

**Behavioural changes associated with chronic intestinal
inflammation: correlation with the changes in tryptophan
metabolism in the gut and brain**

Jeannie Devereaux

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Abstract

Context and purpose of the research project: Inflammatory bowel disease (IBD) encapsulates a chronic and systemic disorder characterised by recurring episodes of inflammation within the gastrointestinal tract. IBD incorporates two primary diseases, namely ulcerative colitis and Crohn's disease. It results in altered motility, ulcer formation, bleeding, diarrhoea, abdominal pain, increased morbidity and is linked with depression and anxiety. IBD's symptomatic burden has a psychosocial negative impact on emotional functioning, and it is believed that negative emotions influence biological disease progression and behavioural changes. Our study hypothesises that depressive and anxiety-like behaviours in IBD patients might be side effects of chronic intestinal inflammatory responses, possibly related to tryptophan metabolism.

Method: This thesis investigated the relationship between behavioural changes and energy metabolism, focusing on the tryptophan synthesis model in the distal colon and brain of the *Winnie* mouse with colitis. The *Winnie* mouse model, which has a *Muc2* missense mutation, exhibits a close synteny of its genome with ulcerative colitis. Using indirect calorimetry, various aspects, including food and water intake, energy metabolism, sleep patterns, and spontaneous physical activity of the *Winnie* mouse, were assessed. Depressive and anxiety-like behaviours were evaluated utilising rodent behavioural tests. In addition, the study employed RNA-sequencing for cytokine activity and immunohistochemistry in the mouse's brain to detect inflammation and structural changes. RNA-sequencing and metabolomics mapped the pathways of glycolysis/gluconeogenesis, fatty acid β -oxidation, and tryptophan *de novo* NAD⁺ synthesis in the distal colons and brains of the *Winnie* mouse. This aimed to clarify the pathophysiology of colitis in relation to behavioural changes.

Major conclusions drawn: Indirect calorimetry results indicated that the *Winnie* mouse consumed less food and water, exhibited perturbed metabolic profiles, showed diminished glucose and lipid oxidation, a paramount indicator of metabolic health, decreased spontaneous physical activity, a hallmark of decreased energy, and increased sleep. Behavioural tests revealed depressive and anxiety-like behaviours, manifested by decreased grooming, a fundamental self-care activity, and reduced exploratory tendencies, a key behavioural metric. Comprehensive gene expression analysis of the

distal colon identified disruptions in energy metabolism, integral detoxification processes, and tryptophan *de novo* NAD⁺ biosynthesis correlating with inflammation. Notably, the transcriptomes of *Winnie* mice and human IBD showed significant homologous similarities. Elevated levels of multiple tryptophan metabolites were detected in the colons of *Winnie* mice, indicating inflammation, compromised detoxification pathways, disrupted metabolism, and altered motility. Brain tryptophan metabolites, key neurotransmitter precursors, showed reduced levels, indicating diminished serotonergic pathway activity and nicotinamide production, central elements that are known to contribute to depression and behavioural changes. Immunohistochemistry revealed changes in brain structure of the *Winnie* mouse evidenced by altered expression of high mobility group box 1 protein, glial fibrillary acidic protein, myelin basic protein, transmembrane protein 119, and neuronal nuclei. In *Winnie* mice brains, pro-inflammatory cytokines increased, while anti-inflammatory cytokines, essential protective agents, decreased. These changes in cytokine activity, glycolysis, and tryptophan metabolism gene expressions, forming a cohesive pattern, correlated with reduced exploratory behaviour, depression, anxiety-like behaviours and behavioural changes, thus supporting the hypothesis.

Declaration

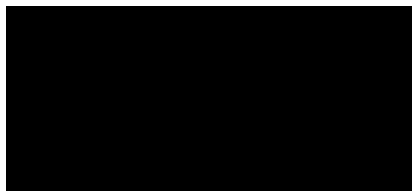
I, Jeannie Devereaux, declare that the PhD thesis entitled "Behavioural changes associated with chronic intestinal inflammation: correlation with the changes in tryptophan metabolism in the gut and brain" is no more than 80,000 words in length excluding quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated in the declaration of contribution to work, this thesis is my own work.

I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures.

Ethics declaration

I, Jeannie Devereaux, have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures. All research procedures reported in the thesis were approved by the Victoria University Animal Experimentation Ethics Committee (AEC-17-016) for all experiments in this thesis.

Signature:



Date: 4th October 2023

Declaration of contribution to work

Under my direction, the following people have made the stated contributions to this thesis:

Chapter 1

Assistance in preparing the manuscript for part of this chapter was provided by supervisors Kulmira Nurgali, Jim Kiatos, and Vasso Apostolopoulos.

Chapter 2

Anne Luxford provided technical assistance and training for the Promethion cages. Rhiannon Filippone and Majid Davidson assisted with tissue extraction. Ramya Ephraim assisted with RNA extraction from the colon tissue. Rhian Stavely contributed to the NGS data. Assistance in preparing the manuscript for this chapter was provided by supervisors Kulmira Nurgali, Jim Kiatos, and Vasso Apostolopoulos.

Chapter 3

Larry Bautista provided the training and assistance in metabolomics. Rhiannon Filippone assisted with tissue extraction. Ramya Ephraim assisted with RNA extraction. Rhian Stavely contributed to the NGS data analysis. Assistance in preparing the manuscript for this chapter was provided by Rhian Stavely and supervisors Kulmira Nurgali, Jim Kiatos, and Vasso Apostolopoulos.

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Assistance with the behaviour studies was provided by Majid Davidson and Esmee Buscher. Narges Dargahi assisted in RNA extraction. Rhian Stavely contributed to the NGS data analysis. During Colin Craig's Honours, Colin Craig and Rhiannon Filippone performed the immunohistochemistry and data analysis. Assistance in preparing the manuscript for this chapter was provided by supervisors Kulmira Nurgali, Jim Kiatos, and Vasso Apostolopoulos.

Chapter 5

Rhiannon Filippone and Majid Davidson assisted with tissue extraction. Narges Dargahi assisted in RNA extraction. Rhian Stavelly contributed to the NGS data analysis. Assistance in preparing the manuscript for this chapter was provided by supervisors Kulmira Nurgali, Jim Kiatos, and Vasso Apostolopoulos.

Chapter 6

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Chapter 7

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Dedication

This thesis is tributed to my daughters, Maria and Christel, as well as the newest family member, my granddaughter, Edie Elizabeth and the second grandchild, yet to be born. I would like to express my heartfelt gratitude to Maria and Christel who have been an endless source of inspiration and light in my life, plus for being such admirable role models. As their mother, I have watched them grow into extraordinary women who have fearlessly pursued their dreams and achieved great success. It is thanks to their unwavering willpower and courage that I have been motivated to follow in their footsteps. I would like to extend a special thank you to my precious granddaughter, Edie, who was born during the time of the thesis. Her delightful videos, from her first attempts at holding up her head to crawling, walking, singing, and blowing kisses, kept me entertained and inspired throughout the process.

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Abbreviations

2-AAA	2- α -aminoadipic acid
3-HAA	3-hydroxyanthranilic acid
3-HAO	3-hydroxyanthranilic acid oxygenase
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HT _{2A}	5-hydroxytryptamine (serotonin) receptor 2A
5-HT _{4R}	5-hydroxytryptamine (serotonin) receptor 4
5-HTP	5-hydroxytryptophan
A	absorbance
α 7nAChR	alpha7 nicotinic acetylcholine receptor
AA	anthranilic acid
ACAT1	acetyl-CoA acetyltransferase 1
ACMS	α -amino- β -carboxymuconate- ϵ -semialdehyde
ACMSD	α -amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase
ACSL	acyl-CoA synthetase
Acyl-CoA	acyl-coenzyme A
ADP	adenosine diphosphate
AFMID	arylformamidase
AhR	aryl hydrocarbon receptor
ALDH	aldehyde dehydrogenase
AMP	adenosine monophosphate
ANOVA	analysis of variance
ANS	autonomic nervous system
ATP	adenosine triphosphate
BBB	blood-brain barrier
BH ₄	tetrahydrobiopterin
BP	biological processes
BPG	bisphosphoglyceric acid
BW	body weight
CAMs	cell adhesion molecules
CC	cellular compartments

CD	Crohn's disease
CD38	cluster of differentiation 38
CD73	ecto-5'-nucleotidase
cDNA	complementary deoxyribonucleic acid
cm	centimetre
CNS	central nervous system
CO ₂	carbon dioxide
CoA	coenzyme A
CPM	counts per million
CRP	c-reactive protein
CSF	cerebrospinal fluid
CYP	cytochrome P450
DALY	disability-adjusted life years
DAMPs	damage-associated molecular patterns
DAO	diamine oxidase
DAPI	4',6-diamidino-2-phenylindole
DEG	differentially expressed gene
DHAP	dihydroxyacetone phosphate
DHTKD1	dehydrogenase E1 and transketolase domain containing 1
DNA	deoxyribonucleic acid
DNBS	dinitrobenzene sulfonic acid
DSS	dextran sulfate sodium
EC	electronic commission
EchoMRI	echo magnetic resonance imaging
EE	energy expenditure
ELISA	enzyme-linked immunosorbent assay
ENS	enteric nervous system
EPM	elevated plus maze
ESR	erythrocyte sedimentation rate
ETC	electron transport chain
FAD	flavin adenine dinucleotide
FADH ₂	flavin adenine dinucleotide + hydrogen ₂
FAO	fatty acid beta (β)-oxidation
FC	fold change

FDR	false discovery rate
FST	forced swim test
g	gram
G3P	glyceraldehyde 3-phosphate
G6P	glucose 6-phosphate
GA-1	glutaric acid type I
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCDH	glutaryl-CoA dehydrogenase
GFAP	glial fibrillary acidic protein
GO	gene ontology
GOBP	gene ontology biological process
GOCC	gene ontology cellular compartment
GOMF	gene ontology molecular function
GOX	glucose oxidation
GSEA	gene set enrichment analysis
GTP	guanine triphosphate
h	hour
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
HADH	hydroxyacyl-CoA dehydrogenase
HCIO ₄	perchloric acid
Het	heterozygous
HIF-1 α	hypoxia-inducible factor-1 α
HMGB1	high mobility group box 1
HPA	hypothalamus–pituitary–adrenal
HPG	hypothalamic/pituitary/gonadal
IBD	inflammatory bowel disease
IDH	isocitrate dehydrogenase
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IFN- γ	interferon gamma
IHC	immunohistochemistry
IL	interleukin

IL-6	interleukin 6
IL-10	interleukin 10
IL-12	interleukin 12
IL-17	interleukin 17
IL-1 β	interleukin 1 beta
IL-21	interleukin 21
IL-22	interleukin 22
IL-23	interleukin 23
IL-23	interleukin 23
IPAA	ileal pouch-anal anastomosis
IR	immunoreactivity
kcal	kilocalories
KEGG	kyoto encyclopedia of genes and genomes
kg	kilogram
KO	knockout
Kyn	kynurenine
KYNA	kynurenic acid
L	litre
LAAO	L-amino acid oxidase/monooxygenase
LAT1	large neutral amino acid transporter
LCAD	long-chain acyl-CoA dehydrogenase
Lcn-2	lipocalin-2
LDH	l-lactate dehydrogenase
LDT	light dark test
LNAA	large neutral amino acids
LOD	limits of detection
LOQ	limits of quantification
LOX	lipid oxidation
LPS	lipopolysaccharide
MAO	monoamine oxidase
MAO-A	monoamine oxidase A
MAO-B	monoamine oxidase B
MBP	myelin basic protein
MCAD	medium-chain acyl-CoA dehydrogenase

MCL	markov cluster algorithm
MF	molecular function
min	minute
mL	millilitre
mM	millimolar
MRI	magnetic resonance imaging
mTORC1	mammalian target of rapamycin complex 1
MUC	Mucin, oligomeric mucus/gel-forming
NA	nicotinic acid
NaAD ⁺	nicotinic acid dinucleotide
NAADP	nicotinic acid adenine dinucleotide phosphate
NAc	nucleus accumbens
NAD ⁺	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide + hydrogen
NADPH	nicotinamide adenine dinucleotide phosphate
NADSYN	nicotinamide adenine dinucleotide synthetase
NAM	nicotinamide
NaMN	nicotinic acid mononucleotide
NAMPT	nicotinamide phosphoribosyltransferase
NAPRT	nicotinate phosphoribosyltransferase
NDS	normal donkey serum
NeuN	neuronal nuclei
NF-κB	nuclear factor kappa light chain enhancer of activated B cells
NGAL	neutrophil gelatinase B-associated lipocalin
NGS	next generation sequencing
ng/μL	nanogram per microlitre
NK	natural killer
NMDA	n-methyl-D-aspartate
NMN	nicotinamide mononucleotide
NMNAT	nicotinamide mononucleotide adenylyl transferases
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain
NR	nicotinamide riboside
NMRK	nicotinamide riboside kinase

O ₂	oxygen
OCT	optimal cutting temperature
OFT	open field test
OGDH	oxoglutarate dehydrogenase
OGDHC	2-oxoglutarate dehydrogenase complex
PARPs	poly [ADP-ribose] polymerase
PBS	phosphate buffered solution
PDHC	pyruvate dehydrogenase complex
PEP	phosphoenolpyruvate
PEPCK	phosphoenol-pyruvate carboxykinase
PFC	prefrontal cortex
pg/mL	picograms per millilitre
PIC	picolinic acid
PPi	protein to protein interaction
PRPP	5'-phosphoribosyl-1-pyrophosphate
psi	per square inch
QoL	quality of life
QPRT	quinolinate phosphoribosyltransferase
QUIN	quinolinic acid
Rheb	ras homolog enriched in brain
REVIGO	reduce and visualise gene ontology
RNA	ribonucleic acid
RNA-Seq	RNA-Sequencing
RNS	reactive nitrogen species
ROS	reactive oxygen species
RQ	respiratory quotient
s	seconds
SAMe	s-adenosyl-l-methionine
SD	standard deviation
SDI	socio-demographic index
SE	standard error
SEM	standard error of the mean
SERT	serotonin transporter
SLC6A19	solute carrier family 6 members 19

SSRIs	selective serotonin reuptake inhibitors
STRING	search tool for retrieval of interacting genes
TCA	tricarboxylic acid
TDO	tryptophan 2,3-dioxygenase
TGF- β 1	transforming growth factor beta 1
Th17	t helper 17
TLR	toll-like receptors
TLR4	toll-like receptor 4
TMEM119	transmembrane protein 119
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF- α	tumour necrosis factor alpha
TPH1	tryptophan hydroxylase 1
TPH2	tryptophan hydroxylase 2
TPI	triose phosphate isomerase
Trp	tryptophan
UC	ulcerative colitis
UFLC	ultra-fast liquid chromatography
VLCAD	very long-chain acyl-CoA dehydrogenase
VCO ₂	rate of carbon dioxide emission in millilitres per minute
VO ₂	rate of oxygen consumption in millilitres per minute
WP	WikiPathways
YLL	years of life lost
μ g	micro grams
μ g/L	microgram per litre
μ L	micro litre

Genes

<i>Acadl</i>	long-chain-acyl-CoA dehydrogenase
<i>Acadm</i>	medium-chain acyl-CoA dehydrogenase
<i>Acadsb</i>	short/branched chain acyl-CoA dehydrogenase
<i>Acadvl</i>	very long chain acyl-CoA dehydrogenase
<i>Acat1</i>	acetyl-CoA acetyltransferase 1
<i>Acox1</i>	acyl-CoA oxidase 1, palmitoyl
<i>Acsf</i>	acyl-CoA synthetase

<i>Adh</i>	alcohol dehydrogenase (NADP ⁺)
<i>Adpgk</i>	ADP:D-glucose 6-phospho-transferase
<i>Afmid</i>	arylformamidase
<i>Akr1a1</i>	aldehyde reductase
<i>Aldh2</i>	aldehyde dehydrogenase (NAD ⁺) 2
<i>Aldh3a1</i>	aldehyde dehydrogenase 3 [NAD(P) ⁺]
<i>Aldh3b1</i>	aldehyde dehydrogenase 3 family member B1
<i>Aldh3b2</i>	aldehyde dehydrogenase (NAD(P) ⁺)
<i>Aoc1</i>	amine oxidase copper containing 1
<i>Aox1</i>	aldehyde oxidase 1
<i>Art2a</i>	ADP-ribosyl transferase 2a
<i>Bdh</i>	3-hydroxybutyrate dehydrogenase
<i>Bpgm</i>	bisphosphoglycerate mutase
<i>Cd38</i>	cluster of differentiation 38
<i>Cd73</i>	ecto-5'-nucleotidase
<i>Cpt1</i>	carnitine O-palmitoyl-transferase 1
<i>Cyp1a1</i>	cytochrome P450 family 1 subfamily A1
<i>Cyp4a10</i>	cytochrome P450, family 4 subfamily a polypeptide 10
<i>Dao</i>	diamine oxidase
<i>Ddc</i>	L-tryptophan decarboxylase
<i>Dhtkd1</i>	dehydrogenase E1 and transketolase domain containing 1
<i>Eci</i>	delta (Δ)3- Δ 2-enoyl-CoA isomerases
<i>Eno</i>	enolase
<i>Fmo</i>	dimethylaniline monooxygenase [N-oxide-forming]
<i>G6pc</i>	glucose 6-phosphate
<i>galM</i>	aldose 1- epimerase
<i>Gapdh</i>	glyceraldehyde 3-phosphate dehydrogenase
<i>Gcdh</i>	glutaryl-CoA dehydrogenase
<i>Gfap</i>	glial fibrillary acidic protein
<i>H2-Ab1</i>	histocompatibility 2, class II antigen A, beta 1
<i>H2-DMb2</i>	histocompatibility 2 class II, locus M beta 2
<i>H2-Eb1</i>	histocompatibility 2, class II antigen E beta 1
<i>H2-Oa</i>	histocompatibility 2 O region alpha locus
<i>Hadh</i>	hydroxyacyl-CoA dehydrogenase

<i>Hadhb</i>	hadh trifunctional multienzyme complex subunit β
<i>Hk</i>	hexokinase
<i>Hk2</i>	hexokinase 2
<i>Hmgb1</i>	high mobility group box 1
<i>Ido</i>	indoleamine 2,3-dioxygenase
<i>Il12a</i>	interleukin 12a
<i>Il12rb2</i>	interleukin 12 receptor subunit beta 2
<i>Il21</i>	interleukin 21
<i>Il22</i>	interleukin 22
<i>Il23a</i>	interleukin 23a
<i>Il23r</i>	interleukin 23 receptor
<i>Il2rb1</i>	interleukin 2 receptor subunit beta 1
<i>Il2rg</i>	interleukin 2 receptor gamma
<i>Il5</i>	interleukin 5
<i>Il8rap</i>	interleukin 18 receptor accessory protein
<i>Inmt</i>	tryptamine N-methyl-transferase
<i>Kynu</i>	kynureninase
<i>LaaO</i>	l-amino acid oxidase/monooxygenase
<i>Lcad</i>	long-chain acyl-CoA dehydrogenase
<i>Ldh</i>	l-lactate dehydrogenase
<i>Ldha</i>	l-lactate dehydrogenase a
<i>Ldhb</i>	l-lactate dehydrogenase b
<i>Mao-a</i>	monoamine oxidase a
<i>Mao-b</i>	monoamine oxidase b
<i>Mbp</i>	myelin basic protein
<i>Me</i>	malic enzyme
<i>Minpp1</i>	multiple inositol-polyphosphate phosphatase 1
<i>Muc2</i>	mucin 2
<i>Nadsyn</i>	nicotinamide adenine dinucleotide synthetase
<i>Nampt</i>	nicotinamide phosphoribosyltransferase
<i>Naprt</i>	nicotinate phosphoribosyl-transferase
<i>Nmnat</i>	nicotinamide mononucleotide adenylyl transferases
<i>Nmrk</i>	nicotinamide ribose kinase
<i>Nnmt</i>	nicotinamide N-methyltransferase

<i>Nod2</i>	nucleotide-binding oligomerisation domain
<i>Nudt17</i>	nucleoside diphosphate-linked moiety X motif 17
<i>Ogdh</i>	oxoglutarate (α -ketoglutarate) dehydrogenase
<i>Parp</i>	poly [ADP-ribose] polymerase
<i>Pck</i>	phosphoenol-pyruvate carboxykinase
<i>Pdha1</i>	pyruvate dehydrogenase E1 subunit α 1
<i>Pepck</i>	phosphoenol-pyruvate carboxykinase
<i>Pfk</i>	6-phosphofructokinase
<i>Pfkm</i>	ATP-dependent 6-phosphofructokinase
<i>Pfkp</i>	6-phosphofructokinase platelet
<i>Pgam1</i>	phosphoglycerate mutase 1
<i>Pgk1</i>	phosphoglycerate kinase 1
<i>Pgm</i>	phosphoglucomutase
<i>Phgdh</i>	D-3-phosphoglycerate dehydrogenase
<i>Pkm</i>	pyruvate kinase
<i>Pnp</i>	purine nucleoside phosphorylase
<i>Ptgis</i>	prostacyclin synthase
<i>Qdpr</i>	dihydropteridine reductase
<i>Rbfox1</i>	RNA binding protein, fox-1 homolog
<i>SAMe</i>	s-adenosyl-l-methionine
<i>Sirt</i>	sirtuins
<i>Slc7a5</i>	solute carrier family 7 member 5
<i>Smad2</i>	mothers against decapentaplegic homolog 2
<i>Sord</i>	sorbitol dehydrogenase
<i>Tgfb1</i>	transforming growth factor beta 1
<i>Tlr2</i>	toll-like receptor 2
<i>Tlr5</i>	toll-like receptor 5
<i>Tmem119</i>	transmembrane protein 119
<i>Tnf</i>	tumour necrosis factor
<i>Tph1</i>	tryptophan hydroxylase 1
<i>Tpi</i>	triose phosphate isomerase
<i>Ugdh</i>	UDP-glucose 6-dehydrogenase

Chapter 1: Literature review

1.1 Summary

Inflammatory bowel disease (IBD) represents a chronic condition characterized by repeated gastrointestinal tract inflammation and primarily encompasses two diseases: ulcerative colitis and Crohn's disease. These conditions are differentiated by specific histopathological changes and the affected regions in the gastrointestinal tract. Ulcerative colitis primarily affects the distal colon mucosa, while Crohn's disease displays non-continuous manifestations throughout the intestinal wall. Indeterminate colitis, accounting for 10% of IBD cases, doesn't clearly fit either ulcerative colitis or Crohn's disease classifications.

The underlying causes of IBD involve over 100 identified genetic defects, environmental factors, and disturbances. While these contributing elements are known, their interrelationships remain unclear. IBD patients not only suffer from physical symptoms like inflammation and discomfort but are also prone to psychological issues like depression and anxiety. Contemporary research has highlighted the potential link between these psychological symptoms and immune-inflammatory responses, with imbalances in tryptophan metabolism playing a potential role. Intriguingly, patients using selective serotonin reuptake inhibitors exhibited fewer IBD incidents, hinting at a possible connection between serotonin, the brain, the gut, and IBD. The importance of understanding tryptophan, a serotonin precursor, cannot be overstated, given its pivotal role in mood and intestinal function.

This research aims to present a holistic view of IBD by examining its epidemiology, pathophysiology, related behavioral shifts, and the critical role of tryptophan metabolism. Additionally, the thesis posits that energy and tryptophan metabolism in the gut and brain impact the behavior of *Winnie* mice with colitis.

1.2 Introduction

The designation “IBD” (inflammatory bowel disease) encapsulates a chronic and pervasive disorder characterised by repeated episodes of inflammation within the gastrointestinal tract. IBD incorporates two primary diseases, namely, ulcerative colitis (UC) and Crohn’s disease (CD) (Fakhoury et al., 2014). The differentiation between UC and CD is based on characteristic histopathological changes, and the anatomical location of disease manifestations within the gastrointestinal tract. Specifically, in UC, inflammation is confined to the distal colon mucosa (Loftus, 2004). In CD the manifestations are non-contiguous, with unaffected areas (skip lesions) alternating with inflamed areas in which the entire intestinal wall is affected (Baumgart & Sandborn, 2012). In addition, a subtype of IBD, known as indeterminate colitis, presents with clinical and histological features that do not allow for differentiation between CD and UC, and accounts for 10% of IBD cases (Loftus, 2004).

Over 100 gene defects, environmental factors, and disturbances have been identified as contributors to the pathophysiology of IBD, however, how these factors interconnect is not yet fully understood. IBD is marked by recurrent inflammation, disrupted gut motility, discomfort, and increased morbidity. Furthermore, it’s associated with depression, anxiety, and behavioural shifts. Recent studies have reframed anxiety and depressive symptoms in IBD patients, suggesting they might be consequences of immune-inflammatory responses and imbalances in tryptophan metabolism. Notably, patients on selective serotonin reuptake inhibitors (SSRIs) have shown a reduced occurrence of CD and UC (Kristensen et al., 2018). This suggests a potential causal link between serotonin (5-HT; 5-hydroxytryptamine), the gut, the brain, and IBD (Yanartaş et al., 2019). Understanding tryptophan, a precursor to serotonin, is crucial since it plays a significant role in mood regulation and intestinal function, influencing conditions such as IBD and depression (Silva et al., 2017).

In order to more fully elucidate the roles and relationships of the disparate and interconnected factors involved in IBD, a more comprehensive approach is required in which IBD epidemiology, pathophysiology, behavioural changes, as well as the role of

tryptophan metabolism, are all examined. To identify the conditions more fully under which tryptophan metabolism is altered, it is necessary to combine models of glycolysis and fatty acid β -oxidation with the tryptophan synthesis model. This thesis hypothesises that chronic intestinal inflammation in the *Winnie* mouse model of spontaneous colitis leads to significant behavioural changes, which are due to changes in energy and tryptophan metabolism within the gut and brain.

The specific aims of this thesis are:

- 1) To evaluate changes in energy metabolism in *Winnie* mice.
- 2) To identify behavioural changes in *Winnie* mice.
- 3) To determine the changes in energy and tryptophan metabolism pathways in the distal colons of *Winnie* mice.
- 4) To determine the changes in energy and tryptophan metabolism pathways in the brains of *Winnie* mice.
- 5). To elucidate the role of energy and tryptophan metabolism associated with behavioural and metabolic alterations in *Winnie* mice.

1.2.1 Epidemiology of IBD

The countries of Australia, the United Kingdom, the United States, and Canada, which have high socio-demographic index (SDI), display a markedly elevated incidence of IBD. Epidemiological studies show the incidence of IBD is increasing and now affects 80,000 Australians and up to 1.4 million people worldwide (Ng et al., 2017). The rates of occurrence and widespread presence of IBD are on the rise across various global regions, signalling its status as an emerging worldwide health issue (Molodecky et al., 2012). According to studies by Molodecky et al. (2012) that examined trends over several decades, 75% of research focusing on CD and 60% examining UC noted statistically significant increases in incidence rates. In terms of annual incidence, Europe recorded the highest numbers for UC at 24.3 cases per 100,000 person-years, followed by North America at 19.2 and Asia and the Middle East at 6.3. For CD, the highest annual incidence was noted in North America at 20.2 cases per 100,000 person-years, followed by Europe at 12.7 and Asia and the Middle East at 5.0. When it comes to prevalence, Europe and

North America had the highest reported figures, with Europe having 505 cases of UC and 322 cases of CD per 100,000 individuals, and North America having 249 cases of UC and 319 cases of CD per 100,000 individuals. Although IBD primarily affects people between the ages of 15 and 30, after age 60, 10% to 15% of individuals develop IBD, constituting a second peak in the distribution. The incidence of CD is slightly higher among females, whereas UC appears to impact both sexes equally (Sumant & Talha, 2016). The rise in IBD prevalence and incidence can be attributed to several factors including diet, medication exposures, urbanisation, pollution, increased IBD awareness among patients and healthcare providers, the use of advanced endoscopic and radiologic diagnostic techniques, and better access to healthcare (Liso et al., 2020; Sumant & Talha, 2016). These observations are corroborated by Liso et al. (2020), who identified urbanisation, pollution, and the excessive use of prescription antibiotics in these areas as potential factors that contribute to the onset of IBD. These factors may explain the observation that IBD incidence has increased notably among immigrants who have migrated from low-risk areas to high-risk regions of western countries.

1.2.2 Quality of life for IBD patients

UC and CD are known to negatively impact Quality of life (QoL). QoL is a crucial aspect of any chronic illness. It comprises discomfort and pain, compromised daily activities, as well as diminished functionality in the physical, social, cognitive, and emotional domains (Matos et al., 2021). In contrast to focusing solely on disease-specific factors, measurement of QoL provides insight on the broader psychosocial implications of a disease. Several factors, including the severity and type of disease, as well as factors independent of the disease, such as gender, psychological disorders, co-morbidities, and coping strategies are linked to a lower QoL in IBD patients (Matos et al., 2021).

Individuals with IBD have a higher mortality rate, ranging from 1.5 to 5 times greater than the general population, with patients diagnosed with CD experiencing the highest levels of morbidity and mortality (Follin-Arbelet et al., 2023). The leading causes of death for IBD patients include infections, disease progression, surgical complications, and multiorgan involvement. In addition, individuals with IBD have a higher risk of developing colorectal malignancy, particularly those with pancolitis, who have the

greatest risk within 20 years. This necessitates frequent diagnostic colonoscopies at intervals of 1 to 2 years (McDowell C. et al., 2023).

The most recent prevalence and burden estimates for IBD varied significantly across regions and were especially high in countries with a high SDI. Despite an increase in prevalence (+6%) from 1990 to 2017, the burden of IBD decreased (disability-adjusted life years (DALY) 12%). This decline was attributable to IBD-related premature mortality (years of life lost (YLL) 26%). In 2017, this metric represented a large proportion of the disease burden in countries with a low SDI (86% of DALYs), whereas disability dominated in countries with a high SDI (71%). Particularly, disease burden decreased in adolescents (DALYs 39% and YLLs 52%) (Piovani et al., 2020).

1.2.3 IBD characteristics

The pathogenesis of colitis commences long before changes become apparent, as a perplexing process within the inflamed gut tissue (Haberman et al., 2019). No single serological antibody has been validated for IBD diagnosis, and instead a combination of multiple markers are used (Cioffi et al., 2015). To identify UC, doctors analyse both blood tests and abdominal imaging studies. They consider a range of factors including anaemia, Erythrocyte Sedimentation Rate (ESR), and C-reactive protein (CRP) levels. Additionally, a biopsy may show lymphocytic infiltration of the mucosa, and procedures like colonoscopy or flexible sigmoidoscopy are often performed for a more comprehensive diagnosis (Coates et al., 2013).

Symptoms reported by UC patients involve mucosal inflammation, which causes oedema, ulcer formation, bleeding, diarrhoea, electrolyte loss, tenesmus, and abdominal pain. Approximately 20% of patients with UC have rectal-only disease, while 15% have pancolitis. UC patients are susceptible to extraintestinal manifestations such as inflammation of the skin, conjunctiva, and inflammatory arthritis. Upon physical examination, patients may report discomfort in the lower or upper left quadrant of the abdomen. Patients presenting with acute abdominal symptoms characterised as guarding, rebound tenderness, or percussion tenderness require an investigation for toxic megacolon (McDowell C. et al., 2023).

The diagnosis of CD relies on a combination of the patient's medical background, radiological and serological examinations, ESR and CRP. If faecal calprotectin is elevated, it indicates the movement of neutrophils to the intestinal mucosa, which occurs during inflammation of the intestines. This measurement serves as a diagnostic tool and a gauge of the severity of the disease. The principal characteristic of CD is that it affects all intestinal layers (transmural) in the colon and ileum, while only 5% of cases affect the gastroduodenal segments (Baumgart & Sandborn, 2012).

1.2.4 Mucins, the intestine, and IBD

Researchers have identified critical features of IBD that result from excessive immune cell activation triggering sustained cell damage within the intestinal epithelium, simultaneous with disruption of the intestinal barrier function (Fakhoury et al., 2014). The intestinal barrier is a complex configuration and comprises four anatomical structures: the intestinal microbiota; the protective mucus layer of the epithelium; the intestinal epithelium barrier and intercellular junctions; and the immunological barrier of which serotonin (derived from tryptophan) is a modulator (Arrieta et al., 2006). The epithelial tissue in the gut is designed to protect against disruptions, pathogens, and inflammation and embody complex microbial systems (Hansson, 2012).

In healthy individuals and mice, the mucin oligomeric mucus/gel-forming gene (MUC), located in the intestinal goblet cells, generates a protective interface mucus gel layer that physically separates the enterocytes and luminal microbial communities within the intestine (**Figure 1.1**) (Arrieta et al., 2006; Hansson, 2012; Tadesse et al., 2017). Mucins refer to a group of heavily glycosylated proteins (glycoconjugates) with high molecular weight that are synthesised by epithelial tissues. These proteins possess a bottlebrush-like structure, which imparts gel-like characteristics to mucus. The mucus gel assembles biological factors secreted from mucosal epithelial cells and subsequently establishes the mucus-lubricating system, which supplies nutrients to the intestinal microbiota and regulates mucosal immunity (Gustafsson et al., 2012).

Distinct mucin genes are expressed in different segments of the gastrointestinal tract. *MUC2* represents the primary mucin gene expressed in the intestines. Additionally, there

exist membrane-bound mucins, namely, MUC1, MUC3, MUC4, MUC13, and MUC17 (Heazlewood et al., 2008). These mucins contribute to the formation of a protective layer on the cell surface, participate in cell signalling processes, and impact the development and progression of various types of cancers (Kang et al., 2022). Perturbations or alterations in mucin production or structure can be linked to a range of diseases. The *MUC2* primary function involves creating a protective barrier that prevents the infiltration of bacteria and potentially harmful substances into the intestinal lining.

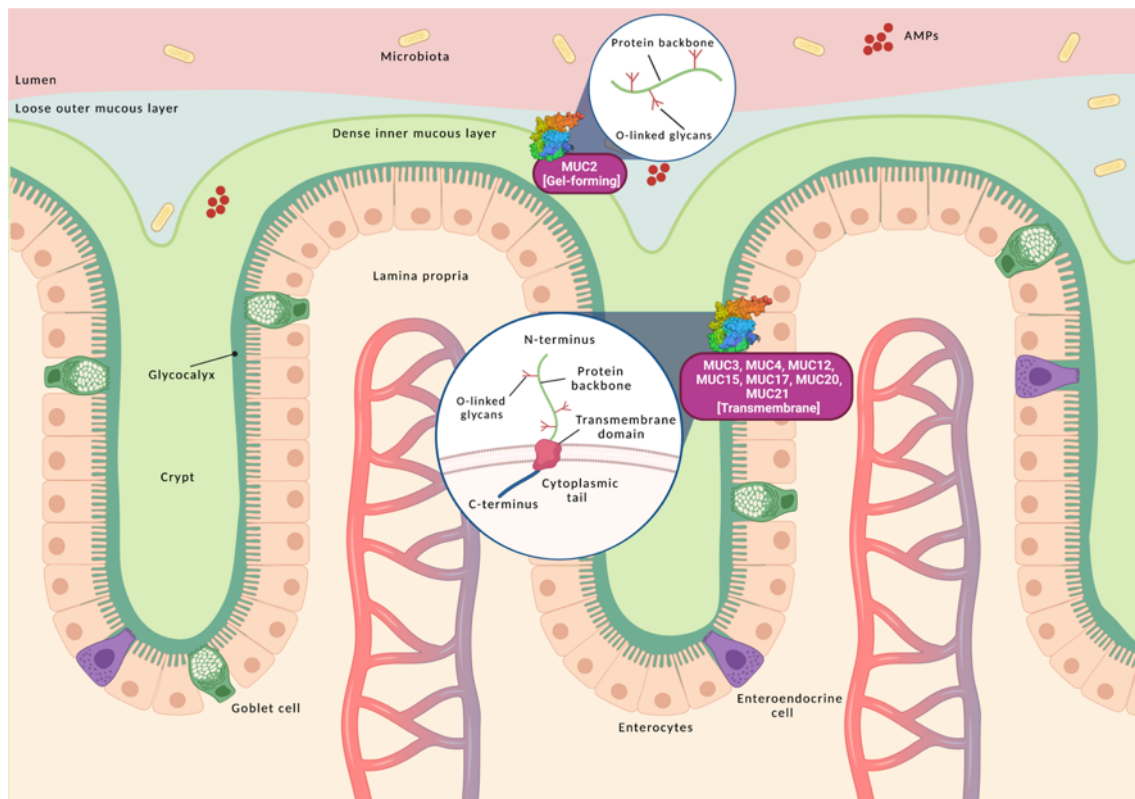
Genetic mutations affecting the *MUC2* gene have been associated with specific forms of IBD (Bankole et al., 2021; Kang et al., 2022). Several studies investigating the pathogenesis of UC have highlighted that intestinal homeostasis disturbances and dysfunctional gut physiology stem from the reduced production and altered expression of the mucin *MUC2* gene (Heazlewood et al., 2008; Tadesse et al., 2017). Mucus glycosylation protects the mucosal barrier because the MUC2 protein core resists proteolytic degradation. Bacterial exoglycosidases release energy-rich monosaccharide residues. Bacteria degrade mucus, but mucus synthesis compensates. Glycosylation alterations can upset this equilibrium, shortening and digesting glycans faster, exposing the protein core and speeding mucus barrier disintegration. Mucus glycosylation defects weaken the mucus layer, reducing pathogen distance from the host (Kang et al., 2022). In UC, MUC2 O-glycosylation changes during remission, although overproduction of abnormal mucin glycosylation are linked to inflammation and disease development. The mucus barrier and tight junction structures share a mutual dependency, such that the loss of one results in the reduction of the other. Mice lacking *claudin 7*, *hepatocyte nuclear factor 4 alpha*, and *Muc2* genes spontaneously develop colitis (Chelakkot et al., 2018). *Muc2* missense mutation in mice, namely the *Winnie* mouse with colitis, contributes to epithelial barrier permeability, mucus abnormalities, and deregulated expression of claudin genes (Eri et al., 2011). Persistent intestinal damage elicits an aberrant immune response, specifically the interleukin (IL)-23/T helper (Th)17 pathway and excess levels of interferon-gamma (IFN- γ), tumour necrosis factor-alpha (TNF- α), IL-6, transforming growth factor beta 1 (TGF- β 1), lipopolysaccharide (LPS), and stress-responsive enzymes. As a result, these cause inflammation and affecting an UC-like phenotype (Heazlewood et al., 2008; Stefanelli et al., 2008). Herewith, the incidence of colorectal cancer

intensifies, prompted by inflammatory bio-reactive factors, subsequently inducing genetic mutations and uncontrollable cancer stem cell differentiation and proliferation (Carpentino et al., 2009). Consequently, significant perturbations of the gastrointestinal tract ecosystem and dysfunctional digestion occur while ingesting nutrients and excreting waste, and subsequently will limit the absorption of macronutrients, water, and electrolytes, causing reduced energy metabolism (Czuber-Dochan et al., 2020).

Figure 1.1 MUC2 in the gastrointestinal tract

MUC gene mutations located in the intestinal goblet cells of the small intestine and colon constitute the underlying mechanisms responsible for intestinal barrier dysfunction.

Abbreviations: MUC, mucin. (Created with BioRender.com).



1.2.5 Neural involvement in IBD

Specifically, the small intestine and proximal colon are innervated by the vagus and splanchnic nerves, while the colon and anal sphincter receive input from the pelvic nerves.

The parasympathetic neurons in the brainstem communicate with the gut through the efferent fibres of the vagus nerve. This enables interactions between these neurons and the resident immune cells in the gastrointestinal tract, forming a complex network of parasympathetic neuron-immune cell communication. In a paracrine manner, nutrient-induced (or lack thereof) neurotransmitters are released via the autonomic nervous system (ANS), subsequently activating acetylcholine, gastrin, cholecystokinin, secretin, ghrelin, motilin, and a gastric inhibitory polypeptide (Drokhlyansky et al., 2020). In response, activated vagal neurons send signals to the brain and surrounding organs, directly affecting metabolic homeostasis, including digestive acid secretion, gut motility, and appetite (Sanger & Lee, 2008). IBD patient enteric neurons within the mucosa, submucosa, and myenteric plexus display abnormalities, which are characteristic of hypertrophy and hyperplasia of ganglia and nerve bundles, amidst glial cell deviations, empty axons, and concentrated neurofibrils. Although biological systems maintain robust, steady states of metabolic pathways via complex feedback systems and allosteric regulators (Mathieu et al., 2017), patients with IBD still endure intestinal insufficiency and homeostatic failure (Kany et al., 2019).

The network of hypothalamic nuclei integrates circulating hunger and satiety signals, reflecting energy stores and nutrient availability, and fosters appropriate behavioural and metabolic responses to fluctuations in energy demand. In addition, signals originating from the gastrointestinal tract connect to the lateral nucleus of the amygdala through corticospinal-thalamic afferent pathways (Joly-Amado et al., 2014). Meanwhile, the central nucleus outputs of the amygdala govern emotional responses, encompassing behavioural, neuroendocrine, autonomic, and the aforementioned gastrointestinal tract responses (Baxter & Croxson, 2012). Neurotransmission loops interconnect the brain, and gut, forming the foundation for the psycho-neuroendocrine-immunity aspects of IBD, rendering the IBD patient psychophysiologically vulnerable (Bonaz & Bernstein, 2013; Mayer & Tillisch, 2011).

1.2.6 Symptoms associated with IBD

It is important to note that the severity and combination of symptoms can vary widely, and not all individuals with IBD experience all of these symptoms. Symptoms may fluctuate over time, with periods of remission and flare-ups (Fakhoury et al., 2014).

1. Persistent or recurrent abdominal pain, often in the lower right or lower left quadrant of the abdomen.
2. Chronic or intermittent diarrhoea in which the stool may be loose, watery, and may contain blood or mucus.
3. Rectal bleeding: blood in the stool can vary in severity, ranging from mild blood streaks to significant bleeding.
4. Persistent urgency to have a bowel movement, even when the rectum is empty.
5. Persistent fatigue and a general feeling of low energy.
6. Unintentional weight loss may occur due to reduced appetite, malabsorption, or increased metabolism associated with IBD.
7. Chronic inflammation and blood loss in the digestive tract can lead to anaemia, resulting in symptoms like weakness, fatigue, and shortness of breath.
8. Some individuals with IBD may experience joint pain, swelling, or stiffness, which can resemble symptoms of arthritis.
9. Skin manifestations, involving rashes, ulcers, or sores, may occur in individuals with IBD.
10. In the later phases of the disease, the fistulas and linear ulcers between the normal mucosa will give the mucosa the appearance of skip lesions. The colon becomes rigid and shortened, while the haustral markings vanish. CD symptoms may vary

based on the underlying cause of inflammation, fistula formation, or stricture formation. In particular, flare-ups of CD may present with pain in the right lower quadrant, weight loss, and diarrhoea without blood. Fistula formation can lead to fecaluria, pneumaturia, and rectovaginal fistulas, while the presence of masses in the right lower quadrant may indicate an abscess (McDowell C. et al., 2023).

1.2.7 Current IBD treatments

The most recent advances in IBD targeted therapies, modify the disease's uncontrolled innate and adaptive immune system to reduce any adverse effects on inflamed mucosal tissue and epithelial barrier integrity (Pithadia & Jain, 2011). The treatment of CD and UC aims to achieve two primary goals: managing symptoms and inflammation and promoting long-term remission to minimise disease activity. The specific treatment approach may vary depending on the severity of the disease, the location of inflammation, and individual factors (Cai et al., 2021). Despite the long-term clinical success of IBD medications, they are limited to eliciting a response in only 60%-80% of patients, do not provide a cure, or are not free of side effects. Here are some of the current treatment options for UC and CD (Cai et al., 2021; Fakhoury et al., 2014):

1. Aminosalicylates: these anti-inflammatory drugs are often used for mild-to-moderate IBD, primarily affecting the colon.
2. Corticosteroids are used for short-term management of moderate-to-severe flare-ups. They can be administered orally, topically, or intravenously.
3. Immunomodulators: medications like azathioprine, mercaptopurine, and methotrexate can help suppress the immune system and reduce inflammation.
4. Biologic therapies: TNF inhibitors (e.g., infliximab, adalimumab), integrin receptor antagonists (e.g., vedolizumab), or IL-12/23 inhibitors (e.g., ustekinumab).
5. Janus kinase inhibitors: drugs like tofacitinib are newer additions to IBD treatment, targeting a pathway involved in inflammation.

6. Nutritional therapy: exclusive enteral nutrition involves consuming a nutritionally complete liquid formula as the sole source of nutrition for a specified period.
7. Surgery for CD involves removing the affected portion of the intestine or repairing strictures, fistulas, or abscesses. For UC, this can involve removing the colon and rectum (proctocolectomy) and creating an ileal pouch-anal anastomosis (IPAA) or creating a permanent ileostomy (McDowell C. et al., 2023).

1.3 IBD and behavioural changes

1.3.1 Depression in IBD: Causation or Correlation?

The burden of IBD symptoms has a significant psychosocial impact on emotional functioning, while negative emotions and psychological stress are believed to influence the biological progression and exacerbations of the disease. Evidence of impaired emotional functioning has primarily been obtained through questionnaires and magnetic resonance imaging studies (Agostini et al., 2011), which do not address the physiological changes. In fact, patients with moderate or severe IBD, compared to those in a quiescent disease state, tend to seek mental health support, exhibit suicidal ideation, exhibit less self-care, and show non-compliance with treatment. These patients experience decreased productivity, increased neuropathic hyperalgesia, and symptomatic flares, indicating the significance of emotional elements (Bhandari et al., 2017). Patients typically exhibit sickness behaviour, which includes depression, anxiety and pathological fatigue (Moulton et al., 2019; Wilkinson et al., 2019). In regard to fatigue, Nocerino et al. (2020) observed that IBD patients are unable to perform even simple daily activities due to a lack of energy and prolonged exhaustion, disproportionate to physical exertion, that are not relieved with rest. Van Langenberg and Gibson (2010) emphasised the need to close the knowledge gap between disease activity and fatigue severity, and the potential pathogenic pathways involved in fatigue pathogenesis. In the absence of treatment for fatigue and mental health and a lack of recognition for its importance, IBD can become a significant encumbrance for the patient.

Although medical interventions involving multiple disciplines have led to advancements in improving the QoL of IBD patients and slowing the progression of the disease, it is

important to acknowledge, diagnose, and manage the emotional and psychological impact of IBD (Keefer & Kane, 2017). While pharmacotherapy has demonstrated some efficacy, medical practitioners are mainly confronted with the difficulty of recognising the functional and medically inexplicable symptoms of IBD, which have psychological origins and manifest in behavioural changes (Baig et al., 2011). The interest in IBD as a psychosomatic illness stems from observations suggesting a potential link between life events, stress, personality type, and the predisposition to develop the disease (Novack et al., 2007). The term "psychosomatic" refers to the relationship between mental phenomena or experiences and their potential influence on biological processes or the development of diseases. In the 20th century, the dominance of the biomedical model has caused a shift in the understanding of psychosomatic illnesses from a primarily psychiatric perspective to a more medical one (Novack et al., 2007). IBD serves as an example of this shift (Tarricone et al., 2017).

1.3.2 Stress and emotional factors in IBD patients

There is evidence that individuals with IBD experience higher rates of affective disorders compared to the general population just prior to their diagnosis, leading to the question of whether these disorders are a component or a cause of IBD (Czuber-Dochan et al., 2014). This has led to the emergence of ambiguous themes, which suggests that IBD-related affective disorders may be independent of the disease and may arise from negative cognitive biases. Another theme suggests that depression and anxiety in these patients may be related to stressors caused by the patient's perceptions of their illness, involving the fear of cancer risks, the anticipation of a poorer QoL, and the fear of embarrassing public episodes (Jordan et al., 2018). In a qualitative study by Jordan et al. (2018), IBD patients expressed apprehension that their reputation might be tarnished due to disease related reduced productivity or absence from work. They worried that this could lead to their demotion or, in the worst-case scenario, termination or redundancy, even though there was no evidence indicating this could be a likely outcome. Participants consistently identified the potential financial consequences of such an event as a significant cause of their anxiety. IBD patients chose to stay at home, citing this as a safety mechanism when the effort required to prevent an unfortunate incident outside the home became too burdensome. They acknowledged that this behaviour could adversely affect their

emotional well-being, as they feared it could lead to social isolation. In addition, patients reported that their perception of others' misunderstanding of IBD often contributed to feelings of depression, which in turn led to social isolation. When they thought others were aware of their disease, they commonly expressed concerns about potential judgement and stigmatisation. Despite expectations, McCombie et al. (2013) found that psychotherapy has had only a minimal impact on improving the QoL of IBD patients and has produced limited effects on restricting disease progression. Likewise, Mikocka-Walus et al. (2017) observed disappointing results in cognitive therapy for IBD patients and recommended a more personal approach.

IBD patients incur high levels of stress (Sun et al., 2019). Psychosocial stress triggers an increase in pro-inflammatory cytokines and cortisol release, causing immunosuppression and dysregulation of energy metabolism, which negatively affects tryptophan metabolism. Researchers are investigating the interactions among the stress system, gut regulation, inflammatory responses, and the corticotropin-releasing factor system to decipher the underlying mechanisms of IBD (Sun et al., 2019). The hippocampus and amygdala are particularly noteworthy in this context (Campbell & Macqueen, 2004).

Indeed, there is a reciprocal relationship between psychological comorbidity and IBD, with each potentially influencing the course of the other. Notably, there is much to be learned about the underlying pathobiology of depression and anxiety and its impact on the pathobiology of IBD (Bernstein, 2016). Given the various causes and shared symptoms of major depressive disorder with frequently co-occurring conditions, comprehending the neurobiological foundations of depression poses a significant challenge for contemporary psychiatry and neurobiology. Ultimately, the intrinsic pathology of depression is remarkably heterogeneous and intricate (Yohn et al., 2017). In the context of IBD, comprehending depression necessitates an examination of genetic changes in both the gut and the brain.

1.3.3 Prevalence of affective disorders in IBD patients

According to a study by Askar et al. (2021), a high percentage of patients with IBD suffer from depression (56.2%) and anxiety (37.1%). The study's findings are consistent with those of other chronic health conditions where there is an increased risk of depression. During remission, 29-35% of IBD patients experience anxiety, while up to 80% of IBD patients experience anxiety during relapses (Askar et al., 2021).

A systematic review by Neuendorf et al. (2016) revealed that the combined prevalence rate for anxiety disorders was 20.5%, while 35.1% of individuals experienced anxiety symptoms. Anxiety levels rose to 75.6% in patients with active IBD compared to those in remission. The aggregate prevalence of depressive disorders stood at 15.2%, with 21.6% experiencing depressive symptoms. Patients with CD exhibited a higher frequency of depressive symptoms (25.3%) in comparison to those with UC, and this prevalence further increased (40.7%) in active IBD cases as opposed to patients in remission (Askar et al., 2021).

In a study of 231 Dutch IBD patients by Bennebroek Evertsz et al. (2012), it was found that up to 43% displayed symptoms of anxiety at a level indicative of a psychiatric disorder. These anxiety symptoms and other psychiatric complaints were not effectively treated. Youths with IBD are at risk of developing anxiety and depressive symptoms demonstrating a prevalence rate of 35.2% (van den Brink et al., 2018).

In another cohort, Choi et al. (2019) compared affective disorders between 15,569 combined CD and UC patients and 46,707 non-IBD controls over six years, demonstrating that CD patients cumulated incidences of anxiety at 11.5%, whereas UC patients scored higher at 16.7%. IBD literature recognises that CD and UC patients vary in their experience of affective disorder symptoms, although the mechanisms that underpin these variants are not yet fully understood (Knowles et al., 2018). In addition, there is increasing evidence that corticosteroids, which are routinely prescribed for IBD patients, contribute significantly to their depression and anxiety (Timmer et al., 2011).

A systematic review by (Alexakis et al., 2017) determined that the available evidence is insufficient to establish a strong correlation between depressive states and a worsening of disease progression in IBD. However, the existing data suggest that such a link is more likely to be present in patients with CD than in those with UC. The degree of disease activity at the outset plays a significant role in this association. Whereas, another study showed that although IBD symptomatology presented a direct effect on depression, the majority of its impact was mediated through chronic illness-related shame and experiential avoidance (Trindade et al., 2016). Byrne et al. (2017) measured depression in 314 IBD patients, anxiety in 322 patients, and depression and/or anxiety in 317 patients. The prevalence of anxiety was 21.1%, 25.5% for depression, and 30.3% for depression and/or anxiety which correlated with an increased disease activity. In CD patients, a reverse correlation existed between the severity of the disease and the connectivity between the amygdala and insula. Symptoms of anxiety and depression were detected in these patients (Fan et al., 2020).

These data highlight the need for additional research to better understand the intricate relationship between mental health and clinical outcomes in IBD. While it is recognised that mental health can have a significant impact on the well-being and QoL of individuals with IBD, the exact mechanisms and effects on disease progression and treatment outcomes remain complex and not fully understood.

1.4 IBD and tryptophan metabolism

Research shows that decreased tryptophan influx to the brain caused by inflammation-induced activation of the kynurenine pathway is a contributing factor to affective disorders and dysregulation of the neuroendocrine stress axis (L.-M. Chen et al., 2021; Höglund et al., 2019; Martin-Subero et al., 2016). Given the frequent occurrence of fatigue and symptoms resembling depression and anxiety in IBD patients, as well as the role of mucosal inflammation and serotonin, there is a persuasive need for research. Specifically, studies should aim to understand how changes in tryptophan metabolism relate to energy metabolism, and how these factors may contribute to sickness behaviour and symptoms of depression and anxiety (Katon, 2003).

Tryptophan metabolism is compartmentalised into five coinciding pathways (**Figure 1.2**): 5-HT and melatonin biosynthesis pathway, tryptamine branch, protein synthesis, kynurenine pathway, and *de novo* nicotinamide adenine dinucleotide (NAD⁺) synthesis (Wnorowski et al., 2021). The main metabolites of tryptophan metabolism include 5-hydroxy indole acetic acid (5-HIAA, serotonin), kynurenine, kynurenic acid (KYNA), anthranilic acid (AA), quinolinic acid (QUIN), picolinic acid (PIC), and nicotinamide. **Figure 1.3** illustrates the molecular formula for these tryptophan metabolites.

1.4.1 Pathway 1, kynurenine pathway leading to *de novo* NAD⁺ biosynthesis

While the majority of past and present studies concentrate on just a few primary tryptophan metabolites and enzymes (Sofia et al., 2018; Tashita et al., 2020), our overview extends beyond that to encompass additional background metabolites and enzymes which may exert a substantial influence on tissue damage and mental health. Firstly, 95% of tryptophan metabolism is the conversion to N-formylkynurenine (kynurenine pathway) via the up-regulation of the rate-limiting enzyme indoleamine 2,3-dioxygenase (IDO) (Roth et al., 2021). In this pathway, tryptophan (**Figure 1.3A**) serves as the precursor to *de novo* NAD⁺ biosynthesis via the kynurenine pathway (Johnson & Imai, 2018).

1.4.1.1 Tryptophan metabolism

Tryptophan is an essential amino acid derived from food, with vitamin B6 as its main cofactor. Tryptophan is a constituent of the *de novo* biosynthesis of proteins and the primary precursor to the kynurenine pathway, NAD biosynthesis, serotonin, and melatonin, all of which are immunological barrier mediators and critical modulators of neurological and physiological stress responses. The equilibrium between tryptophan metabolism and its metabolites is essential for maintaining intestinal homoeostasis and immune function (L.-M. Chen et al., 2021). Because of its critical metabolic positioning, a detailed elaboration of the tryptophan pathways and related gene expressions is displayed in **Figure 1.4**.

Tryptophan synthesises in the enterochromaffin cells of the duodenum mucosa and is absorbed through the sodium-dependent neutral amino acid transporter solute carrier family 6 members 19 (SLC6A19) and partially in the enteric nervous system (ENS). Additionally, tryptophan circulates in the blood, is bound to albumin, and is able to cross the blood brain barrier (BBB) (L.-M. Chen et al., 2021). Increased activity of IDO catalyses the conversion of tryptophan to kynurenine, which can lead to an accumulation of kynurenine. Tryptophan depletion via IDO is part of the cytostatic and antiproliferative activity mediated by IFN- γ in cells (Schröcksnadel et al., 2006).

Through an enzymatic mechanism, a portion of tryptophan produces 5-HT in the serotonergic pathway of the brain (Höglund et al., 2019). A stabilised tryptophan metabolism contributes to overall health via three categories: (1) emotional and cognitive processes; (2) effects on hypothalamic-pituitary-adrenal (HPA), ENS, and pro-inflammatory cytokines, and (3) effects on anxiety, learning, and memory through neurotransmitters (Silva et al., 2017). As tryptophan is a glucogenic or ketogenic amino acid, tryptophan deficiency reciprocally reduces glucose metabolism in living cells (Maria et al., 2018). Hereby, tryptophan metabolism genes and metabolites are critical modulators of neurological and physiological stress responses (Haq et al., 2021). Additionally, tryptophan metabolism reactions between the gastrointestinal tract neural processes and the gamma-aminobutyric acid (GABA), catecholaminergic, and serotonergic systems relay signals throughout the gut-brain axis, subsequently affecting the central nervous system (CNS) (Bonaz & Bernstein, 2013; Martin-Subero et al., 2016).

Figure 1.2 Schematic of tryptophan metabolism

Represented by 5-HT and Melatonin Biosynthesis, Tryptamine branch, Protein Synthesis, Kynurenine Pathway and *De novo* NAD⁺ Synthesis (adapted from Wnorowski et al. (2021)). **Abbreviations:** 3HAA, 3-hydroxyanthranilic acid; 3HKyn, 3-hydroxy-kynurenine; 5HAc, 5-hydroxy-indoleacetaldehyde; 5HFKyn, 5-hydroxy-N-formylkynurenine; 5HKyn, 5-hydroxy-kynurenine; 5HKynN, 5-hydroxykynuramine; 5HTrp, 5-hydroxy-tryptophan; 5HT, 5-hydroxytryptamine (serotonin); AA, anthranilic acid; Acms, 2-amino-3-carboxymuconate semialdehyde; Cin, cinnabaric acid; DHQuin, 4,6-dihydroxy-quinoline; F5HKyn, formyl/-5-hydroxykynurenamine; FKyn, formyl/-kynurenine; FAA, formyl-anthranilic acid; IAA, indole-3-acetic acid; I3P, indole-3-pyruvic acid; I3A, indole-3-aldehyde; ILA, indole-3-lactic acid; IndolAc, indole-3-acetaldoxime; Kyn, kynurenine; Kyna, kynurenic acid; MMTrypta, dimethyl-tryptamine; MTrypta, methyl-tryptamine; NAcSet, N-acetyl-serotonin; NAD, nicotinamide adenine dinucleotide; Nam, nicotinamide; NMN, Nam mononucleotide; NMSer, N-methyl-serotonin; NR, Nam riboside: MNam; Pic, picolinic acid; Quin, quinolinic acid; Trp, tryptophan; Trypta, tryptamine; XA, xanthurenic acid.

Figure 1.2 Schematic of tryptophan metabolism

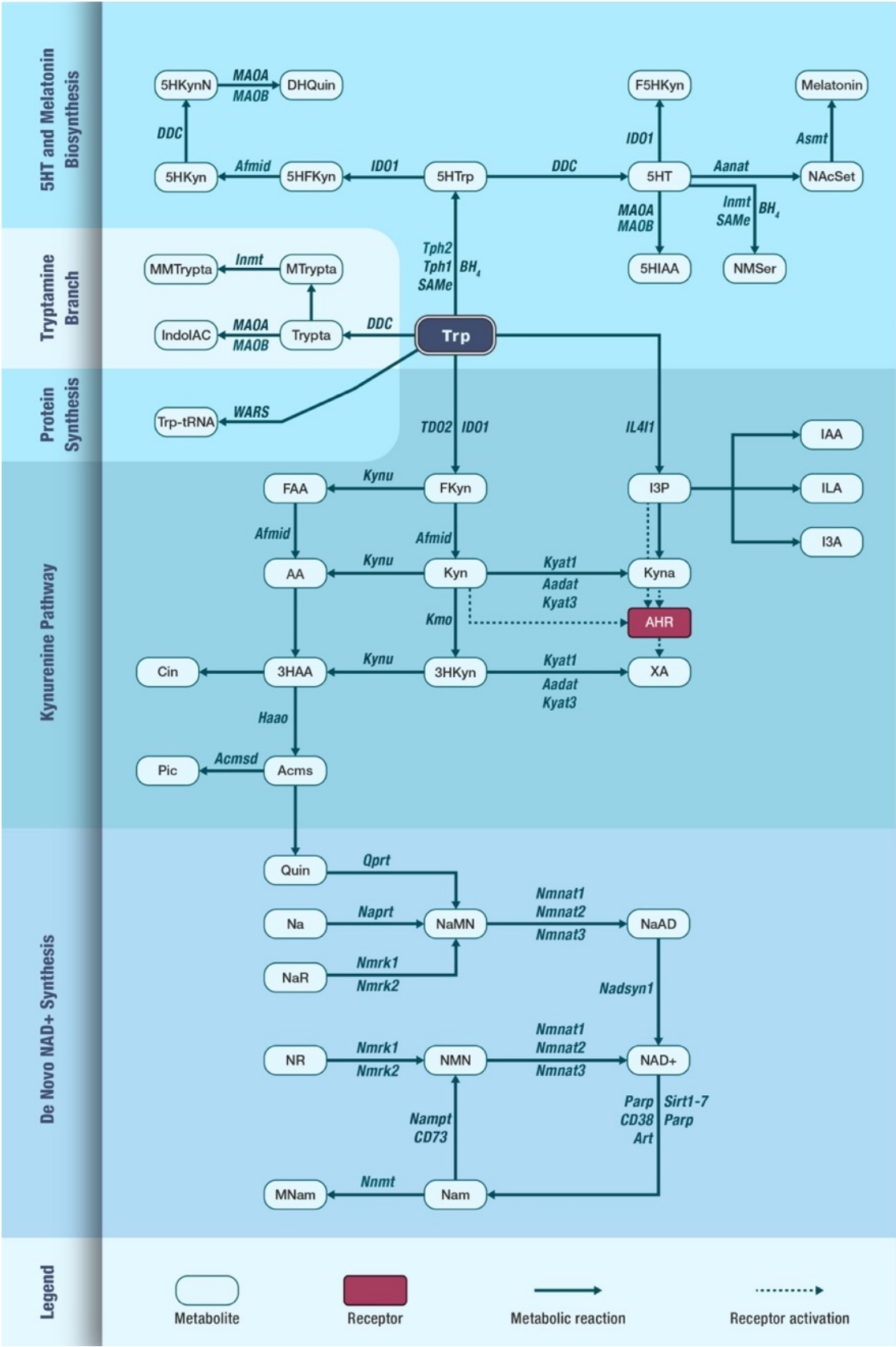
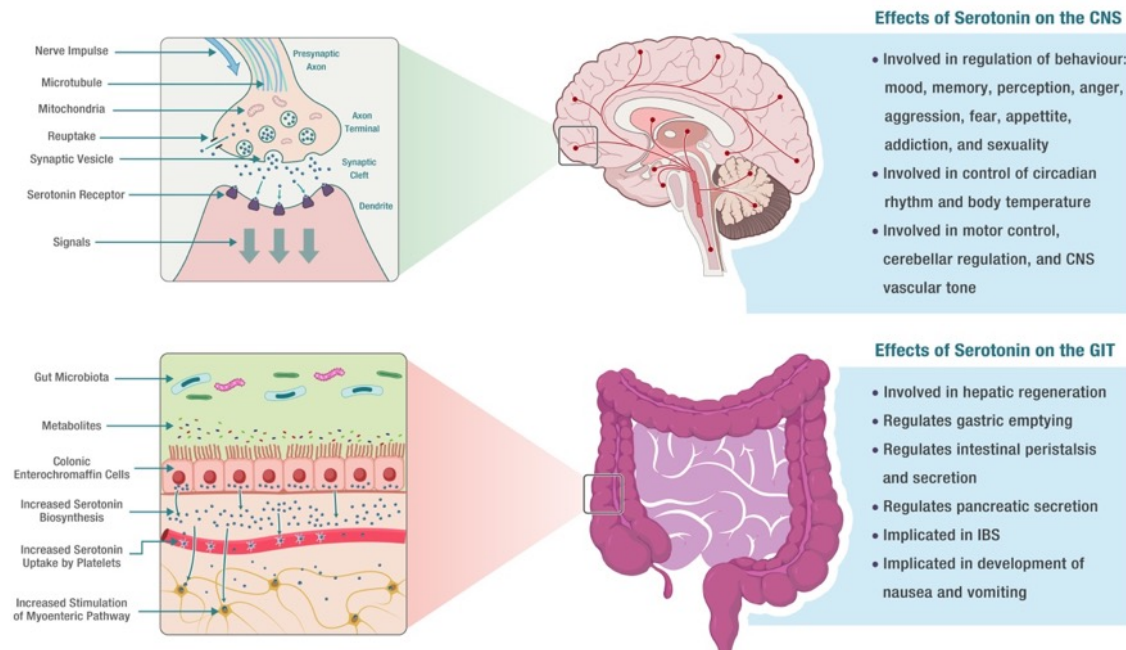


Figure 1.3 Serotonin in the gastrointestinal tract and the central nervous system

The effects of serotonin on the regions of the central nervous system involved in the regulation of behaviour, circadian rhythm, body temperature, motor control, cerebellar regulation and vascular tone. Plus, the effects of serotonin on hepatic regeneration, gastric emptying, intestinal peristalsis, pancreatic secretion, nausea and vomiting



(Created with BioRender.com)

The gut irregularities affect tryptophan transporters and facilitate the reduced production of 5-HT, also known serotonergic dysfunction, which is the key modulator of neurological responses in the brain (Roth et al., 2021). For tryptophan to participate in these processes, it must first cross the BBB through a transport mechanism of membrane-bound carrier proteins called the large neutral amino acid transporter (LAT1). The transport of tryptophan across the BBB is a competitive process, as LAT1 has a limited capacity and transports multiple amino acids. The concentration of tryptophan in the blood, as well as the concentrations of other large neutral amino acids, can impact the rate at which tryptophan is transported across the BBB. The balance of these amino acids is crucial for maintaining proper brain function, as they serve as precursors for neurotransmitters and other essential molecules. Factors that influence these concentrations, like metabolism and diet, can subsequently affect tryptophan's availability in the brain and the subsequent synthesis of serotonin and melatonin (Fukuwatari, 2020).

1.4.1.2 IDO influences tryptophan metabolism

The oxidative cleavage of tryptophan is controlled by the hepatic enzyme tryptophan 2,3-dioxygenase (TDO), which is regulated by glucocorticoids, and the extrahepatic enzymes IDO1 and IDO2 (Ciorba, 2013). The stimulated expression of IDO causes the breakdown of tryptophan into formyl-5-hydroxykynurenine through the kynurenine pathway (Connor et al., 2008). IDO is synthesised by gut microbiota expressed in lamina propria cells and endothelial cells; and by inflammatory markers, namely, TNF- α , IFN- γ , ILs, and arachidonic acid levels (Austin et al., 2009). Given its potent influence on the pathophysiology and severity of gastrointestinal diseases, IDO1 presents as a critical target for pharmaceutical research. Hereby, increased levels of IDO have been observed in the blood and mucosal epithelial cell lines of IBD patients (Gupta et al., 2011). In addition, indole derivatives produced by the gut microbiota, can modulate the immune response and preserve the intestinal mucosal barrier, which plays a crucial role in the pathophysiology of colitis (Agus et al., 2018; Robinson et al., 2016). In addition, elevated expression of IDO impedes the responsiveness of T cells through the induction of anergic state. T-cell-mediated immune function is frequently compromised in immunocompromised conditions, including IBD (Sørensen et al., 2011).

1.4.1.3 Kynurenine, the pivotal metabolite

The kynurenine pathway represents the primary route of tryptophan degradation, accounting for approximately 95% of its catabolism (Castro-Portuguez & Sutphin, 2020). Kynurenine (**Figure 1.3D**) is a key intermediate in the metabolism of tryptophan leading to the generation of several metabolites of the kynurenine pathway. Kynurenine is transaminated by kynurenine aminotransferases and catalysed by arylformamidase (AFMID) to produce KYNA (**Figure 1.3E**), and xanthurenic acid, and/or QUIN, which acts as a source for nicotinamide adenine dinucleotide + hydrogen (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH); leading to *de novo* NAD⁺ synthesis (**Figure 1.3I and 1.3J**) (Dobrovolsky et al., 2005). In the gastrointestinal tract, KYNA acts as an agonist of the G-protein-coupled (GPR35) receptor, which is a glutamate receptor on ENS neurons in the submucosal and myenteric plexuses and plays a direct role in gut motility (Wejksza et al., 2009). In rodents with colitis, KYNA has been shown to inhibit LPS, regulate natural killer (NK) T cells and TNF- α , reduce IL-17 and IL-23 levels in the colon, and subsequently decrease motility and inflammatory activation (Turski et al., 2013). In the brain, KYNA operates as an antagonist for both $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ nAChR) and glycine co-agonist sites on N-methyl-d-aspartate receptors at naturally occurring brain concentrations. An increase in brain levels of KYNA inhibits the release of neurotransmitters like dopamine and glutamate, implicating KYNA in mental health. Consequently, regulation of the kynurenine pathway in the brain is a critical focus for enhancing cognitive performance or developing effective treatments for neurological disorders (Fukuwatari, 2020).

1.4.1.4 Kynurenine pathway, anthranilic acid to quinolinic acid

Kynureninase is a pyridoxal phosphate-dependent enzyme and a member of the aminotransferase family of enzymes that catalyse the cleavage of kynurenine into anthranilate, 3-hydroxykynurenine to 3-hydroxyanthranilic acid (3-HAA), and alanines that synthesise AA and QUIN (**Figure 1.3F**) (Fujigaki et al., 1998; Phillips, 2014). 3-HAA can be subdivided into two pathways. Firstly, additional enzymatic actions synthesise α -amino- β -carboxymuconate- ϵ -semialdehyde (ACMS) intermediate. ACMS decarboxylase (ACMSD) catalyses ACMS to AMS, which generates acetyl-CoA and

subsequently enters the tricarboxylic acid (TCA) cycle via the glutarate pathway to produce CO₂ and H₂O. This pathway also produces PIC (**Figure 1.3H**) (Lugo-Huitrón et al., 2013). Otherwise, in response to ACMSD deficiency, ACMS undergoes cyclisation and produces QUIN (**Figure 1.3G**) via the rate-limiting enzyme quinolinate phosphoribosyltransferase (QPRT), which is further catalysed into nicotinic acid mononucleotide (NaMN) and NAD⁺ (Badawy, 2018a).

1.4.1.5 Quinolinic acid

QUIN converts to NaMN via specific NAD-cleaving enzymes. Numerous variables contribute to QUIN neurotoxicity, including the roles of the rate-limiting enzyme quinolinate phosphoribosyltransferase (QPRT) and 3-hydroxyanthranilic acid oxygenase (3-HAO) in its synthesis and metabolism, as well as influences on the rate of QUIN production in the brain compared to its conversion to NAD⁺. In the brain, QUIN is locally produced and acts as an agonist to N-methyl-D-aspartate (NMDA) receptors, induces selective neuronal damage and acts as a neuroexcitatory toxin. This causes significant calcium influx into neurons and astrocytes, with neurons suffering the most damage. The hippocampus and striatum, which have a high density of NMDA receptors, are the most vulnerable to QUIN neurotoxicity (Braidy et al., 2009). Striatal spiny neurons in the hippocampus containing GABA and Substance P are also vulnerable to QUIN accumulation. In addition, QUIN can enhance glutamate release, limit astrocyte reuptake, and disrupt glutamate-to-glutamine recycling by decreasing glutamine synthase function, causing neurochemical and pathological alterations (Lugo-Huitrón et al., 2013).

Additionally, IFN- γ and bacterial LPS trigger IDO and enhance QUIN synthesis. Astrocytes can enhance this effect since QUIN causes astrogliosis, which increases cytokine expression and release, compromising cell survival. Activated microglia generate and secrete considerable amounts of QUIN in the brain, and QUIN levels rise significantly in both brain homogenates and extracellular fluid during CNS inflammation (Lugo-Huitrón et al., 2013).

Chiarugi et al. (2001) found that cultured human monocyte-derived macrophages produce QUIN during inflammatory responses and continue to do so until the inflammation subsides. Chronic QUIN exposure induces structural alterations in human neurons showing as dendritic beading, microtubular disruption, and reduced organelles. QUIN can also aggravate other excitotoxins' toxicity, leading to progressive mitochondrial dysfunction (Lugo-Huitrón et al., 2013). However, if sufficient NAD^+ is derived from QUIN, it can ameliorate inflammatory biological processes (Gerner et al., 2018).

1.4.1.6 Picolinic acid

PIC is a pyridine derivative and a metabolite towards the end of the kynurenine pathway (**Figure 1.3H**). The neuroprotective role of PIC is thought to be due, in part, to its ability to chelate redox-active metals and prevent their participation in harmful oxidative reactions (Grant et al., 2009). PIC contributes to the solubility, absorption, bioavailability, and transportation of these metal ions to different tissues. This is critical to many biochemical processes, especially within the gastrointestinal tract. In the CNS, PIC and $\text{IFN-}\gamma$ synergistically induce L-arginine-dependent nitric oxide (NO) production through cytotoxic action, aiding in immune modulation and reducing infected cells and viral replication (Grant et al., 2009). Chromium picolinate, a compound formed when PIC binds and chelates chromium, has been shown to have anti-anxiety and antidepressant effects. In animal models of chronic unpredictable stress, it has been demonstrated to reduce plasma corticosterone levels and increase cortical and cerebellar serotonin levels (Coggan et al., 2009; Dubey et al., 2015).

1.4.2 Pathway 2, serotonergic pathway

A small portion of tryptophan will convert to serotonin (5-HT). 5-HT, is a monoamine neurotransmitter in both the ENS and CNS (Haq et al., 2021) (**Figures 1.3B and 1.3C**). In response to numerous stimuli, including the ingestion of nutrients, enterochromaffin cells release 5-HT, where it functions as a hormone, autocrine, or paracrine factor. Given the strategic location of enterochromaffin cells in the mucosal epithelial lining and the emerging role of 5-HT in gut pathology and pathophysiology, it is known that 5-HT

significantly influences the modulation of gut microbial composition, gut motility, glucose absorption, and fluid balance (Kwon et al., 2019).

Because 5-HT cannot directly cross the BBB, the CNS and PNS serotonergic pathway are independent (Pourhamzeh et al., 2022). In the gut, tryptophan hydroxylase 1 (TPH1) converts tryptophan into 5-hydroxytryptophan (5-HTP), which is then decarboxylated to produce 5-HT. Notably, the enterochromaffin cells of the gut mucosa increase in density distally, where the microbial abundance is higher, and produces 90% of the peripheral 5-HT (Roth et al., 2021). In fact, it was determined that TPH1-deficient mice, notable for a marked decrease in 5-HT levels within the gut, show a reduced severity of colitis in established colitis mouse models (Kwon et al., 2021). Additionally, the enzyme aromatic L-amino acid decarboxylase in the brain converts tryptophan to 5-HTP via TPH2, and then 5-HTP to serotonin. TPH2 is an oxygen-labile neuroenzyme and is expressed in the frontal cortex, thalamus, hippocampus, hypothalamus, and amygdala (Invernizzi, 2007; Roth et al., 2021).

Serotonergic neurons in the CNS are located in the dorsal and median raphe nuclei of the brain stem's reticular formation (Kirby et al., 2008). During neuronal development, 5-HT modulates the proliferation, migration, differentiation, maturation, and apoptosis of postmitotic neurons and contributes to synapse formation. Once synthesised, 5-HT is stored in vesicles within the presynaptic neuron. When an action potential reaches the presynaptic neuron, it triggers the release of serotonin into the synaptic cleft. 5-HT molecules then diffuse across the synaptic cleft and bind to 5-HT receptor subtypes, 5-HT₁, -2, -3, -4, and among others, on the membrane of the postsynaptic neuron. This ultimately lead to changes in the activity or function of the target cell (Kirby et al., 2008). The activation of these receptors can either hyperpolarise or depolarise the membrane potential of the neuron, depending on the specific receptor subtype and the downstream signalling pathways, which may result in the activation of enzymes or changes in gene expression (Bockaert et al., 2006). After exerting its effects, 5-HT is taken back up into the presynaptic neuron through a process called reuptake, which is mainly facilitated by the serotonin transporter (SERT). Once back in the presynaptic neuron, serotonin can be repackaged into vesicles for reuse or degraded into the 5-HIAA metabolite by the enzyme monoamine oxidase (MAO) (Yohn et al., 2017). Hereby, serotonergic neurons and 5-HT

levels have long been associated with the development and treatment of neuropsychiatric conditions as well as influencing cognition, memory, and circadian rhythms (Pourhamzeh et al., 2022). Notably, markers of 5-HT deficiency have been found in specific subgroups of individuals with depression, and genetic variations within the 5-HT system have been linked to affective disorders, increased impulsivity, negative effects on cognitive task performance, or decreased inhibitory capacity (Markus & Jonkman, 2007; Sachs et al., 2015).

The gastrointestinal track luminal changes seen in IBD involving increased acidity, pressure, and nutrient concentration, can induce the release of 5-HT. Once released by enterochromaffin cells, 5-HT can bind to serotonergic receptors on adjacent neurons, epithelial cells, and immune cells that interact and influence metabolic homeostasis (Shajib et al., 2019). These interactions can promote the release of various signalling mediators, potentiate neurons in order to improve their responsiveness to another stimulus, or directly initiate an action potential. Hereby, 5-HT plays a particularly critical role in enteroception, secretion, and peristalsis. Serotonin receptor-2 (5-HT_{2A}) receptors on gut smooth muscle cells, when triggered by 5-HT, can stimulate muscle contraction, indirectly activating 5-HT-sensitive mechanosensitive nerve fibres in the gastrointestinal wall (Shajib et al., 2019). 5-HT receptors expressed within the colon epithelium, specifically the G-protein and serotonin receptor-4 (5-HT_{4R}), increase colonic anion, fluid secretion, peristaltic reflex activity, and especially accelerate gastrointestinal transit (Hoffman et al., 2012).

1.4.2.1 MAO, ALDH and 5-HT

MAOs are mitochondrial-bound flavoenzymes that catalyse the oxidative metabolism of biogenic amines, involving 5-HT conversion to 5-hydroxy-3-indolacetaldehyde (5-HIAL), then to 5-HIAA via aldehyde dehydrogenase (NAD⁺) (ALDH) (Bortolato et al., 2010; Prah et al., 2020). The enzymatic action of MAO involves transferring two electrons and two protons from flavin adenine dinucleotide (FAD), oxidising the amine substrate and producing aldehydes, neurotoxic reactive oxygen species (ROS), and ammonia as by-products (Prah et al., 2020). Hence, elevated levels of MAO and 5-HT in the colon can have toxic consequences (Betari et al., 2020). In addition to 5-HT, MAO is

involved in the metabolism of norepinephrine, dopamine, and trace amines (Prah et al., 2020). For this reason, MAO-A inhibitors are prescribed to treat gut disorders associated with elevated 5-HT levels in IBD patients (Ostadkarampour & Putnins, 2021).

ALDH enzymes are a diverse group of NAD- or NADP-dependent enzymes that oxidise aldehydes to carboxylic acid. ALDH supports cellular homeostasis by implementing the detoxification of ROS produced from acetaldehydes resulting from ethanol, drugs, food, food additives, and endogenous metabolism (the pathway of ethanol to acetaldehyde to acetate) (Kim et al., 2017). ALDH enzymes are factors in dopamine metabolism and specifically influence neural function in dopaminergic nerves. However, ALDH inhibition causes the accumulation of 3,4-dihydroxyphenyl acetaldehyde, a neurotoxic dopamine metabolite (Koppaka et al., 2012). High levels of ALDH, otherwise known as acetaldehyde toxicity (disulfiram-ethanol reaction), are symptomatic of faintness, palpitations, nausea, and alcohol flush reactions. Alternatively, under-expressed ALDH is the foundation for alcohol intolerance, an increased risk of cancer, and cardiovascular and neurological diseases (Shortall et al., 2021).

1.4.2.2 SAmE and serotonin

S-Adenosyl-L-methionine (SAmE) plays a critical role in various biochemical processes, involving the production of tetrahydrobiopterin (BH₄). This compound serves as an essential cofactor for TPH, which converts tryptophan into 5-HTP, a precursor of serotonin (Vancassel et al., 2018). SAmE acts as a methyl-donating cofactor in the rate-limiting step of tyrosine hydroxylase, contributing to the synthesis of dopamine and norepinephrine (Bottiglieri, 2002). SAmE normalises metabolic processes, as well as the biosynthesis of phospholipids, proteins, nucleic acids, neurotransmitters, and hormones. The methylation of SAmE produces S-adenosylhomocysteine, which is involved in the conversion of homocysteine to glutathione, a potent antioxidant, within the trans-sulfuration pathway (Mischoulon & Fava, 2002). In colonocytes, the methylation of sulphides, facilitated by thiol methyltransferases, reduces the toxicity of compounds like methanethiol and dimethylsulphide and mitigates acute oxidative damage. In addition, SAmE plays a role in enhancing the fluidity of cell membranes by promoting

phospholipid methylation, which may contribute to cellular flexibility and potentially improve mood regulation in depressed patients (Bottiglieri T, 2000).

1.4.3 Pathway 3, tryptophan to AhR pathway

The tryptamine pathway activates aryl hydrocarbon receptor (AhR) in the intestinal barrier's epithelial, endothelial, and neuronal cells, incurring colonic ion secretion for gastrointestinal tract motility and the transit of food and bacteria along the lumen (Lamas et al., 2018). The conservation of intestinal immune cells relies on the adequate activation of AhR via tryptamine; otherwise, the host is susceptible to the colonisation of enteric pathogens, causing inflammatory disorders and related malignancies (Hoffman et al., 2012; Lamas et al., 2018).

The tryptophan-AhR ligand pathway comprises decarboxylation of tryptophan via the enzymatic actions of gastrointestinal microbes that express tryptophan decarboxylase and yield indole derivatives, specifically tryptamine-containing monoamines (Pohjanvirta, 2017; Sun et al., 2020). 4-6% tryptophan is transformed by bacterial decarboxylase and tryptophanase into a variety of metabolites, including tryptamine and several indole derivatives, namely, indole-3-aldehyde, indole-3-acetic acid, indole-3-propionic acid, and indole-3-acetaldehyde (Pohjanvirta, 2017).

The indole metabolites interact with the AhR on the surface of intestinal immune cells. This interaction is crucial for maintaining immune balance in the intestines, enhancing resistance to pathogens, and protecting the intestinal lining. Indole metabolites, namely indole-3-propionic acid and indole-3-acrylic acid, interact with the pregnane X receptor. This binding aids in maintaining the intestinal epithelium's barrier function and contributes to mucosal homeostasis (Sun et al., 2020). In particular, tryptamine, a β -arylamine, modulates intestinal homeostasis by interacting with the ENS and is the ligand for 5-HT receptors expressed within the colon epithelium. The tryptamine pathway activates AhR, in the intestinal barrier's epithelial, endothelial, and neuronal cells, incurring colonic ion secretion for gastrointestinal tract motility and the transit of food and bacteria along the lumen (Lamas et al., 2018). The conservation of intestinal immune cells relies on the adequate activation of AhR via tryptamine; otherwise, the host is

susceptible to the colonisation of enteric pathogens, causing inflammatory disorders and related malignancies (Hoffman et al., 2012; Lamas et al., 2018). Despite this, kynurenine provides a negative feedback loop through AhR in lymphoid tissues, generating Treg cells and IL-22 (Mezrich et al., 2010; Seok et al., 2018).

1.4.4 Pathway 4, nicotinamide and NAD biosynthesis

One of the most critical roles of nicotinamide is its involvement in the production of NAD⁺, a coenzyme participating in numerous metabolic processes, including energy metabolism, deoxyribonucleic acid (DNA) repair, and cell signalling (Katsyuba et al., 2018). Maintaining NAD⁺ levels is crucial for proper neuronal function, which has been associated with preventing neurodegenerative diseases and maintaining gut homeostasis. Nicotinamide has demonstrated neuroprotective properties through its anti-inflammatory and antioxidant effects, shielding neurons from damage caused by oxidative stress and inflammation (Amjad et al., 2021). For instance, the diminished ratio of NAD⁺/Nicotinamide effectively identified inflammation in UC patients. Additionally, a decrease in mitochondrial density and number in their colonocytes were observed in the mitochondria of UC patients. Collectively, these observations indicate a connection between mitochondrial irregularities, inflammation, and the metabolism of NAD⁺ in colitis (Kang et al., 2021). Furthermore, nicotinamide has been found to promote the production of brain-derived neurotrophic factor, a protein that supports neuronal survival, and synaptic plasticity. Nicotinamide has shown potential benefits in addressing mental health disorders involving anxiety and depression, by modulating neurotransmitter systems and promoting neurogenesis (Cuenoud et al., 2020).

NAD biosynthesis involves five major precursors: primarily tryptophan, followed by nicotinamide (**Figure 1.3I**), nicotinic acid (NA) (**Figure 1.3J**), nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN). During metabolism, NAD-dependent enzymes, such as cluster of differentiation 38 (CD38), consume NAD⁺. To replenish NAD⁺ levels, a complex series of reactions occur across three pathways. These pathways converge at NMN and are further processed by various enzymes to produce NAD⁺ (Braidy et al., 2019; Tempel et al., 2007).

The three pathways include:-

(1) the tryptophan *de novo* NAD⁺ biosynthesis pathway where via the kynurenine pathway tryptophan is converted into nicotinamide, a carboxylic derivative of pyridine that forms parts of NAD⁺ and NADP cofactors.

(2) the salvage pathway, where NAM is converted into NMN by nicotinamide phosphoribosyltransferase (NAMPT), which metabolises NAM and 5'-phosphoribosyl-1-pyrophosphate (PRPP), and NR is metabolised by nicotinamide riboside kinase (NMRK). The majority of NAD⁺ is recycled from NAM, NA, NR, and NMN through the salvage pathway.

(3) the Preiss–Handler pathway where NaMN and NAM are deamidated by intestinal flora and converted into nicotinate through the rate-limiting activity of nicotinate phosphoribosyltransferase (NAPRT). The Preiss-Handler pathway engages NAPRT, nicotinamide mononucleotide adenylyl transferases (NMNAT) and nicotinamide adenine dinucleotide synthetase (NADSYN) (Rodriguez Cetina Biefer et al., 2017).

During the process of glycolysis, NAD⁺ is utilised by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and LDH for the transformation of pyruvate to lactate and NADH back to NAD⁺. Pyruvate is then converted into acetyl CoA through the pyruvate dehydrogenase complex (PDHC), which in turn reduces NAD⁺ to NADH. Throughout the TCA cycle, enzymes including isocitrate dehydrogenase (IDH), oxoglutarate dehydrogenase (OGDH), and malate dehydrogenase, facilitate the reduction of NAD⁺ to NADH. Subsequently, the electron transport chain (ETC) employs NADH to produce adenosine triphosphate (ATP). The availability of NAD⁺ within a cell is determined by the balance between *de novo* NAD⁺ biosynthesis and its rate of consumption (Amjad et al., 2021; Gerner et al., 2018). It follows that NAD⁺, its reduced form NADH, and their phosphorylated counterparts, NADP⁺ and NADPH, are vital cofactors and substrates in the regulation of cellular energy metabolism (Xie et al., 2020).

1.4.4.1 Enzymatic regulation of NAD⁺ biosynthesis

NAPRT, an enzyme localised within the cytoplasm and mitochondria, operates in concert with NAMPT to catalyse the transformation of NA into NAD⁺. These enzymes play a significant role in the modulation and reconfiguration of cellular metabolism, acting through NAD-dependent deacetylases, poly(adenosine diphosphate (ADP)-ribose) polymerases (PARPs), and NADases (Audrito et al., 2020). In contrast to NAMPT, NAPRT remains uninhibited from increased *in vivo* NAD⁺ concentrations, thereby facilitating a considerable enhancement of NAD⁺ levels and offering protection against inflammatory conditions via the prevention of oxidative stress (Imai, 2009). Elevated levels of circulating NAPRT were observed in individuals with progressive colon and rectal cancer (Colombo et al., 2021), and in tissues affected by septic shock and sepsis (Li et al., 2019; Satoh, 2020).

NAMPT, the rate-limiting enzyme in the metabolism of substrates NAM and PRPP into NMN, subsequently facilitates the ATP-dependent conversion of NMN to NAD⁺ (Audrito et al., 2020). At the molecular level, NAMPT activates the nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) signalling pathway and sustains the feedback loops of IL-6 and TNF- α , commonly overexpressed in tissue affected by IBD (Imai, 2009). NAMPT and NAPRT jointly regulate sirtuins (SIRTs), PARPs, CD38, and NADases involved in NAD⁺ consumption (Okabe, 2019).

There are several enzymatic sirtuins of NAD-dependent deacetylases located in subcellular compartments of the nucleus, cytosol, and mitochondria (Machado de Oliveira et al., 2012). SIRT2 arranges chromosomes into a more compact shape through the deacetylation of histones and transcriptional coactivators (Machado de Oliveira et al., 2012). Subsequently, sufficient SIRT2 protects cells against metabolic disturbances through oxidative stress-dependent mechanisms. SIRT2 reduces activity during excess energy supply and increases activity during low-energy states, thereby regulating glucose homeostasis (Kane, 2018).

1.4.4.2 Nicotinamide catabolic enzymes

CD38 incites extracellular and intracellular milieu, maintains Ca^{2+} homeostasis and immune function (Malavasi, 2008). Elevated CD38 expression is shown in senescent cells incurring DNA damage and increased ROS and reactive nitrogen species (RNS), plus in the higher cytolytic potential of macrophages, T-cells, B-cells, IL-6, IL-12, IFN- γ , and NK cells, which are commonly overexpressed in intestinal inflammation (Schneider et al., 2015).

Ecto-5'-nucleotidase (CD73) dephosphorylates NMN into NR and releases NAD biosynthesis nucleoside precursors to neighbouring cells (Allard et al., 2017). The intestinal mucosa, intestinal crypts, and their villus tips exhibit twice the expression of CD73 compared to other tissues. In IBD tissue, stressed and damaged intestinal cells release greater quantities of ATP into the extracellular environment. Subsequently, CD39 then catalyses ATP into adenosine diphosphate (ADP) and successively into extracellular adenosine monophosphate (AMP), which is converted to extracellular adenosine by CD73 (Bynoe et al., 2012; Crane et al., 2007). Within the intestinal mucosa, CD73 regulates adenosine-mediated fluid transport across the luminal aspect of the mucosa (Haskó, 2008).

1.5 Mouse models of IBD and tryptophan metabolism

Research into tryptophan's role is an evolving field with questions surrounding the application of various colitis mouse models. One concern arises from the differential outcomes based on the induction method for colitis, leading to variations in immune-pathophysiology, epithelial permeability, and intestinal changes. Such induced colitis often represents acute rather than chronic inflammatory disease, and certain mouse models exhibit anatomical misalignment of colitis, diminishing their resemblance to human IBD. Hence, the immune pathophysiology differs, and subsequently, the research results vary (Wirtz & Neurath, 2007).

These mouse models include the dextran sulfate sodium (DSS)-induced colitis, where mice are given a chemical that damages the lining of the colon, leading to inflammation

and colitis. Another model is the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, which generates inflammation in the colon. There is the IL-10-deficient colitis model, where mice lacking the interleukin-10 (IL-10) gene develop colitis spontaneously due to immune dysregulation (Boismenu & Chen, 2000; Wirtz & Neurath, 2007).

The *Winnie* mouse model has shown promising parallels associated with colitis. These mice carry a *Muc2* missense mutation, leading to abnormal *Muc2* mucin biosynthesis in goblet cells, which in turn causes spontaneous and progressive chronic intestinal inflammation (Heazlewood et al., 2008). This mirrors human IBD-like symptoms, including endoplasmic reticulum stress, a weakened intestinal epithelial barrier, and structural and functional shifts within the distal colon (Eri et al., 2011). Both IBD patients and *Winnie* mice display significant shifts in faecal microbiome composition, marked by an overabundance of *Actinobacteria* and *Proteobacteria* and diminished levels of *Bacteroidetes* and *Tenericutes* (Robinson et al., 2016). The progression of chronic intestinal inflammation in *Winnie* mice appears from 6 weeks of age and intensifies to severe colitis by 12-16 weeks of age (Eri et al., 2011).

Tryptophan metabolism research in the last decade has escalated in mouse models of colitis. For example, in *Winnie Muc2* and DSS-treated colitis mouse models, the onset and progression of colitis are correlated with increased 5-HT levels in the colon (Bertrand et al., 2009; Stavelly et al., 2018). In a study by Islam et al. (2017), a tryptophan diet reduced colitis symptoms and severity in wild-type mice, but not in AhR-deficient mice. Additionally, this diet decreased the expression of IL-6, TNF- α , IL-1 β and certain chemokines, and increased the expression of IL-22 and Stat3 in the wild-type mice, which contributed to maintaining the integrity of the epithelial layer. A study by Rondeau et al. (2020) found that IBD patients and mice had an altered faecal microbiota with lower AhR activation capacity, and colonisation of mice with microbiota from healthy subjects induced greater AhR activation. Increasing dietary tryptophan content rescued AhR activation capacity and reduced immune cell infiltration in *Muc2* deficient mice.

1.6 Glycolysis and fatty acid β -oxidation links to tryptophan metabolism

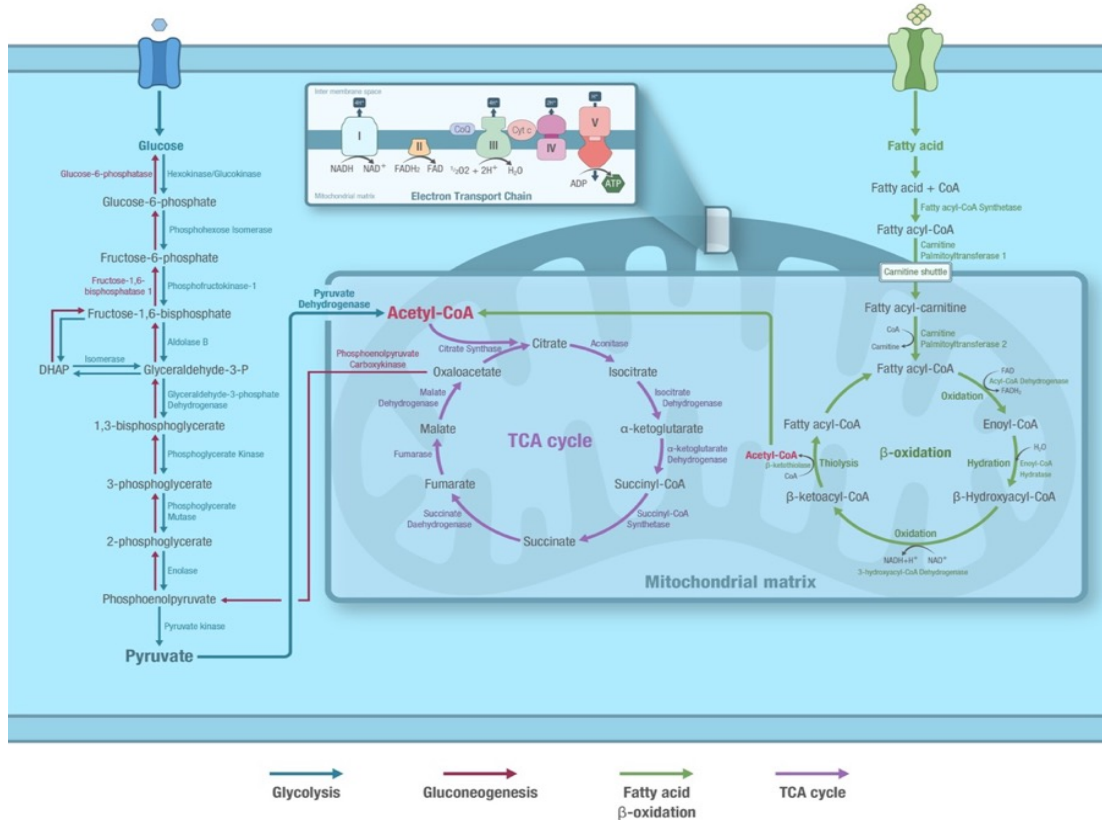
1.6.1 Energy metabolism

Energy metabolism is a major area of interest in determining which factors transfer to biological behaviour in the pathogenesis of IBD. Energy metabolism is the living organism's total chemical reactions used to procure and transform the potential chemical energy of food through stepwise homeostatic cellular processes that synthesise and degrade macromolecules into monomers (Galgani & Ravussin, 2008). Catabolism degrades dietary macromolecules into biomolecules of amino acids, fatty acids, nucleotides, glycerol, and glucose for the release of kinetic energy. Anabolism biosynthesises dietary macromolecules and stores the subsequent potential energy as proteins, glycogen, and triglycerides (Galgani & Ravussin, 2008). These biomolecules are synthesised via glycolysis, fatty acid beta (β)-oxidation (FAO), TCA, the ETC, and oxidative phosphorylation (Galgani & Ravussin, 2008) (**Figure 1.5**).

Figure 1.4 Glycolytic pathways, TCA cycle and fatty acid β -oxidation pathways

Cellular respiration pathways of glycolytic pathways are illustrated in **blue** and **red**; the TCA cycle in **purple** and the fatty acid β -oxidation pathway in **green**.

Abbreviations: tricarboxylic acid, TCA; dihydroxyacetone phosphate, DHAP.



1.6.2 Tryptophan metabolism and glycolysis pathways

In relation to tryptophan metabolism, glycolysis is an oscillatory metabolic process that forms ATP. The glycolytic pathway comprises ten enzymatic reactions (Nakrani et al., 2021). The ten steps comprise: (1) phosphorylation of glucose, (2) isomerisation of glucose 6-phosphate, (3) phosphorylation of fructose (F) 6-phosphate, (4) cleavage of fructose (F)1, 6-biphosphate, (5) isomerisation of dihydroxyacetone phosphate, (6) oxidative phosphorylation of glyceraldehyde 3-phosphate, (7) transfer from 1,3-bisphosphoglycerate to ADP, (8) isomerisation of 3-phosphoglycerate, (9) dehydration of

2-phosphoglycerate to PEP, (10) transfer of phosphate from PEP (**Figure 1.5**) (Chaudhry R., 2022).

Tryptophan *de novo* NAD⁺ biosynthesis is an oscillatory metabolic process, and it is recognised that the metabolites in this pathway influence energy metabolism, mitochondrial respiration and redox status (Lesniak et al., 2013). Tryptophan metabolism links to the glycolysis pathway through the phosphoenolpyruvate (PEP) essential node and to the tryptophan/mammalian target of rapamycin complex 1 (mTORC1) pathway by the GAPDH enzyme binding to Ras homolog enriched in brain (Rheb). At this point, glycolysis determines the efficiency of tryptophan synthesis (Metz et al., 2012). As tryptophan is a glucogenic or ketogenic amino acid, tryptophan deficiency reciprocally reduces glycolysis as observed in living cells (Maria et al., 2018), however this still remains to be fully clarified in humans and mice. This metabolic shift entails an upregulation of anabolic processes involving the synthesis of proteins and lipids, while the catabolic processes, particularly β -oxidation, are actively repressed. A distinctive feature of this metabolic reprogramming is that even though sufficient oxygen is available to facilitate the complete oxidation of glucose, T cells exhibit a shift in ATP production from oxidative phosphorylation to a more rapid glycolytic pathway (Jones & Thompson, 2007). Additionally, immune cells preferentially use glycolysis as a source of energy during the peak of inflammation, at the expense of mitochondrial respiration (Soto-Herederó et al., 2020), described in the Warburg effect (Vander Heiden et al., 2009).

In context to IBD, activated T cells switch to an anabolic metabolic profile characterised by increased rates of nutrient uptake, glycolysis, and biosynthesis and suppression of catabolic pathways including β -oxidation (Chen & Sundrud, 2016). Following activation, T cells experience a transition from a quiescent state to an actively proliferating state. This metabolic shift entails an upregulation of anabolic processes involving the synthesis of proteins and lipids, while the catabolic processes, particularly β -oxidation, are actively repressed. A distinctive feature of this metabolic reprogramming is that even though sufficient oxygen is available to facilitate the complete oxidation of glucose, T cells exhibit a shift in ATP production from oxidative phosphorylation to a more rapid glycolytic pathway (Jones & Thompson, 2007).

1.6.4 Tryptophan metabolism and fatty acid β -oxidation

In mammals, the nervous system regulates energy balance through myriad behavioural, physiological, and metabolic responses elicited by extrinsic and intrinsic cues of food availability and energy demand. During periods of fasting, infection or starvation, the availability of glucose from dietary sources gradually decreases, leading to a reliance on glycogen breakdown and gluconeogenesis to sustain glucose production. However, glycogen stores are finite, and as a result, gluconeogenic precursors such as lactate, pyruvate, glycerol, and certain amino acids become the sole providers of glucose. To prevent excessive degradation of protein mass, which mainly contributes to the production of gluconeogenic precursors, the oxidation of fatty acids becomes crucial as an alternative energy source (Antoni et al., 2017). Therefore, the organism averts the conversion of fats to energy during catabolic states which are involved in many diseases and metabolic disorders (Tolwani et al., 2005). Hereby, ingested triglycerides are enzymatically broken down into smaller-chain fatty acids and monoglyceride molecules by pancreatic lipase, then transported across the intestinal membrane and reformed into triglyceride molecules. (Talley J.T & S.S., 2022; Y. Zhang et al., 2019). Under these conditions, the energy provided by fatty acids maintains energy homeostasis for skeletal muscle, heart muscle, kidneys, and the gastrointestinal tract by supplying acetyl-CoA for ketone body synthesis and for the TCA cycle's functioning (Shores et al., 2011). In addition, up to 50% of fatty acids delivered to the brain are oxidised to acetate, the majority of palmitate and oleate incorporated into brain lipids are derived from circulating fatty acid (Obici et al., 2003).

The FAO process occurs in the mitochondria, where fatty acids are shortened for energy production and involves the following steps. During the TCA cycle, acetyl-CoA combines with carnitine to form the fatty acyl-carnitine molecule, which facilitates the fatty acids crossing of the mitochondrial membrane and is the only energy compound to enter the TCA cycle. Once inside the mitochondria, the fatty acyl-CoA esters undergo β -oxidation via a four-step mechanism involving dehydrogenation, hydration, oxidation, and thiolytic cleavage catalysed by a distinct enzyme (Talley J.T & S.S., 2022). Acyl-CoA dehydrogenase and acyl-CoA oxidase catalyse the first reaction of oxidation in the

mitochondria and peroxisomes. Within each cycle, acetyl-CoA are shortened by two carbons and continue to recycle until the fatty acids are completely degraded. FAD-containing enzymes, dehydrogenases, complete the degradation of fatty acids within the mitochondria. Each cycle finishes with one acetyl-CoA, one FADH₂, one NADH, and H₂O, and the acyl-CoA chain becomes two carbons shorter. Hence, dehydrogenases differ in their acyl chain lengths and substrate specificities. Namely, short-chain acyl-CoA dehydrogenase (SCAD), medium-chain acyl-CoA dehydrogenase (MCAD), long-chain acyl-CoA dehydrogenase (LCAD), and very long-chain acyl-CoA dehydrogenase (VLCAD) (Oey et al., 2006; Yang et al., 2005). FAO is equally essential to ATP production, leading to the majority of palmitate and oleate being derived from circulating fatty acids (Curi et al., 2016; Obici et al., 2003). Accordingly, FAO produces 360 ATP, whereas glucose oxidation produces 32 ATP (Bartlett & Eaton, 2004).

The physiology of enterocytes and the regulation of inflammatory processes are reliant on fatty acids and intracellular lipid deposition, particularly during high metabolic demand when glucose supply is limited (Ma et al., 2019). Serotonin (5-HT) activates neuroendocrine signals that promote the lipolysis of triglycerides and the oxidation of fatty acids. When released by sensory neurons, serotonin interacts with a specific receptor (SER-5) to modulate eating behaviour. However, this SER-5 receptor is not necessary for the serotonin's impact on peripheral fat metabolism. Beyond its role in regulating appetite, enhanced serotonin signalling boosts energy metabolism, which could result in fat loss. If the fatty acids generated from mobilising stored triglycerides are not used in β -oxidation pathways, a homeostatic signal is generated to reduce serotonergic effects, including influences on appetite and eating behaviour (Bouagnon et al., 2019). The molecular underpinnings of these responses and their coordination remain key challenges in understanding energy balance in IBD patients.

1.7 Conclusion

This overview explored the complex interplay of the interconnected topics: the epidemiology and pathophysiology of IBD, the relationship between IBD and behavioural changes, the role of tryptophan metabolism in influencing behaviour, the connection between tryptophan metabolism and IBD. To identify the conditions under

which tryptophan metabolism is altered, the models of glycolysis and fatty acid β -oxidation were discussed with the tryptophan synthesis model. By elucidating these interconnected topics, this overview has laid a foundation for the thesis and a more comprehensive understanding of the intricate relationship between gastrointestinal health, the brain and affective disorders. Studies involving human IBD patients, as well as mouse models of colitis, have demonstrated connections to depression and anomalies in tryptophan metabolism. The realm of tryptophan metabolism, set against the backdrop of IBD and depression, offers a promising avenue for research. By delving deep into the intersections between glycolysis, fatty acid β -oxidation, and tryptophan synthesis, we may unearth novel insights, potentially leading to innovative treatments and therapeutic strategies. Such an exploration would not only benefit IBD patients but could also have broader implications for the fields of gastroenterology, psychiatry, and metabolic research.

Chapter 2: Changes in energy metabolism in the *Winnie* mouse distal colon

2.1 Summary

Inflammatory bowel disease (IBD) is characterised by chronic inflammation that can encompass the entire gastrointestinal tract. Central to managing such conditions is the organism's capacity to maintain intestinal homeostasis, largely hinging on the energy production capabilities of its cells.

Our study offers deeper insights into the metabolic shifts seen in IBD, employing the *Winnie* mouse for real-time metabolic assessments. We utilised the Promethion metabolic multiplexed phenotyping system, a sophisticated tool integrating indirect calorimetry with flow-through respirometry monitors. This enabled us to gauge the *Winnie* mouse's food and water consumption, track the energy derived from the oxidation of various substrates, and ascertain their proportional contributions. From this data, we were able to quantify glucose oxidation (GOX), lipid oxidation (LOX), and determine the respiratory quotient (RQ). Furthermore, to delve into the molecular intricacies of metabolism in IBD, we employed RNA-Sequencing (RNA-Seq). This approach offered a comprehensive view of gene expressions, allowing us to analyse the transcriptome linked to the glycolysis and fatty acid β -oxidation pathways in the *Winnie* mouse's distal colon.

Our findings showed that the *Winnie* mouse exhibited decreased food and water consumption, reduced oxygen intake, and diminished carbon dioxide production. Consequently, we observed a significant decline in energy expenditure, glucose oxidation, and fatty acid oxidation. The transcriptomic data revealed that the pathways for glycolysis and fatty acid β -oxidation were intricately tied to inflammation, leading to metabolic pathway reprogramming. This metabolic shift rendered the *Winnie* mouse more vulnerable to infections, injuries, and inflammatory mediators. Moreover, we identified a compromised ability to metabolise fatty acids and discovered genes adversely impacting tryptophan metabolism.

2.2 Introduction

Inflammatory bowel disease (IBD) is chronic intestinal inflammation, which may affect any segment of the intestinal tract in Crohn's disease (CD), and predominantly the distal colon in ulcerative colitis (UC) (Fakhoury et al., 2014). In IBD, the intestinal barrier undergoes multiple disturbances involving a reduced production and altered expression of mucins, oligomeric mucous/gel-forming proteins, produced by intestinal goblet cells. Dysfunctional mucus and altered mucin-producing function of goblet cells lead to the development of intestinal inflammatory conditions. (Heazlewood et al., 2008). Distinct mucin genes are expressed in different segments of the gastrointestinal tract. There exist membrane-bound mucins termed MUC1, MUC2, MUC3, MUC4, MUC13, and MUC17, which participate in cell signalling processes (Bankole et al., 2021). *MUC2* represents the primary mucin gene expressed in the intestines. Its primary function involves creating a protective barrier within the intestinal lining that prevents the infiltration of bacteria and potentially harmful substances into the intestine (Heazlewood et al., 2008). Reduced *MUC2* production and altered composition lead to intestinal mucosal cell damage and endoplasmic reticulum stress resulting in increased intestinal permeability (Gustafsson & Johansson, 2022). Intestinal permeability allows luminal antigens and toxins to flow passively through the intercellular spaces of the intestinal epithelium, which induces a pro-inflammatory immune response, and the development of chronic inflammation, as observed in IBD. The clinical manifestations of IBD are diarrhoea, abdominal pain, rectal bleeding, mucosal damage/loss, reduced nutrient absorption, and bacterial overgrowth (Baumgart & Carding, 2007).

As a consequence, IBD patients experience a lack of energy or exhaustion out of proportion to physical exertion, which is not relieved with rest, leaving patients unable to perform simple daily activities (Nocerino et al., 2020). Behavioural changes in IBD patients involving depression and fatigue become incipient at disease onset, often remaining unrecognised until they develop into a pernicious, chronic syndrome, reducing the patient's quality of life (QoL) (Borren et al., 2019; Czuber-Dochan et al., 2013). These observations demonstrate the likelihood of associations between intestinal health and energy metabolism (Wachsmuth et al., 2022). Frequently, physicians have inherently

associated the lack of energy with depression rather than considering altered energy metabolism within glycolysis and fatty acid β -oxidation (FAO) (Van Langenberg & Gibson, 2010).

Glycolysis is an oscillatory metabolic process that forms ATP and reduced nicotinamide adenine dinucleotide (NAD^+). Dysregulation of glycolysis affects behaviour and mood regulation by reducing energy expenditure (EE) through the metabolism of tryptophan, which is the main source of serotonin and NAD^+ (Chaudhry R., 2022). Glycolysis is linked to tryptophan metabolism through the phosphoenolpyruvate (PEP) essential node. At this point, glycolysis determines the efficiency of tryptophan metabolism, which has further implications for regulating gut motility and overall gastrointestinal function (Kanova & Kohout, 2021). Furthermore, the abnormalities in the bacterial populations of IBD patients cause alterations in metabolic pathways, compromising amino acid synthesis, monosaccharide conversion to short-chain fatty acids, and FAO (Zheng et al., 2022). It is of interest that serotonin triggers a neuroendocrine response that promotes the breakdown of triglycerides and the oxidation of fatty acids (Martin et al., 2017). Consequently, it is imperative to elucidate the outcomes of metabolic pathways in IBD and to investigate their subsequent impacts on glycolysis and FAO.

In this study, we will utilise the *Winnie* mouse model of spontaneous chronic colitis to investigate energy metabolism, via glycolysis and FAO. Similar manifestations, closely resembling human IBD, are existing in the *Winnie* mouse such as diarrhoea, rectal bleeding/prolapse, weight loss, functional changes in the intestinal tract, changes in the colonic transit time and contractile activity and morphological damage to the mucosa (Rahman et al., 2015; Robinson et al., 2017). The *Winnie* mouse model is due to a missense mutation in the *Muc2* gene, which results in aberrant MUC2 biosynthesis within goblet cells (Eri et al., 2011; Heazlewood et al., 2008). Signs of colitis appear in the *Winnie* mouse from six weeks of age, progressing to severe colitis at 12-16 weeks (Eri et al., 2011).

This study provides an advance in the understanding of the metabolic changes in IBD patients by utilising the *Winnie* mouse. The metabolic multiplexed phenotyping system, involving indirect calorimetry and flow-through respirometry monitors, was used to

measure the food and water intake of the *Winnie* mouse, the energy released from oxidised energy substrates, and their respective ratios. Based on these measurements, glucose oxidation (GOX), lipid oxidation (LOX), and the respiratory quotient (RQ) were determined. To explore metabolic adaptations, RNA-Sequencing (RNA-Seq) provided access to quantitative gene expressions for transcriptome analyses of glycolysis/gluconeogenesis and fatty acid β -oxidation in the *Winnie* mouse.

2.3 Materials and methods

2.3.1 Ethics, animals, and husbandry

This research has been approved by the Victoria University Animal Experimentation Ethics Committee (AEC-17-016) for all experiments in this thesis. All experiments were performed within the Scientific Procedures Premises of Victoria University, licenced by the Department of Economic Development, Jobs, Transport and Resources and in compliance with the Animal Ethics Committee. The Victorian code of practice for the housing and care of laboratory mice, rats, guinea pigs and rabbits govern these facilities. The experiments adhered to Part 3 & 4 – Prevention of Cruelty to Animals Act 1986, Regulations 1997 & 2008, and uphold the Animal Welfare Policy, 2014; and are governed by ‘National Health and Medical Research Council (namely, The Code, 2013).

The *Winnie* mouse model of colitis with a C57BL/6 mice background was obtained from the same colony at Victoria University (Melbourne, Victoria, Australia). The colitis in the *Winnie* mouse is caused by a missense mutation (G9492A, GenBank accession no. AJ511872), by which cysteine is replaced by tyrosine in the D3 67 domain in the N terminus of the mucin 2 (*Muc2*) gene (Heazlewood et al., 2008). Age-matched C57BL/6 mice littermates, obtained from the Animal Resources, constituted the control. C57BL/6 male and female mice (6 weeks and 14 weeks of age, n=8 mice/group) were obtained from the same colony. Female and male *Winnie* mice aged 6 weeks and 14 weeks of age (n=8 mice/group), 14 weeks of age heterozygous (Het) *Winnie* male and female littermates (n=8 mice/group), and 14 weeks of age control C57BL/6 male and female littermates (n=8 mice/group) were compared.

All mice were acclimatised for one week at the Werribee Animal Facility or at Western Centre for Health, Research and Education (Melbourne, Victoria, Australia). All mice were permitted *ad libitum* access to food and water and were housed in a temperature-controlled environment with a 12 hour day/night cycle meeting the National Health and Medical Research Council guidelines for rats and mice. Post *in vivo* experiments, all mice were euthanised at 14 weeks of age via an overdose of injected pentobarbitone (Lethabarb, Virbac Australia) using a 1:16 dilution, 30 g, 100 µL/20 g. The distal portion of the colon tissues were removed and used for subsequent experiments.

2.3.2 Evaluation of colonic inflammation in the *Winnie* mouse

To assess faecal water content, each mouse was placed into a single sterile cage without bedding until defecation occurred. Individual faecal pellets were weighed immediately to determine their wet weight. To remove all the moisture from the faeces, the faecal pellets were oven-fan dried at 60° C for 24 hours and weighed again. The difference between the wet and dry pellet weights determined the faecal water content.

Lipocalin-2 (Lcn-2) is a potent bacteriostatic protein known as neutrophil gelatinase B-associated lipocalin (NGAL). It is stored in neutrophil granules and released at sites of inflammation. Lcn-2 level is elevated in the serum, urine, and the faeces of patients with active IBD, making it a highly-sensitive non-invasive biomarker of intestinal inflammation (Chassaing et al., 2012). Faecal pellets were collected from mice, and immediately frozen in liquid nitrogen and stored at -80° C until used. Faecal pellets were reconstituted in a phosphate buffered solution (PBS) containing 0.1 % tween 20 (100 mg/mL), then vigorously vortexed for 20 minutes to form a homogenous suspension. Samples were centrifuged for 10 minutes at 12,000 revolutions per minute (9660 g force) at 4°C to obtain clear supernatants. Lcn-2 was quantified in the faecal supernatant using a Mouse Lipocalin-2 enzyme-linked immunosorbent assay (ELISA) Kit (NGAL; Abcam; ab119601), according to the manufacturer's instructions. The absorbance of Lcn-2 detection was read at 450nm by the Varioskan Flash Multimode Reader using Skanlt software v2.4.3 (Thermo Fisher Scientific) to calculate levels of faecal Lcn-2. All samples were repeated in duplicate for statistical value. For body weight, scales measured the

mean weight of 20 consecutive measurements of the C57BL/6 mouse compared to the *Winnie* mouse.

2.3.3 Histology

To determine gross morphological damage, distal colons were extracted from euthanised mice, immediately rinsed with PBS to remove faecal debris, and after fixation, tissues were paraffin embedded, and sectioned at 5 μm according to the method outlined by Robinson et al. (2017). Histological sections were evaluated for several parameters to determine the damage score. This included aberrant crypt architecture (score range, 0–5), changes to crypt length (0–5), crypt abscesses (0–5), epithelial damage (0–5), ulceration (0–5), and leukocyte infiltration (0–5). An average of three 500 μm^2 areas were analysed per section. Sections were imaged using the MetaSystems Metafer program and VSlide image stitching software on a Zeiss AxioImager microscope (Zeiss, Germany). All slides were coded, and analysis was performed blindly according to methods described by Filippone et al. (2022).

2.3.4 Whole body composition analysis

Immediately after the Promethion metabolic experiment, the mice' body composition was analysed using an EchoMRI™-1100 scanner (EchoMRI LLC, Houston, TX, USA). The EchoMRI™ is a quantitative magnetic resonance imaging (MRI) system that measures whole body fat mass (all fat molecules in the body), lean tissue mass (tissues containing water, excluding fat, bone minerals, and substances that do not contribute to the nuclear magnetic resonance signal, such as hair and claws), free water (not bound in various tissues, usually mostly urine), and total body water (contained in all the liquids and in a bound state in tissues) in live animals between 20-1100 grams, without the need for anaesthesia or sedation and with minimal impact on animals. Unlike standard MRI, the quantitative magnetic resonance signal process is obtained from the whole body at one time (Kovner et al., 2010). For each scan one mouse was encouraged into and contained within a long plastic tube, which was inserted into the scanner for a rapid and non-invasive, silent scan of body composition in triplicate for 30 seconds (s) time lapses. Upon completion the mice were removed and returned to their home cages. Tubes were disinfected with 70%

ethanol between testing each mouse. Upon completion of the postintervention EchoMRI scan the data is presented as the mean of the 3 scans. All measurements are expressed in grams.

2.3.5 *In vivo* analysis of energy metabolism

The Promethion metabolic high-definition multiplexed respirometry cages for mice (Sable Systems International, Nth Las Vegas, NV, USA) located at the Werribee Animal Facility of Victoria University (Melbourne, Australia) were used for the metabolic studies. Flow-through respirometry monitors metabolic rates, and the sensors evaluate caloric food and water uptake, digestion, and energy efficiency via indirect calorimetry. The Promethion has the following sensors: (1) Food intake monitor measured by mass in grams (g); (2) Water intake monitor measured by mass in grams (g); (3) FAC-1 Access Control Module (access to food and water); (4) WM-1 Running Wheel Module; (5) BXZ-1 Total Activity Monitor, (6) BW-1 Body Weight Module. Mice were placed into the metabolic cages for 6 days to determine real-time energy metabolism, food and water intake, in the following groups: *Winnie* mice versus control C57BL/6 littermates (male and female sexes combined, n=16); in a second study, *Winnie* male mice and *Winnie* female mice (6 weeks and 14 weeks of age, n=6–8 mice/group), control C57BL/6 male and female littermates (6 weeks and 14 weeks of age, n=6–8 mice/group), and Het *Winnie* male and female littermates (14 weeks of age, n=6–8 mice/group). The metabolic cages were set up precisely according to the manufacturer's instructions.

Indirect calorimetry will determine the rate of oxygen consumption (VO_2) in millilitres per minute (mL/min) per mouse and rate of carbon dioxide emission (VCO_2) in mL/min in 4-minute intervals over each 12 hour cycle. Body mass measurements were enabled via a ceiling-mounted 'home-hut', which the mouse could climb into *ad libitum* ("Promethion Metabolic Screening: Revolutionizing Metabolic Measurement," 2019). This is crucial for understanding dynamic metabolic processes and how they respond to different conditions involving exercise, fasting, or various diseases. Overall, indirect calorimetry can give insights into the overall EE, which is important for studies related to weight management, nutrition, physical fitness, and metabolic diseases.

Metabolic data acquisition and instrument control were coordinated by MetaScreen, while the raw data was processed in ExpeData (Sable Systems International, Nth Las Vegas, NV, USA) using an analysis script detailing all aspects of data transformation. After 5 days of acclimatisation, the following final 24 hour data were used for analyses. The data points recorded were summarised by circadian cycle, 0700-1900 hour (light cycle) and 1900-0700 hour (dark cycle). The RQ is analysed using a substance-fixed ratio between the quantities of VO_2 consumption and VCO_2 emission. By analysing the RQ researchers can gain insights into which type of fuel (carbohydrates, fats, or proteins) the body is primarily using for energy (Grobe, 2017). EE was measured using the modified Weir formula (Weir, 1949), with data normalised by dividing the EE by the body weight to generate values of EE/gram, $\text{EE (kcal/kg/BW)} = \text{EE (cal) in 12 hour/1000/body weight}$. The substrate oxidation rates were determined using the calculations of Ferrannini (1988): $\text{EE (kcal/kg/BW)} = (3.94 * \text{VO}_2 \text{ mL in 12 hour dark}) + (1.106 * \text{VCO}_2 \text{ mL in 12 hour dark})$; $\text{GOX (g/kg/BW)} = (4.55 * \text{VCO}_2 \text{ mL in 12 hour dark}) - (3.21 * \text{O}_2 \text{ mL in 12 hour dark})$; $\text{LOX (g/kg/BW)} = (1.67 * \text{VO}_2 \text{ mL in 12 hour dark}) - (\text{VCO}_2 \text{ mL in 12 hour dark})$.

2.3.6 RNA extraction and quality control

RNA-Seq was selected to quantify RNA species in the distal colon, enabling unbiased transcriptome profiling. RNA-Seq determinations of glycolysis/gluconeogenesis and fatty acid β -oxidation within the distal colons of 14-week-old *Winnie* mice (n=6; 4 females and 2 males) were compared to age-matched control C57BL/6 littermates (n=7; 3 females and 4 males).

Distal colons collected from euthanised mice were rinsed with PBS to remove faecal debris, quick frozen in liquid nitrogen, and stored at -80°C until used. Total RNA was extracted using TRIzolTM (Invitrogen, Australia). To ensure the high purity of extracted RNA, the RNeasy Lipid Tissue Mini Kit (cat. no. 74804, Qiagen, Hilden, Germany), which included DNase treatment was used. TissueLyser EXPAND was used to homogenise colon tissues, and the total RNA samples' integrity was determined using the Agilent 2100 Bioanalyser's RNA 6000 Nano chip (Agilent Biotechnologies). All samples passed their test showing no contamination of genomic DNA and 16S ribosomal RNA from bacteria, providing an RNA integrity number >8.5 . The purity of the RNA was

determined using a Denovix DS-11 spectrophotometer to measure the 260/280 and 260/230 ratios, and achieved >1.8. The remaining RNA for each sample was aliquoted into individual 0.5 mL micro-tubes in equal concentrations of 3 µg of RNA per sample (≥100 ng/µl) in nuclease-free water and then stored at -80° C until required for RNA-Seq.

2.3.7 High-throughput RNA-Seq of mRNA

The mRNA samples were submitted to Micromon Genomics (Monash University, Melbourne, Australia) for RNA-Seq using the NGS platform. Micromon Genomics performed RNA quality control with Invitrogen Qubit RNA BR assay (Gene Target Solutions, Sydney, Australia), as well as the polyA purification of mRNA from total RNA samples and the RNA-Seq library construction. All of the samples quantified and analysed with the Qubit and Bio-analyser passed the QNA QC standard. Micromon Genomics conducted the bioinformatics and performed the sequencing on MGITech MGISEQ2000RS hardware (MGISEQ-2000RS High-throughput Sequencing Set). The MGIEasy V2 chemistry set was used to generate the library, with at least 400 m raw reads per lane and paired-end 100b reads for accuracy and processing to compressed FASTQ files. Raw FASTQ files were analysed using the RNAsik pipeline version 1.5.4 (Tsyganov et al., 2018) and the *Mus musculus* reference genome GRCm38 (GenBank accession GCA_000001635.2) (Dobin et al., 2013). Raw read counts were quantified using the featureCounts program (Liao, 2014) and analysed with Degust, a web tool that performs differential expression analysis using limma voom normalisation (Law et al., 2018) to produce counts per million (CPM) library size normalisation and trimmed means of M values and normalisation for RNA composition normalisation (Robinson & Oshlack, 2010). Differential expression of transcripts between the colons of *Winnie* mice and C57BL/6 littermates were determined using via the Degust Webtool. EdgeR was preferred to obtain the total number of genes from Degust. DEGs were identified with an FDR of <0.001 using the Benjamini–Hochberg correction (Benjamini and Hochberg, 1995). Degust also includes a number of quality plots, such as classic multidimensional scaling and MA plots (Powell, 2019). MultiQC which presents the visual output of data gathers it into a single report (Ewels et al., 2016). Genes with changes in expression of $\pm 0.585 \text{ Log}_2\text{FC}$ (fold change), 1.5FC were selected as the cut-off. To analyse differential

gene expression based on overrepresentation analysis software for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways was used (Kanehisa & Goto, 2000).

Heat maps of gene expression values for each sample were visualised using the Morpheus web-based tool, Morpheus, <https://software.broadinstitute.org/morpheus> (accessed on June 14, 2021), and presented as Z-score distributions (across samples) of the CPM values, and hierarchically clustered via Euclidean distance with average linkage.

The visualisation of selected pathways was performed using the R package Pathview using default parameters (Luo & Brouwer, 2013). Pathview downloads the pathway graph data, parses the data file, maps and integrates user data onto the pathway, and renders the pathway graph with the mapped data, <https://r-forge.r-project.org/projects/pathview/> (accessed on June 14, 2021). Input data included significance scores of gene expression values between *Winnie* and C57BL/6 mice, and computed as the $\text{sign Log}_2\text{FC} \times \text{Log}_{10}\text{P}$ values represented in the Kyoto Encyclopedia of Genes and Genomes (KEGG) graph.

2.3.8 STRING protein-protein association networks

Search Tool for Retrieval of Interacting Genes (STRING) database enables comprehensive characterisation of user gene lists and functional genomics datasets, and allows the creation and sharing of highly customised and augmented protein-protein association networks (Szkłarczyk et al., 2021). STRING sources biological pathway databases, specifically gene ontology (GO). Up and downregulated genes were uploaded into the STRING data base to extract biological processes, molecular functions and cellular pathways if applicable. GO biological process (GOBP) comprises extensive cellular compartments related to multiple molecular activities. GO molecular function (GOMF) describes catalytic activity molecularly occurring within the cell. GO cellular compartment (GOCC) indicates the location within cellular anatomy where the gene performs functions.

2.3.9 Homology of genes in *Winnie* mice and IBD patients

Mice are frequently used as model organisms for genetic disease research and the development of novel therapies. By comparing the homology of mouse and human genes, researchers can identify genetic mutations in mice that may have comparable effects in humans, thereby shedding light on the genetic basis of diseases. The National Centre for Biotechnology Information and Gene Expression Omnibus public data source was used to gather gene expression data on the transcriptome of human IBD patients (Barrett et al., 2013; Edgar et al., 2002). For the data source, high-throughput sequencing was used via the Illumina HiSeq 2500 platform to create the expression profile of the inflamed intestine in IBD patients and the healthy colon regions in uninflamed controls undergoing resection of non-obstructive colorectal adenocarcinoma (Peters et al., 2017). Peters et al. (2017) assembled this data as reads per kilobase of transcript, per million mapped reads. Similar to the methods used in this research, poly-A purified mRNA was used by Peters et al. (2017) from RNA isolated using TRIzol obtained from the human colon samples. These data are available at <https://www.ncbi.nlm.nih.gov/geo/> (accessed on March 20, 2023) under the GEO series accession number GSE83687. This dataset includes the data from Crohn's disease patients (n=42), ulcerative colitis patients (n=31) and controls (n=60).

2.3.10 Statistical analysis

Metabolic data comparisons were performed using GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA). A two-way ANOVA and a post hoc Tukey or Holm-Šídák test were applied for multiple group comparisons. Otherwise, an unpaired Student's *t*-test with Welch's correction was applied where applicable. For analyses, $p \leq 0.05$ was considered significant. All data were presented as the mean $\pm$ standard error of the mean (SEM).

2.4 Results

2.4.1 Evaluation of inflammation in the *Winnie* mouse colon

Inflammation was assessed in 14-week-old *Winnie* mice compared to age-matched

control C57BL/6 littermates (**Figure 2.1**). Stool consistency was measured by the faecal water content as a difference between wet weight and dry weight of pellets collected from mice in individual sterile cages prior to culling of 14 weeks of age *Winnie* mice and age-matched control mice. There were non-significant differences in the faecal water content between males and females in both the *Winnie* and control groups, therefore, the pellets of the sexes were merged for analysis. The percentage of faecal water content (%) was higher in *Winnie* mice ($77.30 \pm 1.0\%$, $p < 0.0001$, $n=11$) compared to control mice ($51.5 \pm 7.0\%$, $n=11$) (**Figure 2.1A**). Faecal Lcn-2 measurement was applied as a non-invasive method to determine intestinal inflammation. *Winnie* mice had significantly higher faecal Lcn-2 levels (412.4 ± 12.8 pg/mL, $p < 0.001$, $n=10$ mice/group) compared to control mice (246.9 ± 40.7 pg/mL, $n=6$ mice/group) (**Figure 2.1B**). The *Winnie* male mice weighed less (23.8 ± 0.6 grams, $p < 0.0153$) than control male mice (26.9 ± 0.9 g), while the *Winnie* female mice (21.2 ± 0.4 grams) had a similar weight as control female mice (20.7 ± 0.8 grams). The *Winnie* female mice (21.2 ± 0.4 grams, $p = 0.0117$) weighed significantly less than the *Winnie* male mice (23.8 ± 0.6 grams), $n=8$ mice/group. However, the control female mice (20.7 ± 0.8 grams, $p < 0.0001$) weighed significantly less than the control male mice (26.9 ± 0.9 grams) (**Figure 2.1C**).

Cross sections of the distal colon were stained with haematoxylin and eosin to evaluate gross morphological damage to the colon, including epithelial crypts, leukocyte infiltration, ulcerations, and epithelial damage (Heazlewood et al., 2008). Histological scores were elevated in 14 weeks of age *Winnie* mice distal colons (16.3 ± 0.4 arb. units, $p < 0.0001$) compared to control mice (0.77 ± 0.2 arb. units), $n=5$ mice/group (**Figure 2.1D, 2.1E', 2.1E'', Table 2.1**). These evaluations concur with previous results in *Winnie* mouse studies (Rahman et al., 2015; Robinson et al., 2017).

2.4.2 Whole body composition of the *Winnie* mouse

The body composition involving fat mass, lean tissue mass, free water, and total body water of living *Winnie* mice and control C57BL/6 littermates were measured during a body composition analysis using the EchoMRI™ scanner. The data are presented as the mean $\pm$ SEM of triplicate 30 second scans, with all measurements expressed in grams.

Winnie male mice exhibited non-significant differences in fat mass (0.8 ± 0.05 grams, $p=0.117$) compared to control male mice (0.96 ± 0.1 grams). The percentage in fat mass was also non significantly different in *Winnie* male mice ($0.04 \pm 0.01\%$, $p=0.866$) and control male mice, ($0.04 \pm 0.003\%$). However, *Winnie* male mice demonstrated a statistically significant lower lean mass (21.1 ± 0.4 grams, $p=0.01$) than control male mice (23.4 ± 0.6 grams). The *Winnie* mice males showed non-significant differences in lean mass percentage ($0.9 \pm 0.01\%$, $p=0.093$) compared to control male mice ($0.87 \pm 0.03\%$). *Winnie* female mice demonstrated a statistically higher fat mass (1.6 ± 0.08 grams, $p=0.011$) than control female mice (1.3 ± 0.06 grams), and a higher fat mass percentage ($0.08 \pm 0.003\%$, $p<0.001$) than control female mice ($0.06 \pm 0.003\%$). *Winnie* female mice showed less lean mass (15.3 ± 0.4 grams, $p=0.002$) than control female mice (17.05 ± 0.15 grams) and a lower percentage of lean mass ($0.7 \pm 0.03\%$, $p=0.002$) than control female mice ($0.8 \pm 0.02\%$) (**Figure 2.2**).

Figure 2.1 Evaluation of inflammation in the *Winnie* mouse colon

Faecal water content, Lcn-2 levels, and body weight were evaluated in the 14-week-old *Winnie* mice and age-matched control C57BL/6 littermates. **(A)** The percentage of faecal water content. **(B)** Lcn-2 levels in faecal pellets. **(C)** Weight of the *Winnie* and control male and female mice, n=8 mice/group. **(D)** Quantification of histological scoring in colonic cross sections, colonic crypts, intestinal epithelium, smooth muscle layers, leukocytes. **(E)** Photographical representation of colons was visualised by H&E staining in cross sections from *Winnie* mice and control mice **(E')** control mice and **(E'')** *Winnie* mice (scale bar=50 μ m, n=5 mice/group). Data are expressed as mean $\pm$ SEM, * $p \leq 0.05$, ** $p = 0.0083$, **** $p < 0.0001$. **Abbreviations:** Lcn-2, lipocalin-2; pg/mL, picograms per millilitre; g, grams.

Figure 2.1 Evaluation of inflammation in the *Winnie* mouse colon

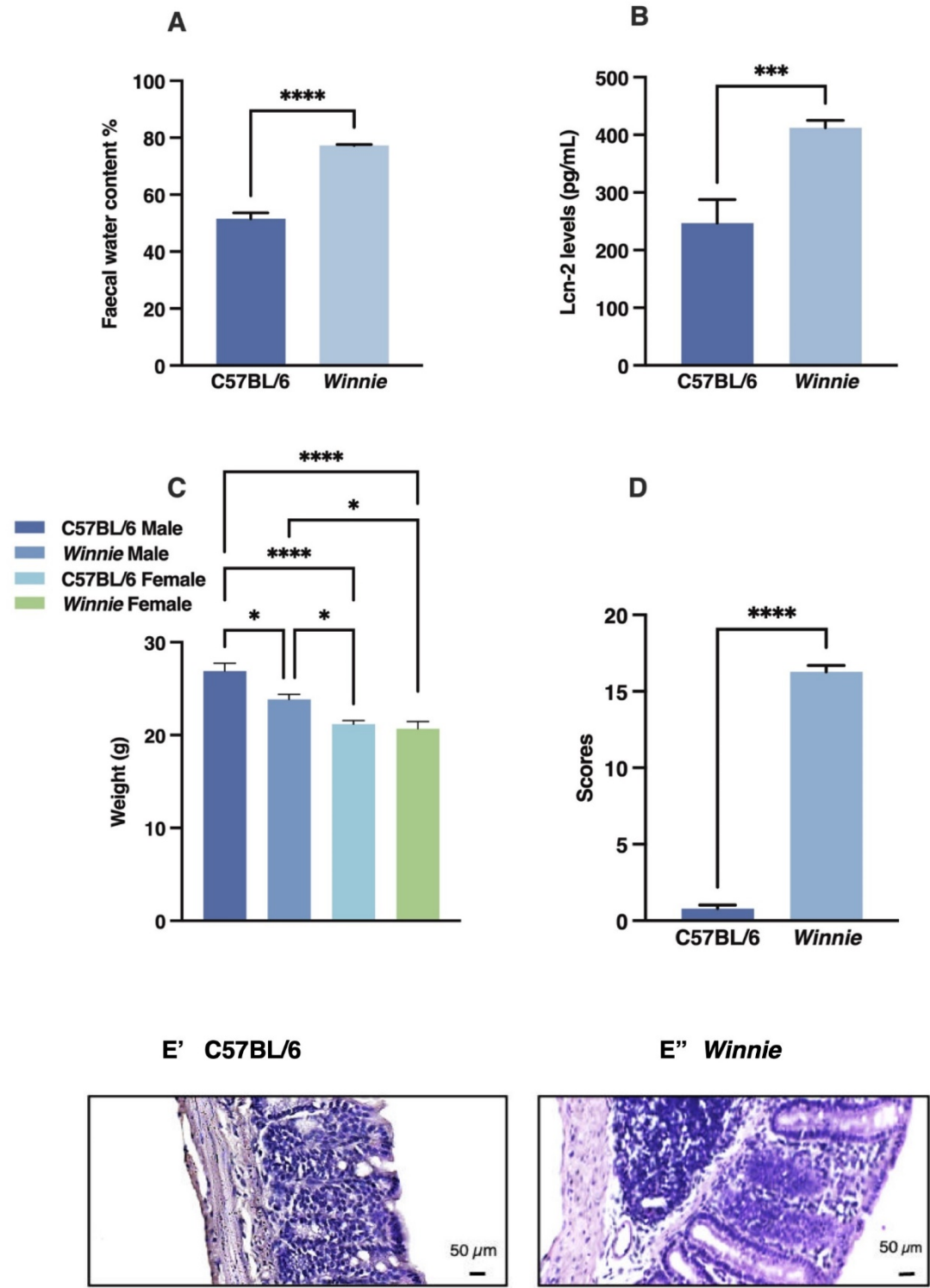


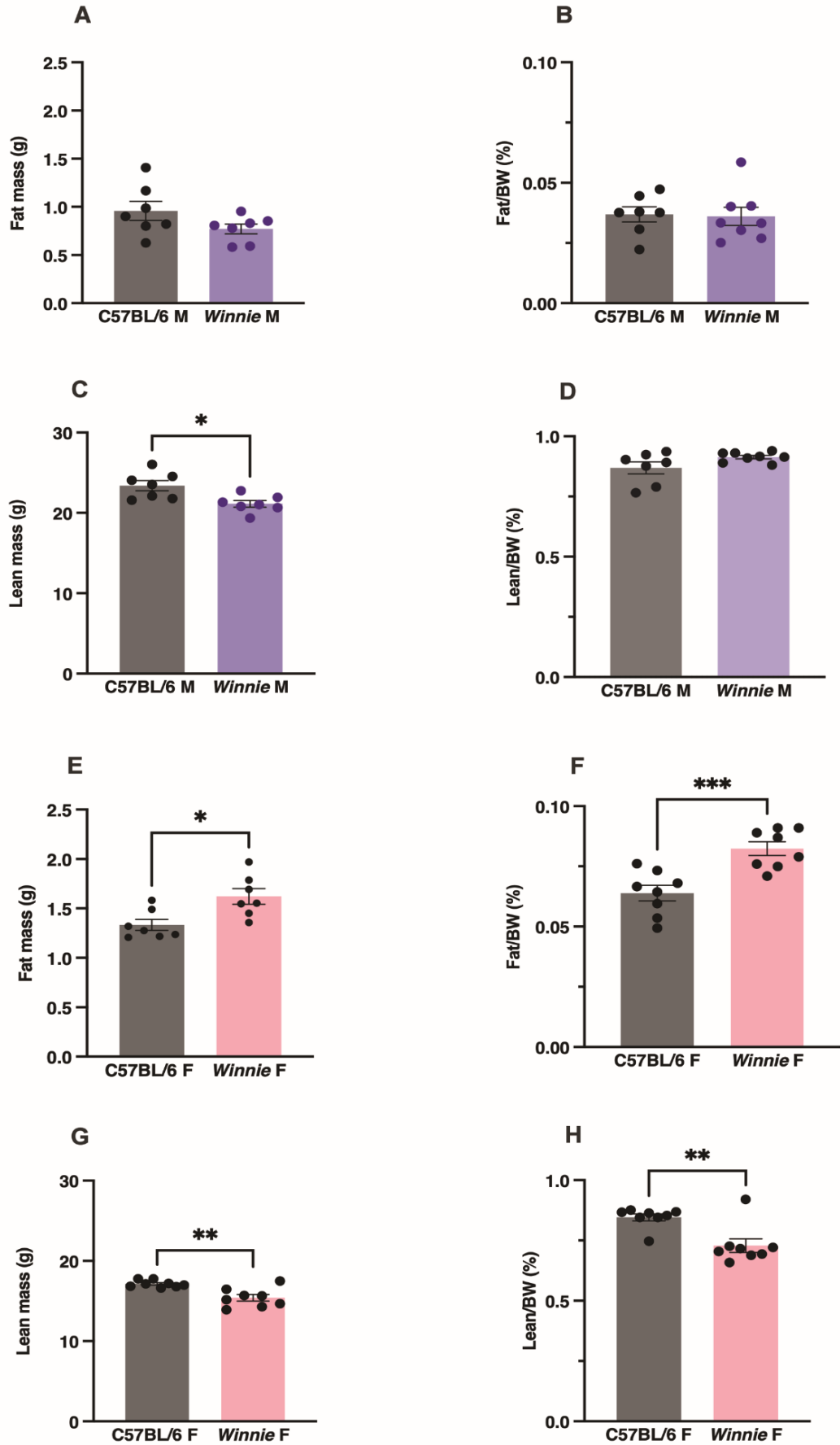
Table 2.1 Histology scoring for *Winnie* mice distal colons

C57BL/6 mice	1			2			3			4			5			Mean	SEM	n	
Colonic crypts	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0.27	0.12	5	
Intestinal epithelium	0	0	0	1	1	0	1	1	1	1	1	1	0	0	0	0.53	0.13	5	
Smooth muscle layer	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0.07	0.07	5	
Leukocytes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00	0.00	5	
Winnie mice	1			2			3			4			5			Mean	SEM	n	p
Colonic crypts	4	4	4	5	5	4	5	5	5	5	5	5	4	4	4	4.53	0.13	5	0.0001
Intestinal epithelium	5	5	4	4	4	5	5	5	5	5	5	5	5	4	4	4.67	0.13	5	0.0001
Smooth muscle layer	3	4	4	2	3	5	2	2	2	3	3	3	5	5	5	3.40	0.31	5	0.0001
Leukocytes	5	3	4	4	5	5	3	3	2	5	3	2	4	4	3	3.67	0.27	5	0.0001

Figure 2.2 Whole body composition of live *Winnie* mice

The body composition analysis, including the fat mass and lean tissue mass, were measured in live *Winnie* male mice and *Winnie* female mice compared to C57BL/6 male and female littermates, utilising the EchoMRI™ scanner. **(A)** Absolute fat mass in male mice in grams. **(B)** Percentage of fat mass in male mice. **(C)** Absolute lean mass in male mice in grams. **(D)** Percentage of lean mass in male mice. **(E)** Absolute fat mass in female mice in grams. **(F)** Percentage of fat in female mice. **(G)** Absolute lean mass in female mice in grams. **(H)** Percentage of lean mass in female mice. Data are expressed as mean $\pm$ SEM, * $p=0.011$, ** $p=0.002$, *** $p<0.001$, $n=8$ mice/group/cycle. **Abbreviations:** g, gram; body weight, BW.

Figure 2.2 Whole body composition of live *Winnie* mice



2.4.3 Consumption and energy metabolism of *Winnie* mice

To determine energy metabolism in *Winnie* mice, indirect calorimetry was used to measure water and food consumption measured by mass in grams (g), assessing energy output mechanisms through the analysis of VO_2 consumption and VCO_2 emission, EE, GOX, and LOX, in which their balance is essential for metabolic homeostasis (Mtaweh et al., 2018). The *Winnie* mice and control C57BL/6 littermates sexes were compared separately, which demonstrated significant reduction in the *Winnie* mouse for all parameters (**Appendix: Figure S 1, Figure S 2, Figure S 3**). 14 weeks of age *Winnie* male mice and age-matched *Winnie* female mice ($n=8$ mice/group) were then compared for variances in metabolism, showing non-significant differences in all tests (**Figure 2.3**). Considering the negligible differences observed between the *Winnie* male mouse and *Winnie* female mouse, we pooled the data from both sexes of *Winnie* mice and juxtaposed it with the unified data from both sexes of control C57BL/6 littermates; with each group comprising $n=16$ mice (**Figure 2.4**). The light cycle showed non-significant differences for all tests, therefore, only the dark cycle is reported. In this study, the *Winnie* mice consumed significantly less water (1.9 ± 0.2 grams, $p < 0.001$) than in control mice (3.05 ± 0.2 grams) (**Figure 2.4A**). The *Winnie* mice attempted fewer drinking bouts (31.9 ± 3.1 bouts, $p = 0.038$) than the control mice (40.9 ± 4.0 bouts) (**Figure 2.4B**). The *Winnie* mice consumed significantly less food (2.0 ± 0.1 grams, $p = 0.011$) than in control mice (3.10 ± 0.2 grams) (**Figure 2.4C**). Accordingly the *Winnie* mice attempted fewer food bouts (16.8 ± 1.9 bouts, $p < 0.001$) than in control mice (26.4 ± 1.2 bouts) (**Figure 2.4D**). VO_2 consumption was significantly reduced in the *Winnie* mice (61.82 ± 2.6 mL/kg/min, $p < 0.0001$) than in control mice (89.08 ± 2.2 mL/kg/min) (**Figure 2.4E**). VCO_2 emission was significantly less in the *Winnie* mice (59.36 ± 2.6 mL/kg/min, $p < 0.001$) than in control mice (82.26 ± 2.5 mL/kg/min) (**Figure 2.4F**). Respiratory quotient showed non-significant differences between the groups (**Figure 2.4G**). Reduced energy metabolism was reflected in the *Winnie* mice displaying significantly lower EE (221 ± 9.3 kcal/kg/BW, $p < 0.001$) than in control mice (309 ± 9.2 kcal/kg/BW) (**Figure 2.4H**). Upon further equations GOX was significantly lower in *Winnie* mice (31.3 ± 2.6 g/kg/BW, $p < 0.001$) than in control mice (51.6 ± 2.7 g/kg/BW) (**Figure 2.4I**). Likewise, LOX in *Winnie* mice (29.9 ± 2.4 g/kg/BW, $p < 0.001$) was significantly lower to control mice (45.1 ± 1.3 g/kg/BW) (**Figure 2.4J**).

Figure 2.3 Energy metabolism of *Winnie* male mice and *Winnie* female mice

There were non-significant differences between 14 weeks of age *Winnie* male mice and *Winnie* female mice for all tests, n=8 mice/group. **(A)** Sum of water (g). **(B)** Number of drinking bouts. **(C)** Sum of food (g). **(D)** Number of food bouts. **(E)** VO_2 mL/kg/min BW. **(F)** VCO_2 mL/kg/min BW. **(G)** Respiratory quotient. **(H)** Energy expenditure kcal/kg BW. **(I)** Glucose oxidation g/kg/BW. **(J)** Lipid oxidation g/kg/BW. Data are expressed as mean $\pm$ SEM. Significance is considered at $p \leq 0.05$. **Abbreviations:** kilo calories per hour, kcal; body weight, BW; kilogram, kg; grams, g; rate of oxygen consumption, VO_2 ; rate of carbon dioxide emission, VCO_2 ; millilitres per kilogram per minute divided by body weight, mL/kg/min BW.

Figure 2.3 Energy metabolism of *Winnie* male mice and *Winnie* female mice

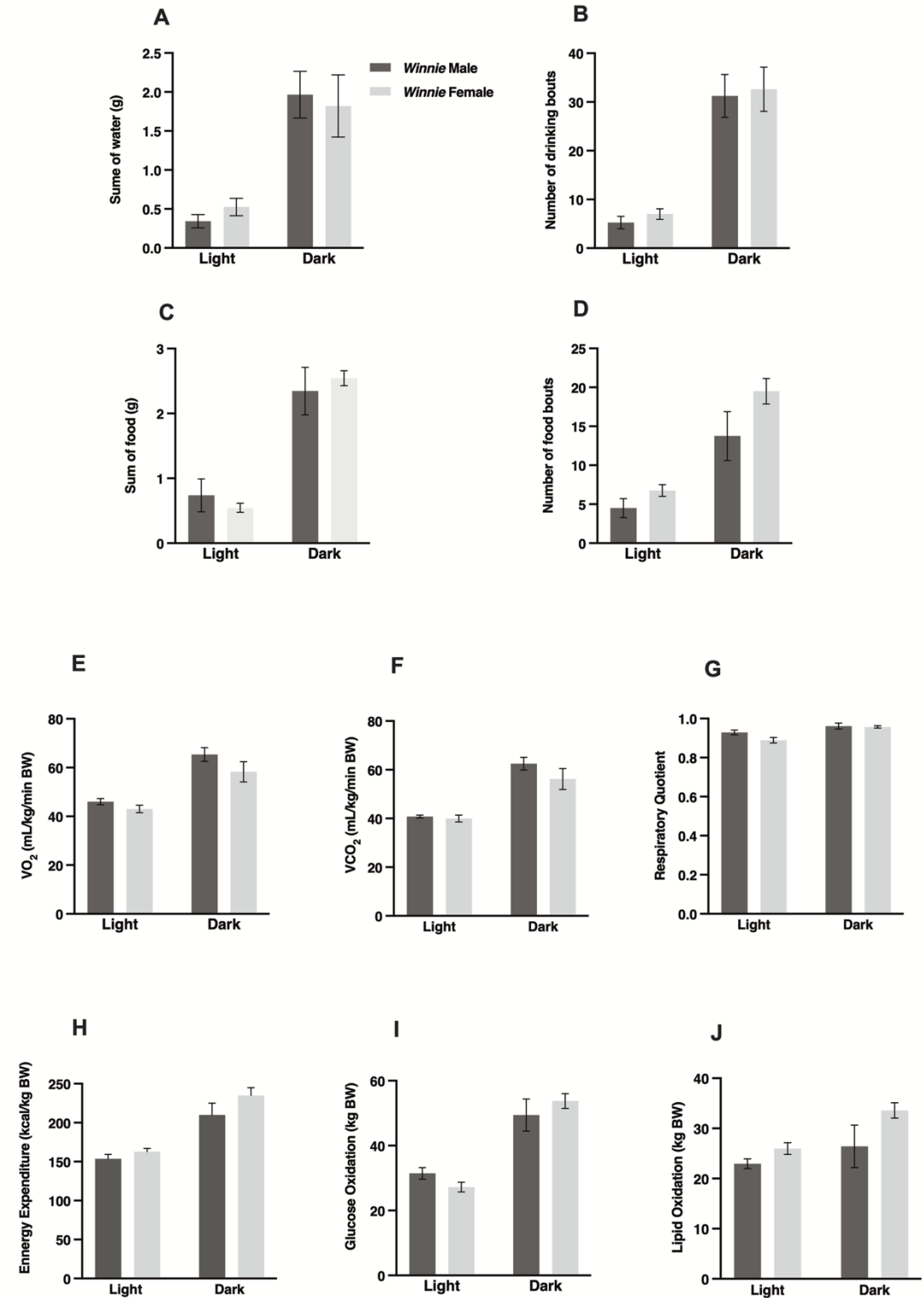
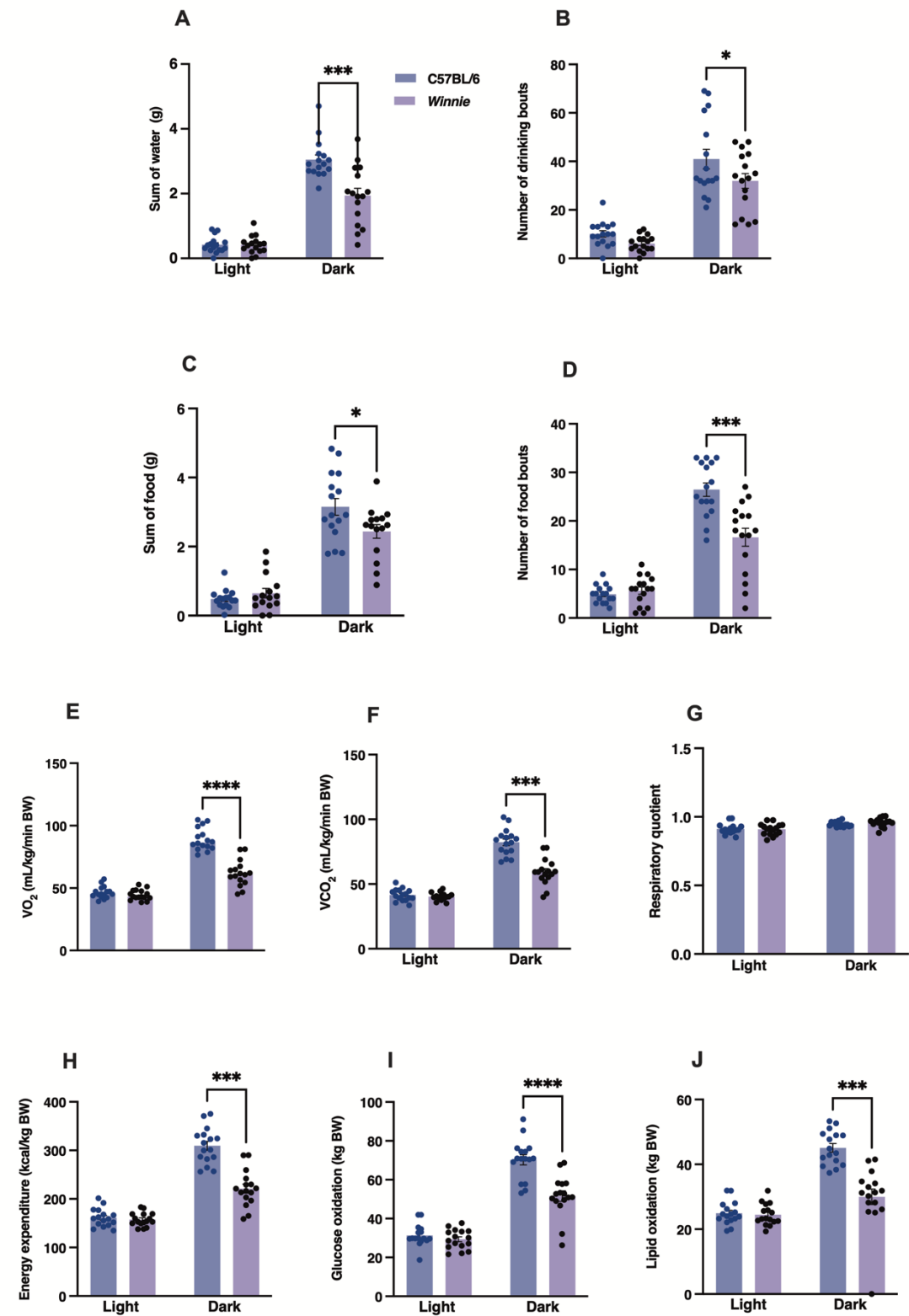


Figure 2.4 Metabolic changes in *Winnie* mice

Winnie mice exhibited significantly lower food consumption and energy metabolism in all tests compared to the control C57BL/6 littermates. **(A)** Sum of water in grams. **(B)** Number of drinking bouts. **(C)** Sum of food in grams. **(D)** Number of food bouts. **(E)** VO_2 mL/kg/min BW. **(F)** VCO_2 mL/kg/min BW. **(G)** Respiratory quotient. **(H)** Energy expenditure kcal/kg BW. **(I)** Glucose oxidation g/kg/BW. **(J)** Lipid oxidation g/kg/BW. Data are expressed as the mean $\pm$ SEM, $*p \leq 0.05$, $***p < 0.001$, $****p < 0.0001$, $n=16$ mice/group. **Abbreviations:** grams, g; kilo calories per hour, kcal; body weight, BW; kilogram, kg; rate of oxygen consumption, VO_2 ; rate of carbon dioxide emission, VCO_2 ; millilitres per kilogram per minute over body weight, mL/kg/min BW.

Figure 2.4 Metabolic changes in *Winnie* mice



2.4.4 6-week *Winnie* mice compared to 14-week old C57BL/6, 14-week old Het *Winnie* and 14-week old *Winnie* mice

In previous experiments in *Winnie* mice, it was determined that progressive chronic intestinal inflammation develops showed signs appearing from 6 weeks of age, with severe colitis apparent by 12-16 weeks of age (Eri et al., 2011). Therefore, in a supplementary experiment, the water and food consumption, and energy metabolism of the male and female 6-week-old *Winnie* mice were compared to age-matched 6 week C57BL/6 male and female mice (n=8 mice/group), which showed non-significant differences in all tests (**Figures 2.5** and **2.6**). To determine any presence of inflammation in 6-week-old *Winnie* mice their water and food consumption, as well as energy metabolism parameters, were assessed and compared to those of 14-week-old C57BL/6 littermates, age-matched Het *Winnie* mice and *Winnie* mice (n=4 females, n=4 males mice/group). All data were analysed within the 12-hour light and dark cycles. Non-significant differences were found for all measured parameters within the light cycle for all group, therefore only the dark cycle is reported (**Figure 2.7**). The 14-week-old *Winnie* mice (1.97 ± 0.3 grams) showed non-significant differences in water consumption compared to 6-week-old *Winnie* mice (2.1 ± 0.3 grams), measured by mass in grams. However, the 6-week-old *Winnie* mice consumed significantly less water (2.1 ± 0.3 grams, $p=0.0008$) compared to the 14-week-old Het *Winnie* mice (3.4 ± 0.4 grams). The 14-week-old C57BL/6 mice (2.8 ± 0.2 grams, $p=0.047$) and the age-matched Het *Winnie* mice (3.4 ± 0.4 grams, $p<0.0001$) drank significantly more water compared to the age-matched *Winnie* mice (1.97 ± 0.3 grams) (**Figure 2.7A**). Drinking bouts were significantly less in 14-week-old *Winnie* mice (49.4 ± 4 bouts, $p<0.0001$) compared to age-matched Het *Winnie* mice (75.5 ± 9 bouts) and 6-week-old *Winnie* mice (49.4 ± 4 $p<0.0001$, $p=0.028$). 14-week-old Het *Winnie* mice attempted more drinking bouts to age-matched C57BL/6 mice (46.4 ± 5.0 bouts, $p<0.0001$) (**Figure 2.7B**).

Food consumption showed non-significant differences between 14-week-old *Winnie* mice, age-matched Het *Winnie* mice and age-matched C57BL/6 littermates. However, food consumption was significantly lower in the 6-week-old *Winnie* mice (1.05 ± 0.5 grams) compared to 14-week-old C57BL/6 mice (3.1 ± 0.4 grams, $p<0.0001$), 14-week-old Het *Winnie* mice (3.0 ± 0.3 grams, $p=0.0003$) and 14-week-old *Winnie* mice (2.5 ± 0.3

grams, $p=0.0098$). The 6-week-old *Winnie* mouse consumed less possibly due to the shorter length of the small intestine and the longer gastric emptying time in young mice compared to adult mice (Yu et al., 2014) (**Figure 2.7C**). The 6-week-old *Winnie* mice attempted less food bouts (20 ± 3 bouts, $p<0.0001$) compared to 14-week-old C57BL/6 mice (46.4 ± 5 bouts) and 14-week-old Het *Winnie* mice (30.4 ± 3 bouts, $p=0.0248$). Although the 14-week-old *Winnie* mice consumed similar food amounts as 14-week-old C57BL/6 mice, the *Winnie* mice made significantly fewer feeding bouts (17.1 ± 1.5 bouts, $p<0.0001$) compared to 14-week-old C57BL/6 mice and age-matched Het *Winnie* mice. The 6-week-old *Winnie* mice (20 ± 3 bouts) and 14-week-old *Winnie* mice (17.1 ± 1.5 bouts) showed non-significant differences in food bouts. The 14-week-old C57BL/6 mice made a significantly higher number of food bouts (46.4 ± 5 bouts, $p < 0.0001$) compared to the other groups (**Figure 2.7D**).

In the indirect calorimetry test, 6-week-old *Winnie* male and female mice exhibited no statistically significant differences in comparison to their 6-week-old C57BL/6 counterparts across all parameters. Subsequently, these 6-week-old *Winnie* mice were contrasted with 14-week-old Het *Winnie* mice, 14-week-old C57BL/6 mice, and 14-week-old *Winnie* mice. The 6-week-old mice demonstrated predominantly non-significant variations when compared to the 14-week-old Het *Winnie* and 14-week-old C57BL/6 groups. An exception was observed in the reduced food consumption by the 6-week-old *Winnie* mice, an outcome that was anticipated. In contrast, the 14-week-old *Winnie* mice displayed diminished values in all assessed metrics compared to the other groups, indicating a decline in food and water intake as well as a reduced energy metabolism. These results truly indicate the progression of the disease in 14-week-old *Winnie* mice.

The results are as follows. Energy metabolism (**Figure 2.8**) shows the VO_2 in 14-week-old *Winnie* mice (63.5 ± 4 kcal/kg/BW) was significantly lower compared to 6-week-old *Winnie* mice (80.8 ± 4 , $p=0.0004$ kcal/kg/BW), 14-week-old C57BL/6 mice (80.8 ± 2.6 kcal/kg/BW, $p=0.0004$), and 14-week-old Het *Winnie* mice (77.6 ± 4 kcal/kg/BW, $p<0.0001$) (**Figure 2.8A**). Likewise, VCO_2 emission in 14-week-old *Winnie* mice (65.5 ± 2.6 kcal/kg/BW) was significantly lower compared to 6-week-old *Winnie* mice (79.3 ± 5.4 kcal/kg/BW, $p=0.0006$), C57BL/6 mice (80.8 ± 2.6 kcal/kg/BW, $p=0.0001$) and

Het *Winnie* mice (77.6 ± 4 kcal/kg/BW, $p=0.0021$) (**Figure 2.8B**). RQ showed non-significant differences between groups (**Figure 2.8C**). EE in 14-week-old *Winnie* mice (209.8 ± 15 kcal/kg/BW, $p<0.0001$) was significantly lower than each control group (6 week *Winnie* mice (298.2 ± 19 kcal/kg/BW), 14-week-old C57BL/6 mice (296 ± 12 kcal/kg/BW) and 14-week-old Het *Winnie* mice (295.5 ± 16 kcal/kg/BW) mice (**Figure 2.8D**). Further calculations established that 14-week-old *Winnie* mice (50.84 ± 2.89 g/kg/BW) displayed lower GOX than 6-week-old *Winnie* mice (72.4 ± 2.3 g/kg/BW, $p<0.0001$), 14-week-old C57BL/6 mice (64.4 ± 3.39 g/kg/BW, $p=0.0137$, 14-week-old Het *Winnie* mice (62.5 ± 5.6 g/kg/BW, $p=0.0422$) (**Figure 2.8E**). Similarly, 14-week-old *Winnie* mice (26.6 ± 1.12 g/kg/BW, $p<0.0001$) showed a significant reduction in LOX compared to 14-week-old C57BL/6 mice (41.95 ± 1.2 g/kg/BW), 14-week-old Het *Winnie* mice (44.5 ± 2.5 g/kg/BW), and 6-week-old *Winnie* mice (47.8 ± 1.8 g/kg/BW, $p=0.02$) which exhibited higher LOX to 14-week-old C57BL/6 mice (41.95 ± 1.23 g/kg/BW) (**Figure 2.8F**).

Figure 2.5 Energy metabolism in 6 week *Winnie* male mice

There were non-significant differences between 6-week-old *Winnie* mice and 6-week-old C57BL/6 mice for all tests. **(A)** Sum of food (g). **(B)** Food bouts. **(C)** Sum of water (g). **(D)** Water bouts. **(E)** VO_2 mL/kg/min BW. **(F)** VCO_2 mL/kg/min BW. **(G)** Energy expenditure kcal/kg BW. **(H)** Glucose oxidation g/kg/BW. **(I)** Lipid oxidation g/kg/BW. Data are expressed as mean $\pm$ SEM. Significance is considered at $p \leq 0.05$, $n=8$ mice/group/cycle. **Abbreviations:** kilo calories per hour, kcal; body weight, BW; kilogram, kg; grams, g; rate of oxygen consumption, VO_2 ; rate of carbon dioxide emission, VCO_2 ; millilitres per kilogram per minute divided by body weight, mL/kg/min BW.

Figure 2.5 Energy metabolism in 6 week *Winnie* male mice

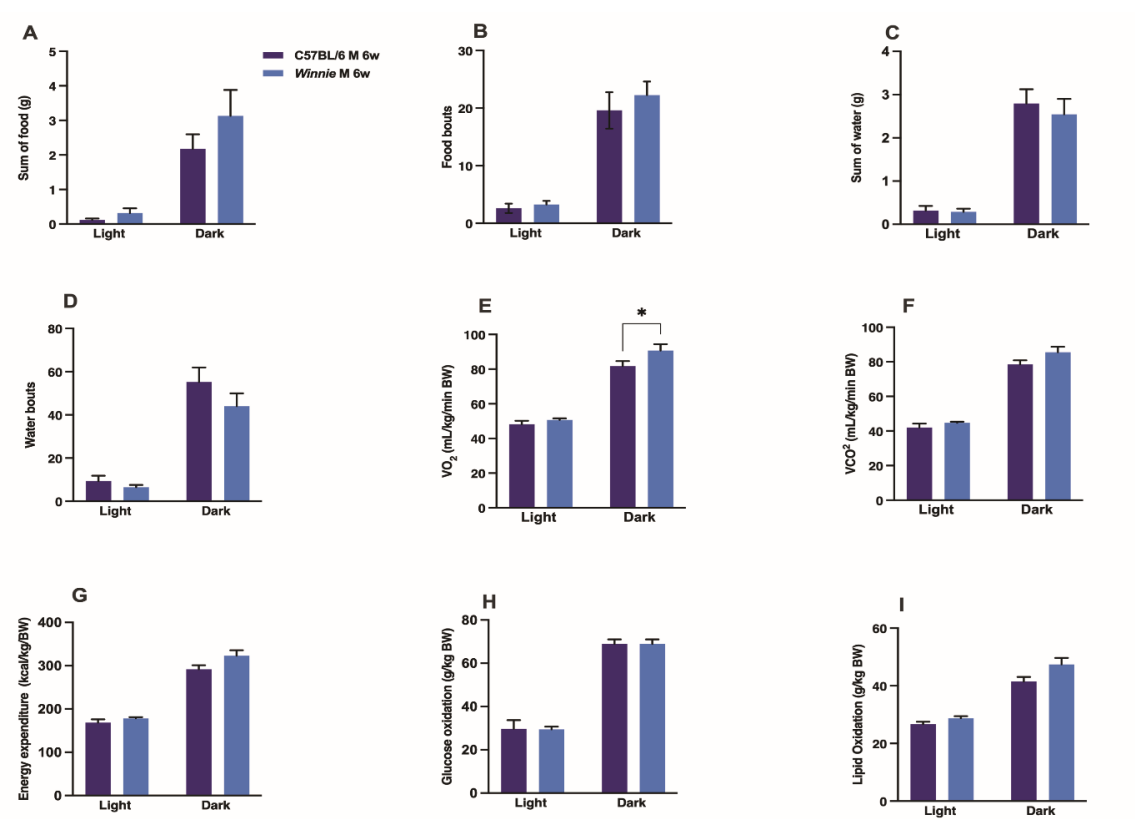


Figure 2.6 Energy metabolism in 6 week *Winnie* female mice

There were non-significant differences between 6-week-old *Winnie* mice and 6-week-old C57BL/6 mice for all tests. **(A)** Sum of food (g). **(B)** Food bouts. **(C)** Sum of water (g). **(D)** Water bouts. **(E)** VO_2 mL/kg/min BW. **(F)** VCO_2 mL/kg/min BW. **(G)** Energy expenditure kcal/kg BW. **(H)** Glucose oxidation g/kg/BW. **(I)** Lipid oxidation g/kg/BW. Data are expressed as mean $\pm$ SEM. Significance is considered at $p \leq 0.05$, $n=8$ mice/group/cycle. **Abbreviations:** kilo calories per hour, kcal; body weight, BW; kilogram, kg; grams, g; rate of oxygen consumption, VO_2 ; rate of carbon dioxide emission, VCO_2 ; millilitres per kilogram per minute divided by body weight, mL/kg/min BW; gram per kilogram divided by bodyweight, g/kg/BW.

Figure 2.6 Energy metabolism in 6 week *Winnie* female mice

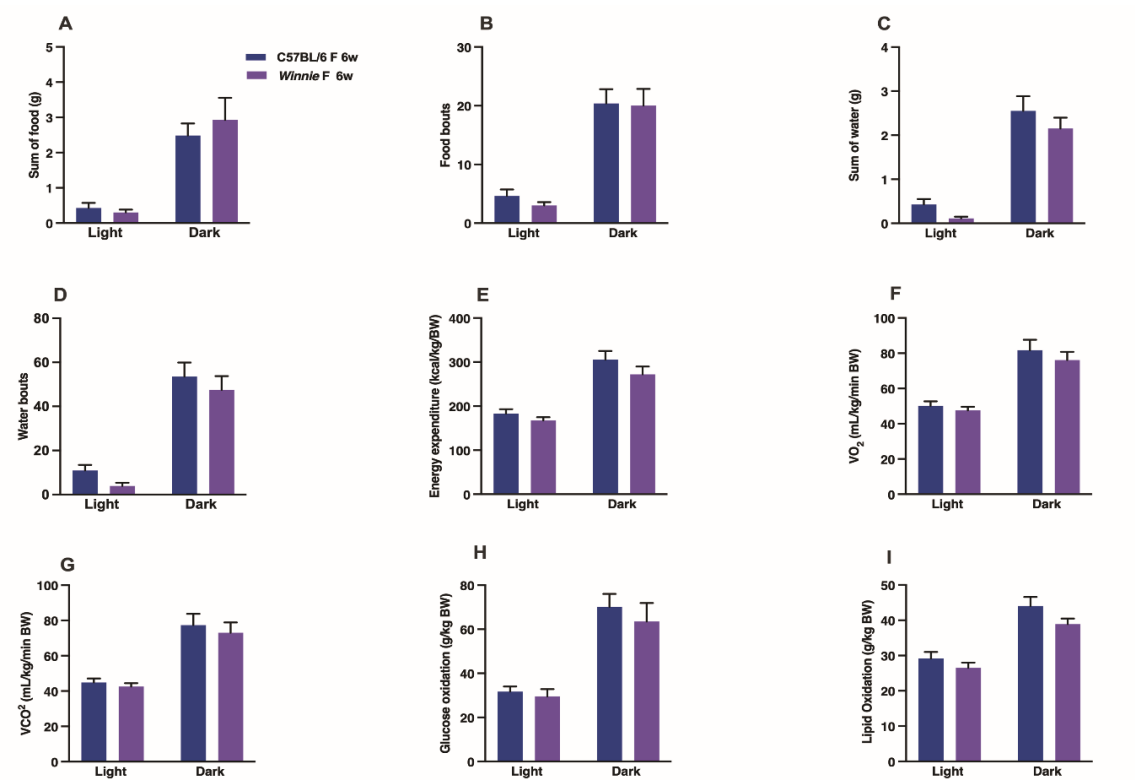


Figure 2.7 Food and water intake of 6 week *Winnie*, 14 week Het *Winnie*, 14 week C57BL/6 and 14 week *Winnie* mice

The *Winnie* mouse showed significant reductions in food and water intake compared to the C57BL/6 mouse. **(A)** Sum of water (g). **(B)** Number of drinking bouts. **(C)** Sum of food (g). **(D)** Number of food bouts. Data are expressed as mean $\pm$ SEM, * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$, n=8 mice/group/cycle. **Abbreviations:** grams, g.

Figure 2.7 Food and water intake of 6 week *Winnie*, 14 week Het *Winnie*, 14 week C57BL/6 and 14 week *Winnie* mice

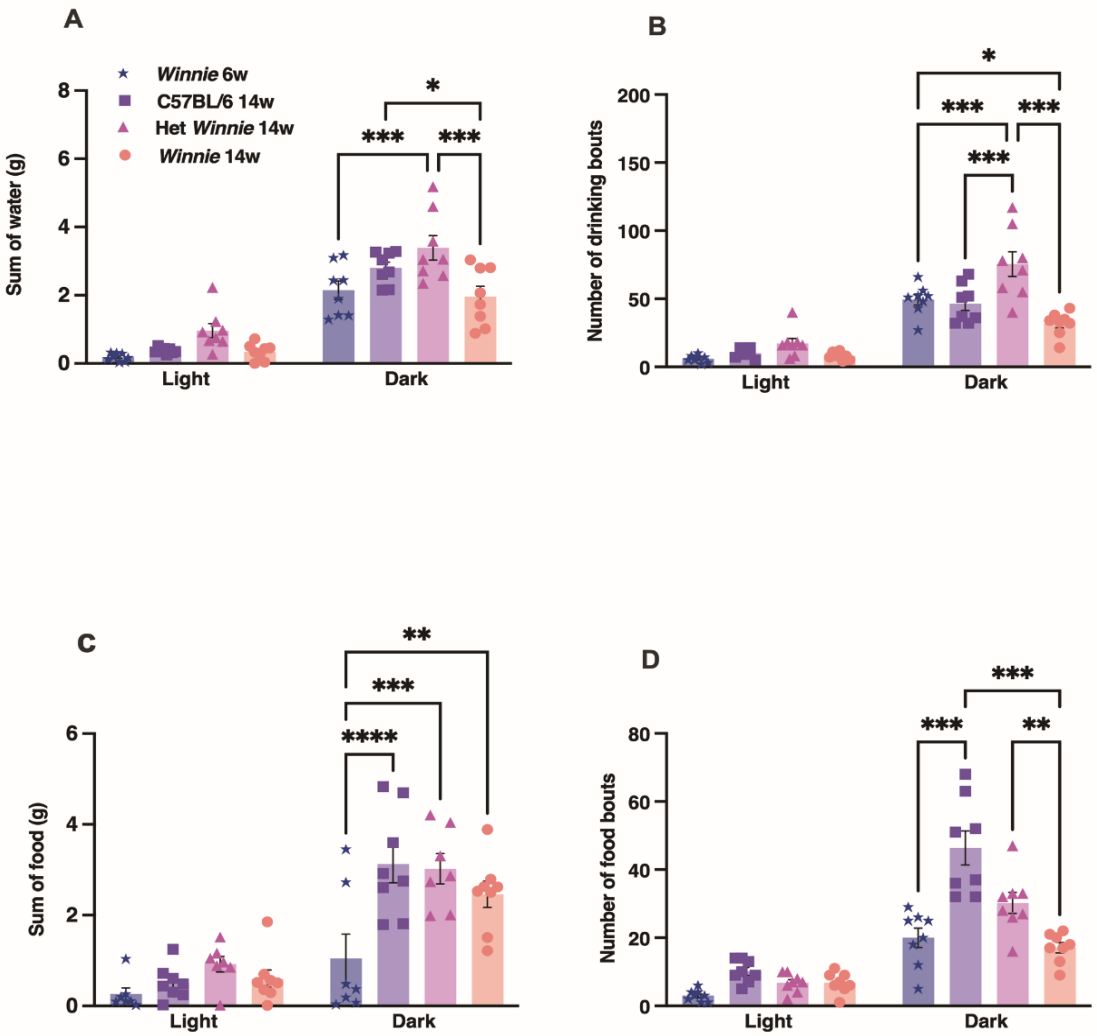
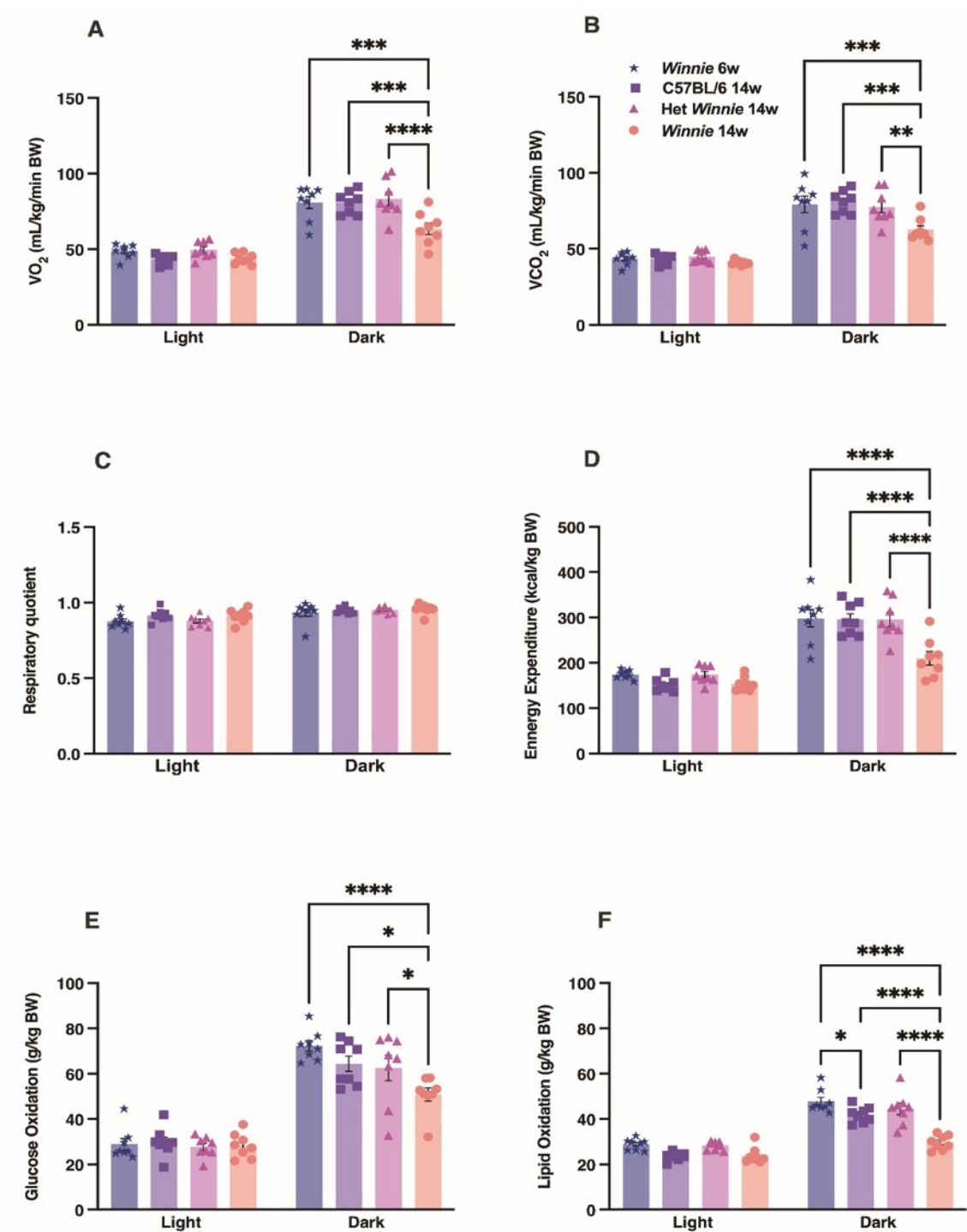


Figure 2.8 Energy metabolism in 6 week *Winnie*, 14 week C57BL/6 mice, 14 week Het *Winnie* and 14 week *Winnie* mice

The *Winnie* mouse showed significant reductions in energy metabolism compared to compared to the other mice groups. **(A)** VO_2 mL/kg/min BW. **(B)** VCO_2 mL/kg/min BW. **(C)** Respiratory quotient. **(D)** Energy expenditure kcal/kg BW. **(E)** Glucose oxidation g/kg/BW. **(F)** Lipid oxidation g/kg/BW. Data are expressed as mean $\pm$ SEM, * $p \leq 0.05$, ** $p = 0.002$, *** $p \leq 0.001$, **** $p \leq 0.0001$, n=8 mice/group/cycle. **Abbreviations:** grams, g; body weight, BW; kilocalories, kcal; kilogram, kg; rate of oxygen consumption, VO_2 ; rate of carbon dioxide emission, VO_2 ; millilitre per kilogram per minute over body weight; mL/kg/min BW; gram per kilogram divided by body weight, g/kg/BW.

Figure 2.8 Energy metabolism in 6 week *Winnie*, 14 week C57BL/6, 14 week Het *Winnie* mice and 14 week *Winnie* mice



2.4.5 Gene expressions of glycolysis/gluconeogenesis in *Winnie* mice colons

Due to the significance of the lower EE, GOX and LOX, further investigation was undertaken into the expression of genes associated with glycolysis/gluconeogenesis and fatty acid β -oxidation via high-throughput RNA-Seq performed on mRNA extracted from the distal colons of 14-week-old *Winnie* mice and age-matched C57BL/6 littermates.

The first step in cellular respiration is glycolysis, which generates adenosine triphosphate (ATP) both aerobically, and anaerobically via ten enzyme-mediated steps (Nakrani et al., 2021). The genes associated with glycolysis/gluconeogenesis included downregulation of 6-phosphofructokinase (*Pfk9*), aldose 1-epimerase (*galM*), glucose 6-phosphate (*G6pc*), enolase (*Eno1*), L-lactate dehydrogenase (*Ldh*), alcohol dehydrogenase (*Adh*), aldehyde dehydrogenase nicotinamide adenine dinucleotide (NAD⁺) (*Aldh2*), and phosphoenolpyruvate carboxykinase (*Pepck*) associated with gluconeogenesis.

Transcriptome analysis included the upregulation of gene expressions, hexokinase (*Hk2*), adenosine diphosphate: D-glucose 6-phospho-transferase (*Adpgk*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), phosphoglycerate kinase (*Pgk1*), phosphoglucomutase (*Pgm*), multiple inositol-polyphosphate phosphatase 1 (*Minpp1*), phosphoglycerate mutase 1 (*Pgam1*), bisphosphoglycerate mutase (*Bpgm*), pyruvate kinase (*Pkm*), aldo-keto reductase family 1 member A1 (*Akr1a1*), and aldehyde dehydrogenase (NAD(P)⁺) (*Aldh3b2*). Results are displayed in the KEGG graph rendered by Pathview (**Figure 2.9A**, **Table 2.2** Glossary of terms for glycolysis/gluconeogenesis-associated gene expressions in *Winnie* mouse brains extracted from <https://www.genome.jp/pathway/map00010> (accessed on 15th March, 2022), Kanehisa and Goto (2000)).

The heat map of glycolysis associated genes demonstrated non-significant differences between *Winnie* male mice and *Winnie* female mice (**Figure 2.9B**). The homology in expression of target genes involved in glycolysis and gluconeogenesis was investigated in both *Winnie* mice and patients with IBD compared to their respective uninflamed control groups. Genes failing the initial cut-off ± 0.5 LogFC are represented as black (non-significant differences). In the case of *Winnie* mice, the results showed a 65%

concordance in the expression of genes associated with glycolysis and gluconeogenesis when compared to UC patients. This indicates a significant overlap in gene expression patterns related to these metabolic pathways between *Winnie* mice and UC patients (**Figure 2.9C**). Additionally, when comparing *Winnie* mice with patients diagnosed with CD, the concordance in the expression of glycolysis and gluconeogenesis associated genes was found to be 53% (**Figure 2.9C**). Although slightly lower than the concordance observed in UC patients, it still suggests a substantial similarity in the expression patterns of these metabolic genes between *Winnie* mice and CD patients.

Figure 2.9 Changes in glycolysis/gluconeogenesis gene expressions in *Winnie* mice

(A) Pathview plot for the glycolysis and gluconeogenesis from the KEGG database comparing *Winnie* mice distal colons to control mice. Rectangular nodes indicate gene expression data by RNA-Seq. Gene expression data (**green** to **red**) presented as significance scores for gene expression analysis between the *Winnie* mice and control mice. **Green** represents downregulated gene expressions comprising electronic commission (EC):2.7.1.11, *Pfk9*; EC:5.1.3.3, *galM*; EC:3.1.3.9, *G6pc*; EC:4.2.1.11, *Eno1*; EC:1.1.1.27, *Ldh*; EC:1.1.1.1, *Adh*; EC:1.2.1.3, *Aldh2*; EC:4.1.1.32, *Pepck*. **Red** represents upregulated gene expressions comprising EC:2.7.1.1, *Hk2*; EC:2.7.1.147, *Adpgk*; EC:1.2.1.12, *Gapdh*; EC:2.7.2.3 *Pgk1*; EC:5.4.2.2, *Pgm*; EC:3.1.3.80, *Minpp1*; EC:5.4.2.1.1, *Pgam1*; EC:5.4.2.4, *Bpgm*; EC:2.7.1.40, *Pkm*; EC:1.1.1.2, *Akr1a1*; EC:1.2.1.5, *Aldh3b2*.

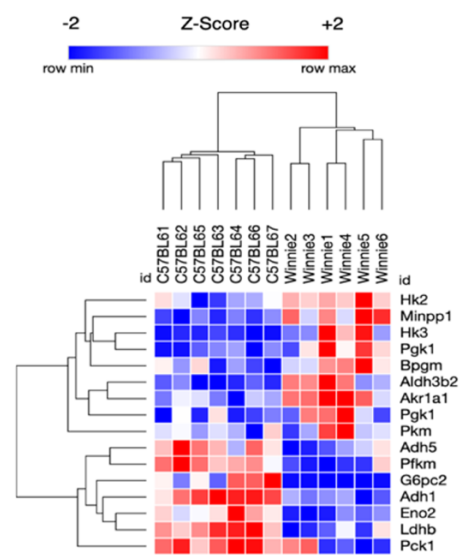
(B) Heat map representation of upregulated genes (**red**), downregulated genes (**blue**) and unchanged genes (**black**) associated with colonic inflammation determined by RNA-Seq. Target genes of glycolysis/gluconeogenesis in the *Winnie* mouse were identified.

(C) The homology in expression of these genes was determined in *Winnie* mice and IBD patients compared to controls. Target genes of glycolysis/gluconeogenesis in the *Winnie* mouse determined by RNA-Seq were identified. Heat map representation of upregulated genes (**red**), downregulated genes (**blue**). Genes below the initial cut off $\pm 0.5\log FC$ are represented as **black**.

Figure 2.9 Changes in glycolysis/gluconeogenesis gene expressions in *Winnie* mice



B



C



Table 2.2 Glossary of terms of glycolysis/gluconeogenesis-associated genes

Upregulated change			
Downregulated change			
Unchanged			
Reaction	Substrate	Product	Comment
Step 1: Phosphorylation of glucose			
Hexokinase (<i>Hk2</i>) EC:2.7.1.1			
ATP:D-glucose 6-phosphotransferase. ATP + D-Glucose $\rightleftharpoons$ ADP + D-Glucose 6-phosphate	ATP, D-glucose	ADP, D-glucose 6-phosphate	<i>Hk</i> catalyses the phosphorylation of D-glucose into glucose 6-phosphate (G6P) for the pentose phosphate shunt and glycogen synthesis. Activator: AMP/ADP Inhibitor: G6P accumulation
ADP:D-glucose 6-phospho-transferase (<i>Adpgk</i>) EC:2.7.1.147			
ADP+D glucose $\rightleftharpoons$ AMP+D-glucose 6-phosphate	ADP, D-glucose, D-glucos-amine	AMP, D-glucose 6-phosphate, D-glucosamine 6-phosphate	<i>Adpgk</i> catalyses the ADP-dependent phosphorylation of glucose to G6P. Co factor: magnesium
Step 2: Isomerisation/Conversion of Glucose-6-phosphate to d-F 6-phosphate (F6P)			
Glycose-6-phosphate isomerase (<i>Pgi</i>) EC:5.3.1.9			
α -D-Glucose 6-phosphate = β -D-F 6-phosphate	α -D-glucose 6-phosphate	β -D-fructo-furanose 6-phosphate	<i>Pgi</i> catalyses the reversible isomerisation of G6P to D-F 6-phosphate (F6P). Activator: AMP, F 2,6 phosphate
Step 3: Phosphorylation of F 6-phosphate consumes -1 ATP			
6-phosphofructokinase (<i>Pfk</i>) EC:2.7.1.11			
ATP + β -D-F 6-phosphate = ADP + β -D-F 1,6-bisphosphate + H ⁺	ATP; β -D-fructo-furanose 6-phosphate	ADP; β -D-fructo-furanose 1,6-bisphosphate	<i>Pfk</i> catalyses the phosphorylation of D-F6-phosphate to F1,6-bisphosphate by ATP.

			Activator: AMP, F2,6 biphosphate Inhibitor: ATP, Citrate
Step 4: Aldolase : Cleavage of F1, 6-biphosphate			
Aldose 1-epimerase (<i>galM</i>) EC 5.1.3.3			
α -D-glucose = β – D-glucose	α -D-glucose	β -D-glucose	<i>galM</i> catalyses the cleavage of F1,6 biphosphate into two isomers, three-carbon molecules G3P and DHAP.
Step 5: Isomerisation of dihydroxyacetone phosphate			
Triose phosphate isomerase (<i>Tpi</i>) EC:5.3.1.1			
D-glyceraldehyde 3-phosphate = Glycerone phosphate	D-glyceraldehyde 3-phosphate	Glycerone phosphate	<i>Tpi</i> catalyses the conversion of the ketose of dihydroxyacetone phosphate into an aldose of glyceraldehyde 3-phosphate, via an intramolecular redox reaction.
Step 6: Glyceraldehyde 3-phosphate – produces +2 NADH			
Glyceraldehyde 3-phosphate dehydrogenase, (<i>Gapdh</i>) EC:1.2.1.12			
D-glyceraldehyde 3-phosphate + NAD ⁺ + phosphate = 3- phosphor-D-glycerophosphate + NADH + H ⁺	D-glycer-aldehyde 3-phosphate, phosphate; NAD ⁺	3-phospho-D-glyceroyl phosphate, NADH; H ⁺	<i>Gapdh</i> catalyses D-G3P into 3-phospho-D-glyceroyl phosphate. Inhibitor: Fumarate
Step 7: Transfer from 1,3-bisphosphoglycerate to ADP – produces +2 ATP			
Phosphoglycerate kinase, (<i>Pgk</i>) EC:2.7.2.3			
(2R)-ATP + 3-phospho-D-glycerate = (2R) ADP + 3-phospho-D-glyceroyl phosphate	ATP, 3-phospho-D-glycerate	ADP; 3-phospho-D-glyceroyl phosphate. ATP:3-phospho-D-glycerate 1-phospho-transferase.	<i>Pgk</i> catalyses the interconversion of 1,3 diphosphoglycerate and 3-PGA.
Step 8: Isomerisation of 3-phosphoglycerate			
Multiple inositol-polyphosphate phosphatase 1 (<i>Minpp1</i>) EC:3.1.3.80			

2,3-bisphospho-D-glycerate + H ₂ O = 2-phospho-D-glycerate + phosphate	2,3-bisphospho-D-glycerate, H ₂ O	2-phospho-D-glycerate, phosphate	Rapoport-Luebering shunt. <i>Minpp1</i> hydrolyses the abundant metabolites inositol pentakisphosphate and inositol hexakisphosphate.
Phosphoglucomutase (<i>Pgm</i>) EC:5.4.2.2			
α-D-glucose 1-phosphate = D-glucose 6-phosphate	α-D-glucose 1, phosphate	D-glucose 6-phosphate	<i>Pgm</i> facilitates the interconversion of G1P and G6P. Co factor: Magnesium
Phosphoglycerate mutase (<i>Pgam</i>) EC:5.4.2.11			
2-phospho-D-glycerate = 3-phospho-D-glycerate	2-phospho-D-glycerate	3-phospho-D-glycerate	Activated by 2,3-bisphospho-D-glycerate by transferring a phosphate to histidine. This phosphate can be transferred to the free OH of 2-phospho-D-glycerate, followed by transfer of the phosphate already on the phosphoglycerate back to the histidine
Bisphosphoglycerate mutase (<i>Bpgm</i>) EC:5.4.2.4			
3-phospho-D-glyceroyl phosphate = 2,3-bisphospho-D-glycerate	3-phospho-D-glyceroyl phosphate	2,3-bisphospho-D-glycerate	<i>Bpgm</i> maintains the concentration of 2,3-diphospho-D-glycerate. Cofactor: Magnesium Inhibitor: Citrate
Step 9: Dehydration of 2-phosphoglycerate to PEP			
Enolase (<i>Eno</i>) EC:4.2.1.11			
2-phospho-D-glycerate = PEP + H ₂ O	2-phospho-D-glycerate	Phosphoenol-pyruvate, H ₂ O	<i>Eno</i> catalyses 2-phosphoglycerate to PEP.
Phosphoenol-pyruvate carboxykinase (<i>Pepck</i>) EC:4.1.1.32			
GTP + oxaloacetate = GDP + PEP + CO ₂	Oxaloacetate GTP	GDP, PEP, CO ₂	<i>Pepck</i> is critical for initiating gluconeogenesis, in which oxaloacetate is converted to PEP.

Step 10: Transfer of phosphate from PEP			
Pyruvate kinase (<i>Pkm</i>) EC:2.7.1.40			
ADP + Phosphoenol-pyruvate $\rightleftharpoons$ ATP + pyruvate	ATP, pyruvate, ATP: pyruvate 2-O-phospho-transferase	ADP, Phosphoenol-pyruvate	<i>Pkm</i> catalyses the conversion of PEP and ADP into pyruvate and ATP. Activator: AMP/ADP, F-1,6-BP. Inhibitor: ATP, acetyl CoA, alanine.
Lactate Fermentation: Anaerobic Metabolism			
L-lactate dehydrogenase, (<i>Ldh</i>) EC:1.1.1.27			
Pyruvate + NADH + H ⁺ = (S)-lactate + NAD ⁺	(S)-lactate, NAD ⁺	Pyruvate, NADH, H ⁺	<i>Ldh</i> catalyses the interconversion of pyruvate to lactate, reduces NADH to NAD ⁺ , increases the speed of reaction.
Alcohol Fermentation			
Aldo-keto reductase family 1 (<i>Akr1a1</i>) EC:1.1.1.2			
Ethanol + NADP ⁺ = an Aldehyde + NADPH + H ⁺	Ethanol, NADP ⁺	Aldehyde, NADPH, H ⁺	<i>Akr1a1</i> catalyses the oxidation and reduction of alcohols and aldehydes.
Aldehyde dehydrogenase (NAD(P)⁺) (<i>Aldh3a1</i>) EC:1.2.1.5			
Acetaldehyde + NAD ⁺ + H ₂ O = Acetate + NADH + H ⁺	Aldehyde, NAD ⁺ , NADP ⁺ , H ₂ O	Carboxylate, NADH, NADPH, H ⁺	<i>Aldh3a1</i> encodes for detoxification via the NAD(P) ⁺ -dependent oxidation of numerous endogenous and exogenous aldehyde substrates.
Alcohol dehydrogenase (<i>Adh</i>) EC:1.1.1.1			
1). Ethanol + NAD ⁺ + H ₂ O = Acetaldehyde + NADH + H ⁺	Ethanol, NAD ⁺ , secondary alcohol.	Aldehyde, NADH, H ⁺ , ketone.	<i>Adh</i> detoxifies alcohol-derived acetaldehyde; metabolises corticosteroids, biogenic amines, neurotransmitters, and lipid peroxidation.
Aldehyde dehydrogenase (NAD⁺) (<i>Aldh2</i>) EC:1.2.1.3			

Aldehyde + NAD ⁺ + H ₂ O = Acetate + NADH + H ⁺	Aldehyde; NAD ⁺ , NADP ⁺ , H ₂ O	NADH, NADPH, H ⁺	<i>Aldh</i> encodes enzymes that are required for detoxification via the NAD(P) ⁺ -dependent oxidation of numerous aldehyde substrates.

STRING identified biological pathways and the enrichment terms from GOBP, GOMF and GOCC from the *Winnie* mice DEGs. The lists of the top 10 up- and downregulated are listed in **Table 2.3 downregulated DEGs** and **Table 2.4 upregulated DEGs**. Downregulated DEGs GOBP terms included pyruvate metabolic process, carbohydrate metabolic process, organic acid metabolic process, carbohydrate catabolic process, hexose catabolic process, NAD metabolic process, monocarboxylic acid metabolic process, oxidation-reduction process, lactate biosynthetic process from pyruvate, and nicotinamide adenine dinucleotide + hydrogen (NADH) metabolic process. Downregulated DEGs GOMF terms included L-lactate dehydrogenase activity, oxidoreductase activity acting on the CH-OH group of donors NAD or NADP as acceptor, NAD binding, oxidoreductase activity, identical protein binding.

Upregulated DEGs GOBP terms included monosaccharide metabolic process, carbohydrate metabolic process, hexose metabolic process, carbohydrate catabolic process, carbohydrate derivative metabolic process, organophosphate metabolic process, fructose 6-phosphate metabolic process and monosaccharide catabolic process. Upregulated DEGs GOMF terms included glucokinase activity, intramolecular transferase activity, phosphotransferases, mannokinase activity, magnesium ion binding, phosphotransferase activity alcohol group as acceptor, kinase activity.

Table 2.3 Enrichment terms of downregulated glycolysis /gluconeogenesis genes

Category	Term ID	Term description	Observed gene count	Back ground gene count	Strength	FDR
GOBP	GO:0006090	Pyruvate metabolic process	6	63	2.32	2.01E-09
GOBP	GO:0005975	Carbohydrate metabolic process	8	398	1.65	3.46E-09
GOBP	GO:0016052	Carbohydrate catabolic process	5	85	2.11	3.01E-07
GOBP	GO:0019320	Hexose catabolic process	4	21	2.62	3.01E-07
GOBP	GO:0019674	NAD metabolic process	4	20	2.64	3.01E-07
GOBP	GO:0032787	Monocarboxylic acid metabolic process	7	461	1.52	3.01E-07
GOBP	GO:0055114	Oxidation-reduction process	8	917	1.28	3.84E-07
GOBP	GO:0019244	Lactate biosynthetic process from pyruvate	3	4	3.22	2.02E-06
GOBP	GO:0006734	NADH metabolic process	3	27	2.39	0.00014
GOBP	GO:0046034	ATP metabolic process	4	172	1.71	0.00038
GOMF	GO:0004459	L-lactate dehydrogenase activity	3	6	3.04	1.82E-05
GOMF	GO:0016616	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	4	123	1.86	0.00023
GOMF	GO:0051287	NAD binding	3	62	2.03	0.0016
GOMF	GO:0016491	Oxidoreductase activity	5	710	1.19	0.0036
GOMF	GO:0042802	Identical protein binding	6	1962	0.83	0.031

Table 2.4 Enrichment terms of upregulated glycolysis/gluconeogenesis genes

Category	ID	Term description	Observed gene count	Back-ground gene count	Strength	FDR
GOBP	GO:0005996	Monosaccharide metabolic process	10	153	2.01	4.61E-15
GOBP	GO:0005975	Carbohydrate metabolic process	11	398	1.64	1.32E-13
GOBP	GO:0019318	Hexose metabolic process	9	128	2.04	1.32E-13
GOBP	GO:0016052	Carbohydrate catabolic process	8	85	2.17	6.95E-13
GOBP	GO:1901135	Carbohydrate derivative metabolic process	9	814	1.24	1.29E-07
GOBP	GO:0019637	Organophosphate metabolic process	8	724	1.24	1.58E-06
GOBP	GO:0006002	Fructose 6-phosphate metabolic process	3	14	2.53	5.07E-05
GOBP	GO:0046365	Monosaccharide catabolic process	2	26	2.08	0.0376
GOMF	GO:0004340	Glucokinase activity	3	5	2.98	3.67E-05
GOMF	GO:0016868	Intramolecular transferase activity, phosphotransferases	3	9	2.72	3.67E-05
GOMF	GO:0019158	Mannokinase activity	3	5	2.98	3.67E-05
GOMF	GO:0000287	Magnesium ion binding	4	208	1.48	0.0023
GOMF	GO:0016773	Phosphotransferase activity, alcohol group as acceptor	5	659	1.08	0.0097
GOMF	GO:0016301	Kinase activity	5	714	1.04	0.0131

2.4.6 Gene expressions of fatty acid β -oxidation in *Winnie* mice colons

The fatty acid degradation (β -oxidation) pathway was assessed by high-throughput RNA-Seq performed on mRNA extracted from the distal colon of 14-week-old *Winnie* mice versus age-matched C57BL/6 littermates.

The transcriptome analysis included downregulated gene expressions: carnitine O-palmitoyl-transferase (*Cpt1*), medium-chain acyl-coenzyme A (acyl-CoA) dehydrogenase (*Acadm*), very long chain acyl-CoA dehydrogenase (*Acadvl*), hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit β (*Hadhb*), short/branched chain acyl-CoA dehydrogenase (*Acadsb*), glutaryl-CoA dehydrogenase (*Gcdh*), enoyl-CoA delta isomerase 1 (*Eci1*), alcohol dehydrogenase (*Adh1*), aldehyde dehydrogenase (NAD⁺) (*Aldh2*), and cytochrome P450, family 4 subfamily a polypeptide 10 (*Cyp4a10*).

Upregulated gene expression comprises acyl-CoA synthetase (*Acsf*), long-chain acyl-CoA dehydrogenase (*Lcad*, *Acadl*), and acetyl-CoA acetyltransferase 1 (*Acat1*). Results are displayed in the KEGG graph rendered by Pathview (**Figure 2.10A**, **Table 2.5**). Glossary of terms for fatty acid β -oxidation-associated gene expressions in *Winnie* mouse brains are extracted from <https://www.genome.jp/pathway/map00071>, (accessed on April 4, 2022), Kanehisa and Goto (2000)).

STRING identified biological pathways and the enrichment terms from GOBP, GOMF and GOCC from the *Winnie* mice distal colon downregulated and upregulated DEGs (**Table 2.6** and **Table 2.7**).

Figure 2.10 Changes of fatty acid β -oxidation gene expressions in *Winnie* mice

(A) Pathview plot for the FAO from the KEGG database illustrates rectangular nodes that indicate gene expression data by RNA-Seq. Gene expression data **green** to **red** presented as significance scores for gene expression analysis between the *Winnie* mice and C57BL/6 littermates.

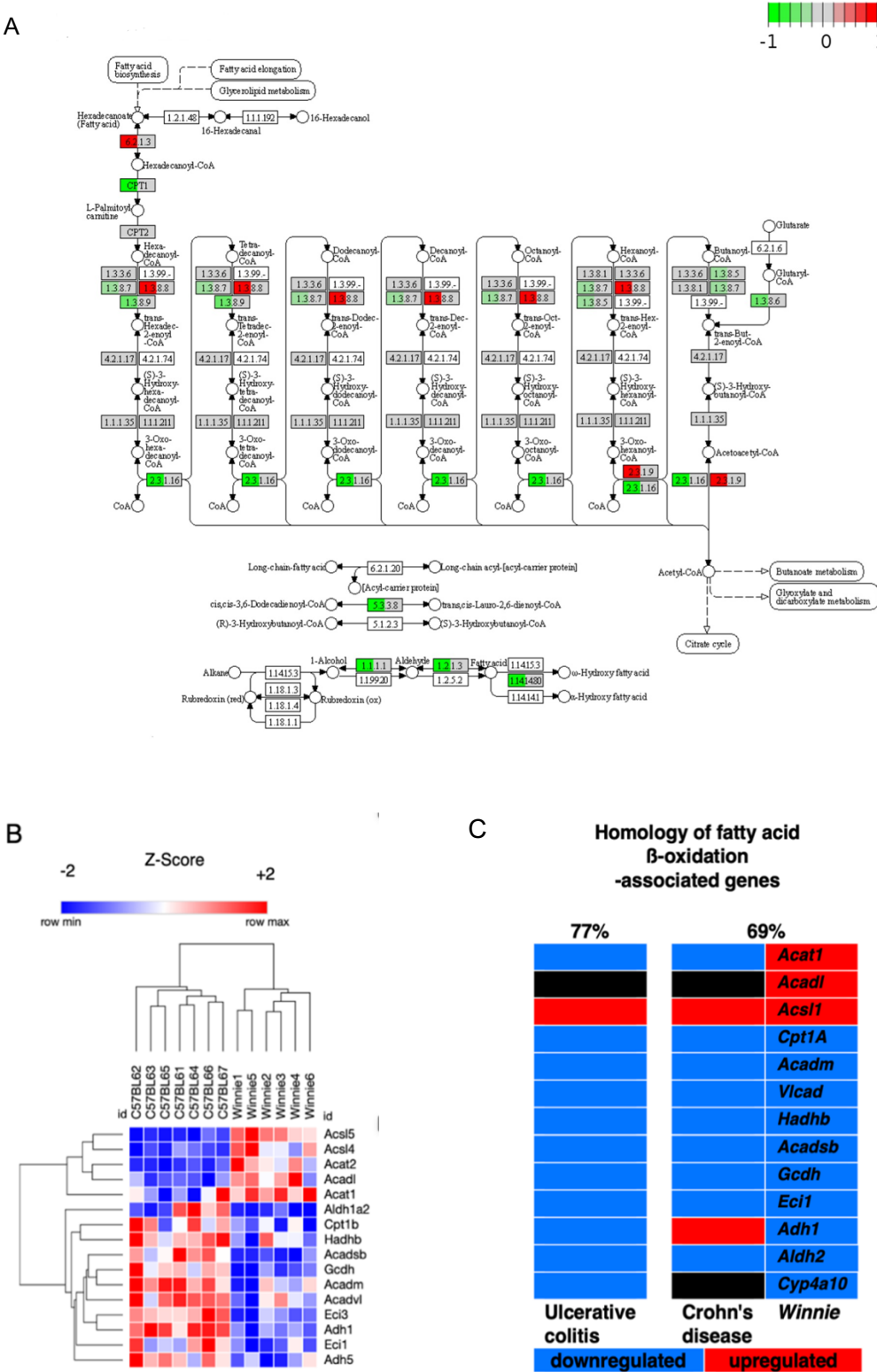
Green downregulated gene expressions comprising EC:CPT1, *Cpt1*; EC:1.3.8.7, *Acadm*; EC:1.3.8.9, *Acadvl*; EC:1.3.8.5, *Acadsb*; EC:1.3.8.6, *Gcdh*; EC:2.3.1.16, *Hadhb*; EC 5.3.3.8, *Eci*; EC:1.1.1.1, *Aldh2*; EC 1.2.1.3, *Adh1*; EC:1.14.14.80, *Cyp4a10*.

Red represents upregulated gene expressions of EC:6.2.1.3, *Acsf*; EC:1.3.8.8, *Acadl*; EC:2.3.1.9, *Acat1*.

(B) Heat map representation of upregulated genes (**red**), downregulated genes (**blue**) and unchanged genes (**black**) associated with colonic inflammation determined by RNA-Seq. Target genes of FAO in the *Winnie* mouse were identified.

(C) The homology in expression of these genes was determined in *Winnie* mice and IBD patients compared to their respective uninflamed controls. Heat map representation of upregulated genes (**red**), downregulated genes (**blue**) and unchanged genes (**black**) associated with colonic inflammation determined by RNA-Seq. Target genes of FAO in the *Winnie* mouse were identified. Genes failing the initial cut off $\pm 0.5\log FC$ are represented as black (non-significant differences).

Figure 2.10 Changes of fatty acid β -oxidation gene expressions in *Winnie* mice



Downregulated DEGs GOBP terms included fatty acid metabolic process, fatty acid catabolic process, oxidation-reduction process, FAO using acyl-CoA dehydrogenase, carnitine metabolic process, cellular detoxification of aldehyde, ethanol catabolic process, medium-chain fatty acid metabolic process. Downregulated DEGs GOMF terms included acyl-CoA dehydrogenase activity, carnitine O-palmitoyltransferase activity, oxidoreductase activity, flavin adenine dinucleotide binding, fatty-acyl-CoA binding, transferase activity transferring acyl groups other than amino-acyl groups, aldehyde dehydrogenase (NAD⁺) activity. Downregulated DEGs GOCC terms included mitochondrion, organelle membrane, intracellular membrane-bounded organelle, cytoplasm, mitochondrial outer membrane, mitochondrial nucleoid.

Upregulated DEGs GOBP terms included fatty-acyl-CoA metabolic process, fatty acid metabolic process, long-chain fatty acid metabolic process, fatty-acyl-CoA biosynthetic process, regulation of lipid biosynthetic process, long-chain fatty-acyl-CoA metabolic process, fatty acid transport, positive regulation of lipid biosynthetic process. Upregulated DEGs GOMF terms included acyl-CoA ligase activity, long-chain fatty acid-CoA ligase activity, arachidonate-CoA ligase activity, decanoate-CoA ligase activity. Upregulated DEGs GOCC terms included mitochondrial membrane.

In the homology of genes in *Winnie* mice and IBD patients associated with FAO, the results exhibited a high concordance with IBD patients. Specifically, there was a 77% concordance in gene expression between *Winnie* mice and UC patients (**Figure 2.10C**). When comparing the expression of these genes between *Winnie* mice and patients diagnosed with CD, the concordance was found to be 69% (**Figure 2.10C**). The similarities in gene expression patterns further support the utility of the *Winnie* mouse in investigating the underlying molecular mechanisms associated with glycolysis, gluconeogenesis and fatty acid β -oxidation in the context of IBD.

Table 2.5 Glossary of terms for fatty acid β -oxidation-associated genes

Upregulated – change

Downregulated – change

Unchanged

Reaction	Substrate	Product	Comment
Activation of fatty-acid			
Acyl CoA Synthase (<i>Acsf</i>) EC:6.2.1.3			
ATP + a long-chain fatty-acid + CoA = AMP + diphosphate + an acyl-CoA.	ATP, long-chain fatty-acid, CoA.	AMP, diphosphate, acyl-CoA.	<i>ACSL</i> diffuses into the mitochondria membrane and is then converted by carnitine acyltransferase-1 to fatty-acid chain.
Mitochondrial carnitine cycle			
Carnitine O-palmitoyl-transferase (<i>Cpt1</i>) EC:CPT			
Palmitoyl-CoA + L-carnitine = CoA + L-palmitoyl carnitine	Palmitoyl-CoA, L-carnitine	CoA, L-palmitoyl carnitine	CPT1 adds on carnitine and rids CoA, and then becomes palmitoyl.
Step 1: Dehydrogenation Oxidation of FAD			
Medium-chain acyl-CoA dehydrogenase (<i>Acadm</i>) EC:1.3.8.7			
A medium-chain acyl-CoA + electron-transfer flavoprotein = a medium-chain trans-2,3-dehydroacyl-CoA + reduced electron-transfer flavoprotein	Medium-chain acyl-CoA, electron-transfer flavoprotein	Medium-chain trans-2,3-dehydroacyl-CoA, reduced electron-transfer flavoprotein	<i>Acadm</i> catalyses the initial step in each cycle of fatty-acid β -oxidation. <i>ACAD</i> transfers electrons to FAD and reduces FAD to form FADH ₂ .
Long-chain-acyl-CoA dehydrogenase (<i>Acadl</i>) EC:1.3.8.8			

A long-chain acyl-CoA + electron-transfer flavoprotein $\rightleftharpoons$ a long-chain trans-2,3-dehydroacyl-CoA + reduced electron-transfer flavoprotein.	Long-chain acyl-CoA, electron-transfer flavoprotein	Long-chain trans-2,3-dehydroacyl-CoA, reduced electron-transfer flavoprotein	<i>Acadl</i> must use carnitine transport to enter the mitochondria.
Step 1. Very-long-chain acyl-CoA dehydrogenase (<i>Acadvl</i>) EC:1.3.8.9			
A very-long-chain acyl-CoA + electron-transfer flavoprotein = a very-long-chain trans-2,3-dehydroacyl-CoA + reduced electron-transfer flavoprotein.	A very-long-chain acyl-CoA, electron-transfer flavoprotein.	A very-long-chain trans-2,3-dehydroacyl-CoA, reduced electron-transfer flavoprotein.	<i>Acadvl</i> is an enzyme that catalyses the first step in fatty-acids β -oxidation.
Short-chain acyl-CoA dehydrogenase (<i>Acadsb</i>) EC:1.3.8.5			
Hexanoyl-CoA + FAD $\rightleftharpoons$ trans-hex-2-enoyl-CoA + FADH ₂	2-methylbutanoyl-CoA, electron-transfer flavoprotein.	(E)-2-methylbut-2-enoyl-CoA, reduced electron-transfer flavoprotein, H ⁺	<i>Acadsb</i> catalyses short-chain 2-methyl fatty-acids and isoleucine degradation.
Glutaryl-CoA dehydrogenase (<i>Gcdh</i>) EC:1.3.8.6			
Glutaryl-CoA + FAD $\rightleftharpoons$ FADH ₂ + crotonoyl-CoA + CO ₂ + reduced electron-transfer flavoprotein	Glutaryl-CoA; electron-transfer flavoprotein, (E)-glutaconyl-CoA	Crotonyl-CoA; CO ₂ ; reduced electron-transfer flavoprotein, (E)-glutaconyl-CoA.	<i>Gcdh</i> catalyses the oxidation of glutaryl-CoA to glutaconyl-CoA and the decarboxylation of the latter to crotonyl-CoA in the mitochondria.
Step 2: Hydration catalysed by enoyl-CoA hydratase			
Step 3: Oxidation by NAD⁺ inside the mitochondria. Dehydrogenation catalysed by 3-hydroxyacyl-CoA dehydrogenase, which generates NADH.			
Step 4: Thiolitic Cleavage of CoA 3			
Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit β (<i>Hadhb</i>) EC:2.3.1.16			

Acyl-CoA + acetyl-CoA = CoA + 3-oxoacyl-CoA	Acyl-CoA, acetyl-CoA, [acetyl-CoA C-acyltransferase]-S-acyl-L-cysteine; [acetyl-CoA C-acyltransferase]-L-cysteine.	CoA, 3-oxoacyl-CoA, [acetyl-CoA C-acyltransferase]-L-cysteine; [acetyl-CoA C-acyltransferase]-S-acyl-L-cysteine.	<i>Hadhb</i> acts on 3-oxoacyl-CoAs to produce acetyl-CoA and an acyl-CoA shortened by two carbon atoms.
Acetyl-CoA acetyltransferase 1 (<i>Acat</i>) EC:2.3.1.9			
1). Acetyl-CoA $\rightleftharpoons$ CoA + acetoacetyl-CoA 2). Acetyl-CoA + butanoyl-CoA $\rightleftharpoons$ CoA + 3-oxohexanoyl-CoA	Acetyl-CoA; [acetyl-CoA C-acyltransferase]-L-cysteine; [acetyl-CoA C-acyltransferase]-S-acyl-L-cysteine	CoA; acetoacetyl-CoA; [acetyl-CoA C-acyltransferase]-S-acyl-L-cysteine; [acetyl-CoA C-acyltransferase]-L-cysteine	<i>Acat</i> catalyses the Claisen condensation of an acetyl-CoA and an acyl-CoA leading to the formation of an acyl-CoA.
Δ^3-Δ^2-enoyl-CoA isomerases, dodecenoyl-CoA isomerase (<i>EciI</i>) EC 5.3.3.8			
3-cis-Dodecenoyl-CoA $\rightleftharpoons$ 2-trans-dodecenoyl-CoA	(3E)-alk-3-enoyl-CoA	(2E)-alk-2-enoyl-CoA	<i>EciI</i> , isomerises the bond to a trans bond at position C2. The enzyme can catalyse the isomerisation of 3-acetylenic fatty acyl thioesters to 2,3-dienoyl fatty acyl thioesters.
Alcohol Fermentation			
Alcohol dehydrogenase; aldehyde reductase, (<i>AdhI</i>) EC:1.1.1.1			
A primary alcohol + NAD ⁺ = an aldehyde + NADH + H ⁺ . A secondary alcohol + NAD ⁺ = a ketone + NADH + H ⁺ .	Primary alcohol NAD ⁺ , secondary alcohol	Aldehyde, NADH, H ⁺ , ketone	<i>AdhI</i> catalyses the first step in ethanol metabolism.

Aldehyde Dehydrogenase (NAD ⁺) (<i>Aldh2</i>) EC 1.2.1.3			
An aldehyde + NAD ⁺ + H ₂ O <=> Fatty-acid + NADH + H ⁺	Aldehyde, NAD ⁺ , H ₂ O	Carboxylate, NADH, H ⁺	Wide specificity, including oxidation of D- glucuronolactone to D-glucarate. CoA-independent aldehyde dehydrogenase.
Cytochrome P450, family 4, subfamily a poly peptide 10 (<i>Cyp4a10</i>) EC:1.14.14.80			
A long-chain fatty-acid + [reduced NADPH--- hemoprotein reductase] + O ₂ = an omega-hydroxy-long-chain fatty-acid + [oxidised NADPH---hemoprotein reductase] + H ₂ O	Long-chain fatty- acid; [reduced NADPH--- hemoprotein reductase], O ₂ .	Omega-hydroxy- long-chain fatty- acid, [oxidised NADPH--- hemoprotein reductase], H ₂ O	<i>Cyp4a10</i> acts on laurate, myristate, palmitate, oleate, and arachidonate.

Table 2.6 Enrichment terms of fatty acid β -oxidation downregulated genes

Category	Term ID	Term description	Observed Gene count	Back-ground gene count	Strength	FDR
GOBP	GO:0006631	Fatty acid metabolic process	11	310	1.81	3.8E-16
GOBP	GO:0009062	Fatty acid catabolic process	9	79	2.32	3.8E-16
GOBP	GO:0055114	Oxidation-reduction process	12	917	1.38	4.16E-14
GOBP	GO:0033539	FAO using acyl-CoA dehydrogenase	3	10	2.74	1.88E-05
GOBP	GO:0009437	Carnitine metabolic process	3	13	2.63	3.54E-05
GOBP	GO:0110095	Cellular detoxification of aldehyde	2	9	2.61	0.006
GOBP	GO:0006068	Ethanol catabolic process	2	10	2.57	0.007
GOBP	GO:0051791	Medium-chain fatty acid metabolic process	2	12	2.49	0.0088
GOMF	GO:0003995	Acyl-CoA dehydrogenase activity	4	10	2.87	1.62E-07
GOMF	GO:0004095	Carnitine O-palmitoyltransferase activity	3	4	3.14	6.94E-06
GOMF	GO:0016491	Oxidoreductase activity	7	710	1.26	1.65E-05
GOMF	GO:0050660	Flavin adenine dinucleotide binding	4	85	1.94	4.83E-05
GOMF	GO:0000062	Fatty-acyl-CoA binding	3	25	2.34	0.00014
GOMF	GO:0004029	Aldehyde dehydrogenase (NAD ⁺) activity	2	17	2.33	0.0093
GOCC	GO:0005739	Mitochondrion	11	1776	1.06	1.8E-08
GOCC	GO:0031090	Organelle membrane	9	2973	0.75	0.00042
GOCC	GO:0043231	Intracellular membrane-bounded organelle	12	9507	0.37	0.007
GOCC	GO:0005737	Cytoplasm	12	10283	0.33	0.0153
GOCC	GO:0042645	Mitochondrial nucleoid	2	47	1.89	0.0314

Table 2.7 Enrichment terms of fatty acid β -oxidation upregulated genes

Category	Term ID	Term description	Observed gene count	Back-ground gene count	Strength	FDR
GOBP	GO:0035337	Fatty-acyl-CoA metabolic process	3	22	2.88	6.63E-05
GOBP	GO:0006631	Fatty acid metabolic process	4	310	1.85	0.00026
GOBP	GO:0001676	Long-chain fatty acid metabolic process	3	95	2.24	0.00085
GOBP	GO:0046949	Fatty-acyl-CoA biosynthetic process	2	12	2.96	0.0019
GOBP	GO:0046890	Regulation of lipid biosynthetic process	3	187	1.95	0.002
GOBP	GO:0035336	Long-chain fatty-acyl-CoA metabolic process	2	17	2.81	0.0032
GOBP	GO:0015908	Fatty acid transport	2	62	2.25	0.0168
GOBP	GO:0046889	Positive regulation of lipid biosynthetic process	2	98	2.05	0.0352
GOMF	GO:0003996	Acyl-CoA ligase activity	2	13	2.93	0.0053
GOMF	GO:0004467	Long-chain fatty acid-CoA ligase activity	2	13	2.93	0.0053
GOMF	GO:0047676	Arachidonate-CoA ligase activity	2	11	3	0.0053
GOMF	GO:0102391	Decanoate-CoA ligase activity	2	10	3.04	0.0053
GOCC	GO:0031966	Mitochondrial membrane	4	647	1.53	0.0013

2.5 Discussion

The significance of this study resides in the novel data and the investigation of rarely studied gene expressions that have provided new insights into the complex mechanisms and physiological shifts within glycolysis and FAO. The results of our study provide an *in vivo* evaluation of energy metabolism in the *Winnie* mice of colitis, as well as transcriptome analysis of glycolysis and FAO pathways. The results confirmed the inflammatory activity in *Winnie* mice by assessing body weight, histological features,

immune cell infiltration, Lcn-2 levels, and stool composition. These indications may have negatively influenced body mass, food and water bouts, food and water intake, and energy metabolism. The 14 week *Winnie* mice interacted fewer times with the water and food vessels, and consumed less water and food than control mice. This behaviour suggests potential food and water aversion, or perhaps a decreased capability to reach these hoppers. The underlying reasons could range from gastrointestinal discomfort or pain to a suppressed appetite, lowered energy levels, or depressive-like symptoms. In accordance, dietary behaviour in people with IBD showed that less interaction with food was the most commonly reported symptom in 83% of the studies (Day et al., 2021). In particular, CD patients demonstrated less desire to eat than healthy controls (Day et al., 2021). Also, during active disease in CD patients, food exclusion rates were 69% higher than in remission (Bergeron et al., 2018). As such, variations in food consumption and body composition are determinants of daily EE. Analysis of the energy metabolism indirect calorimetry data revealed a reduction in all energy metabolism parameters in *Winnie* mice compared to controls, namely, VO_2 consumption and VCO_2 emission, EE, GOX and LOX. In the comparative analysis between *Winnie* mice and the control group at the onset of the disease (6 weeks), the lack of metabolic alterations suggests that the variations detected in 14 week *Winnie* mice are a consequence of inflammatory processes rather than a causative factor. Similar to IBD patients, this reduced energy metabolism in the *Winnie* mouse colon may reflect an impaired nutritional status, resulting in diminished functional ability (Scaldaferri et al., 2017). It is also of interest that in paediatric IBD patients inflammation, anorexia, and malnutrition have been linked to the loss of lean body mass, which was observed in the *Winnie* mouse (Werkstetter et al., 2012). In addition, in adult IBD patients, sarcopenia have been linked to the loss of lean body mass (Bryant et al., 2015). To elucidate the underlying mechanisms of these energy metabolism changes in the *Winnie* mouse, the glycolysis/gluconeogenesis and FAO gene expressions were determined by means of RNA-Seq of the distal colon samples.

2.5.1 Glycolysis/gluconeogenesis in *Winnie* mice colons

Glycolysis directs the actions of inflammatory cells (Vermeulen et al., 2011). Immune cells preferentially use glycolysis as a source of energy during the peak of inflammation at the expense of mitochondrial oxidative phosphorylation, which can lead to

tricarboxylic acid (TCA) cycle disruption and metabolic reprogramming (Vermeulen et al., 2011). Specifically, glycolytic enzymes are linked to the severity of UC are implicated in epithelial transformation (Vermeulen et al., 2011). Factors identified in this study linked to glucose metabolism dysregulation in the distal colon of *Winnie* mice included the downregulation of genes, *Pfk9*, *galM*, *G6pc*, *Eno1*, *Ldh*, *Adh*, *Aldh2*, and *Pepck*, alongside upregulation of *Hk2*, *Adpgk*, *Gapdh*, *Pgk1*, *Pgm*, *Minpp1*, *Pgam1*, *Bpgm*, *Pkm*, *Akr1a1*, and *Aldh3b2*. We will explore each of these findings and discuss their implications.

The *Winnie* mice expressed upregulated *Hk2*, the gene encoding the rate-limiting first step of glycolysis. Although our study did not test the insulin levels in *Winnie* mice, it is shown in human colorectal cancer that the carbohydrate-induced rise in glucose triggers insulin secretion, which increases HK and 6-phosphofructokinase activity (He et al., 2016). HK upregulation enhances the rate of glucose transport across the cell membrane, stimulates glycogen synthesis, and decreases glycogen breakdown, forming glucose 6-phosphate (G6P) and adenosine diphosphate (ADP) (Dimitriadis et al., 2011). HK2 is highly expressed in gut epithelium, contributes to immune responses, and is upregulated during inflammation. To determine the role of epithelial HK2 for intestinal inflammation, *Hk2*^{ΔIEC} mice demonstrated HK2 levels increased during the course of colitis. Specifically, HK2 expression was induced in the epithelial tips of both ileum and colon by the microbiota (Hinrichsen et al., 2021). Successively, ADPGK, which was upregulated in the *Winnie* mouse, catalyses the ADP-dependent phosphorylation of G6P under hypoxic and ischemic conditions, subsequently priming glycolysis if ATP is limited. The upregulation of *Adpgk* initiates the Warburg phenotype in activated immune cells, characterised by upregulated glycolysis with preferential fermentation of glucose to lactate (Kornberg, 2020). These actions lead to cell dysfunction and, eventually, premature apoptosis (Imle et al., 2019). These implications impair metabolite changes throughout the glycolysis pathway (Liberti & Locasale, 2016), which becomes evident throughout the gene expression changes in the *Winnie* mouse.

Our results demonstrated the downregulation of *Pfk* in *Winnie* mice. In humans and mice, PFK is a key enzyme in ATP production in the glycolytic pathway by converting 1,3-

bisphosphoglycerate (1,3-BPG) to 3-phosphoglycerate (3-PG). *In vitro* and human studies have shown that the inhibition of PFK reflects low ATP levels (Chiarelli et al., 2012; Liao et al., 2022). Conversely, studies of the intestinal mucosa in the mouse model of (dextran sulfate sodium) DSS-induced colitis show high quantities of ATP in the extracellular environment (Wan et al., 2016). Downregulated *PFK* suppresses lipogenesis, thereby reducing EE and lean body mass, as observed in the mouse model of PFK deficiency (Getty-Kaushik et al., 2010; Richard et al., 2007) and in the indirect calorimetry results of the *Winnie* mice. Our results correlate with Kwon et al. (2021) that found reduced lipogenesis in DSS-induced colitis mice and that lipid metabolism overall was disrupted, causing fat accumulation in the liver and adipose tissues, leading to metabolic disorders.

Alterations in glucose metabolism in the *Winnie* mice can be attributed to the downregulation of the *G6pc* gene, leading to aberrant glycogen storage patterns. The primary diagnostic indicators in humans include hepatomegaly, hypoglycaemia, hyperlactacidemia, and increased hyperlipidaemia (Froissart et al., 2011). The implied cycle of an intolerance to fasting repeats in *Winnie* mice with a *Pgm2* deficiency. Adequate activity of *Pgm2* is crucial for efficient glycolysis when the body is in a fasting state because activated forms of glucose and galactose are necessary for proper protein glycosylation. This results in a normalising overall galactosylation and sialylation levels, making the *Winnie* mouse less susceptible to hypoglycaemia (Balakrishnan et al., 2019). However, its compensatory regulation may be insufficient. Intracellular glycan production, exposure to glycosidases, or substrate glycoprotein expression can change cell surface glycosylation. In UC and CD patients' intestinal mucosa, inflammatory-induced alterations in epithelial glycoprotein expression and glycan modifying enzyme activity, including sialidases, have been found (Balakrishnan et al., 2019). Furthermore, *GalM* downregulation situates the *Winnie* mouse and the IBD patient at risk of developing galactosemia (Kikuchi et al., 2021).

A compromise in ATP synthesis and energy metabolism in the *Winnie* mouse occurs through the *Gapdh* upregulation, which reduces NAD^+ to nicotinamide adenine dinucleotide + hydrogen (NADH). Subsequently, the reducing equivalent, nicotinamide

adenine dinucleotide phosphate (NADPH), is transported to the mitochondria via the glyceraldehyde 3-phosphate (G3P) shuttle rather than NADH itself (S. Wang et al., 2020), and as a result compromising ATP synthesis and EE in the *Winnie* mouse colon (Getty-Kaushik et al., 2010; Richard et al., 2007). Although our results suggest diminished ATP in the *Winnie* mouse colon, the upregulation of *Pgk1*, which is induced by hypoxia-inducible factor-1 α (HIF-1 α), typically generates ATP and 3-PG (Michelson et al., 1983). Multiple studies have focused on the overexpression of HIF1 α in the inflamed mucosa of both UC and CD, however, the literature is unable to establish the cause or its pathological relevance (Glover & Colgan, 2011). This factor, in turn, upregulates glycolysis-associated genes to adapt to low oxygen levels (Giatromanolaki et al., 2003).

Pgam upregulation was found in the *Winnie* mouse. Overexpressed *Pgam* has been found to increase HIF-1 α in low O₂ tissue, which has a protective role during mucosal inflammation (Glover & Colgan, 2011; Sharif et al., 2019). The *Winnie* mouse overexpressed *Bpgm* and *Minpp1* of the Rapoport-Luebering glycolytic bypass, which promotes the release of O₂ to tissue from erythrocytes to produce ATP energy. This is crucial for effectively satisfying tissue-oxygen demands and facilitating a critical physiological response to O₂ deprivation (Cho et al., 2008). These results may play a compensatory role to changes in IBD pathophysiology. Hereby, intestinal blood flow increases after eating and alters local oxygen partial pressure. Consequently, the oxygenation profile of the intestinal mucosa exhibits dynamic changes throughout the day due to repeated significant blood perfusion and metabolic fluctuations. Even in the absence of inflammation, the mucosal epithelial cells experience a state of "physiologic hypoxia" as part of their baseline condition. However, in the pathophysiology of IBD, this metabolic profile is disrupted. The presence of inflammation modifies this normal metabolic state, resulting in a rapid depletion of nutrients and oxygen within the actively inflamed mucosa. This depletion contributes to the development of hypoxia, hypoglycaemia, and acidosis, exacerbating the condition (Glover & Colgan, 2011).

Eno1 was downregulated in *Winnie* mice. At variance with our findings, other studies have shown that the downregulation of the *Eno1* gene results in a decreased incidence of hyperglycemia and hyperlipidaemia (Jung et al., 2013), which accounts for the stability

of the mitochondrial membrane observed in those studies, and involves the dehydration of 2-phospho-D-glycerate to PEP (Jung et al., 2013). Through the PEP essential node, *Pkm* exhibiting upregulation in *Winnie* mice serves as a crucial link between glycolysis, the TCA cycle, and tryptophan metabolism. The upregulation of *Pkm* was observed in mucosal biopsies from mice with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, which correlated with villus atrophy and epithelial obstruction in the colon (Tang et al., 2015). These findings align with the pathophysiology observed in *Winnie* mice colons (Heazlewood et al., 2008). While *Pkm* overexpression has been associated with tissue destruction, its upregulation can serve as a mechanism of allosteric regulation to support NAD⁺ generation for tissue repair. Specifically, *Pkm* diverges via *Ldh* to facilitate the interconversion of pyruvate to lactate and NADH⁺ to NAD⁺ (Z. Zhang et al., 2019). However, in the case of *Winnie* mice, this compensatory mechanism may not have been adequately activated due to the downregulation of *Ldh*. The deficiency of LDH affects the breakdown of glucose for energy, particularly in muscle cells, leading to symptoms of fatigue, muscle pain, and cramps (Farhana A, 2021). Numerous studies highlight that a significant number of IBD patients experience abdominal muscle cramping and pain due to functional intestinal disorders (Coates et al., 2013). Further investigation into the repercussions of *Ldh* deficiency in *Winnie* mice could offer insights for addressing this issue.

The *Winnie* mice revealed the downregulation of *Pepck*, an enzyme critical for initiating gluconeogenesis, in which oxaloacetate is converted to PEP. This downregulation directly impacts serotonin levels in the gut by influencing tryptophan synthesis (Kanova & Kohout, 2021). *Pepck* plays a crucial role in energy generation during periods of reduced food intake and prolonged fasting, as the brain relies exclusively on glucose derived from non-carbohydrate sources like lactate, alanine, glycerol, and glutamine in living cells (Maria et al., 2018). Insulin inhibits gluconeogenesis and suppresses the transcription of *Pepck* (Yang et al., 2009), which aligns with the upregulation of *Hk2* and *Adpgk* in the *Winnie* mice. This mechanism possibly represents allosteric regulation in the *Winnie* mouse (He et al., 2016; Imle et al., 2019).

Adh1, *Aldh2*, and *Cyp4a10* downregulated in *Winnie* mice, suggesting a compromised state in their detoxification pathways. Concurrently, *Aldh3a1* presents a correlation with DNA damage responses (Koppaka et al., 2012). Under conditions of homeostasis, *Adh1* is implicated in the metabolism of alcohol and fatty acids, thereby providing a safeguard against oxidative stress and toxicity. *Adh1* downregulation indicates diminished NAD⁺ (Edenberg & Bosron, 2010). In a human IBD study (Magnusson et al., 2016), the levels of ALDH⁺ dendritic cells and macrophages were found to be lower in the colons of UC active patients and those in remission compared to healthy controls. Coupled with the fact that the proportion of ALDH⁺ monocytes in the blood was similar between UC patients in remission and healthy individuals, this indicates that ALDH activity is modulated by the UC intestinal environment, regardless of the disease's active status. Since NAD⁺ levels are affected by the microbiota and NAD⁺ serves as a necessary cofactor for aldehyde dehydrogenases, it plays a crucial, rate-limiting role, along with magnesium, in the conversion of retinol to retinoic acid. Subsequently, the microbiota associated with the disease could potentially influence ALDH activity. A deficiency in ALDH2 predisposes the *Winnie* mouse to aldehyde-induced harm, diminished ROS detoxification capacity and elevated risk for colon cancer (Kim et al., 2017). These implications are coupled with the accumulation of neurotoxic dopamine metabolites, thereby impacting the functionality of dopaminergic nerves (Koppaka et al., 2012). The combination of diminished lean body mass and increased fat mass observed in *Winnie* mice aligns with the under-expression of detoxification pathways, given that adipose tissue serves as a principal reservoir for bioaccumulation of toxicants (Jackson et al., 2017; Werkstetter et al., 2012). In addition, in *Winnie* mice the upregulation of *Akr1a1* is implicated in the tryptophan to AhR pathway, leading to the generation of neurotoxic electrophiles. This might potentially form protein adducts resultant from lipid peroxidation, thereby inducing acetaldehyde-associated DNA damage in the *Winnie* mouse colon (Stewart, 2007). Crucially, these outcomes suggest a connection to the pathophysiology of IBD, the functional alterations concomitant with this condition (Vermeulen et al., 2011).

2.5.2 Fatty acid β -oxidation in *Winnie* mice colons

Abnormalities in lipid utilisation and metabolism in IBD lead to defective mucous production, altered fatty acid uptake, and overproduction of inflammatory signalling molecules, contributing to IBD pathogenesis (Shores et al., 2011). The importance of determining FAO gene expression is to elucidate defects in metabolism that may affect homeostasis and energy metabolism that correlate with human IBD symptoms. Factors identified in this study linked to FAO dysregulation in the distal colon of *Winnie* mice include downregulation of genes such as *Cpt1*, *Acadm*, *Acadvl*, *Acadsb*, *Gcdh*, *Hadhb*, *Eci*, *Aldh2*, *Adh1*, *Cyp4a10*, alongside upregulation of *Acs1*, *Acadl*, and *Acat1*. We will explore each of these findings and discuss their implications.

The results show an overexpression of *Acs1*, necessary for the acetylation of fatty acids. In context, ACSL1 and ACSL4 levels in the terminal ileum and colon of IBD patients were found to be significantly higher compared to non-diseased tissue. This suggests that in IBD pathophysiology, enterocyte fatty acids mostly enter the phospholipid route where they function as precursors for inflammatory mediators. Phospholipids may be absorbed into the afflicted intestine's plasma membranes, maintaining membrane integrity and epithelial barrier function (Heimerl et al., 2006). Although this study noted that ASCL facilitated cell proliferation along the intestinal crypt-villus axis (Heimerl et al., 2006), if the influx of acyl-CoAs generated by mobilising stored triglycerides is not utilised by β -oxidation pathways, a homeostatic signal is generated to reduce serotonergic effects, including influences on feeding (Bouagnon et al., 2019). Further investigation is needed to identify if this consequence influenced the *Winnie* mouse's reduced food consumption.

In addition, *Cpt1* downregulation is inhibited by the accumulation of malonyl-CoA, and thereby inhibits ketogenesis by blocking the transport of fatty acids into mitochondria. When there is an excess of carbohydrates available, insulin signalling promotes the dephosphorylation and activation of acetyl-CoA carboxylase, which stimulates the use of carbohydrates as an energy source (Foster, 2012). Humans with *CPT1* deficiency are unable to fast due to incurring hypoglycaemia and reduced ketone production (Jones & Bennett, 2017). The symptoms of *Acadm* deficiency and *Cpt1* deficiency exhibit similarities, leading to hypoketotic hypoglycaemia during fasting or starvation (Boonsimma et al., 2021). These side-effects are further exacerbated by *Acadm*

downregulation, which can cause medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, an enzyme crucial for ketone bodies synthesis which offers an energy source during times of extended fasting or increased energy requirements. When a mammal is in a catabolic state or confronts increased metabolic demands, this deficit may cause acute metabolic decompensation (Grosse et al., 2006). Typically, hypoglycaemia is identified via plasma as a glucose concentration that is significantly lower than normal (Houten et al., 2013). Further investigation into the plasma levels of the *Winnie* mouse is needed to elucidate glucose levels and determine if the upregulation of *Hk2* and *Adpgk* is an allosteric response to these gene expressions.

The cycle of intolerance to fasting continues throughout FAO in *Winnie* mice, and this is particularly concerning due to the fact that the *Winnie* mice consumed significantly less food compared to the control group. During catabolic and fasting states, *Acadvl* downregulation prevents the conversion of long-chain fatty acids to energy, resulting in acylcarnitine and triglyceride accumulation (Diekman et al., 2021). There is limited literature on *Acadvl* in mice, however in catabolic illnesses triggered by fever or fasting combined with (very long-chain acyl-CoA dehydrogenase) VLCAD deficiency, the catabolic illness typically progresses into hypoketotic hypoglycaemia, muscle weakness, and increased hydrogen peroxide (H_2O_2) (Diekman et al., 2021). Typically, colonocytes express high levels of H_2O_2 and diffuse the excess into extracellular spaces, causing oxidative stress, which is considered to be one of the core foundations of colitis (Pravda, 2005). In human and mouse studies, overexpressed *Lcad* generates high levels of H_2O_2 , which inhibits the TCA cycle particularly aconitase, alpha-ketoglutarate dehydrogenase, and succinate dehydrogenase, and subsequently reduces energy metabolism (Y. Zhang et al., 2019).

Another contributing factor to reduced energy metabolism in *Winnie* mice is *Hadhb* downregulation, which plays a role in the assembly of the mitochondrial trifunctional protein complex. This complex contains long-chain 3-keto-acyl-CoA thiolase, an enzyme responsible for metabolising long-chain fatty acids into energy (Rector et al., 2008). Humans born with *Hadhb* deficiency, also known as Charcot-Marie-Tooth disease, experience motor and sensory neuropathy and exhibit smaller and weaker muscles (Guan

et al., 2021). Reduced energy metabolism has also been linked to a decrease in *Acadshb*, as observed in the *Winnie* mice, which regulates the metabolism of free fatty acids, triglycerides, and cholesterol (Jiang et al., 2021). In a study conducted on colonic specimens from post-surgical resections of patients with IBD, downregulated *Acadshb* led to a reduction in the TCA cycle and oxidative phosphorylation, thereby decreasing ATP production (Moriggi et al., 2017). Another example of diminished *Acadshb* causes short/branched chain acyl-CoA dehydrogenase deficiency, specifically affecting isoleucine catabolism (Alfardan et al., 2010). This deficiency is characterised by lethargy and muscle weakness and is reminiscent of the fatigue experienced in IBD, and may be the source of reduced food and water bouts of the *Winnie* mouse (Alfardan et al., 2010).

In *Winnie* mice, *Acat1*, the enzyme involved in the final step of ketolysis, was found to be upregulated. *Acat1* breaks down dietary proteins and fats and induces Claisen condensation, contributing to EE homeostasis (Liu et al., 2005). *In vitro* studies have shown that *Acat1* is allosterically regulated by increasing cholesterol content within the endoplasmic reticulum. This regulation correlates with the downregulation of *Acadshb* and *Hadhb*, as well as the imbalanced lipid levels generated by *Acs1* in the *Winnie* mouse (Liu et al., 2005). However, our findings differ from a study conducted in acute DSS-induced colitis mice and human IBD data, which reported reduced *Acat1* expression in the colonic mucosa during active disease. This implies a potential defect in ketone utilisation (Huang et al., 2022). The disparity between these findings could be attributed to differences in disease activity, diet, cholesterol levels in the participants, and the fact that the acute colitis mouse model does not exhibit abnormal dyslipidaemia (Kwon et al., 2021). The data used in this research is derived from chronic colitis in *Winnie* mice, where the downregulation of *Hadhb* and *Acadshb* and upregulation of *Acs1* was observed. These findings suggest that the *Winnie* mouse may express a homeostatic response to cholesterol levels. To gain further clarity, it would be beneficial to perform serum testing of cholesterol levels in *Winnie* mice.

Due to the prominence of hypoglycaemia and metabolism defects in colitis mouse models and IBD patients, the literature recommends personalised dietary plans for IBD patients and highlights gene-nutrient interactions associated with nutritional deficiencies (Laing et al., 2019). Just one of many examples is through *Eci1* downregulation in the *Winnie*

mice, which plays a significant role in the oxidation pathways of oleic acid and linoleic acid (Fillgrove & Anderson, 2001). One study demonstrated that *Eci1*-deficient mice, when fed an olive oil-rich diet, accumulated acylcarnitine, while the control group, which underwent overnight fasting, experienced ketotic hypoglycaemia (van Weeghel et al., 2012). As such, IBD patients are advised to consume a carbohydrate-rich diet to prevent occurrences of hypoglycaemia (Laing et al., 2019). In terms of specific dietary requirements, IBD patients may be at risk of developing toxicity, as indicated by *Aldh2* downregulation in *Winnie* mice. Downregulated *Cyp4a10* in *Winnie* mice functions to metabolise and eliminate endogenous and exogenous molecules, including steroids and cholesterol. It is of interest that conjugated reactive xenobiotic metabolites are implicated as one of the causes of UC. *Cyp4a10* downregulation increases the risk of infection and elevated IFN- γ levels in *Winnie* mice and IBD patients (Okita & Okita, 2001; Urlacher & Girhard, 2012).

Gcdh, which is responsible for the oxidation of L-lysine, L-hydroxylysine, and L-tryptophan, was downregulated in the *Winnie* mice. Unlike other colitis mouse models, the *Winnie* mouse is characterised by missense mutations in which *Gcdh* deficiency is detected (Kölker et al., 2006). GCDH plays a critical role in tryptophan metabolism by converting glutaryl-CoA to glutaconyl-CoA and crotonyl-CoA. When GCDH is deficient, metabolic disorders can occur, leading to the toxic build-up of glutaric acid type I (GA-1). GA-1 metabolism results in an increased production of quinolinic acid via the kynurenine pathway, and higher levels of glutaryl-CoA (Kölker et al., 2006). Additionally, studies show elevated levels of total protein glutarylation in cells lacking GCDH and in fasting mice (Trefely et al., 2020), which correlates to the decreased food intake of the *Winnie* mice. If GCDH deficiency is left untreated, it can lead to catabolic-induced enteritis and a febrile response (Kölker et al., 2006), which are symptoms experienced by patients with IBD (Eri et al., 2011).

In our study, we observed substantial alterations in gene sets that are correlated with disease severity. Our findings suggests that even at the point of diagnosis, the genomic, epigenetic, and transcriptomic landscapes are likely already skewed. Our data also reflects the metabolic responses during chronic inflammation of acute and chronic DSS-induced colitis mice (Melgar et al., 2007), however the *Winnie* mouse model of chronic

colitis exhibited a close synteny of its genome with UC and CD in humans. Specifically, genes and pathways in the *Winnie* mouse linked to the severity of UC are implicated in epithelial transformation.

In IBD patients, inflammation plays a significant and enduring role, which correlates with its extent, duration, and severity. The risk of developing colitis-associated colorectal cancer is significantly high in humans with IBD (Manninen et al., 2013), which correlates to the glycolytic gene changes in the *Winnie* mice initiating the Warburg effect (Vander Heiden et al., 2009) coupled with the metabolic reprogramming of FAO (Bouagnon et al., 2019). Research focusing on the non-cancerous intestinal mucosa in IBD suggests that the pathogenesis of colorectal cancer commences long before neoplastic changes become apparent, as a hidden process within the inflamed gut tissue (Haberman et al., 2019).

2.6 Conclusion

This research discerned that the *Winnie* mice exhibited diminished food and water intake, culminating in reduced energy metabolism. This observation was corroborated by analysing glycolysis and FAO gene expressions responsible for generating ATP energy. The data revealed oscillating gene expressions, thereby inciting metabolic aberration associated with numerous adverse effects, inclusive of heightened susceptibility to infection, toxic accumulation of H_2O_2 , an inability to metabolise medium- and long-chain fatty acids, mitochondrial dysfunction, and an intolerance to fasting. Crucially, these outcomes suggest a connection to the pathophysiology of IBD, the functional alterations concomitant with this condition. Additionally, tryptophan metabolism, a critical process for the synthesis of the neurotransmitter serotonin, was found to associate with the reshaping of metabolic pathways. This reshaping could potentially influence behaviour, considering the established relationship between the gut and the brain. In light of these findings, the researchers advocate for the investigation and identification of early alterations in metabolic processes of IBD patients demonstrating depleted energy. By acknowledging these changes as potential contributors to IBD pathogenesis, it could be plausible to enhance energy management in these patients. This might be accomplished

through the formulation of specific guidelines and the advancement of targeted therapies, potentially forestalling or mitigating further disease progression.

Chapter 3: Tryptophan *de novo* NAD⁺ synthesis in the *Winnie* mouse colon

3.1 Summary

Inflammatory bowel disease (IBD) is a chronic, systemic illness leading to inflammation throughout the gastrointestinal tract. Those afflicted with IBD endure periodic inflammation, disruptions in gut motility, and visceral discomfort. A consistent observation in IBD patients is the dysregulation in tryptophan metabolism, which subsequently affects 5-hydroxytryptamine, a pivotal signalling molecule in the enteric nervous system. This molecule is essential in regulating enterochromaffin cells, crucial for intestinal homeostasis. Notably, tryptophan serves as a precursor for several vital compounds, including serotonin, melatonin, kynurenine, and nicotinamide adenine dinucleotide (NAD⁺).

The onset of IBD is intricately tied to genetic interplays within the inflamed tissue. Leveraging RNA-sequencing and metabolomics, our study explored alterations in the serotonergic and *de novo* NAD⁺ synthesis pathways of tryptophan metabolism and its related metabolites in the distal colons of *Winnie* mice.

Our investigation unveiled a transcriptome reflective of inflammation, with critical implications for the *Winnie* mice. The changes in the concentration levels of tryptophan metabolites is reflected in changes in the expression of genes involved in these pathways. The genes and metabolites shed light on the physiological alterations in tryptophan metabolism, specifically its diversion towards the kynurenine pathway and the consequential effects on *de novo* NAD⁺ synthesis in the context of chronic intestinal inflammation.

3.2 Introduction

Inflammatory bowel disease (IBD) is a systemic and incurable disease that causes chronic inflammation of the gastrointestinal tract. It comprises two archetypal pathologies: ulcerative colitis (UC) and Crohn's disease (CD). CD and UC are differentiated histopathologically and via disease localisation within the gastrointestinal tract (Lee et al., 2018). More than 100 gene defects, environmental factors, and disturbances have been implicated in IBD pathophysiology, although how these relate remains only partially understood (Burisch & Munkholm, 2015). Typical manifestations of IBD include diarrhoea, abdominal pain, rectal bleeding, abscesses, bowel perforations, and toxic megacolon. With time, IBD patients develop intestinal malabsorption, anaemia, bacterial overgrowth, and energy metabolism impairment (Fakhoury et al., 2014).

These IBD symptoms deemed to be associated with 5-HT, a tryptophan derivative, which is an essential amino acid synthesised by mammals that must be externally sourced, and is an enteric nervous system signalling molecule (Gershon & Tack, 2007). 5-hydroxytryptamine (5-HT, serotonin) regulates the energy production in intestinal cells, which is necessary for maintaining intestinal homeostasis (Gershon & Tack, 2007). In response to numerous stimuli, including the ingestion of nutrients, enterochromaffin cells release 5-HT, which regulates gut motility, glucose absorption, and fluid balance. Recent evidence indicates that peripheral 5-HT is involved in multiple metabolic control pathways (Wyler et al., 2017).

Although clinical studies indicate that tryptophan metabolism is associated with IBD severity, much uncertainty still exists. Studies have partially elucidated intestinal tryptophan metabolism and its role in regulating symptoms and behaviour in IBD patients and mouse colitis models (Gershon, 2013). However, published studies have limited their focus to just a few tryptophan metabolites, or only indoleamine 2,3-dioxygenase (IDO), the first rate-limiting enzyme of tryptophan catabolism (Ciorba, 2013). Additionally, studies have typically employed cell culture, plasma, and faecal samples, due to their methodological ease (Dudzińska et al., 2019; Gupta et al., 2011; Nikolaus et al., 2017; Wu et al., 2013). Studies on the interplay between tryptophan, bacteria, the host's

metabolome and the immune system revealed a unique function for tryptophan metabolism in the pathogenesis of IBD (Lavelle & Sokol, 2020) .

Despite evident connections between chronic intestinal inflammation and tryptophan metabolism, but few studies have looked into the intrinsic molecules involved in tryptophan metabolism that might distinguish the downstream physiological alterations in IBD patients. For instance, 95% of tryptophan catabolises into the kynurenine pathway, otherwise known as tryptophan *de novo* NAD⁺ biosynthesis (Sofia et al., 2018), which depends on expressions of interferon (IFN)- γ , tumour necrosis factor (TNF)- α , bacterial lipopolysaccharides (LPS), and the pro-inflammatory cytokine interleukin-6 (IL-6), all of which are key pathogenic factors in IBD (Sofia et al., 2018). Part of the remaining tryptophan enters the serotonergic pathway, which processes tryptophan mainly via tryptophan hydroxylase 1 (TPH1) within enterochromaffin cells or TPH2 within the brain into 5-HT and melatonin (Agus et al., 2018). NAD⁺ plays an important role in energy metabolism, homeostasis, and, more recently found, in the regulation of immune responses. It has been shown that the immunoregulatory properties of NAD⁺ are strongly related to the overexpression of TPH (Rodriguez Cetina Biefer et al., 2017). Indole, or the aryl hydrocarbon receptor (AhR) pathway metabolises the remaining tryptophan (1-2%) into indole derivatives via gut microbiota, activating AhR (Zhang et al., 2021). The past research on NAD⁺ synthesis has been restricted to comparisons of just one or two genes (Dobrovolsky et al., 2005). As this pathway relies on downstream tryptophan metabolism effectors, analysing the *de novo* NAD⁺ synthesis pathway will contribute to a deeper understanding of tryptophan metabolism, and its association with chronic intestinal inflammation.

Since IBD development is closely related to gene interactions within diseased tissue, by utilising RNA-sequencing (RNA-Seq) and metabolomics, this study aimed to determine changes in the tryptophan *de novo* NAD⁺ synthesis within the *Winnie* mouse of chronic colitis. The *Winnie* mice are affected by a missense mutation of the *Muc2* gene, resulting in aberrant *Muc2* mucin biosynthesis within the goblet cells. Consequently, progressive chronic intestinal inflammation develops, with signs appearing from 6 weeks of age, develop severe colitis apparent by 12-16 weeks of age, and a histopathological outcome

similar to human IBD (Eri et al., 2011). Previous evaluations of lipocalin-2, faecal water content, weight differences, and structural morphological changes within the colon demonstrated inflammation in the *Winnie* mouse distal colon (Rahman et al., 2015; Robinson et al., 2016; Robinson et al., 2017; Sahakian et al., 2021). Herein, we evaluated tryptophan *de novo* NAD synthesis in the distal colon of 14 weeks of age *Winnie* mice compared to age-matched control C57BL/6 littermates.

3.3 Materials and methods

3.3.1 Ethics, animals and animal husbandry

This study was approved by the Victoria University Animal Experimentation Ethics Committee (AEC-17-016). All animal experiments complied with the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes described in [Section 2.3.1](#). The *Winnie* mouse model of colitis with a C57BL/6 mice background was obtained from the same colony at Victoria University (Melbourne, Victoria, Australia). The colitis in *Winnie* mouse models is caused by a missense mutation (G9492A, GenBank accession no. AJ511872), by which cysteine is replaced by tyrosine in the D3 67 domain in the N terminus of the mucin 2 (*Muc2*) gene (Heazlewood et al., 2008). Age-matched C57BL/6 littermates, obtained from the Animal Resources, constituted the control. Post *in vivo* experiments, all mice were euthanised at 14 weeks of age via an overdose of injected pentobarbitone (Lethabarb, Virbac Australia) using a 1:16 dilution, 30 g, 100 µL/20 g. The brain tissues were removed and used for subsequent experiments.

3.3.2 Evaluation of inflammation

As described in Chapter 2, [Section 2.4.2](#), the clinical signs of colonic inflammation in *Winnie* mice, were determined by body weight, faecal water content, lipocalin 2 levels (Lcn-2), neutrophil infiltration, and changes in colonic mucosal structure.

3.3.3 RNA extraction and quality control

RNA-Seq determination of tryptophan and nicotinamide metabolism within the distal colons of 14-week-old *Winnie* mice ($n=6$, 3 females and 3 males) was compared to age-matched controls C57BL/6 littermates ($n=8$, 4 females and 4 males). Chapter 2, [Section 2.4.7](#) details the process that was followed. To ensure RNA was produced with minimal impurities, the RNeasy Lipid Tissue Mini Kit (cat.no.74804, Qiagen) and TRIzolTM (Invitrogen, Australia), which included DNase treatment was used. The absorbance (A) scores for all samples demonstrated that nucleotide purity was high, and between 1.8-2.0 for A260/A280 ratios and 2.0-2.2 for A260/A230 ratios and the RNA integrity number > 8.5. The remaining samples of Poly-A tail purified mRNA were submitted to Micromon Genomics (Monash University) for RNA-Seq using the next generation sequencing platform.

3.3.4 Data visualisation

Gene lists for tryptophan and nicotinamide-associated gene expressions were generated by merging related gene lists from multiple databases accessed via the molecular signature database (MSigDB) (<http://www.broadinstitute.org/gsea/msigdb/>). This included the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene ontology biological processes (GOBP) *de novo* NAD biosynthetic process, GOBP tryptophan catabolic process to kynurenine, KEGG tryptophan metabolism, REACTOME tryptophan catabolism, Wikipathways (WP) tryptophan catabolism leading to NAD production, WP tryptophan metabolism, GOBP *de novo* NAD biosynthetic process, GOBP NAD biosynthesis via nicotinamide riboside salvage pathway, GOBP NAD biosynthetic process, GOBP NAD metabolic process, GOBP nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH) metabolic process, GOBP NADH oxidation, GOBP nicotinamide adenine dinucleotide phosphate (NADPH) oxidation, Gene Ontology Molecular Function (GOMF) NAD binding and GOBP L-kynurenine metabolic process. Reactome offers curated data that are cross-referenced to other sources to identify proteins, genes, microRNAs or chemicals; and the parent-child relationships and processes shared between pathways (Fabregat et al., 2017). KEGG links sequenced molecular genomes and some partial genomes functions and map graphical

representations of cellular processes for their interpretation (Kanehisa & Goto, 2000). WikiPathways is a curated database used to analyse and integrate experimental transcriptomics, proteomics and metabolomics data in biological pathways (Slenter et al., 2018).

Heat maps of tryptophan associated and nicotinamide differentially expressed genes (DEGs) values for each colon sample were visualised using the Morpheus web-based tool (<https://software.broadinstitute.org/morpheus>) and presented as Z-score distributions (across samples) of the counts per million (CPM) values. Data are hierarchically clustered by Euclidian distance.

Visualisation of selected pathways was performed using the R package Pathview using default parameters (<https://r-forge.r-project.org/projects/pathview/>) (Luo & Brouwer, 2013). Input data included significance scores of gene expression values between *Winnie* and C57BL/6 littermates and computed as the sign Log_2FC (fold change) $\times \text{Log}_{10}\text{P}$ is represented in the KEGG graph.

3.3.5 Homology of genes in *Winnie* mice and IBD patients

Mice are frequently used as model organisms for genetic disease research and the development of novel therapies. By comparing the homology of mouse and human genes, researchers can identify genetic mutations in mice that may have comparable effects in humans, thereby shedding light on the genetic basis of diseases. The National Centre for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) public data source was used to gather gene expression data on the transcriptome of human IBD patients (Barrett et al., 2013; Edgar et al., 2002). High-throughput sequencing was used to create the expression profile of the inflamed intestine in IBD patients and the healthy colon regions in uninflamed controls undergoing resection of non-obstructive colorectal adenocarcinoma. Peters et al. (2017) assembled this data as reads per kilobase of transcript, per million mapped reads. Poly-A purified mRNA was used from RNA isolated using TRIzol obtained from *Winnie* mice colon samples. These data are available at <https://www.ncbi.nlm.nih.gov/geo/> (accessed on March 20, 2023) under the GEO

series accession number GSE83687. This dataset includes CD patients (n=42), UC patients (n=31) and controls (n=60).

3.3.6 STRING protein-protein association networks

As described in Chapter 2, [Section 2.4.9](#), the search tool for retrieval of interacting genes (STRING) database was employed (Szklarczyk et al., 2021). Up and downregulated genes were uploaded into the STRING database to extract biological processes, molecular functions and cellular pathways.

3.3.7 Tryptophan metabolite concentrations

Metabolomics provides an account of the metabolic state of a biological system. Integrating metabolomics and RNA-Seq in the same study can provide a more comprehensive understanding of cellular processes and its regulation at both the transcriptional and metabolic levels.

The following reference standards were purchased from Sigma–Aldrich (Castle Hill, Australia): L-tryptophan; 5-hydroxyindole-acetic acid (5-HIAA), kynurenic acid (KYNA), L-kynurenine 98%, 2,3-pyridinedicarboxylic acid (quinolinic acid, QUIN), nicotinamide, and 2-picolinic (PIC) acid. Each standard was accurately weighed using the calculation, molecular weight * $(1 * 10^{-3}) * 0.02$. The standards were dissolved individually into the mobile phase composition of 15% acetonitrile, 0.2% trifluoroacetic acid, and 84.8% Mill-Q purified water, degassed under vacuum. The eluents were filtered through a Millipore Membrane Filter (type HA, pore size 0.45 µm, Billerica, MA, USA). 0.5 mL of eluent was aliquoted into a 2 mL dark-coloured glass bottle and injected into the UFLC (Ultra-Fast Liquid Chromatography).

3.3.8 Chromatographic conditions

The Shimadzu Prominence UFLC consisted of variable wavelength UV-Visible detectors. The high precision 6-port valve achieves 0.3% or less area repeatability in 100,000 cycle endurance tests. The measurements for simultaneous tryptophan

compounds were obtained via the isocratic, ion-pair, reversed-phased C18 (250mm length x 4.6mm internal diameter) analytical column in the aforementioned mobile phase composition. The chromatogram was run under multistep gradient conditions with a pump flow rate of 0.8 mL/minute, a column temperature of 25° C and an injection volume of 10 µL/minute. The observed analysis column pump pressure fluctuated around 2500 pounds per square inch (psi). Elution was monitored by the photodiode array detector (collecting UV–Vis spectra from 190 to 800 nm), which provides chromatograms at the desired wavelength in this range. Standard retention times were monitored using the 254nm wavelength. Retention times for standards were recorded which are illustrated in **Appendix Figure S 4**.

3.3.9 Standard solutions calibration curve and method validation

The UFLC analytical method was validated for the parameters of selectivity, specificity, linearity, precision, accuracy (% recovery), sensitivity, limits of detection (LOD), and limits of quantification (LOQ) in calibrating the solutions (Trullols et al., 2004). Selectivity was tested by examining chromatographic blanks for retention time analyte peaks. Quantification was performed by the peak area method, which was calculated against the calibration plot. Calibration curves determined the linearity of each standard by employing five dilutions (quality control (QC) points), with the mobile phase of stock solutions utilising a 1:2 serial dilution. The calibration curve was performed in triplicate by plotting the ratio of the peak area of the mixed working solution to IS (y) against the standard concentration (x), respectively. The linearity graph was plotted as ‘concentration vs. peak area response’ via the Lab Solutions calibration curve function, achieving excellent linearity with a correlation coefficient ranging from 0.9995 - 1.000 for all compounds. The intra-day reproducibility was assessed using 5 solutions daily, while the inter-day reproducibility was determined by measuring the same solutions on 3 consecutive days. Intra-day accuracy and precision were calculated from the % bias [% (measured–theoretical)/theoretical concentration], and relative standard deviation [%RSD = % standard deviation/mean] for the 5 replicates of each QC point. LOD and LOQ values were calculated by expressing the noise to signal ratio of the lowest known concentration of linearity samples in µg/mL or ppm. The calculation for LOD is derived

using the standard deviation of the response (S_y) and the slope (S) of the calibration curve. Specifically, the formula is: $LOD = 3.3(S_y/S)$. The standard deviation of the response, S_y , can be deduced from the standard deviation of y-intercepts of regression lines. The formula for LOQ is: $LOQ = 10(S_y/S)$. S_y can be determined by examining the standard deviation of y-intercepts from regression lines (Trullols et al., 2004). Following analysis, the output chromatogram displayed the constituents of standard solutions as a peak against the retention time. The retention times were utilised for quantitative analysis and detection of unknown concentrations in the biological samples (Table 3.1, Appendix Table S1 - S14).

Table 3.1 Calibration curve preparation and quality controls

Accuracy, slope, intercept, linearity range, correlation coefficient, standard error (SE) of intercept, standard deviation (SD) of intercept, LOD, LOQ for each metabolite.

Compound	Accuracy $\pm$ SD	Slope	Intercept	Linearity range $\mu\text{g/L}$	Correlation coefficient (r)	SE of intercept	SD of intercept	LOD $\mu\text{g/L}$	LOQ $\mu\text{g/L}$
Trp	$2.17\text{e}+12 \pm 1.09\text{e}+11$	$3\text{e}-05$	-0.0835	0.25 - 4.1	0.9995	$7.16\text{e}+03$	$1.60\text{e}+04$	$1.76\text{e}+09$	$5.34\text{e}+09$
5-HIAA	$8.00\text{e}+15 \pm 2.82\text{e}+14$	$1\text{e}-07$	0.0002	0.011-0.190	0.9997	$8.15\text{e}+03$	$1.82\text{e}+04$	$6.02\text{e}+11$	$6.08\text{e}+09$
KYNA	94.53 ± 2.73	$2\text{e}+06$	-0.0039	0.02-0.16	0.9997	$3.59\text{e}+03$	$7.19\text{e}+03$	$3.39\text{e}+10$	$1.03\text{e}+11$
KYN	$3.19\text{e}+16 \pm 3.57\text{e}+15$	$6\text{e}-08$	0.0058	0.0131–0.21	0.9997	$2.67\text{e}+04$	$5.33\text{e}+04$	$2.93\text{e}+12$	$8.89\text{e}+12$
QUIN	$1.66\text{E}+14 \pm 8.74\text{e}+12$	$4\text{e}-08$	-0.0004	0.0075 – 0.12	0.9999	$7.00\text{e}+03$	$1.56\text{e}+04$	$2.93\text{e}+12$	$3.91\text{e}+12$
PIC	$1.66\text{e}+14 \pm 8.74\text{e}+12$	$8\text{e}-07$	-0.0211	0.16 – 2.56	1.000	$5.41\text{e}+03$	$1.21\text{e}+04$	$4.99\text{e}+10$	$1.51\text{e}+11$
NAM	$6.25\text{e}+16 \pm 122\text{e}+15$	$4.00\text{E}-08$	0.0004	0.0075-0.21	0.9999	$2.86\text{E}-04$	$6.40\text{E}-04$	$5.28\text{E}+04$	$1.60\text{E}+02$

Abbreviations: Lower limits of detection, LOD; lower limits of quantification, LOQ; standard deviation, SD; standard error, SE; Trp, tryptophan; 5-HIAA 5 hydroxy indole acetic acid; KYNA, kynurenic acid; KYN, kynurenine; QUIN, quinolinic acid; PIC, picolinic acid; NAM, nicotinamide; microgram per litre, $\mu\text{g/L}$.

3.3.10 Preparation of biological sample for UFLC

Biological samples were weighed and placed into individual, labelled Safe-Lock tubes, containing ice-cold 0.2 M perchloric acid (HClO_4) solutions (10 $\mu\text{L}/\text{mg}$ of tissue) in preparation for solubilisation. Homogenisation was performed in the pre-cooled

homogenising bead beater (TissueLyser LT, Qiagen, Melbourne, Australia). Two beads and five 60 second pulses, with 45 second intervals between pulses to prevent tissue heating, were sufficient to form a homogeneous tissue suspension. The homogenates were retained at 5° C for 15 minutes to aid the precipitation of proteins, cell debris, and microscopic tissues. Next, the homogenates were centrifuged at 12,000 revolutions (9660 g force) per minute at 4° C for 15 minutes to extract the metabolites from the matrix. The supernatants were immediately separated from the precipitates and transferred into a sterile 5 mL microtube. The supernatants were adjusted to a ratio of 4:1 v/v with ice-cold 0.1M HClO₄, vortexed for 30 seconds, and filtered into a new tube, ready for use (Li & Bartlett, 2014).

3.3.11 Analysis of tryptophan pathway metabolites

Biological samples of the *Winnie* mouse and control C57BL/6 littermates distal colons were injected into the UFLC (n=8 mice/group). The concentrations of each tryptophan metabolite in the biological samples were determined using analyte: internal standard peak height ratios using Shimadzu software. The concentrations of each metabolite recorded in the Shimadzu acquired 'American Standard Code for Information Interchange' text file was analysed by Origin Pro, Version 2021b (OriginLab Corporation, Northampton, MA, USA). All peak fitting, surface fitting, and signal processing capacities reached Chi-Sqr tolerance values of 1E-6.

3.3.12 Statistical analysis

GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA) was used to analyse the concentrations of tryptophan metabolites in the biological samples. Significant differences were determined using an unpaired *t*-test, Welch's test, and ratio paired *t*-test where applicable. For these analyses, $p \leq 0.05$ was accepted as significant, and the data are presented as the mean $\pm$ standard error of the mean (SEM).

3.4 Results

3.4.1 Changes in tryptophan metabolism associated genes

RNA-Seq was performed to quantify RNA species in the distal colon of 14-week-old *Winnie* mice (n=6, 3 females and 3 males) versus age-matched C57BL/6 littermates (n=8, 4 females and 4 males). Firstly, gene lists for tryptophan and nicotinamide-associated gene expressions were generated by merging related gene lists from multiple databases accessed via the molecular signature database (MSigDB) (**Figure 3.1A**). All pathways displayed gene expression changes except for the GOBP_L-kynurenine metabolic processes. The remaining pathways comprised downregulated genes of NAD synthetase 1 (*Nadsyn1*), nicotinamide phosphoribosyltransferase (*Nampt*), nicotinate phosphoribosyltransferase (*Naprt*), poly [ADP-ribose] polymerase 9 (*Parp9*), prostaglandin I2 synthase (*Ptgis*), ATP(adenosine triphosphate)-dependent 6-phosphofructokinase (*Pfkm*), phosphoglycerate kinase (*Pgk*), phosphoglucomutase 2 like 1 (*Pgm2l1*), nudix hydrolase 17 (*Nudt17*), valosin containing protein (*Vcp*), oxoglutarate dehydrogenase (*Ogdh*), malic enzyme 1,-2,-3 (*Me1*, *Me2*, *Me3*), UDP-glucose 6-dehydrogenase (*Ugdh*), sorbitol dehydrogenase (*Sord*), NAD-dependent protein deacetylase sirtuin-3, -4 (*Sirt3*, *Sirt4*), dihydropteridine reductase (*Qdpr*), D-3-phosphoglycerate dehydrogenase (*Phgdh*), solute carrier family 36 family 4 member, (*Slc36a4*), solute carrier family 7 member 5 (*slc7a5*).

The upregulated genes in **Figure 3.1A** comprised aldehyde dehydrogenase 1 family member A1 (*Aldh1a1*), aldehyde dehydrogenase 1 family member A2 (*Aldh1a2*), 24-dehydrocholesterol reductase (*Dhcr24*), aldehyde dehydrogenase 1 family member A2 (*Aldh9a1*), amine oxidase copper containing 1 (*Aoc1*), monoamine oxidase-b (*Mao-b*), acetyl-CoA acetyltransferase 2 (*Acat2*), aldehyde dehydrogenase 1 family member B1 (*Aldh1b1*), aldehyde dehydrogenase 1 family member 13 (*Aldha13*), 3-hydroxybutyrate dehydrogenase type 2 (*Bdh2*), 15-hydroxyprostaglandin dehydrogenase (*Hpgd*), glyoxylate and hydroxypyruvate reductase (*Grhpr*), dimethylaniline monooxygenase [N-oxide-forming] 2 and -5 (*Fmo2*, *Fmo5*), cluster of differentiation 38 (*Cd38*), gamma-enolase 2 (*Eno2*), gamma-enolase 3 (*Eno3*), bisphosphoglycerate mutase (*Bpgm*),

hexokinase 3 (*Hk3*), hexokinase 2 (*Hk2*), glycerol phosphate dehydrogenase 2 (*Gpd2*), malate dehydrogenase 2 (*Mdh2*), indoleamine 2,3-dioxygenase1 (*Ido1*).

Heat maps of gene expression values for each colon sample were visualised using the web-based tool Morpheus. The heat map visualisation of the distal colons from 14-week-old *Winnie* mice (n=6, 3 females and 3 males) and age-matched C57BL/6 littermates (n=8, 4 females and 4 males) shows distinct differences. The heat maps shows the relationship and an obvious visual cue that is distinct between the upregulation and downregulation of gene expressions in the tryptophan metabolism pathway (**Figure 3.1B**), as well as nicotinate and nicotinamide pathway genes heat map (**Figure 3.1C**). The comparison showed non-significant differences between the sexes; therefore the sexes were merged for all further analysis.

Figure 3.1 Heat map representation of tryptophan and nicotinamide genes

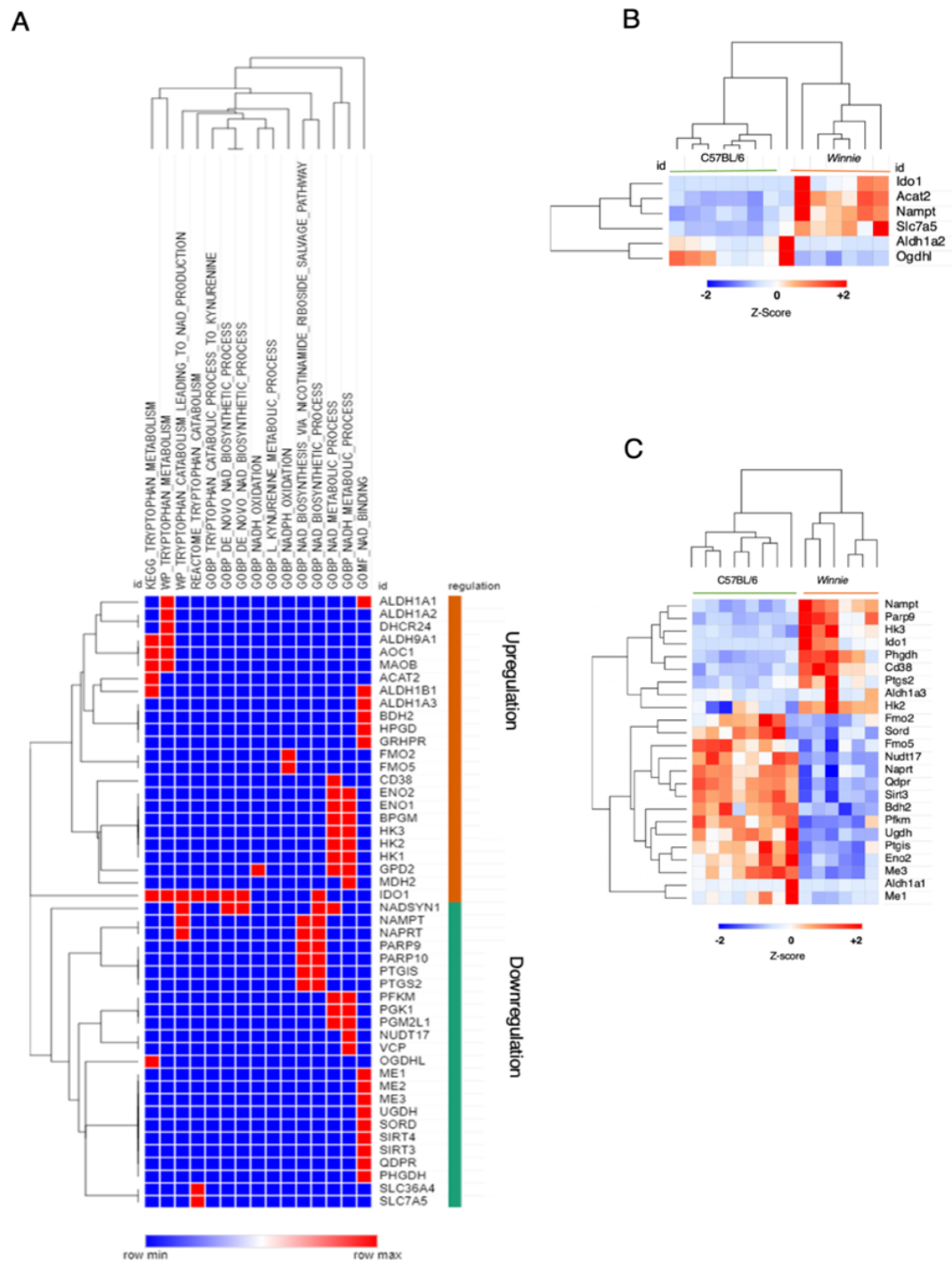
(A) Gene lists for tryptophan and nicotinamide-associated gene expressions were generated by merging related gene lists from multiple databases accessed via the molecular signature database (MSigDB). Heat map representation of upregulated and downregulated genes shows the relationship and an obvious visual cue that is distinct between the up and downregulation of genes.

(B) Heat map representation of tryptophan gene expressions. Plotted data indicate gene expression values from DEGs analysis between the colons of C57BL/6 mice and *Winnie* mice. Upregulated gene expressions (**red**), downregulated gene expressions (**blue**) and gene expressions that did not attain the cut-off (white).

(C) Heat map representation of nicotinamide gene expressions. Plotted data indicate gene expression values from DEGs analysis between the colons of C57BL/6 mice and *Winnie* mice. Nicotinamide metabolism upregulated gene expressions (**red**), downregulated gene expressions (**blue**) and gene expressions that did not attain the cut-off (white).

Abbreviations: acetyl-CoA acetyltransferase, *Acat2*; *Aldh1a1*; aldehyde dehydrogenase 1 family member A2, *Aldh1a2*; aldehyde dehydrogenase family 1 member A3, *Aldh1a3*; 3-hydroxybutyrate dehydrogenase type 2, *Bdh2*; cluster of differentiations, *Cd38*; gamma-enolase 2, *Eno2*; dimethylaniline monooxygenase [N-oxide-forming] 2, *Fmo2*; dimethylaniline monooxygenase [N-oxide-forming] 5, *Fmo5*; hexokinase 2, *Hk2*; hexokinase 3, *Hk3*; indoleamine 2,3-dioxygenase1, *Ido1*; nicotinamide phosphoribosyltransferase, *Nampt*; malic enzyme 1, *Me1*; malic enzyme 3, *Me3*; nucleoside diphosphate-linked moiety X motif 17, *Nudt17*; nicotinate phosphoribosyltransferase, *Naprt1*; oxoglutarate dehydrogenase, *Ogdh*; retinal dehydrogenase 1, poly [ADP-ribose] polymerase 9, *Parp9*; ATP-dependent 6-phosphofructokinase, *Pfkm*; D-3-phosphoglycerate dehydrogenase, *Phgdh*; prostacyclin synthase, *Ptgis*; dihydropteridine reductase, *Qdpr*; solute carrier family 7 member 5, *Slc7a5*; NAD-dependent protein deacetylase sirtuin-3, *Sirt3*; sorbitol dehydrogenase, *Sord*; UDP-glucose 6-dehydrogenase, *Ugdh*.

Figure 3.1 Heat map representation of tryptophan and nicotinamide DEGs



Input data included significance scores of gene expression values between *Winnie* and C57BL/6 littermates, computed as the sign $\text{Log}_2\text{FC} \times \text{Log}_{10}\text{P}$ values, and the relative expression of UFLC data. Pathview plots for tryptophan and nicotinamide pathways from the KEGG database and tryptophan compounds concentrations are represented in the KEGG graph. In *Winnie* mice, the mRNA transcripts included four upregulated gene expressions and seven downregulated gene expressions. Downregulated gene expressions comprise aryl-formamidase (*Afmid*); L-tryptophan decarboxylase (*Ddc*); S-adenosyl-L-methionine (*SAMe*); amine oxidase copper containing 1 (*Aoc1*); aldehyde dehydrogenase (NAD^+) (*Aldh2*); dehydrogenase E1 and transketolase domain containing 1 (*Dhtkd1*); glutaryl-CoA dehydrogenase (*Gcdh*). Upregulated gene expressions comprise indoleamine 2,3-dioxygenase1 (*Ido1*), monoamine oxidase-b (*Mao-b*), kynureninase (*Kynu*), and acetyl-CoA acetyltransferase 1 (*Acat1*). (Figure 3.2A, Table 3.2 Glossary of terms for tryptophan metabolism-associated gene expressions in *Winnie* mouse brains are extracted from <https://www.genome.jp/pathway/map00380>, Kanehisa and Goto (2000).

STRING identified biological pathways and the enrichment terms from GOBP, GOMF and GOCC from the *Winnie* mouse distal colon downregulated DEGs. The lists of the top 10 downregulated DEGs (Table 3.3) and the top 10 upregulated DEGs (Table 3.4). This process illuminates the extensive reach of tryptophan metabolism into metabolic pathways. Enrichment terms associated with tryptophan downregulated DEGs revealed amine metabolic process, indole-containing compound metabolic process metabolic pathways, histidine metabolism, fatty acid degradation, and lysine degradation, arginine and proline metabolism, metabolism of amino acids and derivatives. Enrichment terms associated with tryptophan upregulated DEGs revealed nucleotide biosynthetic process, tryptophan catabolic process to kynurenine, de novo NAD biosynthetic process from tryptophan, alpha-amino acid catabolic process, cellular nitrogen compound catabolic process, heterocycle catabolic process, aromatic compound catabolic process, cellular catabolic process.

Target genes of tryptophan in the *Winnie* mouse were identified. The homology in expression of these genes was determined in *Winnie* mice and IBD patients compared to

their respective uninflamed controls. In *Winnie* mice, the results showed 81% concordance in the expression of genes associated with tryptophan metabolism when compared to UC patients (**Figure 3.2C**). When comparing *Winnie* mice with patients diagnosed with CD, the concordance in the expression of tryptophan associated genes was 81% (**Figure 3.2C**). The similarities in gene expression patterns further support the utility of the *Winnie* mouse model in investigating the underlying molecular mechanisms associated in the context of IBD.

Figure 3.2 Tryptophan metabolism gene expressions

Pathview plot from the KEGG database produced the tryptophan gene expressions in the KEGG graph, representing the distal colon of 14-week-old *Winnie* mice (n=6 mice, 3 females and 3 males) versus age-matching C57BL/6 littermates (n=8 mice, 4 females and 4 males).

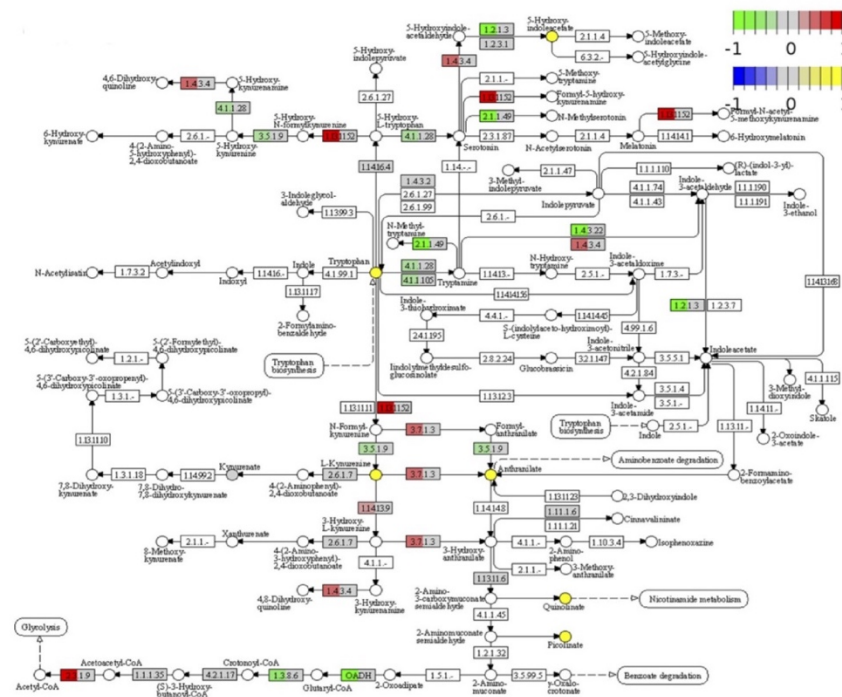
(A) Rectangular nodes indicate gene expression data by RNA-Seq. Gene expression data (**green** to **red**) presented as significance scores for gene expression analysis between the colons of *Winnie* mice and C57BL/6 littermates. **Green** represents downregulated genes expressions comprising electronic commission (EC):3.5.1.9, *Afmid*; EC:4.1.1.28 and EC:4.1.1.105, *Ddc*; EC:2.1.1.49, *SAMe*; EC:1.4.3.22, *Aoc1*; EC:1.2.1.3, *Aldh2*; OADH, *Dhtkd1*; EC:1.3.8.6, *Gcdh*. **Red** represents upregulated genes expressions; EC:1.4.3.4, *Mao-b*; EC:1.13.11.52, *Ido1*; EC:3.7.1.3, *Kynu*; EC:2.3.1.9, *Acat1*. Circular nodes indicate relative expression of compounds by UFLC (**blue** to **yellow**) between the distal colons of *Winnie* and C57BL/6 littermates (n=8 mice/group). White represents metabolites and gene expressions showing non-significant differences. **Blue** represents statistically higher concentrated tryptophan metabolites measured by UFLC, including tryptophan, 5-HIAA, kynurenine, quinolinate and picolinate.

(B) Homology of tryptophan-associated genes in *Winnie* mice and IBD patients determined by RNA-Seq. Heat map representation of upregulated (**red**), downregulated (**green**), unchanged (**black**) genes associated with colonic inflammation.

Abbreviations: aryl-formamidase, *Afmid*; L-tryptophan decarboxylase, *Ddc*; S-adenosyl-L-methionine, *SAMe*; amine oxidase copper containing 1, *Aoc1*; aldehyde dehydrogenase (NAD⁺), *Aldh2*; dehydrogenase E1 and transketolase domain containing 1, *Dhtkd1*; glutaryl-CoA dehydrogenase, *Gcdh*; indoleamine 2,3-dioxygenase1, *Ido1*; monoamine oxidase, *Mao-b*; kynureninase, *Kynu*; acetyl-CoA acetyltransferase 1, *Acat1*.

Figure 3.2 Tryptophan metabolism gene expressions in Winnie mice distal colons

A



B

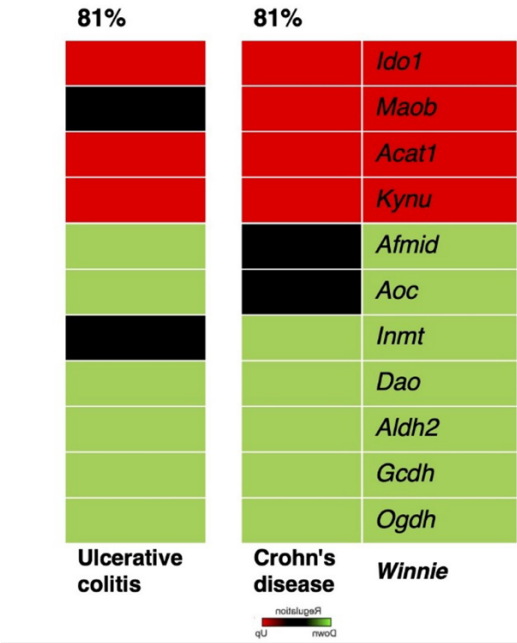


Table 3.2 Glossary of terms for tryptophan metabolism-associated genes

Reaction	Substrate	Product	Comment
Downregulated gene expressions			
L-tryptophan decarboxylase (<i>Ddc</i>) EC 4.1.1.28 EC 4.1.1.105			
5-hydroxy-kynurenamine decarboxylase. 5-hydroxy-L-tryptophan = 5-HT + CO ₂	5-hydroxy-L-tryptophan	CO ₂ , 5-hydroxy-tryptamine	<i>Ddc</i> synthesises dopamine and serotonin via decarboxylation of L-3,4-dihydroxyphenyl-alanine and L-5-hydroxytryptophan.
S-Adenosyl-L-methionine (<i>SAMe</i>) EC:2.1.1.49			
1). S-Adenosyl-L-methionine + serotonin <=> S-adenosyl-L-homocysteine + N-methylserotonin 2). S-Adenosyl-L-methionine + tryptamine <=> S-adenosyl-L-homocysteine + N-methyltryptamine	S-adenosyl-L-methionine, amine	S-adenosyl-L-homocysteine, methylated amine	<i>SAMe</i> primary, secondary and tertiary amines act as acceptors, including tryptamine, aniline, nicotine, and xenobiotics.
Amine oxidase copper containing 1, diamine oxidase (<i>Aoc</i>) EC:1.4.3.22			
Histamine + H ₂ O + O ₂ = (imidazol-4-yl) acetaldehyde + NH ₃ + H ₂ O ₂	Histamine, H ₂ O, O ₂	(Imidazol-4-yl) acetaldehyde, NH ₃ , H ₂ O ₂	<i>Aoc</i> breaks down diamines, histamines and monoamines.
Aldehyde dehydrogenase (NAD⁺) (<i>Aldh2</i>) EC 1.2.1.3			

5-Hydroxy-indoleacetaldehyde + NAD ⁺ + H ₂ O <=> 5-Hydroxyindoleacetate + H ⁺ + NADH	Aldehyde; NAD ⁺ , H ₂ O	Carboxylate; NADH, H ⁺	<i>Aldh</i> catalyse the reduction of indole-3-acetaldehyde to indole-3-ethanol with the oxidation of NAD(P)H to NAD(P) ⁺ .
Aryl-formamidase (<i>Afmid</i>) EC:3.5.1.9			
1). N-formyl-L-kynurenine + H ₂ O <=> Formate + L-kynurenine	N-formyl-L-kynurenine, H ₂ O	Formate, L-kynurenine	<i>Afmid</i> catalyses the hydrolysis of N-formyl-L-kynurenine to L-kynurenine. Required for elimination of toxic metabolites.
Dehydrogenase E1 and transketolase domain containing 1 (<i>Dhtdk1</i>) OADH			
Succinyl-CoA + enzyme N6-(dihydrolipoyl)lysine = CoA + enzyme N6-(S-succinyl dihydrolipoyl)lysine	Succinyl-CoA; enzyme N6-(dihydrolipoyl)lysine	CoA; enzyme N6-(S-succinyl dihydrolipoyl)lysine	A multimer forms the core of the multienzyme complex, and binds tightly to oxoglutarate dehydrogenase and dihydrolipoyl dehydrogenase.
Upregulated gene expressions			
Monoamine oxidase (<i>Mao</i>) EC 1.4.3.4			
1). 5-HT + H ₂ O + O ₂ <=> 5-Hydroxyindole-acetaldehyde + NH ₃ + H ₂ O ₂ 2). 3-Hydroxy-kynurenamine + O ₂	RCH ₂ NHR'; H ₂ O, O ₂	RCHO, R'NH ₂ , H ₂ O ₂	<i>Mao</i> - mitochondrial-bound flavoenzymes catalyse the oxidative metabolism of

$\rightleftharpoons$ 4,8-Dihydroxyquinoline + NH ₃ + H ₂ O ₂ 3). Tryptamine + H ₂ O + O ₂ $\rightleftharpoons$ Indole-3-acetaldehyde + NH ₃ + H ₂ O ₂			biogenic amines, 5-HT. Inhibitor: chlorgyline, 1-deprenyl and pargyline.
Indoleamine 2,3-dioxygenase (<i>Idol</i>) EC:1.13.11.52			
1). L-tryptophan + O ₂ $\rightleftharpoons$ N-formyl-L-kynurenine	D-tryptophan, O ₂ , L-tryptophan	N-formyl-D-kynurenine, N-formyl-L-kynurenine	Pathological and host-defense mechanisms induce IDO activity.
Kynureninase (<i>Kynu</i>) EC:3.7.1.3			
1). L-kynurenine + H ₂ O = anthranilate + L-alanine	L-kynurenine, H ₂ O	Anthranilate, L-alanine	Kynureninase - aminotransferase superfamily. Mitochondrial flavoprotein Cofactor: B6

Table 3.3 Enrichment terms of tryptophan downregulated genes

Category	Term ID	Term description	Observed gene count	Back ground gene count	Strength	False discovery rate (FDR)
GOBP	GO:0009308	Amine metabolic process	4	52	2.38	1.67E-05
GOBP	GO:0042430	Indole-containing compound metabolic process	3	23	2.61	.00033
GOBP	GO:0006568	Tryptophan metabolic process	2	11	2.76	.0289

Table 3.4 Enrichment terms of tryptophan upregulated genes

Category	Term ID	Term description	Observed gene count	Back ground gene count	Strength	FDR
GOBP	GO:0009165	Nucleotide biosynthetic process	3	188	1.94	.0033
GOBP	GO:0019441	Tryptophan catabolic process to kynurenine	2	5	3.34	.0033
GOBP	GO:0034354	<i>De novo</i> NAD ⁺ biosynthetic process from tryptophan	2	6	3.26	.0033
GOBP	GO:1901606	Alpha-amino acid catabolic process	3	86	2.28	.0033
GOBP	GO:0044270	Cellular nitrogen compound catabolic process	3	279	1.77	.0041
GOBP	GO:0046700	Heterocycle catabolic process	3	282	1.77	.0041
GOBP	GO:0019439	Aromatic compound catabolic process	3	297	1.75	.0045
GOBP	GO:0006084	Acetyl-CoA metabolic process	2	32	2.54	.0052
GOBP	GO:0044248	Cellular catabolic process	4	1445	1.18	.0063
GOBP	GO:0032787	Monocarboxylic acid metabolic process	3	461	1.55	.0102

3.4.2 Changes in nicotinamide metabolism associated genes

The *Winnie* mouse distal colons exhibited downregulated gene expressions of nicotinamide mononucleotide adenylyl transferases (*Nmnat 2* and *Nmnat3*), silent information regulator 2 (*Sirt2*), nicotinamide ribose kinases 1 and 2 (*Nnmt1*, *Nnmt2*), nicotinamide adenine dinucleotide synthetase 1 (*Nadsyn1*) and nicotinate phosphoribosyltransferase (*Naprt*). The upregulated gene expressions of the nicotinamide pathways included cluster of differentiation 38 (*Cd38*), nicotinamide phosphoribosyltransferase (*Nampt*), adenosine diphosphate (ADP)-ribosyl transferase2a (*Art2a*), ecto-5'-nucleotidase (*Cd73*), and purine-nucleoside phosphorylase (*Pnp*) (**Figure 3.3A**, **Table 3.5** Glossary of terms for nicotinamide-associated gene expressions in *Winnie* mouse brains are extracted from <https://www.genome.jp/pathway/map00760n> (accessed on 30th September, 2022), Kanehisa and Goto (2000)).

STRING was employed to seek enrichment terms associated with downregulated DEGs in nicotinamide metabolism uncovered NAD biosynthetic process, organonitrogen compound metabolic process, nicotinamide-nucleotide adenylyltransferase activity. GO molecular functions downregulated DEGs enrichment terms uncovered nicotinamide-nucleotide adenylyltransferase activity, nucleotide binding, Transferase activity, transferring pentosyl groups, ligase activity, forming carbon-nitrogen bonds, anion binding, catalytic activity, nicotinate and nicotinamide metabolism, metabolic pathways, and nicotinate metabolism.

The homology in expression of nicotinamide genes was determined between *Winnie* mice and IBD patients and their respective uninflamed controls. The results revealed that the expression of nicotinamide metabolism-associated genes in *Winnie* mice exhibited a 64% concordance with UC patients (**Figure 3.3B**). When comparing these genes between *Winnie* mice and CD patients, the concordance of nicotinamide metabolism-associated genes was 45% (**Figure 3.3B**). Although slightly lower than the concordance with UC patients, it still suggests a similarity in the expression patterns of these metabolic genes between *Winnie* mice and CD patients.

Figure 3.3 Nicotinamide gene expressions in *Winnie* mice distal colons

(A) Pathview plot from the KEGG database produced the nicotinate and nicotinamide gene expressions in the KEGG graph, representing the distal colon of 14-week-old *Winnie* mice ($n=6$ mice/group) versus age-matching control C57BL/6 littermates ($n=7$ mice/group). Rectangular nodes indicate gene expression data by RNA-Seq. Gene expression data (green to red) presented as significance scores for gene expression analysis between the colons of *Winnie* and C57BL/6 littermates. Circular nodes indicate the relative expression of compounds by UFLC (blue to yellow) between the distal colons of *Winnie* and C57BL/6 littermates.

Green represents downregulated gene expressions comprising EC:2.3.1.286, *Sirt2*; EC:2.7.7.1, *Nmnat2*; EC:2.7.7.18, *Nmnat3*; EC:2.7.1.22, *Nmrk1*; EC:2.7.1.173, *Nmrk2*; EC:6.3.5.1, *Nadsyn1*; EC:6.3.4.21, *Naprt*. Red represents upregulated gene expressions comprising EC:2.4.99.20, *Cd38*; EC:3.2.2.6, *Cd38*; EC:3.2.2.5, *Art2a*; EC:2.4.2.12, *Nampt1*; EC:3.1.3.5, *Cd73*; EC:2.4.2.1, *Pnp*. Blue represents statistically highly concentrated tryptophan metabolites of quinolinate measured by UFLC.

(B) Homology of nicotinamide-associated genes in *Winnie* mice and IBD patients. Heat map representation of upregulated (red), downregulated (green), unchanged (black) genes associated with colonic inflammation determined by RNA-Seq.

Abbreviations: nicotinamide mononucleotide adenylyl transferases, *Nmnat*; silent information regulator 2, *Sirt2*; nicotinamide ribose kinases, *Nmrk*; nicotinamide adenine dinucleotide synthetase1, *Nadsyn1*; nicotinate phosphoribosyltransferase, *Naprt*; cluster of differentiation 38, *Cd38*; nicotinamide phosphoribosyltransferase, *Nampt*; ADP-ribosyl transferase2a, *Art2a*, *Parp2*; ecto-5'-nucleotidase, *Cd73*, *Nt5e*; purine-nucleoside phosphorylase, *Pnp*.

Figure 3.3 Nicotinamide metabolism gene expressions in *Winnie* mice distal colons

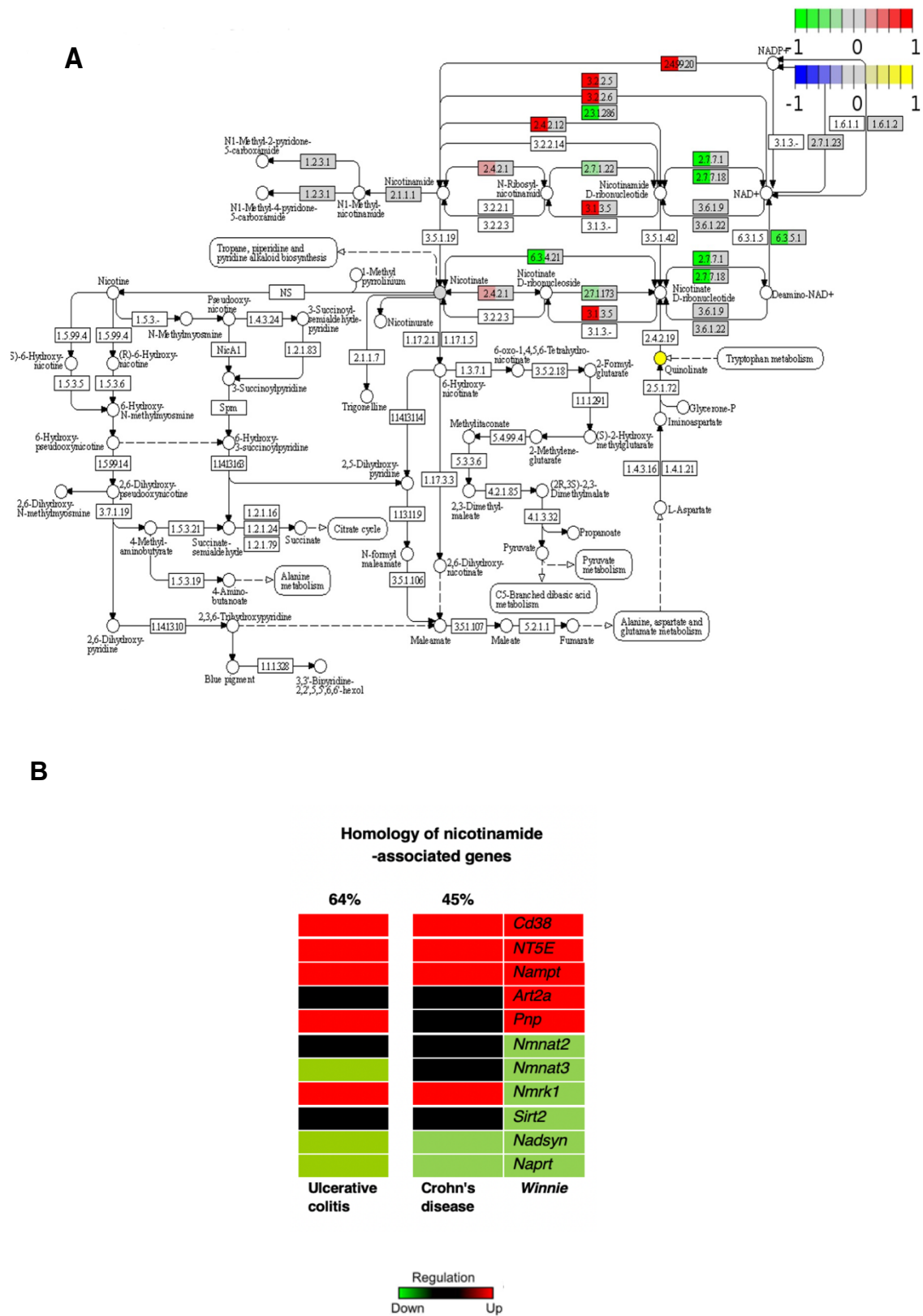


Table 3.5 Glossary of terms for nicotinamide-associated genes

Reaction	Substrate	Product	Comment
Downregulated gene expressions			
Sirtuin2 (<i>Sirt2</i>) EC:2.3.1.286			
[protein]-N6-acetyl-L-lysine + NAD ⁺ + H ₂ O <=> [protein]-L-lysine + 2"-O-acetyl-ADP-D-ribose + nicotinamide	[protein]-N6-acetyl-L-lysine; NAD ⁺ , H ₂ O	[protein]-L-lysine, 2"-O-acetyl-ADP-D-ribose, Niacinamide	<i>Sirtuins</i> regulate deacetylating proteins. NAD ⁺ dependent protein deacetylase.
Nicotinamide mononucleotide adenylyl transferase (<i>Nmnat2</i> - Golgi apparatus; <i>Nmnat3</i> - mitochondria) EC:2.7.7.1 EC:2.7.7.18			
ATP + nicotinamide ribonucleotide = diphosphate + NAD ⁺	ATP, β-nicotinate D-ribonucleotide	Diphosphate, NAD ⁺	<i>Nmnat</i> catalyses the formation of NAD ⁺ from NMN and ATP. Cofactor: Magnesium
Nicotinamide riboside kinase (<i>Nmrk1</i>) EC:2.7.1.22			
ATP + 1-(β-D-ribofuranosyl)-nicotinamide <=> ADP + β-nicotinamide D-ribonucleotide	ATP, 1-(β-D-ribofuranosyl)-nicotinamide	ADP, β-nicotinamide D-ribonucleotide	<i>Nmrk</i> encodes for NR/NMN-driven NAD ⁺ synthesis. ATP:N-ribosylnicotinamide 5'-phosphotransferase
Nicotinamide riboside kinase (<i>Nmrk2</i>) EC:2.7.1.173			
Nicotinate D-ribonucleotide + ADP <=> Nicotinate D-ribonucleoside + ATP	ATP, β-D-ribosylnicotinate	ADP, nicotinate β-D-ribonucleotide	<i>Nmrk</i> encoding for NR/NMN-driven NAD ⁺ synthesis; ATP: β-D-ribosylnicotinate 5-phosphotransferase
NAD synthetase 1 - glutamine-hydrolysing (<i>Nadsyn1</i>) EC:6.3.5.1			
ATP + Deamido - NAD ⁺ + L-Glutamine + H ₂ O <=> AMP + Diphosphate + NAD ⁺ + L-Glutamate	ATP, Deamido-NAD ⁺ , L-glutamine, H ₂ O	AMP, Diphosphate, NAD ⁺ , L-glutamate	<i>Nadsyn</i> the ATP-dependent amidation of deamido-NAD ⁺ using L-glutamine as a nitrogen source.
Nicotinate phosphoribosyl-transferase; niacin ribonucleotidase (<i>Naprt</i>) EC:6.3.4.21			

Nicotinate D-ribonucleotide + Diphosphate + ADP + Orthophosphate $\rightleftharpoons$ Nicotinate + 5-Phospho-alpha-D-ribose 1-diphosphate + ATP + H ₂ O + H ⁺	Nicotinate; 5-phospho-alpha-D-ribose 1-diphosphate, ATP, H ₂ O	β -nicotinate D-ribonucleotide, diphosphate, ADP, phosphate	<i>Naprt</i> , pyridine nucleotide recycling, can form β -nicotinate D-ribonucleotide and diphosphate from nicotinate and 5-phospho-alpha-D-ribose 1-diphosphate in the absence of ATP.
Upregulated gene expressions			
Cluster of differentiation 38 (<i>Cd38</i>) EC:2.4.99.20 EC:3.2.2.6			
1). NADP ⁺ + Nicotinate $\rightleftharpoons$ NAM + NaAD dinucleotide phosphate	1). NADP ⁺ , Nicotinate, 2'-phospho-cyclic ADP-ribose	1). NADH, NAM, 2'-phospho-cyclic ADP-ribose	<i>CD38</i> catalyses the removal of NAM from NADP ⁺ , forming 2'-phospho-cyclic ADP-ribose, and the addition of nicotinate to the cyclic product, forming NADP ⁺ .
ADP-ribosyl-transferase 2a (<i>Art2a</i>) EC:3.2.2.5			
NAD ⁺ + H ₂ O = ADP-D-ribose + NAM	NAD ⁺ , H ₂ O	ADP-D-ribose, NAM	<i>Art2a</i> catalyses the hydrolysis of NAD ⁺ , without associated ADP-ribosyl cyclase activity.
Nicotinamide phosphoribosyl-transferase (<i>Nampt1</i>) EC:2.4.2.12			
Nicotinamide D-ribonucleotide + diphosphate = NAM + 5-phospho-alpha-D-ribose 1-diphosphate	Nicotinamide D-ribonucleotide, diphosphate	NAM, 5-phospho-alpha-D-ribose 1-diphosphate	<i>Nampt</i> catalyses the condensation of NAM with 5-phosphoribosyl-1-pyrophosphate to yield NMN.
Purine-nucleoside phosphorylase (<i>Pnp</i>) EC:2.4.2.1			
Nicotinate D-ribonucleoside: orthophosphate ribosyl transferase.	Purine ribonucleoside; phosphate, purine 2'-deoxyribo-nucleoside.	Purine; alpha-D-ribose 1-phosphate, 2-deoxy-alpha-D-ribose 1-phosphate	<i>Pnp</i> catalyses the phosphorolytic breakdown of the N-glycosidic bond in the β - (deoxy) ribonucleoside molecules, with the formation of free

			purine bases and pentose-1-phosphate.
5',3'-Nucleotidase (Cd73) EC:3.1.3.5			
1).Nicotinamide D-ribonucleotide + H ₂ O <=> Nicotinamide- β-riboside + Orthophosphate	5'-ribonucleotide, H ₂ O	Ribonucleoside, phosphate	Dephosphorylates the 5' and 2'(3')- phosphates of uracil and thymine deoxyribonucleotides, protects mitochondrial DNA replication from excess dTTP.

Table 3.6 Enrichment terms of nicotinamide metabolism downregulated genes

Category	Term ID	Term description	Gene count	Back-ground gene count	Strength	FDR
GOBP	GO:0009435	NAD biosynthetic process	5	16	3.06	3.62E-11
GOBP	GO:1901564	Organonitrogen compound metabolic process	6	4475	0.69	.0323
GOMF	GO:0000309	Nicotinamide-nucleotide adenylyl-transferase activity	2	3	3.39	.002
GOMF	GO:0000166	Nucleotide binding	5	2021	0.96	.0292
GOMF	GO:0016740	Transferase activity	5	2088	0.94	.0292
GOMF	GO:0016763	Transferase activity, transferring pentosyl groups	2	50	2.17	.0347
GOMF	GO:0016879	Ligase activity, forming carbon-nitrogen bonds	2	48	2.19	.0347
GOMF	GO:0043168	Anion binding	5	2678	0.84	.0463
GOMF	GO:0003824	Catalytic activity	6	5224	0.63	.0485

Table 3.7 Enrichment terms of nicotinamide metabolism upregulated genes

Category	Term ID	Term description	Gene count	Back-ground gene count	Strength	FDR
GOMF	GO:0016763	Transferase activity, transferring pentosyl groups	3	50	2.42	.00042
KEGG	mmu00240	Pyrimidine metabolism	2	57	2.19	.0116
KEGG	mmu01100	Metabolic pathways	4	1536	1.06	.0124
KEGG	mmu00230	Purine metabolism	2	134	1.82	.031

3.4.3 Tryptophan pathway metabolites in the *Winnie* mouse

Metabolomics analysis was employed to quantify the tryptophan metabolites associated with the transcriptome. The metabolites of tryptophan, 5-HIAA, KYNA, kynurenine, QUIN, nicotinamide, and PIC in the distal colons of *Winnie* mice compared to C57BL/6 littermates were measured by UFLC (**Figure 3.4**). Non-significant differences were identified between the males and females; therefore, the data for both sexes were merged for further analysis. Significantly higher concentrations of tryptophan ($p=0.010$), 5-HIAA ($p=0.010$), kynurenine ($p=0.002$), QUIN ($p<0.001$), and PIC ($p=0.012$) were found in the *Winnie* mouse colons compared to controls, whereas non-significant differences in concentrations of KYNA and nicotinamide were observed (**Table 3.8**).

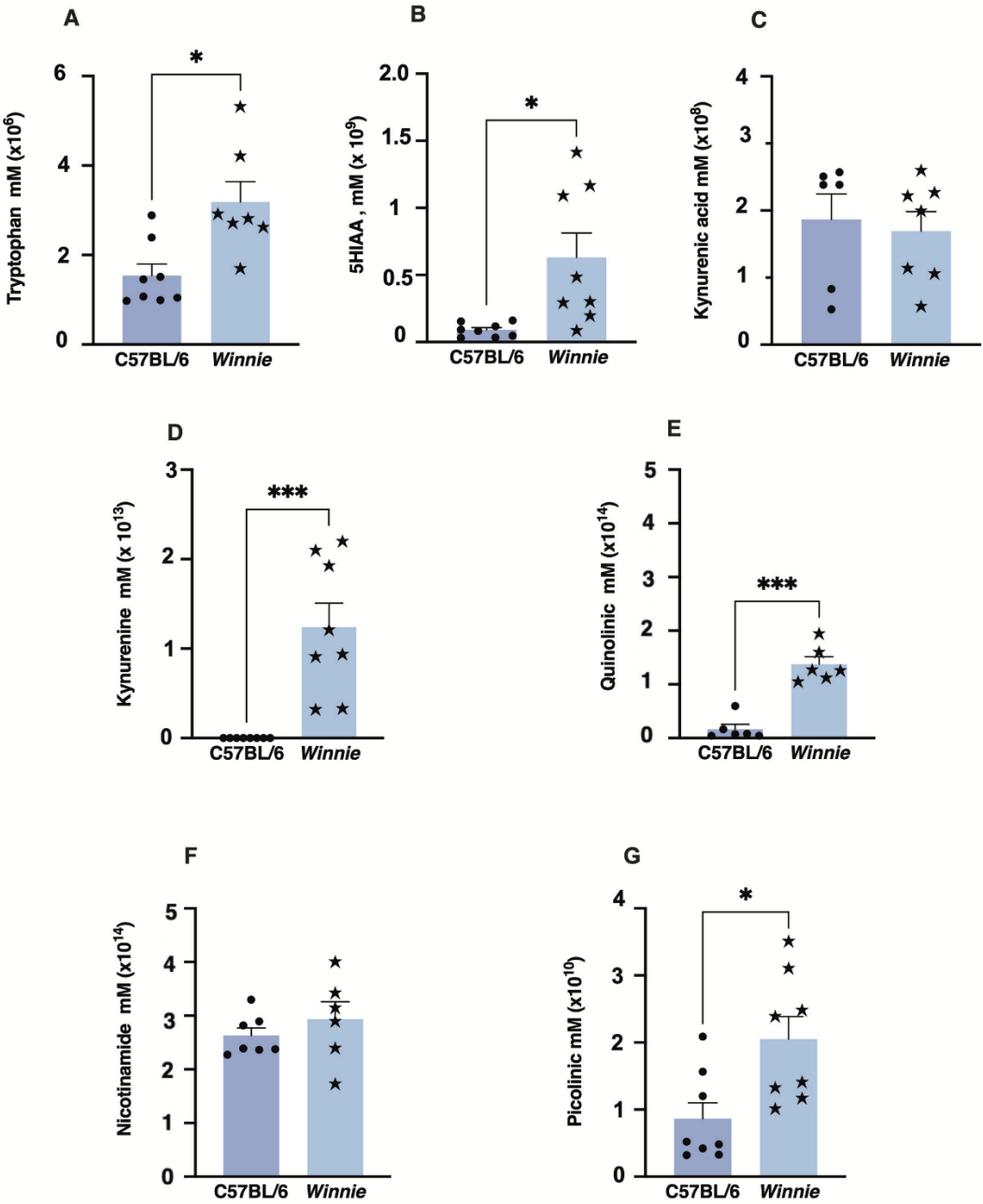
Table 3.8 Tryptophan metabolite concentrations in *Winnie* mouse colons

Metabolite mM	C57BL/6	SEM	<i>n</i>	<i>Winnie</i>	SEM	<i>n</i>	<i>p</i> value
Tryptophan	1.5421E+06	2.5446E+05	n=8	3.1898E+06	4.5154E+05	n=6	0.010
5-HIAA	9.0011E+07	1.8232E+07	n=7	6.3066E+08	1.8146E+08	n=6	0.010
Kynurenine	5.6546E+07	2.2829E+07	n=8	6.2113E+12	1.3378E+12	n=8	0.002
KYNA	4.6700E+07	9.4818E+06	n=8	4.2403E+07	7.2018E+06	n=7	ns
QUIN	1.6638E+13	8.8030E+12	n=5	1.3758E+14	1.3767E+13	n=6	<0.001
Nicotinamide	2.6301E+14	1.4396E+13	n=8	2.9351E+14	3.2542E+13	n=8	non-significant
PIC	1.7323E+10	4.7378E+09	n=8	4.1064E+10	6.7288E+09	n=8	0.012

Figure 3.4 Tryptophan metabolites in *Winnie* mice distal colons

Tryptophan metabolites in *Winnie* mice distal colons (14-week-old , n=8 mice/group) compared age-matched C57BL/6 littermates were measured using UFLC. **(A)** Tryptophan. **(B)** 5-HIAA. **(C)** Kynurenine. **(D)** Kynurenic acid. **(E)** Quinolinic acid. **(F)** Nicotinamide. **(G)** Picolinic acid. * $p < 0.02$, *** $p < 0.001$. Data are expressed as mean $\pm$ SEM. **Abbreviations:** 5-HIAA, 5-Hydroxy indoleacetic acid; mM millimolar.

Figure 3.4 Tryptophan metabolites in the *Winnie* mice distal colons



3.5 Discussion

The premise of this study was to identify changes in the tryptophan metabolism leading to *de novo* NAD⁺ synthesis transcriptome and the associated metabolites within the distal colon of the *Winnie* mouse. By analysing the transcriptome in the *Winnie* mouse, we were able to establish the upregulated and downregulated genes. Hereby, dysregulation in tryptophan metabolism within the distal colon of *Winnie* mice revealed the upregulation of genes such as *Ido1*, *Mao-b*, *Kynu*, and *Acat1*, and the downregulation of *Afmid*, *Ddc*, *SAME*, *Aoc1*, *Aldh2*, *Dhtkd1*, and *Gcdh*. These gene alterations corresponded to changes in tryptophan metabolite concentrations, leading to elevated levels of tryptophan, 5-HIAA, kynurenine, QUIN, and PIC in the examined *Winnie* mice. The significance and implications of these genetic and metabolic shifts will be further elucidated.

3.5.1 Upregulated tryptophan-associated DEGS in the *Winnie* mice distal colons

In homeostatic states, IDO1 expression is low within the normal murine and human intestinal lamina propria, however in active IBD IDO1 is highly expressed (Ciorba, 2013). In the *Winnie* mouse distal colon, *Ido1* expression upregulated which correlates with the overexpression of pathogenic factors namely IFN- γ , TNF- α , IL-6, and bacterial LPS (Ciorba, 2013). Likewise, *Ido1*^{+/+} mice with dextran sulfate sodium (DSS)-induced colitis expressed elevated levels of IL-1 β and TNF- α and incidentally developed more severe colitis than the *Ido1*^{-/-} mice (Shon et al., 2015). Overall, elevated IDO1 signifies the enhanced degradation of tryptophan, thereby impeding the responsiveness of T cells through the induction of anergic state. T-cell-mediated immune function is frequently dysregulated in immunocompromised conditions, including IBD, and chronic illnesses (Mow et al., 2004). Overexpression of IDO1 in ulcerated colon sites in humans, has been associated with a range of adverse effects, including shortened colon length, intestinal bleeding, diarrhoea, and reduced microbial proliferation; factors considered instrumental in shaping gastrointestinal diseases (Ciorba, 2013). Such observations align with the pathophysiological patterns observed in *Winnie* mice and other murine models of colitis (Ciorba, 2013; Robinson et al., 2017).

Similar to IDO1, the *Mao* genes are linked to IL-1 β , IL-6, IFN- γ and TNF- α (Billett, 2004). Our study revealed the upregulation of *Mao-b* in the *Winnie* mouse colon, which correlates with the immunological characteristics of severe chronic inflammation in this mouse model (Eri et al., 2011). In the gut, MAO-B overexpression is prominent in tissues and oxidative stress and tissues from colorectal cancer patients (Yang et al., 2020). Regardless of its location, the overexpression of *Mao* results in the production of adverse aldehydes, neurotoxic levels of H₂O₂, ammonia-producing oxidative stress molecules, and elevated reactive oxygen species (ROS); leading to accumulative mitochondrial damage and premature neuronal apoptosis. Such biochemical changes disrupt the epithelial barrier permeability, enabling luminal pathogen invasion (Bortolato et al., 2010; Prah et al., 2020; Robinson et al., 2017). It is of interest that *Winnie* mouse faeces revealed significantly higher levels of tyramine, the first known *Mao* substrate (Robinson et al., 2016). In humans and DSS-treated colitis mouse model, elevated levels of tyramine and 5-HIAA in the gut cause gastrointestinal dysmotility, resulting in spasms and visceral discomfort (Bertrand et al., 2009; Hansen et al., 2008; Rafehi et al., 2019). These alterations mirror the impaired smooth muscle contractility and altered colonic function seen in the *Winnie* mouse (Robinson et al., 2017; Stavely et al., 2018).

We observed an upregulation of *Kynu*, an enzyme that catalyses hydrolytic cleavage of L-kynurenine into L-alanine or anthranilic acid metabolites. If kynurenine and hydroxykynurenine are produced at a rate surpassing *Kynu* metabolic capacity then kynurenine aminotransferase may increase the production of KYNA and xanthurenic acid (Fujigaki et al., 2017). This study found non-significant differences in KYNA concentrations between *Winnie* mice and controls. This finding suggests that the upregulation of *Kynu* promoted the conversion of kynurenine towards QUIN, which is significantly higher in concentration in the *Winnie* mouse than controls. Although limited, existing literature connects *Kynu* overexpression with similar patterns seen in both skin and gut epithelial tissue (Gallo & Hooper, 2012). For example, in psoriasis, *Kynu* upregulation was detected in skin epithelial tissue; earning *Kynu* the title of a "molecular scar gene" (Harden et al., 2016). These preliminary findings suggest a potential role of *Kynu* in the epithelial hyperplasia observed in the colon of the *Winnie* mouse (Rahman et al., 2015).

This study observed an upregulation of mitochondrial *Acat1* in *Winnie* mice. It is noteworthy that increased cholesterol content in the endoplasmic reticulum triggers an allosteric regulation of ACAT1 (Liu et al., 2005). Yet, our findings contrast with a study involving acute DSS-induced colitis mice and data from human IBD cases, where the study reported a reduction in ACAT1 in the colonic mucosa during active disease, hinting at a potential defect in ketone utilisation (Huang et al., 2022). This discrepancy might be explained by differences in IBD activity, dietary habits, and cholesterol levels in humans, and the absence of abnormal dyslipidaemia in acute colitis-induced mice. As *Winnie* mice develop chronic colitis, we propose that ACAT1 may respond to cholesterol levels in *Winnie* mice through a homeostatic upregulation; a possibility that warrants further investigation for confirmation (Kwon et al., 2021).

3.5.2 Downregulated tryptophan-associated DEGs in *Winnie* mice distal colons

Afmid, a gene encoding arylformamidase, an enzyme that hydrolyses N-formylkynurenine, the precursor to kynurenine, was downregulated in *Winnie* mice. The distribution of kynurenine, contingent on IDO1 expression, dictates the ratio between neuroprotective KYNA and neurotoxic QUIN (Dobrovolsky et al., 2005). Downregulated *Afmid* correlates with higher aldehyde, butanal, and propanol levels, which have been previously observed in *Winnie* mice faeces (Robinson et al., 2016). This aberration seems to be triggered by the upregulation of *Mao-b* aldehyde synthesis and the downregulation of *Aldh2*, thereby compromising aldehyde detoxification and increasing the susceptibility of *Winnie* mice to aldehyde-induced damage and a limited capacity to detoxify ROS (Kim et al., 2017). Shifts in tryptophan metabolism towards the kynurenine pathway bear significant implications for *de novo* NAD⁺ synthesis. In this context, upregulation of 3-hydroxy-kynurenamine (a precursor to QUIN), 3-hydroxyanthranilic acid (3-HAA) (a precursor to anthranilic acid), or indol-3-yl pyruvic acid (a precursor to IDO1) inhibit *Aldh2* expression, corroborating the findings of this study (Badawy & Morgan, 2007; Koppaka et al., 2012).

The downregulation of *Ddc* constrains the enzymatic activity of gastrointestinal microbes, thereby causing an imbalance in intestinal microbiome composition (Williams et al., 2014). Under normal conditions, enzymatic gut microbes, specifically *Firmicutes*,

catalyse tryptophan decarboxylation, generating tryptamine-containing monoamines, thereby promoting intestinal immunity by activating AhR in epithelial, endothelial, and neuronal cells (Williams et al., 2014). The *Ddc* downregulation corroborates previous findings of altered *Bacteroidetes* and *Firmicutes* diversity in *Winnie* mouse faecal samples, alongside increased *Actinobacteria* and *Bifidobacterium* (Robinson et al., 2016). The intestinal microbiota partakes directly in protein and free amino acid metabolism, yielding metabolites that influence epithelial cell signalling pathways and play a direct role in inflammation (Robinson et al., 2016).

Downregulated *SAMe* suggests an enhanced bacterial production of anionic sulphide, culminating in acute oxidative damage to colon tissues (Schmedes et al., 2004). For instance, in healthy colonocytes, the expression of *SAMe* and methionine typically suppress sulphide methylation, thereby mitigating the toxicity of methanethiol and dimethylsulphide. This formation of *SAMe* serves as a protective mechanism against harmful luminal sulphides (Bottiglieri, 2002). In context, severely ulcerated human colons express *SAMe* downregulation, which inhibits hydrogen sulphide detoxification (Schmedes et al., 2004). Oxidative stress is a principal factor contributing to tissue damage in colitis. Patients with active CD and UC have demonstrated decreased mucosal antioxidant capacity and plasma antioxidants (Wang et al., 2016).

Downregulated *Aoc1* encodes for the enzyme diamine oxidase (DAO) and is responsible for regulating rapidly proliferating intestinal mucosal cells synthesised on the apical surfaces of enterocytes (Sánchez-Pérez et al., 2022). *AOC1* catalyses the breakdown of putrescine into gamma-aminobutyraldehyde (Sánchez-Pérez et al., 2022). However, *AOC1* downregulation diverts ornithine metabolism away from putrescine, corroborating previous research that identified reduced putrescine levels in *Winnie* mouse faecal samples (Robinson et al., 2016). This reaction hampers the digestion of biogenic amines produced by bacterial decarboxylation of food within the gastrointestinal tract. Dysregulation of amines has been associated with inflammation and a host of clinical disorders. An imbalance between *AOC1* and ingested biogenic amines can result in disturbed gastrointestinal activity, known as dietary histaminosis or enteral histaminosis (Schnedl et al., 2019). Histamine reactions can impede intestinal mucosal cell

proliferation, exacerbate intestinal permeability and visceral hypersensitivity (Honzawa et al., 2011), and increase the cortisol to dehydroepiandrosterone ratio (Miyake et al., 2014).

Gcdh, encodes for the critical enzyme GCDH in the mitochondrial oxidation of L-lysine, L-hydroxylysine, and L-tryptophan (Kölker et al., 2006), and was downregulated in the *Winnie* mouse. During L-tryptophan catabolism, GCDH catalyses the oxidative decarboxylation of glutaryl-CoA to glutaconyl-CoA, and glutaminy-CoA to crotonyl-CoA in rodents (Braissant et al., 2017). GCDH deficiency results in the toxic accumulation of glutaric acid. If untreated, catabolic-induced gastroenteritis and a febrile response occur (Kölker et al., 2006), which are routinely experienced in IBD patients (Fakhoury et al., 2014; Torisu et al., 2017). Limited research in IBD has explored GCDH due to its predominant association with missense mutations in the *Gcdh* gene and the prominent presence of the *Muc2* mutation in the colons of *Winnie* mice, setting them apart from other mouse models of colitis (Eri et al., 2011; Kölker et al., 2006). Consequently, our identification of *Gcdh* downregulation in *Winnie* mice represents a distinctive and noteworthy contribution to the existing literature.

Dhtkd1, which is downregulated in the *Winnie* mouse colon, is a component of the mitochondrial 2-oxoglutarate (α -ketoglutarate) dehydrogenase complex (OGDHC) (Leandro et al., 2020), consequently impeding cellular homeostasis (Nemeria et al., 2014). Although research on the relationship between OGDH and IBD is limited, one study in CD patients demonstrated that downregulated OGDH contributes to hypotonia and metabolic acidosis due to increased D-lactate levels or gastrointestinal bicarbonate loss (Jacobi et al., 2010). The CD patients suffered diarrhoea, which can be accompanied by acid-base imbalances (Jacobi et al., 2010). In particular, *Dhtkd1* is a key gene in the amino acid metabolite α -aminoadipic acid (2-AAA) catabolic pathway, encoding part of a super complex that catalyses the conversion of 2-oxoadipate to glutaryl-CoA; in the degradation pathways of L-lysine, L-hydroxylysine, and L-tryptophan (Boyko et al., 2022; Danhauser et al., 2012). Altered expression of the DHTKD1 protein could potentially affect tryptophan metabolism and the balance of neuroactive metabolites (Xu et al., 2013).

3.5.3 Nicotinate and nicotinamide metabolism

In this research, anomalies in nicotinamide metabolic pathways in the distal colon of *Winnie* mice manifested as increased expression of genes, notably *Cd38*, *Art2a*, *Nampt1*, *Cd73*, and *Pnp*, concomitant with decreased expression of *Sirt2*, *Nmnat2*, *Nmnat3*, *Nmrk1*, *Nmrk2*, *Nadsyn1*, and *Naprt*. A deeper exploration is needed to be undertaken to comprehend the ramifications and importance of these genetic and metabolic deviations.

In this pathway, tryptophan serves as the precursor to *de novo* NAD⁺ biosynthesis via the kynurenine pathway (Xie et al., 2020), which converts to nicotinic acid mononucleotide (NaMN), and transforms to nicotinic acid dinucleotide (NaAD⁺) by NMN/NaMN nicotinamide mononucleotide adenylyl transferases (NMNAT) and then is amidated to NAD⁺ by NADSYN (Johnson & Imai, 2018). Deviations from NAD⁺ homeostasis distort NAD-dependent enzyme levels and impact DNA repair, neuron regeneration, and inflammation resistance, and as a result contribute to the development of a spectrum of diseases (Johnson & Imai, 2018).

3.5.4 Upregulated nicotinamide DEGs in *Winnie* mice distal colons

The observed upregulation of *Nampt* in *Winnie* mice triggers the activation of the nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) signalling pathway in the IL-6 and TNF- α feedback loop, leading to an escalation in IDO1 activity (Ciorba, 2013). This substantiates a mechanistic association between tryptophan catabolism and NAD synthesis (Imai, 2009). Although this study did not assess the *Winnie* mouse for cancer, human IBD tissue and mouse models of colitis show that upregulated NAMPT is associated with colitis-associated cancer (Garten et al., 2009).

Upregulated *Cd38*, the gene encoding for CD38, one of the main NAD-degrading enzymes, was found in *Winnie* mice colons. LPS and TNF- α , both prevalent in IBD disease and *Winnie* mice, are potent inducers of CD38 expression (Piedra-Quintero et al., 2020). CD38 signalling contributes to the persistence of LPS, as noted in human colonic mucosa immune cells within the lamina propria (Piedra-Quintero et al., 2020). DSS-colitis-induced mice showed neutrophils and monocytes recruited to the inflamed

intestinal mucosa showing relatively high levels of cell surface CD38 (Schneider et al., 2015).

NAD-consuming *Art2a* was upregulated in *Winnie* mice, which involves poly [ADP]-ribose polymerase (PARPs). In response to DNA damage, PARPs use NAD molecules as donors to transfer ADP-ribose to substrate proteins, including histones, lamins, and nucleic acids (Luo & Kraus, 2012). This reaction consumes NADP and incidentally generates nicotinic acid adenine dinucleotide phosphate (NAADP), and as a result depletes NAD⁺ availability causing DNA damage (Decker & Muller, 2002; Luo & Kraus, 2012), thus consistent with the conditions observed in *Winnie* mice (Eri et al., 2011).

In the *Winnie* mice colons, there is an upregulation of genes associated with the purine salvage pathway, specifically *Cd73* and *Pnp*. The presence of purinergic receptors along the gastrointestinal tract plays a significant role in maintaining homeostasis of intestinal secretions and promoting relaxation of the intestinal smooth muscle (Grubišić et al., 2019). CD73 is predominantly expressed in the intestinal crypts, mucosa, and tips of villi (Fletcher et al., 2017). CD73 facilitates the translocation of adenosine-mediated fluid across the luminal aspect of the mucosa (Bynoe et al., 2012). Adenosine, through the action of CD73, helps in promoting the production of IL-10, offering a protective mechanism against cellular damage within the intestine. This finding in the *Winnie* mouse is particularly important in the context of colitis, where cellular damage is often observed (Haskó, 2008; Iyer & Cheng, 2012). However, under the pathologic conditions of intestinal mucosa damage of DSS-induced colitis mice, purines are liberated as damage-associated-molecular-patterns (DAMPs) (Grubišić et al., 2019). This phenomenon disrupts the synthesis of adenosine (Crittenden et al., 2018); thereby providing a protective effect for cancerous cells by inhibiting the activity of cytotoxic T cells and natural killer (NK) cells (Harvey et al., 2020).

In addition, PNP converts guanosine to guanine, inosine to hypoxanthine, and deoxyguanine to guanine, thereafter, releasing ribose phosphate, all of which are the building blocks for DNA and RNA (Nyhan, 2014). *Pnp* gene upregulation has been linked with the modulation of specific immune cells and inflammatory markers, namely regulatory T cells, IL-17, and t helper 17 (Th17), which are implicated in the pathogenesis

of colitis (Forrest et al., 2002). The presence of these markers are found in *Winnie* mice and in human IBD colon tissue (Eri et al., 2011). Purine analysis in DSS colitis-induced mice indicated that perturbations in purines correlate with elevated IL-22 levels and lymphoid cell counts, coupled with a compromised defence mechanism against intestinal inflammation and damage (Crittenden et al., 2018). In *Winnie* mice, dysmotility arises from aberrations in cholinergic and purinergic signal transmission to smooth muscle cells (Robinson et al., 2017). The data gathered from these investigations strongly suggest that the upregulation of *Cd73* and *Pnp* in *Winnie* mice has precipitated an energy-altered milieu conducive to the propagation of inflammation further reinforcing the role of these genes in the pathogenesis of colitis.

3.5.5 Downregulated nicotinamide DEGs in *Winnie* mice distal colons

Nmnat2 and *Nmnat3*, which were downregulated in the *Winnie* mouse, protect enteric neurons against injury-induced axonal degeneration (Brazill et al., 2017; Sasaki & Milbrandt, 2010). However, in the case of the *Winnie* mouse, which experiences frequent attacks from cytokines, inhibiting *Nmnat* may lead to an increased susceptibility to axonal degeneration in the gut (Eri et al., 2011; Sasaki & Milbrandt, 2010). Additionally, studies on *Nmnat*-deficient mice have revealed a correlation between chronic inflammation, reduced mitochondrial activity, and diminished cellular protection against DNA damage (Pan et al., 2014). Subsequently, we propose that the downregulation of *Nmrk1* and *Nmrk2* observed in the *Winnie* mouse may contribute to neuronal damage in the colon tissue. In homeostatic conditions, *Nmrk* allosterically upregulates to safeguard neurons against axonopathy if NAD⁺ is depleted or under attack by NAD-consuming enzymes (Ratajczak et al., 2016).

NAMPT and NAPRT reciprocally synthesise NAD, and play significant roles in regulating and reprogramming cellular metabolism (Audrito et al., 2020). However, *Naprt* was downregulated while *Nampt* was upregulated. In a cohort of 261 colorectal cancer patients, it was discovered that high NAMPT or NAPRT expression is linked to vascular invasion and an advanced malignant tumour stage (Li et al., 2019), whereas, *Naprt* downregulation inhibits the production of NAD⁺ and prevents the protection of tissues from oxidative stress (Colombo et al., 2021). Niacin, the precursor to NAMPT or

NAPRT, protects against colitis in mice through its anti-inflammatory and anti-angiogenic properties (Salem & Wadie, 2017). In the context of gut physiology, the pattern and extent of leukocyte migration from the intravascular to the interstitial space are tightly regulated by the microcirculation and its endothelial lining, which is essential for maintaining mucosal immunological homeostasis. Comparatively, the microvessels in chronically inflamed IBD show substantial alterations in microvascular physiology and function when compared to healthy unaffected gut vessels (Deban et al., 2008).

The pathophysiology of IBD exhibits symptoms resembling glutamine deficiency, such as tight junction damage, early cell death, and cellular stress responses (Kim & Kim, 2017). *Nadsyn1*, the gene encoding as a glutamate-dependent enzyme, was downregulated in *Winnie* mice, which suggests its downregulation was in response to glutamine deficiency (Lin et al., 2021). A study in acute DSS-induced colitis mice found that glutamine sufficiency is required for the colonic epithelium to develop a cell-protective, antiapoptotic, and anti-inflammatory response to inflammatory damage (Xue et al., 2011). Further investigation into glutamine deficiencies in the *Winnie* mouse would determine if its supplementation would improve NAD⁺ levels and benefit the epithelial lining of the gut.

Sirt2 was downregulated in the *Winnie* mouse colon. In homeostatic conditions, SIRT2 supports the cell's longevity by facilitating chromosomal compaction through histone and transcriptional coactivator deacetylation, as well as aiding axon myelination. Subsequently, the downregulation of *Sirt2* in *Winnie* mice renders the extrinsic innervation of the digestive tract more susceptible to damage (Beirowski et al., 2011; Goluba et al., 2022). In UC patients and mouse models of colitis, structural and biochemical changes have been observed, including increased intestinal permeability and reduced expression of certain brush border membrane enzymes (DeRoche et al., 2014; Randall-Demllo et al., 2016). Although the exact mechanisms underlying this dysfunction are not fully understood, they are believed to involve heightened neuronal excitability, neuronal injury, and alterations in neuropeptidergic innervation and receptors (Mourad et al., 2017).

3.5.6 Concentrations of tryptophan metabolites in *Winnie* mice distal colons

A metabolomic analysis was conducted on distal colon samples from *Winnie* mice to examine the tryptophan metabolite profiles. By combining metabolomics and RNA-Seq data, this study gained insights into the complex relationships between the tryptophan metabolic pathways and gene expression regulation. The study revealed significant differences in the concentrations of several metabolites in the *Winnie* mice compared to controls. Specifically, tryptophan, 5-HIAA, kynurenine, QUIN and PIC showed higher concentrations in *Winnie* mice, indicating their association with chronic intestinal inflammation. On the other hand, KYNA and nicotinamide exhibited non-significant differences in concentration between the *Winnie* mice and controls. This section explores the relationship between gene expressions and metabolites in the context of chronic intestinal inflammation in *Winnie* mice.

The tryptophan concentration was significantly higher in the *Winnie* mouse colon compared to control mice. One possible explanation is that tryptophan is released from tissue reservoirs or circulating proteins when food intake is reduced (Klaessens et al., 2022). However, a study analysing microarray-derived data from human IBD intestinal mucosa found significantly lower levels of tryptophan compared to non-IBD tissue (Wnorowski et al., 2021). Additionally, in a study supplementing tryptophan in colitis-induced mice, a non-significant correlation was observed between tryptophan supplementation and changes in colonic morphology or survival rates compared to control mice (Chen et al., 2018). The observed disparities in the effects of tryptophan could be ascribed to variances in tryptophan content within dietary sources, variations in the severity of the underlying diseases, metabolic divergence among individuals, and the influence of *Gcdh* downregulation, as observed in the *Winnie* mouse.

5-HIAA, the surrogate of serotonin, was significantly more concentrated in the *Winnie* mouse distal colon compared to controls. These findings align with a previous study in *Winnie* mice, which observed an increased release of 5-HT (serotonin) in the intestinal mucosa and the subsequent under-expression of SERT (serotonin transporter) responsible for metabolising 5-HT (Stavely et al., 2018). Several studies have highlighted a link between high levels of 5-HT and gastrointestinal symptoms of nausea, abdominal

cramping, and diarrhoea (Banskota et al., 2019). These symptoms are believed to be a manifestation of altered gastrointestinal motility and peristaltic reflexes (Stavely et al., 2018). This association is consistent with the abnormal gut morphology and function observed in *Winnie* mice and IBD patients, involving impaired movement of contents through the digestive tract (Liso et al., 2020; Robinson et al., 2017). The available evidence suggests that an imbalance in 5-HT levels could be a key factor in gastrointestinal disorders, potentially through its role in regulating motility and the overall structural integrity of the gut. This combination of findings supports the conceptual premise that MAO inhibitors, prescribed for IBD patients, resolve painful gut disorders and that the associated depression and anxiety are reduced, demonstrating the cross-talk between the gut and brain (Mikocka-Walus, 2017).

Increased kynurenine was observed in *Winnie* mice colons, as well as the colon biopsies of patients with IBD, indicating its association with intestinal inflammation (L.-M. Chen et al., 2021). Notably, kynurenine concentrations were found to be 1.5 times higher in colorectal cancer tissue compared to healthy colon samples. Inhibition of kynurenine binding to the AhR receptor reduced cancer cell proliferation, suggesting the role of kynurenine as an oncometabolite (Venkateswaran & Conacci-Sorrell, 2020). Kynurenine has been implicated in inhibiting reactive effector T and NK cell responses, potentially contributing to an immune-tolerant tumour environment (Bishnupuri et al., 2019). Earlier research found that *Winnie* mice exhibit dysregulated reactive effector T and NK cell responses (Eri et al., 2011). Additionally, kynurenine can be transaminated by kyn-aminotransferase, with *Afmid* catalysing the conversion of kynurenine to the neuroprotective KYNA (Phillips et al., 2019). KYNA is crucial for inflammatory activation and gut motility, as it blocks LPS-mediated modification of NK T cells and TNF- α , lowers IL-17 and IL-23, and prevents further disease activity (Turski et al., 2013). However, the gene downregulation of *Afmid* and the upregulation of *Ido1* shift the conversion of kynurenine away from KYNA and towards QUIN (Phillips et al., 2019), as observed in the *Winnie* mouse in our study.

QUIN, which is in higher concentrations in the *Winnie* mouse colon, has been extensively studied for its role as an agonist to N-methyl-D-aspartate (NMDA) receptors as a neuroexcitatory toxin in the central nervous system (CNS) (Lugo-Huitrón et al., 2013).

However, since the gastrointestinal tract is innervated, and demonstrates neurogenesis processes similar to those in the central nervous system (CNS), hypothetically, there may be similarities in QUIN's role in the colon as in the brain. Biological psychiatric studies demonstrated that activated monocytes produce QUIN during inflammatory processes (Myint & Kim, 2014). During inflammation, large quantities of leukocytes stockpile QUIN in order to sustain an adequate supply of NAD⁺ (Fricker et al., 2018). These findings are consistent with another *Winnie* mice colon study, which detected high monocyte levels circumventing the periphery of the ganglia (Stavely et al., 2018). This is especially true in the presence of bacteria secreting NAD glycohydrolases of CD38, CD73 and ART2A (Moffett et al., 2020), which are all upregulated in the *Winnie* mice.

Winnie mice exhibited high levels of PIC. These findings suggest that the flux of tryptophan metabolites is so pronounced in *Winnie* mice that the dehydrogenase competing with the non-enzymatic cyclisation of aminomuconic semialdehyde becomes saturated with its substrate (Badawy, 2017). There are few studies on PIC and IBD, however in the serum of diabetes and chronic hepatitis C patients, increased PIC levels have been correlated with inflammatory responses. For instance, PIC has biological activity on the outer membrane of inflammatory macrophages, activating IFN- γ and inducing L-arginine-dependent nitric oxide (NO) production (Zuwała-Jagiello et al., 2012), which aligns with the inflammatory activity observed in *Winnie* mice (Eri et al., 2011).

Based on our findings, indicating an increase in the activity of NAD-consuming enzymes (Braidly et al., 2019), it is proposed that the *Winnie* mice relied on *de novo* NAD⁺ synthesis from tryptophan metabolism. Supplementing *Winnie* mice with nicotinamide could provide insights into the potential alleviation of chronic colonic inflammation. Studies have shown that nicotinamide ameliorates pathological angiogenesis in a mouse model of colitis (Salem & Wadie, 2017). Nicotinamide supplementation has been found to inhibit the expression of pro-inflammatory cytokines of IL-6, IL-12, IL-1 β , TNF- α , IFN- γ and IL-21 while increasing the anti-inflammatory IL-10. Nicotinamide supplementation reduced disease activity in DSS-induced colitis mice, leading to improvements in body weight, colon length, disease activity index score, myeloperoxidase activity, and mucosal healing (Kang et al., 2021).

3.6 Conclusion

The significance of this study resides in the novel data and the investigation of rarely studied gene expressions that have provided new insights into the complex mechanisms and physiological shifts within tryptophan metabolism. In addition, this study is exploratory and interpretative and revealed the transcriptome in alignment with chronic intestinal inflammation in *Winnie* mice. The changes in the concentration levels of tryptophan metabolites in the *Winnie* mouse colon is reflected in changes in the expression of genes involved in these pathways. The genes and metabolites shed light on the physiological alterations in tryptophan metabolism, specifically its diversion towards the kynurenine pathway and the consequential effects on *de novo* NAD⁺ synthesis in the context of chronic intestinal inflammation. This study broadens our comprehension of the multifaceted roles of the signalling molecule 5-HIAA in preserving normal gut function and instigating intestinal inflammation. These results may help distinguish between the beneficial and detrimental effects of 5-HIAA, leading to innovative approaches to counteract the undesirable effects of IBD. In *Winnie* mice, reduced homeostasis restoration ability could have significant consequences, including metabolic reprogramming that promotes inflammation, decreased energy metabolism, and reduced nutrition due to decreased food intake. The findings from this investigation hold significant potential to enhance the management of early-onset IBD, contributing to the development of targeted therapeutic interventions that could potentially retard disease progression and augment the quality of life for individuals afflicted with IBD.

Chapter 4: Behavioural changes, sleep patterns and cytokines in *Winnie* mice

4.1 Summary

Inflammatory bowel disease (IBD) is an incurable systemic disease characterised by recurrent idiopathic inflammation of the gastrointestinal tract, which includes two major pathologies, ulcerative colitis and Crohn's disease. Patients with IBD experience recurrent inflammation, altered gut motility, visceral discomfort, and a rise in comorbid affective disorders, in particular depression and anxiety. To elucidate mechanisms underlying changes in behaviour associated with chronic intestinal inflammation, this study conducted a series of behavioural tests to analyse emotional processes, sleep patterns and spontaneous physical activity in the *Winnie* mouse model of chronic colitis, which closely represents the pathophysiology of IBD. In addition, this study determined changes in cytokine activity within *Winnie* mice brains by utilising RNA-sequencing and the relation of these changes to behavioural changes. Immunohistochemical analysis of neurons, oligodendrocytes, astrocytes, and microglial cells in the prefrontal cortex and hippocampus was performed to decipher morphological changes in the *Winnie* mice brains.

The *Winnie* mice displayed reduced spontaneous physical activity coupled with symptoms of hypersomnia and atypical depression. The results demonstrated increased transcription of the innate immunity-related gene responses and structural changes in the brain, which might underlie the depressive and anxiety-like behaviours observed in *Winnie* mice. Immunohistochemistry revealed changes in structure in *Winnie* mice brains evidenced by altered expression of high mobility group box 1 protein, glial fibrillary acidic protein, myelin basic protein, transmembrane protein 119, and neuronal nuclei. In the brains of *Winnie* mice, pro-inflammatory cytokines increased, while anti-inflammatory cytokines decreased. Protein expressions illustrated structural changes, in particular to the hippocampus, which correlated with behavioural changes. These findings might shed light on mechanisms implicated in the comorbidity of intestinal inflammation and affective disorders, which might lead to the development and implementation of early interventions to alleviate the onset and progression of neuropsychiatric disorders associated with IBD.

4.2 Introduction

Inflammatory bowel disease (IBD) is an incurable systemic disease characterised by recurrent idiopathic inflammation of the gastrointestinal tract, which includes ulcerative colitis (UC) and Crohn's disease (CD) (Fakhoury et al., 2014). Patients with IBD experience intermittent inflammation, altered gut motility, visceral discomfort, and comorbid affective disorders, in particular, depression and anxiety (Baumgart & Carding, 2007; Fakhoury et al., 2014). Our study aims to elucidate behavioural changes associated with depression and anxiety in the *Winnie* mouse. Depressive symptoms, which are delineated from sadness and bereavement, are characteristic of anhedonia, dysthymia, restlessness, limited physical activity, insomnia, hypochondriasis, a poorer quality of life, and suicide ideation (Yanartaş et al., 2019). Anxiety is typified by worry, intolerance to uncertainty, cognitive avoidance, an irrational fear and the cognitive response to this disposition (Mossman et al., 2017). Depression and anxiety are complex in nature involving genetic factors, personality type, biochemical factors, illness, ageing and stress, leading to metabolic, endocrine, cardiovascular, neurological, and immunological dysfunction (Sankowski et al., 2015). Depression and anxiety have been reclassified as clinical manifestations of activated immunological and pro-inflammatory cytokines, oxidative and nitrosative stress pathways, including dysregulated tryptophan metabolism, autoimmune disease, and gut-brain connections (Martin-Subero et al., 2016). Although depression and anxiety are known to represent two different constructs, they are highly inter-correlated and share biological and inflammatory pathways inter-related in the gut and brain, which consequently disrupt gastrointestinal homeostasis and perpetuate intestinal as well as systemic inflammation (Abautret-Daly et al., 2018; Choi et al., 2019).

IBD patients present with somatic symptoms and depressed behaviours exhibiting neuropathic hyperalgesia, weakness, headaches, fibromyalgia, fatigue, and alexithymia more so than expected just prior to their IBD diagnosis (Martino et al., 2020). In particular, alexithymia exists in up to 35% of IBD patients, and is directly proportional to clinical severity of gastrointestinal pathologies (Martino et al., 2020). For these individuals differentiating and expressing emotions, thoughts, and physiological responses is confounding and affects their ability to manage IBD, subsequently

leading to worse perceived quality of life (QoL) and contributing to comorbid depression and anxiety (Yanartaş et al., 2019). Another study indicates that the depression and anxiety experienced by IBD patients might be linked to the stress stemming from their views on their illness, including concerns about potential cancer risks, expectations of a diminished QoL, and apprehension about public incidents that might be humiliating (Jordan et al., 2018). There is evidence which shows that patients with IBD express increased stress, decreased productivity, increased systemic and gut pain, and symptomatic flares (Sun et al., 2019).

One explanation for behavioural changes associated with colitis could be explicated through the immunoregulatory effects of inflammation and changes to brain structure. For example, at a damaged brain site of dextran sulfate sodium (DSS) colitis mouse models, inflammatory mediators and cytokines initiate a cascade of cell recruitment, proliferation, and activation (Han et al., 2018). To elucidate depressive and anxiety-like behaviours that are linked to colitis, a series of behavioural tests were conducted on the *Winnie* mouse, and compared to control mice. Throughout each behavioural test, depressive and anxiety-like states are interpreted by measuring spontaneous rodent behaviour and selected actions (Van Meer & Raber, 2005) as well as sleep patterns and spontaneous physical activity using Promethion metabolic cages. The Promethion metabolic cages are designed to capture highly precise and exhaustive metabolic and behavioural data. These cages offer a versatile array of measurement options, including general movement tracking, wheel running analysis and resting behaviour observation (Speakman, 2013).

Studies in animal models of intestinal inflammation have generally focused on male mice sample populations to avoid the variability within the oestrus cycle associated with female animals (Nyuyki et al., 2018). In order to represent the variances of both sexes, this study ascertained the behavioural differences of both male and female *Winnie* mice compared to age- and sex-matched control littermates. RNA-sequencing (RNA-Seq) of *Winnie* and control mice brains was performed to analyse cytokine gene expression activity. Immunohistochemistry (IHC) was utilised to measure the presence and distribution of specific proteins or antigens within the brain tissue (Ramos-Vara, 2017), namely neurons, oligodendrocytes, and astrocytes in the prefrontal cortex and hippocampus. The

information derived from these tests will provide some understanding of the mechanisms underlying affective disorders, which are comorbid with intestinal inflammation and might lead to the development of early interventions to alleviate the onset and progression of neuropsychiatric disorders associated with IBD.

4.3 Materials and Methods

4.3.1 Ethics, animals, and animal husbandry

This study was approved by the Victoria University Animal Experimentation Ethics Committee (AEC-17-016). All animal experiments complied with the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes described in [Section 2.3.1](#). The *Winnie* mouse model of colitis with a C57BL/6 mice background was obtained from the same colony at Victoria University (Melbourne, Victoria, Australia). The colitis in *Winnie* mouse models is caused by a missense mutation (G9492A, GenBank accession no. AJ511872), by which cysteine is replaced by tyrosine in the D3 67 domain in the N terminus of the mucin 2 (*Muc2*) gene (Heazlewood et al., 2008). Age-matched C57BL/6 littermates, obtained from the Animal Resources, constituted the control. Post *in vivo* experiments, all mice were euthanised at 14 weeks of age via an overdose of injected pentobarbitone (Lethabarb, Virbac Australia) using a 1:16 dilution, 30 g, 100 µL/20 g. The brain tissues were removed and used for subsequent experiments.

4.3.2 Spontaneous physical activity and sleep data acquisition

The Promethion metabolic high-definition multiplexed respirometry cage with indirect calorimetry method (Sable Systems International, Nth Las Vegas, NV, USA) was used to measure the real-time spontaneous physical activity and sleeping behaviour of the *Winnie* mice compared to controls. The Promethion cages were set up according to the manufacturer's instruction and as described in [Section 2.3.5](#) ("Promethion Metabolic Screening: Revolutionizing Metabolic Measurement," 2019). MetaScreen coordinated data acquisition and instrument control. ExpeData (Sable Systems International, North

Las Vegas, NV, USA) processed the raw data using an analysis script outlining all aspects of data transformation.

Winnie mice of 14 week of age (w) were separated by their biological sex and compared to C57BL/6 male and female mice, n=8 mice/group. In additional experiments, the *Winnie* male mice and *Winnie* female mice were compared, and then merged to form an n=16 mice/group for each test. After 5 days of acclimatisation, the following 24-hour data were used for statistical analyses. The data points were recorded from 0700 to 1900 hour (light cycle) and 1900 to 0700 hour (dark cycle).

The distance in wheel metres, the percentage of wheel running time, the wheel speed, walking activity percentage and sleeping behaviour of *Winnie* mice were evaluated. The wheel distance in metres (m) and speed (mean speed during wheel running, metres/second) were measured. The run percentage is the sum of the animal's time spent running on the wheel, as a percentage of the light and dark cycle. The walk percentage is the sum of the animal's time spent in ambulatory locomotion outside of the wheel, as a percentage of the light and dark cycle.

Sleep hours are the sum of an animal's sleep in hours and as a percentage of the light and dark cycle. The animal is considered sleeping when it has been "quiet" for more than 40 seconds. The still percentage, or the animal's "quiet" time is when the animal is not engaged in eating, drinking, grooming, or locomotion as a percentage of the light and dark cycle. The percentage of still time is always greater than the percentage of sleep time. To obtain the time the animal is "quiet" but not asleep, the percentage of still time was subtracted from the percentage of sleep time ("Promethion Metabolic Screening: Revolutionizing Metabolic Measurement," 2019).

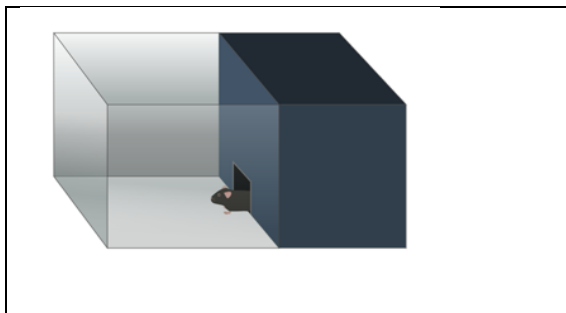
4.3.3 Behavioural study tests

Four behavioural tests were used to phenotype depressive and anxiety-like behaviours of the *Winnie* mice by interpreting and measuring spontaneous rodent behaviour and selected actions. The light-dark test (LDT), open field test (OFT), and elevated plus maze (EPM) demonstrate anxiety-like behaviour, whereas the forced swim test (FST) and

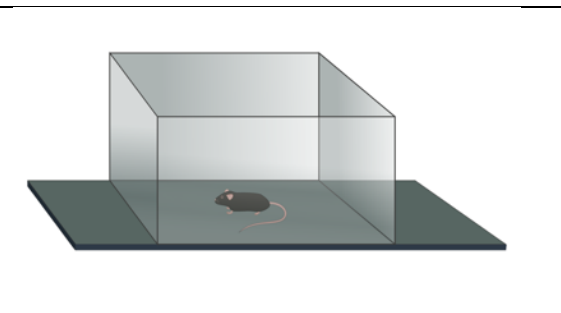
grooming behaviour (OFT) reveal depressive-like behaviour (**Figure 4.1**) (Belovicova et al., 2017; Bourin & Hascoët, 2003; Can et al., 2012). *Winnie* mice of 14-week-old were separated by their biological sex and compared to control C57BL/6 male and female littermates, n=8 mice/group. A second analysis merged the sexes to form n=16 mice/group. Behavioural tests were performed between 0900 hours and 1300 hours. In order for acclimatisation to reduce stress, the animals were transferred from the animal facility to the experimental room one hour before commencing the behavioural tests. The mice cages were positioned so that the remaining mice could not view the behavioural test. The behavioural tests were performed once. All behavioural test equipment was cleaned with 70% ethanol after each test to prevent a bias based on olfactory cues. The behaviour tests were recorded by a video camera (D-link camera). All measurements for each test were analysed using in-house-developed Advanced Move Tracker (AMT) software (Davidson et al., 2023).

Figure 4.1 Behavioural tests models

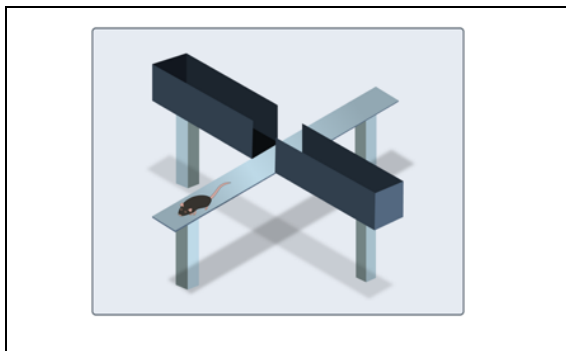
A. Light dark test



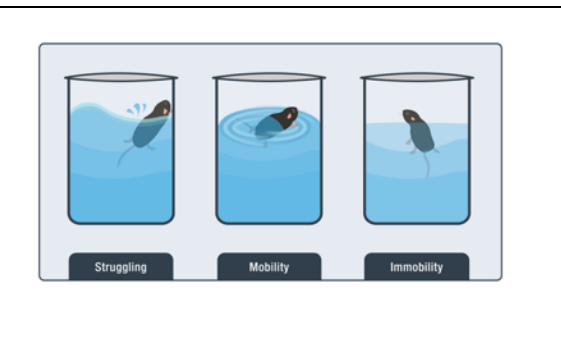
B. Open field test



C. Elevated plus maze



D. Forced swimming test



4.3.4 Light-dark test

During LDT (Bourin & Hascoët, 2003), mice exhibiting higher levels of anxiogenic-like behaviour make fewer transitions between the white and black boxes. For example, mice naturally have an aversion to open and brightly illuminated spaces and prefer dark, protected spaces. However, an inherent conflict between their desire to explore the unprotected area and their risk-avoidance behaviour inhibits their exploratory drive. Increased exploratory behaviour is associated with increased time spent in the white box area, whereas naïve mice prefer the black box. The parameters comprised the number of entries into the white box (defined as the placement of all four paws in the box), the total time spent in each box, the distance travelled in centimetres (cm) within the white box, and the latency (poking) to enter the white box. Poking is time spent in risk assessment that involves a stretch-attend posture with the head and fore-paws extended into the white box while the body remains in the black box (Arrant et al., 2013). The LDT box is made up of the white box (28 x 20 x 20 cm) and the black box (15 x 20 x 20 cm). The black box top is covered to prevent light input. The opening between the black and white boxes is 5 x 5 x 5 cm, which is closed off by a black shutter (**Figure 4.1A**). The mouse was first

placed in the white box and five seconds later, the shutter between the boxes was opened. The mouse moves between the two boxes for 10 minutes, which is recorded for analysis.

4.3.5 Open field test

The OFT (Seibenhener & Wooten, 2015) determines anxiety-like behaviour by examining environmental exploration, locomotor activity, adaptation, fear, shifted activity, and reaction to an unknown environment. The most commonly used measures of overall exploratory and locomotor activity is the frequency of central square entries, the corners, peripheries of the field, unprotected areas, and measurements of reduced grooming. The open field was constructed using a white, high-density, non-porous plastic board to erect a wall-enclosed area measuring 72 x 72 x 33 cm (**Figure 4.1B**). The field had a central zone of 36 x 36 cm. The mouse was placed in the central zone and was able to move freely in the entire open field. After two minutes of acclimation, the free movements of mice were tracked using a video computer system for up to four minutes. For data analysis, Factor 1 parameters measured the time duration of thigmotaxis, the time duration and number of entries into the central zone, and general exploratory locomotion.

During the OFT, the grooming behaviour of *Winnie* mice was measured. Self-grooming is an innate animal behaviour performed in cephalocaudal progression. It involves the microstructure of licking and caressing fur and skin while stationary for hygiene maintenance, thermoregulation, communication, and de-arousal, known as a synaptic chain pattern (Kalueff et al., 2016). Instead of considering rodent self-grooming as a direct reflection of a specific symptom, it is more fitting to regard it as an indirect indicator of various behavioural aspects that might be pertinent to human brain disorders and to increase our understanding of the neural processes governing hierarchical motor control such as motor action sequences and intricate motor activity patterns. In a novel environment, grooming of the mouse is expected as a displacement response. Depressed mice typically show reduced self-grooming activity and grooming pattern rigidity (Kalueff et al., 2016). For analysis, Factor 2 measured the total duration of grooming and the number of groomings.

4.3.6 Elevated plus maze test

The EPM (Schneider et al., 2011) determines anxiety-like behaviour based on the natural aversion of mice to exploring open and elevated areas and its instinct to explore novel environments. The intersection of the platform measures 5 x 5 cm with two expanding opposed open arms (5 x 30 cm) with a small 5 mm lip that protects the mouse from falling and two opposed closed arms with walls (16 cm) that are open at the top. The two opposing standing arms do not interfere with the central zone, and the apparatus is raised 45 cm from the floor (**Figure 4.1C**). A mouse was placed in the middle area of the EPM, facing one of the open arms, and can move freely into each section, which is recorded for five minutes. The total number of entries and time spent in each arm constitute the index of primary anxiety. The time spent in avoidance of the arms is due to fear of open and elevated spaces. Mice exhibiting higher levels of anxiogenic-like behaviour make fewer transitions between the open arm and the closed arm. Subsequently, this approach–avoidance conflict correlates with increases in physiological stress indicators. Increased time spent in open arms indicates a lower degree of “anxiety” in the animal.

4.3.7 Forced swimming test

The FST uses immobility as a marker of behavioural despair and depressive-like behaviour in rodents. A commonly chosen source of motivation is fear, such as the fear of drowning. The mouse was placed in the two litre beaker containing 1.4 litres of fresh water at 24° C in front of a white background for recording purposes. The mouse was not able to reach the bottom of the beaker and was not able to escape (**Figure 4.1D**). The movement of the mouse was video recorded for six minutes, with the last 4 minutes included in the analysis. Following the initial frantic escape movements, rodents develop an immobile posture. Immobility and mobility in the water in a stressful and inescapable environment were measured for behavioural despair. The movements counted as *bona fide* mobility are as follows: (1) code the duration of time spent as “immobile” if the mouse is floating with the absence of any movement except for those necessary for keeping the nose above water; (2) code the duration of time spent as “swimming” if the movement of forelimbs or hind limbs in a paddling fashion was observed. The operational definition for mobility in the FST was ‘any movements other than those necessary to

balance the body and keep the head above the water.’ Mice generally float in water readily, however, they still manifest small movements to balance their bodies and keep their heads above the water. These behaviours are not an attempt to escape and were not scored as mobility. After a single bout of mobility, even though they are essentially immobile, mice can still drift in the water because of momentum, which is not scored as mobility (Can et al., 2012).

4.3.8 Biological samples for high-throughput RNA-Sequencing

Winnie mice and control C57BL/6 littermates (n=10 mice/group, 5 females and 5 males per group) were euthanised at 14 weeks of age. After brain extraction and washing with phosphate buffered solution (PBS), the tissues were frozen in liquid nitrogen and stored at -80° C until required. To ensure RNA was produced with minimal impurities the RNeasy Lipid Tissue MiniKit (cat. no. 74804, Qiagen) containing the QIAzol® Lysis Reagent was used. After extraction, the quality of RNA was assessed using a 2100 Bioanalyser (Agilent Technologies, USA) microfluidics platform with the RNA 6000 Nano Kit (Agilent Technologies). All samples were free from contamination of genomic deoxyribonucleic acid (DNA) and 16S ribosomal RNA from bacteria, providing an RNA integrity number > 8.5/10. Upon completion, one 4 µL aliquot of each RNA sample was placed into the Nanodrop (Denovix, Melbourne, Australia) spectrophotometer to assess RNA quality and concentration. The results demonstrated that 260/280 nm and 260/230 nm measurements were >1.8.

4.3.9 High-throughput RNA-Seq of mRNA

The mRNA samples were submitted to Micromon Genomics (Monash University, Melbourne, Australia) for RNA-Seq using the NGS platform and bonafide methods (Benjamini & Hochberg, 1995; Dobin et al., 2013; Liao, 2014; Powell, 2019; Tsyganov et al., 2018). Micromon Genomics performed RNA quality control with Invitrogen Qubit RNA BR assay (Gene Target Solutions, Sydney, Australia), as well as the polyA purification of mRNA from total RNA samples and the RNA-Seq library construction. All of the samples quantified and analysed with the Qubit and Bio-analyser passed the QNA QC standard. Micromon performed the sequencing on MGITech MGISEQ2000RS

hardware (MGISEQ-2000RS High-throughput Sequencing Set). The MGIEasy V2 chemistry set was used to generate the library, with at least 400 m raw reads per lane and paired-end 100b reads for accuracy and processing to compressed FASTQ files. Raw FASTQ files were analysed using the RNAsik pipeline version 1.5.4 (Tsyganov et al., 2018) and the *Mus musculus* reference genome GRCm38 (GenBank accession GCA_000001635.2) (Dobin et al., 2013). Raw read counts were quantified using the featureCounts program (Liao, 2014) and analysed with Degust, a web tool that performs differential expression analysis using limma voom normalisation (Law et al., 2018) to produce counts per million (CPM) library size normalisation and trimmed means of M values and normalisation for RNA composition normalisation (Robinson & Oshlack, 2010). Differential expression of transcripts between the colons of C57BL/6 littermates and *Winnie* mice were determined using via the Degust Webtool. EdgeR was preferred to obtain the total number of genes from Degust (Robinson, 2010). DEGs were identified with an FDR of <0.001 using the Benjamini–Hochberg correction (Benjamini and Hochberg, 1995). Degust also includes a number of quality plots, such as classic multidimensional scaling and MA plots (Powell, 2019). MultiQC which presents the visual output of data gathers it into a single report (Ewels et al., 2016). To analyse differential gene expression based on overrepresentation analysis software for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways was used (Kanehisa & Goto, 2000).

Pathview plots for the IBD pathway from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database presented as significance scores ($\text{sign Log}_2\text{FC}(\text{fold change}) \times \text{Log}_{10}\text{P values}$) for gene expression analysis between the brains of *Winnie* mice and C57BL/6 littermates.

Heat maps of the expression of IBD pathway genes from the KEGG database from individual mice values for each sample were visualised. Plotted data indicate leading-edge genes identified in Gene Set Enrichment Analysis (GSEA) analysis (Subramanian et al., 2005), between the brains of *Winnie* mice and C57BL/6 littermates. GSEA includes gene set annotation, statistical analysis, and pathway interpretation. Data are represented as Z-scores of CPM values across samples. Data are hierarchically clustered by Euclidian distance.

4.3.10 STRING protein-protein association networks

As described in Chapter 2, [Section 2.4.9](#), the Search Tool for Retrieval of Interacting Genes (STRING) database was utilised to identify clusters and excerpt biological processes, molecular functions and cellular pathways (Szkłarczyk et al., 2021).

4.3.11 Transcardiac perfusion of the brain tissue

Brain perfusion was performed on 14-week-old control C57BL/6 littermates (n=6) and age-matched *Winnie* mice (n=6) to ensure brain tissue preservation and to remove proteins in the blood to prevent any interaction with antibodies during IHC, according to Wu et al. (2021). Coronal cross-sections: 20 µm thickness; prefrontal cortex (PFC): bregma +2.58 mm, hippocampus: bregma -1.94 mm, nucleus accumbens (NAc): bregma +1.54 mm. The prepared 20 µm brain sections were then subjected to an antigen-blocking agent with 10% normal donkey serum (NDS) (Merck Millipore, Bayswater, Australia) diluted in PBS-0.1% Triton X-100 for one hour at room temperature.

The optimal cutting temperature compound (OCT) embedded perfused *Winnie* mouse and C57BL/6 mouse brains, which were used to investigate myelination of oligodendrocytes, neurons, and microglial activation. IHC will identify, *in vitro*, epitopes expressed by a single protein target in tissues through antigen-antibody interactions visible through powerful microscopy (Ramos-Vara, 2017). Sections for the immune labelling included HMGB1, neuronal nuclei (NeuN), myelin basic protein (MBP), transmembrane protein 119 (TMEM119) and glial fibrillary acidic protein (GFAP) were then incubated with primary and secondary antibodies (**Table 4.1**) using the method described by Davidson et al. (2022). Secondary antibodies (**Table 4.1**), diluted in 2% NDS and PBS-0.1% Triton X-100, were then added to each section and tissues were incubated for 2 hours in a humidified container. Subsequently, specimens were washed with PBS-0.1% Triton X-100 (3x10 minutes) and mounted onto glass slides using a fluorescent mounting medium (DAKO, Australia).

Images were taken using a Nikon Eclipse Ti2 multichannel confocal laser scanning system (Nikon, Japan). The confocal microscopy allowed visualisation of

immunolabelled sections through the filter combinations appropriate for the specific fluorophores consisting of Alexa 594, FITC, and Alexa 647 (488 or 594 or 640 nm excitation). Images (512 x 512 pixels) were obtained at 20 x (dry, 0.75) or 40 x magnification. Images were captured at 20 x (PFC and NAc), scale bars 100 μ m and 40 x (hippocampus), scale bars 50 μ m. All images will be captured at identical acquisition exposure-time conditions and are calibrated to standardised minimum baseline fluorescence. Images were converted to binary and changes in fluorescence from baseline were measured using the software Image J (National Institute of Health, Bethesda, MD). In the brain sections, astrocytes, microglia and oligodendrocytes in the PFC, NAc and hippocampus were measured by immunoreactivity (IR) density % of an area. A representation of secondary antibody labelling using donkey anti-rabbit Alexa Fluor 488 was applied to determine any evidence of non-specific labelling. Scale bars were 50 μ m, and images were captured at 40 x.

Table 4.1 Immune labelling of HMGB1, NeuN, MBP, TMEM119 and GFAP

Target	Primary Antibody	Host species	Titration	Supplier	Secondary Antibody (Host species)	Titration
Nuclear protein	Anti-HMGB1	Rabbit	1:1000	Abcam	Alexa fluor 488 - Donkey anti-Rabbit	1:400
Neuronal cell bodies	Anti-NeuN	Mouse	1:500	Abcam	Alexa fluor 647 - Donkey anti-Mouse	1:400
Oligodendrocytes	Anti-MBP	Rabbit	1:1000	Abcam	Alexa fluor 488 - Donkey anti-Rabbit	1:450
Microglial cell	Anti-TMEM119	Rabbit	1:1000	Abcam	Alexa fluor 594 - Donkey anti-Rabbit	1:450
Astrocytes	Anti-GFAP	Goat	1:1000	Abcam	Alexa fluor 488 - Donkey anti-Rabbit	1:450
Abbreviations: High mobility group box protein 1 (HMGB1), Neuronal nuclei (NeuN), Myelin basic protein (MBP), Transmembrane protein 119 (TMEM119), Glial fibrillary protein (GFAP).						
*All secondary antibodies were purchased from Jackson Immuno Research						

4.3.12 Statistical analysis

GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA) was utilised to analyse the comparisons of the controls C57BL/6 littermates versus *Winnie* mice groups. For multiple comparisons, either a one-way ANOVA or a two-way ANOVA and a post hoc Holm-Šídák or Tukey test was applied, or a Student's *t*-test where applicable. Alpha was set at 0.05, and significant was considered at *p* value ≤ 0.05 . All data are presented as mean $\pm$ standard error of the mean (SEM). Outliers were determined using a box and whiskers plot.

4.4 Results

4.4.1 Spontaneous physical activity and sleep behaviour

Using ExpeData parameters spontaneous physical activity including wheel meters, wheel speed (metres/second), and wheel running percentage and walking activity percentage (time spent in ambulatory locomotion outside of the wheel, as a percentage of the cycle) were measured in *Winnie* mice compared to the control mice. In the same experiment, sleep behaviour was assessed, which is either a characteristic of, or a precursor of anxiety and depression (Strekalova et al., 2008). All parameters were measured and analysed during the daylight and night (dark) cycles (12 hours each cycle). The light cycle showed non-significant differences for all tests, therefore, only the dark cycle is reported on.

4.4.2 *Winnie* male mice and *Winnie* female mice physical activity and sleep behaviour

Physical activity, sleep behaviour of the *Winnie* male mice and *Winnie* female mice versus control C57BL/6 male and female littermates were compared separately which demonstrated significantly reduced physical activity and longer sleep hours in *Winnie* mice for both groups (**Appendix Figure S 5, Figure S 6**). From this data, the 14-week-old *Winnie* male mice and age-matched *Winnie* female mice were compared to determine if there were any differences between the sexes in spontaneous physical activity and sleep behaviour. The results showed minimal differences between sexes, although the female *Winnie* wheel running percentage was at a higher speed during the light and dark cycles (n=8 females/group, and n=8 males/group) (**Appendix Figure S 7**). Considering the negligible differences observed between the *Winnie* male mouse and *Winnie* female mouse, we pooled the data from both sexes of *Winnie* mice and juxtaposed it with the unified data from both sexes of control C57BL/6 littermates; with each group comprising n=16 mice.

4.4.3 Reduced spontaneous physical activity and increased sleep in *Winnie* mice

Merged sexes of 14-week-old *Winnie* mice and age-matched controls (n=16 mice/group) were measured for spontaneous physical activity. The *Winnie* mice achieved significantly less distance travelled in the wheel (wheel metres) (2932 ± 617 metres, $p < 0.001$) compared to controls (8512.6 ± 987 metres) (**Figure 4.2A**). The *Winnie* mice performed slower wheel speed (0.3 ± 0.03 metres/second, $p < 0.001$) than the controls (0.48 ± 0.04 metres/second) (**Figure 4.2B**). Likewise, the wheel running percentage was significantly less in the *Winnie* mice ($22.08 \pm 3.7\%$, $p < 0.001$) than controls ($50.9 \pm 2.6\%$) (**Figure 4.2C**). There were non-significant differences in walking activity percentage (the time spent in ambulatory locomotion outside of the wheel), between *Winnie* mice ($15.82 \pm 1.6\%$) and controls ($21.49 \pm 5.2\%$) (**Figure 4.2D**).

In humans, the diagnosis of depression or atypical depression includes leaden paralysis, extended time in bed, reduced and broken sleep, or hypersomnia (Ohayon & Roberts, 2015). This study measured sleep hours, sleep percentage, the percentage of still time, and the percentage of quiet time without sleep of the *Winnie* mice compared to controls. The data points were recorded within light and dark cycles from 0700 hour to 1900 hour and 1900 to 0700 hour. Non-significant differences were observed in the sleeping patterns of the *Winnie* mice and controls during the light hours. Hence, these results will only focus on the nocturnal hours, a period when mice are typically awake. Hereby, the *Winnie* mice slept significantly longer hours (6.1 ± 0.5 hours, $p < 0.001$) compared to controls (3.5 ± 0.4 hours) (**Figure 4.2E**). The *Winnie* mice sleep percentage of the dark cycle was significantly higher ($50.5 \pm 3.9\%$, $p < 0.001$) than controls ($28.9 \pm 3.2\%$) (**Figure 4.2F**). For *Winnie* mice, the percentage of still time of the dark cycle was significantly higher ($53.3 \pm 4.0\%$, $p < 0.001$) than controls ($35.4 \pm 3.7\%$) (**Figure 4.2G**). The percentage of quiet time without sleeping of the dark cycle was significantly lower for the *Winnie* mice ($2.2 \pm 0.5\%$, $p < 0.001$) than the controls ($6.5 \pm 0.6\%$) (**Figure 4.2H**).

4.4.4 Behaviour of *Winnie* male mice compared to control male mice and *Winnie* female mice compared to control female mice

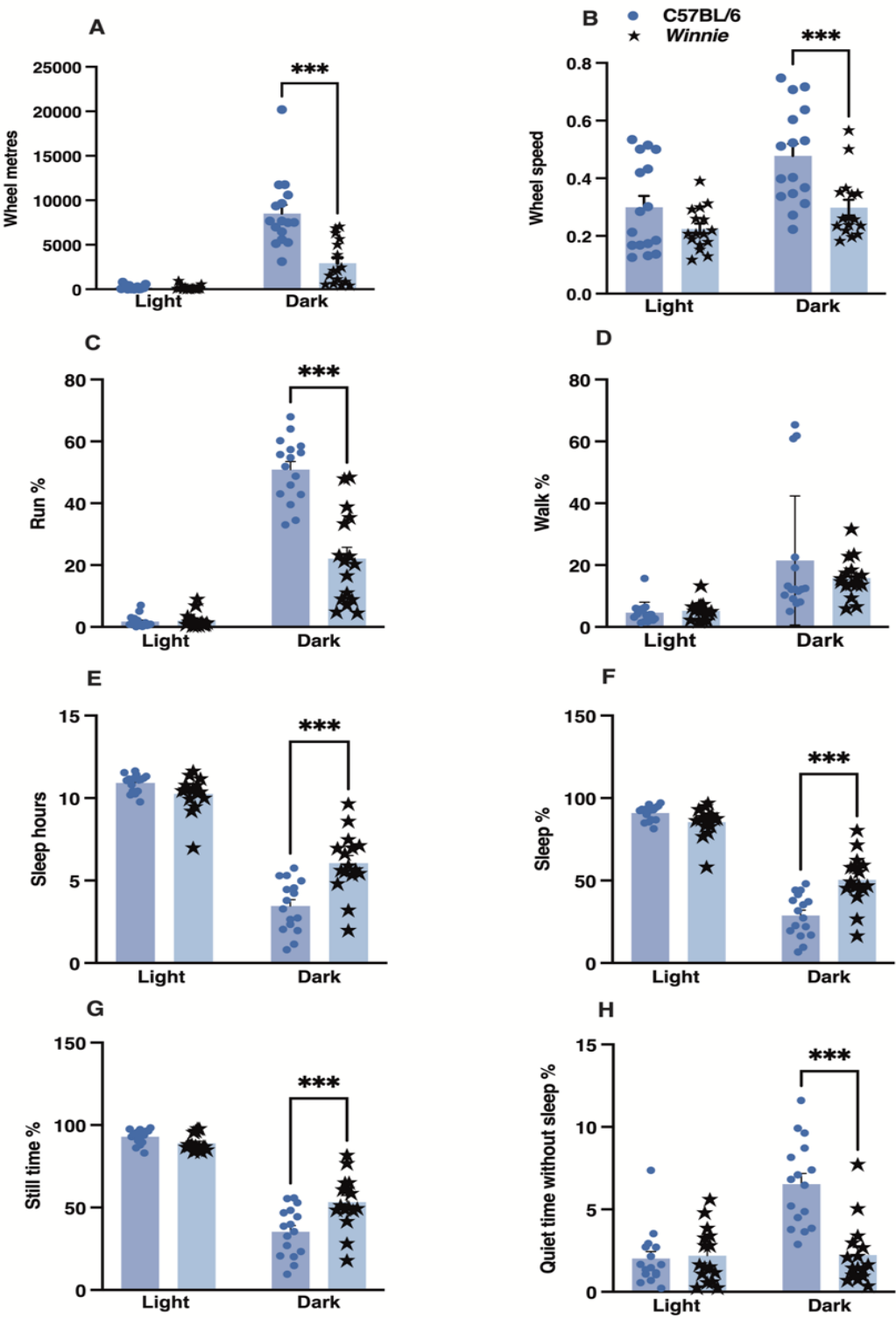
Behavioural tests were used to assess anxiety- and depressive-like behaviours in 14-week-old *Winnie* male mice and age-matched *Winnie* female mice, $n=8$ mice/group (**Appendix Figure S 8**). The *Winnie* male mice and female mice showed non-significant differences in all parameters of the OFT, and therefore displaying non-significant differences in anxiety or grooming activity (**Appendix Figure S 8.1**). In the LDT there were non-significant differences between *Winnie* male and female mice in the time spent in the white or black box within ten minutes. However, the *Winnie* female mice showed less entries in the white box (16.4 ± 3.6 units, $p=0.035$) versus *Winnie* male mice (20.6 ± 1.2 units). The *Winnie* female mice made more pokes from the black box (25 ± 3 units, $p=0.017$) versus *Winnie* male mice (15.43 ± 1.6 units) (**Appendix Figure S 8.2**). In the EPM, the *Winnie* female mice spent less time in the open arm (5.5 ± 6.7 seconds, $p=0.005$) compared to *Winnie* male mice (35.3 ± 27.8 seconds); however, the *Winnie* female mice spent more time at the centre (146.3 ± 35.4 seconds, $p=0.001$) than *Winnie* male mice (87.7 ± 19.9 seconds). The time in the closed arm and the number of entries in the closed arm and the open arm were non-significantly different between groups (**Appendix Figure S 8.3**). The *Winnie* mice sexes showed non-significant differences in the FST tests (**Appendix Figure S 8.4**).

Considering the negligible differences observed between the *Winnie* male mice and *Winnie* female mice groups, it remains inconclusive whether the female *Winnie* mice displayed higher levels of depression or anxiety than their male counterparts. Consequently, we pooled the data from both sexes of *Winnie* mice and juxtaposed it with the unified data from both sexes of C57BL/6 mice; with each group comprising $n=16$ mice.

Figure 4.2 Reduced spontaneous physical activity and increased sleep in *Winnie* mice

Spontaneous physical activity and sleep behaviour were compared between *Winnie* mice and C57BL/6 littermates. The data points were recorded within light and dark cycles from 0700 to 1900 hour and 1900 to 0700 hour. **(A)** Wheel metres. **(B)** Wheel speed (metres/second). **(C)** Run percentage. **(D)** Walk percentage. **(E)** Sleep hours. **(F)** Sleep percentage. **(G)** Still time percentage. **(H)** Quiet time without sleeping. For analyses, $p \leq 0.05$ was considered significant. All data are presented as mean $\pm$ SEM, n=16 mice/group, *** $p < 0.001$. **Abbreviations:** %, percentage.

Figure 4.2 Reduced physical activity and increased sleep in *Winnie* mice



4.4.5 Anxiety-like behaviours expressed in *Winnie* mice

In the LDT, increased exploratory behaviour is associated with increased time spent in the white box, whereas anxious mice prefer the black box; and make fewer transitions between the white and black box; indicating anxiety (Bourin & Hascoët, 2003). During the LDT, the *Winnie* mice spent significantly less time in the white box (226.7 ± 15.3 seconds, $p=0.001$) compared to controls (307.2 ± 16.1 seconds) within ten minutes (**Figure 4.3A**). The *Winnie* mice entered the white box significantly fewer times (18.6 ± 1.3 units, $p=0.005$) compared to controls (23.8 ± 1.3 units) within ten minutes (**Figure 4.3B**). The *Winnie* mice spent significantly more time in the black box (362.1 ± 16.6 seconds, $p=0.005$) compared to controls (299.7 ± 12.9 seconds) within ten minutes (**Figure 4.3C**). There were non-significant differences in the distance travelled within the white box between groups over ten minutes, *Winnie* mice (1249.5 ± 94.2 cm) versus controls (1169.5 ± 67.8 cm) (**Figure 4.3D**). Risk assessment was measured by units of poking, which was greatly significantly higher in *Winnie* mice (20.2 ± 2.1 units, $p<0.001$) than controls (7.4 ± 1 units) (**Figure 4.3E**).

In the OFT, the measures of overall exploratory and locomotor activity is the frequency of central square entries, the corners, peripheries of the field, unprotected areas, indicating anxiety. The measurements of reduced grooming indicate depression (Seibenhener & Wooten, 2015). The *Winnie* mice spent more time in the centre of the field (15.5 ± 2.0 seconds, $p=0.036$) than controls (9.45 ± 1.9 seconds) within four minutes (**Figure 4.4A**). There were non-significant differences in the number of times in the centre of the field between the *Winnie* mice (13.5 ± 4.2 seconds, $p=0.07$) and controls (13.6 ± 7.08 seconds) within four minutes (**Figure 4.4B**); indicating non-significant differences in anxiety-like behaviour between the groups. Anxious mice spend significantly more time exploring the field's periphery, usually in thigmotaxis than in the unprotected centre area (Seibenhener & Wooten, 2015). The *Winnie* mice spent significantly less time in the corners (169.2 ± 7.7 seconds, $p=0.006$) than the controls (203.9 ± 8.7 seconds) within four minutes (**Figure 4.4C**). However, there were non-significant differences in the number of times in the corners between the *Winnie* mice (47.4 ± 2.3 units, $p=0.07$) and controls (56.7 ± 3.9 units) (**Figure 4.4D**). Based on the observed data, it remains inconclusive whether the *Winnie* mice manifest symptoms consistent with anxiety.

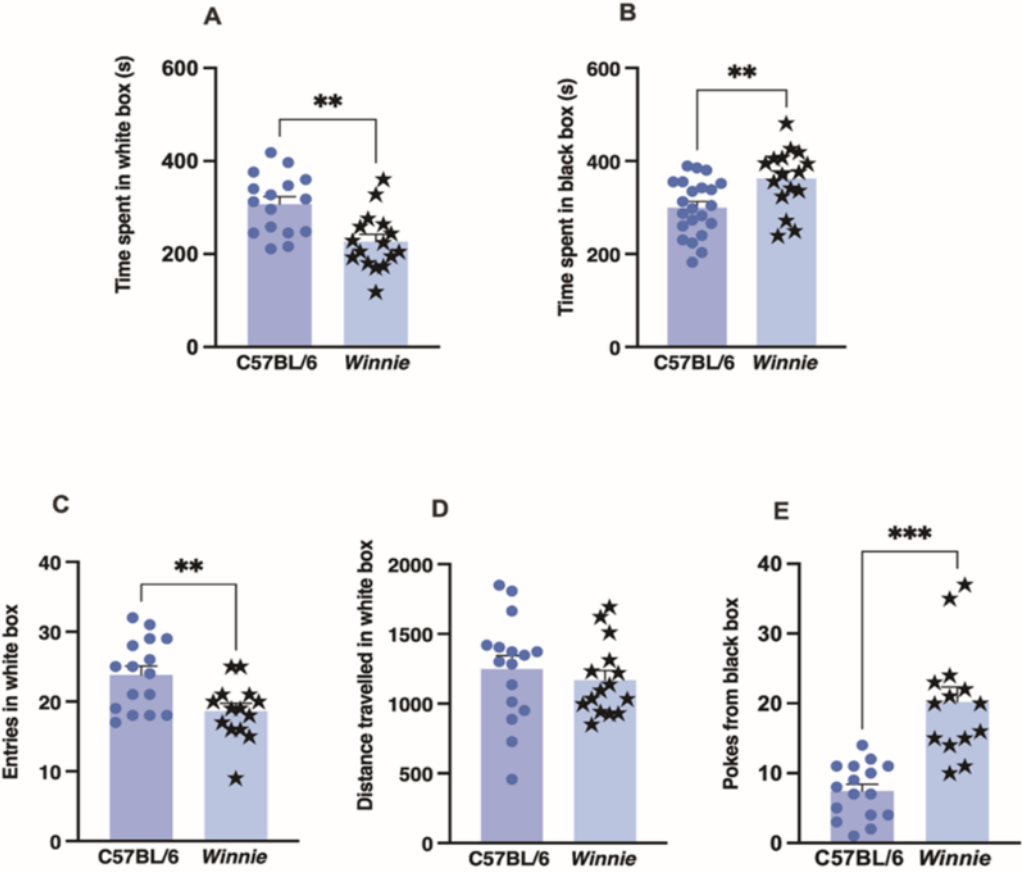
4.4.6 Reduction of exploratory activity in *Winnie* mice

The EPM determines anxiety-like behaviour based on the natural aversion of mice to exploring open and elevated areas and its instinct to explore novel environments (Schneider et al., 2011). Non-significant differences were displayed in the time spent in the closed arm, *Winnie* mice (159.0 ± 11.2 seconds, $p=0.8$) versus controls (156.3 ± 7.9 seconds) within five minutes (**Figure 4.5A**). However, the *Winnie* mice spent significantly less time in the open arm (21.1 ± 6.1 seconds, $p=0.002$) than controls (58.02 ± 8.8 seconds) within five minutes (**Figure 4.5B**) indicating anxiety-like behaviour. There were non-significant differences in time spent in the centre, *Winnie* mice (117.04 ± 10.3 seconds) versus controls (104.32 ± 8.4 seconds) within five minutes (**Figure 4.5C**). In addition, there were non-significant differences in entries into the closed arm, *Winnie* mice (11.2 ± 0.8 units) versus controls (12.4 ± 0.9 units) within five minutes (**Figure 4.5D**). There were non-significant differences in entries into the open arm, *Winnie* mice (5.15 ± 1.2 units) versus controls (6.1 ± 0.6 units) within five minutes (**Figure 4.5E**).

Figure 4.3 *Winnie* mice exhibit anxiety-like behaviours

Mice exhibiting higher levels of anxiogenic-like behaviour make fewer transitions between the white and black box. **(A)** Time spent in the white box (seconds). **(B)** Time spent in the black box (seconds). **(C)** The number of entries into the white box. **(D)** Distance travelled within the white box (cm). **(E)** The number of pokes from the black box. For analyses, $p \leq 0.05$ was considered significant. All data are presented as mean $\pm$ SEM, $n=16$ mice/group, $**p < 0.005$, $***p < 0.001$. **Abbreviations:** seconds, s.

Figure 4.3 *Winnie* mice exhibit anxiety-like behaviours



4.4.7 Depressive-like behaviour expressed in *Winnie* mice

Depressed mice, as well as those suffering from neurodegenerative disorders, often display notable changes in their self-grooming behaviours. Specifically, there is a pronounced reduction in their self-grooming activity. This decrease in self-grooming can serve as an observable symptom or indicator of their underlying conditions (Kalueff et al., 2016). Our findings distinctly suggests the presence of depression in *Winnie* mice. The grooming time in *Winnie* mice was significantly lower (48.8 ± 8.1 seconds, $p=0.001$) compared to controls (98.9 ± 6.9 seconds) within four minutes (Figure 4.4E). The number of grooming sessions was significantly reduced in *Winnie* mice (123.1 ± 13.4 seconds, $p<0.001$) compared to controls (197.0 ± 11.2 seconds) (Figure 4.4F).

The FST uses immobility as a marker of despair and depressive-like behaviour in rodents (Can et al., 2012). The *Winnie* mice demonstrated significantly less swimming mobility (57.4 ± 4.1 seconds, $p=0.0013$) than controls (103.2 ± 12.2 seconds) within four minutes (Figure 4.6A). The *Winnie* mice swimming immobility was significantly higher (182.5 ± 4.1 seconds, $p=0.007$) than controls (151.4 ± 9.9 seconds) within four minutes (Figure 4.6B). Our results from the FST clearly indicate the manifestation of depressive symptoms in the *Winnie* mice.

Figure 4.4 *Winnie* mice exhibit anxiety and reduced grooming behaviour

During the OFT high exploratory behaviour, anxiety levels and self-grooming behaviour were measured in 14-week-old *Winnie* mice and compared to age-matched control C57BL/6 littermates. **(A)** Time spent in the centre. **(B)** Number of times in the centre. **(C)** Time spent in the corners **(D)** Number of times in the corners. **(E)** Grooming time. **(F)** Number of groomings. All data are presented as mean $\pm$ SEM, n=16 mice/group; * $p \leq 0.05$; ** $p < 0.006$; *** $p \leq 0.001$. **Abbreviations:** seconds, s.

Figure 4.4 *Winnie* mice exhibit anxiety and depressive-like behaviours

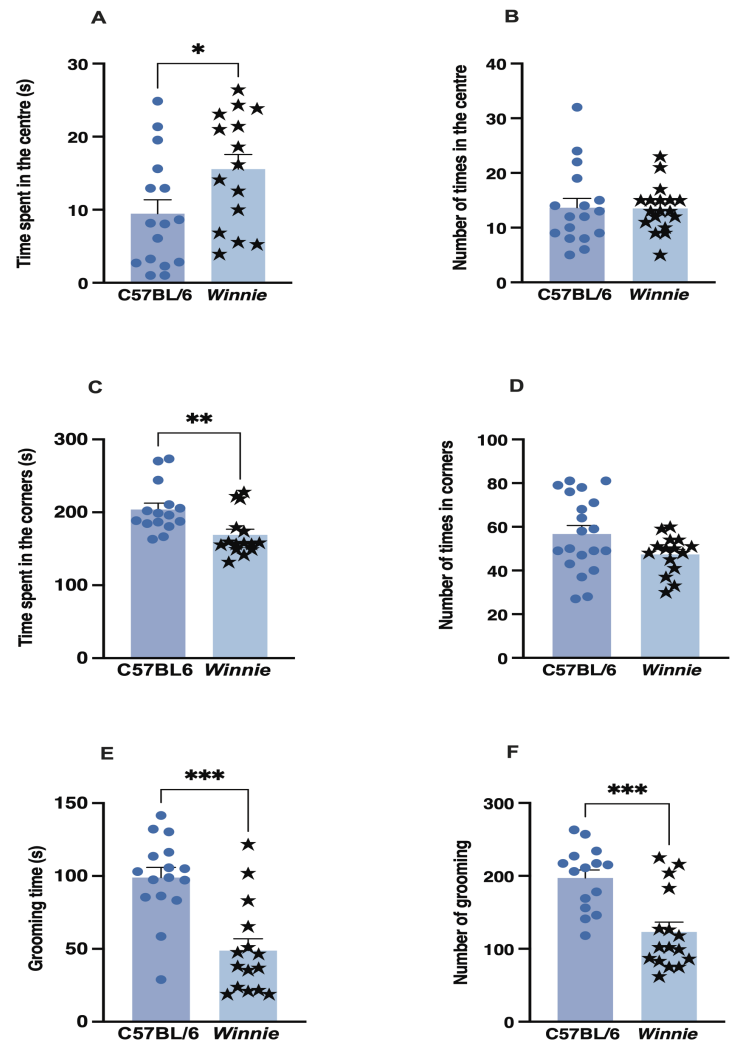


Figure 4.5 Reduction of exploratory activity in *Winnie* mice

EPM comparisons were conducted using the total time scores and the number of entries across the test session for each behavioural measure. **(A)** Time in the closed arm (s). **(B)** Time in the open arm (s). **(C)** Time spent in the centre (s). **(D)** Entries in closed arm. **(E)** Entries in open arm. All data are presented as mean $\pm$ SEM, n=16 mice/group, ** $p < 0.002$.

Abbreviations: seconds, s.

Figure 4.5 Reduction of exploratory activity in *Winnie* mice

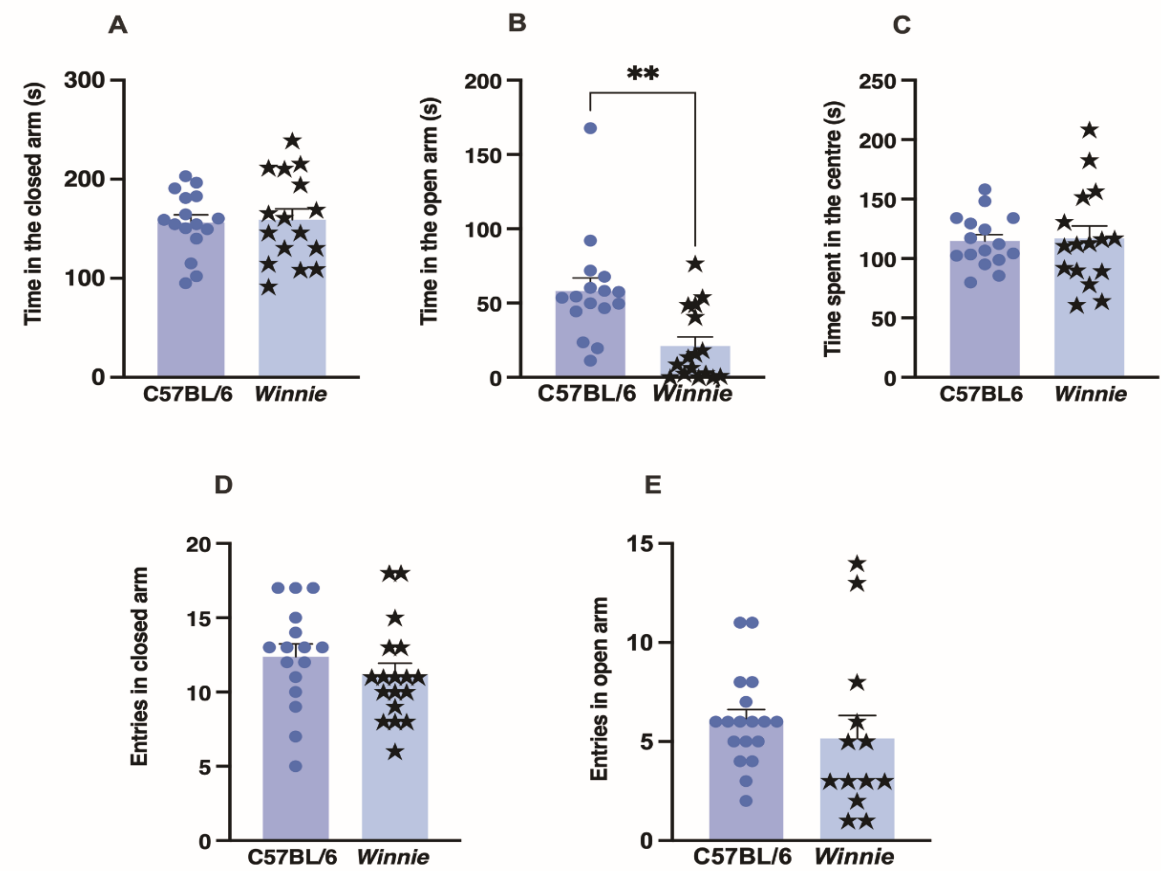
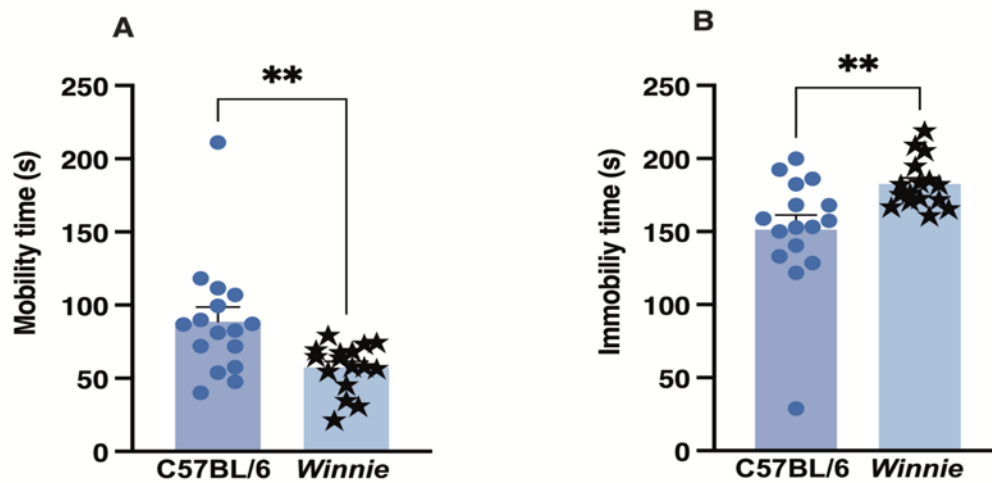


Figure 4.6 *Winnie* mice exhibit depressive behaviour

The FST uses swimming immobility as a marker of behavioural despair and is the standard test of depressive-like behaviour in rodents. **(A)** Mobility time (s). **(B)** Immobility time (s). All data are presented as mean $\pm$ SEM, $n=16$ mice/group, $**p \leq 0.007$. **Abbreviations:** seconds, s.



4.4.8 Expressions of inflammatory cytokines in *Winnie* mice brains

Cytokines are involved in the pathophysiology of depression and anxiety, and can be upregulated in response to inflammation (Felger & Lotrich, 2013). This study performed RNA-Seq analysis to measure cytokine transcripts in *Winnie* mouse brains compared to the control C57BL/6 littermates. Via the Pathview plot from the KEGG database gene expression data presented as significance scores (sign $\text{Log}_2\text{FC}(\text{fold change}) \times \text{Log}_{10}\text{P}$ values) for analysis between the brains of *Winnie* mice and controls. The analysis revealed the downregulated DEGs of interleukin 22 (*Il22*), interleukin 12a (*Il2a*), and interleukin 12 receptor β 2 (*Il2rb2*). Upregulation DEGs included transforming growth factor β 1 (*Tgfb1*), interleukin 2 receptor gamma (*Il2rg*), interleukin 5 (*Il5*), mothers against decapentaplegic homolog 2 (*Smad2*), interleukin 21 (*Il21*), interleukin 23 α (*Il23a*), interleukin 12 receptor β 1 (*Il2rb1*), toll-like receptor 5 (*Tlr5*), toll-like receptor 2 (*Tlr2*) and tumour necrosis factor (*Tnf*) (**Figure 4.7A**).

Data visualisation of a heat map of DEGs mapped from the IBD pathway from the KEGG database from individual mice was generated (**Figure 4.7B**) showed non-significant differences between male and female mice. The represented genes included *Il23a*, *Il2rb1*, *Il21*, histocompatibility 2, O region alpha locus (*H2-Oa*), interleukin 18 receptor accessory protein (*Il8rap*), interleukin 23 receptor (*Il23r*), interleukin 5 (*Il5*), *Tnf*, nucleotide-binding oligomerisation domain (*Nod2*), histocompatibility 2 (*H2-DMb2*), *Tlr2*, histocompatibility 2, class II antigen E beta (*H2-Eb1*), *Il2rg*, *Tlr5* and histocompatibility 2, class II antigen A, beta 1 (*H2-Ab1*).

Figure 4.7 Inflammatory cytokines in *Winnie* mice brains

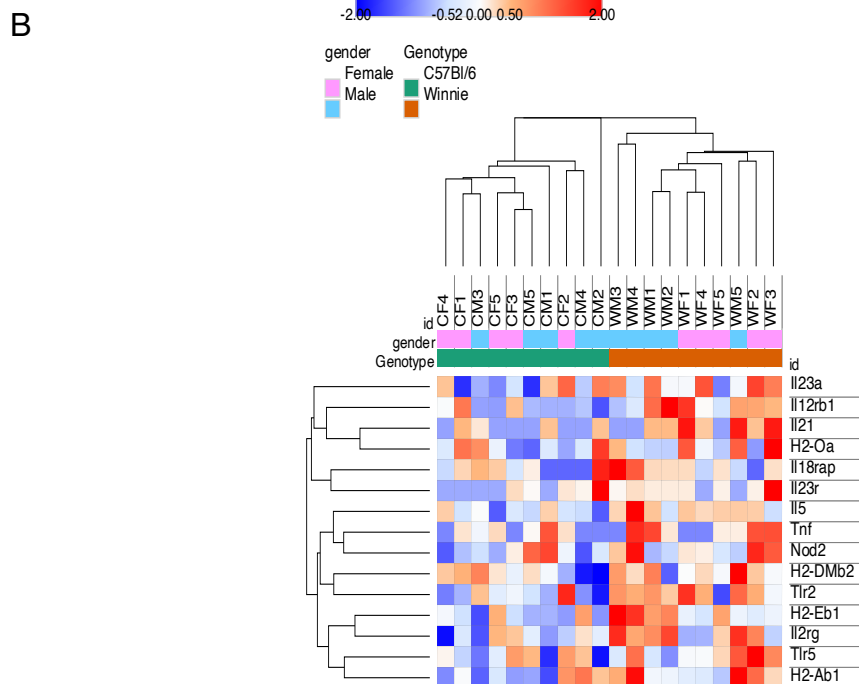
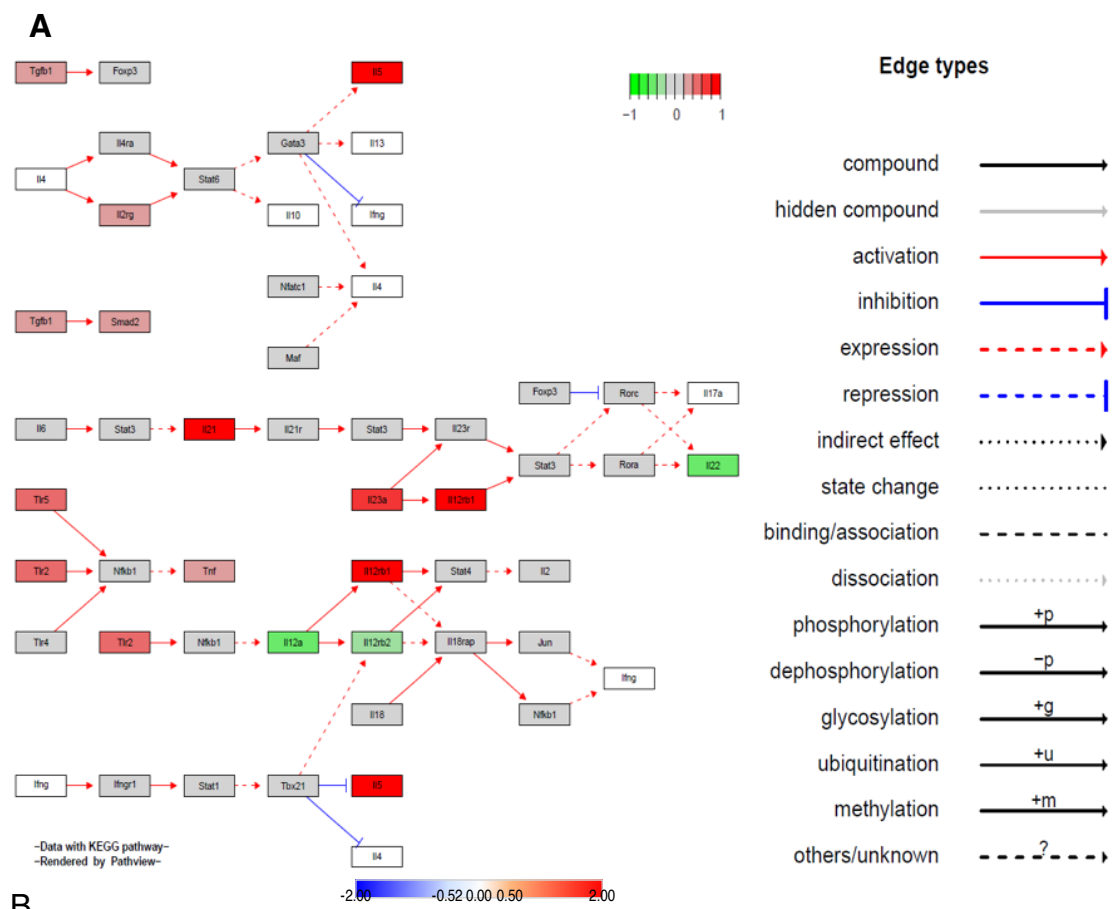
Pathview plot for the Inflammatory Bowel Disease pathway from the KEGG database.

(A) Data (**Green** to **Red**) presented as significance scores (sign $\text{Log}_2\text{FC} \times \text{Log}_{10}\text{P}$ values) for cytokine gene expression analysis between the brains of control C57BL/6 littermates and *Winnie* mice. **Green** represents downregulated gene expressions included *Il22*, *Il12a*, *Il12rb2*. **Red** represents upregulated genes of *Tgfb1*, *Smad2*, *Il2rg*, *Il5*, *Il21*, *Il23a*, *Il12rb1*, *Tlr5*, *Tlr2*, *Tnf*.

(B) Heat map representation of upregulated genes (**red**) and downregulated genes (**blue**) associated with cytokine gene expressions in *Winnie* mice determined by RNA-Seq. Data are represented as Z-scores of CPM values across samples.

Abbreviations: interleukin 22, *Il22*; interleukin 12a, *Il2a*; and interleukin 12 receptor β 2, *Il2rb2*; transforming growth factor β 1, *Tgfb1*; interleukin 2 receptor gamma, *Il2rg*; interleukin 5, *Il5*; mothers against decapentaplegic homolog 2, *Smad2*; interleukin 21, *Il21*; toll-like receptor 5, *Tlr5*; toll-like receptor 2, *Tlr2*; tumour necrosis factor, *Tnf*; interleukin 23 a, *Il23a*; interleukin 12 receptor β 1, *Il12rb1*; interleukin 21, *Il21*; histocompatibility 2 O region alpha locus, *H2-Oa*; interleukin 18 receptor accessory protein, *Il8rap*; interleukin 23 receptor, *Il23r*; interleukin 5, *Il5*; nucleotide-binding oligomerisation domain, *Nod2*; histocompatibility 2 class II, locus Mb2, *H2-DMb2*; histocompatibility 2, class II antigen E beta, *H2-Eb1*; histocompatibility 2, class II antigen A, beta 1, *H2-Ab1*.

Figure 4.7 Inflammatory cytokines in *Winnie* mice brains



4.4.9 *Winnie* mice exhibit protein expressions related to inflammation

To evaluate for alterations in the expression of microglia, astrocytes, and oligodendrocyte cells in the PFC, hippocampus, and NAc, IHC was performed on coronal slices of these brain areas. This study determined IR of HMGB1, NeuN, GFAP, MBP, and TMEM119 in the *Winnie* mice brains.

4.4.9.1 *Changes in HMGB1 in the hippocampus*

HMGB1 is a non-histone chromatin-associated protein and is primarily known for its function in the immune system's response to inflammation, the promotion of axonal growth and remyelination following injury or disease by activating glial cells, and its ability to regulate neural stem cell differentiation and neurogenesis (Paudel et al., 2020). In coronal cross-sections of the hippocampus were labelled with a nuclei marker, DAPI and anti-HMGB1 antibody. The quantitative analysis of HMGB1 IR in a 2 mm² area demonstrated the following results (**Figure 4.8 A and B**). The total HMGB1 in the hippocampus of *Winnie* mice (5.2 ± 0.8 IR area%, $p=0.0067$, $n=5$) demonstrated a significantly higher level of HMGB1 expression compared to controls (1.7 ± 0.7 IR area%, $n=5$) (**Figure 4.8 C**). Although this study demonstrated changes in HMGB1 IR, *Hmgb1* showed non-significant differences in the RNA-Seq results in *Winnie* mice brains.

4.4.9.2 *Neuronal changes in the hippocampus*

The expression of NeuN facilitates the examination of neurogenesis within the dentate gyrus region of the hippocampus. Anti-NeuN is utilised in diagnostic pathology for determining neuronal phenotype and identifying neuronal differentiation (Gittins & Harrison, 2004). To label *Winnie* mice and control mice the hippocampus was stained with DAPI and an anti-NeuN antibody separately, followed by merged staining of DAPI and an anti-NeuN antibody (**Figure 4.9**). To quantify the data an analysis of NeuN IR in a 2 mm² area was conducted in the hippocampus of *Winnie* mice compare to control mice. *Winnie* mice exhibited significantly lower NeuN expression in the total hippocampus (4.1 ± 0.4 IR area%, $p=0.0257$, $n=4$) compared to the control mice (6.0 ± 0.6 IR area%, $n=4$). *Rbfox1*, in association with NeuN protein expression, did not display any change in the RNA-Seq analysis of *Winnie* mice brains.

4.4.9.3 Myelin basic protein changes in the prefrontal cortex

Oligodendrocyte lineage cells react swiftly to various brain damage, especially demyelination damage by altering its morphology, proliferation, and differentiation rates. MBP, abundantly expressed in oligodendrocyte-produced myelin, aids in the organisation and structure of the myelin sheath inside the central nervous system (CNS), and regulates brain inflammation. The main function or dysfunction of MBP in the brain is demyelination (Harauz et al., 2009). MBP plays an essential role in the creation of new neuronal connections, thereby promoting synaptic plasticity. This process is pivotal for managing emotions, facilitating learning, and forming memories and protect against neurodegenerative diseases (Zhou et al., 2021). An MBP-specific antibody was used to mark oligodendrocytes, enabling the detection of myelination variations **Figure 4.10**. In comparison to controls (21.4 ± 1.2 IR area%, $n=5$), MBP IR was significantly lower in the prelimbic area of the PFC in *Winnie* mice (15 ± 0.9 IR area%, $n=4$) (Welch's correction, $p=0.0022$). The MBP IR shows non-significant differences between *Winnie* mice (17.6 ± 0.7 IR area%, $n=5$) and controls (20.4 ± 1.1 IR area%, $n=5$) in the hippocampus ($p=0.0633$). There were non-significant differences in MBP IR between groups in the NAc (*Winnie* 20.5 ± 1.3 IR area%, $n=5$; controls 21.0 ± 1 IR area%, $n=4$) (Welch's correction, $p=0.8$). While the study revealed alterations in MBP IR, *Mbp* did not exhibit significant differences in the RNA-Seq analysis of *Winnie* mice brains compared to the controls.

Figure 4.8 HMGB1 expression in *Winnie* mice brains

In coronal cross-sections with a thickness of 20 μm and a bregma -1.94 mm, the hippocampus was labelled with a nuclei marker, HMGB1 and DAPI. Confocal images represent distinct differences between the control C57BL/6 littermates and *Winnie* mice in 4',6-diamidino-2-phenylindole (DAPI) (**blue**) and HMGB1 (**red**) IR. The hippocampus was labelled with nuclei marker DAPI (**blue**) (A), an anti-HMGB1 antibody (**red**) (A'), and combined DAPI and HMGB1 staining (**blue** and **red**) (A''). Hippocampal tissues from *Winnie* mice were stained with DAPI (**blue**) (B), anti-HMGB1 antibody (**red**) (B'), and combined DAPI and HMGB1 staining (**blue** and **red**) (B''). (C) HMGB1 in the hippocampus, ** $p=0.0067$. All data are presented as mean $\pm$ SEM, $n=5$ /mice/group. **Abbreviations:** immunoreactivity, IR; 4',6-diamidino-2-phenylindole, DAPI; high mobility group box 1, HMGB1.

Figure 4.8 Changes in HMGB1 expression in the hippocampus

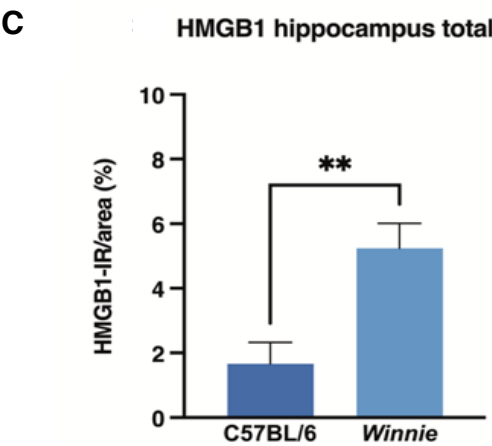
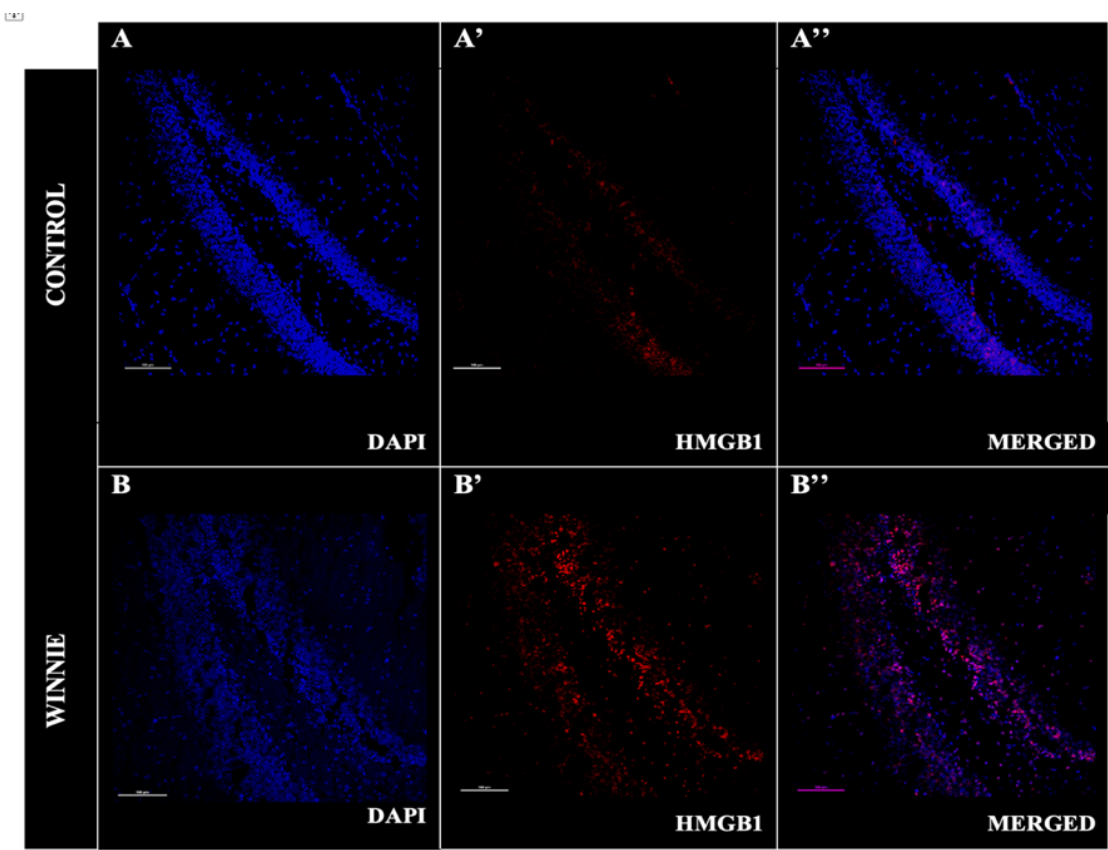


Figure 4.9 Changes in neuronal expression in the hippocampus

In coronal cross-sections with a thickness of 20 μm and a bregma -1.94 mm, labelling of hippocampal cells from control mice were stained with DAPI (**blue**) and anti-NeuN antibody (**green**). In controls (**A**) an anti-NeuN antibody (**green**). In *Winnie* mice, (**A'**) an anti-NeuN antibody (**green**). In controls (**B**) DAPI (**blue**). In the *Winnie* mice (**B'**) DAPI staining (**blue**). In controls (**C**) merged staining of DAPI and NeuN (**blue** and **green**). In *Winnie* mice, (**C'**) merged DAPI and NeuN staining (**blue** and **green**). Coronal cross-sections, hippocampus: bregma -1.94 mm. Images were captured at 40 x (Hippocampus), scale bars 50 μm . Quantitative differences are displayed in (**D**) NeuN hippocampus ($*p=0.0257$). All data are presented as mean $\pm$ SEM, n=4-5 animals/group. **Abbreviations:** immunoreactivity, IR; neuronal nuclei, NeuN; 4',6-diamidino-2-phenylindole, DAPI.

Figure 4.9 Changes in neuronal expression in the hippocampus

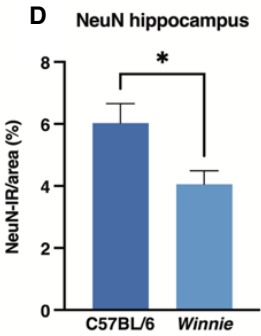
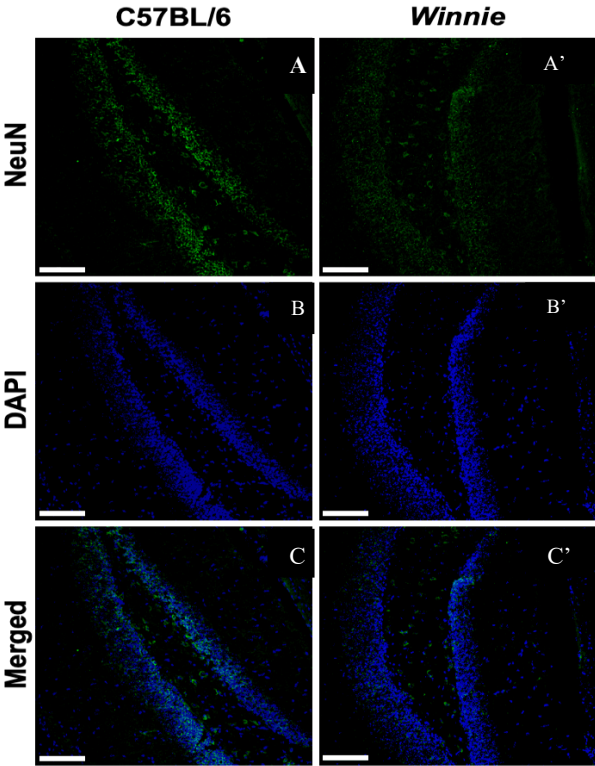
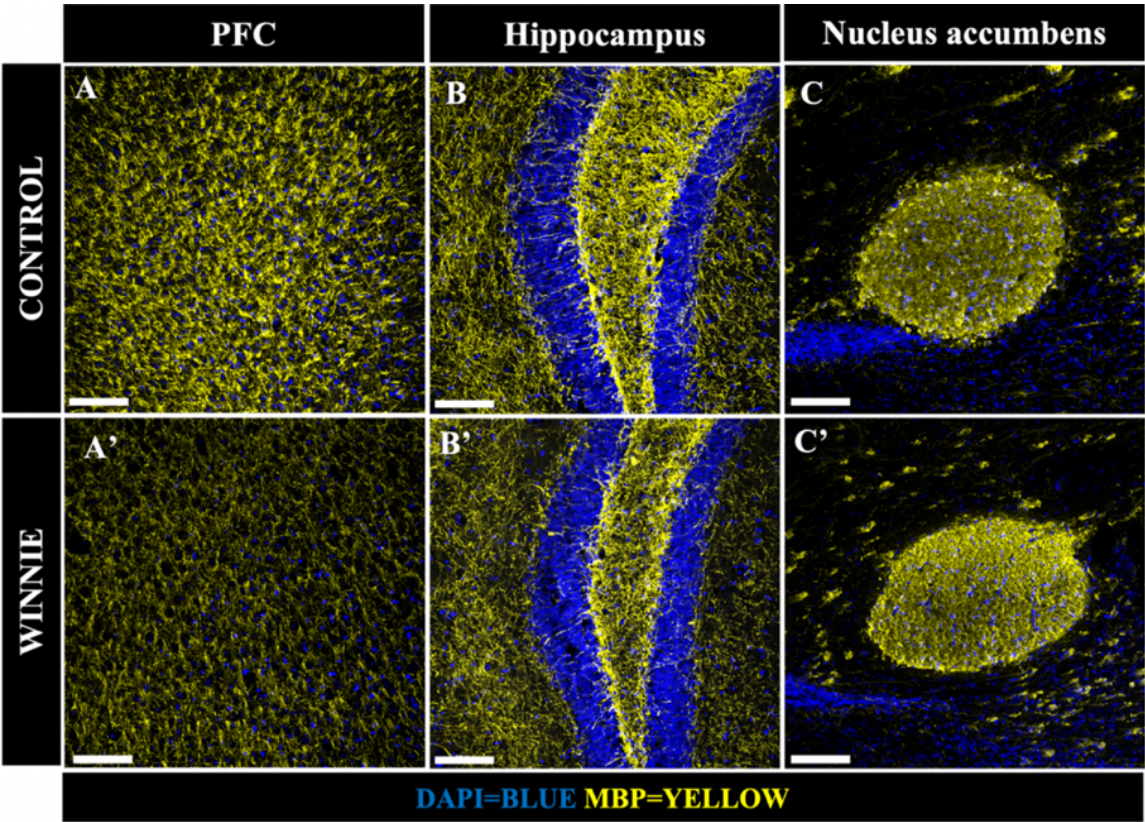


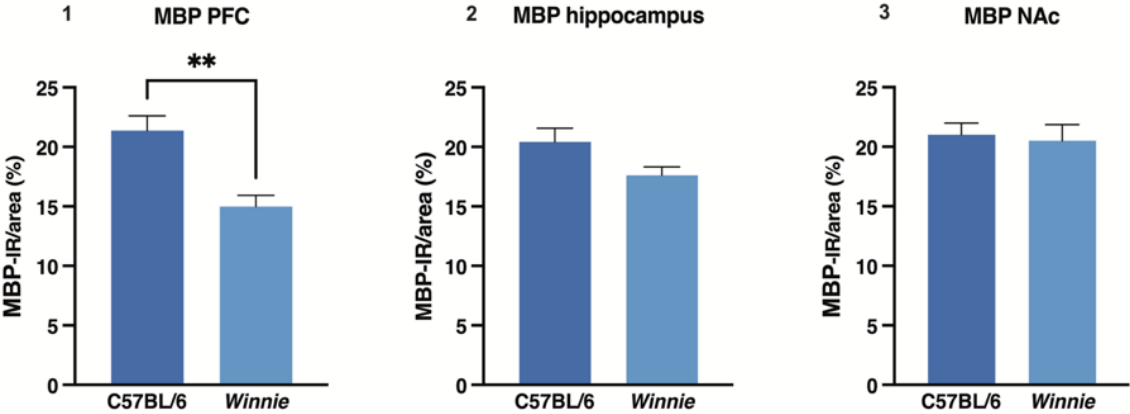
Figure 4.10 Myelin basic protein in *Winnie* mice brains

DAPI (**blue**) and MBP (**yellow**) staining of the nuclei and myelin in the PFC from the controls (**A**) and *Winnie* mice (**A'**) displaying. DAPI and MBP staining of the nuclei and myelin in the hippocampus from the controls (**B**) and *Winnie* mice (**B'**). DAPI and MBP staining of the nuclei and myelin in the NAc from the controls (**C**) and *Winnie* mice (**C'**). Coronal cross-sections: 20 μm thickness; PFC: bregma +2.58 mm, hippocampus: bregma -1.94 mm, NAc: bregma +1.54 mm. Images were captured at 20 x (PFC and NAc), scale bars 100 μm and 40 x (hippocampus), scale bars 50 μm . **(D1)** MBP in the PFC, $**p=0.0022$; **(D2)** MBP in the hippocampus, $p=0.0633$; **(D3)** MBP in the NAc, $p=0.8$. All data are presented as mean $\pm$ SEM, $n=4-5$ mice/group. **Abbreviations:** immunoreactivity, IR; nucleus accumbens, NAc; prefrontal cortex PFC; myelin basic protein, MBP; 4',6-diamidino-2-phenylindole, DAPI.

Figure 4.10 Myelin basic protein in *Winnie* mice brains



D



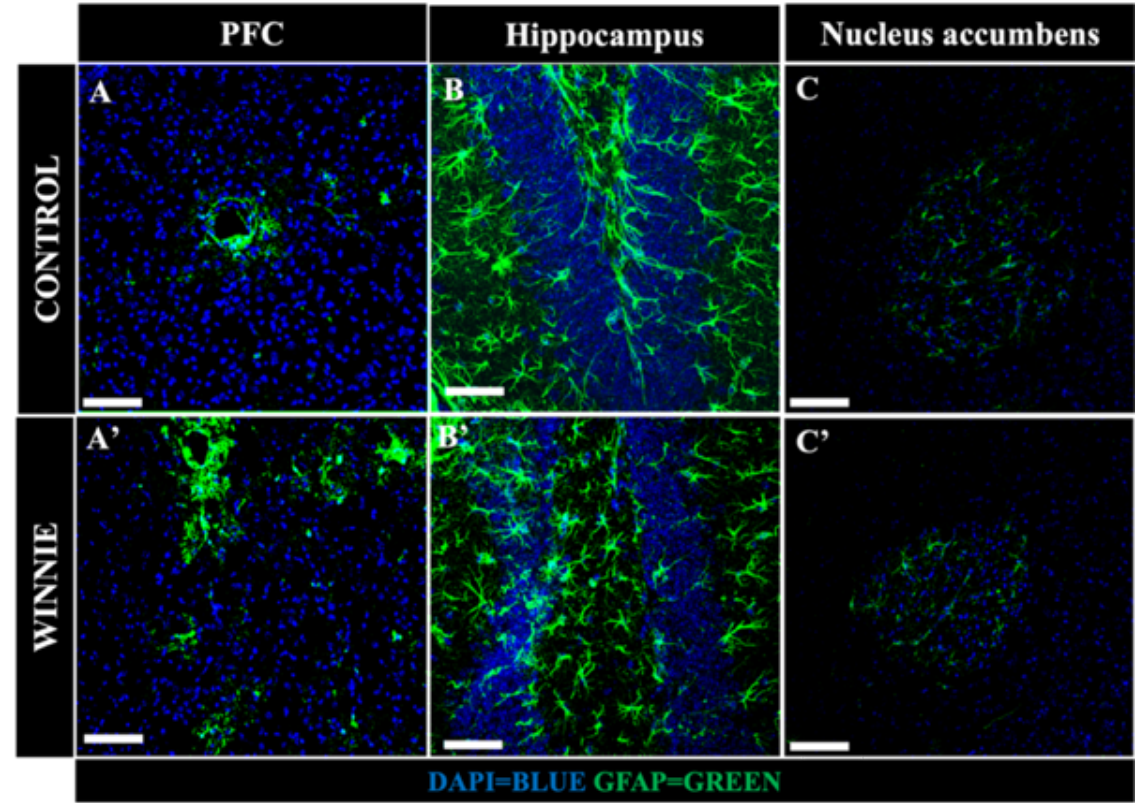
4.4.9.4 Changes in glial fibrillary acidic protein

The GFAP antibody was employed to identify astrocytes in the coronal brain slices. GFAP, an intermediate filament protein, is found in astrocytes and provides structural integrity and metabolic support for neurons and the neural network. Its expression increases during astrogliosis and inflammation in the CNS (Carson et al., 2006). By forming new synapses, GFAP benefits emotions, learning and memory (Yang & Wang, 2015). After staining with DAPI (**blue**) and GFAP (**green**) antibody, different patterns of microglia expression were found in the PFC, hippocampus and NAc in the *Winnie* mice brains compared to control C57BL/6 littermates (**Figure 4.11**). When compared to controls (0.8 ± 0.3 IR area%, $n=4$), *Winnie* mice exhibited significantly higher GFAP IR within the PFC (2.7 ± 0.5 IR area%, $p=0.017$, $n=4$). In the hippocampus, there were non-significant differences in GFAP IR between the *Winnie* (4.2 ± 0.8 IR area%, $n=4$) and controls (4.8 ± 0.6 IR area%, $n=5$) (Welch's correction, $p=0.58$). There were non-significant differences in GFAP IR in the NAc in *Winnie* mice (3.9 ± 0.5 IR area%, $n=4$) and controls (4.7 ± 0.8 IR area%, $n=5$) (Welch's correction, $p=0.44$). While the study revealed alterations in GFAP IR, *Gfap* did not exhibit significant differences in the RNA-Seq analysis of *Winnie* mice brain samples.

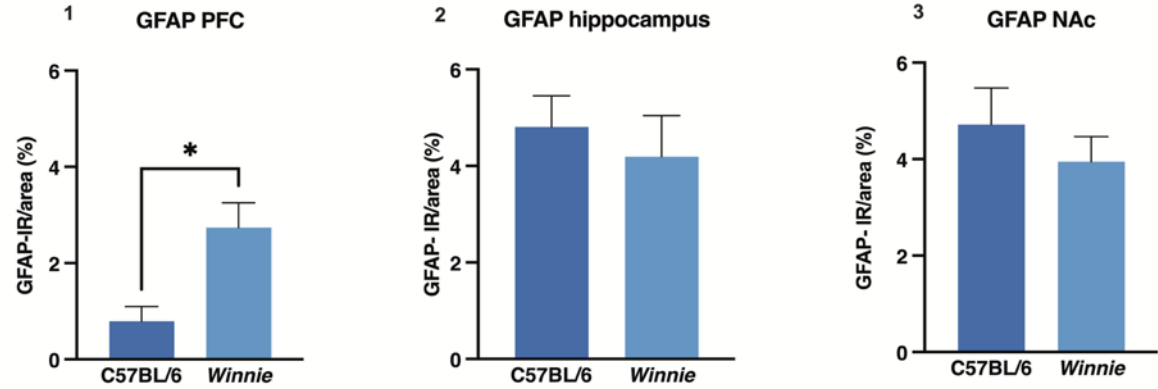
Figure 4.11 Glial fibrillary acidic protein in *Winnie* mice

Images display differences in DAPI (blue stain) and GFAP (green stain) IHC staining in the controls and *Winnie* mice brains. **(A)** PFC staining of DAPI (**blue**) and GFAP (**green**) in controls. **(A')** PFC staining of DAPI (**blue**) and GFAP (**green**) in *Winnie* mice. **(B)** Hippocampus staining of DAPI (**blue**) and GFAP (**green**) in controls. **(B')** Hippocampus staining of DAPI (**blue**) and GFAP (**green**) in *Winnie* mice. **(C)** DAPI (**blue**) and GFAP (**green**) staining in the NAc of controls. **(C')** DAPI (**blue**) and GFAP (**green**) staining in the NAc of *Winnie* mice. Coronal cross-sections: 20 μ m thickness; PFC: bregma +2.58 mm, hippocampus: bregma -1.94 mm, NAc: bregma +1.54 mm. Images were captured at 20 x (PFC and NAc), scale bars 100 μ m and 40 x (Hippocampus) scale bars 50 μ m. Quantitative analysis of **(D1)** GFAP PFC, $*p=0.017$ (n=4); **(D2)** GFAP hippocampus, $p=0.58$, *Winnie* (n=4), controls (n=5); **(D3)** GFAP NAc, $p=0.44$, *Winnie* (n=4), Control mice (n=5). All data are presented as mean $\pm$ SEM. Significance was considered at p value 0.05. **Abbreviations:** immunoreactivity, IR; prefrontal cortex, PFC; glial fibrillary acidic protein, GFAP; nucleus accumbens, NAc, 4',6-diamidino-2-phenylindole, DAPI.

Figure 4.11 Glial fibrillary acidic protein in *Winnie* mice brains



D



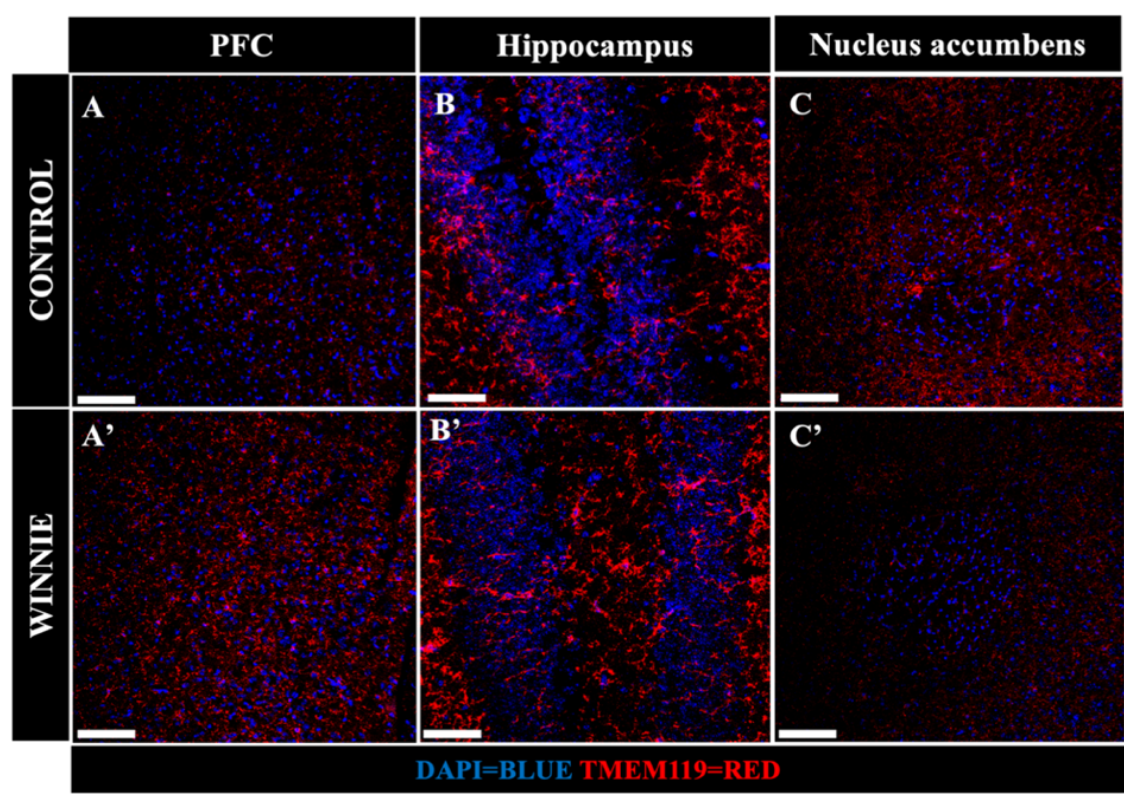
4.4.9.5 Changes in TMEM119 expression

Microglia were identified via the TMEM119 antibody. TMEM119 is located in the hippocampus and upregulates in association with microgliosis and neuroinflammation. TMEM119 regulates the maturation of astrocytes and is a critical indicator for behaviour, memory and learning (Ruan & Elyaman, 2022). Notably, the immune response of microglia showed varied expression patterns across different brain areas in the *Winnie* mice compared to control C57BL/6 littermates (**Figure 4.12**). DAPI (**blue**) staining distinguishes from the TMEM119 (**red**) staining. In the PFC, the images display clear distinctions of TMEM119 IR between the *Winnie* mice and controls. The quantitative analysis of TMEM119 IR in the prelimbic area of the PFC was significantly higher in the *Winnie* mice (8.5 ± 0.5 IR area%, $p=0.0002$, $n=4$) than controls (2.8 ± 0.6 IR area%, $n=4$). The hippocampus showed non-significant differences in TMEM119 IR between controls and *Winnie* mice. In the hippocampus, there were non-significant differences in TMEM119 IR between the *Winnie* mice (8.7 ± 0.9 IR area%, $n=4$) and controls (7.7 ± 1.4 IR area%, $n=5$) (Welch's correction, $p=0.55$). Control mice illustrate the significant difference to *Winnie* mice in the NAc. A significant decrease in TMEM119 IR was revealed in the *Winnie* mice NAc (65.1 ± 2.5 IR area%, $p=0.016$, $n=4$) when compared to controls (78.4 ± 2.8 IR area%, $n=4$). While the study revealed alterations in TMEM119 IR, *Tmem119* did not exhibit statistically significant differences in the RNA-Seq analysis of *Winnie* mice brain samples compared to controls.

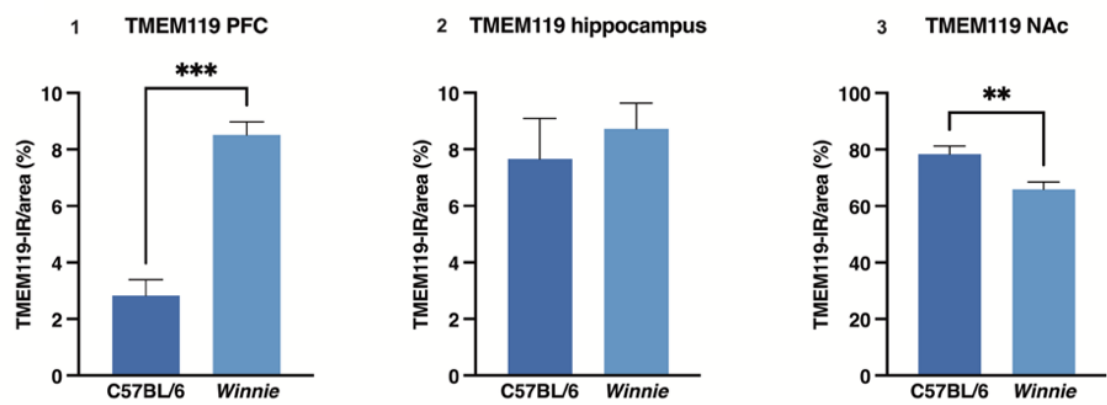
Figure 4.12 Changes in microglia expression in *Winnie* mice brains

DAPI (**blue**) and TMEM119 (**red**) staining of the nuclei and microglia in the PFC from the controls C57BL/6 littermates (**A**) and *Winnie* mice (**A'**) is displaying. DAPI and TMEM119 staining of the nuclei and microglia in the hippocampus from the control mice (**B**) and *Winnie* mice (**B'**). DAPI and TMEM119 staining of the nuclei and microglia in the NAc from the controls (**C**) and *Winnie* mice (**C'**). Coronal cross-sections: 20 μm thickness; PFC: bregma +2.58 mm, hippocampus: bregma -1.94 mm, NAc: bregma +1.54 mm. Images were captured at 20 x (PFC and NAc), scale bars 100 μm and 40 x (hippocampus), scale bars 50 μm . **(D1)** TMEM119 PFC, *** $p=0.0002$; **(D2)** TMEM119 hippocampus, $p=0.55$, *Winnie* (n=4), controls (n=5); **(D3)** TMEM119 NAc, ** $p=0.016$. All data are presented as mean $\pm$ SEM. Significance was considered at p value 0.05. **Abbreviations:** transmembrane protein 119, TMEM119; immunoreactivity, IR; prefrontal cortex, PFC; nucleus accumbens, NAc; 4',6-diamidino-2-phenylindole, DAPI.

Figure 4.12 Changes in microglia expression in *Winnie* mice brains



D



4.5 Discussion

Behavioural phenotyping of *Winnie* mice observed anxiety- and depressive-like behavioural changes in exploration, risk-taking, reduced motivation, reduced spontaneous physical activity and increased sleep time. This study gathered information from the hippocampus section of the brains of *Winnie* mice at a molecular level involving cytokine gene expressions and protein levels. We will explore each of these findings and discuss their implications.

4.5.1 *Winnie* mice exhibit reduced spontaneous physical activity and increased sleep

Voluntary wheel running behaviour serves as a sensitive indicator of disturbed wellbeing, as observed in the DSS-induced colitis mouse model (Weegh et al., 2020). The *Winnie* mice exhibited reduced distance in wheel metres, the wheel running speed, the percentage of wheel running, and walking activity percentage. The *Winnie* mice showed physical activity patterns similar to DSS-induced colitis mice, which suggested that reduced spontaneous physical activity is associated with neuroinflammation (Truyens et al., 2023). Similarly, *Muc2*^{-/-} colitis mouse model demonstrated reduced spontaneous physical activity (Morozova et al., 2022). A plausible interpretation could be that the inflammatory reaction incited by colitis may instigate changes in neurotransmitter systems and hormonal disparities, potentially influencing the motivation and behaviour of the mouse (Jain et al., 2015). Another cause could be hyperalgesia inhibiting the *Winnie* mouse's motivation, as noted in DSS-induced colitis mice that exhibit mechanical hyperalgesia of the abdominal skin and of the plantar skin as well as heightened visceral nociception (Jain et al., 2015). Although hyperalgesia was not measured in this study, it is warranted that the discomfort and pain associated with colitis may discourage the *Winnie* mouse from participating in exercise or activities that exacerbate its symptoms (Verma-Gandhu et al., 2007).

The *Winnie* mouse slept for a significantly longer time during the waking hours. Despite being nocturnal, the *Winnie* mice did not show variation in the light hours of sleep activity when compared to the control mice. In contrast, up to 82% of IBD patients experience sleep problems, night-time awakenings, and nonrestorative sleep (Kinnucan et al., 2013),

however humans have comorbidities such as sleep apnoea, type II diabetes, and social stresses (Sharma & Kavuru, 2010). Additionally, sleep studies in colitis mouse models tend to focus on orchestrating sleep disruption and examining sleep quality and how these may affect disease progression (Kinnucan et al., 2013; Tang et al., 2009). The lack of sleep data in untreated colitis models severely limits our understanding in this area. The findings offer support for the novel hypothesis that *Winnie* mice may incur hypersomnia, characterised by excessive sleepiness (Kaplan & Harvey, 2009). In mouse brain studies, C. R. Chen et al. (2021) demonstrated that suppression of the paraventricular nucleus of the hypothalamus contributed to hypersomnia. Another study by Veasey et al. (2004), reported significant oxidative injuries in the sleep-wake regions of mouse brains and biochemical changes in association with marked hypersomnolence, and forebrain redox alteration in wake-promoting brain regions were consistent with persistent sleepiness. In humans, hypersomnia is a critical symptom of atypical depression, distinguished by mood reactivity, leaden paralysis, or rejection sensitivity (Kaplan & Harvey, 2009).

In regards to behavioural changes in humans, research studies have consistently demonstrated a bidirectional relationship between extended sleep, sleep disturbances and psychiatric conditions, with evidence suggesting that dysregulated sleep patterns can contribute to the development and exacerbation of anxiety disorders and depression (Wulff, 2010). Additionally, dysregulated sleep can influence neurobiological processes, including alterations in neurotransmitter systems, hormonal imbalances, and dysregulation of the circadian rhythm, which impact emotional dysregulation, cognition, impaired concentration and memory, decreased motivation, and reduced resilience to stress (Kaplan & Harvey, 2009). To gain a clearer understanding, further investigation is needed into the sleep quality of the *Winnie* mouse. Elucidating these factors could provide valuable insights into the fatigue experienced by approximately 80 % of individuals with active IBD (Borren et al., 2019).

4.5.2 *Winnie* mice express depressive and anxiety-like behaviours

In the LDT, the *Winnie* mice exhibited an inherent conflict between its inclination to investigate the unprotected region (exploratory drive) and its risk-avoidance behaviour (measured as poking), thereby displaying anxiety-like behaviour (Bourin & Hascoët,

2003). Previous studies involving rodents have revealed that the administration of anxiolytic drugs resulted in increased transitions between light and dark boxes, while not affecting the preference for spending more time in the black box (Heredia et al., 2014). Our results concur with previous colitis-mice behavioural studies (Haj-Mirzaian et al., 2017; Matisz et al., 2020).

The *Winnie* mice displayed reduced grooming. Depressed mice and those with neurodegenerative disorders typically display reduced self-grooming and grooming pattern rigidity (Kalueff et al., 2016). Reduced grooming implies a lack of energy for self-care and reduced self-efficacy of Bandura's theory (Williams, 1995). The *Winnie* mice, facing defeat during the FST, showed a higher immobility percentage, indicating depressive-like behaviour (Can et al., 2012). In concordance with studies in colitis-induced mice (Truyens et al., 2023; Wu et al., 2023), our results suggest that depressive-like behaviours are reliably induced by chronic intestinal inflammation in *Winnie* mice.

4.5.3 Cytokine activity in *Winnie* mice brains

Intestinal inflammation is recognised to function as an induction mechanisms for depression-related behaviours (Gadotti et al., 2019). Kronfol and Remick (2000) proposed several mechanisms for cytokine-mediated brain signalling involving the passive transport of cytokines into the brain at circumventricular sites with leaky regions of the blood-brain barrier (BBB). Secondly, the binding of cytokines to cerebral vascular endothelium; carrier-mediated transport of cytokines into the brain across the BBB and the activation of peripheral afferent nerve terminals at the site of cytokine release. Lastly, a biological pathway in which activated microglia and CNS-expressed adhesion molecules attract activated peripheral cell types to the meninges and brain parenchyma. From these results, it is proposed that the imbalanced cytokine activity triggered microglial activation and caused neuroinflammation, plausibly causing the depression- and anxiety-like behaviour in the *Winnie* mice and as observed in IBD patients (Frenkel et al., 2020).

Given the substantial prevalence of affective disorder comorbidities in mice with colitis (Do & Woo, 2018; Wu & Endres, 2022), we propose that cytokine modulating factors

may played a significant role in shaping the clinical presentation of affective disorders. Previous studies in DSS colitis-induced mice have conferred the activation of microglial cells and over-expression of multiple inflammatory cytokines correlated with behavioural changes (Dong et al., 2021). However the DSS-induced colitis mouse is an acute model of colitis, whereas the *Winnie* mouse is a chronic colitis mouse model (Eri et al., 2011). Hereby, the mRNA investigation mapped the *mus musculus* IBD KEGG pathway of cytokines in the brain of the *Winnie* mouse. Cytokine activity in *Winnie* mice brains presented an increased expression of pro-inflammatory genes, namely, *Tgfb1*, *Smad2*, *Il2r γ* , *Il5*, *Il21*, *Il23a*, *Il12rb1*, *Tlr5*, *Tlr2*, and *Tnf*. Downregulated gene expressions included *Il22*, *Il12a*, and *Il12rb2*.

Our findings demonstrated that behavioural anomalies emerge due to cytokine activity of the brain, accompanied by alterations in the activation of neuroimmune responses. For instance, in patients with major depressive disorder and neuropathic hyperalgesia, IL-23a stimulates TNF- α release, implicating the kynurenine pathway (Felger & Lotrich, 2013; Savitz, 2015). In accordance, *Il23a* and *Tnf- α* were upregulated in the *Winnie* mice brains. In correlation, another study in DSS-induced colitis mice by Nyuyki et al. (2018), investigated the association of intestinal inflammation and hyperalgesia, in which nociceptive responses were meticulously recorded, and the resulting hyperalgesia was quantified. Notably, the experimental group exhibited a significant increase in both mechanical and thermal sensitivity when compared to the respective control groups, providing valuable insights into the potential role of hyperalgesia as a marker for disease progression (Nyuyki et al., 2018). Another association with hyperalgesia is the revelation of upregulated *Il2r γ* , which is a principal IL-21 signalling component that has an essential function in promoting plasma cell differentiation of terminal B cells and in T helper 17 (Th17) cells (Spolski & Leonard, 2014). This response is implicated in the pathophysiology of human autoimmune illnesses and promotes the development of intestinal inflammation and exacerbates pain in humans (Bernstein et al., 2019).

Upon investigating the impact of systemic inflammatory challenges on the expression of enzymes in the kynurenine pathway in rat brains, there was an observed association of TNF- α with the inflammatory response (Connor et al., 2008). *Tnf- α* was upregulated in the *Winnie* mice. However, in acute DSS-treated colitis mice, TNF- α was not significantly

expressed (Gadotti et al., 2019). Due to the significant roles that these cytokines play in modulating the BBB permeability and influencing the phenotypic changes of infiltrating leukocytes, further investigation into the BBB of *Winnie* mice is required to clarify the disparities between acute and chronic models of colitis. In regard to behaviour, anti-TNF- α therapy in IBD alters brain activity reflecting visceral sensory function and cognitive-affective biases (Gray et al., 2018).

Cytokine activity in the brain increases the onset of neuropathic hyperalgesia in mice and humans (Felger & Lotrich, 2013). In the *Winnie* mice, *Il5* was upregulated. IL-5 participates in humoral inflammation and increases microglial cell metabolism and proliferation. Spinal glial activation is necessary for the onset of neuropathic hyperalgesia (Felger & Lotrich, 2013). Immobilisation stress exposure in colitis-induced mice showed an increase of activated microglial cells in the hippocampus (Jang et al., 2019). In context, microglia are the brain's principal pathologic event sensor and express all toll-like receptors (TLR), and its activation can result in chronic inflammation and neuronal damage in mice (Ifuku et al., 2020). *Tlr5*, found upregulated in *Winnie* mice brains, are pattern recognition receptors, and activated by pathogen and host-derived, potentially damage-associated chemical patterns (Ifuku et al., 2020). Recent research in *Tlr5*^{-/-} mice has linked human TLR5 to various CNS illnesses, namely Alzheimer's disease, hyperalgesia, and cerebral ischemia (Ifuku et al., 2020). Similarly, upregulated *Tlr2*, which upregulated in *Winnie* mice, are recognition molecules for multiple pathogens and signals tissue damage in the form of pain and necrosis, as well as increases TNF- α production during acute processes (de Oliveira Nascimento et al., 2012). These findings are corroborated by an investigation involving dinitrobenzene sulfonic acid (DNBS)-treated mice, which revealed elevated IL1 β , TNF- α , TLR4, TLR2, and IL-6 in the hippocampus (Haj-Mirzaian et al., 2017). In addition, behavioural alterations indicative of depressive and anxiety-like symptoms in colitis-induced mice were observed (Haj-Mirzaian et al., 2017), which corroborates the findings of depression in the *Winnie* mice of this study.

Tgf- β /Smad2 was upregulated in the *Winnie* mice brains. Activation of TGF- β /Smad2 signalling appears to have a protective role in preventing BBB breakdown and limiting CNS infiltration by inflammatory agents, as shown in a mouse model of hepatic

encephalopathy (McMillin et al., 2015). In a study of acute colitis mouse models (Mitchell, Kim, Howe, Lee, et al., 2022), dysregulated immune responses led to increased BBB permeability, facilitating the translocation of pro-inflammatory cytokines and immune cells from the gut into the brain. Further evidence of BBB permeability in TNBS colitis rodent brains (Natah et al., 2005) and DSS-induced colitis mice brains (Han et al., 2018) has been observed.

In *Winnie* mice, *Il23a* was upregulated, a marker associated with the IL-23/IL-17 pathways (Noviello et al., 2021). A genome-wide association study identified IL23R as an IBD gene (Duerr et al., 2006). Although, colitis mice studies of the brain displaying *Il23a* are sparse, *Il23a* upregulation is consistent with characteristics of inflammation found in both experimental mice with colitis and the colons of patients with IBD (Noviello et al., 2021). This underscores the importance of the IL-23/IL-17 pathway as a key component in the communication network between the gut and the brain (Noviello et al., 2021). It is of interest that while TGF- β is known to suppress macrophages in rodent brains (Zhang & An, 2007), it also promotes the production of IL-23. The latter, in turn, may instigate the synthesis of inflammatory cytokines by macrophages within the brains of mice (Cua et al., 2003). In IBD patients, the IL-23R chain pairs with IL-12R β 1 to create a heterodimeric complex (Sewell & Kaser, 2022). Of note, *Il12rb1*, which was upregulated in *Winnie* mice brains, is implicated in autoimmune inflammation of the mouse brain (Cua et al., 2003).

The *Winnie* mice brains revealed *Il12a* downregulation, which belongs to the inflammatory IL-6/12-like family (Jones & Vignali, 2011). Albeit, insufficient IL-6 influences a reduction in IDO (Savitz, 2015). Reduced IDO in rodents display decreased exploratory behaviour (Too et al., 2016), which corroborates the *Winnie* mice behavioural study. Subsequently, investigations involving rodents and depression indicate that the absence of *Il2rb*, which was downregulated in the *Winnie* mice, was associated with impairments in spatial learning and memory (Wu et al., 2010). In accordance, DSS-induced colitis mice did not exhibit novel object recognition or object location memory (Lee et al., 2023; Matisz et al., 2020). Our data aligns with earlier findings, indicating that acute colitis negatively affects memory functions that rely on the hippocampus and prefrontal cortex (Barker & Warburton, 2011). These cognitive deficiencies contribute to

the manifestation of depressive behaviours and deficits in prepulse inhibition of the acoustic startle reflex and reactivity (Wu et al., 2010).

We hypothesised that the increase in cytokines over the course of the disease might trigger changes in the CNS, leading to behavioural alterations (H. Wang et al., 2020). From our findings, the distinct immune response observed in the *Winnie* mice challenges the notion that colitis-associated pain in humans is solely attributable to psychosocial factors. In fact, neuropathic hyperalgesia can be seen as a significant manifestation of inflammation, driven by the sensitisation of primary afferent neurons by signals from mucosal tissue, inflammatory cytokines, and mediators (Sweeney et al., 2018). It is worth noting that pain is reported in approximately 70% of IBD patients (Bielefeldt et al., 2009). Our results suggests that imbalances in cytokine levels in *Winnie* mice brains may have a profound impact on the immune responses related to anxiety, depression, and neuropathic hyperalgesia. This notion is confirmed in a chronic DSS-induced colitis mice study (Truyens et al., 2023), which exhibited increased expression of inflammatory genes associated with pathways involving neutrophil and complement activation in the frontal cortex, hippocampus, and choroid plexus (Truyens et al., 2023). From these results we propose that it is plausible that specific alterations in cytokine levels could be associated with distinct subtypes of depression, potentially exacerbating the condition.

4.5.4 Immunohistochemistry of *Winnie* mice brains

In the current study, IHC measured HMGB1, NeuN, MBP, TMEM119 and GFAP in *Winnie* mice brains compared to control C57BL/6 littermates. Significant changes were observed in in the PFC, NAc, and hippocampus. A deeper exploration will now be undertaken to comprehend the ramifications and importance of these proteins and their deviations in the *Winnie* mouse brain.

4.5.5 HMGB1 and NeuN expression in *Winnie* mice brains

Recently, an increase in the levels of HMGB1, a damage-associated molecular pattern (DAMP) marker, was observed in the *Winnie* mice colon. HMGB1 is responsive to changes in the redox environment and oxidative stress (Stavely et al., 2022). HMGB1 was likewise observed in other chronic mouse models of colitis (Mitchell, Kim, Howe,

Patel, et al., 2022), which proposed that HMGB1, released from an inflamed intestine, can potentially traverse to the brain via the bloodstream. The researchers demonstrated that elevated HMGB1, in the presence of low levels of endogenous lipopolysaccharides, activates caspase-mediated inflammatory responses in the brain. These results imply that chronic intestinal inflammation could lead to changes in brain activity in mice, which associated with long-term memory loss (Mitchell, Kim, Howe, Lee, et al., 2022). One study indicated that HMGB plays a role in the development of chronic-stress-induced depression via the kynurenine pathway (Wang et al., 2018). In line with these studies, our study observed a significant increase in HMGB1 in the hippocampus of *Winnie* mice.

Fang et al. (2012) stated that microglia, in response to infection or damage, can initiate proinflammatory responses. This can either actively release HMGB1 from activated immune cells or passively discharge it from damaged or necrotic cells, thereby acting as a necrotic cell death signal. In this context, *Winnie* mice brains demonstrated upregulated *Tlr2* and *Tlr5*. In human monocyte cells, these toll-like receptors interact with HMGB1, initiating a cascade of cytokines and chemokines through the activation of signal transduction pathways (Gardella et al., 2002). In the mouse models induced by stress, HMGB1-driven inflammation is associated with the activation of the nucleotide-binding oligomerisation domain (NOD)-like receptor protein 3 and disrupts the kynurenine pathway. This leads to an imbalance between neuroprotective and neurotoxic elements, which has been implicated in depression-like behaviours (H. Zhang et al., 2019). This idea finds support in a recent study of DNBS-treated mice, where an increased expression of HMGB1 in the hippocampus was associated with depressive-like behavioural changes (Haj-Mirzaian et al., 2017).

The *Winnie* mouse brain revealed reduced NeuN IR in the hippocampus, which is shown to contribute to cognitive decline in DSS-induced colitis mice (Lee et al., 2023). In humans and rodents neuronal loss inhibits hippocampus-dependent learning while disrupting olfactory learning (Armstrong & Barker, 2001). Although NeuN is reduced in the *Winnie* mouse, *Tgfb β /Smad* signalling is upregulated, which facilitates the recruitment of new neurons from neural stem cells after neuronal death in mice (Vogel et al., 2009). If the *Tgfb β /Smad* signalling had not been upregulated in the *Winnie* mouse, the neuronal loss may have been potentially more severe.

4.5.6 MBP expression in *Winnie* mice brains

The *Winnie* mouse exhibited MBP IR in the PFC, showing non-significant differences in the NAc or hippocampus. In humans and mice, MBP plays a vital role in developing new neuronal connections and synaptic plasticity, which govern emotion, learning, and memory formation, and regulates inflammation, offering potential protection against neurodegenerative diseases (Zhou et al., 2021). The neurotoxic metabolites of kynurenine have been shown to promote white matter damage by activating microglia, leading to inflammation and the destruction of myelin (Schwarcz et al., 2012). Kynurenine receptors, located in diverse tissues, play a pivotal role in modulating the central nervous system (CNS), immune responses, and metabolic processes in a tissue-specific manner (Schwarcz et al., 2012).

4.5.7 TMEM 119 expression in *Winnie* mice brains

In the hippocampus, the *Winnie* mice showed non-significant differences in TMEM119 compared to controls. The *Winnie* mice NAc demonstrated a marked reduction in TMEM119. The *Winnie* mice exhibited a significant increase in TMEM119 IR within the prelimbic cortex of the medial PFC. The literature in TMEM119 in colitis mouse models is sparse, however it was reported that the reduced development of neurons in the NAc of mice is connected to reduced voluntary running activity (Roberts et al., 2014), as observed in the *Winnie* mouse. In another study by Batra et al. (2022), TMEM119 expression revealed significant increases during the acute phase after DSS treatment. Subsequently, Batra et al. (2022) provided evidence supporting the notion that colon injury and signalling during colitis play a role in inducing cellular changes and immune cell infiltration in the brain, potentially leading to pathogenic consequences.

Studies involving both human subjects and rodents have reported an increase in TMEM119 in conjunction with microgliosis and neuroinflammation (Ruan & Elyaman, 2022). Brain tissue from individuals with major depressive disorder was examined post-mortem in which microglia were extracted (Böttcher et al., 2020). This research showed an upregulated expression of TMEM119 and TGF- β 1 in the microglia (Böttcher et al., 2020), which was similarly observed in *Winnie* mice brains. Böttcher et al. (2020)

proposed that the interaction between neurons and microglia could serve as an indicator of neuroinflammation linked to mood disorders in humans.

4.5.8 GFAP expression in *Winnie* mice brains

Our study examined astrogliosis as a marker of neuroinflammation by measuring GFAP in the *Winnie* mice brains. Non-significant differences were shown in the NAc and hippocampus of the *Winnie* mice, however, GFAP-positive cells were significantly higher in the PFC, which resembles reactive astrocytes. Overexpressed GFAP in the PFC of *Winnie* mice increases during astrogliosis and inflammation in the CNS. Astrogliosis, characterized by notable alterations in astrocyte morphology and functionality, is a hallmark of numerous neurological disorders, including neurotrauma, ischemic stroke, and neurodegenerative conditions (Orre et al., 2014). This phenomenon significantly influences both the progression of the disease and the subsequent recuperation (Al Shweiki et al., 2017; Kanai et al., 2009). Our findings contrast with DSS-induced colitis mice, which demonstrated an elevated expression of GFAP in the hippocampus on the 3rd day post-treatment, but not on the 7th day (Do & Woo, 2018). One important distinction is that the mice in this study employed a younger acute model of colitis (Do & Woo, 2018), which does not compare to the 14-week-old *Winnie* mouse model of colitis (Robinson et al., 2017). Nevertheless, research involving chronic DSS-induced colitis mice has shown an increased GFAP level, suggesting activated astroglia (Lee et al., 2023; Zonis et al., 2015).

4.6 Conclusion

The results supported the hypothesis that chronic intestinal inflammation is associated with behavioural changes. The *Winnie* mouse displayed less spontaneous physical activity coupled with symptoms associated with hypersomnia and depression, which are known to precede psychiatric disorders, fatigue, and sickness behaviour. The increased cytokines in *Winnie* mice brains significantly influences the co-occurrence of neurodegenerative disease with neurological illnesses, as well as depressive and anxiety-like behaviours. Protein expressions illustrated structural changes, in particular to the hippocampus, which correlated with behavioural changes. The results might provide

insight into a putative etiological mechanism implicated in the comorbidity of depression in IBD patients. Paying attention to emerging psychiatric symptoms and early intervention by physicians and allied health professionals may alleviate the onset of chronic intestinal inflammation or slow the disease's progression. The physiological, emotional, and psychological effects of chronic intestinal inflammation and its associated affective disorders must be recognised, diagnosed, and treated.

Chapter 5: Tryptophan de novo NAD⁺ synthesis in the *Winnie* mouse brain

5.1 Summary

Inflammatory bowel disease (IBD) is a persistent, non-communicable ailment of the gastrointestinal tract, manifesting either as Crohn's disease or ulcerative colitis. Concurrent with its onset are behavioural shifts, including depression, anxiety, and fatigue. Often, become incipient at disease onset and remain unrecognised until the behaviours become pernicious symptoms and develop into a chronic syndrome. Central to this is the disruption in tryptophan metabolism, which, when inflamed, activates the kynurenine pathway associated with numerous mood disorders.

The progression of IBD is deeply intertwined with genetic interactions in the affected tissue. Harnessing the capabilities of RNA sequencing and metabolomics, our study discerned alterations in genes related to glycolysis and tryptophan's *de novo* NAD⁺ synthesis in the brains of *Winnie* mice with spontaneous chronic colitis. Subsequent metabolomic analyses quantified tryptophan metabolite concentrations in the brain, examining their correlation with gene expression patterns.

The transcriptome of *Winnie* mice brought to light disruptions in serotonergic pathways, analogous to those linked with human depression. Regarding fatigue, reduced glucose metabolism was evident from the transcriptomic data. These observations imply that both glycolysis disruptions and tryptophan deficiencies might play a more pronounced role in influencing the serotonin pathway than previously acknowledged. Validating our hypothesis, the study highlighted gene anomalies and diminished tryptophan metabolite levels that seemed to negatively impact the behavioural traits of *Winnie* mice.

5.2 Introduction

Inflammatory bowel disease (IBD) is a chronic non-communicable disease of the gastrointestinal tract that can manifest as Crohn's disease (CD) or ulcerative colitis (UC) (Fakhoury et al., 2014). IBD is typically characterised by dysfunctional colon physiology, specifically a decrease in *MUC2* (mucin) expression, causing increased intestinal permeability, vascular wall irregularities, and vessel lumen obstruction (Heazlewood et al., 2008). Similarly, *Muc2* knockout mice show signs of colon inflammation and colitis (Niv, 2016).

The burden of IBD symptoms has a negative psychosocial impact on patients, and it is believed that negative emotions and psychological stress influence biological disease progression (Jordan et al., 2018). For example, depression and anxiety become incipient at disease onset, remaining unrecognised until the behaviours become pernicious symptoms and develop into a chronic syndrome (Jordan et al., 2018). In addition, Nocerino et al. (2020) stated that IBD symptoms initially present as a lack of energy or exhaustion out of proportion to physical exertion, which is not relieved with rest, with patients unable to perform simple daily activities. Although fatigue can have multiple causes, physicians associate the lack of energy with depression (Borren et al., 2019). Other manifestations include anhedonia, decreased physical activity, hypochondriasis, alexithymia, somatisation and sleep disturbances, all of which contribute to the aetiology of pathological fatigue and sickness behaviour (Choi et al., 2019). Additionally, the interest in IBD as a psychosomatic illness stems from observations suggesting a potential link between life events, stress, personality type, and the genetic predisposition to develop the disease (Choi et al., 2019).

Research into the gut-brain axis, which constitutes bidirectional communication via neural, hormonal, and immune pathways, as well as the gut microbiome, has gained much attention in affective disorder research (Abautret-Daly et al., 2018; Do & Woo, 2018). The gut-brain axis is facilitated by the nervous supply of the gastrointestinal tract. Specifically, the small intestine and proximal colon are innervated by the vagus and splanchnic nerves, while the colon and anal sphincter receive input from the pelvic nerves. Bonaz and Bernstein (2013) consider the interplay between the brain and gut to be likely

a key factor in the onset of IBD. This intricate relationship encompasses multiple components: 1) the autonomic nervous system, 2) the central nervous system, 3) the stress regulation system, known as the hypothalamic-pituitary-adrenal axis, 4) the gastrointestinal hormone system related to stress, and 5) the intestinal reaction, which involves the gut lining, microbiota, and immune responses within the intestine. The gut-brain axis forms the foundation for the psycho-neuroendocrine-immunity aspects of IBD, rendering IBD patients psychophysiologically vulnerable (Bonaz & Bernstein, 2013; Mayer & Tillisch, 2011).

In studies involving both humans and rodents that explore the connection between the gut and the brain, evidence suggests that dysregulated tryptophan metabolism may contribute to anxiety and depressive symptoms in IBD patients. This dysregulation could establish a link between intestinal inflammation and neurological disturbances (L.-M. Chen et al., 2021; Shajib et al., 2019; Silva et al., 2017). Hereby, IBD can be characterised as a systemic disorder that involves the gut and brain (Roth et al., 2021). As early as the 1960s, Coppen (1968) posited the serotonin hypothesis of depression, suggesting that a reduction in serotonergic neurotransmission in the brain lays the groundwork for depression. Later studies, such as Lestage et al. (2002), linked depression to the inflammation-triggered activation of the kynurenine pathway. The neurotoxic metabolites of kynurenine have shown to promote white matter damage by activating microglia, leading to inflammation and the destruction of myelin. Kynurenine receptors, located in diverse tissues, play a pivotal role in modulating the CNS, immune responses, and metabolic processes in a tissue-specific manner (Schwarcz et al., 2012).

Studies in both humans with colitis and mouse models of colitis show that nicotinamide adenine dinucleotide (NAD^+) synthesis from tryptophan in colon tissue follows an oscillatory metabolic pattern (Nikolaus et al., 2017). Maria et al. (2018) showed in living cells that the glycolytic process impacts the efficiency of tryptophan's conversion to NAD^+ through the crucial phosphoenolpyruvate (PEP) node. By integrating the glycolysis and tryptophan *de novo* NAD^+ synthesis models in the *Winnie* mouse with colitis, one can better understand the conditions that dictate the rate of NAD^+ production from tryptophan and how these metabolic routes affect behaviour. This study will utilise

the *Winnie* mouse, which has chronic intestinal inflammation caused by a missense mutation in the (*Muc2*) gene (Heazlewood et al., 2008). Consequently, progressive chronic intestinal inflammation develops with signs of diarrhoea, rectal bleeding, and pain appearing from 6 weeks of age, and with severe colitis apparent by 12-16 weeks of age, with a histopathological outcome similar to human IBD. (Eri et al., 2011).

5.3 Materials and Methods

5.3.1 Ethics, animals and husbandry

This study was approved by the Victoria University Animal Experimentation Ethics Committee (AEC-17-016). All animal experiments complied with the guidelines of the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes described in [Section 2.3.1](#). The *Winnie* mouse model of colitis with a C57BL/6 mice background was obtained from the colony at the Victoria University Werribee Animal Facility (Melbourne, Victoria, Australia). The colitis in the *Winnie* mouse model is caused by a missense mutation (G9492A, GenBank accession no. AJ511872), by which cysteine is replaced by tyrosine in the D3 67 domain in the N terminus of the mucin 2 (*Muc2*) gene (Heazlewood et al., 2008). Age-matched C57BL/6 littermates, obtained from the same colony, constituted the control. Post *in vivo* experiments, all mice were euthanised at 14 weeks of age via an overdose of injected pentobarbitone (Lethabarb, Virbac Australia) using a 1:16 dilution, 30 g, 100 µL/20 g. The brain tissues were removed and used for subsequent experiments.

5.3.2 Biological samples for High-throughput RNA-Sequencing

As described in Chapter 4, [Section 4.4.8](#), the brains of *Winnie* mice and control C57BL/6 littermates (n=10 mice/group, 5 females and 5 males per group) were prepared for ribonucleic acid (RNA)-Sequencing of the glycolysis/gluconeogenesis pathway, tryptophan metabolism and the nicotinamide and nicotinate metabolism pathways.

5.3.3 High-throughput RNA-Sequencing

As described in Chapter 4, [Section 4.4.8](#), RNA was extracted from brain tissues using the RNeasy Lipid Tissue MiniKit (cat. no. 74804, Qiagen) containing the QIAzol® Lysis Reagent, exactly according to the manufacturer's instructions. Upon completion, one 4 µL aliquot of each RNA sample was placed into the Nanodrop (Denovix, Melbourne, Australia) spectrophotometer to assess RNA quality and concentration. The results demonstrated that 260/280 nm and 260/230 nm measurements were >1.8.

5.3.4 RNA-Sequencing Data Analysis

As described in Chapter 4, [Section 4.4.8](#), the remaining samples of Poly-A tail purified mRNA were submitted to Micromon Genomics (Monash University, Melbourne Australia) for RNA-sequencing (RNA-Seq) using the Next Generation Sequencing platform. Micromon performed quantification with Qubit, the polyA purification of mRNA from total RNA samples, RNA-Seq library preparation and high-throughput sequencing using a 100-base pair single-end read protocol on the Illumina HiSeq 2500 System. Each sample was analysed by Bioanalyser (Monash) and passed its QNA quality control standard. Raw FASTQ files were analysed using the RNAsik pipeline version 1.5.4 (Tsyganov et al., 2018) using the STAR aligner, and the *Mus musculus* reference genome GRCm38 (GenBank accession GCA_000001635.2) (Dobin et al., 2013). Raw read counts were quantified using the featureCounts programme (Liao, 2014) and analysed with Degust Webtool to produce counts per million (CPM) library size normalisation, and differentially expressed genes (DEGs) analysis (Powell, 2019).

5.3.5 Data visualisation

Pathview plot for the tryptophan metabolism pathway and nicotinate and nicotinamide pathway from the KEGG database presented as significance scores (sign $\text{Log}_2\text{FC}(\text{fold change}) \times \text{Log}_{10}\text{P}$ values) for gene expression analysis between the brains of control *Winnie* mice and control C57BL/6 littermates.

5.3.6 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) includes gene set annotation, statistical analysis, and pathway interpretation. First, functional information of gene ontology words or pathway information is annotated to the genes in the input list. The input list is then statistically tested to see if any functional categories or pathways are considerably overrepresented in comparison to the reference set. If a route is strongly enriched in the input gene list, it indicates that the genes in the list are functionally connected and may be involved in a certain biological process. GSEA was performed against the KEGG database (Kanehisa & Goto, 2000) using the tool WebGestalt gene set analysis toolkit (Liao et al., 2019). Gene lists were pre-ranked by the relative expression between the brains of *Winnie* mice and control C57BL/6 littermates. GSEA parameters were performed with 1000 permutations. Cut-offs for significantly enriched pathways included low identification of leading-edge genes (<5) and a p value >0.05 .

As described in Chapter 2, the search tool for retrieval of interacting genes (STRING) was used to enter up and downregulated genes into the STRING database to establish gene ontology biological processes (GOBP), gene ontology molecular functions (GOMF) and gene ontology cellular pathways (GOCC).

g:Profiler employs the g:shortest common supersequence algorithm, for computing multiple testing corrections. An adjusted p-value threshold of ≤ 0.05 is considered significant for the p-values obtained from gene ontology (GO) and pathway enrichment analysis. The generated gene lists were processed using g:Profiler's g:Gost functional profiling instrument with an Ensemble gene list consisting of both upregulated and downregulated genes. The organism was set to *Mus musculus*, and the query was set to highlight driver terms in GO. In the advanced options, only annotated genes were included in the statistical domain scope, and the significance threshold was set to the g:SCS threshold set at 0.05. Numeric IDs were handled as ENTREZGENE_ACC. The selected data sources included GO biological process (GOBP), GO molecular function (GOMF) and KEGG pathways (Reimand et al., 2019).

REVIGO (Reduce and Visualise Gene Ontology) summarises lists of GO terms by identifying a representative group of terms through a clustering algorithm that considers its semantic similarity. REVIGO uses the Resnik or Lin measure, to quantify the similarity between GO terms. These measures are based on the hierarchical structure of the GO and the information content associated with each term. Multidimensional scaling and graph-based visualisations accurately display the divisions and semantic connections within the data (Supek et al., 2011).

5.3.7 Tryptophan reference standards

The method described in Chapter 3, [Section 3.4.7](#) was used to measure the reference standards of L-tryptophan; 5-hydroxyindole-acetic acid (5-HIAA), kynurenic acid (KYNA), L-kynurenine 98%, 2,3-pyridinedicarboxylic acid (quinolinic acid, QUIN), nicotinamide, and 2-picolinic acid (PIC) in *Winnie* mice brains. The Shimadzu Prominence Ultra-Fast Liquid Chromatography (UFLC) was employed using the same parameters and validation method as described in Chapter 3, [Section 3.4.8](#), (**Figure S 4** Tryptophan metabolites chromatogram, **Table S1 – S14**).

5.3.8 Preparation of biological sample for UFLC

Each biological sample was accurately weighed and placed into its labelled Safe-Lock tube containing ice-cold 0.2 M HClO₄ solutions (10 µL/mg of tissue) in preparation for solubilisation. Homogenisation was performed in the pre-cooled homogenising bead beater (TissueLyser LT, Qiagen, Melbourne, Australia). After preparation, the homogenates were centrifuged at 12,000 revolutions per minute at 4°C (9660 g force) for 15 minutes to extract metabolites from the matrix. The supernatants were immediately separated from the precipitates and transferred into a sterile 5 mL microtube. The supernatants were adjusted to a ratio of 4:1 v/v with ice-cold 0.1M HClO₄ and vortexed for 30 seconds, then filtered into a new tube, ready for use (Li & Bartlett, 2014).

5.3.9 Tryptophan pathways metabolite analysis

Winnie mice brain samples were injected into the UFLC (n=8 mice/group). The concentrations of each tryptophan metabolite in the biological samples were determined using analyte: internal standard peak height ratios utilising Shimadzu software. The ASC11 data file was analysed by Origin Pro, Version 2021b (OriginLab Corporation, Northampton, MA, USA). All peak fitting, surface fitting, and signal processing capacities reached Chi-Sqr tolerance values of 1E-6.

5.3.10 Statistical analysis

GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA) was used for the analytical comparisons. For multiple comparisons, a two-way ANOVA and a post hoc Holm-Šidák or Tukey test for multiple comparisons were applied, or a Student's t-test where applicable. Significance was considered at $p \leq 0.05$. All data are presented as mean $\pm$ standard error of the mean (SEM).

5.4 Results

5.4.1 Changes in gene expressions associated with glycolysis/gluconeogenesis

The mRNA transcriptome in the brains of *Winnie* mice was compared to control C57BL/6 littermates, which identified genes associated with glycolysis/gluconeogenesis and tryptophan *de novo* NAD⁺ synthesis. Data from the KEGG database was used to create Pathview plots for each metabolic pathway presented as significance scores (sign Log₂FC $\times$ Log₁₀P values) for gene expression analysis between the groups.

Data included downregulated gene expressions, such as, triose phosphate isomerase (*Tpi*), phosphoglycerate kinase 1 (*Pgk1*), bisphosphoglycerate mutase (*Bpgm*), phosphoglycerate mutase (*Pgam*), pyruvate dehydrogenase E1 subunit α 1 (*Pdha1*), lactose dehydrogenase (*Ldha*), aldehyde dehydrogenase 3 [NAD(P)+] (*Aldh3a1*), aldehyde dehydrogenase 3 family member B1 (*Aldh3b1*) and alcohol dehydrogenase (*Adh 1/7*) (Figure 5.1A). The upregulated gene expression included only hexokinase 2

(*Hk2*) (**Figure 5.1A**, **Table 5.1** Glossary of terms for glycolysis/gluconeogenesis-associated gene expressions in the *Winnie* mouse brains are extracted from <https://www.genome.jp/pathway/map00010> (accessed on July 21, 2022), Kanehisa and Goto (2000)).

In **Table 5.2** Gene ontology (GO) biological process (GOBP) included pyruvate metabolic process, cellular aldehyde metabolic process, carbohydrate catabolic process, carbohydrate metabolic process, NAD metabolic process, alcohol catabolic process, purine ribonucleotide metabolic process, and canonical glycolysis. GO molecular functions (GOMF) included aldehyde dehydrogenase (NAD⁺) activity, oxidoreductase activity, and benzaldehyde dehydrogenase (NAD⁺) activity.

Figure 5.1 DEGs associated with glycolysis/gluconeogenesis in *Winnie* mice brains

(A) Data on Kegg rendered by Pathview plot of the leading edge genes representing glycolysis/gluconeogenesis in the brain of *Winnie* mice versus control C57BL/6 littermates using high-throughput RNA-Seq of mRNA. Gene expression data (**green** to **red**) presented as significance scores (sign $\text{Log}_2\text{FC} \times \text{Log}_{10}\text{P}$ values).

Green represents downregulated transcriptome comprising electronic commission (EC):5.3.1.1, *Tpi1*; EC:1.2.1.12, *Pgk1*; EC:5.4.2.4, *Bpgm*; EC:5.4.2.11, *Pgam*; EC:1.2.4.1 *Pdha1*; EC:1.1.1.27, *Ldha*; EC:1.2.1.3, *Aldh3a1*; EC:1.2.1.5 *Aldh3b1*; EC:1.1.1.1, *Adh 1/7*. **Red** represents the upregulated gene EC:2.7.1.1, *Hk2*.

Abbreviations: triose phosphate isomerase 1, *Tpi1*; phosphoglycerate kinase 1, *Pgk1*; bisphosphoglycerate mutase, *Bpgm*; phosphoglycerate mutase 1, *Pgam1*; pyruvate dehydrogenase E1 subunit α 1, *Pdha1*; lactose dehydrogenase, *Ldha*; aldehyde dehydrogenase 3 [NAD(P)+], *Aldh3a1*; aldehyde dehydrogenase 3 family member B1, *Aldh3b1*; alcohol dehydrogenase 5, *Adh5*; hexokinase 2, *Hk2*.

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Table 5.1 Glossary of terms of the glycolysis/gluconeogenesis-associated genes

Reaction	Substrate	Product	Comment
Upregulated DEGs			
Step 1: Phosphorylation of glucose			
STAGE 1: Investment and preparation			
Hexokinase (<i>Hk2</i>) EC:2.7.1.1.			
adenosine triphosphate (ATP) + D-Glucose $\rightleftharpoons$ ADP + D- Glucose 6-phosphate	ATP; D-Glucose	ADP; D-glucose 6- phosphate	<i>Hk2</i> catalyses D- glucose into glucose- 6-phosphate. Activator: AMP/ADP Inhibitor: G6P
Downregulated DEGs			
Step 5 - Isomerisation of dihydroxyacetone phosphate			
Stage 2 – Cleavage			
Triose phosphate isomerase (<i>Tpi</i>) EC:5.3.1.1			
D-Glyceraldehyde 3- phosphate $\rightleftharpoons$ Glycerone phosphate	D-glyceraldehyde 3-phosphate	Glycerone phosphate	TPI catalyses the ketose of dihydroxyacetone phosphate into and aldose of G3P, 2,3- BPG. Inhibitor: Citrate.
Step 7: Transfer from 1,3-bisphosphoglycerate to ADP			
Phosphoglycerate kinase 1 (<i>Pgk1</i>) EC:2.7.2.3.			
(2R)-ATP + 3-Phospho-D- glycerate $\rightleftharpoons$ (2R) ADP + 3- Phospho-D-glyceroyl phosphate	ATP; 3-phospho-D- glycerate	ADP; 3-phospho-D- glyceroyl phosphate	PGK catalyses 1,3 diphosphoglycerate interconversion 3- phosphoglycerate; synthesises pyruvate from D- glyceraldehyde 3- phosphate.
Step 8: Isomerisation of 3-phosphoglycerate			

Phosphoglycerate mutase (<i>Pgam</i>) EC:5.4.2.11			
2-phospho-D-glycerate $\leftrightarrow$ 3-phospho-D-glycerate	2-phospho-D-glycerate	3-phospho-D-glycerate	Activated by 2,3-bisphospho-D-glycerate by transferring a phosphate to histidine.
Bisphosphoglycerate mutase (<i>Bpgm</i>) EC:5.4.2.4			
(2R) - 3-phospho-D-glyceroyl phosphate = (2R) 2,3-bisphospho-D-glycerate + H ⁺	3-phospho-D-glyceroyl phosphate	2,3-bisphospho-D-glycerate	BPGM maintains the concentration of 2,3-diphospho-D-glycerate (2,3-BPG). Inhibitor: Citrate
Pyruvate oxidation, pyruvate $\Rightarrow$ acetyl-CoA			
Pyruvate dehydrogenase E1 subunit α 1 (<i>Pdha</i>) EC:1.2.4.1			
Pyruvate + Enzyme N6-(lipoyl)lysine $\rightleftharpoons$ [dihydrolipoyllysine-residue acetyltransferase] S-acetyldihydrolipoyllysine; CO ₂	Pyruvate. [dihydrolipoyllysine-residue acetyltransferase] lipoyllysine	[dihydrolipoyllysine-residue acetyltransferase] S-acetyldihydrolipoyllysine; CO ₂	PDHA converts pyruvate to acetyl-coA, increases the influx of acetyl-coA into the TCA cycle. Co factors: B1, B2, B3, B5, Lipoic Acid
Lactate fermentation; anaerobic metabolism			
Lactate dehydrogenase (<i>Ldha</i>) EC:1.1.1.27			
Pyruvate + NADH + H ⁺ = (S)-lactate + NAD ⁺	(S)-lactate; NAD ⁺	Pyruvate; NADH; H ⁺	LDHA catalyses the inter-conversion of pyruvate to lactate and NADH to NAD ⁺ .
Aldehyde dehydrogenase (<i>Aldh2</i>) EC:1.2.1.3			
An aldehyde + NAD ⁺ + H ₂ O $\rightleftharpoons$ Carboxylate + NADH + H ⁺	Aldehyde, NAD ⁺ , NADP ⁺ , H ₂ O	Carboxylate, NADH, NADPH, H ⁺	ALDH encodes enzymes for detoxification via the NAD(P) ⁺
Aldehyde dehydrogenase 3 [NAD(P)+] (<i>Aldh3a1</i>) EC:1.2.1.5			

1). Indole-3-acetaldehyde + $\text{NAD}^+ + \text{H}_2\text{O} \rightleftharpoons \text{Indole-3-acetate} + \text{NADH} + \text{H}^+$	Aldehyde; NAD^+ ; NADP^+ ; H_2O	Carboxylate; NADH , NADPH , H^+	ALDH3A1 detoxifies alcohol-derived acetaldehyde; metabolises corticosteroids, biogenic amines, neurotransmitters, and lipid peroxidation.
Alcohol dehydrogenase (<i>Adh 1/7</i>) EC:1.1.1.1			
A primary alcohol + $\text{NAD}^+ =$ an aldehyde + $\text{NADH} + \text{H}^+$	Primary alcohol; NAD^+ ; secondary alcohol	Aldehyde; NADH ; H^+ ; ketone	Detoxification and metabolism of ethanol.

Table 5.2 Enrichment terms of glycolysis/gluconeogenesis downregulated genes

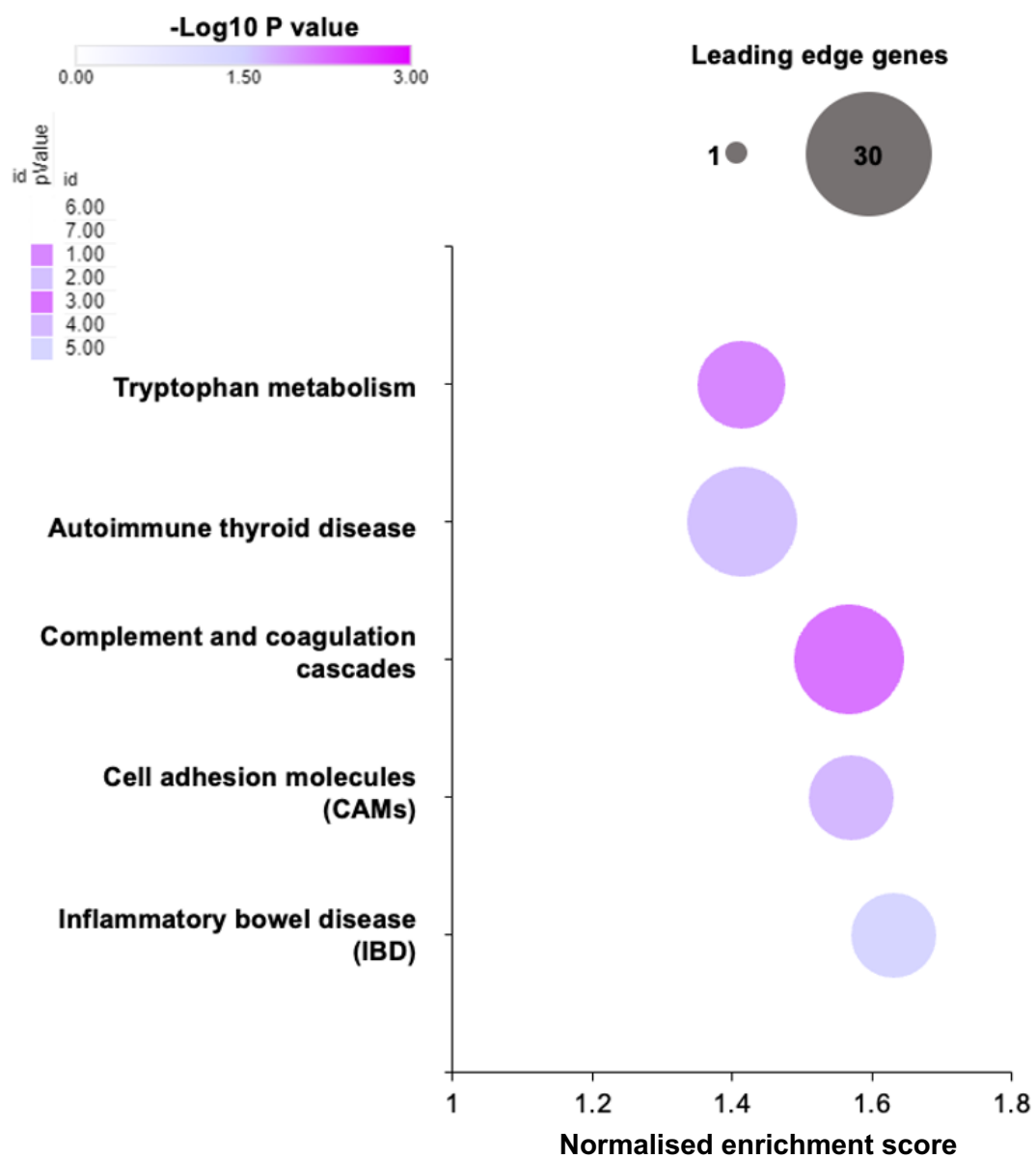
Category	Term ID	Term description	Observed gene count	Back-ground gene count	Strength	FDR
GOBP	GO:0006090	Pyruvate metabolic process	5	73	2.27	4.48E-07
GOBP	GO:0006081	Cellular aldehyde metabolic process	4	60	2.26	2.45E-05
GOBP	GO:0016052	Carbohydrate catabolic process	4	110	2	0.00015
GOBP	GO:0005975	Carbohydrate metabolic process	5	457	1.48	0.00026
GOBP	GO:0019674	NAD metabolic process	3	32	2.41	0.00026
GOBP	GO:0046164	Alcohol catabolic process	3	55	2.17	0.00071
GOBP	GO:0009150	Purine ribonucleotide metabolic process	4	390	1.45	0.0034
GOBP	GO:0061621	Canonical glycolysis	2	17	2.51	0.0074
GOMF	GO:0004029	Aldehyde dehydrogenase (NAD ⁺) activity	3	17	2.68	5.98E-05
GOMF	GO:0016491	Oxido-reductase activity	6	787	1.32	6.06E-05
GOMF	GO:0018479	Benzaldehyde dehydrogenase (NAD ⁺) activity	2	4	3.14	0.0014

5.4.2 Enrichment terms associated with tryptophan metabolism-gene expressions

In GSEA, gene lists were pre-ranked by the relative expression between the brains of *Winnie* mice and control C57BL/6 littermates. A bubble plot was produced of the top enriched pathways from GSEA analysis against the KEGG database. The enrichment terms included tryptophan metabolism, autoimmune thyroid disease, complement and coagulation cascades, cell adhesion molecules (CAMs), and IBD (**Figure 5.2** and **Table S 15**). In context, gene mutations in mice associated with hypothyroidism demonstrate significant intestinal defects (Bao et al., 2018). In humans and mice, both complement and coagulation system components are associated with key structures in the CNS and peripheral nervous system. The potential interplay between these two systems effects neuroinflammation-associated alterations in synaptic transmission and neuronal conduction (Berkowitz et al., 2021). Human and mouse studies emphasised the significance of neuronal CAMs belonging to the immunoglobulin superfamily, and are emerging as likely mediators of the cognitive and neural changes induced by chronic stress (Albert et al., 2012).

Figure 5.2 The top enriched pathways from GSEA analysis

Bubble plot of the top enriched pathways from GSEA analysis against the KEGG database. The X-axis represents positive normalised enrichment scores, colour scales represent the significance of enrichment ($-\log_{10}P$ values), and the size of the bubbles represents the number of identified leading edge genes. Enrichment plots include tryptophan metabolism, autoimmune thyroid disease, complement and coagulation cascades, CAMs, and IBD.



5.4.3 DEGs associated with tryptophan metabolism in *Winnie* mice brains

Transcriptome analysis associated with tryptophan metabolism in *Winnie* mice brains revealed downregulated gene expressions of indoleamine 2,3-dioxygenase (*Ido1*), aldehyde dehydrogenase NAD⁺ (*Aldh2*), and hydroxyacyl-CoA dehydrogenase (*Hadh*) genes. Upregulated gene expressions included arylformamidase (*Afmid*), S-Adenosyl-L-methionine (*SAMe*), aldehyde oxidase (*Aox1*), amine oxidase copper containing 1 (*Aoc1*), L-tryptophan hydroxylase (*TpH1*), L-amino acid oxidase/monooxygenase (*LaaO*), dehydrogenase E1 and transketolase domain containing 1 (*Dhtkd1*), and cytochrome P450 family 1 subfamily A1 (*Cyp1a1*). The Pathview plot for tryptophan metabolism DEGs is represented in the KEGG graph (**Figure 5.4A**, **Table 5.3** Glossary of terms for tryptophan-associated gene expressions in the *Winnie* mouse brain are extracted from <https://www.genome.jp/pathway/map00380> (accessed on 28th July, 2022). Kanehisa and Goto (2000)).

STRING enrichment pathways for downregulated DEGs involve small molecule catabolic processes, fatty acid degradation, valine leucine and isoleucine degradation, and lysine degradation. STRING enrichment pathways for upregulated DEGs include the amine metabolic process, oxidoreductase activity metabolic pathways and metabolism (**Table 5.4** and **Table 5.5**).

Figure 5.3 DEGs associated with tryptophan metabolism in *Winnie* mice brains

Pathview plot for the tryptophan metabolism pathway from the KEGG database. Rectangular nodes indicate gene expression data by RNA-Seq.

(A) Gene expression data (green to red) presented as significance scores (sign $\text{Log}_2\text{FC} \times \text{Log}_{10}\text{P}$ values) for gene expression analysis between the brains of control C57BL/6 mice and *Winnie* mice.

Green represents downregulated gene expressions of the tryptophan metabolism: EC:1.13.11.52, *Ido1*, EC:1.2.1.3, *Aldh2*; EC:1.1.1.35, *Hadh*.

Red represents the upregulated gene expressions of EC:3.5.1.9, *Afmid*; EC:2.1.1.49, *SAMe*; EC:1.4.3.22, *Aoc1*; EC:1.14.16.4, *TpH1*; EC:1.4.3.2, *LaaO*; EC:1.2.3.1, *Aox*; OADH, *Dhtkd1*; and EC:1.14.14.1, *Cyp1a1*.

Circular nodes indicate relative expression of tryptophan compounds (blue to yellow) between the brains of control C57BL/6 mice and *Winnie* mice. The blue circles represent the metabolites of tryptophan, kynurenine, KYNA, 5-HIAA and PIC.

Abbreviations: indoleamine 2,3-dioxygenase, *Ido1*; aldehyde dehydrogenase NAD^+ 2, *Aldh2*; and hydroxyacyl-CoA dehydrogenase, *Hadh*; arylformamidase, *Afmid*; tryptamine N-methyl-transferase, *Inmt*; S-Adenosyl-L-methionine *SAMe*; aldehyde oxidase, *Aox1*; amine oxidase copper containing 1, *Aoc1*; L-tryptophan hydroxylase, *TpH1*; L-amino acid oxidase/monooxygenase, *LaaO*; dehydrogenase E1 and transketolase domain containing 1, *Dhtkd1*; cytochrome P450 family 1 subfamily A1, *Cyp1a1*.

A



Table 5.3 Glossary of terms for tryptophan metabolism-associated genes

Reaction	Substrate	Product	Comment
Downregulated DEGs			
Indoleamine 2,3-dioxygenase (<i>Ido1</i>) EC:1.13.11.52			
L-tryptophan + O ₂ <=> N-formyl-L-kynurenine	O ₂ ; L-tryptophan	N-formyl-L-kynurenine	<i>IDO</i> activity implicated in tryptophan catabolism.
Aldehyde dehydrogenase (NAD⁺) (<i>Aldh2</i>) EC:1.2.1.3			
5-Hydroxyindoleacetaldehyde + NAD ⁺ + H ₂ O <=> 5-Hydroxyindoleacetate + H ⁺ + NADH	Aldehyde; NAD ⁺ ; H ₂ O	Carboxylate; NADH; H ⁺	<i>Aldh</i> detoxifies alcohol-derived acetaldehyde; metabolises corticosteroids, biogenic amines, neurotransmitters, and lipid peroxidation.
Hydroxyacyl-CoA dehydrogenase (<i>Hadh</i>) EC:1.1.1.35			
(S)-3-hydroxyacyl-CoA + NAD ⁺ = 3-oxoacyl-CoA + NADH + H ⁺	(S)-3-hydroxyacyl-CoA; NAD ⁺	3-oxoacyl-CoA; NADH; H ⁺	<i>Hadh</i> -Broad specificity to acyl chain-length.
Upregulated DEGs			
Arylformamidase (<i>Afmid</i>) EC:3.5.1.9			
N-formyl-L-kynurenine + H ₂ O <=> Formate + L-kynurenine	N-formyl-L-kynurenine, H ₂ O	Formate, L-kynurenine	<i>Afmid</i> converts N-formylkynurenine to kynurenine.
S-Adenosyl-L-methionine (<i>SAMe</i>) EC:2.1.1.49			
S-Adenosyl-L-methionine + Tryptamine <=> S-Adenosyl-L-homocysteine + N-Methyltryptamine	S-adenosyl-L-methionine; amine	S-adenosyl-L-homocysteine; methylated amine	<i>SAMe</i> acts as acceptors, including tryptamine, aniline, nicotine and xenobiotics.

Amine oxidase copper containing 1 (<i>Aoc</i>) EC:1.4.3.22			
Tryptamine + H ₂ O + O ₂ <=> Indole-3-acetaldehyde + NH ₃ + H ₂ O ₂	Tryptamine; H ₂ O; O ₂	Acetaldehyde; NH ₃ H ₂ O ₂	<i>Aoc</i> oxidises histamine and primary monoamines.
L-tryptophan hydroxylase (<i>Tph1</i>) EC 1.14.16.4			
L-tryptophan + tetrahydropteridine + O ₂ <=> 5-hydroxy-L-tryptophan + a 4a-hydroxy-tetrahydropteridine	L-tryptophan; tetrahydropteridi- ne; O ₂	5-hydroxy-L- tryptophan; 4a-hydroxy- tetrahydropteridi- ne	Serotonin biosynthetic process.
L-amino-acid oxidase (<i>Lao</i>) EC:1.4.3.2			
L-tryptophan + H ₂ O + O ₂ <=> Indole pyruvate + NH ₃ + H ₂ O ₂ peroxide	L-amino acid ; H ₂ O; O ₂	2-oxo carboxylate, NH ₃ , H ₂ O ₂	A flavoprotein
Aldehyde oxidase (<i>Aox</i>) EC 1.2.3.1			
An aldehyde + H ₂ O + O ₂ = a carboxylate + H ₂ O ₂	Aldehyde; H ₂ O; O ₂	Carboxylate; H ₂ O ₂	<i>Aox</i> regulates ROS homeostasis; catalyses nitric oxide (NO) production.
Dehydrogenase E1 and transketolase domain containing 1 (<i>Dhtkd1</i>) EC:OADH			
Succinyl-CoA + enzyme N6- (dihydrolipoyl)lysine = CoA + enzyme N6-(S-succinyl dihydrolipoyl)lysine	Succinyl-CoA; enzyme N6- (dihydrolipoyl) lysine	CoA; enzyme N6- (S-succinyl dihydrolipoyl) lysine	<i>Dhtkd1</i> is a critical gene in the catabolic pathway of the amino acid metabolite α- amino adipic acid.
Cytochrome P450 family 1 subfamily A1 (<i>Cyp1a1</i>) EC:1.14.14.1			
RH + [reduced NADPH--- haemoprotein reductase] + O ₂ = ROH + [oxidised NADPH--- haemoprotein reductase] + H ₂ O	RH; [reduced NADPH--- haemoprotein	ROH; [oxidised NADPH--- haemoprotein	<i>Cyp1a1</i> metabolises foreign chemicals, arachidonic acids, eicosanoid

	reductase]; O ₂	reductase]; H ₂ O	biosynthesis; cholesterol, sterol, bile acid biosynthesis; vitamin D3 synthesis and catabolism; retinoic acid hydroxylation; biogenic amine, neuroamine metabolism.

Table 5.4 Enrichment terms of tryptophan metabolism downregulated genes

Category	Term ID	Term description	Observed gene count	Back-ground gene count	Strength	FDR
GOBP	GO:0044282	Small molecule catabolic process	3	318	1.84	0.0394
KEGG	mmu00071	Fatty acid degradation	2	50	2.47	0.0027
KEGG	mmu00280	Valine, leucine and isoleucine degradation	2	55	2.43	0.0027
KEGG	mmu00310	Lysine degradation	2	61	2.38	0.0027

Table 5.5 Enrichment terms of tryptophan metabolism upregulated genes

Category	Term ID	Term description	Observed gene count	Back-ground gene count	Strength	FDR
GOBP	GO:0009308	Amine metabolic process	3	52	2.33	0.0038
GOMF	GO:0016491	Oxido-reductase activity	5	710	1.41	0.00067

5.4.4 Pathway enrichment analysis, g.Profiler and REVIGO

The overall enrichment analysis of genes was conducted by contrasting the expression levels of both upregulated and downregulated genes collectively (**Figure 5.5A**), downregulated genes (**Figure 5.5B**), and upregulated genes (**Figure 5.5C**). The GO pathways were grouped according to GOBP and GOMF, which were sourced from g:Profiler and then processed using the REVIGO tool. The first list contained the GO term ID, while the second had the associated P-value, which was originally produced by g:Profiler. The default configurations were applied when using the REVIGO tool. **Table 5.6** provides the enrichment terms associated with downregulated DEGs associated with tryptophan. **Table 5.7** exhibits the enrichment terms relevant to upregulated DEGs associated with tryptophan.

Figure 5.5 Enrichment terms of tryptophan metabolism associated DEGs

Up and downregulated genes were analysed for pathway enrichment using G: profiler online interface encompassing GOMF, GOBP, GOCC, and KEGG Pathways within a Manhattan plot illustrating enrichment analysis. X-axis = functional term names. Y-axis=adjusted enrichment p values in negative logscale. Circles sizes correspond to term size. **(A)** Down- and upregulated DEGs. **(B)**. Downregulated DEGs (Corresponds to **Table 5.6**). **(C)** Upregulated DEGs (Corresponds to **Table 5.7**).

Figure 5.4 Enrichment terms graph of tryptophan associated DEGs

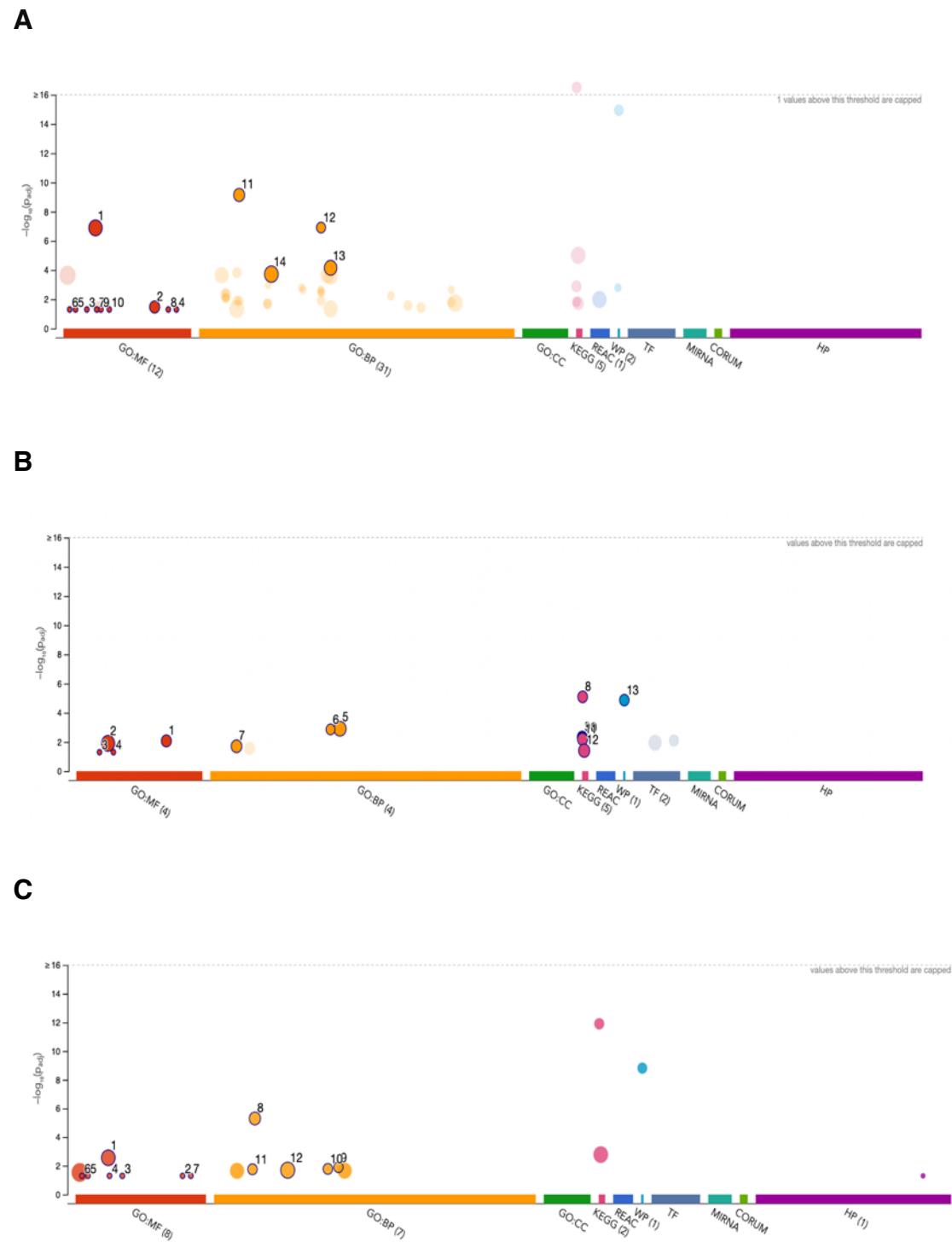


Table 5.6 Enrichment terms of tryptophan metabolism downregulated genes

ID	Source	Term ID	Term name	P _{adj} (query_1)
1	GOMF	GO:0051287	NAD binding	8.446E-3
2	GOMF	GO:0016491	Oxidoreductase activity	1.239E-2
3	GOMF	GO:0008957	Phenyl-acetaldehyde dehydrogenase activity	4.965E-2
4	GOMF	GO:0018547	Nitroglycerin reductase activity	4.965E-2
5	GOBP	GO:0044282	Small molecule catabolic process	1.237E-3
6	GOBP	GO:0042430	Indole-containing compound metabolic process	1.380E-3
7	GOBP	GO:0006576	Biogenic amine metabolic process	1.951E-2
9	KEGG	KEGG:00071	Fatty acid degradation	4.543E-3
10	KEGG	KEGG:00280	Valine, leucine and isoleucine degradation	5.274E-3
11	KEGG	KEGG:00310	Lysine degradation	6.473E-3
12	KEGG	KEGG:01240	Biosynthesis of cofactors	3.902E-2

Table 5.7 Enrichment terms of tryptophan metabolism upregulated genes

ID	Source	Term ID	Term Name	P _{adj} (query_1)
1	GOMF	GO:0016491	Oxidoreductase activity	2.74352E-03
2	GOMF	GO:0003824	Catalytic activity	2.89829E-02
3	GOMF	GO:0098615	Dimethyl selenide methyltransferase activity	4.98562E-02
4	GOMF	GO:0030748	Amine N-methyltransferase activity	4.98562E-02
5	GOMF	GO:0016711	Flavonoid 3'-monooxygenase activity	4.98562E-02
6	GOMF	GO:0004790	Thioether S-methyltransferase activity	4.98562E-02
7	GOMF	GO:0004061	Arylformamidase activity	4.98562E-02
8	GOMF	GO:0102707	S-adenosyl-L-methionine: beta-alanine N-methyltransferase activity	4.98562E-02
9	GOBP	GO:0009308	Amine metabolic process	5.01919E-06
10	GOBP	GO:0042430	Indole-containing compound metabolic process	1.25275E-02

11	GOBP	GO:0035902	Response to immobilisation stress	1.64318E-02
12	GOBP	GO:0009072	Aromatic amino acid metabolic process	1.74902E-02
13	GOBP	GO:0019752	Carboxylic acid metabolic process	2.02112E-02
14	GOBP	GO:0043436	Oxoacid metabolic process	2.18708E-02
15	GOBP	GO:0006082	Organic acid metabolic process	2.24748E-02

5.4.5 REVIGO pathway visualisation for gene ontology

The REVIGO scatterplot presents the GO terms in a semantic space, with closely related terms appearing near each other. The colour intensity of each bubble indicates the *p*-value from the g:Profiler assessment. The bubble's size displays the specificity of the GO terms: smaller bubbles symbolise more precise terms, while larger bubbles indicate broader terms. The Tree Map illustrates a dual-layer hierarchy of GO terms through colour-coded tiles (Supek et al., 2011).

The GOBP relevant to all tryptophan metabolism-associated DEGs involves the amine metabolic process, aromatic amine metabolic process, carboxylic acid metabolic process, indole-containing amine metabolic process, small molecule catabolic process, biogenic amine metabolic process, and immobilisation to stress (**Figure 5.6A** Scatterplot, **Figure 5.6B** Tree Map). The GOBP relevant to the downregulated tryptophan metabolism-associated DEGs involves the indole-containing amine metabolic process, biogenic amine metabolic process and small molecule catabolic process (**Figure 5.7A** Scatterplot, **Figure 5.7B** Tree Map). The GOMF of downregulated tryptophan metabolism-associated genes involves oxidoreductase activity, nitroglycerin reductase activity, phenylacetaldehyde dehydrogenase activity and NAD binding (**Figure 5.8A** Scatterplot, **Figure 5.8B** Tree Map). The GOBP relevant to the upregulated tryptophan metabolism-associated DEGs involves amine metabolic processes, carboxylic acid metabolic process, indole-containing amine metabolic process and aromatic amine metabolic process (**Figure 5.9A** Scatterplot, **Figure 5.9B** Tree Map). The GOMF of upregulated tryptophan metabolism-associated genes involves catalytic activity, oxidoreductase activity, arylformamidase activity, thioether S-methyltransferase activity, flavonoid 3'-monooxygenase activity, amine N-methyltransferase activity, dimethyl selenide methyltransferase activity, and S-

adenosyl-L-methionine:beta-alanine N methyltransferase activity (**Figure 5.10A** Scatterplot, **Figure 5.10B** Tree Map).

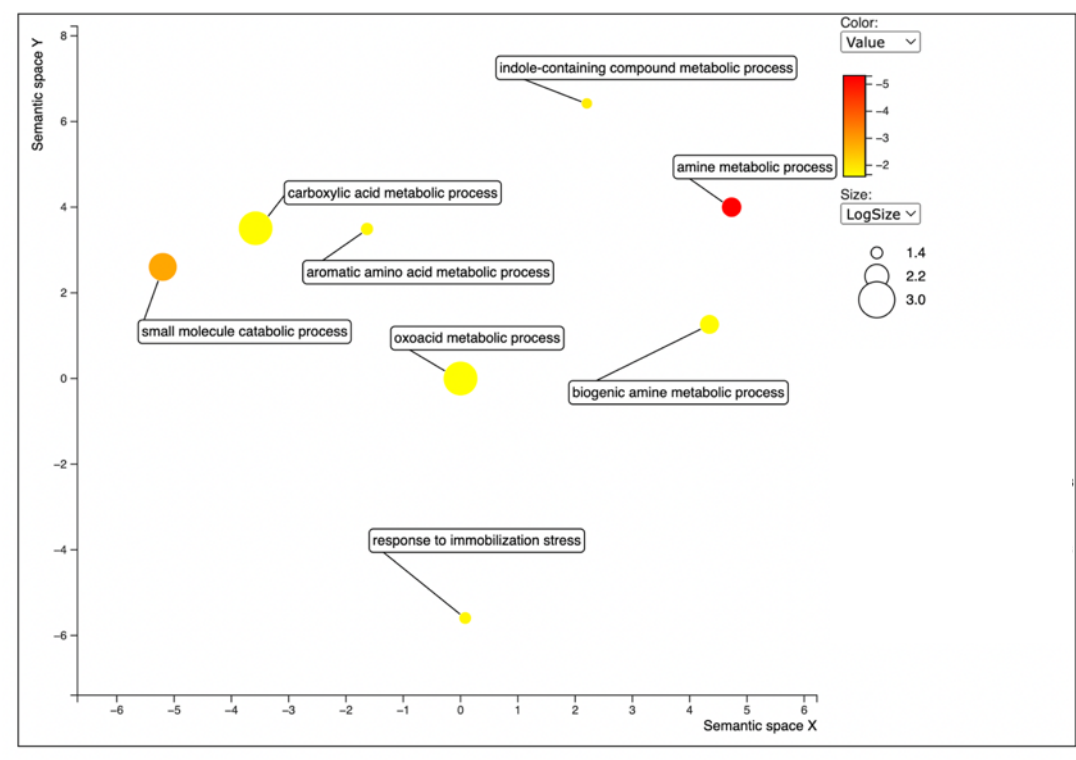
5.4.6 DEGs associated with nicotinamide metabolism in *Winnie* mice brains

The tryptophan pathway leads to the production of NAD⁺, a critical cofactor involved in numerous biochemical reactions and homeostasis (Houtkooper et al., 2010). In the absence of sufficient dietary niacin, tryptophan can be used as a source to produce NAD⁺. Transcriptome analysis of *Winnie* mice brains included gene expression changes associated with nicotinamide metabolism. Downregulated gene expressions included purine-nucleoside phosphorylase (*Pnp*), and cluster of differentiation 38 (*Cd38*). Upregulated genes included nicotinamide/nicotinate riboside kinase (*Nmrk1*), and aldehyde oxidase (*Aox1*) (**Figure 5.11**, **Table 5.8** Glossary of terms for nicotinamide-associated gene expressions in *Winnie* mice brains are extracted from <https://www.genome.jp/pathway/map00760>, (accessed on 1st August, 2022), Kanehisa and Goto (2000)).

STRING presented one cluster in red nodes of *Cd38*, *Nmrk* and *Pnp*. STRING enrichment terms associated with all DEGs related to nicotinamide (**Table 5.9**) listing metabolic pathways, pyrimidine metabolism, purine metabolism and nicotinate metabolism.

Figure 5.5 Up and downregulated tryptophan DEGs biological processes

A

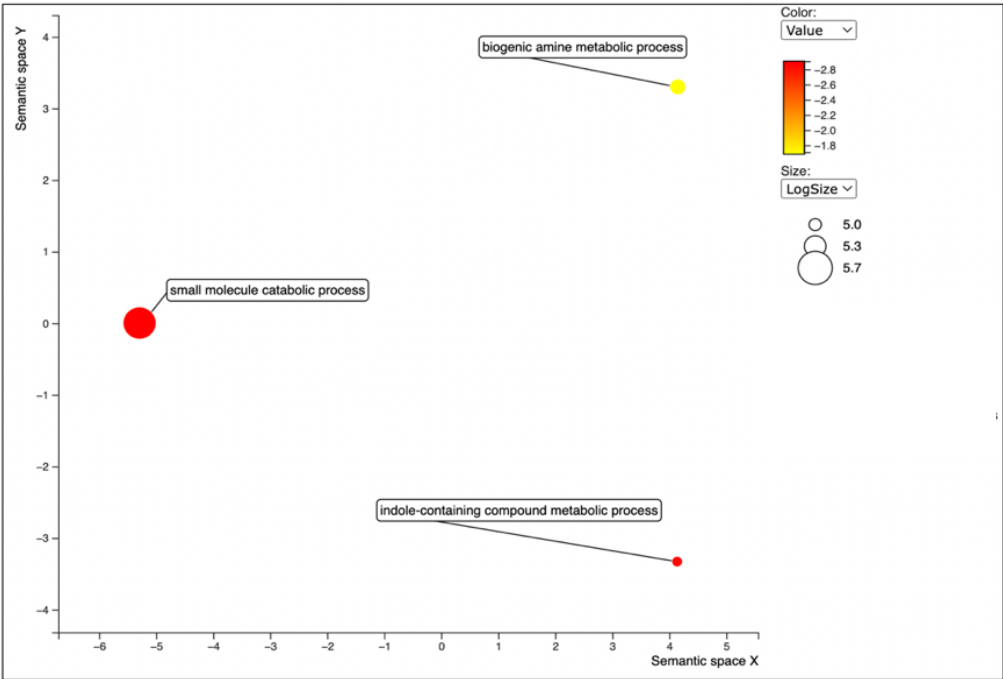


B



Figure 5.6 Downregulated tryptophan DEGs biological processes

A

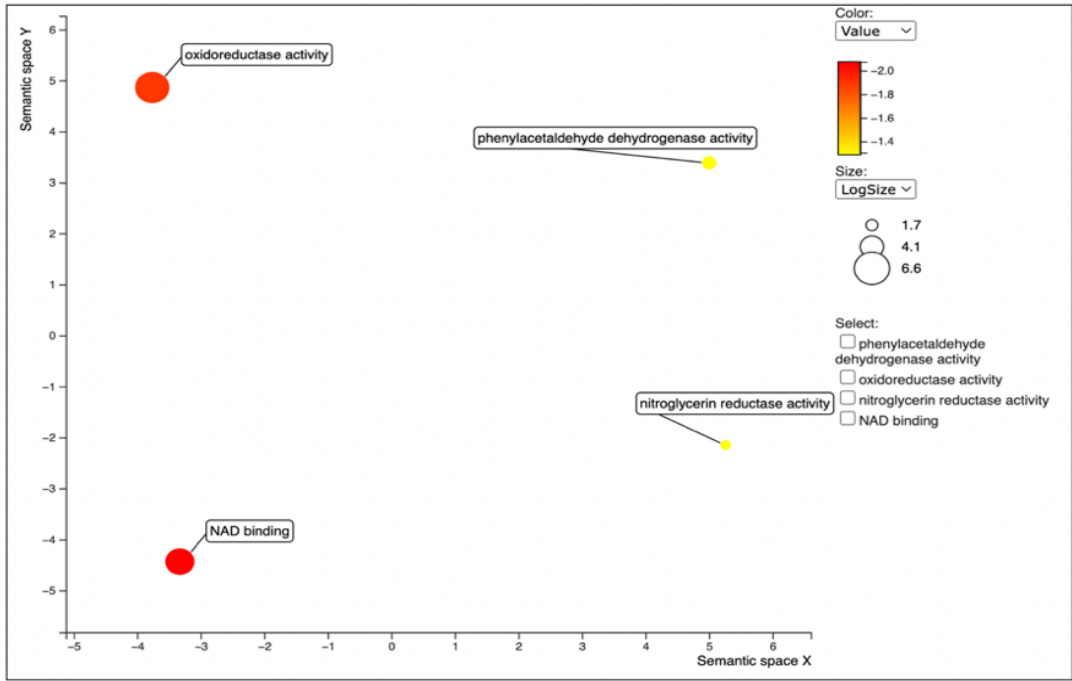


B



Figure 5.7 Downregulated tryptophan DEGs molecular functions

A



B

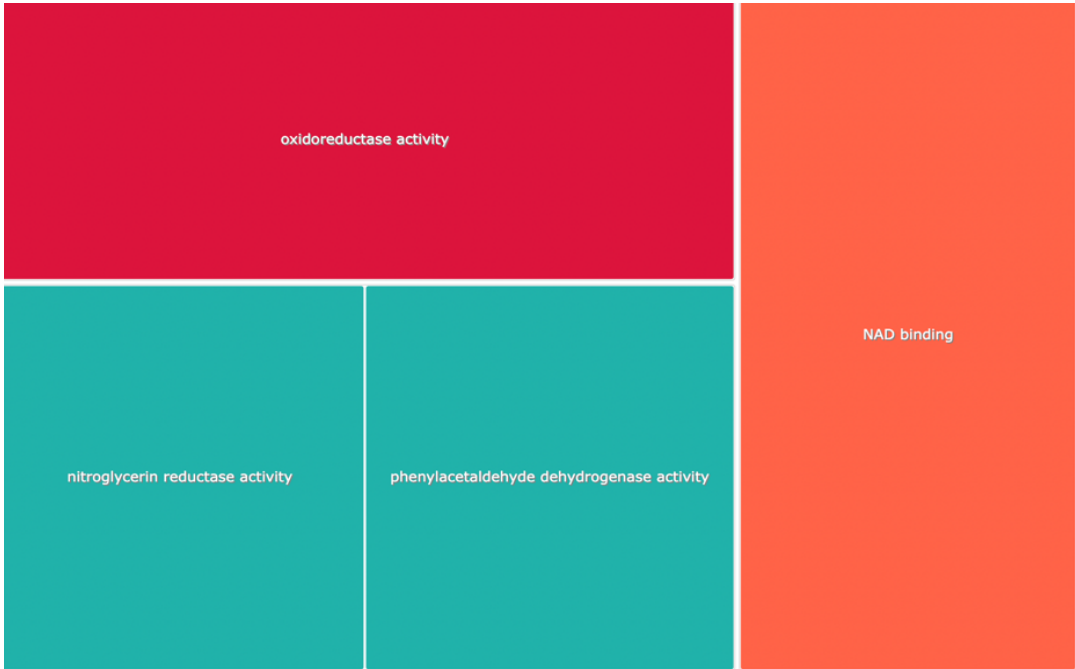


Figure 5.8 Upregulated tryptophan DEGs biological processes

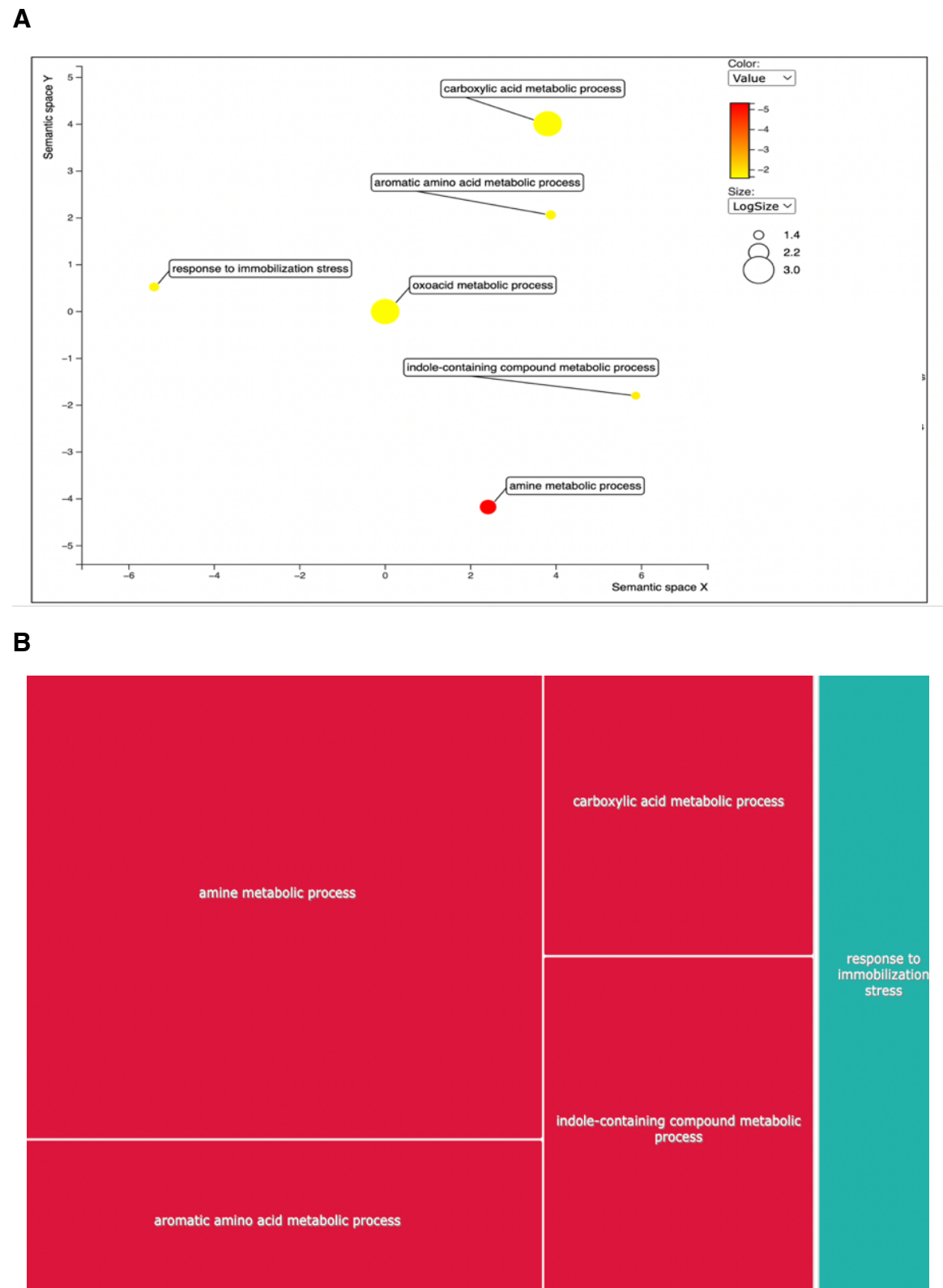


Figure 5.9 Downregulated tryptophan DEGs molecular functions

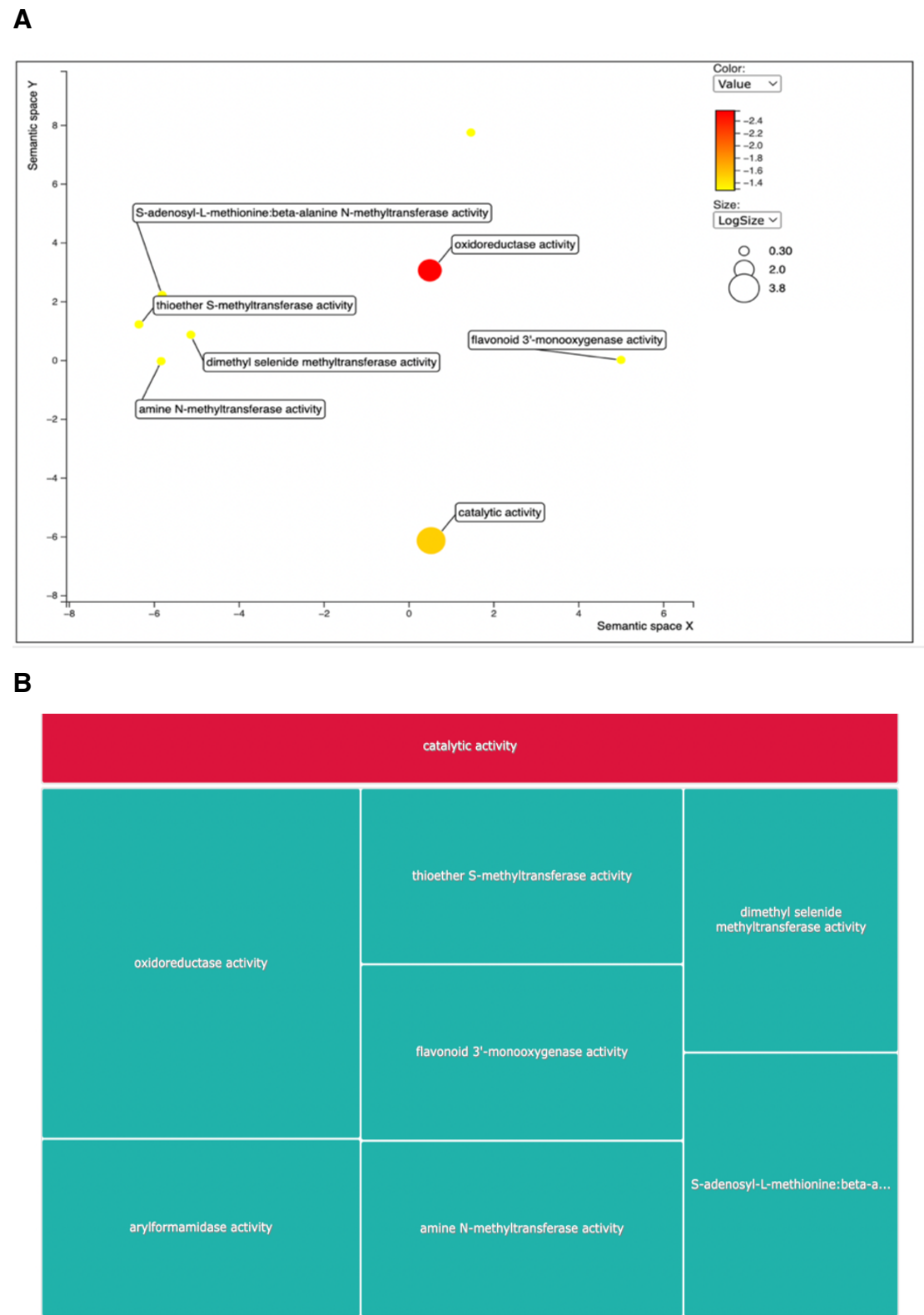


Figure 5.10 DEGs representing nicotinamide metabolism in *Winnie* mice brains

Pathview plot for the nicotinate and nicotinamide pathway from the KEGG database. Rectangular nodes indicate gene expression data by RNA-Seq.

(A) Gene expression data (**green** to **red**) presented as significance scores (sign $\text{Log}_2\text{FC} \times \text{Log}_{10}\text{P}$ values) for gene expression analysis between the brains of control C57BL/6 mice and *Winnie* mice. **Green** represents downregulated gene expressions comprising EC:2.4.2.1 *Pnp*; EC:3.2.2.6 and EC:249920, *Cd38*. **Red** represents upregulated gene expressions comprising EC:2.7.1.22 and EC:2.7.1.173, *Nmrk1*, and EC:1.2.3.1, *Aox*. Circular nodes indicate relative expression of compounds by UFLC (**blue** to **yellow**) between the brains of control C57BL/6 mice and *Winnie* mice. **Blue** represents the UFLC results of nicotinamide.

Abbreviations: phosphorylase, *Pnp*; cluster of differentiation 38, *Cd38*; nicotinamide/nicotinate riboside kinase 1, *Nmrk1*; aldehyde oxidase, *Acox1*.

A



Table 5.8 Glossary of terms for nicotinamide metabolism-associated genes

Reaction	Substrate	Product	Comment
Downregulated DEGs			
Purine-nucleoside phosphorylase (<i>Pnp</i>) EC:2.4.2.1			
Nicotinate D-ribonucleoside: orthophosphate ribosyl transferase.	Purine ribonucleoside; phosphate, purine 2'-deoxyribo-nucleoside	Purine; alpha-D-ribose 1-phosphate, 2-deoxy-alpha-D-ribose 1-phosphate	<i>Pnp</i> catalyses N-glycosidic bond in the β-(deoxy) ribonucleoside molecules, with the formation of free purine bases and pentose-1 phosphate.
ADP-ribosyl cyclase 1; NAD⁺ nucleosidase (<i>Cd38</i>) EC:3.2.2.6. EC:249920			
1). NAD ⁺ + H ₂ O = ADP-D-ribose + nicotinamide 2). NADP ⁺ + nicotinate = NADPH + nicotinamide	1). NAD ⁺ ; H ₂ O; cyclic ADP-ribose. 2). NADP ⁺ ; nicotinate; 2'-phospho-cyclic ADP-ribose.	1). ADP-D-ribose; nicotinamide; cyclic ADP-ribose. 2). NADPH; nicotinamide; 2'-phospho-cyclic ADP-ribose.	<i>Cd38</i> removes nicotinamide from NADP ⁺ , forming 2'-phospho-cyclic ADP-ribose, and the addition of nicotinate to the cyclic product, forming NADP ⁺
Upregulated DEGs			
Ribosyl-nicotinamide kinase (<i>Nmrk1</i>) EC:2.7.1.22 EC:2.7.1.173			
ATP + Nicotinamide-β-riboside <=> ADP + Nicotinamide D-ribonucleotide, then converted to NAD ⁺	ATP; 1-(β-D-ribofuranosyl)-nicotinamide	ADP; β-nicotinamide D-ribonucleotide	<i>Nmrk1</i> catalyses nicotinamide riboside and nicotinic acid riboside to form nicotinamide mononucleotide and nicotinic acid mononucleotide.
Aldehyde oxidase (<i>Aox</i>) EC 1.2.3.1			

An aldehyde + H ₂ O + O ₂ = a carboxylate + H ₂ O ₂	Aldehyde; H ₂ O; O ₂	Carboxylate; H ₂ O ₂	<i>Aox</i> reduces nitrite to nitric oxide with NADH or aldehyde as electron donor.

Table 5.9 Enrichment terms of all nicotinamide-associated genes

Category	Term ID	Term description	Observed gene count	Back-ground gene count	Strength	FDR
KEGG	mmu00760	Nicotinate and nicotinamide metabolism	4	39	2.66	2.08E-08
KEGG	mmu01100	Metabolic pathways	5	1536	1.16	0.00027
KEGG	mmu00240	Pyrimidine metabolism	2	57	2.19	0.0077
KEGG	mmu00230	Purine metabolism	2	134	1.82	0.031

5.4.7 Changes in concentration of tryptophan metabolites

Metabolomics analysis was employed to quantify the tryptophan metabolite concentrations of tryptophan, 5HIAA, kynurenine, KYNA (kynurenic acid), QUIN (quinolinic acid), PIC (picolinic acid) and nicotinamide. Metabolomics provides an account of the metabolic state of a biological system, which may shed insight into cellular processes and their regulation at both the transcriptional and metabolic levels. Changes in the concentration levels of tryptophan metabolites are reflected in changes in the expression of genes involved in these pathways.

The tryptophan metabolite concentrations were measured in the brains of control mice and compared to the *Winnie* mice according to the method described in Chapter 3, [Section 3.4.7](#). Non-significant differences were identified between the males and females. Therefore, the mouse sexes were merged for analysis. Tryptophan concentration was significantly lower ($p < 0.001$), which coincided with a suboptimal serotonergic pathway as demonstrated by the lower concentration of 5-HIAA ($p = 0.010$). Kynurenine was significantly lower in concentration ($p = 0.03$). KYNA was significantly lower in

concentration ($p=0.010$). Nicotinamide was significantly lower in concentration ($p=0.015$). PIC was significantly lower in concentration ($p=0.019$) in *Winnie* mouse brains. However, QUIN, which does not cross the blood-brain barrier (BBB) and is typically produced by astrocytes showed non-significant differences between the *Winnie* mice and control C57BL/6 littermates brains (Table 5.10 and Figure 5.12).

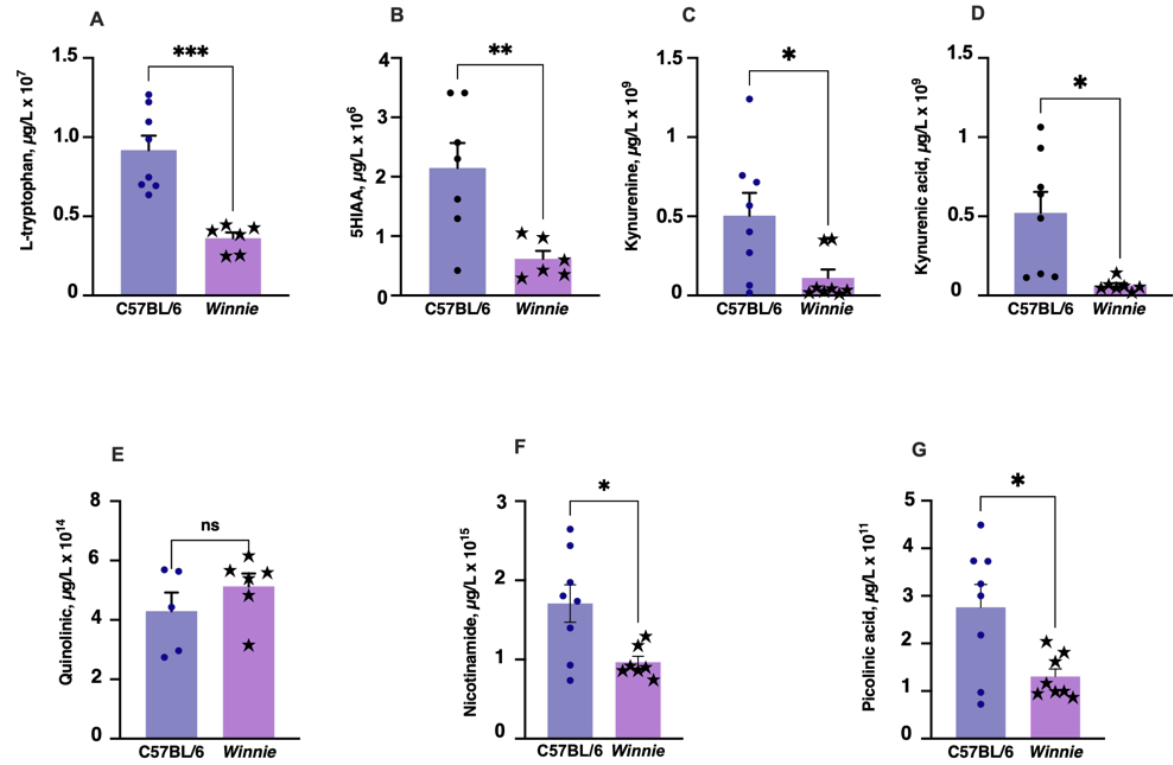
Table 5.10 Tryptophan metabolite concentrations in *Winnie* mice brains

Metabolite mM	Control C57BL/6	SEM	<i>Winnie</i>	SEM	<i>p</i> value
Tryptophan	9.1873E+06	9.0523E+05	3.6232E+06	3.5812E+05	***<0.001
5-HIAA	2.1478E+06	4.1931E+05	6.1870E+05	1.3292E+05	**0.010
Kynurenine	5.0462E+09	1.4336E+09	1.1139E+09	5.3018E+08	*0.030
KYNA	5.2167E+08	1.3227E+08	6.3519E+07	1.4461E+07	**0.010
QUIN	4.2935E+14	6.3110E+13	5.1333E+14	4.3324E+13	non- significant
Nicotinamide	1.7079E+15	2.3686E+14	9.6624E+14	7.38339E+13	*0.017
PIC	2.76003E+11	47898170341	1.30621E+11	1.6010E+10	*0.019
Data is expressed as the mean $\pm$ SEM, $p \leq 0.05$.					
Abbreviations: millimolar, mM; 5-hydroxyindoleacetic acid, 5-HIAA; kynurenic acid, KYNA; quinolinic acid, QUIN; picolinic acid, PIC.					

Figure 5.11 Tryptophan metabolite concentrations in *Winnie* mice brains

Tryptophan metabolites were detected in *Winnie* mice brains compared to control C57BL/6 mice brains. **(A)** Tryptophan. **(B)** 5- hydroxyindole acetic acid. **(C)** Kynurenine. **(D)** Kynurenic acid. **(E)** Quinolinic acid, n=6. **(F)** Nicotinamide. **(G)** Picolinic acid. Data is expressed as mean $\pm$ SEM, * p <0.05, ** p =0.010, *** p <0.001. **Abbreviations:** 5IHAA, 5-indole hydroxy acetic acid, mM, millimolar.

Figure 5.11 Tryptophan metabolite concentrations in *Winnie* mice brains



5.5 Discussion

The brain in the chronic colitis-induced mouse model has not been extensively studied due to an emphasis on understanding the pathophysiology of the colon (Baydi et al., 2021). Therefore, this study determined the glycolysis/gluconeogenesis and tryptophan *de novo* NAD⁺ synthesis gene expressions in the brains of *Winnie* mice compared to control C57BL/6 littermates.

5.5.1 Dysregulation of glycolysis/gluconeogenesis in *Winnie* mice brains

Glycolysis pathway changes in the *Winnie* mice brains demonstrated allosteric regulation, which is a key mechanism for meeting the changing metabolic needs of cellular function and homeostasis (Tanner et al., 2018). Several factors contributing to the glucose metabolism dysregulation were identified in the *Winnie* mice of this study, including downregulated *Tpi1*, *Pgk1*, *Bpgm*, *Pgam*, *Pdha1*, *Ldha*, *Aldh3a1*, *Aldh3b1* and *Adh 1/7* and upregulated *Hk2*. Each of these findings and their significance will be discussed.

First, in *Winnie* mice brains, upregulation of *Hk2* was noted, which is responsible for initiating the phosphorylation of glucose for the supply of glucose to all bodily tissues (Hinrichsen et al., 2021). HK2 is the prototypic inducible isoform of all HK family members given its ability to be amplified by diverse environmental influences and signalling pathways, especially during inflammatory conditions such as IBD (Hinrichsen et al., 2021). In brain cells, Hu et al. (2022) established that HK2 is elevated in pro-inflammatory microglia, and by utilising HK2, microglia are able to achieve maximal glycolytic flux and adaptive capacity. It is of interest that following lipopolysaccharide stimulation in mouse microglia, HK2 levels were significantly increased (Hu et al., 2022). In correlation, dextran sulfate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis mouse models show alterations of microglial phenotypes, as well as emotional-affective behaviours and stress-induced neuronal activation in the limbic system (Nyuyki et al., 2018; Riazi et al., 2008). Similar results were demonstrated in MRI studies of IBD patients (Tynan et al., 2010), which explains one of the underlying mechanisms of stress-associated depression (Bernstein, 2016).

Tpi1, which is downregulated in *Winnie* mice, converts DHAP into glyceraldehyde 3-phosphate (G3P) (Soto-Herederó et al., 2020). At this junction, glycolysis is linked to the tryptophan/ mammalian target of rapamycin complex 1 (mTORC1) pathway by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) binding to Ras homolog enriched in brain (Rheb) (Kanová & Kohout, 2021). In context, a murine model of TPI deficiency presented with anaemia and severe neuromuscular dysfunction, a markedly shortened lifespan and decreased body weight (Myers et al., 2022). Humans with TPI deficiency manifest intellectual disability, however behaviour tests, designed to detect changes in CNS function in mice with inhibited TPI1 observed no noteworthy differences (Segal et al., 2019). Hence, in mice, a reduction in TPI1 activity is not enough to induce symptoms of TPI deficiency (Segal et al., 2019). Separately, the *Pgk1* gene, which was downregulated, encodes the phosphoglycerate kinase enzyme and generates adenosine triphosphate (ATP). Humans presenting with *Pgk1* mutations present with haemolytic anaemia and or brain dysfunction (Spiegel et al., 2009). TPI1 and PGK inhibition associates with haemolytic anaemia in IBD patients, with a frequency of 62.1% in CD patients and 55.7% in UC patients (Atuğ et al., 2016). It was noted that treatment of IBD-associated anaemia with iron supplementation led to improvement in patient quality of life (QoL) (Wells et al., 2006).

Winnie mice showed *Bpgm* and *Pgam* downregulation of the Luebering-Rapoport glycolytic bypass reactions. *Bpgm* regulates haemoglobin oxygen affinity by controlling its allosteric effector 2,3-(bisphosphoglyceric acid) BPG (Cho et al., 2008). Consequently, 2,3-BPG shifts the oxygen dissociation curve to the right, enabling erythrocytes to generate ATP. Therefore, *Bpgm* inhibition triggers a decrease in oxygen release (Oslund et al., 2017). A study by Agostini et al. (2011) in IBD patients noted that the blood oxygen level-dependent signal was significantly reduced in the amygdala, thalamus, and cerebellum relative to controls. Consequently, the group-related differences in brain activity observed in UC patients were associated with decreased sensitivity to positive-content emotions (Agostini et al., 2011). In the *Muc2* knockout colitis mice, the same behavioural phenotypes were observed and correlated with the brain glycinergic system (Morozova et al., 2022). BPGM activity is the source for 2,3-BPG to activate PGAM1 phosphorylation (Oslund et al., 2017). BPGM and PGAM1 deficiency leads to increased *de novo* serine biosynthesis and ultimately glycine and

purine production (Oslund et al., 2017). Although the *Winnie* mouse genes are associated with depression and anxiety, earlier studies have indicated that glycine can improve symptoms of anxiety, obsessive-compulsive disorder, and regulate sensorimotor gating in mice (Cleveland et al., 2009).

Inhibition of LDH restricts ATP generation and accumulates mitochondrial-toxic compounds, thereby reducing mitochondrial membrane potential and limiting energy production (Hall et al., 2012). Consequently, LDH deficiency leads to nicotinamide adenine dinucleotide + hydrogen (NADH) accumulation, causing NAD⁺ depletion and energy shortage (Farhana A, 2021). Hence, in mice, spatial working memory was impaired in association with LDH inhibition (Harris et al., 2019). LDH downregulation affects *Pdhal* (downregulated in *Winnie* mice), which is required for acetyl-CoA synthesis in the hippocampus of rodents, which in turn impedes aerobic glycolysis required for memory acquisition (Harris et al., 2019). MRI studies in UC patients showed a lower amplitude of low-frequency fluctuation in the hippocampus, which correlated with memory loss, defective executive function and attention network (Fan et al., 2019) and suicide ideation (Fan et al., 2013). Together with the reduced levels of BPGM and LDHA, the *Winnie* mice may lack the ability to meet short-term energy needs when oxygen is insufficient. This combination could potentially explain IBD-associated fatigue, affecting physical function and cognitive performance (Bryce et al., 2019).

Several members of the *Aldh* gene family (*Adh5* and *Aldh2*, *Aldh3a1*, and *Aldh3b1*), which are associated with detoxification (Hodges & Minich, 2015), are downregulated in *Winnie* mice. *Adh* protects cells from oxidative stress and enhances the first phase of ethanol metabolism; oxidises noradrenaline, dopamine and serotonin; metabolises retinol, intermediates in the process of lipid peroxidation, and also plays a role in leukotriene, and prostaglandin metabolism (Edenberg & Bosron, 2010; Höög et al., 2001). Downregulated *Aldh* renders the *Winnie* mice vulnerable to aldehyde-induced damage and a reduced capacity to detoxify reactive oxygen species (ROS). This impedes the pathway of ethanol to acetaldehyde to acetate (Isse et al., 2005), which contributes to 'aldehydic load' and links to the pathology of neurodegenerative diseases (Chen et al., 2009). Specifically, poor ethanol detoxification is associated with an accumulation of 3,4-dihydroxy phenylacetaldehyde, a neurotoxic dopamine metabolite, that affects neural function in

dopaminergic nerve function (Koppaka et al., 2012; Shortall et al., 2021). In human gastrointestinal conditions, diminished ALDH2 resulted in increased facial blood flow, rosacea and migraines, implicating the gut-brain-skin axis (Chen et al., 2009). Serotonergic neurons need ALDH2 to complete 5-hydroxytryptamine (5-HT) production and metabolism processes (Bortolato & Shih, 2011), which may be one of the factors that led to the lower levels of 5-HIAA in *Winnie* mice. It is of interest that mice deficient in brain 5-HIAA display exaggerated aggression (Mosienko et al., 2012).

Hadh, which is downregulated in *Winnie* mice, participates in fatty acid β -oxidation as well as tryptophan metabolism (Schönfeld & Reiser, 2013). The literature on *Hadh* in mice with colitis is scarce, however, it is shown that *Hadh*^{-/-} mice are in good health and show normal grooming and exploratory behaviours (Schulz et al., 2011). Nonetheless, in humans, *Hadh* serves as a multifunctional dehydrogenase involved in androgenic regulation of the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic/pituitary/gonadal (HPG) axis (He et al., 2000). This regulation significantly influences autonomic and neuroendocrine function, feeding and metabolism, as well as stress-related behaviours (He et al., 2000). Recognising that imbalanced levels of androgens can affect cognitive performance in both humans and animals highlights the significance of comprehending these intricate molecular interactions (Edinger & Frye, 2007). Androgens can impact cortisol secretion, which, in turn, inhibits the synthesis of 5-HT by reducing the availability of tryptophan to the brain (Badawy, 2018b). It is likely that this mechanism has influenced the lower 5-HIAA levels in *Winnie* mice brains, contributing to a compromised serotonergic pathway.

Cytochrome P450 (*Cyp11a1*), which is upregulated in *Winnie* mice, is responsible for converting cholesterol into hormones (Lu et al., 2023). Heme deficiency leads to increased accumulation of CYP11A1 in the cytosol of mouse brains (Meyer et al., 2005). Rat brain cells lose complex IV after induction of heme deficiency in primary hippocampal neurons, suggesting an association with loss of mitochondrial quality and the aging process, including neurodegeneration (Atamna et al., 2002). CYP enzymes bear resemblance to proinflammatory cytokines and interferon (IFN), which contribute to the activation of sensory neurons that generate neuropathic hyperalgesia (Stavropoulou et al., 2018), which is commonly experienced by IBD patients (Bielefeldt et al., 2009). Further

research focusing on the specific sites of these actions in *Winnie* mouse brains will provide valuable insights into the role of androgens in the stress response and how this action influences neuropathic hyperalgesia in patients with IBD.

5.5.2 Tryptophan metabolism dysregulation in *Winnie* mice brains

Several factors contributing to the dysregulation of tryptophan metabolism were identified in the *Winnie* mice. These included reduced tryptophan, 5-HIAA, kynurenine, KYNA, PIC and NAD levels, however non-significant differences in QUIN levels. Also, downregulated gene expressions included *Idol* and *Afmid* whilst *Tph1*, *Cyp1a1*, *Laaol*, *SAME* and *Dhtkd1* were upregulated.

Dysregulation of tryptophan metabolic pathways potentially influences behaviour, considering the established relationship between the gut and the brain in the systemic disorder of IBD patients (Roth et al., 2021). Tryptophan metabolism is pivotal in the production of serotonin (5-HT), which plays a significant role in regulating mood, sleep, appetite, and other behavioural aspects (Jacobsen et al., 2012; Wang et al., 2022). In the *Winnie* mice brains, tryptophan level was significantly lower than in controls. Tryptophan deficiency can inhibit the signalling of the mTORC1, which is integral to neuronal growth development (Takei & Nawa, 2014). Another example showed that the depletion of tryptophan in the prefrontal cortex-amygdala circuits of human brains is associated with facial signals of aggression (Passamonti et al., 2012). Reduced tryptophan is a phenomenon that can be attributed to several key factors. In particular, the availability of tryptophan in the brain is contingent upon its plasma concentration and its competitive relationship with large neutral amino acids, namely L-valine, L-leucine, L-isoleucine, L-phenylalanine, and L-tyrosine. These amino acids enter the CNS as well as tryptophan and kynurenine for transport across the BBB via the large neutral amino acid transporter (LAT-1) (Feurté et al., 2001). Additionally, the metabolism of tryptophan, via the kynurenine pathway, serves as a critical link between peripheral inflammation and CNS alterations (Roth et al., 2021). Hereby, *Idol* was reduced in the *Winnie* mice brains, which can diminish the availability of tryptophan, thereby exerting both positive and negative effects on brain function (Hochstrasser et al., 2011). To better understand the interplay of

these factors and establish a definitive correlation, future research should aim to compare both plasma and brain tryptophan levels in the *Winnie* mouse.

5-HIAA was less concentrated in *Winnie* mice. In TNBS colitis-induced rodents, the marked suppression of 5-HT release from the hypothalamus contributed to the depressive-associated disorder of anorexia (Ballinger et al., 2000; El-Haj et al., 2002). In the context of behavioural changes, socially stressed mice show decreased 5-HIAA levels in the hippocampus, amygdala, and nucleus accumbens in depressive-like states (Avgustinovich et al., 2004) and also exhibited aggressive behaviour (Mosienko et al., 2012). In suicidal humans reduced 5-HT_{1A} receptors in the frontal cortex and amygdala correlated with decreased 5-HIAA levels in the hippocampus, hypothalamus, amygdala, and nucleus accumbens during the onset of depression (Bach-Mizrachi et al., 2008). In humans, a low 5-HIAA concentration in cerebrospinal fluid (CSF) has been identified in impulsive violent offenders, a condition that has also been associated with disturbances in diurnal activity rhythms (Popova, 2006). Given these findings, investigations into aggressive behaviour in the *Winnie* mouse model may prove valuable. In a clinical context, patients with moderate to severe IBD are more likely to exhibit suicidal ideation, and seek mental health care plans, potentially highlighting the broader implications of serotonergic dysregulation in these patients (Bhandari et al., 2017). Selective serotonin reuptake inhibitors (SSRIs) are prescribed to treat IBD patients for depressive disorder and anxiety. Coincidentally, these patients show a reduced incidence of bowel disease (Kristensen et al., 2018), which indicates a causal relationship between serotonin, the gut, the brain, and colitis (Roth et al., 2021).

In this study, significantly low kynurenine concentration was noted in *Winnie* mice. This is in contrast to the notably elevated kynurenine levels observed in humans with major depressive disorder (Réus et al., 2015) as well as in DSS-induced colitis mice (Zhao et al., 2023). This contradiction may be explained through a gerbil study in which when there is no general inflammation, approximately 80% of the brain's kynurenine comes from blood transport, whereas during immune activation localised in the CNS, more than 98% of kynurenine is produced directly within the brain (Kita et al., 2002). Therefore, the observed differences might arise from the absence of gut inflammation in humans without colitis. Whereas in DSS-induced colitis mice, acute inflammation seems to stimulate the

tryptophan-kynurenine-KYNA pathway in the brain (Zhao et al., 2023). In contrast, chronic colitis in *Winnie* mice appears to have a diminished impact on this pathway. Aside, while high concentrations of kynurenine can induce apoptosis through the generation of ROS, optimal levels of kynurenine may protect macromolecules from oxidative modifications (Mor et al., 2021; Peyrot & Ducrocq, 2008). Cerebral oxidative stress in mice with DSS-induced colitis correlates with depressive and anxiety-like behaviours (Dong et al., 2021). It is postulated that the low kynurenine levels observed in *Winnie* mice may not be sufficient to defend against oxidative damage. This is particularly pertinent given that neuroprotective KYNA concentrations were lower in *Winnie* mice. *Cyp11a1* upregulation assists the *Winnie* mouse to process foreign chemicals and pollutants, while facilitating the metabolism of biogenic amines and neuroamines (Lu et al., 2023; Nelson, 2004). The measurement of oxidative stress in the *Winnie* mouse brain needs to be determined to confirm our postulations.

Early studies have indicated that KYNA exhibits restricted permeability across the BBB. Within the brain, KYNA synthesis predominantly occurs in astrocytes. The cellular energy requisites associated with KYNA synthesis can impede its production in rat brains. (Fukui et al., 1991; Gramsbergen et al., 1997). On the positive side, reduced levels of KYNA in mouse brains enhance their cognitive abilities and are associated with increased levels of extracellular glutamate (Potter et al., 2010). In the rodent prefrontal cortex, inhibition of KYNA synthesis has shown to increase extracellular levels of gamma-aminobutyric acid (GABA), which is an inhibitory neurotransmitter that plays a role in reducing anxiety (Beggiato et al., 2014). We, therefore, propose that the low KYNA levels shown in the *Winnie* mouse brain aid in counteracting gene expressions that promote anxiety.

PIC concentration is significantly lower in *Winnie* mice, while QUIN concentrations showed non-significant differences in concentration compared to the controls. In human brains, an adequate concentration of PIC is known to help protect against the neurotoxic effects of QUIN without inhibiting QUIN's neuroexcitatory properties (Grant et al., 2009). QUIN, produced mainly by infiltrating macrophages and then astrocytes (Guillemin et al., 2003), acts as an agonist of N-methyl-D-aspartate (NMDA) receptors,

especially in the presence of ROS, as well as inhibits gluconeogenesis and mitochondrial MAO activity, and functions as a neuroexcitatory toxin (Ganzella et al., 2006; Lugo-Huitrón et al., 2013). Adequate levels of KYNA have the potential to counteract QUIN's neurodegenerative effects and protect neurons against its excitotoxicity (Hilmas et al., 2001). Previous research has identified an imbalance between the neuroprotective properties of KYNA and the neurotoxic effects of QUIN in major depressive disorder (Réus et al., 2015). Non-significant differences in QUIN levels do not necessarily mean that *Winnie* mice are not depressed, as there are multiple factors contributing to affective disorders in both mice and humans (Savitz, 2015; Savitz et al., 2015).

In *Winnie* mice, *Tph1* is notably upregulated. In post-mortem brain studies of humans who died a sudden death, TPH1 was predominantly found in the pineal gland, cortex, hypothalamus, thalamus, cerebellum, and amygdala. These areas are critically involved in the development and manifestation of human psychiatric disorders (Zill et al., 2007). TPH1 is sensitive to stress-induced alterations, underlining its potential role in the onset of stress-related psychopathologies (Abumaria et al., 2008). In contrast, TPH2 levels in the brain remain unresponsive to repeated stress exposure (Zill et al., 2007). The expression of TPH1 is subject to regulation by adrenal glucocorticoids, which presents a plausible mechanism through which stress may exert its effects on serotonin synthesis. This regulatory pathway is of considerable interest, as changes in serotonin levels are intimately tied to fluctuations in mood and emotional well-being (Abumaria et al., 2008). In addition, stress is recognised as a significant factor affecting the physiological and psychological well-being of individuals with IBD. Research has observed that repeated exposure to stress can yield the emergence of depressive-like behaviours (Colonnello & Agostini, 2020).

The expression of *Laaol* was upregulated in the *Winnie* mice brains. Specifically, *Laaol* acts to regulate and reduce the ratio of tryptophan to large neutral amino acids (LNAA) in plasma. This altered ratio is consequential, as it is associated with the upregulation of serotonin synthesis (Usuda et al., 2018). LAAO1 is a pivotal player in the modification of neurotransmitter levels within the hippocampus, a brain region integral to learning and memory. It is involved in regulating fear learning and contextual fear conditioning memory. This regulatory role of LAAO1 extends to the production of acetylcholine and

serotonin, as well as to the metabolism of phenylalanine, thereby illustrating its broad influence on brain chemistry (Usuda et al., 2018). To elucidate the interrelationships among these neurotransmitters and delineate their distinct roles in cognitive processes, it is required to conduct more comprehensive behavioral studies, particularly emphasizing learning and memory, using the *Winnie* mouse model.

Ido1 expression was downregulated in the *Winnie* mice. In both mice and humans, IDO1 expression can be influenced by interferon (IFN), tumour necrosis factor (TNF), interleukin-6 (IL-6), bacterial lipopolysaccharides (LPS) and the expression of stress-responsive enzymes in mice and humans (Kiank et al., 2010). Rodent studies have shown that IDO1 deficiency can affect exploratory behaviour and learning capabilities, leading to sustained hypo-locomotion during initial exploration phases (Too et al., 2016). In another study, inhibiting IDO1 was associated with changes in fear learning and memory in mice (Fuertig R, 2016). Our results conflict with the DSS mouse model of acute colitis, in which IDO1 expression was upregulated in the cerebral cortex (Zhao et al., 2023). However, the *Winnie* mouse model represents chronic disease which explains this discrepancy. A prospective investigation aimed at measuring and manipulating IDO1 expression in *Winnie* mice could be a promising avenue of research in exploratory behaviours.

In the *Winnie* mice, *Afmid* that codes for an enzyme arylformamidase, that catalyses the transformation of kynurenine into the neuroprotective compound KYNA, was upregulated (Dobrovolsky et al., 2005). Despite *Afmid* upregulation, KYNA was low in concentration. Whilst studies specifically in colitis mouse models are limited, AFMID is known to play a role in toxin elimination (Phillips et al., 2019). *Winnie* mice DEGs enrichment terms stated that AFMID is involved in responses to immobilisation stress. Excessive exposure to stress factors like immobilisation negatively impacts the gut's immune system and alters its microbial makeup via the HPA axis (Jang et al., 2019). In mouse models of colitis, disruptions can lead to a combination of symptoms. These include inflammation in the gastrointestinal tract, mental health issues such as anxiety and depression, inflammation in the hippocampus, and an imbalance in the gut microbiota (Jang et al., 2019).

SAMe, which upregulated in *Winnie* mice, acts as a protective mechanism against toxic luminal sulfides, and serves as a precursor in the biosynthesis of spermidine and spermine (Roje, 2006). Notably, *SAMe* administration in rodents led to increased 5-HT levels in the hippocampus and frontal cortex, and activation of postsynaptic 5-HT_{1A} receptors, suggesting potential antidepressant effects of *SAMe* (Sales et al., 2023). We speculate whether 5-HIAA in *Winnie* mice would be further reduced if *SAMe* were not upregulated. The administration of *SAMe* to rodents 24 hours before a forced swim test, noted an improved swimming mobility response, indicating a lack of depression (Saunderson et al., 2016). However, earlier human studies on *SAMe* and depression have noted rapid mood shifts in some patients, particularly in those with suspected bipolar major affective disorder (Carney et al., 1987). In the context of histamine metabolism, *SAMe* plays a role, as histamine acts as a neurotransmitter or neuromodulator in the CNS and is involved in allergic reactions and inflammatory processes (Criconia et al., 1994; Mischoulon & Fava, 2002). Separately, *Aoc* is involved in the metabolism of several amine compounds, such as exogenous histamine, and also deaminates putrescine, GABA, and spermidine (Shan et al., 2015). In humans and mice, reduced levels of AOC potentially lead to altered neurotransmitter levels, and impact learning, cognition, and memory (Yoshikawa et al., 2019).

Perhaps, in an adaptive effort to optimise mitochondrial energy metabolism, *Winnie* mice allosterically upregulated *Dhtkd1*. It encodes a component of a complex that facilitates the conversion of 2-oxoadipate to glutaryl-CoA, a key step in the degradation pathways of L-lysine, L-hydroxylysine, and L-tryptophan (Boyko et al., 2022; Danhauser et al., 2012). Variations in the *Dhtkd1* gene have the potential to impact tryptophan metabolism and the equilibrium of neuroactive metabolites. Such disruptions could contribute to the onset of depression or other neurological disorders (Xu et al., 2013).

5.5.3 Nicotinamide metabolism dysregulation in the *Winnie* mouse

Several factors contributing to the dysregulation *de novo* NAD⁺ synthesis were identified in the *Winnie* mice, including reduced nicotinamide. Also, downregulated gene expressions comprised *Pnp* and *Cd38* and upregulated gene expressions comprised *Nmrk1* and *Aox*. Each of these findings and their significance will be discussed.

In the *Winnie* mice brains, nicotinamide was significantly lower. Reduced level of nicotinamide is associated with depression,, anxiety, psychological stress in humans (Gasperi et al., 2019). Notably, the administration of nicotinamide in mouse models of depression has restored ATP, neuronal function, and NADH levels, which consequently reduced depression-like behaviour in mice (Liu et al., 2020). In a study by Liu et al. (2021), mice with chronic cerebral hypoperfusion displayed symptoms of depression, anxiety, cognitive decline, and hopelessness. Nicotinamide treatment significantly ameliorated all of these symptoms in mice. In addition, nicotinamide has been reported to enhance sensory, motor, and cognitive abilities. This was evidenced by the improved performance of animals on bilateral tactile adhesive removal and locomotor placing tasks (Fricker et al., 2018). Reduced nicotinamide has been shown to contribute to neurodegeneration and compromise cellular defense against stress and damage (Klaidman et al., 2001; Liu et al., 2021). A comprehensive study is required to elucidate the synthesis of NAD⁺ and its interplay with QUIN and nicotinamide in *Winnie* mice.

The activity of NAD⁺-consuming enzymes remained largely unaltered, with the exception of *Cd38* expression, which is downregulated in the *Winnie* mice brains. Research has demonstrated that mice deficient in *Cd38* exhibit elevated levels of NAD⁺, as well as accelerated rates of oxidative phosphorylation (Aksoy et al., 2006). Changes in spatial learning ability were observed in *Cd38*^{-/-} mice (Young & Kirkland, 2008). However, despite downregulated *Cd38*, the underlying cause of the reduced nicotinamide levels in *Winnie* mice remains unclear. Adding to the complexity of this situation is the observed upregulation of *Nmrk1*, an enzyme known to increase its activity in response to ATP and NAD⁺ depletion (Diguët et al., 2018).

Pnp downregulation, observed in the *Winnie* mice brains, has significant implications for initiating and sustaining astrogliosis (Ciccarelli et al., 2001). In humans, PNP deficiency leads to an accumulation of toxic deoxynucleotides, which can disrupt neural cell division, as well as hinder DNA synthesis and repair processes. Such disruptions can give rise to a spectrum of neurological disorders and the development of autoimmune diseases (Walker et al., 2011). A comprehensive understanding of the role and regulatory

mechanisms of PNP is essential for identifying potential therapeutic strategies aimed at addressing these conditions.

Lastly, *Aox*, which is upregulated in the *Winnie* mice brains, plays a role in purine degradation, xenobiotic metabolism, and the conversion of aldehyde groups into carboxylic acids (Dalvie & Di, 2019). Research suggests that AOX plays a role in counteracting ROS and other compounds that cause oxidative stress (Szibor et al., 2017). While AOX produces hydrogen peroxide (H_2O_2), it can also, under specific circumstances, catalyse the creation of superoxide. This function serves as a protective measure in tissue (El-Khoury et al., 2013).

5.6 Conclusion

Our study revealed gene and metabolite irregularities in the brain, which adversely affected the behaviour of *Winnie* mice, providing a link between chronic intestinal inflammation and brain functional changes. The key findings observed were a susceptibility for the *Winnie* mice to incur compromised mitochondrial function, reduced ATP metabolism, neuromuscular dysfunction, hindered memory formation, neuropathic hyperalgesia, and neurodegeneration. Additionally, *Winnie* mice are susceptible to aggressive behaviour and manifestations of stress-related disorders. Our data indicated compromised exploratory behaviour as well as symptoms of depression and anxiety. These findings suggest that glycolysis and tryptophan metabolism inhibition may exert a greater influence on behaviour than previously recognised. The results shed light on the medically unexplained affective disorders seen in IBD patients, offering a valuable opportunity to deepen the understanding of these manifestations. QoL would be positively impacted by addressing the fatigue and mental health issues associated with IBD. The focus on gene expression and metabolic alterations associated with IBD represents a significant shift in the understanding of IBD and potentially opens new avenues for the treatment and management of this debilitating condition. These findings provide a compelling foundation for undertaking further detailed clinical investigations into IBD-associated depression and other disorders.

Chapter 6: General discussion, future recommendations

6.1 Introduction

A heightened prevalence of comorbid affective disorders and associated symptoms has been documented among patients with inflammatory bowel disease (IBD). These manifestations prominently encompass depression, anxiety, anhedonia, fatigue, suicidal ideation, and actual suicide (Yanartaş et al., 2019), thus affecting their quality of life (QoL) (Knowles et al., 2018). It is of interest that a diminished incidence of IBD has been reported in patients administered selective serotonin reuptake inhibitors (SSRIs) (Mikocka-Walus et al., 2016). This suggests a potential causal linkage between tryptophan metabolism, specifically the serotonergic pathway, in the gut and brain and the pathophysiology of IBD (Yanartaş et al., 2019). Tryptophan metabolism governs the production of serotonin, which in turn, significantly modulates behavioural aspects such as mood, sleep, and appetite. Studies in both IBD patients and mouse models of colitis show that nicotinamide adenine dinucleotide (NAD^+) synthesis from tryptophan follows an oscillatory metabolic pattern (Nikolaus et al., 2017). By integrating the models of glycolysis, fatty acid β -oxidation (FAO) and tryptophan *de novo* NAD^+ synthesis in *Winnie* mice with chronic colitis, one could better understand the conditions that determine the rate of NAD^+ production from tryptophan. Furthermore, we were able to establish how these metabolic routes affected behavioural changes, which formed the hypothesis of this thesis.

We chose the *Winnie* mouse model because it develops progressive chronic intestinal inflammation, with severe colitis becoming apparent between 12 and 16 weeks of age. This closely mimics the histopathological outcomes seen in human IBD (Eri et al., 2011). For this study, energy metabolism, behaviour, and changes in the distal colons and brains of 14-week-old *Winnie* mice and age-matched control C57BL/6 littermates were examined.

In this thesis, **Chapter 1** provides an overview of IBD, tryptophan *de novo* NAD^+ synthesis and energy metabolism. **Chapter 2** presents data on the changes in energy metabolism in *Winnie* mice with chronic colitis, *in vivo*, using indirect calorimetry and the changes in glycolysis/gluconeogenesis and fatty acid β -oxidation using RNA-Seq. **Chapter 3** demonstrates the changes in tryptophan metabolism pathways correlating with

chronic intestinal inflammation in *Winnie* mice with chronic colitis. **Chapter 4** investigates changes in behaviour of *Winnie* mice with colitis, employing the Promethion system to determine spontaneous physical activity and sleep patterns and determines the changes in the cytokine transcriptome and protein expression in the brain of *Winnie* mice. **Chapter 5** determines the changes in glycolysis/gluconeogenesis and tryptophan metabolism pathways in the brain of *Winnie* mice with colitis and how these changes are associated with behaviour.

6.1.1 The *Winnie* mice consumption and energy metabolism

In IBD, inflammation plays a significant and enduring role, which correlates with the extent, duration, and severity of the disease. In **Chapter 2**, we verified inflammatory activity in the distal colons of *Winnie* mice compared to control mice ([Figure 2.1](#)). This was achieved through parameters of faecal water content, Lcn-2 levels, histological morphological features, *in vivo* body weight and total body composition ([Figure 2.2](#)). Subsequently, we assessed the effects of colitis on energy metabolism in *Winnie* mice using Promethion metabolic cages and indirect calorimetry. It is noteworthy that there were no statistically significant differences observed between the male and female *Winnie* groups across all tests ([Figure 2.3](#)). Therefore we pooled the data from both sexes of *Winnie* mice and compared them with the combined data from both sexes of control C57BL/6 littermates; with each group comprising n=16 mice ([Figure 2.4](#)). A notable observation was the reduced attempts by *Winnie* mice to access the water and food hoppers compared to the controls. This behaviour suggests potential food and water aversion, or perhaps a decreased capability to reach these hoppers. The underlying reasons could range from gastrointestinal discomfort or pain to a suppressed appetite or lowered energy levels. As a result, the *Winnie* mice consumed significantly less food and water. This might have contributed to the lower weight in male *Winnie* mice and reduced lean body mass in both *Winnie* sexes. Lower food and water intake, coupled with altered body composition, impacts daily energy expenditure (EE). Our indirect calorimetry results highlighted significantly decreased oxygen consumption rates, carbon dioxide emission rates, as well as diminished EE, glucose oxidation (GOX), and lipid oxidation (LOX) in the *Winnie* mice. Reduced EE, GOX and LOX gave reason to investigate the underlying genetic causes, thus glycolysis/gluconeogenesis and FAO.

In the indirect calorimetry test, 6-week-old *Winnie* male and female mice exhibited no statistically significant differences in comparison to 6-week-old C57BL/6 mice across all parameters (Figure 2.5, Figure 2.6). Subsequently, these 6-week-old *Winnie* mice were contrasted with 14-week-old Het *Winnie* mice, 14-week-old C57BL/6 mice, and 14-week-old *Winnie* mice (Figure 2.7, Figure 2.8). The 6-week-old *Winnie* mice demonstrated non-significant variations when compared to the 14-week-old Het *Winnie* and 14-week-old C57BL/6 groups. An exception was observed in the reduced food consumption by the 6-week-old *Winnie* mice, an outcome that was anticipated. In contrast, the 14-week-old *Winnie* mice displayed diminished values in all assessed metrics compared to the other groups, indicating a decline in food and water intake as well as a reduced energy metabolism. These results truly indicate the progression of the disease in 14-week-old *Winnie* mice.

6.1.2 Changes in glycolysis and fatty acid β -oxidation in *Winnie* mice colons

Tryptophan metabolism links to the glycolysis pathway through the phosphoenolpyruvate (PEP) essential node and to the tryptophan/mammalian target of rapamycin complex 1 (mTORC1) pathway by the GAPDH enzyme binding to Ras homolog enriched in brain (Rheb). At this point, glycolysis determines the efficiency of tryptophan synthesis (Metz et al., 2012). Serotonin (5-HT) activates neuroendocrine signals that promote the lipolysis of triglycerides and the oxidation of fatty acids. (Bouagnon et al., 2019). To explore metabolic adaptations related to chronic intestinal inflammation, RNA-Sequencing produced quantitative gene expressions of glycolysis/gluconeogenesis and fatty acid β -oxidation in *Winnie* mice distal colons (Chapter 2, Figure 2.9, Figure 2.10).

The data supported the premise that a reprogramming of the glycolytic and FAO pathways are intrinsically associated with chronic intestinal inflammation in *Winnie* mice. For instance, mitochondrial dysfunction, aldehyde-induced harm, generation of high levels of hydrogen peroxide (H_2O_2), diminished reactive oxygen species (ROS) detoxification capacity, generation of neurotoxic electrophiles, reduced adenosine triphosphate (ATP) production, increased hypoxia-inducible factor-1 α (HIF-1 α), reduced lipogenesis, an inability to metabolise medium- and long-chain fatty acids. Consequently, the gene

changes contribute to villus atrophy and epithelial obstruction in the colon, hypoglycaemia, hyperlactacidemia, infection, intolerance to fasting, inhibition of ketogenesis and an elevated risk for colon cancer (Froissart et al., 2011; Getty-Kaushik et al., 2010; Richard et al., 2007). Our results corroborated the risk of developing colitis-associated colorectal cancer, which is of high risk in IBD patients (Manninen et al., 2013). Furthermore, the glycolytic gene expression changes correlate to signalling the onset of the Warburg effect in *Winnie* mice (Vander Heiden et al., 2009). Our findings in the *Winnie* mouse reveal that even at the point of diagnosis, the genomic, epigenetic, and transcriptomic landscapes are already in a diseased state. Crucially, these outcomes suggested a connection to the pathophysiology of IBD, the metabolic alterations concomitant with this condition. In light of these findings, this thesis advocates for the investigation and identification of early alterations in the metabolic processes of IBD patients.

6.1.3 Changes in tryptophan *de novo* NAD⁺ synthesis of *Winnie* mice colons

This thesis determined tryptophan metabolism-associated genes and tryptophan pathway metabolites comparing the distal colons of *Winnie* mice to control mice, and their role in *de novo* NAD⁺ synthesis (**Chapter 3**) ([Figure 3.2](#)). The results showed *Ido1* upregulation, resulting in higher concentrations of kynurenine and the neurotoxic compound quinolinic acid (QUIN) ([Figure 3.4](#)). Additional observations included the overexpression of *Mao-b*, which potentially produces harmful aldehydes, elevated ROS, and mitochondrial damage (Prah et al., 2020). Likewise, *Afmid* downregulation occurred, implicating increased aldehyde-induced damage and a reduced capacity to detoxify ROS (Kim et al., 2017). QUIN production was further intensified by *kynureninase* upregulation, which theoretically correlates to dysregulated epithelial hyperplasia in humans (Harden et al., 2016). However, the observed higher concentration of picolinic acid (PIC) potentially counteracts the neurotoxic effects of QUIN by preventing harmful oxidative reactions (Grant et al., 2009). Although QUIN was higher in *Winnie* mice, nicotinamide showed non-significant differences in levels between groups. The distal colon metabolites revealed higher concentrations of tryptophan and 5-hydroxy indoleacetic acid (5-HIAA), which is associated with the onset and progression of colitis and increased gastrointestinal dysmotility in *Winnie* mice and humans (Hansen et al., 2008; Stavely et al., 2018). While

other neuroactive compounds are released by luminal stimulation, the release of 5-HIAA from enterochromaffin cells is important because it represents the earliest intercellular event in the transduction of mucosal stimuli that initiates the neuronal reactions responsible for visceral sensation and peristalsis (Robinson et al., 2017; Stavely et al., 2018). Our results therefore could facilitate the differentiation between the advantageous and deleterious effects of 5-HIAA.

In the distal colons of *Winnie* mice, the gene expressions associated with tryptophan *de novo* NAD⁺ synthesis were concomitant with genes of the glycolysis and FAO pathways. This concurrence substantiates the integration of the glycolysis and FAO models with the tryptophan synthesis model during evaluations of the tryptophan model's dynamics. These genes included glutaryl-CoA dehydrogenase (*Gcdh*), for the oxidation of L-lysine, L-hydroxylysine, and L-tryptophan (Braissant et al., 2017); acetyl-CoA acetyltransferase 1 (*Acat1*), the last step in ketolysis (Kolb et al., 2021); oxoglutarate dehydrogenase (*Ogdh*), which encodes the distribution of 2-oxoglutarate, the malate-aspartate shuttle system, and the oxidative deamination of glutamate (Nemeria et al., 2014); hydroxyacyl-CoA dehydrogenase (*Hadh*) involved in androgen metabolism (He et al., 2000); and *Aldh*, which plays a role in 5-HIAA metabolism (Badawy & Morgan, 2007).

The genes associated with nicotinamide metabolism in *Winnie* mice distal colons exhibited NAD⁺ catalytic activity, as evidenced by the upregulation of *Cd38*, *Cd73*, and *Art2a*, and the concomitant downregulation of *Nmrk1* and *Nmrk2* (Figure 3.3). Maintaining NAD⁺ levels is crucial for proper neuronal function through its anti-inflammatory and antioxidant effects (Katsyuba et al., 2018). Overall, nicotinamide gene expressions implicated oxidative stress, a diminished ability to restore homeostasis, a compromised purine salvage pathway and overall may have inhibited the production of NAD⁺.

In *Winnie* mice, the diminished capacity to restore homeostasis may culminate in significant repercussions, including metabolic reprogramming advancing inflammation, and decreasing energy metabolism. The data revealed a heightened susceptibility to infection, toxic accumulation of H₂O₂, an inability to metabolise medium- and long-chain fatty acids, mitochondrial dysfunction, an intolerance to fasting, a metabolic preference

for carbohydrates, lethargy, muscle weakness, hypoketotic hypoglycaemia, toxic production of glutaric acid and ineffective detoxification pathways (Kölker et al., 2006). The findings from *de novo* NAD⁺ synthesis have provided compelling evidence that imbalances in energy metabolism and tryptophan metabolism are linked to inflammation in the gastrointestinal tract. By understanding a person's metabolic profile, healthcare providers can assess their predisposition to IBD and implement preventive measures to reduce the risk or delay the onset of episodes.

6.1.4 *Winnie* mice gene expressions show similarities to human IBD genes

This thesis investigated the homology in the expression of target genes involved in glycolysis/gluconeogenesis (Figure 2.9), fatty acid β -oxidation (Figure 2.10) and tryptophan *de novo* NAD⁺ synthesis (Figure 3.2, Figure 3.3) in the colon of *Winnie* mice and IBD patients compared to their respective control groups (Barrett et al., 2013; Edgar et al., 2002; Peters et al., 2017). The similarities in gene expression patterns supported the utility of the *Winnie* mouse model of chronic colitis in investigating the underlying molecular mechanisms associated with IBD. In the *Winnie* mice distal colons, the results showed 81% concordance with tryptophan metabolism-associated genes when compared to UC and CD patients; 64% concordance with nicotinamide-associated genes when compared to UC; 45% concordance with nicotinamide-associated genes when compared to CD; 64% concordance with glycolysis/gluconeogenesis-associated genes when compared to UC patients; 52% concordance with glycolysis and gluconeogenesis associated genes when compared to CD patients; 77% concordance with fatty acid β -oxidation-associated genes when compared to UC patients; 69% concordance with fatty acid β -oxidation-associated genes when compared to CD patients.

6.1.5 Behavioural changes, spontaneous physical activity and sleep behaviour

It is unclear whether behavioural changes in IBD patients are psychological or whether they involve biological factors, specifically the active inflammatory state of IBD and its intersection with the pathobiology of mood and anxiety disorders (Bernstein, 2016). To explore the nature of this nexus, we employed Promethion metabolic cages and rodent

behavioural tests to measure spontaneous physical activity, sleep behaviour and behavioural changes in *Winnie* mice (**Chapter 4**).

The *Winnie* mice' spontaneous physical activity showed a reduced distance of wheel metres, reduced percentage of wheel running as well as a slower wheel running speed ([Figure 4.2](#)). This correlates to the clinical evidence that as depressive-like symptoms intensify in IBD patients, there is decreased activity and increased fatigue (Van Langenberg & Gibson, 2010). Additionally, *Winnie* mice slept significantly longer than the control mice during the dark (wake) cycle, and showed a significant quieter time without sleep, however sleep time during the light (sleep) cycle did not differ ([Figure 4.2](#)). The association between extended sleep in waking hours and affective disorders underscores the importance of prioritising sleep as a fundamental aspect of mental health (Belovicova et al., 2017). Furthermore, it is proposed that the reduced physical activity may explain the reduced energy expenditure referred to in Chapter 2.

The behavioural tests focused on evaluating the *Winnie* mouse's activity, reactions, and movement patterns to discern depression and anxiety-like tendencies. During the light-dark test, *Winnie* mice notably spent significant time in the dark box, which is indicative of anxiety-related tendencies ([Section 4.5.2, Figure 4.3](#)). A significant marker of anxiety, the risk assessment, was measured through poking actions. The frequency of poking was notably higher in *Winnie* mice ([Figure 4.3](#)). The elevated maze plus test ([Section 4.4.6, Figure 4.5](#)), highlighted the *Winnie* mice's anxiogenic tendencies by their hesitance to explore higher regions, coupled with increased risk assessment actions. Mice exhibiting symptoms of depression often manifest as decreased self-grooming tendencies and rigid grooming patterns. During the open field test ([Section 4.4.5, Figure 4.4](#)), the duration and instances of grooming were notably reduced in *Winnie* mice than controls, indicating depression. In situations simulating despair, such as the inescapable forced swimming test, mice with depressive behaviour remain immobilised longer than their respective control group, which was demonstrated by *Winnie* mice ([Chapter 4.4.7, Figure 4.6](#)).

The depressive-like behaviour and reduced physical activity observed in *Winnie* mice align with the current findings in IBD patients ([Chapter 1, Section 1.3](#)). In fact, depression

affects 25-29.3%, and anxiety affects up to 30% of IBD patients compared to a 16.2% lifetime prevalence in the general population (van den Brink et al., 2018).

6.2 Changes in cytokine activity and protein expression in *Winnie* mice brains

Anxiety and depressive-like behaviours can be linked to the limbic regions of the brain, where cytokine receptors are densely distributed on both neurons and glial cells. Cytokines influence the neurocircuitry and neurotransmitter systems, which is crucial in the connection between neurological diseases and tryptophan catabolism (Myint et al., 2013; H. Wang et al., 2020). In the literature review (**Chapter 1**), we found that the research on the brains of mouse colitis models is limited due to the primary focus on understanding the colon-related pathophysiology. Furthermore, studies have primarily employed acute colitis mouse models, which fail to accurately replicate the ulcerative colitis pathophysiology in humans (Baydi et al., 2021). Therefore, we explored cytokine activity and protein immunoreactivity, the gene and protein expression changes of glycolysis and tryptophan metabolism in the brain of the *Winnie* mouse model of chronic colitis (**Chapter 4**).

The cytokines in the brains of *Winnie* mice compared to controls uncovered anomalies in inflammatory and anti-inflammatory cytokines that distinctively correlated to behavioural changes in *Winnie* mice (**Figure 4.7**). The inflammatory cytokines indicated the likelihood of neuronal damage, increased microglial cell metabolism and proliferation, inflammatory response of the blood-brain barrier, which contributes to neuropathic hyperalgesia, mechanical and thermal sensitivity, major depressive disorder and autoimmune inflammation. To support these findings, we explored proteins in the *Winnie* mouse brain, such as high mobility group box 1 protein (HMGB1), neuronal nuclei (NeuN), transmembrane protein 119 (TMEM119), myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) (**Chapter 4**). The RNA-Seq determined non-significant differences in *Hmgb1*, *Rbfox1*, *Tmem119*, *Mbp* and *Gfap* gene expressions in the *Winnie* mouse brains of this study, although we observed notable changes in the proteins. The higher levels of HMGB1 in the *Winnie* mouse brains (**Figure 4.8**) are associated with the limbic dysfunction and emotional lability, often seen with painful mental health states in humans (Sankowski et al., 2015; Xu et al., 2020). *Winnie* mice revealed reduced NeuN

in the hippocampus, which contributes to the net neuronal loss (Figure 4.9) (Gittins & Harrison, 2004), and correlates to the inhibition of hippocampus-dependent learning (Armstrong & Barker, 2001). Over expressed TMEM119 in *Winnie* mice (Figure 4.10) is indicative of neuroinflammation in mood disorders (Böttcher et al., 2020), and is a critical indicator for changes in memory and learning (Ruan & Elyaman, 2022). The *Winnie* mice exhibited higher MBP in the prefrontal cortex (PFC) (Figure 4.11), which plays a vital role in synaptic plasticity, governs emotions, learning, and memory formation (Zhou et al., 2021). Overexpressed GFAP in the PFC of *Winnie* mice (Figure 4.12) increases during astrogliosis and inflammation in the CNS and is found in numerous neurological disorders (Orre et al., 2014) .

6.2.1 Changes in glycolysis and tryptophan metabolism in *Winnie* mice brains

This thesis provides a broader understanding of the transcriptome's complexity and dynamism of the glucose/gluconeogenesis metabolism, tryptophan *de novo* NAD⁺ synthesis in *Winnie* mice brains (Chapter 5). The glycolysis/gluconeogenesis gene expressions in *Winnie* mice brains revealed mostly downregulated gene expressions with the exception of one upregulated gene expression of hexokinase 2 (*Hk2*) (Figure 5.1, Table 5.1). The changes in the expression of glycolysis genes in the *Winnie* mouse brain are associated with haemolytic anaemia, neuromuscular dysfunction, reduced ATP production, neuronal death, aldehyde-induced damage, a decrease in oxygen, reduced mitochondrial membrane potential, and compromised detoxification pathways. The genes indicated behavioural changes associated with decreased sensitivity to positive-content emotions, neurobiological dysfunctions, impaired spatial working memory and cognitive performance.

6.2.2 Tryptophan metabolism dysregulation in *Winnie* mice brains

The effects of the tryptophan metabolism-associated gene expressions in the *Winnie* mouse brain represent the complex interplay between immune response, metabolism, and behaviour (Figure 5.3).

This study found opposing differences in tryptophan metabolites between the brains and distal colons of *Winnie* mice. In the brains, concentrations of tryptophan, 5-HIAA, kynurenine, kynurenic acid (KYNA), PIC, and nicotinamide were significantly lower than in control mice (Figure 5.11). Whereas the *Winnie* mouse distal colon exhibited higher concentrations of tryptophan, 5-HIAA, kynurenine, QUIN and PIC. Research indicates that inflammation can activate the kynurenine pathway in peripheral tissues, reducing the flow of tryptophan to the brain. This likely explains the higher concentration of metabolites in the colon and the lower concentration of metabolites in the brain of *Winnie* mice. Such changes have been linked to mood disorders and disruptions in the body's stress response system (Höglund et al., 2019).

Furthermore, *Winnie* mice exhibited compromised brain pathways related to serotonin, a neurotransmitter associated with depression and suicidal thoughts in IBD patients. The *Winnie* mice genes showed increased susceptibility to stress, impulsivity, and decreased cognitive performance (Markus & Jonkman, 2007; Sachs et al., 2015). Additionally, the genes showed an inclination to impede the breakdown of androgens, leading to reduced 5-HIAA. This may influence cognitive performance and stress reactions (He et al., 2000). The reduced metabolite levels suggest a weaker defense against damage from oxidation, neurotoxins, or inflammation brought about by these gene changes (Figure 5.3).

The *Winnie* mouse brain gene expressions showed *Idol* downregulation, which contradicted previous studies, that have associated *Idol* upregulation with depression (Myint & Kim, 2014). However, it was also observed that downregulated *Idol* is also associated with depression, high-risk avoidance behaviour and inhibited exploratory drive (Too et al., 2016). *Winnie* mice brains revealed a compromised serotonergic pathway, with significantly reduced 5-HIAA, which is associated with depression and suicidal ideation in humans (Boldrini, 2005). The results showed an impeded breakdown of androgens, feasibly leading to reduced 5-HIAA, and possible alterations in cognitive performance and stress-related behaviours (He et al., 2000). KYNA was significantly lower in *Winnie* mice brains compared to controls. On the positive side, reduced levels of KYNA in mouse brains enhance their cognitive abilities, and are associated with increased levels of extracellular glutamate (Potter et al., 2010). Although nicotinamide showed a significant reduction in *Winnie* mice brains, the activity of NAD⁺-consuming

enzymes remains largely unaltered, with the exception of downregulated *Cd38* expression. In opposition, *Ido1* and NAD⁺-consuming enzymes were upregulated in the *Winnie* mice colons.

Overall, the gene expressions are associated with a heightened susceptibility to infection, toxic accumulation of H₂O₂, neurotoxicity, immunotoxicity, mitochondrial dysfunction, reduced ATP metabolism, neurodegeneration, and hypoglycaemia. Further key findings associated with severe neuromuscular dysfunction, reduced lifespan, diminished body weight, hindered memory formation, prolonged neuropathic hyperalgesia, aggressive behaviour and indications of stress-related disorders, compromised exploratory behaviour and learning abilities, effects on cognition, impaired memory acquisition, impaired spatial working memory, abrupt mood shifts, impulsivity symptoms of depression, anxiety, and a sense of despair.

6.3 Significance of the thesis

In *Winnie* mice, both the colon and brain exhibited changes in the expressions of genes associated with tryptophan metabolism, impacting the normal functioning of the gut and subsequently leading to gastrointestinal, neuropathological, and potential affective disorders. There have been many unanswered questions concerning affective disorders and fatigue symptoms in IBD patients and whether the indicators are secondary psychological responses or pathophysiological. However, our data implied that disruptions in energy and tryptophan metabolism play a more pronounced role in influencing the serotonin pathway and its by-products than previously believed. Hereby, instead of viewing behavioural changes as simple emotional expressions, this study suggests that gene expressions might be central contributors to these shifts, not just responses to the ailment. We, therefore, emphasise to address fatigue and mental health concerns in IBD patients, underscoring their role in ensuring a satisfactory QoL. If not addressed, IBD could be an overwhelming challenge for the afflicted. Such a fresh outlook aims to chart a course towards creating specific treatments, potentially reducing the disease's impact.

6.4 Future recommendations

Our results identified numerous factors affecting a pattern of damaging consequences. The observed changes in gene expressions emphasised the importance of considering metabolic disorders in the differential diagnosis of IBD patients presenting with relatively non-specific symptoms.

Our study utilised four behavioural tests, however, there is a scope to research deeper into our observations, related to depression and anxiety, by refining the choice of behavioural tests for specificity. Alternative rodent behavioural tests include fear conditioning, marble burying (to gauge anxiety and perseverative behaviour); the Morris water maze spatial (learning and memory); passive avoidance and novel object recognition tests (learning and memory); pre-pulse inhibition (sensory and perceptual abilities); and thermal sensitivity (latency to the initial adverse reaction).

From our results, we strongly recommend personalised dietary strategies for IBD patients, highlighting gene-nutrient interactions. Furthermore, by understanding a person's gene and metabolic profile, healthcare providers can assess their predisposition to IBD and IBD-associated co-morbidities, and implement preventive measures to reduce the risk or delay the onset of episodes. This concept is particularly important as it relates to specific therapeutic strategies, which ultimately need to be individualised, rather than the current generic approach. Besides, there is a reciprocal relationship between psychological comorbidity and IBD, with each potentially influencing the course of the other. A shift to a personalised approach targeting specific mechanisms in individual patients will be key to reducing the increasing disease burden of IBD.

Identifying IBD-related etiological pathology in clinical gastroenterology remains ambiguous due to psychosomatic ambiguity and the absence of diagnostic biomarkers. These indicators include assessing disease activity, predicting prognosis or risk levels, and determining the effectiveness of treatment. Therefore, evaluating if the link between tryptophan metabolism changes in the colon and brain can be used as a specific diagnostic marker for IBD. Our results provided great potential for the development of new

biomarkers that can detect pathological genomic variances, proteomic changes, and metabolomic profiles.

We suggest that it is important to focus on specific emotional and cognitive dysfunctions in translational psychiatric research aimed at improved understanding of the aetiopathophysiology and the development of novel pharmacotherapies for specific psycho-pathologies. Incorporating psychological support, such as cognitive-behavioural therapy, mindfulness techniques, or stress reduction strategies, as part of comprehensive care for individuals with IBD may improve mental well-being and potentially impact disease outcomes.

This thesis touches upon a fundamental in biomedical research: the complex cause-and-effect relationship between metabolic changes and inflammatory conditions. The problem is rendered more complex when deciphering the pathophysiological processes in animal models of disease. There are several ways by which future studies could avoid some of the resultant limitations of the current study. For instance, interventional studies, where one parameter (e.g., metabolic function) is deliberately altered to observe the resultant effect on the another (e.g., inflammation), could provide insights into causality. Furthermore, experimental designs incorporating pair-fed controls could help elucidate whether reduced food and water intake due to inflammation-associated pain contributes to the observed metabolic changes. Conversely, the use of agents known to induce metabolic dysfunction could help determine if these changes can instigate inflammatory responses in the model used. It is also pertinent to consider the potential bidirectional nature of such relationships. Metabolic changes and inflammation often influence each other, creating a cycle that perpetuates both conditions. Investigating the possibility of a feedback loop being present, rather than a linear cause-and-effect relationship may, may enrich the analysis.

6.5 Conclusion

The novelty and significance of this study lie in its comprehensive examination of gene expressions related to energy and tryptophan metabolic pathways, utilising the *Winnie*

mouse model of chronic colitis. The objective of this study was to discern whether these molecular shifts had consequent behavioural ramifications. Our findings elucidate several manifestations attributable to underlying colitis, including tendencies for food and water aversion, attenuated energy metabolic activity, alterations in body composition, and their plausible correlations with pathophysiological processes observed in IBD patients. Subsequent investigations delved into the behavioural manifestations resulting from these metabolic perturbations in *Winnie* mice, evidencing decreased physical activity, proclivity towards enhanced sleep duration, and manifestations suggestive of depressive and anxiety disorders. The data thus garnered lends credence to the posited hypothesis: the synergistic interplay of energy and tryptophan metabolism in the *Winnie* mouse model's gut and brain precipitates these behavioural presentations. The implications of this research are manifold; it not only demystifies the pathophysiology underlying IBD and its concomitant behavioural anomalies but also furnishes a robust foundation for the conceptualisation of innovative therapeutic paradigms targeting affective disorders prevalent in IBD patients. The insights procured from this rigorous investigation are poised to significantly refine early IBD management protocols, ushering in bespoke interventions with the potential to decelerate disease evolution and enhance the life quality of IBD patients. Our research offers a deeper understanding of IBD's pathophysiological intricacies, signposting promising therapeutic trajectories.

Chapter 7: References

7.1 References

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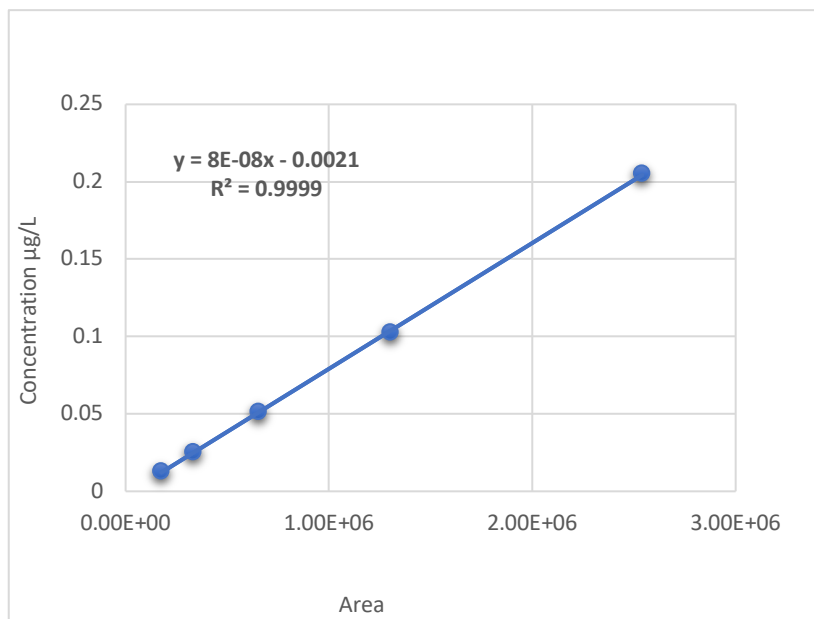
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Appendix: Supplementary Data

Table S 1 Calibration curve of L-tryptophan



Standard Concentration ug/L	Peak Area	Found Concentration ug/L	Recovery %
0.25	1.76E+05	5.86E+09	2.34E+12
0.5012	3.32E+05	1.11E+10	2.21E+12
1.025	6.54E+05	2.18E+10	2.13E+12
2.05	1.30E+06	4.34E+10	2.12E+12
4.1	2.54E+06	8.47E+10	2.07E+12
Mean (n=5)			2.17E+12
SD			1.09E+11
SE of Intercept			7.16E+03
SD of Intercept			1.60E+04
LOD			1.76E+09
LOQ			5.34E+09

Table S 2 Method validation of L-tryptophan

Summary Output

Regression Statistics

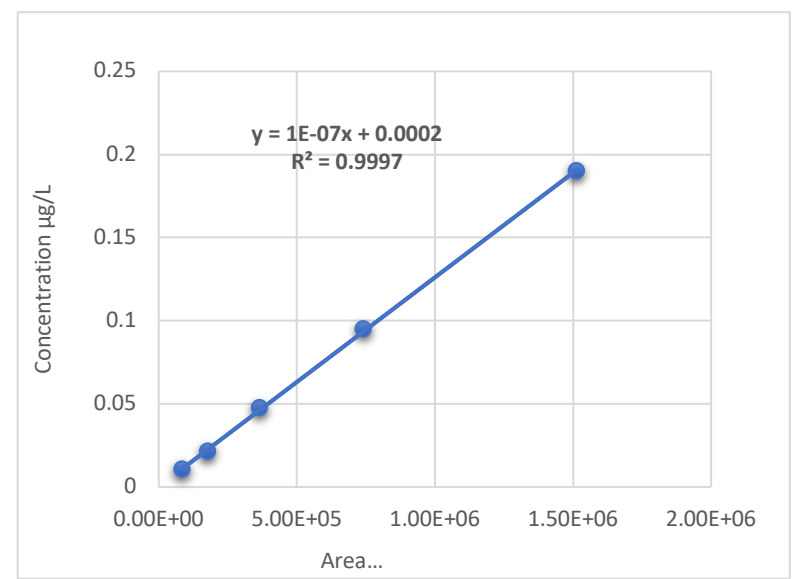
Multiple R	0.99995453
R Square	0.99990907
Adjusted R Square	0.99987875
Standard Error	10607.3841
Observations	5

ANOVA

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	3.7117E+12	3.7117E+12	32987.7923	3.6804E-07
Residual	3	337549791	112516597		
Total	4	3.712E+12			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	26423.7507	7163.84276	3.68848837	0.03455279	3625.20576	49222.2956	3625.20576	49222.2956
X Variable 1	615047.172	3386.34967	181.625417	3.6804E-07	604270.296	625824.048	604270.296	625824.048

Table S 3 Calibration curve of 5 hydroxy indole acetic acid (5-HIAA)



Standard Concentration ug/L	Peak Area	Found Concentration ug/L	Recovery %
0.011	8.68E+04	8.68E+11	8.15E+15
0.021	1.78E+05	1.78E+12	8.40E+15
0.048	3.65E+05	3.65E+12	7.69E+15
0.095	7.41E+05	7.41E+12	7.80E+15
0.190	1.51E+06	1.51E+13	7.97E+15
Mean (n=5)			8.00E+15
SD			2.82E+14
SE of Intercept			8.15E+03
SD of Intercept			1.82E+04
LOD			6.02E+11
LOQ			6.08E+09

Table S 4 Method validation of 5 hydroxy indole acetic acid (5-HIAA)

Summary Output

Regression Statistics

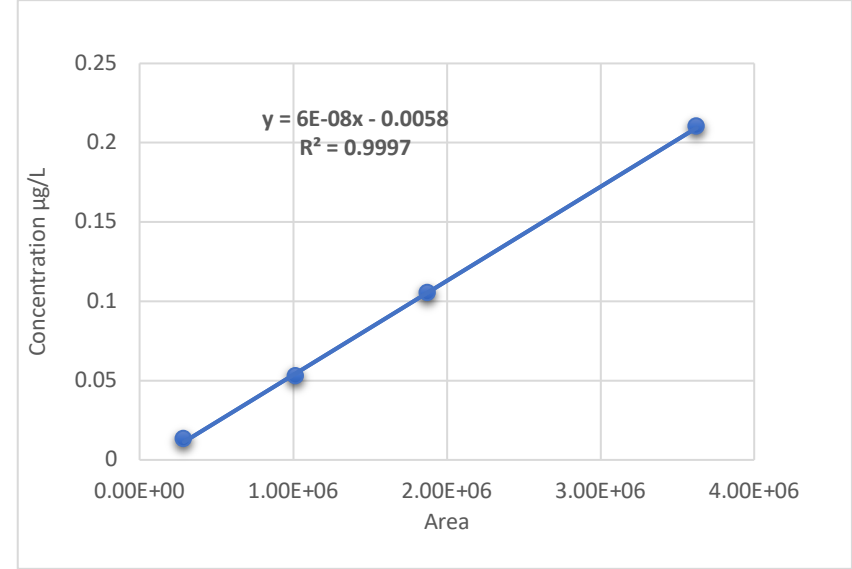
Multiple R	0.9998353
R Square	0.99967064
Adjusted R Square	0.99956085
Standard Error	12176.4434
Observations	5

ANOVA

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	1.35E+12	1.35E+12	9105.47133	2.5371E-06
Residual	3	444797319	148265773		
Total	4	1.3505E+12			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	-1716.4451	8153.0456	-0.2105281	0.84674401	-27663.075	24230.1848	-27663.075	24230.1848
X Variable 1	7944724.37	83258.3153	95.4225934	2.5371E-06	7679759.25	8209689.48	7679759.25	8209689.48

Table S 5 Calibration curve of L-kynurenine



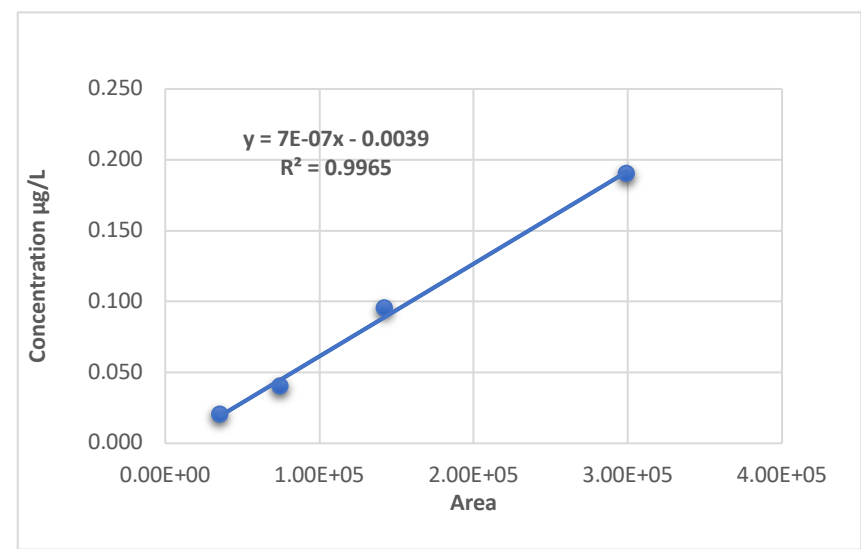
b	y	$x=(y-b)/a$	$x/b*100$
Standard Concentration ug/L	Peak Area	Found Concentration ug/L	Recovery %
0.0131	2.89E+05	4.82E+12	3.68E+16
0.0525	1.02E+06	1.69E+13	3.22E+16
0.105	1.87E+06	3.12E+13	2.98E+16
0.21	3.62E+06	6.04E+13	2.88E+16
Mean (n=4)			3.19E+16
SD			3.57E+15
SE of Intercept			2.67E+04
SD of Intercept			5.33E+04
LOD			2.93E+12
LOQ			8.89E+12

Table S 6 Method validation of L-kynurenine

Summary Output

Regression Statistics								
Multiple R	0.999827343							
R Square	0.999654715							
Adjusted R Square	0.999482073							
Standard Error	32705.36273							
Observations	4							
ANOVA								
	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>			
Regression	1	6.19356E+12	6.1936E+12	5790.31871	0.00017266			
Residual	2	2139281503	1069640751					
Total	3	6.1957E+12						
	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	98572.13438	26661.63002	3.69715334	0.06599841	-16143.601	213287.87	-16143.601	213287.87
X Variable 1	16840544.04	221311.9593	76.0941438	0.00017266	15888315.5	17792772.5	15888315.5	17792772.5

Table S 7 Calibration curve of kynurenic acid



Standard Concentration ug/L	Peak Area	Found Concentration ug/L	Recovery %
0.020	3.58E+04	5.12E+10	2.56E+14
0.040	7.46E+04	1.07E+11	2.66E+14
0.080	1.42E+05	2.03E+11	2.54E+14
0.160	2.99E+05	4.28E+11	2.67E+14
Mean (n=4)			2.61E+14
SD			6.88E+12
SE of Intercept			3.59E+03
SD of Intercept			7.19E+03
LOD			3.39E+10
LOQ			1.03E+11

Table S 8 Method validation of kynurenic acid

Summary Output

Regression Statistics

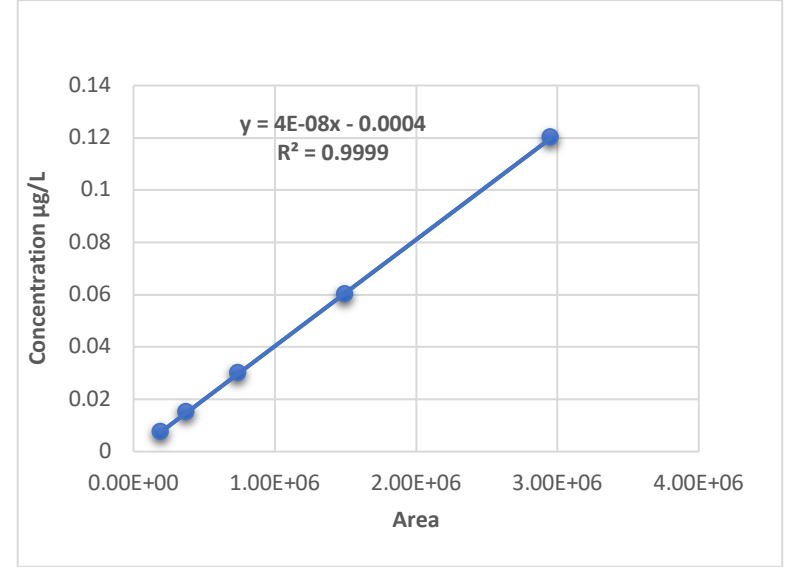
Multiple R	0.99956899
R Square	0.99913816
Adjusted R Square	0.99870724
Standard Error	4180.93173
Observations	4

ANOVA

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	4.053E+10	4.053E+10	2318.62419	0.00043101
Residual	2	34960380.3	17480190.2		
Total	3	4.0565E+10			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	-2731.1717	3594.4597	-0.7598282	0.52670749	-18196.884	12734.5401	-18196.884	12734.5401
X Variable 1	1877324.46	38987.3894	48.1520944	0.00043101	1709575.26	2045073.65	1709575.26	2045073.65

Table S 9 Calibration curve of quinolinic acid



Standard Concentration ug/L	Peak Area	Found Concentration ug/L	Recovery %
0.0075	1.94E+05	4.84E+12	6.46E+16
0.015	3.73E+05	9.33E+12	6.22E+16
0.03	7.42E+05	1.85E+13	6.18E+16
0.06	1.50E+06	3.75E+13	6.24E+16
0.12	2.95E+06	7.38E+13	6.15E+16
Mean (n=5)			6.25E+16
SD			1.22E+15
SE of Intercept			7.00E+03
SD of Intercept			1.56E+04
LOD			8.61E+11
LOQ			3.91E+12

Table S 10 Method validation of quinolinic acid**Summary Output***Regression Statistics*

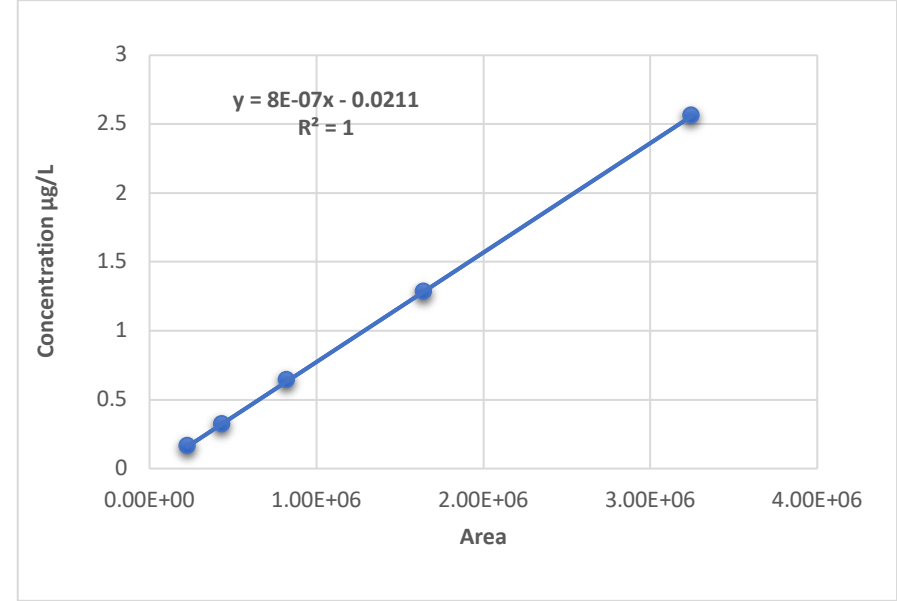
Multiple R	0.999968248
R Square	0.999936497
Adjusted R Square	0.999915329
Standard Error	10336.29301
Observations	5

ANOVA

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	5.0469E+12	5.0469E+12	47238.7468	2.1478E-07
Residual	3	320516859	106838953		
Total	4	5.0473E+12			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	9564.40625	6997.70345	1.36679217	0.26510692	-12705.409	31834.2217	-12705.409	31834.2217
X Variable 1	24555640.51	112980.133	217.344765	2.1478E-07	24196087.3	24915193.7	24196087.3	24915193.7

Table S 11 Calibration curve of picolinic acid



Standard Concentration ug/L	Peak Area	Found Concentration ug/L	Recovery %
0.16	2.30E+05	2.87E+11	1.79E+14
0.32	4.33E+05	5.42E+11	1.69E+14
0.64	8.21E+05	1.03E+12	1.60E+14
1.28	1.64E+06	2.06E+12	1.61E+14
2.56	3.25E+06	4.06E+12	1.59E+14
Mean (n=5)			1.66E+14
SD			8.74E+12
SE of Intercept			5.41E+03
SD of Intercept			1.21E+04
LOD			4.99E+10
LOQ			1.51E+11

Table S 12 Method validation of picolinic acid

Summary Output

Regression Statistics

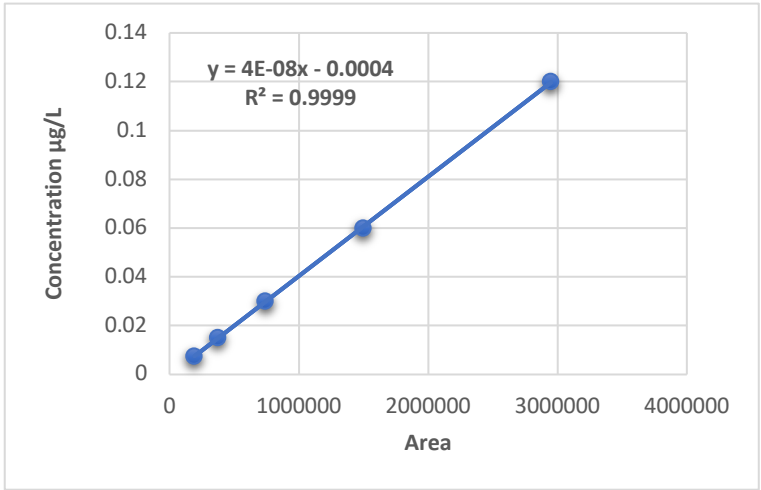
Multiple R	0.999984101
R Square	0.999968202
Adjusted R Square	0.999957603
Standard Error	7998.32399
Observations	5

ANOVA

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	6.0354E+12	6.0354E+12	94343.3045	7.6101E-08
Residual	3	191919560	63973186.6		
Total	4	6.0356E+12			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	26537.52778	5414.89094	4.90084252	0.01625971	9304.92812	43770.1274	9304.92812	43770.1274
X Variable 1	1258731.457	4098.05275	307.153552	7.6101E-08	1245689.62	1271773.29	1245689.62	1271773.29

Table S 13 Calibration curve of nicotinamide



Standard Concentration ug/L	Peak Area	Found Concentration ug/L	Recovery %
0.0075	193682	4.84E+12	6.46E+16
0.015	373157	9.33E+12	6.22E+16
0.03	741500	1.85E+13	6.18E+16
0.06	1498395	3.75E+13	6.24E+16
0.12	2950274	7.38E+13	6.15E+16
Mean (n=5)			6.25E+16
SD			1.22E+15
SE of Intercept			2.86E-04
SD of Intercept			6.40E-04
LOD			5.28E+04
LOQ			1.60E+02

Table S 14 Method validation of nicotinamide

Summary Output

Regression Statistics

Multiple R	0.99996825
R Square	0.9999365
Adjusted R Square	0.99991533
Standard Error	0.00042092
Observations	5

ANOVA

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	0.00836947	0.00836947	47238.7468	2.1478E-07
Residual	3	5.3152E-07	1.7717E-07		
Total	4	0.00837			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	-0.0003865	0.00028631	-1.3500255	0.26983197	-0.0012977	0.00052463	-0.0012977	0.00052463
X Variable 1	4.0721E-08	1.8736E-10	217.344765	2.1478E-07	4.0125E-08	4.1318E-08	4.0125E-08	4.1318E-08

Table S 15 Gene set enrichment analysis associated with tryptophan metabolism

Gene Set	Description	Enrichment score	Normalised enrichment score	p value	FDR	Size	Leading edge #	Genes
mmu00380	Tryptophan metabolism	0.594	0.594	0	0.422	21	3	<i>Aoc1, Ftc1, Hal</i>
mmu05320	Autoimmune thyroid disease	0.586	1.591	0.017	0.384	37	14	<i>Cd80, Cga, Ctla4, Fas, Fas1, Gzmb, H2-Ab1, H2-B1, H2-DMb2, H2-Eb1, H2-Oa, H2-Q2, H2-Q6, Il5</i>
mmu04610	Complement and coagulation cascades	0.530	1.696	0.019	0.298	60	23	<i>Bdkrb1, Bdkrb2, C1s2, C3, C4b, C7, Cd46, Cf1, Cf1, Cr2, F13a1, F2, F2rl2, F5, F9, Hc, Itgax, Plg, Serpinb2, Serpine1, Serpin1, Vwf, Zp3r</i>
mmu05321	Inflammatory bowel disease	0.488	1.516	0.0351	0.481	51	15	<i>H2-Ab1, H2-DMb2, H2-Eb1, H2-Oa, Il12rb1, Il18rap, Il21, Il23a, Il23r, Il2rg, Il5, Nod2, Tlr2, Tlr5, Tnf</i>
mmu04514	Cell adhesion molecules (CAMs)	0.433	1.442	0	0.698	127	23	<i>Cd2, Cd80, Cldn14, Cldn20, Cldn9, Ctla4, H2-Ab1, H2-B1, H2-DMb2, H2-Eb1, H2-Oa, H2-Q2, H2-Q6, Icos, Itga4, Mpz, Ncam2, Sdc4, Sele, Selp, Siglec1, Tigit, Vcan</i>

Figure S 1 Food and water consumption, male mice group and female mice group

14-week-old *Winnie* male mice were compared to age-matched C57BL/6 male littermates for ingestive behaviour. In addition, 14-week-old *Winnie* female mice were compared to age-matched C57BL/6 female littermates for ingestive behaviour. The data points of sleep behaviour were recorded within light and dark cycles from 0700 to 1900 hour and 1900 to 0700 hour. For ingestive behaviour, *Winnie* male mice consumed significantly less food in grams (g) (2.6 ± 0.4 g, $p=0.007$) than C57BL/6 male mice (3.2 ± 0.4 g), which correlates to the *Winnie* male mice having significantly fewer feeding bouts (13.8 ± 3 g, $p<0.001$) compared to the C57BL/6 male mice (29 ± 1.7 g). Water intake was significantly reduced in *Winnie* male mice (1.96 ± 0.3 g, $p<0.001$) compared to C57BL/6 male mice (3.3 ± 0.3 g). Accordingly, the *Winnie* mice (31.3 ± 4 g, $p=0.013$) attempted significantly fewer drinking bouts (53 ± 9 g) than the C57BL/6 mice. The food consumption of *Winnie* female mice (2.5 ± 0.4 g, $p=0.01$) was significantly lower than that of C57BL/6 female mice (3.4 ± 0.3 g), however, the differences in feeding bouts were not different over 24 hours (*Winnie* female mice, 19.5 ± 1.6 ; controls, 24 ± 2). The *Winnie* female mice consumed less water (1.9 ± 0.4 g, $p=0.03$) than C57BL/6 female mice (2.8 ± 0.1 g); the *Winnie* female mice attempted fewer drinking bouts (31.2 ± 1 , $p=0.006$) than C57BL/6 mice (53 ± 14) over 24 hours.

(A) Sum of food, males. (B) Sum of food, females. (C) Number of feeding bouts, males. (D) Number of feeding bouts, females. (E) Sum of water, males. (F) Sum of water, females. (G) Number of drinking bouts, males. (H) Number of drinking, females. For analyses, $*p \leq 0.05$ was considered significant, $**p=0.01$, $***p=0.001$. All data were presented as the mean $\pm$ SEM, $n=8$ mice/group.

Figure S 1 Food and water consumption, male mice group and female mice group

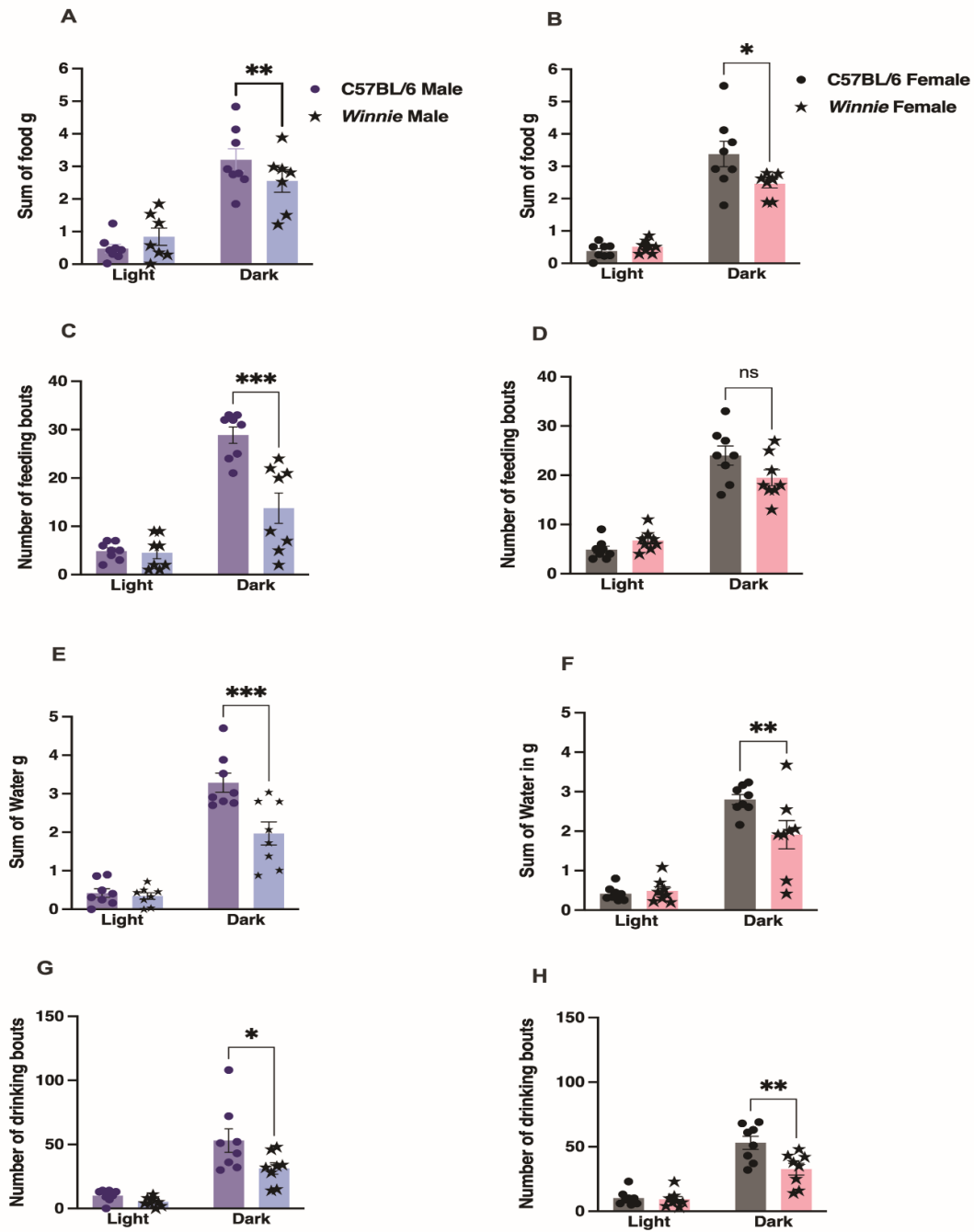


Figure S 2 Energy expenditure, male mice group and female mice group

14-week-old *Winnie* male mice were compared to age-matched controls C57BL/6 male littermates for energy expenditure. Thereafter, 14-week-old *Winnie* female mice were compared to age-matched controls, C57BL/6 female littermates for energy expenditure. The data points of sleep behaviour were recorded within light and dark cycles from 0700 to 1900 hour and 1900 to 0700 hour. VO_2 consumption (58 ± 4 mL/kg/min, $p < 0.001$) and VCO_2 emission (56 ± 4 mL/kg/min, $p < 0.001$) were significantly lower in *Winnie* male mice to controls, VO_2 consumption (80.8 ± 2.6 mL/kg/min) and VCO_2 emission (77.2 ± 2.5 mL/kg/min). *Winnie* female mice showed significantly less VO_2 consumption (65.4 ± 3 mL/kg/min, $p < 0.001$) than female control mice (91 ± 3 mL/kg/min). VCO_2 emission was significantly lower in *Winnie* female mice (62.5 ± 3 mL/kg/min, $p < 0.001$) than female control (87 ± 3 mL/kg/min). RQ levels did not differ between groups. EE was greatly significantly lower in *Winnie* male mice (209 ± 15 kcal/kg BW, $p < 0.001$) than in male control mice (294 ± 12 kcal/kg BW) as well as *Winnie* female mice (233.5 ± 10 kcal/kg BW, $p < 0.001$) being greatly significantly lower than female control mice (325 ± 1 kcal/kg BW).

(A) VO_2 mL/kg/min BW, males. (B) VO_2 mL/kg/min BW, females. (C) VCO_2 mL/kg/min BW, males. (D) VCO_2 mL/kg/min BW, females. (E) Respiratory quotient, males. (F) Respiratory quotient, females. (G) Energy expenditure kcal/kg BW, males. (H) Energy expenditure kcal/kg BW, females. For analyses, $p \leq 0.05$ was considered significant, *** $p = 0.001$. All data were presented as the mean $\pm$ SEM, $n = 8$ mice/group.

Figure S 2 Energy expenditure, male mice group and female mice group

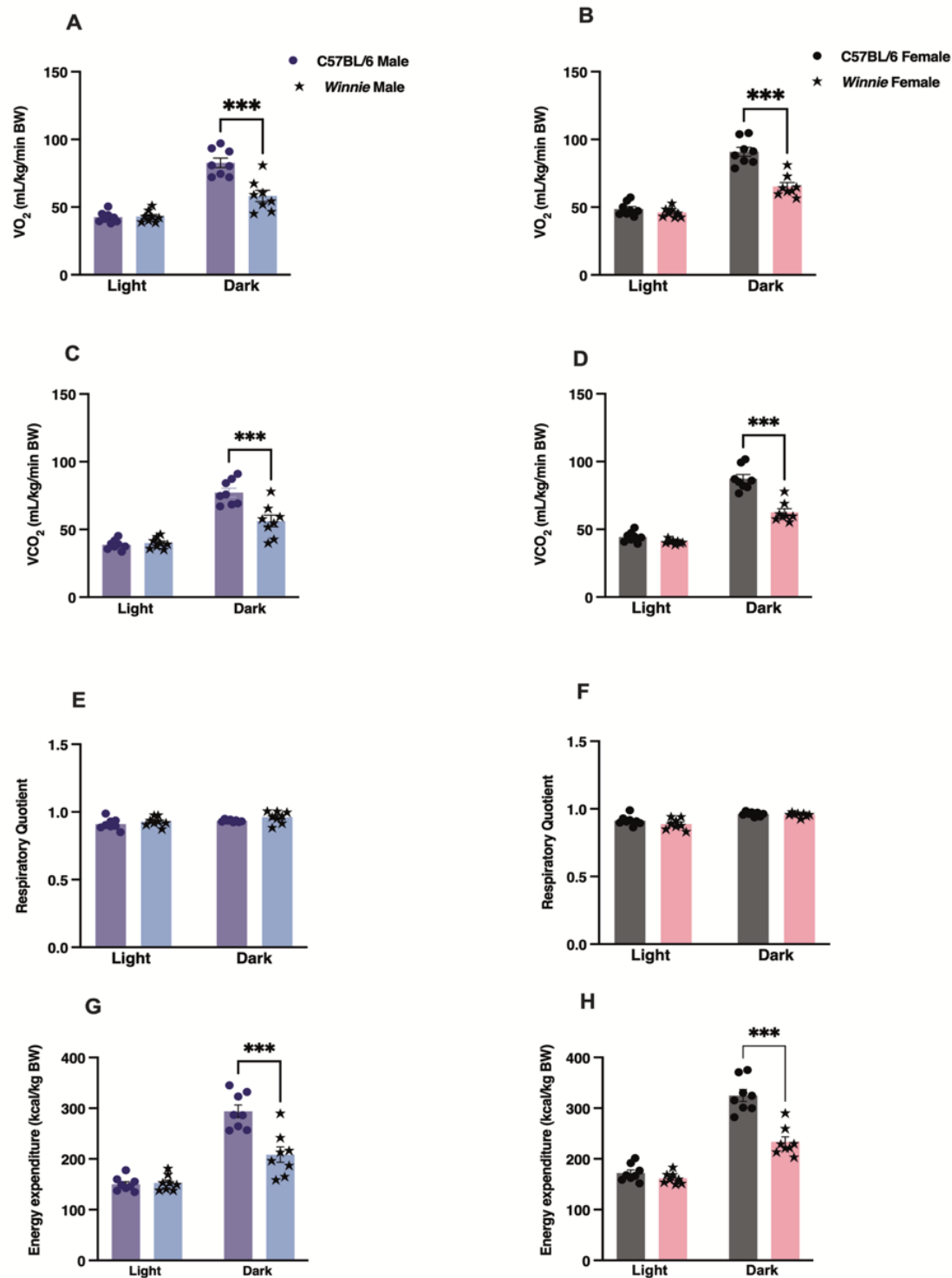


Figure S 3 Glucose oxidation and lipid oxidation of the male mice group and female mice group

14-week-old *Winnie* male mice were compared to age-matched controls, C57BL/6 male littermates for glucose and lipid oxidation. Thereafter, 14-week-old *Winnie* female mice were compared to age-matched controls, C57BL/6 female littermates for glucose and lipid oxidation. The data points of sleep behaviour were recorded within light and dark cycles from 0700 to 1900 hour and 1900 to 0700 hour. **(A)** Glucose oxidation g/kg/BW, males. *Winnie* male mice (0.05 ± 0.005 g/kg/BW, $p=0.017$). **(B)** Glucose oxidation, g/kg/BW, *Winnie* female mice (0.05 ± 0.002 g/kg/BW, $p<0.001$) displayed significantly lower levels of glucose oxidation than controls (male controls 0.06 ± 0.003 g/kg/BW, female controls 0.08 ± 0.003 g/kg/BW). **(C)** Lipid oxidation g/kg/BW, males. Lipid oxidation was significantly lower in *Winnie* male mice (0.03 ± 0.001 g/kg/BW, $p<0.001$) and **(D)** Lipid oxidation g/kg/BW, females. *Winnie* female mice (0.03 ± 0.002 g/kg/BW, $p<0.001$) compared to the male control mice (0.04 ± 0.001 g/kg/BW) and female control mice (0.05 ± 0.002 g/kg/BW). For analyses, $p \leq 0.05$ was considered significant, *** $p=0.001$. All data were presented as the mean $\pm$ SEM, n=8 mice/group.

Figure S 3 Glucose and lipid oxidation of male mice versus female mice groups

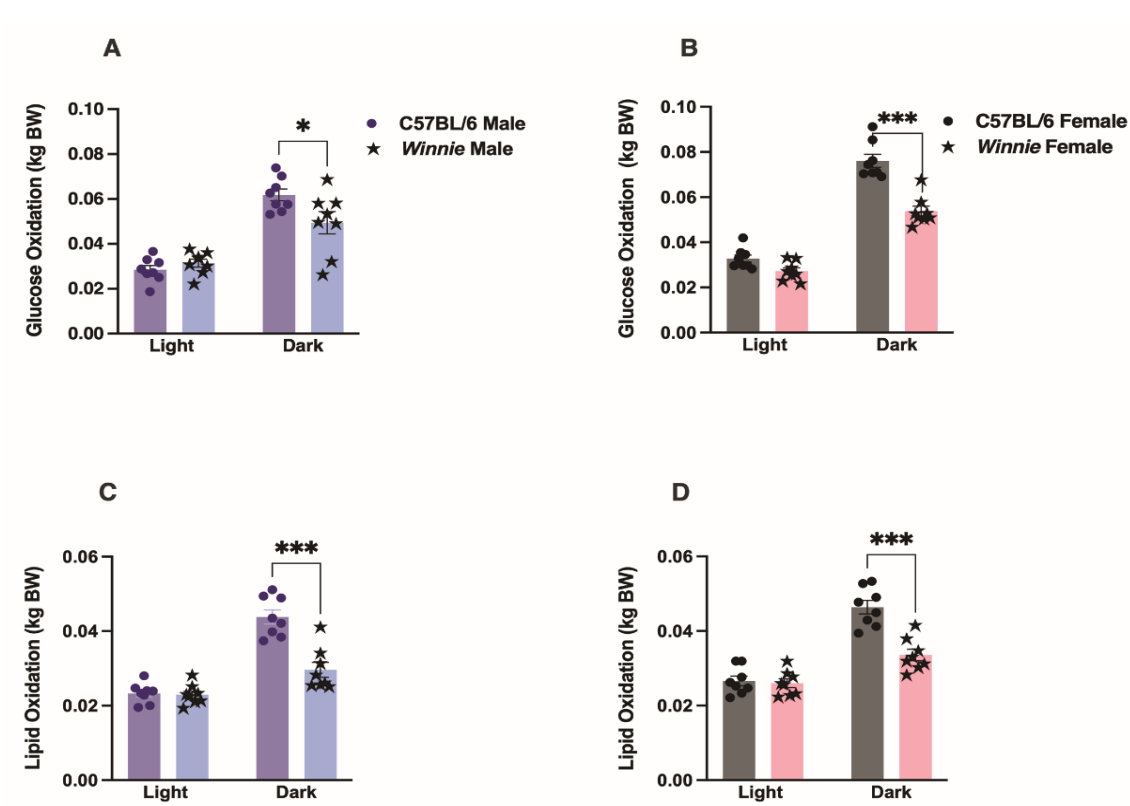


Figure S 4 Tryptophan metabolite chromatograms

Peak graphs demonstrate the compound concentrations of tryptophan metabolite standards correlating to retention times detected. Retention times are recorded in minutes for each metabolite: tryptophan 10.68 minutes, 5-HIAA 7.68 minutes, kynurenic acid 6.21 minutes, kynurenine 5.47 minutes, quinolinic 3.17 minutes, nicotinamide 3.00 minutes and picolinic acid 2.94 minutes. **Abbreviations:** mAU, milli-absorbance unit; min, minutes

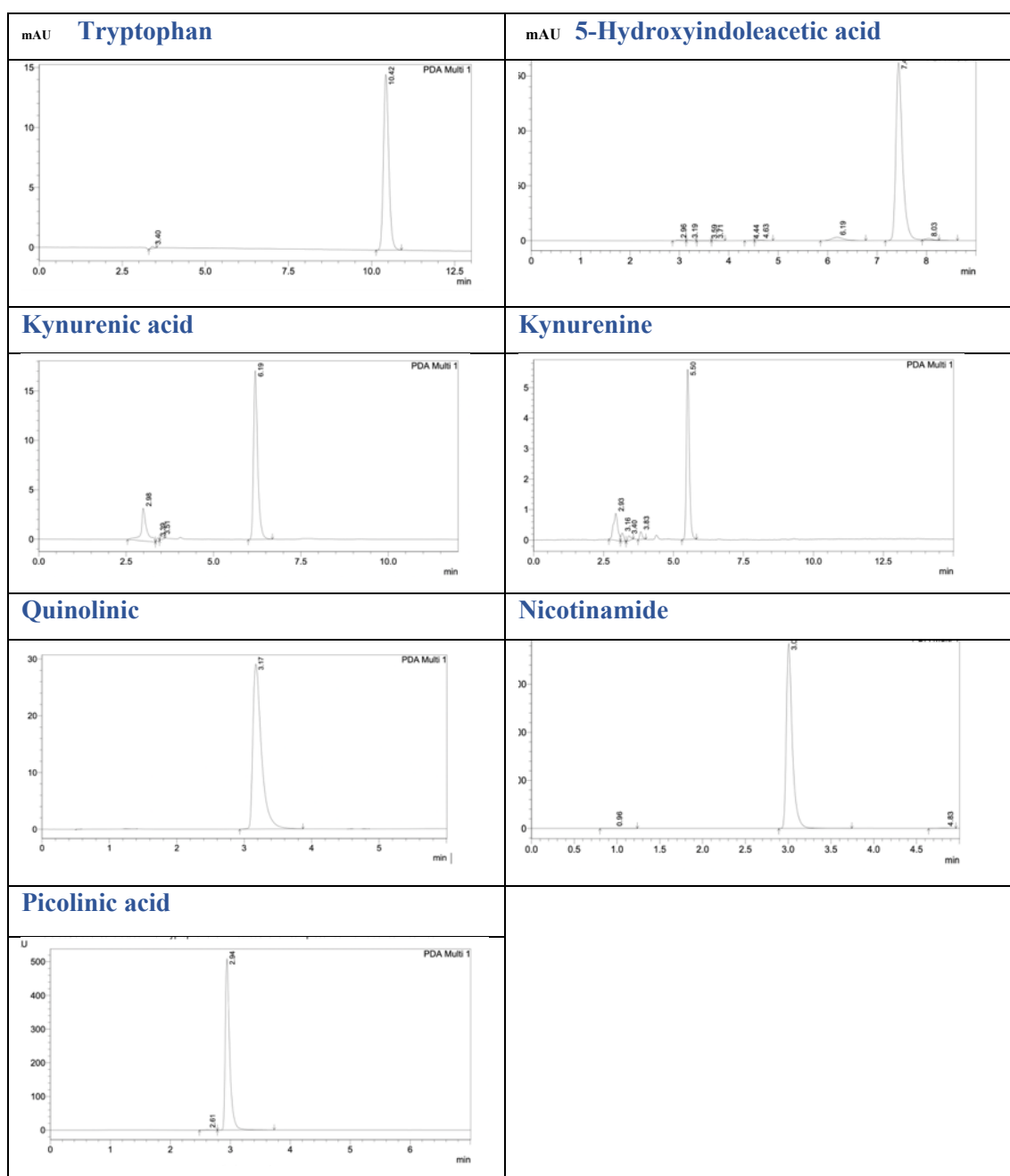


Figure S 5 *Winnie* mice sleep longer hours

14-week-old *Winnie* male mice were compared to age-matched controls, C57BL/6 male littermates for sleep behaviour. Likewise, 14-week-old *Winnie* female mice were compared to age-matched controls, C57BL/6 female littermates for sleep behaviour. The data points of sleep behaviour were recorded within light and dark cycles from 0700 to 1900 hour and 1900 to 0700 hour. **(A)** Sleep hours, male control mice (3.4±0.7 hours) versus *Winnie* male mice (6.3±0.7 hours, ** $p=0.002$). **(B)** Sleep hours, female control mice (3.6±0.4 hours) versus *Winnie* female mice (5.8±0.7 hours, ** $p=0.002$). **(C)** Sleep percentage over a 24 hour per, male control mice (27.9±5.8 hours) versus *Winnie* male mice (52.5±5.5 hours, ** $p=0.002$). **(D)** Sleep percentage over a 24 hour period, female control mice (29.7 ±3.4%) versus *Winnie* female mice (48.4±5.8%, ** $p=0.002$). **(E)** Still percentage over a 24 hour period, C57BL/6 male mice (33.9±6.5%) versus *Winnie* male mice (55.6±5.5%, ** $p=0.003$). **(F)** Still percentage over a 24 hour period, female control mice (36.9±3.9%) versus *Winnie* female mice (50.9±6.1%, * $p=0.029$). **(G)** Quiet time without sleep percentage, male control mice (5.9±0.9%) versus *Winnie* male mice (1.9±0.8%, *** $p<0.001$). **(H)** Quiet time without sleep percentage, female control mice (7.2±0.8%) versus *Winnie* female mice (2.6±0.4%, *** $p<0.001$). For analyses, $p\leq 0.05$ was considered significant, ** $p\leq 0.003$, *** $p<0.001$ All data were presented as the mean ± SEM, n=8 mice/group.

Figure S 5 *Winnie* mice sleep longer hours

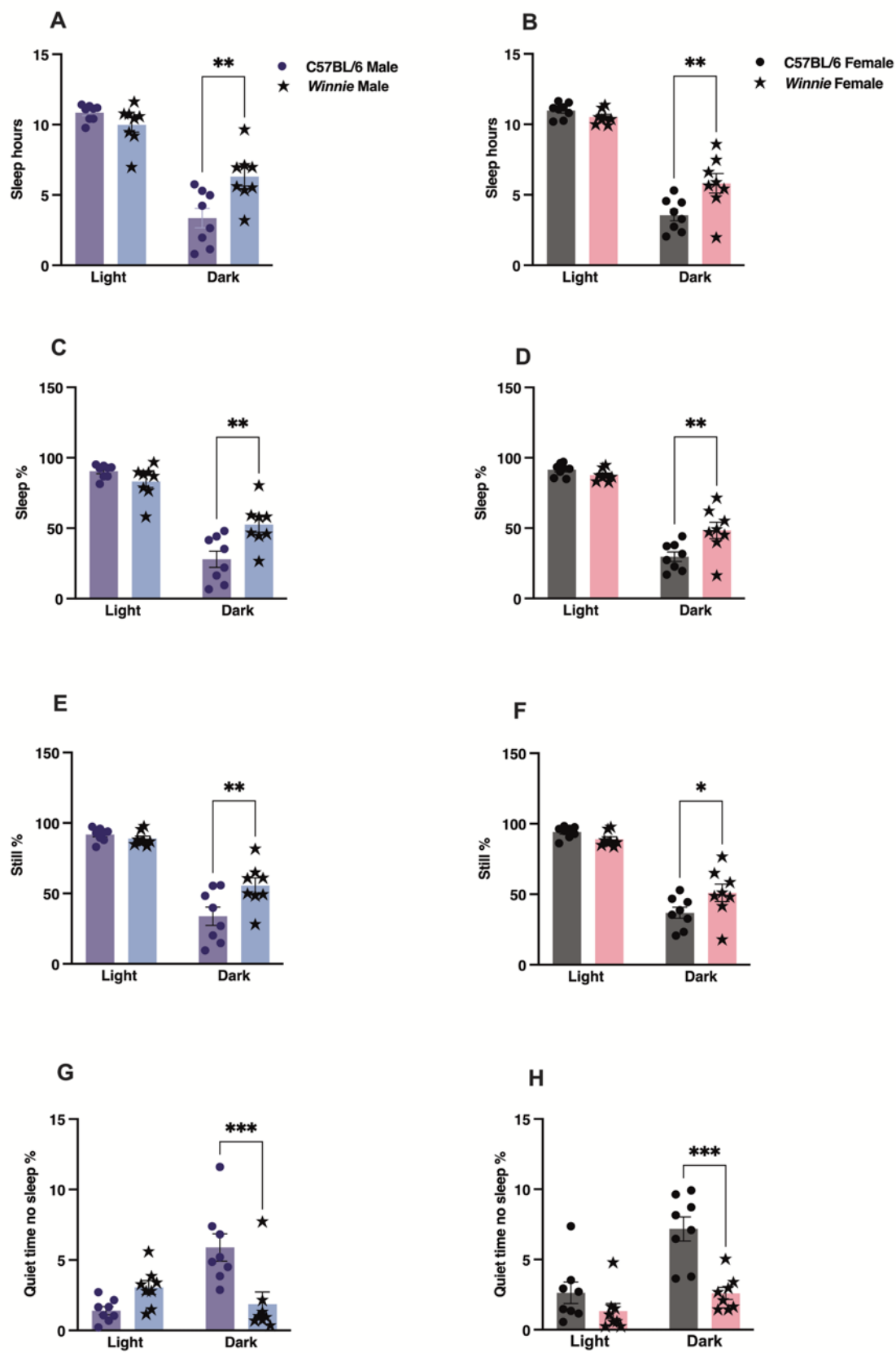


Figure S 6 Wheel activity of *Winnie* male mice versus controls and *Winnie* female mice versus controls

Wheel activity data points comparing *Winnie* male mice and *Winnie* female mice were recorded within light and dark cycles from 0700 to 1900 hour and 1900 to 0700 hour and then compared to C57BL/6 male and female littermates. **(A)** *Winnie* male mice attained significant fewer wheel metres (1957 ± 818 metres, $p < 0.001$) than male control mice (9455 ± 1913 metres). **(B)** Less wheel metres were observed in *Winnie* female mice (3909 ± 831 metres, $p < 0.001$) to female control mice (7570 ± 509 metres). **(C)** The *Winnie* male mice were significantly slower in wheel speed (0.2 ± 0.02 metres/second, $p = 0.23$) than the male control mice, during the light hours (0.5 ± 0.06 metres/second). In the dark hours, the *Winnie* male mice were significantly slower in wheel speed (0.2 ± 0.02 metres/second, $p = 0.003$) than male control mice (0.3 ± 0.05 metres/second). **(D)** The *Winnie* female mice showed non-significant differences in wheel speed to the female control mice. For analyses, $*p \leq 0.05$ was considered significant, $**p = 0.01$, $***p = 0.001$. All data were presented as the mean $\pm$ SEM, $n = 8$ mice/group.

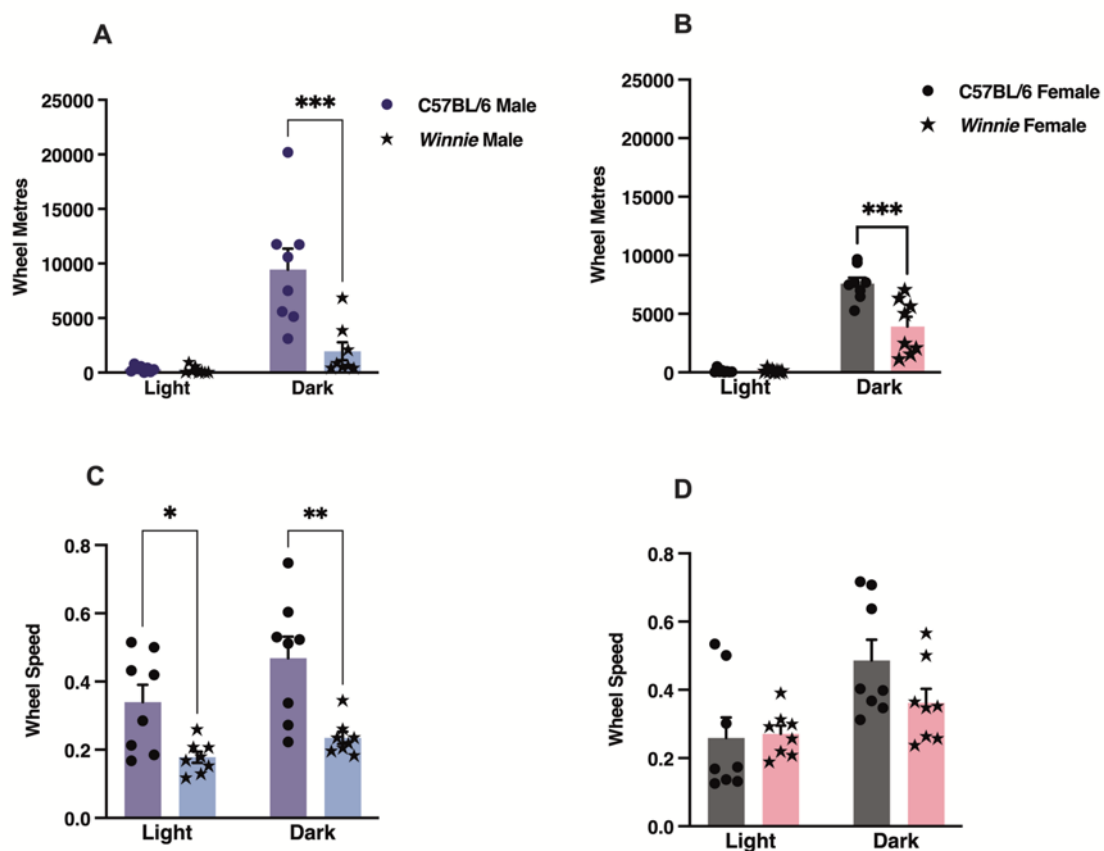


Figure S 7 *Winnie* male mice compared to *Winnie* female mice in physical activity and sleep behaviour

The *Winnie* male mice and *Winnie* female mice were compared separately, which showed non-significant differences between groups; except that the female *Winnie* mice ran on their wheels at a higher rate during the light and dark cycles (14-week-old, n=8 females/group, and n=8 males/group) The data points were recorded within light and dark cycles from 0700 to 1900 hour and 1900 to 0700 hour. **(A)** Sleep hours. **(B)** Sleep percentage. **(C)** Still time percentage. **(D)** Quiet time with no sleep. **(E)** Walk percentage. **(F)** Run percentage. **(G)** Wheel metres. **(H)** Wheel speed. *Winnie* male mice speed light hours (0.18 ± 0.02 metres/second) compared to *Winnie* female mice light hours (0.27 ± 0.36 metres/second), $*p=0.041$, n=8 mice/group. *Winnie* male mice speed dark hours (0.23 ± 0.02 metres/second) and *Winnie* female mice dark hours (0.361 ± 0.04 metres/second), $**p=0.005$. For analyses, $*p \leq 0.05$ was considered significant, $**p=0.005$. All data were presented as the mean $\pm$ SEM, n=8 mice/group.

Figure S 7 *Winnie* male mice versus *Winnie* female mice in physical activity and sleep behaviour

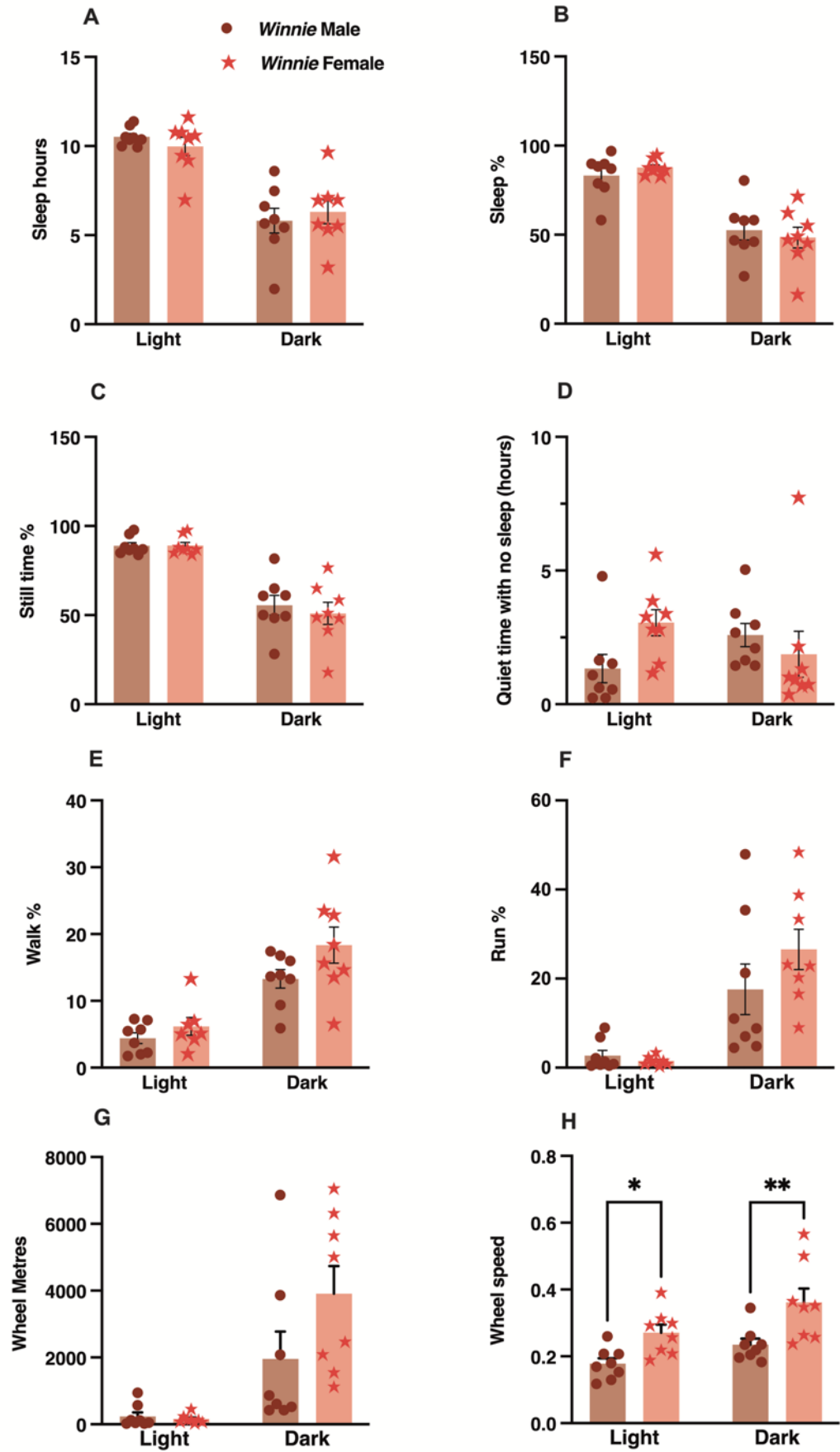


Figure S 8 Behaviour tests between *Winnie* male mice and *Winnie* female mice

(1). Open field test. (1A) Time spent in the centre in seconds. (1B) Number of times in the corners. (1C) Number of times in the centre. (1D) Grooming time in seconds. (1E) Grooming number. (2). Light Dark Test (2A) Time in the white box in seconds. (2B) Entries into the white box, $*p=0.035$. (2C) Time in the black box in seconds. (2D) Pokes from the black box, $*p=0.017$. (2E) Distance travelled in the white box in centre metres. (3). Elevated maze plus. (3A) Time in the closed arm in seconds. (3B) Time in the open arm in seconds, $**p=0.005$. (3C) Entries in closed arm. (3D) Entries in the open arm. (3E) Time spent in the centre in seconds, $p=0.001$ (4). Forced swimming test. (4A) Swimming mobility time in seconds. (4B) Immobility time in seconds. (4C) Swimming percentage. For analyses, $*p\leq 0.05$ was considered significant, $**p\leq 0.001$. All data were presented as the mean $\pm$ SEM, $n=8$ mice/group.

Figure S 8 Behaviour of *Winnie* male mice versus *Winnie* female mice

