimentally induced gut disorders have critically developed our understanding of the mechanisms underlying central changes induced by disruptions in gut homeostasis. Disorders of the gut and chronic inflammation often result in psychological abnormalities, such as anxiety and depression (Nyuyki and Pittman, 2015). Additionally, physiological responses can be induced by stress, for instance triggering relapse in experimental colitis (Bernstein et al., 2010).

Great interest has recently been paid to the importance of gut health on mental health and vice versa. This has become particularly evident in the continual expansion of anecdotal evidence on the central comorbidities associated with various gut disorders, particularly in IBS and IBD (Drossman et al., 1999; Whitehead et al., 2002). Disorders of the gut are commonly associated with poorer mental health. For instance, 54–94% of IBS patients actively seeking treatment also present with emotional, psychological and cognitive comorbidities (Whitehead et al., 2002) as do chemotherapy recipients. The literature presented above provides clear evidence that gut disorders often occur simultaneously with central comorbidities, aligning with our hypothesis that gut-brain axis dysregulation may be mediating both chemotherapy-induced mucositis and neurological changes. Therefore, it is pivotal that we determine the *direct* and *indirect* central consequences of drug-induced gut disorders, such as chemotherapy-induced mucositis. Chemotherapy induces a range of peripheral and central side-effects, significantly reducing quality of life. In the gut this has been termed chemotherapy-induced mucositis and in the CNS, chemotherapy-induced cognitive impairment (CICI). The current review will now explore whether mucositis and CICI are linked and whether they exacerbate other symptoms, such as pain associated with mucositis, or cognitive impairment which are often experienced simultaneously in the chemotherapy setting.

CHEMOTHERAPY FROM THE BOTTOM-UP: THE GUT AND COGNITION

Chemotherapy drugs can be considered paradoxical at the most basic level. Primarily, they offer recipients' survivorship as they target malignant cells in an attempt to rid the host of cancer. On the other hand, due to their non-selective nature, they also target healthy cells and induce a range of side-effects reducing patient quality of life. The organ where their actions are perhaps often first noticed is the GIT due to its high regenerative capacity. Mucositis occurs in up to 70% of chemotherapy recipients and may manifest anywhere along the alimentary tract, termed oral or intestinal mucositis (**Figure 4**; Scully et al., 2003). It is one of the most significant dose-limiting side-effects of intensive anti-cancer therapy due to the painful nature of the disorder.

Sonis classified the pathogenesis of mucositis into five stages (Sonis, 2004). Hallmark characteristics of mucositis include villus atrophy, shallow crypts, inflammation and ulceration. Mucositis results in a high inflammatory response via the up-regulation

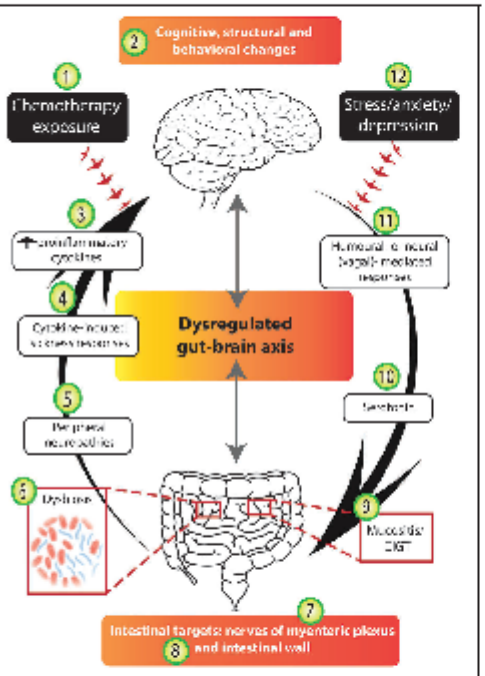

FIGURE 4 | Chemotherapy disrupts several stages of the gut-brain axis. The arrows highlight the bidirectional nature of the gut-brain axis; in an unbalanced system mechanisms from the bottom-up (and vice versa) are disrupted. We suggest that chemotherapy-induced gut-brain axis dysregulation plays a major role in the intestinal, psychological and neurological complications experienced by many cancer patients. Chemotherapy exposure (1) often results in molecular and structural changes in the brain (2), e.g., hippocampal changes as identified in rodent models. Chemotherapy exposure causes cognitive and behavioral changes (2) to a subset of patients and these findings have been supported by some experimental models. The altered immune profile of chemotherapy recipients results in increased circulating pro-inflammatory cytokines (3) which have been reported to cause cytokine-induced sickness-like responses (4) which mimic chemotherapy-induced side-effects. Damage to peripheral nerves resulting in peripheral neuropathies (5) are experienced by some chemotherapy recipients. Chemotherapy targets the intestines and its microbial contents causing dysbiosis (6), impairing the nerves of the myenteric plexus (7), damaging intestinal wall parameters (8), and resulting in mucositis (9). Serotonin dysregulation under chemotherapy conditions (10) may play a role in chemotherapy-induced intestinal and neurological changes. Finally, both humoral and neural/vagal peripheral-to-central immune signaling pathways (11) may mediate chemotherapy-induced gut-brain axis dysregulation. Importantly, we acknowledge that the stress, anxiety and depression associated with cancer diagnosis and treatment (12) may contribute to both bottom-up and top-down pathways, having negative effects in the gut and the brain.

and activation of various transcription factors, ultimately causing elevations in circulating proinflammatory cytokines (**Figure 4**), in particular IL-1β and TNF-α (Sonis, 2004). Whilst mucositis is an acute phenomenon which usually resolves upon cessation of chemotherapy treatment, clinical symptoms generally begin 5–10 days post-chemotherapy exposure and include significant pain, abdominal bloating, nausea and vomiting, diarrhea and/or constipation (Gibson and Keefe, 2006). Although guidelines for the prevention and treatment of mucositis exist, they fail to include effective treatment options (Gibson et al., 2013). Novel complementary treatment approaches are showing positive results utilizing naturally sourced products, such as Emu Oil and Rhubarb extract (Mashtoub et al., 2013; Bajic et al., 2016a). Although these treatment strategies show promise, to date they are still in the pre-clinical stages.

Our understanding of the central consequences of drug-induced gut disorders, such as mucositis remains elusive, yet evidence on CICI is expanding as various mechanisms underlying its pathogenesis are becoming clearer. CICI occurs in 15–45% of patients undergoing anti-cancer therapy (**Figure 4**; Vardy and Tannock, 2007). Subjective (self-report) report rates are considerably higher than objective measures with some studies reporting 95% of patients experiencing changes in cognitive performance (Downie et al., 2006). Subjective measures are nonetheless important as they identify the impact of cognitive impairment and the strain it places on patients' lives and daily functioning (Shilling and Jenkins, 2007). The breast cancer population forms the majority of the CICI literature as they offer researchers completion of extensive retrospective studies due to their typically good prognosis (Ahles et al., 2010). Regardless, CICI has been investigated in a range of other cancer types including myeloma and testicular cancer (Schagen et al., 2008; Potrata et al., 2010).

The cognitive domains most commonly reported in CICI are executive functioning, attention and concentration, processing speed, reaction time and motor speed and dexterity (Asher, 2011). Perceived cognitive impairment affects various facets of the patient's life, including relationships, employment, self-esteem/worth, finances and independence. The CICI experience leaves patients feeling distressed, anxious, frustrated, irritable, depressed and embarrassed, often reducing confidence (Mitchell, 2007; Von Ah et al., 2013). Current estimates on the duration of CICI are varied with some studies identifying deficits up to 20 years post-chemotherapy cessation, yet most indicate improvements up to 12 months later (Collins et al., 2009; Koppelmans et al., 2012). Neuroimaging studies have confirmed molecular and structural changes in the gray matter of the frontal and temporal lobes and the cerebellum of breast cancer patients following chemotherapy exposure (Silverman et al., 2007; McDonald et al., 2010). Additionally, chemotherapy induces white matter tract changes and the reorganization of global brain networks, which have undoubtable associative if not causal impacts on cognitive performance (Abraham et al., 2008; Bruno et al., 2012).

Animal studies have begun to unravel various mechanisms underlying the pathogenesis of CICI and involve structural and behavioral changes. Hippocampal and frontal cortical alterations have correlated with behavioral memory changes in various rodent models (**Figure 4**; Yang et al., 2010, 2012; Wigmore, 2012). Neurogenesis occurs in the dentate gyrus and cellular

proliferation is critical in hippocampal circuit plasticity and memory consolidation (Deng et al., 2010; Ming and Song, 2011). CICI models have reported on the vulnerability of stem cells to proliferate in the dentate gyrus irrespective of chemotherapy drug class (Briones and Woods, 2011; El Beltagy et al., 2012). Considering the pivotal role neural stem cells in this region have to divide into new neurons or astrocytes, disruptions in this process present as a direct mechanism which may be contributing to CICI. More recently, neuroimmunological manifestations, such as glial dysregulation and neuroinflammation, have been reported to contribute to CICI (Briones and Woods, 2013; Bajic et al., 2016b).

Currently, effective prevention strategies or treatment approaches for CICI remain undetermined although two evidence-based guidelines are available to assist oncologists in addressing cognitive deficits (Network, 2015). Other interventions for CICI are broadly categorized into cognitive training, compensatory strategies, pharmacological, and complementary and integrative medicines (Vance et al., 2017). Recently, Toll-like receptors (TLRs) have been suggested as a common component in the pathology of neuropathy/pain and chemotherapy-induced gut toxicity, presenting a novel and much needed therapeutic approach in the treatment of chemotherapy-induced toxicities (Wardill et al., 2015). TLRs have profound homeostatic effects, tightly regulating innate immune and gut functions, modulating pain behaviors (Akira and Takeda, 2004; Rakoff-Nahoum et al., 2004; Doyle and O'Neill, 2006; Hutchinson et al., 2010, 2012; Gibson et al., 2016). Wardill et al. (2015) hypothesized that TLR-4 mediates glial activation and neuropathy driven by the molecular signals released from chemotherapy-induced gut toxicity. Primary studies have indicated that an altered TLR expression profile may contribute to chemotherapy-induced pain and diarrhea (Gibson et al., 2016). This study importantly highlights the need for further research examining both peripheral and central toxicities associated with chemotherapy treatment. Interestingly, the selective serotonin reuptake inhibitor, fluoxetine, has shown promising results in a rat model of CICI. Fluoxetine co-administration with the chemotherapy drug improved cognitive performance in rats assessed by object location recognition (Lyons et al., 2012). Whilst cellular proliferation in the dentate gyrus significantly reduced in the chemotherapy group, co-administration with fluoxetine reversed this reduction. Regardless of the evidence presented here indicating CNS changes following chemotherapy exposure, it is important to note that some studies have reported no structural or cognitive changes (Fremouw et al., 2012; Wilson and Weber, 2013). Various cytotoxic insults have revealed no morphological changes to neurons located in the CNS (Ginos et al., 1987; Gangloff et al., 2005). These negative findings could result from a range of factors, including differences in species, drug, dose, type of administration and cognitive parameters examined; but importantly, suggests that more complex mechanisms are likely to play a role in CICI.

Whilst the direct mechanisms presented here reflect the complex etiology of CICI, they fail to acknowledge the influence other simultaneously occurring side-effects may be having on CICI symptoms. Many of the CICI models described above utilized chemotherapy drugs that are also often used to examine mucositis, for example 5-Fluorouracil (5-FU), methotrexate and oxaliplatin. Although mucositis would have most likely been present in these models, the gut tissue would not have been examined and thus, the potential for *indirect* mechanisms relating to gut-brain axis dysregulation would have been ignored. In doing so, we may be missing critical mechanisms contributing to or exacerbating CICI pathogenesis. In order to explore this theory, we will now consider the influence chemotherapy exposure has on the gut-brain axis, opening novel hypotheses surrounding how mucositis may contribute to the etiology of CICI.

CHEMOTHERAPY INTERRUPTS SEVERAL STAGES OF THE GUT-BRAIN AXIS

As presented above, the two organs most vulnerable to the toxicities of chemotherapy treatment are the gut and the brain. Therefore, it is plausible that several stages of the gut-brain axis may become dysregulated in the chemotherapy setting (**Figure 4**). Here, we propose that chemotherapy exposure influences the gut-brain axis via several mechanisms which include: altering intestinal microbiota composition and function; upsetting the balance of "beneficial" and "detrimental" bacteria in the lumen, deleteriously affecting the gut lining, impairing the ENS and activating neuroimmune and pain signaling pathways (**Figure 4**). The interaction of the gut-brain axis and the neuropsychological comorbidities associated with specific gut disorders have been extensively reviewed, for example depression/cognitive deficits and IBS/IBD (Whitehead et al., 2002; Attree et al., 2003; Filipovic and Filipovic, 2014; Fond et al., 2014; Padhy et al., 2015). However, this angle of research is yet to be reviewed in the context of chemotherapy exposure and cognitive impairment. Research in this area will continue to develop as we begin to appreciate that chemotherapy-induced side-effects involving the gut-brain axis may continue to linger for some time after treatment cessation, placing significant strain on health care and importantly, patient quality of life.

The Microbiome

It has been estimated that our gut contains 100-fold more genes than the human genome and approximately 1,000 bacterial species (Ley et al., 2006; Qin et al., 2010). Our gut microbiome coevolves with us (Ley et al., 2008) and changes may be either beneficial or detrimental to human health. In healthy individuals, the gut microbiota is responsible for a number of health benefits, such as pathogen protection, nutrition, host metabolism and immune modulation (O'Hara and Shanahan, 2006). Although a core microbial population has been established in individuals, changes can be caused by many factors including age, diet, antibiotic and analgesic use and environmental factors (Jalanka-Tuovinen et al., 2011). The microbiome facilitates intestinal homeostasis and more specifically, has the capacity to influence inflammation and immunity, both at the local (mucosal) and systemic levels (Clemente et al., 2012). Commensal bacteria play important roles in anti-viral immunity, regulating systemic

immune activation (Abt et al., 2012). Signals released by commensal bacteria assist in immune development and thereby, have important implications for infectious and inflammatory disease susceptibility (Ichinohe et al., 2011; Abt et al., 2012), such as in the case of chemotherapy-induced mucositis. Consequently, dysbiosis can heavily influence pathological intestinal conditions with an inflammatory component, for example in experimentally-induced IBD (García-Lafuente et al., 1997; Dalal and Chang, 2014; Touchefeu et al., 2014; Håkansson et al., 2015). Critically, IBD patients reported microbial composition changes with major shifts in genomic landscape and functional outcomes (Morgan et al., 2012). Undoubtedly, the implications of such IBD studies have heavily impacted oncology, raising many questions specifically relating to the intestinal microbiota, immune, malignancy and anti-cancer treatment interactions.

Whilst Sonis' five-phase model of mucositis (Sonis, 2004) lacked any potential influence on the microbiota, unequivocal research has indeed confirmed that intestinal inflammation modulates microbiome composition and function (Morgan et al., 2012; Touchefeu et al., 2014). As intestinal inflammation is a common characteristic of mucositis, it makes sense that chemotherapy induces functional and compositional changes to the microbiome. It has been suggested that mucositis development is influenced by commensal bacteria in multiple pathways involving inflammation and oxidative stress, intestinal permeability (discussed below), mucus layer composition, epithelial repair mechanisms and via the release of immune effector molecules (van Vliet et al., 2010). Indeed, research has begun to unravel the complexities surrounding the interactions between the host and the intestinal microbiota following chemotherapy exposure and consequently, several excellent reviews exist (van Vliet et al., 2010; Touchefeu et al., 2014; Dzutsev et al., 2015; Vanhoecke et al., 2015; Zitvogel et al., 2015). Commonly used chemotherapy drugs, such as 5-FU and irinotecan report drastic shifts in intestinal microflora, from commensal bacteria which maintain a symbiotic relationship with the host, to elevated levels of *Escherichia* spp., *Clostridium* spp., and *Enterococcus* spp. which can be associated with several pathologies involving inflammation and infection (Von Bültzingslöwen et al., 2003; Stringer et al., 2007, 2009; Lin et al., 2012; **Table 1**). Several clinical studies have supported pre-clinical findings describing alterations in fecal microbial composition following chemotherapy treatment. Literature reveals a general decrease in the overall diversity of bacteria in the microbiota of cancer patients undergoing anti-cancer treatment when compared to healthy individuals, irrespective of cancer type or chemotherapy regime (Manichanh et al., 2008; Zwielehner et al., 2011; Montassier et al., 2014; see **Table 1**).

In addition to the direct effects microorganisms and their enzymes have on cancer initiation and progression (Sears and Garrett, 2014; Gagnière et al., 2016), the microbiota also modifies drug absorption and metabolism via gene expression changes (Carmody and Turnbaugh, 2014; Wilson and Nicholson, 2017). This has become a pivotal research angle in oncology as chemotherapy-microbiota-immune interactions have identified microbial-mediated innate and adaptive immune responses and their effect on the efficacy of cancer immunotherapy and chemotherapy drugs (Iida et al., 2013; Viaud et al., 2013; Sivan et al., 2015; Vétizou et al., 2015). Two crucial studies in *Science* Iida et al. (2013); Viaud et al. (2013) reported that microbiota disruption by antibiotic treatment impaired chemotherapy drug efficacy on tumors, utilizing cyclophosphamide and oxaliplatin. More recent studies have illustrated the important role certain microbial strains (*Bifidobacterium*) play in anti-tumor immunity (Sivan et al., 2015; Vétizou et al., 2015). Although these studies were performed in mice, their findings indicate the potential risks associated with the use of antibiotics during chemotherapy treatment. The growing field of microbiome research has raised a lot of questions and comments on the complex interplay and interwoven relationships between microbes and cancer, including anti-cancer treatments (Pennisi, 2013; Bordon, 2014; Greenhill, 2014; Lokody, 2014; Mukaida, 2014). Further, some of the above studies (Sivan et al., 2015; Vétizou et al., 2015) have implications for microbial therapy in cancer immunotherapy. As our understanding of these interactions continues to progress, new knowledge in this area will open up possibilities of novel paradigm shifts in treatment approaches which may improve anticancer efficacy and even prevent toxicity. The studies presented in this section suggest a role for chemotherapy-induced dysbiosis in intestinal disease pathogenesis and chemotherapy-induced gut-brain axis dysregulation (**Figure 4**). As mentioned, commensal bacteria are critical in regulating intestinal homeostasis and more specifically, intestinal integrity. In fact, the effects commensal bacteria have on intestinal integrity and vice versa, go hand-in-hand. Accordingly, the reciprocal relationship shared between commensal bacteria and the intestinal wall will be presented together in the following section. Chemotherapy compromises intestinal integrity and leads to profound effects on the gut lining, eventually leading to a dysbiotic microbial community and consequently risking microbial invasion into the systemic circulation.

Chemotherapy Impairs Intestinal Barrier-Microbiota Interactions

The even comprehensively described pathogenesis of mucositis (Sonis, 2004) is unable to fully encapsulate the mechanisms underlying the pathogenesis of chemotherapy-induced gut damage. Although it covers many essential aspects of the pathological processes underlying mucositis, such as epithelial barrier damage. More recently, some research groups have re-defined gut damage caused by chemotherapy as chemotherapy-induced gut toxicity (CIGT). The proposed term includes additional pathological manifestations caused by chemotherapy treatment, such as abnormalities in tight junctions, immune dysfunction and microbiota influences (Montassier et al., 2014; Touchefeu et al., 2014; Wardill et al., 2014).

Nonetheless, the epithelial barrier lining of the GIT is fundamental in ensuring the maintenance of intestinal integrity. As well as forming a mechanical barrier to separate the inside of the body from the outside world, it is heavily involved in the communication shared between the body

TABLE 1 | Summary of key papers highlighting chemotherapy-microbiota-immune interactions.

Study	Subjects	Treatment	Commentary
Lin et al., 2012	Tumor bearing rats	Irinotecan alone Irinotecan/5-FU	Increased abundance *clostridial clusters I, XI*, and *Enterobacteriaceae*.
Von Bültzingslöwen et al., 2003	Rats	5-FU	Increased facultative and anaerobic bacteria from the oral cavity. Increased facultative anaerobes in large intestine. Proportion of facultative gram-negative rods increased in both oral cavity and intestine.
Stringer et al., 2009	Rats	Irinotecan	Increased jejunal samples of *Escherichia* spp., *Clostridium* spp., *Staphylococcus* spp. Increased colonic samples of *Escherichia spp.*, *Clostridium spp.*, *Enterococcus spp.*, *Serratia spp.*, *Staphylococcus spp.* No changes in fecal flora except *E. coli*.
Stringer et al., 2007	Rats	Irinotecan	Extensive changes were evident in stomach, jejunum, colon and feces. Most significant changes were in colon, indicating a relationship between colon bacteria modification and diarrhea incidence.
Montassier et al., 2014	Patients with non-Hodgkin's lymphoma	Bone marrow transplantation with chemotherapy conditioning	Steep reduction in alpha diversity during chemotherapy. Decrease in *Firmicutes bacteria* and *Bifidobacterium* whilst *Bacteriodes* and *Estarichia* were increased
Manichanh et al., 2008	Patients with abdominal tumors	Pelvic radiotherapy	Fecal samples reported significant microbiota profile changes in patients with post-radiotherapy diarrhea. Not all patients reported diarrhea. Importantly, this study suggests initial microbial colonization may be linked to susceptibility or protection against diarrhea following radiotherapy treatment.
Zwielehner et al., 2011	Patients with various malignancies	Chemotherapy and antibiotic treatment	Chemotherapy decreased *Clostridium cluster IV* and *XIVa*. *C. difficile* was present in three out of seventeen patients and was accompanied by a decrease in the genera *Bifidobacterium*, *Lactobacillus*, *Veillonella* and *Faecalibacterium prausnitzii*. *Enterococcus faecium* increased following chemotherapy.
Iida et al., 2013	Tumor bearing mice	Oxaliplatin and cisplatin	Chemotherapy-induced dysbiosis impairs response to immunotherapy and chemotherapy.
Viaud et al., 2013	Tumor bearing mice	Cyclophosphamide	Jejunal and fecal samples reported dysbiosis and induces translocation of specific Gram-positive bacteria to secondary lymphoid organs whereby they stimulate subsets of T cells. These results suggest that the gut microbiota may affect anticancer immune response.
Sivan et al., 2015	Tumor bearing mice	Co-housing, fecal transfer, programmed cell death protein 1 ligand 1 (PD-L1)–specific antibody therapy (checkpoint blockade), oral *Bifidobacterium*	Changes to anti-tumor immunity were eliminated by co-housing and fecal transfer. Oral *Bifidobacterium* administration improved tumor control to same degree as PDL-1 therapy; combination treatment nearly abolished tumor outgrowth.
Vétizou et al., 2015	Tumor bearing mice and metastatic melanoma patients	Ipilimumab (CTLA-4 blocker) regulates T cell activation and improves survivability of metastatic melanoma patients.	CTLA-4 blockade is influenced by the microbiota. Changes in *B. fragilis* and/or *B. thetaiotaomicron* and *Burkholderiales* affects immune response facilitating tumor control in mice and patients.

and the intestinal contents (Powell, 1981). Tight junctions are intertwined throughout the epithelial barrier and regulate diffusion of solutes according to strict size and charge limitations (Balda and Matter, 2016). Chemotherapy exposure increases intestinal permeability and the most widely studied mechanisms to date have included apoptosis of intestinal crypts and villous atrophy (Keefe et al., 2000; Carneiro-Filho et al., 2004). Early clinical studies assessing the severity of intestinal damage in high dose regimes reported abnormalities in intestinal permeability and defects in tight-junction integrity (**Figure 4**; Keefe et al., 1997). Convincing rodent evidence has implicated mucosal barrier injury and tight junction deficits with gut toxicity induced by various chemotherapy drugs, including irinotecan and methotrexate (Beutheu Youmba et al., 2012; Wardill et al., 2014). However, it should be noted that rodent model application in gut immunity and microbiome research has serious limitations

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and pitfalls due to compositional microbiota differences between species. Whilst the rodent microbiome shares some common features with human commensal bacteria, unique commensals in rodents have differential effects in immune responses and disease pathogenesis (Nguyen et al., 2015). Consequently, animal models of inflammation are different to human models of inflammation in terms of microbial colonization, morphology of lesions and clinical manifestations. Nonetheless, research in experimental models continues to provide critical insight into complex interactions between the host, microbiota and immune responses. More recently, it has been becoming more evident the impact intestinal integrity has on the microbiota and vice versa, especially under chemotherapy conditions. The health and stability of the intestinal wall influences the microbiota and vice versa.

Commensal bacteria in the microbiota have a protective effect on intestinal integrity, interacting with TLR and Nuclear Factor kappa B pathways, ensuring the development of an innate immune response (Doyle and O'Neill, 2006). These components of innate immunity in the gut and the activation of these pathways are pivotal in maintaining barrier function, promoting mucosal repair and protecting the gut against injury (Rakoff-Nahoum et al., 2004; Cario, 2008). Chemotherapy exposure alters commensal microbial composition in the microbiota, thus negatively affecting barrier function, repair pathways and compromising intestinal integrity (Stringer et al., 2009). Accordingly, further investigations are required to fully appreciate the role chemotherapy-induced intestinal permeability changes play in gut-brain axis dysregulation. As intestinal integrity becomes compromised under chemotherapy conditions, it is not surprising that nerves of the myenteric plexus and peripheral nerve endings become damaged as these neural components also reside outside of the blood-brain barrier (BBB) as will be discussed below.

Chemotherapy Results in Peripheral and Enteric Neuropathy

The PNS is particularly vulnerable to the cytotoxic nature of different chemotherapy drug classes, including platinum analogs, antitubulins, proteasome inhibitors, immunomodulatory agents and some newer biologics, such as brentuximab (Cavaletti and Marmiroli, 2015). Chemotherapy-induced peripheral neuropathy (CIPN) is experienced by 30–40% of chemotherapy recipients and is often responsible for early cessation of treatment, decreasing chemotherapeutic efficacy and causing higher relapse (Wang et al., 2012; Areti et al., 2014). Typically, sensorimotor symptoms are more common than motor involvement, presenting in a bilateral "glove-and-stocking" distribution in the hands and feet to include paraesthesia, numbness, burning pain, allodynia and hyperalgesia (Windebank and Grisold, 2008). However, the development of motor and autonomic neuropathic symptoms may also occur, such as sensory ataxia, pain, weakness of distal muscles, reduced deep tendon reflexes and severe numbness that can severely affect the patient's ability to function and their quality of life (Park et al., 2013). Often symptoms fail to improve after cessation of treatment, referred to as a "coasting" phenomenon (Windebank and Grisold, 2008).

The pathogenesis of CIPN is primarily related to axonopathy and neuronopathy in which dorsal root ganglia (DRG) are involved. Peripheral nerves and their ganglia are particularly susceptible to chemotherapy-induced damage due to their location as they lack the protective defenses associated with the BBB (Furness et al., 2014). For example, chemotherapy interrupts the cell cycle, inducing structural and functional changes in DRG which partly explain the development of sensory symptoms in CIPN (Gill and Windebank, 1998; Cavaletti et al., 2000). Many pathophysiological mechanisms mediating chemotherapy-induced peripheral nerve damage have been identified. Some examples include, but are not exclusive to dysregulated axonal transport and trophic factor support via microtubule structural changes (Theiss and Meller, 2000), mitochondrial stress (McDonald and Windebank, 2002; Chen et al., 2007) and reduced blood supply to nerves (Theiss and Meller, 2000; Isoardo et al., 2004). Further changes contributing to CIPN pathogenesis include dysregulated ion channels, neurotransmitter release and receptor sensitivity (Descoeur et al., 2011; Mihara et al., 2011; Tatsushima et al., 2011). The evidence presented here clearly describes mechanisms by which the PNS is damaged following chemotherapy exposure, forming an important element of the proposed central hypothesis (**Figure 4**).

In addition to peripheral neuropathies, neurons residing within the ENS are also susceptible to the deleterious effects of various chemotherapy drugs, including cisplatin, oxaliplatin and more recently, 5-FU (Vera et al., 2011; Wafai et al., 2013; McQuade et al., 2016). Systemic administration of these chemotherapy drugs induces structural and functional changes to myenteric neurons (**Figure 4**), consequently resulting in downstream negative effects on GI motility. Interestingly, acute exposure of 5-FU increases intestinal transit whilst prolonged treatment decreases transit time (McQuade et al., 2016). These findings outline the complex nature chemotherapy drugs have on enteric neurons and altered motility patterns. Here, we highlight that chemotherapy results in damage to neurons and ganglia residing outside of the BBB, exerting functional maladaptations in both the PNS and ENS. So far we have described several mechanisms relating to chemotherapy-induced gut-brain axis dysregulation, yet we have not identified how immune signals from the intestinal cavity may communicate to the brain and potentially contribute to the pathogenesis of CICI. In the following section we present peripheral-to-central immune pathways as being critical in the transmission of signals from the gut to the brain following chemotherapy exposure.

Peripheral-to-Central Immune Signaling Pathways Mediating Chemotherapy-Induced Gut-Brain Axis Dysregulation

Historically there has been controversy surrounding the theory that a communication system existed between the immune system and the CNS (Dantzer and Kelley, 2007). Traditionally

it was assumed that proinflammatory cytokines were unable to pass through the BBB due to their size. However, the humoral route explained that cytokines expressed in the periphery could in fact cross the BBB at leaky circumventricular organs through fenestrated capillaries. At these sites blood-borne cytokines act on parenchymal astrocytes that express secondary mediators, such as nitric oxide and prostaglandins which freely diffuse to nearby brain regions, such as the hypothalamus to mediate the effects of pyrogenic and corticotropic cytokines (Katsuura et al., 1990). Whilst this hypothesis leads toward the existence of a communication system between the immune system and the CNS, it was unable to fully account for other contributing pathways that may be mediating physiological responses. Consequently, it is now widely accepted that peripheral cytokines signal the brain and in turn, this triggers sickness behavior responses (Dantzer, 2004).

Central or peripheral immune challenges trigger a range of physiological, behavioral and motivational changes to assist the host in healing (**Figures 2**, **4**). Non-specific symptoms which accompany sickness behaviors include, but are not exclusive to fever, depressed activity, a loss of interest in regular activities (appetite, sexual, cleaning, hygiene), weakness, malaise, listlessness and cognitive changes (Dantzer and Kelley, 2007). As demonstrated by Dantzer and Kelley (Dantzer and Kelley, 2007), the last two decades of research on this phenomenon have confirmed that local or systemic proinflammatory cytokines expressed at physiological levels, during both acute and chronic inflammatory responses, serve as true communication molecules between the immune system and brain. For example, direct administration of IL-1β or TNF-α to the lateral ventricle decreased social exploration and feeding behavior in rats (Kent et al., 1992). In the chemotherapy setting, this phenomenon may be related to either the systemic nature of the drugs themselves or localized inflammatory responses occurring as a result of the toxicities associated with their use, such as in the case of mucositis (see **Figure 2**).

Interestingly, whilst IL-1β and TNF-α are key proinflammatory cytokines instigating sickness behavior responses, they also play a pivotal role in the pathogenesis of mucositis. Since both proinflammatory cytokines play an important role in the pathogenesis of mucositis and sickness behaviors which involve cognitive deficits, it is plausible that these cytokines and the pathways mediating their activation may present as key mechanisms underlying the central hypothesis in this review (**Figure 4**). Various animal models have identified that sickness behavior responses may be induced by a range of clinical conditions, such as systemic or central administration of lipopolysaccharide (active fragment of gram negative bacteria) or recombinant proinflammatory cytokines (Tomas et al., 1984; Goehler et al., 1999; Dantzer and Kelley, 2007). Furthermore, many symptoms associated with cytokine-induced sickness responses mimic the cluster of chemotherapy-induced side-effects, including fatigue, depression, reduced appetite, heightened sensitivity to pain and cognitive impairment (**Figure 4**). As previously mentioned, up to 70% of chemotherapy recipients experience mucositis (Scully et al., 2003) and an altered immune profile due to the systemic nature of anti-cancer treatments, yet whether these side-effects may be contributing to CICI remains elusive. Accordingly, we present pathways which may be enabling the communication of peripheral immune signals to the brain, more specifically defining how mucositis-driven inflammation may signal the brain via vagal- and neural-mediated mechanisms and contribute to the pathogenesis of CICI.

Information from proinflammatory cytokines and mediators expressed under chemotherapy-induced mucositis conditions may signal the brain via a vagal communication pathway (**Figure 4**). Dendritic cells (DCs) are a specialized subset of immune cells located within the vagus nerve and surrounding paraganglia (Goehler et al., 1999). The signals (proinflammatory cytokines, chemokines and mediators) expressed by DCs are capable of communicating to the brain (Banchereau and Steinman, 1998; Reis e Sousa et al., 1999). Vagal immunosensation requires primary afferent neuron activation as the initial interface triggering the brain. Following chemotherapy exposure, proinflammatory cytokines and mediators, such as those from the IL-1 family arise from mucositis-induced inflammation. IL-1 binds to receptors on the paraganglia surrounding vagal afferents and release neurotransmitters onto the vagus, consequently activating vagal fibers. A vagal-mediated neural signal is then carried to the nucleus tractus solitarius which projects the message to higher order brain regions, such as the hypothalamus and hippocampus, whereby, IL-1 production is increased and other neural cascading events are initiated to produce sickness behavior responses (Dantzer and Kelley, 2007; Wardill et al., 2015). Whilst this evidence clearly demonstrates the role that vagal afferent nerves play in peripheral-to-central transmission of immune messages from the abdominal cavity, to date these pathways have not been examined under chemotherapy conditions and are therefore presented as potential mechanisms contributing to gut-brain axis dysregulation.

DCs play a role in immunomodulation and neuroimmune regulation, crucially bridging innate and immune adaptive processes. Importantly, DCs express pattern recognition receptors for a range of chemicals (e.g., TLRs), chemokines, microorganisms and neurotransmitters, such as serotonin (Banchereau and Steinman, 1998; Reis e Sousa et al., 1999). Damage to surroundings GIT tissues and increased levels of proinflammatory mediators, such as cytokines and chemokines induce maturation of DCs (Ricart et al., 2011). Matured DCs migrate to secondary lymphoid organs to initiate a localized immune response via interacting with naïve T cells (Banchereau and Steinman, 1998). Recent data implies an emerging role for DC-expressed serotonin and receptor activation in regulating innate and immune responses associated with gut inflammatory conditions (Holst et al., 2015; Szabo et al., 2018). Mechanisms underlying DC-mediated serotonin and receptor-sub types affect various levels of localized inflammation, even having anti-inflammatory effects preventing excess inflammation and tissue damage (Szabo et al., 2018). These findings coupled with the aforementioned positive cognitive effects of fluoxetine in a rat model of CICI (Lyons et al., 2012), serotonin presents as a new therapeutic approach for inflammatory disorders, having effects

in both the gut and the brain. Accordingly, serotonin is presented as an underestimated contributing factor potentially implicated in chemotherapy mediated gut-brain axis dysregulation (see Wigmore, 2012).

CONCLUSION

From the bottom-up, the gut and the brain are the two primary organs most susceptible to toxicity associated with the non-selective nature of chemotherapy drugs. As chemotherapy exposure induces cognitive decline and mucositis in a subset of recipients, it makes sense that several stages of the gut-brain axis are prone to negative effects in this setting. The gut-brain axis is largely responsible for the maintenance of homeostasis and achieves this delicate balance by integrating a vast array of signals and information from many systems, as described above and shown in the figures. In this regard, upsetting the balance of any stage in the gut-brain axis following chemotherapy treatment has the potential to exacerbate side-effects, such as in the case of mucositis and CICI. The findings from our review support our main hypotheses that chemotherapy treatment causes severe and prolonged psychosocial impacts on the survivor. Secondly, the gut-brain axis is an important mediator of a diverse range of cognitive and emotional disorders similar to those experienced by cancer survivors. Evidently, chemotherapy affects the gut-brain axis at several key stages which are outlined above. Collectively, we conclude that the psycho-social side-effects of chemotherapy treatment may be caused by the effects of chemotherapy on the gut-brain axis.

Apart from chemotherapy treatments crossing the BBB and *directly* causing damage to specific regions, peripheral inflammatory responses from either the malignancy or systemic treatment also *indirectly* cause cellular changes in the spinal cord. We recently demonstrated glial dysregulation in the thoracic region of rats with 5-FU-induced intestinal mucositis indicating an indirect regional-specific neuroimmune response to CIGT (Bajic et al., 2015). Our data provides evidence that experimentally-induced jejunal toxicity *indirectly* downregulates thoracic astrocytic expression. In addition to this recent finding, the evidence presented here suggests a role for chemotherapy-induced dysbiosis in intestinal inflammation. This further complicates intestinal inflammation and ulceration induced by chemotherapy exposure which may potentially influence CICI. Neurons in both the ENS and the PNS are also vulnerable to the cytotoxic nature of chemotherapy treatments. The implications of co-administration of pharmacological interventions (e.g., fluoxetine) with chemotherapy drugs remains undetermined, although preliminary studies showing improvements in cognitive performance warrants further investigation. In view of the aforementioned data, we conclude that several stages of the gut-brain axis become dysregulated following chemotherapy exposure and may be implicated in the pathogenesis of CICI. Harnessing our understanding of the role gut-brain axis dysregulation plays in modulating brain function may offer clues for more targeted therapeutic strategies to prevent CICI and warrants further investigation.

AUTHOR CONTRIBUTIONS

JB wrote this manuscript to form part of her thesis and it is her second review. IJ, GH, and MH contributed equally at various points throughout the course of this manuscript, adding valuable input and critically reviewing each draft made by JB.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

ACKNOWLEDGMENTS

JB is supported by the Australian Government Research Training Program Scholarship and the Freemason's Society Supplementary Scholarship. MH is funded by an Australian Research Council, Australian Research Fellowship. The authors would like to especially thank Kelsi Dodds (Doctoral candidate) for assistance with the figure artwork. We would also like to thank Dr. Eliazbeth Beckett and Dr. Nicholas Bajic for their assistance with the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix Three

Understanding the side-effects of chemotherapy: a cross-sectional correlational study of gastrointestinal symptoms and cognitive impairment in an Australian breast cancer cohort

Welcome to My Survey

Thank you for participating in our survey. Your feedback is important.

Thank you for choosing to participate in the study titled, "Understanding the side-effects of chemotherapy: a cross-sectional correlational study of gastrointestinal symptoms and cognitive impairment in an Australian breast cancer cohort" being undertaken by The University of Adelaide.

By now, you will have received an email from the Breast Cancer Network Australia detailing information about the study, including several attachments:

1. Participant Information Sheet,
2. Inclusion and Exclusion Check list,
3. Contacts for Information on Project and Complaints Procedure documents and
4. A consent form.

From this information you will understand that this is a retrospective study which is strictly voluntary. The questionnaire approach asks you to detail some of the side-effects you may have experienced when you were undergoing anti-cancer treatment.

If you are reading this, then you have selected the on line option. You will not need to sign and return the consent form to the researchers alternatively, your consent will be given at the bottom of this page in the form of checking the "Yes, I give consent" box.

If at any time, you should feel distressed and alone, please don't hesitate to call the Cancer Council on 13 11 20 for support, or alternatively you might prefer to contact Lifeline on 13 11 14.

If, at any time throughout this study, you wish to withdraw from the study, please see the 'Contacts for Information on Project and
Complaints Procedure' document. You may also choose to withdraw from the study at any time, without notifying the researchers.

Once again, the researchers thank you for your time and willingness to help us understand more about the side-effects of anti-cancer treatments. Hopefully, one day our research will lead to the development of more targeted therapies that reduce the negative effects of anti-cancer treatments and thus, improve the quality of life of cancer patients.

1. By checking the box below, you are willing and volunteering to participate in this survey, thereby giving consent for the investigators of this study to use your answers in this study. Your answers will remain entirely anonymous. If you do not wish to participate, please do not complete the survey.

- ○ Yes, I give consent
- ○ No, I don't give consent

Understanding the side-effects of chemotherapy: a cross-sectional correlational study of gastrointestinal symptoms and cognitive impairment in an Australian breast cancer cohort

Demographic information

2. What is your age?

- 40 to 50
- 50 to 60
- 60 to 70
- 70 or over

3. What is your gender?

- Female
- Male

4. Which of the following best describes your current relationship status?

- Married
- Widowed
- Divorced
- Separated
- In a domestic partnership or civil union
- Single, but cohabiting with a significant other
- Single, never married

5. Which of the following categories best describes your employment status?

- Employed, working full-time
- Employed, working part-time
- Not employed, looking for work
- Not employed, NOT looking for work
- Retired
- Disabled, not able to work

6. Please specify your occupation (or previous occupation if retired or not working)

1.

2.

3.

7. What is the highest level of education you have completed?

8. Please specify completed course(s) and/or degree(s)

Course(s)

Degree(s)

9. How important is exercise to you?

- Extremely important
- Very important
- Moderately important
- Slightly important
- Not at all important

10. In a typical week, how many days do you exercise?

- I don't regularly exercise
- Once a week
- 2 to 4 days a week
- 5 to 7 days a week

Understanding the side-effects of chemotherapy: a cross-sectional correlational study of gastrointestinal symptoms and cognitive impairment in an Australian breast cancer cohort

Questions about your medical history, including your cancer and treatment

11. Please indicate your primary breast cancer diagnosis.

- ☐ Ductal carcinoma in situ
- ☐ Lobular carcinoma in situ
- ☐ Paget's disease of the nipple
- ☐ Inflammatory breast cancer
- ☐ Locally advanced breast cancer
- ☐ Early breast cancer
- ☐ Secondary breast cancer

12. Please enter the date of your diagnosis.

	DD	MM	YYYY
Date / Time	/	/	

13. Please indicate your treatment type(s). More than one option may be selected.

- ☐ Surgery (partial mastectomy)
- ☐ Surgery (full mastectomy)
- ☐ Chemotherapy regime
- ☐ Radiotherapy
- ☐ Other (please specify)

14. Please indicate your total length of treatment time.

- ○ Less than 6 months
- ○ Less than 12 months
- ○ More than 1 year
- ○ More than 2 years

15. Please indicate the time you have spent in remission (a decrease in or disappearance of signs and symptoms of cancer).

- ○ Less than 1 year
- ○ Less than 2 years
- ○ More than 2 years

16. Did your cancer diagnosis and/or treatment prevent you from working/studying?

- ○ Yes
- ○ No

If yes, please detail timeframe

17. Please indicate the most suitable reasons why you could no longer work. You may select more than one option.

- ☐ Levels of depression/anxiety increased
- ☐ Cognitive issues
- ☐ Side-effects of treatment
- ☐ Pain
- ☐ Employment was terminated by employee
- ☐ Please comment.

18. Did your cancer diagnosis and treatment regime prevent you from your normal daily activities (other than work which was previously specified)?

- ○ Yes
- ○ No

Please comment.

Understanding the side-effects of chemotherapy: a cross-sectional correlational study of gastrointestinal symptoms and cognitive impairment in an Australian breast cancer cohort

The following questions are regarding pain.

Pain is defined by the International Association for the Study of Pain as "an unpleasant sensory and emotional experience in association with actual or potential tissue damage, or described in terms of such damage".

19. Prior to your cancer diagnosis, how often did you experience pain?

- ○ Daily
- ○ A couple of times a week
- ○ 3-4 times a week
- ○ Once a week
- ○ Once a fortnight
- ○ Once a month
- ○ Rarely
- ○ Comment (please specify pain types), e.g. neck, headache, knee, etc.

20. After your cancer diagnosis and treatment commencement, did you notice an increase in your sensitivity to pain? The frequency of pain?

- ○ Yes
- ○ No

21. The following are a list of "habits" that may influence pain. Please indicate the answers that most accurately reflect your use.

	Currently, yes	Currently, no	Previously, yes	Previously, no
Dieting	☐	☐	☐	☐
Alcohol	☐	☐	☐	☐
Tobacco	☐	☐	☐	☐
Marijuana	☐	☐	☐	☐
Amphetamines	☐	☐	☐	☐
Cocaine	☐	☐	☐	☐
Ecstasy	☐	☐	☐	☐
Herion	☐	☐	☐	☐
Vitamin/mineral supplements	☐	☐	☐	☐
Caffeine	☐	☐	☐	☐

22. Stressors. Please rate your level of stress in relation to the following areas:

	No stress	Intermittent stress	Persistent stress	Overwhelming stress
Marriage/partner	☐	☐	☐	☐
Work	☐	☐	☐	☐
Parents	☐	☐	☐	☐
Children	☐	☐	☐	☐
Family	☐	☐	☐	☐
Co-workers	☐	☐	☐	☐
Financial	☐	☐	☐	☐
Health	☐	☐	☐	☐

Other (please specify)

Understanding the side-effects of chemotherapy: a cross-sectional correlational study of gastrointestinal symptoms and cognitive impairment in an Australian breast cancer cohort

Cognition

23. Did you experience changes in your cognition prior to cancer diagnosis?

- ○ Yes
- ○ No

Comment (please specify, e.g. memory, learning new things, etc.)

24. Did you notice any changes in your cognitive ability after your cancer diagnosis?

- ○ Yes
- ○ No

25. At what time-point did you notice changes in your cognitive ability?

- ○ After cancer diagnosis but before treatment
- ○ After treatment commenced
- ○ After treatment ceased

26. Do you continue to experience changes in your cognitive ability even after your treatment has ceased?

- ○ Yes
- ○ No

27. Do you feel that your cognitive ability has changed since being diagnosed with cancer and undergoing treatment?

- ○ Yes
- ○ No

28. Did you feel your cognitive changes were more noticeable after each chemotherapy and/or radiation treatment session?

- ○ Yes
- ○ No

29. Have you ever noticed your cognitive impairments improving? If so, please detail when?

- Yes
- No

Comments (please specify)

30. Do you feel as if your cognitive ability has returned to how it was previous to your cancer diagnosis?

Please comment.

- Yes
- No

Comments (please specify)

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Gastrointestinal concerns

31. Did you experience any gastrointestinal upsets throughout your treatment?

- ○ Yes
- ○ No

32. Please indicate your main gastrointestinal complaints.

	All the time	Frequently	Sometimes	Not very often	Not at all
Pain	☐	☐	☐	☐	☐
Bloating	☐	☐	☐	☐	☐
Diarrhoea	☐	☐	☐	☐	☐
Constipation	☐	☐	☐	☐	☐
Nausea	☐	☐	☐	☐	☐
Vomitting	☐	☐	☐	☐	☐
Wind/excessive farting	☐	☐	☐	☐	☐

33. Did you consult your doctor?

- ○ Yes
- ○ No

34. Did your doctor provide you with any pain relief or coping strategies? If so, please detail?

- ○ Yes
- ○ No

If yes, please detail (e.g. analgesics, breathing, avoiding certain food types, etc.)

35. Were you diagnosed with chemotherapy-induced mucositis (oral or intestinal)?

- ○ Yes
- ○ No
- ○ If yes, please detail

36. Did your gastrointestinal upsets appear more severe after each chemotherapy/radiation treatment session?

- ○ Yes
- ○ No

Understanding the side-effects of chemotherapy: a cross-sectional correlational study of gastrointestinal symptoms and cognitive impairment in an Australian breast cancer cohort

Allergies and other medications

37. Are you susceptible to allergies? If so, please specify what triggers your allergies (e.g. penicillin, codeine, pollen, animals, hay, spring time, etc.)?

- ○ Yes
- ○ No

Other (please specify)

38. Please specify when your allergies are most severe.

- ○ All year
- ○ Spring time
- ○ Upon contact with triggers (e.g. patting a cat)

Other (please comment)

39. Do you take any medication to relieve your allergies?

- ○ Yes
- ○ No

Comment (please specify medication and if it is effective)

40. Are you on any other regular medications or herbal remedies (e.g. hormone therapy, St. John's Wort, pain medication like ibuprofen, aspirin, etc.)?

- ○ Hormone therapy
- ○ Pain medication
- ○ Anti-depressants

Other (please specify)

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Depression, anxiety and stress scale

41. Please read each statement and check the box which indicates how much the statement applied to you during your anti-cancer treatment. There are no right or wrong answers. Do not spend too much time on any statement.

	Did not apply to me all	Applied to me to some degree, or some of the time	Applied to me a considerable degree	Applied to me very much, or most of the time
I found myself getting upset by quite trivial things	○	○	○	○
I was aware of dryness of my mouth	○	○	○	○
I couldn't seem to experience any positive feeling at all	○	○	○	○
I experienced breathing difficulty (e.g., excessively rapid breathing, breathlessness in the absence of physical exertion)	○	○	○	○
I just couldn't seem to get going	○	○	○	○
I tended to over-react to situations	○	○	○	○
I had a feeling of shakiness (e.g., legs going to give way)	○	○	○	○
I found it difficult to relax	○	○	○	○
I found myself in situations that made me so anxious I was most relieved when they ended	○	○	○	○
I felt that I had nothing to look forward to	○	○	○	○
I found myself getting upset rather easily	○	○	○	○
I felt that I was using a lot of nervous energy	○	○	○	○
I felt sad and depressed	○	○	○	○

	Did not apply to me at all	Applied to me to some degree, or some of the time	Applied to me a considerable degree	Applied to me very much, or most of the time
I found myself getting impatient when I was delayed in any way (e.g., lifts, traffic lights, being kept waiting)				
I had a feeling of faintness				
I felt that I had lost interest in just about everything				
I felt I wasn't worth much as a person				
I felt that I was rather touchy				
I perspired noticeably (e.g., hands sweaty) in the absence of high temperatures or physical exertion				
I felt scared without any good reason				
I felt that life wasn't worthwhile				
I found it hard to wind down				
I had difficulty in swallowing				
I couldn't seem to get any enjoyment out of the things I did				
I was aware of the action of my heart in the absence of physical exertion (e.g., sense of heart rate increase, heart missing a beat)				
I felt down-hearted and blue				
I found that I was very irritable				
I felt I was close to panic				
I found it hard to calm down after something upset me				
I feared that I would be "thrown" by some trivial but unfamiliar task				

I was unable to become enthusiastic about anything	○	○	○	○
I found it difficult to tolerate interruptions to what I was doing	○	○	○	○
I felt I was pretty worthless	○	○	○	○
I felt terrified	○	○	○	○
I was intolerant of anything that kept me from getting on with what I was doing	○	○	○	○
I could see nothing in the future to be hopeful about	○	○	○	○
I felt that life was meaningless	○	○	○	○
I found myself getting agitated	○	○	○	○
I was worried about situations in which I might panic and make a fool of myself	○	○	○	○
I experienced trembling (e.g., in the hands)	○	○	○	○
I found it difficult to work up the initiative to do things	○	○	○	○

PERCEIVED COGNITIVE IMPAIRMENTS

42. Below is a list of statements that other people with your condition have said are important. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Never	About once a week	Two to three times a week	Nearly every day	Several times a day
I have had trouble forming thoughts	○	○	○	○	○
My thinking has been slow	○	○	○	○	○
I have had trouble concentrating	○	○	○	○	○
I have had trouble finding my way to a familiar place	○	○	○	○	○
I have had trouble remembering where I put things, like my keys or my wallet	○	○	○	○	○
I have had trouble remembering new information, like phone numbers or simple instructions	○	○	○	○	○
I have had trouble recalling the name of something to someone	○	○	○	○	○
I have had trouble finding the right word(s) to express myself	○	○	○	○	○

	Never	About once a week	Two to three times a week	Nearly every day	Several times a day
I have used the wrong word when I referred to an object	○	○	○	○	○
I have had trouble saying what I mean in conversations with others	○	○	○	○	○
I have walked into a room and forgotten what I meant to get or do there	○	○	○	○	○
I have had to work really hard to pay attention or I would make a mistake	○	○	○	○	○
I have forgotten names of people soon after being introduced	○	○	○	○	○
My reactions in everyday situations have been slow	○	○	○	○	○
I have had to work harder than usual to keep track of what I was doing	○	○	○	○	○
My thinking has been slower than usual	○	○	○	○	○
I have had to work harder than usual to express myself clearly	○	○	○	○	○
I have had to use written lists more often than usual so I would not forget things	○	○	○	○	○
I have trouble keeping track of what I am doing if I am interrupted	○	○	○	○	○
I have trouble shifting back and forth between different activities that require thinking	○	○	○	○	○

COMMENTS FROM OTHERS

43. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Never	About once a week	Two to three times a week	Nearly every day	Several times a day
Other people have told me I seemed to have trouble remembering information	○	○	○	○	○
Other people have told me I seemed to have trouble speaking clearly	○	○	○	○	○
Other people have told me I seemed to have trouble thinking clearly	○	○	○	○	○
Other people have told me I seemed confused	○	○	○	○	○

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FACT-Cognitive function (Version 3)

PERCEIVED COGNITIVE ABILITIES

44. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Not at all	A little bit	Somewhat	Quite a bit	Very much
I have been able to concentrate	○	○	○	○	○
I have been able to bring to mind words that I wanted to use while talking to someone	○	○	○	○	○
I have been able to remember things, like where I left my keys or wallet	○	○	○	○	○
I have been able to remember to do things, like take medicine or buy something I needed	○	○	○	○	○
I am able to pay attention and keep track of what I am doing without extra effort	○	○	○	○	○
My mind is as sharp as it has always been	○	○	○	○	○
My memory is as good as it has always been	○	○	○	○	○
I am able to shift back and forth between two activities that require thinking	○	○	○	○	○
I am able to keep track of what I am doing, even if I am interrupted	○	○	○	○	○

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FACIT-Fatigue Scale (Version 4)

IMPACT ON QUALITY OF LIFE

45. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Not at all	A little bit	Somewhat	Quite a bit	Very much
I have been upset about these problems	○	○	○	○	○
These problems have interfered with my ability to work	○	○	○	○	○
These problems have interfered with my ability to do things I enjoy	○	○	○	○	○
These problems have interfered with the quality of my life	○	○	○	○	○

46. Below is a list of statements that other people with your condition have said are important. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Not at all	A little bit	Somewhat	Quite a bit	Very much
I feel fatigued	○	○	○	○	○

I feel weak all over	○	○	○	○	○
I feel listless ("washed out")	○	○	○	○	○
I feel tired	○	○	○	○	○
I have trouble starting things because I am tired	○	○	○	○	○
I have trouble finishing things because I am tired	○	○	○	○	○
I have energy	○	○	○	○	○
I am able to do my usual activities	○	○	○	○	○
I need to sleep during the day	○	○	○	○	○
I am too tired to eat	○	○	○	○	○
I need help doing my usual activities	○	○	○	○	○
I am frustrated by being too tired to do the things I want to do	○	○	○	○	○

PHYSICAL WELL BEING

47. Below is a list of statements that other people with your condition have said are important. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Not at all	A little bit	Somewhat	Quite a bit	Very much
I have a lack of energy	○	○	○	○	○
I have nausea	○	○	○	○	○
Because of my physical condition, I have trouble meeting the needs of my family	○	○	○	○	○

I have pain	○	○	○	○	○
I am bothered by side effects of treatment	○	○	○	○	○
I feel ill	○	○	○	○	○
I am forced to spend time in bed	○	○	○	○	○

SOCIAL/FAMILY WELL BEING

48. Below is a list of statements that other people with your condition have said are important. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Not at all	A little bit	Somewhat	Quite a bit	Very much
I feel close to my friends	○	○	○	○	○
I get emotional support from my family	○	○	○	○	○
I get support from my friends	○	○	○	○	○
My family has accepted my illness	○	○	○	○	○
I am satisfied with family communication about my illness	○	○	○	○	○
I feel close to my partner (or the person who is my main support)	○	○	○	○	○

49. Regardless of your current level of sexual activity, please answer the following question. If you prefer not to answer it, please put an X in the box below and go to the next section.

	Not at all	A little bit	Somewhat	Quite a bit	Very much
I am satisfied with my sex life	○	○	○	○	○

EMOTIONAL WELL BEING

50. Below is a list of statements that other people with your condition have said are important. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Not at all	A little bit	Somewhat	Quite a bit	Very much
I feel sad	○	○	○	○	○
I am satisfied with how I am coping with my illness	○	○	○	○	○
I am losing hope in the fight against my illness	○	○	○	○	○
I feel nervous	○	○	○	○	○
I worry about dying	○	○	○	○	○
I worry that my condition will get worse	○	○	○	○	○

FUNCTIONAL WELL BEING

51. Below is a list of statements that other people with your condition have said are important. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Not at all	A little bit	Somewhat	Quite a bit	Very much
I am able to work (include work at home)	○	○	○	○	○
My work (include work at home) is fulfilling	○	○	○	○	○
I am able to enjoy life	○	○	○	○	○
I have accepted my illness	○	○	○	○	○
I am sleeping well	○	○	○	○	○
I am enjoying the things I usually do for fun	○	○	○	○	○
I am content with the quality of my life right now	○	○	○	○	○

52. Below is a list of statements that other people with your condition have said are important. Please check one number per line to indicate the response which would most accurately describe how you felt during your anti-cancer treatment.

	Not at all	A little bit	Somewhat	Quite a bit	Very much
I have cramps in my stomach area	○	○	○	○	○
I have pain in my stomach area					
Stomach pain interferes with my daily functioning	○	○	○	○	○
I have control of my bowels	○	○	○	○	○
I move my bowels more frequently than usual	○	○	○	○	○
I am afraid to be far from a toilet	○	○	○	○	○
I have to limit my social activity because of diarrhoea	○	○	○	○	○
I have to limit my physical activity because of diarrhoea	○	○	○	○	○
I have to limit my sexual activity because of diarrhoea	○	○	○	○	○
I am embarrassed by having diarrhoea	○	○	○	○	○
I have abdominal cramps or discomfort due to my diarrhoea	○	○	○	○	○
My problem with diarrhoea keeps/ wakes me up at night	○	○	○	○	○
I must move my bowels frequently to avoid accidents	○	○	○	○	○
I wear pads or protection to prevent soiling my underwear	○	○	○	○	○

Thank you for taking the time to participate in this survey.

Initial email sent to Breast Cancer Network Australia Review and Survey Group.

Subject: Share your experiences about how chemotherapy affects your well-being

Dear %%First Name%%

Women who have chemotherapy as part of their breast cancer treatment may experience a number of side effects. These include nausea, pain and vomiting, as well as cognitive changes, such as difficulty concentrating, feeling somewhat confused or having trouble with memory.

Researchers at the University of Adelaide are inviting you to participate in a study exploring possible links between the gastrointestinal side effects of chemotherapy (e.g. nausea, diarrhoea, bloating and constipation) and women's experiences of changes to their cognition.

Who can take part?
You can participate in this study if you:

- are aged 30 years or above
- are post-menopausal
- are currently undergoing chemotherapy treatment, or have had chemotherapy within the past 24 months
- have noticed difficulties with your cognition (e.g. having trouble concentrating, memory difficulties, feeling mildly confused)
- are a non-smoker
- are not diabetic
- have not had any pre-existing brain disorders or mental health conditions (such as major depression, brain injury, chronic pain, dyslexia, epilepsy, etc.)
- have not had a history of inflammatory gastrointestinal disorders (such as Irritable Bowel Disease, Crohn's Disease, ulcerative colitis, repeated stomach ulcers)
- are not on any medication that may affect your cognition (such as opioids)

What does the study involve?
Participation involves completing a survey, either online or by hardcopy posted to you.

The survey will take between 30 and 40 minutes to complete and your responses will be anonymous.

The survey will ask some questions about you, your diagnosis and treatment, side effects of chemotherapy you have experienced and the impact that these side effects have on your physical and emotional health and wellbeing.

To take the survey online, please visit: https://www.surveymonkey.com/r/H-2015-167_Understandingtheside-effectsofchemotherapy_UofA.

Or alternatively, if you wish to fill out a hardcopy of the survey, please email Ms Juliana Bajic at the University of Adelaide, Juliana.bajic@adelaide.edu.au, and she will mail out all the documents, including a stamped self-addressed envelope so you don't incur any costs.

For more information, please read the Participant Information Statement and Participant Inclusion and Exclusion Check List. If you have any questions or wish to find out more about the study, please contact Ms Juliana Bajic by emailing Juliana.bajic@adelaide.edu.au.

Privacy
The researchers will make sure that the information you provide is anonymous (your name and contact details will not be collected or stored with your survey answers). By taking this survey you are providing your consent (agreement) for the researchers to collect and store your survey answers on Survey Monkey's overseas server (large computer).

Click here to view BCNA's privacy policy.
Click here to view Survey Monkey's privacy policy.

Thanks very much for taking the time to consider this opportunity.

Warm regards,

Lisa Morstyn
Policy Officer

Appendix Five

This appendix includes the ethics approval numbers for the animal and human research undertaken throughout my PhD. As specifically stated in each chapter, some of the animals reported in these works, formed the controls of larger studies and accordingly, these studies have been acknowledged and cited appropriately. Nonetheless, I have included each reference and reference number, as it appears throughout my thesis, followed by the ethics approval numbers. All animal ethics approval was granted by the Animal Ethics Committee of The University of Adelaide. Permission was granted by the lead investigators of the larger studies mentioned here, prior to data collation, molecular work, statistical analysis and the write up of each chapter. The Human Research Ethics Committee of The University of Adelaide granted approval for the final research chapter of this thesis.

Chapter Three:

References

108. Mashtoub, S., C.D. Tran, and G.S. Howarth, Emu oil expedites small intestinal repair following 5-fluorouracil-induced mucositis in rats. Exp Biol Med (Maywood), 2013. 238(11): p. 1305-17.

Ethics approval # **S-2009-091A**

110. Bajic, J.E., et al., Rhubarb extract partially improves mucosal integrity in chemotherapyinduced intestinal mucositis. World J Gastroenterol, 2016. 22(37): p. 8322-8333.

Ethics approval # **S-2010-111**

286. Mashtoub, S., et al., Emu Oil Combined with Lyprinol Reduces Small Intestinal Damage in a Rat Model of Chemotherapy-Induced Mucositis. Nutr Cancer, 2016. 68(7): p. 1171-80.

Ethics approval # **S-2012-236A**

287. Whittaker, A.L., et al., Differential Effectiveness of Clinically-Relevant Analgesics in a Rat Model of Chemotherapy-Induced Mucositis. PLoS One, 2016. 11(7): p. e0158851.

Ethics approval # **S-2014-073A**

Chapter Four: Ethics approval # **M-2015-085B**

Chapter Five:

References

378. Chartier, L.C., et al., Emu Oil Improves Clinical Indicators of Disease in a Mouse Model of Colitis-Associated Colorectal Cancer. Dig Dis Sci, 2018. 63(1): p. 135-145.

Ethics approval # **M-2017-109**

379. Ghaemi, R., et al., Emu Oil Prevents Bodyweight Loss in a Mouse Model of Chronic Ulcerative Colitis. J Nutr Inter Metab, 2016. 4 (Supp): p. 6-47.

Ethics approval # **M-2015-252A**

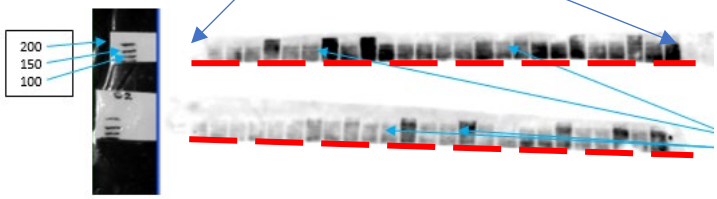
Chapter Six: Ethics approval # **H-2015-167**

Appendix Six

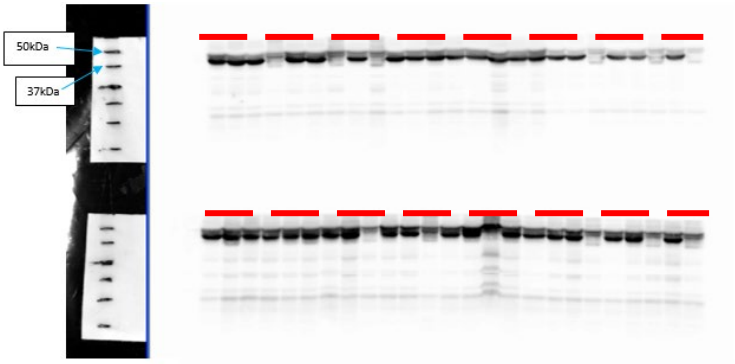
Please find here examples of a full western blot from each chapter. Following on from the examples, I have provided all full western blots used throughout this thesis. As some proteins were developed differently (using skim milk or BSA), it was necessary to cut the full blots to ensure the right developing specifications (see below).

Example of the same blot. Blots may be cut at 75kDa. A represents the top half of the blot (75-200kDa) and B represents the bottom half of the blot (15-75kDa).

A

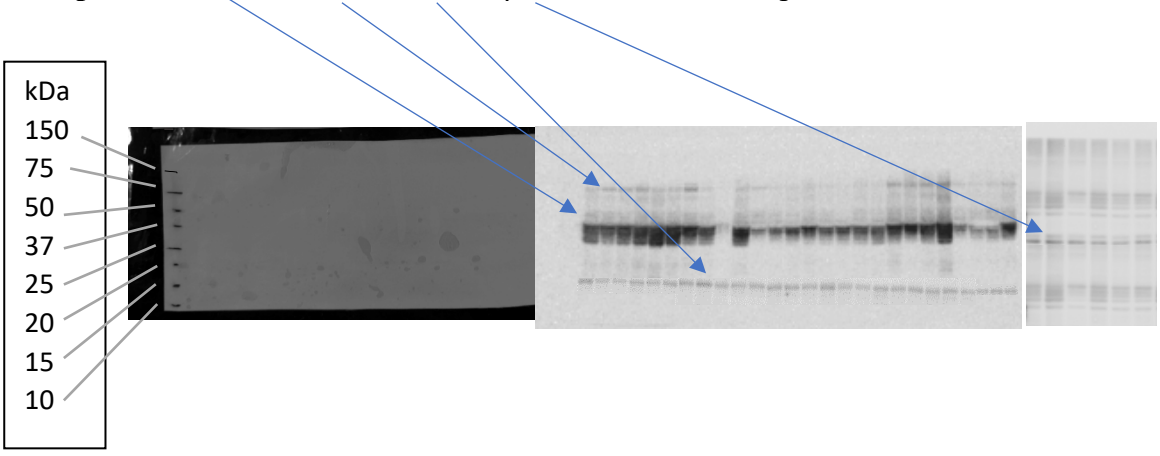


B

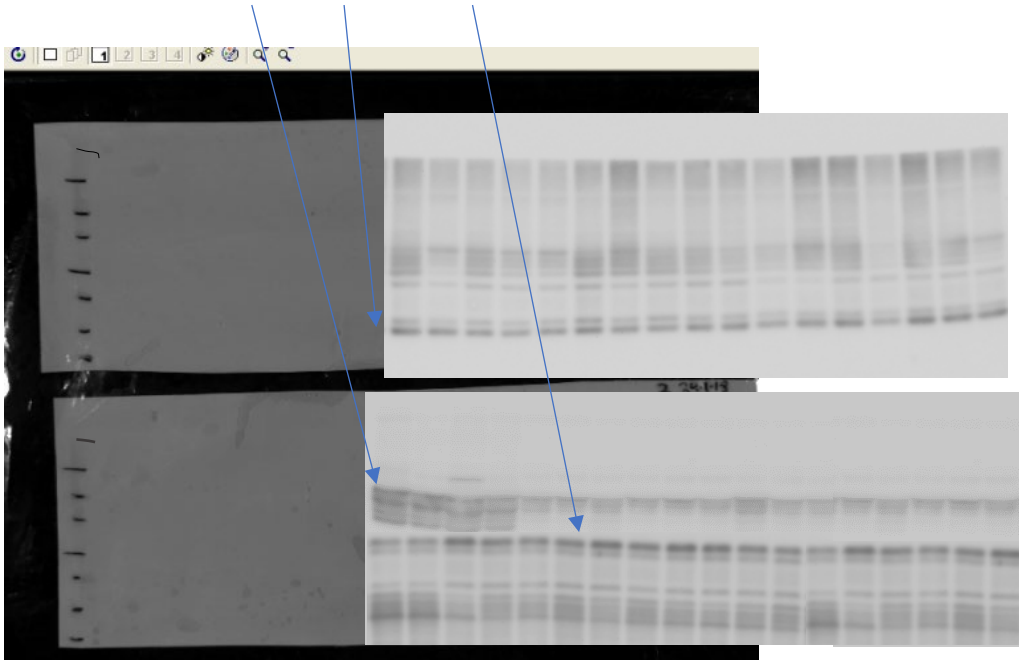


Western blot examples:

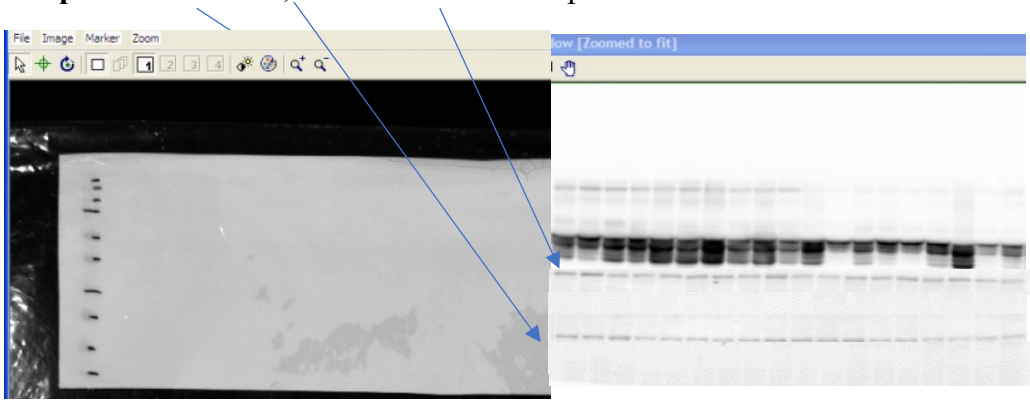
Chapter Three: GFAP, CD11b, IL-1β and actin blot examples.



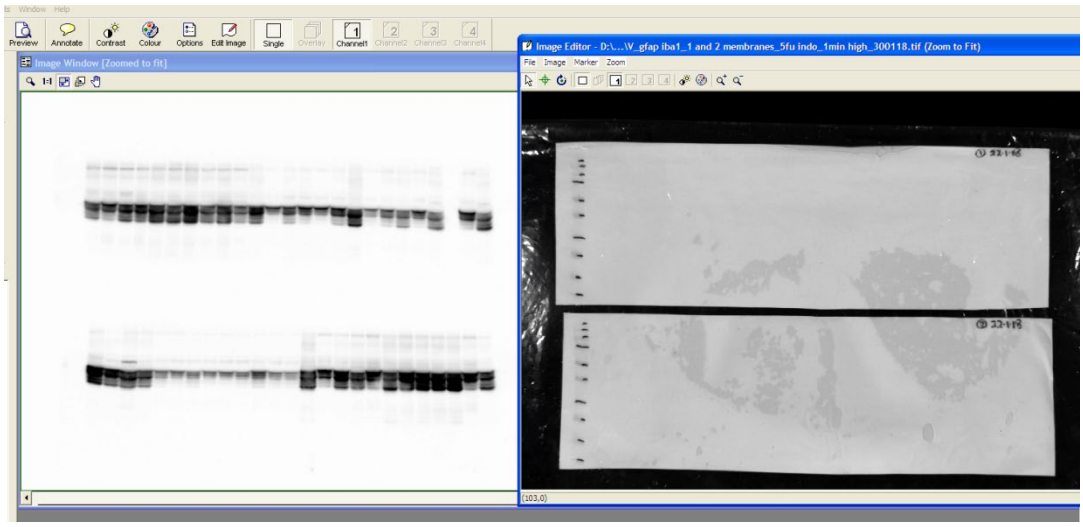
Chapter Four: GFAP, IL-1β and actin examples.



Chapter Five: GFAP, Iba-1 and actin examples.



All blots: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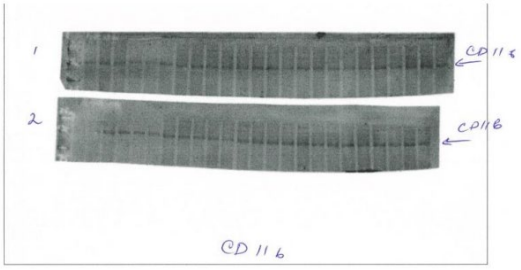
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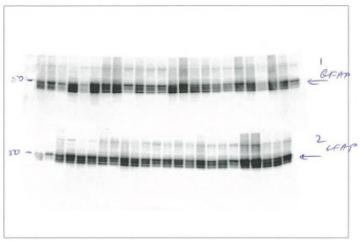
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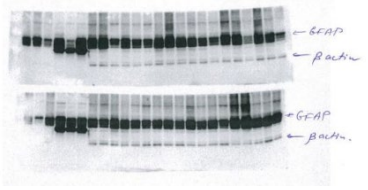
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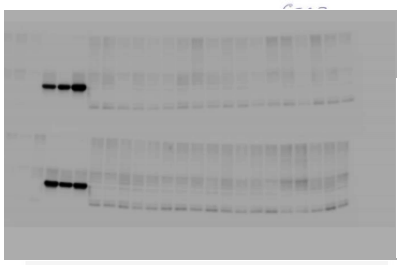

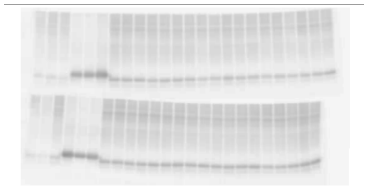

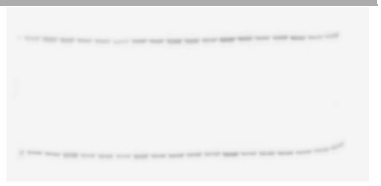

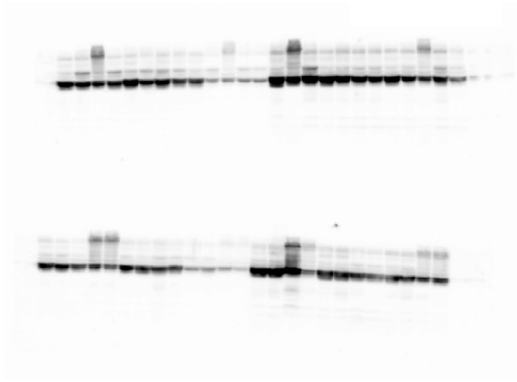

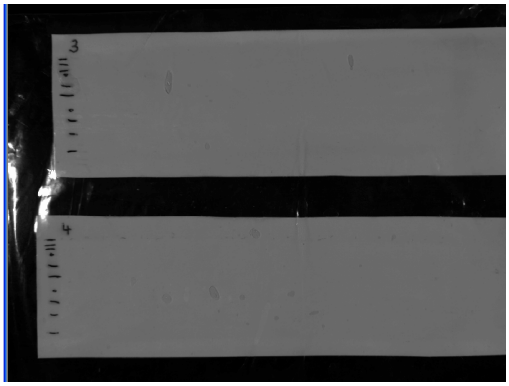
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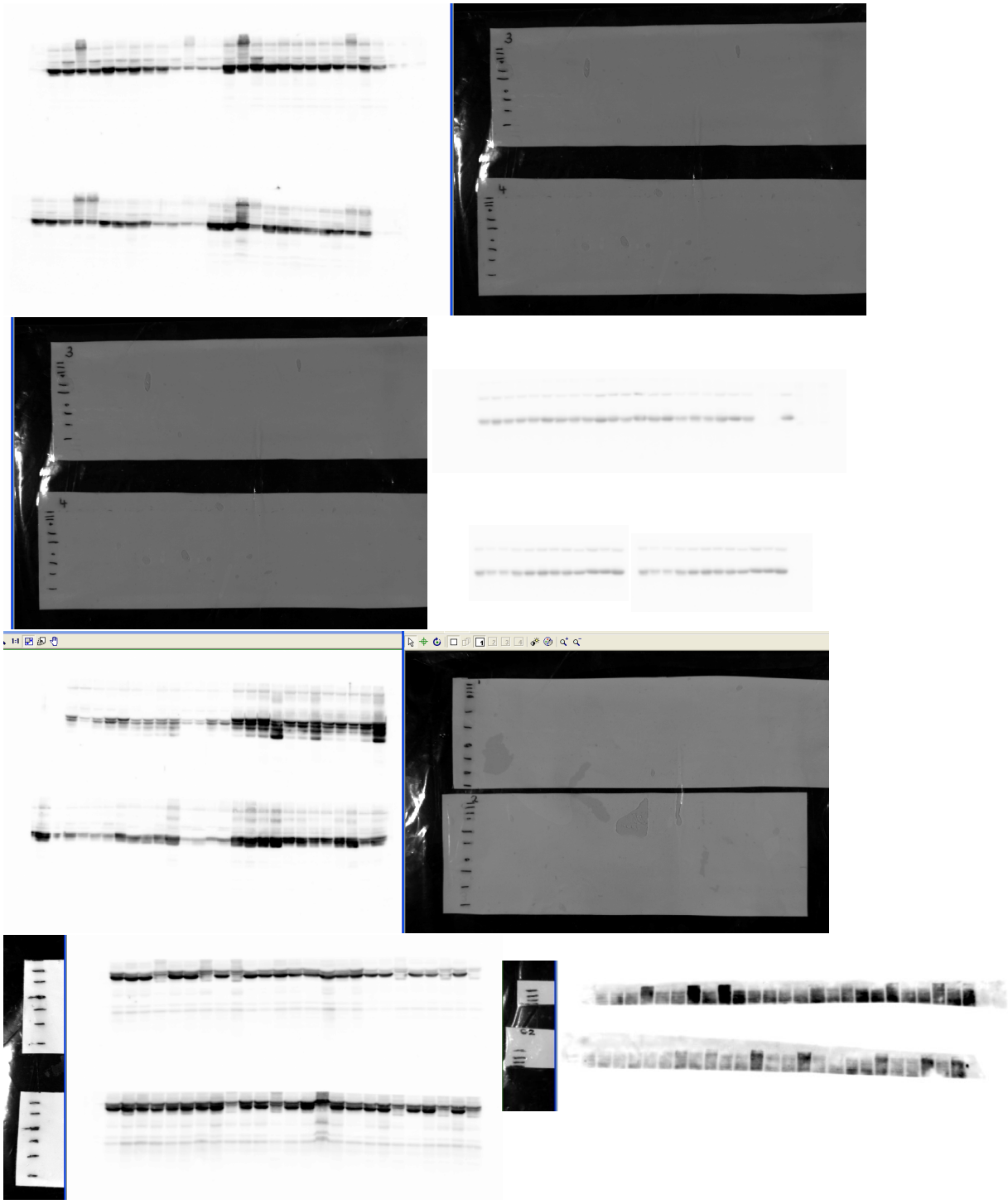

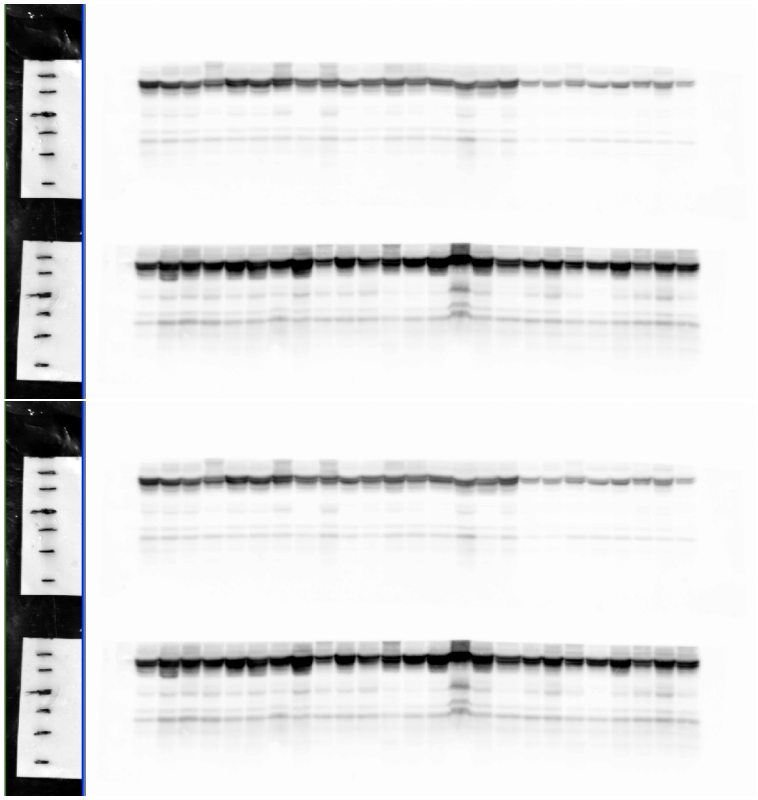

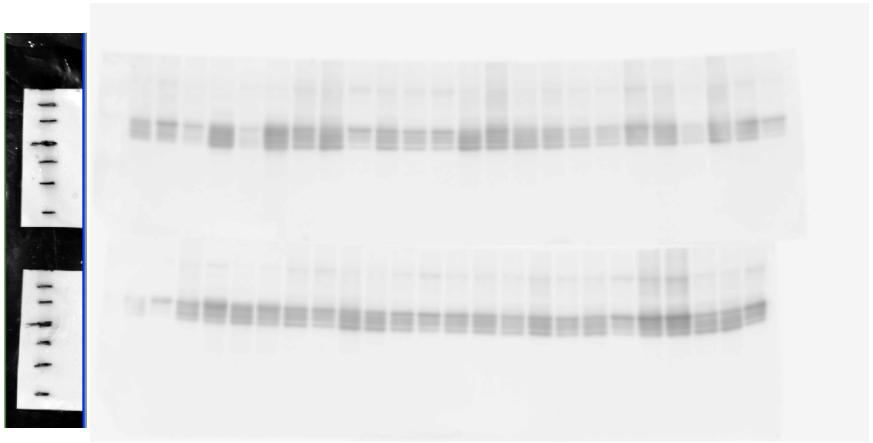

Thank you for taking the time to read my thesis!
Yours sincerely, Juliana Bajic.