UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Defining the Role of Inflammation and Effective Therapies for the Commonest Cause of Stroke in Children: Cerebral Cavernous Malformation (CCM)

by Hamidreza Sadegh

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

under the supervision of

Prof Philip Hansbro
Dr Jaesung Peter Choi
Dr Nicole Gower Hansbro

University of Technology Sydney
Faculty of Science

July 2023

Certificate of Original Authorship

I, *Hamidreza Sadegh*, declare that this thesis is submitted in fulfilment of the requirements for the award of *Doctor of Philosophy*, in the *School of Life Sciences* at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Acknowledgement

During the last three and a half years of my journey, I have traversed a path filled with valuable lessons, unforeseen challenges, and novel experiences that have profoundly shaped both my academic knowledge and personal growth. This pursuit of a doctoral degree has been more than a mere academic endeavour; it has been an opportunity for me to expand my intellectual horizons while simultaneously nurturing my character.

In reflecting upon this transformative journey, I am reminded of a Persian proverb that resonates deeply within me: “You never become an astonishing statue unless you endure the axe hits.” This proverb encapsulates the essence of my doctoral experience, where resilience, perseverance, and determination were integral to my progress. As I bring my thesis to completion, I am profoundly grateful to those who have played a significant role in my academic and personal development. Their unwavering support, guidance, and encouragement have been instrumental throughout this arduous but rewarding endeavour.

First and foremost, I extend my heartfelt appreciation to my esteemed supervisors, Prof Philip Hansbro, Dr Peter Choi and Dr Nicole Hansbro. Their expertise, mentorship, and scholarly guidance have been invaluable in shaping the direction of my research and nurturing my intellectual curiosity. Their insightful feedback, constructive criticism, and valuable suggestions alongside of their unwavering belief in my abilities and their dedication to my success have been a constant source of motivation.

My sincere appreciation extends to my research colleagues at Centre for Inflammation, (UTS), Centenary Institute who have provided an intellectually stimulating environment throughout my doctoral studies. Dr Caitlin Gillis, Dr Saima Firdous Rehman, Tayyaba Sadaf, Ridhima Wadhwa, Dr Annalicia Vaughan, Dr Matt Johansen, Dr Nadia Amorim, Piyush Jha, Shatarupa Das, Dr Christina Nalkurthi, Linda Tong, Duc Nguyen and all people who supported and inspired me, both directly and indirectly, during my PhD studies. Though too numerous to name individually, their impact on my personal and professional growth has been immeasurable.

I would also like to acknowledge Dr Matthew Foley, Deputy Facility Manager, Analysis and X-Ray Microscopy Manager at Sydney Microscopy and Microanalysis (SMM), University of Sydney, for their assistance and generous support in various aspects of my

research, whether it was providing access to resources, facilitating administrative processes, or offering technical expertise. His efforts have been pivotal in enabling a conducive research environment.

Furthermore, I am grateful to my family for their unwavering encouragement, love, and understanding during this challenging and demanding phase of my life. Their unwavering belief in my abilities, emotional support, and patience have been an anchor that kept me grounded and motivated throughout this journey. Dad, Mom, I love you so much.

This doctoral journey has been a remarkable period of intellectual exploration, personal growth, and self-discovery. The lessons I have learned, the challenges I have overcome, and the experiences I have gained have undoubtedly transformed me into a more resilient and knowledgeable individual. I am deeply grateful to all those who have been part of this extraordinary odyssey, and I look forward to the continued pursuit of knowledge and making meaningful contributions to my field.

Thank you all for being an integral part of my journey.

Hamidreza Sadegh

Conference proceeding:

14th international conference of Cerebral Vascular Biology (CVB) – Uppsala, Sweden 18th - 22nd June 2023

- Rapid oral presentation and poster

Title: "*Preventing stroke from cerebral cavernous malformations by targeting the microbiome*"

Centenary institute symposium 2021 – December 2021

- Oral presentation

Title: "*Identification of effect of high-fibre and high fat diet on experimental mice model of cerebral cavernous malformation (CCM)*"

Table of Contents

Table of Figures

List of Tables

Abbreviations

CCM	Cerebral cavernous malformation
CNS	Central nervous system
AVMs	Arteriovenous malformations
dAVFs	Dural arteriovenous fistulas
KRIT1	Krev Interaction Trapped 1
PDCD10	Programmed Cell Death Protein 10 (aka CCM3)
ICH	Intracerebral haemorrhage
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Alpha
MAP3k3	Mitogen-Activated Protein Kinase Kinase Kinase 3 (aka MEKK3)
IL-6R	Interleukin-6 Receptor
MSR1	Macrophage Scavenger Receptor 1 (aka CD204)
CD14	Cluster of Differentiation 14
TGFBR2	Transforming growth factor beta receptor 2
IGH	Immunoglobulin heavy chain
TLR4	Toll-like receptor 4
STRIPAK	Striatin-Interacting Phosphatase and Kinase
Rhoa	Ras homolog family member A
MAPK	Mitogen-Activated Protein Kinase

KLF2	Krüppel-like transcription factors 2
KLF4	Krüppel-like transcription factors 4
HEG1	Heart-of-glass receptor 1
Rap1	Ras-related protein 1
ICAP1	Integrin cytoplasmic domain-associated protein 1
TGF-β	Transforming Growth Factor Beta
EndMT	Endothelial-to-Mesenchymal Transition
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
ROCK	Rho-Associated Protein Kinase
GFP	Green Fluorescent Protein
YFP	Yellow Fluorescent Protein
RFP	Red Fluorescent Protein
CFP	Cyan Fluorescent Protein
GI	Gastrointestinal tract
SCFAs	Short-Chain Fatty Acids
NAFLD	Non-Alcoholic Fatty Liver Disease
GOS	Galacto-Oligosaccharides
FOS	Fructo-Oligosaccharides
GPCR	G-Protein Coupled Receptor
FFAR3	Free Fatty Acid Receptor 3

iGABA	Gamma-Aminobutyric Acid
KD	Ketogenic Diet
TMA	Trimethylamine
TMAO	Trimethylamine N-Oxid
FMT	Faecal microbiota transplant
BALF	Bronchoalveolar Lavage Fluid
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
4HT	4-hydroxyTamoxifen
BSA	bovine serum albumin
GNB	Gram-negative bacteria
HDAC	histone deacetylases
PPAR	peroxisome proliferator-activated receptors
VN	vagus nerve
ENS	enteric nervous system
GF	Griseofulvin
ACTH	adrenocorticotropic hormone (ACTH)
HPA	hypothalamus-pituitary-adrenal
SCA	Subclinical Carotid Atherosclerosis
LBP	Lipopolysaccharide-binding protein

PCoA	Principal coordinate analysis
PCA	Principal Component analysis
KT	kalkitoxin (KT)
NFATC1	nuclear factor of activated T cell 1
C3	complement 3
MNC	mononuclear cells
DSS	dextran sulphate sodium
PAR	prejunctional-actinomyosin ring
NVU	neurovascular unit
CDI	*Clostridium difficile* infection
S1P	Sphingosine-1-phosphate
eNOS	endothelial nitric oxide synthase
ER	Estrogen receptor

Thesis Abstract

Cerebral cavernous malformations (CCMs) are enlarged blood vessels that develop abnormally, posing a non-cancerous but potentially life-threatening condition that can result in a haemorrhagic stroke. Therefore, there is an urgent requirement for innovative, non-surgical treatment alternatives for CCM. Recent studies have identified the dysbiosis of gut microbiome and the innate immune response (TLR4-MEKK3-KLF signalling pathway) as a critical stimulant of CCM in mouse models. The gut barrier has since been identified as a major determinant of the disease course in CCM, whereby its disruption augments CCM formation in mouse models. These studies provide strong evidence that a novel and innovative strategy to treat CCM is to target the gut microbiome.

In this study, we established a CCM1 knockout mouse model through the utilization of *Cdh5CreERT2* (hereafter referred to as *Ccm1iECKO*) to assess the impact of diet-induced modifications to the microbiome on CCM development across two distinct temporal phases: short-term and long-term. Evaluation of brain lesion burden encompassed measurements of size and quantity through micro-CT imaging, while histological analysis was employed to examine gut morphology and integrity. Metagenomics and metabolomics sequencing were performed on collected faecal samples to track alterations in the gut microbiome and associated metabolites. Lastly, blood LPS levels were scrutinized following dietary interventions to elucidate the role of LPS in CCM.

In summary, a diet rich in plant-based fats leads to a reduction in CCM lesion burden in the short term, with this effect observed only in female mice. Conversely, dietary modifications such as high-fibre and low-fibre, while enhancing gut integrity, did not exert any influence on CCM lesions. Additionally, no discernible impact on blood LPS levels was observed across all dietary interventions. This study findings indicate that the

alteration of the gut microbiome through a high fat diet intervention can mitigate or regress CCM lesions, irrespective of circulating LPS levels. Given that surgical resection stands as the sole recourse for treating CCM patients afflicted with severe clinical symptoms, these findings serve as a foundational step toward studies to develop a non-surgical microbiome-targeted therapies. These therapies hold promise as safe and effective alternatives for the treatment of CCM, potentially circumventing the need for invasive surgical interventions, particularly in cases where severe clinical symptoms necessitate intervention. Furthermore, this study unveils that the influence of the gut microbiome on CCM disease extends beyond the LPS-TLR4 interaction, operating through distinct mechanisms.

1. Chapter 1: Introduction and Literature review

1.1. Cerebral Cavernous Malformations (CCMs)

Cerebral cavernous malformations or cavernous haemangiomata are vascular abnormalities that are predominantly in the central nervous system (CNS) which affect approximately 35 million people worldwide annually (~0.5% prevalence) (1-3). Abnormal epithelial cells cause slow-moving blood vessels that then form mulberry-shaped cavities, so-called lesions. The lesions can appear anywhere but the main clinical impact of CCM is in the CNS (4). In some cases, brain and spinal cord haemorrhages result, due to leaking of blood from the lesions into surrounding tissues, leading to severe clinical symptoms (5, 6). In terms of genetic susceptibility, CCMs are divided broadly into two different conditions: Familial and Sporadic. In Sporadic CCM, lesion formation occurs in patients with no familial history, and usually only a single lesion is formed. On the other hand, the familial form occurs in patients who carry mutated CCM causative genes, and multiple lesions form and develop (7-9).

Despite numerous clinical and molecular investigations into CCM disease, research efforts are ongoing to elucidate the fundamental mechanisms and pathogenesis. CCM is one of five different types of vascular malformations, namely arteriovenous malformations (AVMs) (10), dural arteriovenous fistulas (dAVFs) (11), developmental venous anomaly (DVA) (12), capillary telangiectasias (13). The lack of direct arteriovenous communication and non-intervening brain tissue are defining traits of CCM lesions (11). A better understanding of CCM development necessitates a multidisciplinary investigation by different groups, including cardiologists and vascular biologists, human geneticists, and cellular and molecular biologists. Previous studies have revealed that genetic mutations in three key genes result in lesion formation: *CCM1 (KRIT1)* (14), *CCM2* (15), which encodeds Malcaverin protein and *CCM3* or *PDCD10* (16). The cause of CCMs can be congenital, sporadic mutations or environmental factors; however, they can develop during a lifetime mostly between 20s to 50s years old age (16). CCM lesions vary greatly in size, from spotted lesions (<0.5cm) to several centimetres in diameter (<5cm) (17). The precise location, quantity, and size of CCM lesions are critical determinants of symptom severity in affected individuals. Specifically, larger lesions have been shown to be associated with more pronounced clinical manifestations, irrespective of lesion number. (18).

Similarly, the clinical symptoms of CCM disease are highly variable, ranging from headache and weakness or numbness to more severe ones such as seizures, focal neurological deficits and stroke

(18, 19). This variety can be seen even among identical mutations and identical family history (20). Some affected individuals may experience a stroke during childhood, and some remain asymptomatic for their entire life. Recent studies revealed that in addition to these core genetic mutations, environmental factors such as comorbidity condition, race and genetic background, radiation exposure, life-style and gut microbiome composition could affect signalling pathways that modify disease development (21, 22).

1.2. The etiopathogenesis of CCM: genetic drivers and the two-hit model

The knowledge of the etiopathology of CCM remains limited. Firstly, there is a lack of profound understanding of molecular mechanisms and physiological stimuli that underlay CCM lesion development. Secondly, we do not know the modifiers of disease conditions and why some patients progress to intracranial haemorrhage. A heterozygous mutation in any CCM causative genes passed from parents to the child by autosomal dominant pattern is responsible for Familial CCM. A variety of mutations, ranging from nonsense and missense to canonical splice site and frameshift mutations, within all three causative genes, has been reported (14, 15, 23). It remains undefined whether one specific mutation is sufficient to cause disease, or whether there is an amplification of disease susceptibility or severity with accumulated mutations (24). Although mutations in *CCM2* and *PDCD10* are also common, up to 50 per cent of symptomatic familial CCM are the result of heterozygous mutations in *CCM1*(25).

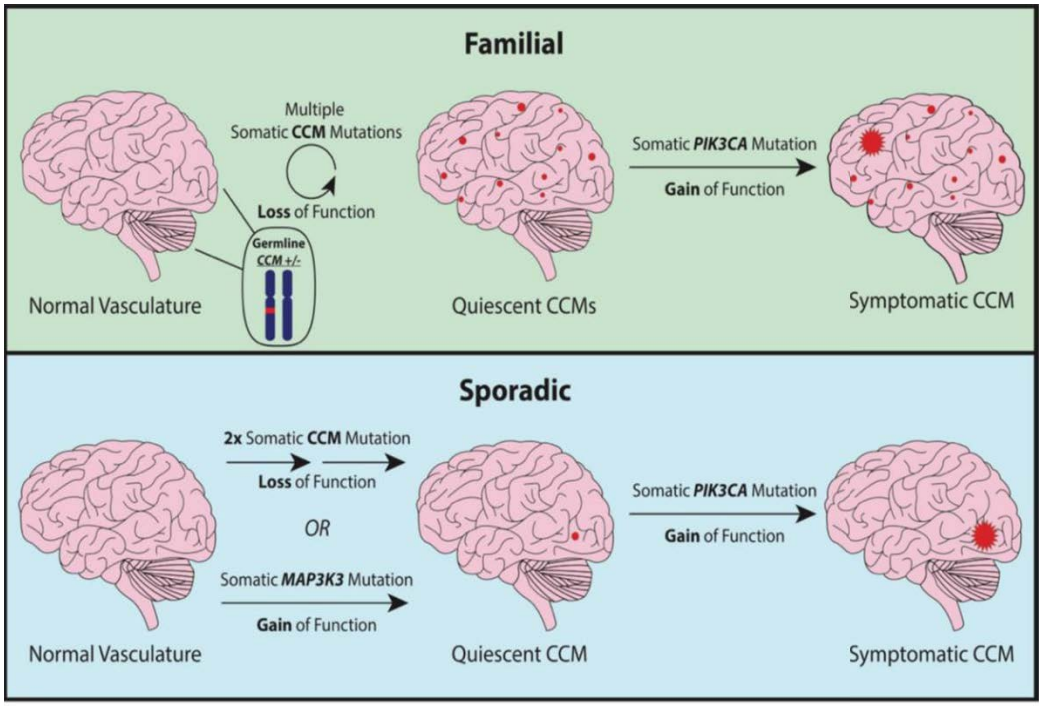


Figure 1.1 Familial and sporadic form of CCM *- Familial: 1x germline mutation + 1x somatic mutation / Sporadic: 2x somatic mutations on same CCM gene) extracted from (26).*

If the haploinsufficiency of CCM causative genes leads to the formation of the lesion(s), why does the CCM phenotype emerge as focal lesions instead of systemic vascular lesions? This question has raised the hypothesis that a second somatic mutation is necessary for CCM causative genes to show a phenotype and some people however carry genetic mutations in CCM causative genes but remain asymptomatic throughout their entire lifespan (27, 28). The two-hit hypothesis, or the Knudson hypothesis suggests that most tumour suppressors need mutations or epigenetic factors to silence both alleles of their genes; otherwise, no phenotype is observed (29). Similarly, CCM pathogenesis also requires the two-hit hypothesis. Individuals carry heterozygous germline mutations in one of the CCM genes in familial forms. Still, CCM lesions only appear when a second mutation (somatic mutation) affects another allele of the same gene (30, 31). Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) gene mutations have been linked to the occurrence of CCM. Such mutations can activate the PI3K/Akt/mTOR signalling pathway, which triggers angiogenesis, and further promotes the formation of CCMs. The simultaneous presence of mutations in both *PIK3CA* and *CCM1* genes can synergistically augment CCM formation. (32-34).

Furthermore, some findings elaborated a link between CCM clinical severity and mutations in *PIK3CA*. In a mouse model of CCM, it was observed that lesions usually develop early but can also form anew in adulthood when mutations in *PIK3CA* occur (30, 35). Mouse models of CCM

without mutations in *PIK3CA* still demonstrated lesion formation and development but showed less lesion burden. Many sporadic patients have a specific mutation in Mitogen-activated protein kinase kinase kinase 3 (*MAP3k3*) (p.I441M) which leads to upregulation of *MEKK3* (32, 33). Some data has shown that mutations in *MAP3k3* and *PIK3CA* happened simultaneously, but initial data showed that CCM progression and *MAP3K3* are entirely separate (32). In other words, loss of function mutation in CCM genes and *MAP3K3* mutations that cause upregulation of *MEKK3* have the same consequences, and are sufficient to initiate the formation of CCM lesions. In contrast, mutations in *PIK3CA* merely causes more significant lesions and severe symptoms, not initiation of CCM lesions.

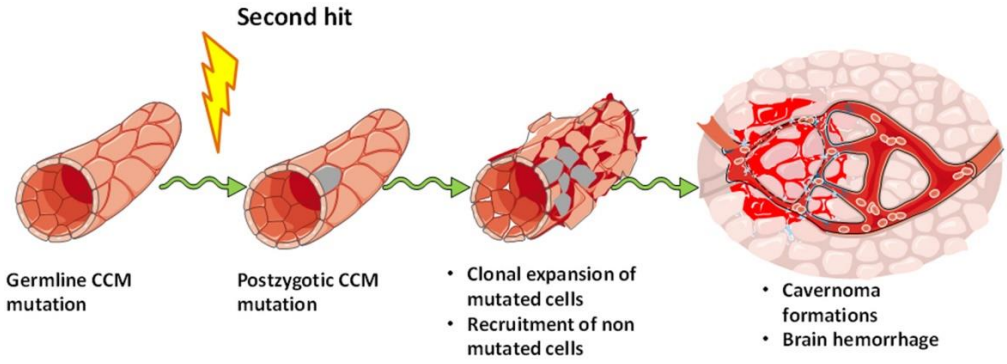


Figure 1.2 The two-hit hypothesis of CCM lesion formation *- The development of CCM relies on a second event, where individuals with one mutated CCM gene only develop the malformations when a second mutation affects the other allele of the same gene. extracted from (36).*

1.3. CCM Lesion Formation and haemorrhage

The lesion burden and speed of formation of CCM lesions in patients are highly variable; even after the initial formation, the number and size of the lesion(s) can alter during the lifetime of the affected individual (26). Several key steps lead to forming and establishing CCM lesions within the brain. Initially, genetic mutations occur in one of three CCM causative genes (KRIT1, CCM2 and PDCD10). Endothelial cells, which form a monolayer along the luminal surface of blood vessels, play a pivotal role in preserving the structural integrity and proper functioning of the vasculature. Mutation in CCM genes in vascular endothelial cells within the brain disrupts the normal functioning of endothelial cells and cell-cell interactions, leading to malformation of the inner walls of blood vessels (37). These malformations lack normal structural components like smooth muscle and have thin walls prone to leaking and bleeding (38). Over time, these small vascular lesions formed with several endothelial cells start to grow and expand and lead to larger diluted blood vessels called cavernous lesions (39, 40).

Once expanded, larger lesions cause an alteration of normal blood flow within the brain. Sluggish and stagnate blood flow in these lesions increases pressure and damage surrounding brain parenchyma, leading to seizure and neurological deficits in CCM patients (41). In some severe cases, CCM lesions due to thin vascular walls and high blood pressure can cause blood leakage and microhemorrhages within brain tissue (42). The emergence of cavernous lesions or potential haemorrhages can induce an inflammatory response in the brain tissue around the lesions. This inflammation is also associated with the growth and establishment of lesions, increased brain tissue damage, and increased risk of stroke (43). In response to bleeding and inflammation, the brain may attempt to repair the damaged areas by forming scar tissue. The formation of scar tissue can further disrupt the normal structure and function of the brain tissue, contributing to the establishment of cavernous lesions. No specific medication can treat CCMs directly, but medications may be prescribed to manage symptoms such as seizures or headaches. In severe cases, surgical removal of the CCM lesion is the only option, and this is especially indicated when the lesion is located in a surgically accessible area, is causing significant symptoms and/or poses a risk of bleeding (44).

1.4. CCM lesion outcomes

Although CCM lesions are referred to as established lesions after formation and extension, the lesions can continue to change in size, number and even chance of bleeding. Previous clinical studies with CCM patient follow-up suggested five scenarios for formed lesions (45) (Figure 1.5). In some cases, there would be no change in size or number of lesions and the lesion may persist for the rest of life. Clinical symptoms may vary depending on the location of these stable lesions within the brain: clinically this scenario is referred to as lesion stabilisation. Alternatively, some lesions can shrink in size or completely vanish during a certain time. Although the reasons for lesion regression are not fully understood, some studies suggest that non-genetic factors and patient lifestyle play a role (46, 47). Lesion burden in familial cases may increase in number during a lifetime, although predictive markers of this progression are unknown. Some patients who present initially asymptomatic or with mild clinical symptoms develop more severe clinical symptoms due to the new formation of more lesions, or lesion haemorrhages (48). Similarly, some lesions can grow in size and put more pressure on brain tissue, consequently causing more severe clinical symptoms. Occasionally, lesion extension leads to leaking blood to brain tissue and bleeding (Symptomatic Haemorrhage or CASH), resulting in neurological deficits or stroke depending on the haemorrhage location and brain tissue damage (49).

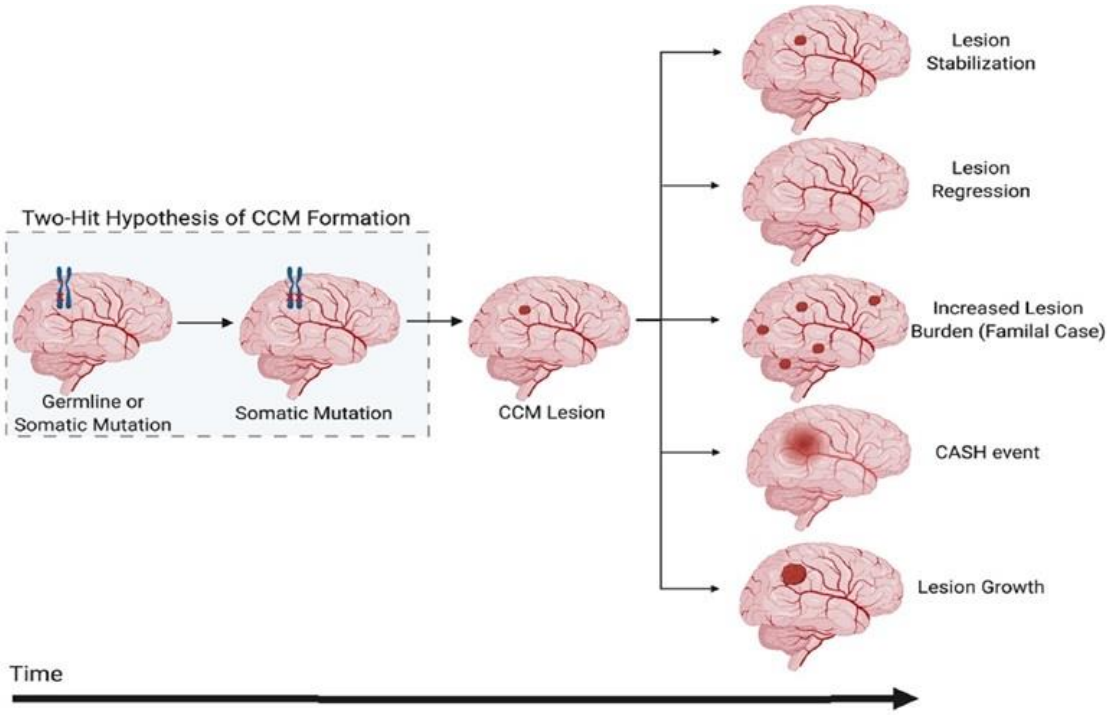


Figure 1.3. The different development of CCM lesions over time *- The progression of CCM disease is believed to result in five important clinical outcomes, including stabilization (No difference in volume and number), regression (shrinkage or vanishing), increased number (Only in familial form), Cavernous Angioma with Symptomatic Haemorrhage (CASH), and lesion growth) extracted from (26).*

1.5. CCM protein complex interactions

KRIT1 (Krev interaction trapped protein 1), also known as CCM1, CCM2, and CCM3 (Programmed cell death protein 10, PDCD10) are proteins involved in cellular scaffolding. They play a crucial role in regulating multiple cellular functions such as cell adhesion, survival, division, polarity, and proliferation (50-52). KRIT1 interacts with multiple signalling proteins to modulate these processes, while CCM2 interacts with KRIT1 and regulates the STRIPAK complex, which is involved in cell polarity and proliferation (53, 54). PDCD10 is a signalling protein critical for cellular processes such as apoptosis, cell adhesion, and angiogenesis. PDCD10 can affect Ras homolog family member A (RhoA) and inhibit its activity, leading to increased cell-cell junction stability and vascular integrity. (55). Moreover, PDCD10 possesses the ability to modulate the activity of MST4, a protein kinase that triggers the activation of the p38 MAPK pathway. This pathway, in turn, hinders both cell migration and angiogenesis processes (56). Additionally, all three CCM proteins can interact with other signalling pathways that affect angiogenesis, such as the Wnt/β-catenin and Notch pathways (57, 58). Altogether, these

biochemical and preclinical animal studies provide significant evidence for an important role for CCM proteins in vascular integrity and angiogenesis.

Loss of function mutations in CCM proteins can lead to the disruption of endothelial cell-cell junctions, thereby increasing vascular permeability and the risk of haemorrhage. (59). Previous studies showed that the interaction of CCM proteins could make a triplex signalling platform (51-53) in which CCM2 protein is located at the centre and interacts with other CCM proteins (KRIT1 and PDCD10) by separate binding sites at the same time (53, 54). There is strong evidence that mutation in PDCD10 leads to more severe clinical symptoms in CCM patients compared to a mutation in KRIT1 or CCM2 (51, 55). Moreover, PDCD10 is more highly conserved than KRIT1 and CCM2 during evolution from humans to insects, but KRIT1 and CCM2 are conserved from humans to fish (51, 56-58).

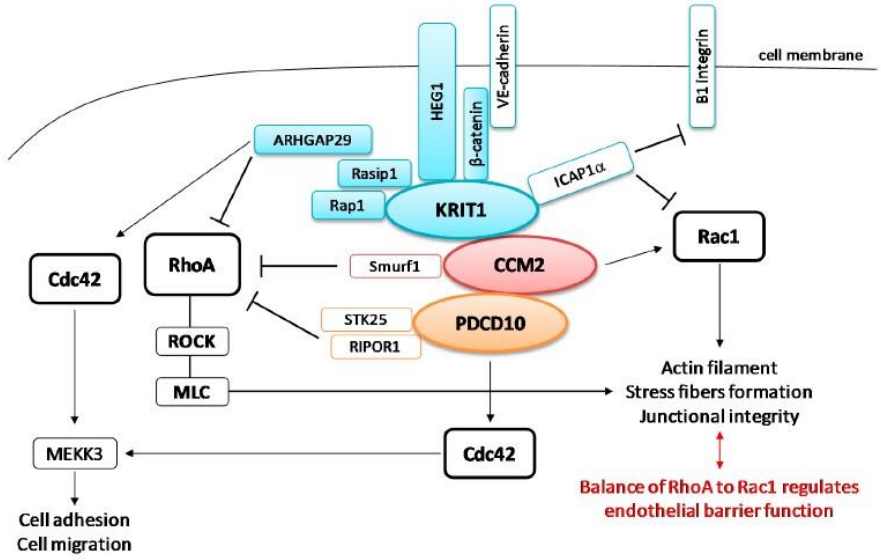


Figure 1.4 Interaction of CCM proteins complex within the cell *- The CCM signalling complex participates in Rho GTPase signalling. In this process, KRIT1 attaches to the cell membrane through beta-catenin and HEG1. Subsequently, it recruits ARHGAP29 to regulate the RhoA and Cdc42 pathways. CCM2 inhibits RhoA signalling by associating with Smurf1, while PDCD10 can potentially control RhoA through interactions with RIPOR1 or STK25. All three CCM proteins (KRIT1, CCM2, and PDCD10) play a negative regulatory role in the RhoA-ROCK pathway, which helps prevent excessive stress fibre formation and endothelial barrier dysfunction observed in CCM lesions. extracted from (60).*

1.6. Signalling pathways causing CCM

The signalling pathways of CCM disease pathogenesis are complex, and understanding of the underlying mechanisms is currently incomplete. Several main pathways likely play an essential role in initiating CCM disease. In addition to the main pathways involved in CCM pathogenesis, there is evidence that other signalling pathways such as the Wnt signalling pathway and the VEGF signalling pathway, play a role in CCM disease progression (57, 61). All of these pathways are associated with regulating blood vessel formation and maintenance.

i. MEKK3 (mitogen-activated protein kinase kinase 3)

MEKK3, a mitogen-activated protein kinase kinase 3, is a signalling pathway that modulates a range of cellular processes, encompassing inflammation, cell proliferation, and angiogenesis. Recent research findings have indicated a potential involvement of MEKK3 in the regulation of Cdc42, a small GTPase critical for governing cell migration, polarity, and cytoskeletal organization. Rho GTPase activating protein 29 (ARHGAP29) and PDCD10, two components of the STRIPAK complex that are involved in Cdc42 regulation, have both been shown to interact with MEKK3. Therefore, it is possible that dysregulation of the MEKK3-Cdc42 pathway may contribute to the development of CCM lesions (62). Furthermore, studies have found that loss of any of the three CCM genes (*CCM1/KRIT1, CCM2 or CCM3/PDCD10*) leads to dysregulation of the MEKK3-KLF2/4 (Krüppel-like transcription factors) pathway, resulting in endothelial dysfunction, increased permeability, and dysmorphic vessel formation (57). Overall, the MEKK3-KLF2/4 signalling pathway appears to be an important regulator of endothelial function and vascular integrity, and dysregulation of this pathway may contribute to the development of CCM lesions.

ii. Rho GTPase signalling

A strictly regulated cascade that essentially engages to promote and regulate cell adhesion, shape and motility (63, 64). Rho GTPases comprise a group of small GTP-binding proteins that plays a molecular switch role via inactive and active GTP-binding sites. In an active state, Rho GTPases induce various downstream proteins to regulate different cell activities by protein activation or modulating protein function (63, 65). Studies have shown that loss of function mutation in CCM causative genes (*KRIT1* and *CCM2*) affects the normal cellular activity of Rho GTPases, leading to downregulation of RhoA, Rac1, and Cdc42 (66). RhoA protein plays a crucial role in cell adhesion and migration by stimulating the formation of stress fibres and focal adhesions (67-69).

On the other hand, Rac1 and Cdc42 affect lamellipodia and filopodia, which are essential in cell-cell interaction and motility (70). Therefore, negative regulation of RhoA, Rac1 and Cdc42 in the context of CCM mutations, likely mediates the increased endothelial permeability and disruption

in endothelial cell-cell junctions that precedes the formation of CCM lesions (66). Structural biology studies on *KRIT1, CCM2* and RhoA GTPases have revealed more detailed insights into the physical interactions of these proteins, and with other proteins involved in cell junction formation and endothelial permeability, such as β1 integrin, ICAP1, and Par3 (71-73). Some strong experimental evidence showed the interaction of the Rho GTPase signalling pathway in CCM pathogenesis. One study in mice found a significant increase in RhoA activation and formation of CCM lesions in mice brains subsequent to the deletion of *KRIT1* and *CCM2* genes (74).

iii. ***TGF-β signalling***

TGF-β (transforming growth factor beta) signalling is a complicated cascade associated with several key activities such as tissue remodelling, cell differentiation and growth (75, 76). TGF-β type I and type II receptors are cell surface receptors for TGF-β ligands, belonging to the TGF-β superfamily. These receptors interact with various ligands, including TGF-β itself and bone morphogenetic proteins (BMPs), to initiate multiple downstream signalling pathways. TGF-beta ligands regulate the expression of pro-angiogenic factors and extracellular matrix components, affecting endothelial cell behaviour and vessel formation. TGF-beta signalling influences angiogenesis and vascular remodelling processes, which are dysregulated in CCM. One pathway downstream of TGF-β receptors is the Smad pathway, which affects the regulation of gene expression by phosphorylation and nuclear translocation of Smad proteins (77). A previous study revealed that loss of function mutations in KRIT1 or CCM2 leads to increased Smad protein phosphorylation due to enhanced TGF-β signalling activity (78). Unusual activity of the TGF-β signalling pathway resulting from mutations in CCM causative genes can confound the endothelial cell-cell junction and induce endothelial-to-mesenchymal transition (EndMT), leading to abnormal blood vessels, which have thin cell walls and are susceptible to leakage, and bleeding. This is an initial step to CCM lesion formation (78, 79). There is a great deal of evidence about the role of TGF-β signalling in CCM pathogenesis (57, 79, 80). For instance, one study on a mouse model of CCM showed that deletion of KRIT1 and CCM2 resulted in a significant increase in TGF-β signalling activity and caused the formation of CCM lesions within mice brains (79).

iv. ***Notch signalling***

The Notch signalling pathway is a highly conserved pathway involved in cell homeostasis and fate determination (81, 82). Furthermore, the Notch signalling pathway is considered to play a role in endothelial cell-cell junctions and vascular permeability (83). (83). Evidence from human patients and animal models of CCM disease indicates changes in expression of Notch receptors and their ligands and dysregulation of the Notch signalling pathway within brain endothelial cells

of CCM lesions (84, 85). The Notch signalling pathway can also interact with other signalling pathways involved in CCM pathogenesis. For example, some studies suggest that Notch signalling can regulate the expression of TGF-β receptors and Smad proteins, leading to modulation of TGF-β-induced endothelial-to-mesenchymal transition (EndMT) (86). Moreover, Notch can also affect Rho GTPase activity, one of the critical pathways in CCM development (78). However, the exact mechanisms through which Notch signalling is involved in CCM development and progression are still being investigated.

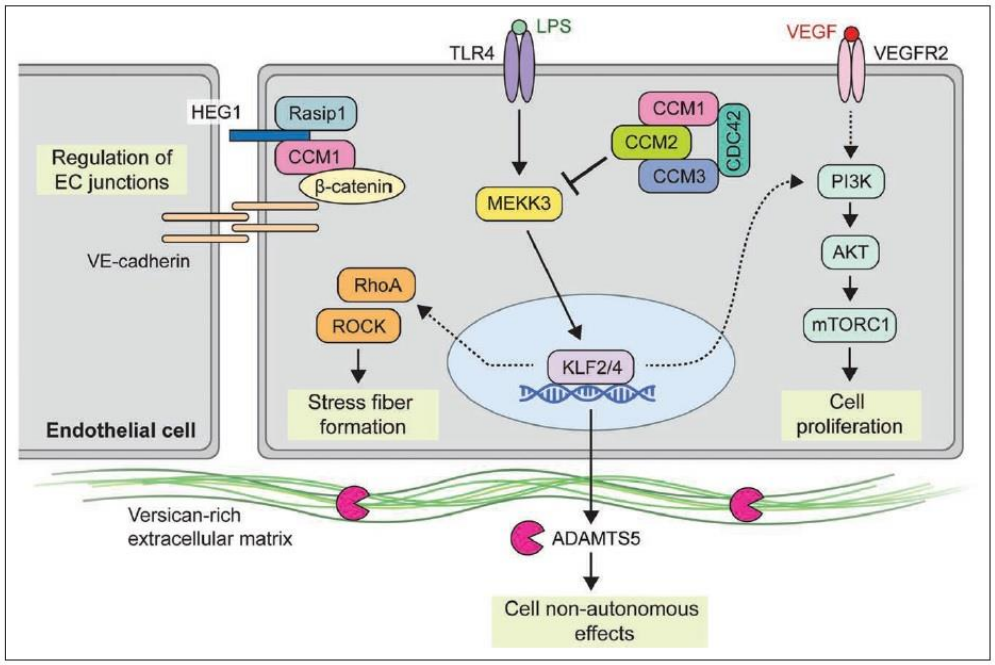


Figure 1.5 Signalling pathways involved in cerebral cavernous malformation (CCM) *- The upstream and downstream effectors involved in CCM signalling. extracted from (26).*

1.7. Animal models of CCMs

CCM disease pathogenesis remains enigmatic, and there are no effective therapies. Due to the haemorrhage of CCM lesions, clinical symptoms in patients would be severe and complicated. So, studying the onset of lesion formation and understanding the mechanism of lesion formation is crucial (87, 88). A large part of our understanding of CCM disease arises from the analysis of animal models, which enable researchers to interrogate pathogenic pathways and evaluate diverse therapeutic approaches aimed at mitigating CCM disease progression. Several animal models have been established to simulate human CCM disease.

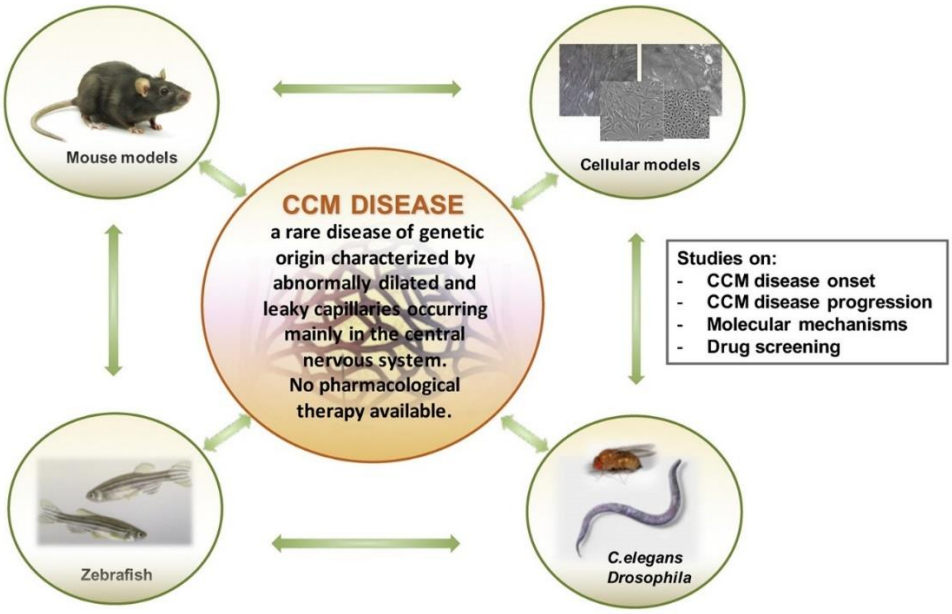


Figure 1.6 Potential animal models of CCM disease *- The worm, zebrafish and mice models of CCM have been used for stimulation of human CCM disease (89)*

C. elegans and zebrafish *(Danio rerio)* animal models have been used to investigate the molecular basis of CCM. The *C. elegans* model has identified signalling pathways that regulate endothelial cell behaviour, and mutations in the mec-4 gene, which is human homolog of transient receptor potential cation channel subfamily M member 2 (TRPM2), have been found to cause aberrant blood vessel formation (90). The zebrafish model has also been valuable in studying the role of various genes in CCM pathogenesis, such as mutations in the ccm2 gene that lead to abnormal blood vessel formation in zebrafish embryos (91-93). With developments in genetic modification techniques and the ability to create targeted mutations in animals, various murine models of CCM have been developed. Mouse models of CCM have enabled researchers to interrogate the CCM disease mechanism by enabling quantifying lesion burden within the brain and tracking the progression or regression of CCM lesions. There are several murine models for simulating CCM disease, such as global deletion of CCM genes, sensitised CCM models, conditional deletion and inducible deletion of CCM genes.

Complete global deletion of CCM genes leads to deadly embryonic effects because of disruption in angiogenesis (66, 69, 94, 95). Although the global deletion of CCM genes in animal models is not feasible for whole organism studies, these approaches have helped decipher the role of CCM proteins in cellular activities. For instance, the deletion of Krit1 causes an increased stretch in arteries, shrinkage of branchial arch arteries and reduced arterial markers and Notch-related gene expression (66). Complete deletion of Ccm2 on a global scale revealed the indispensable role of CCM2 in various vascular developmental processes, including angiogenesis, lumen formation

within the first branchial arch artery, and embryonic heart development (95-97). All CCM causative genes (*Krit1, Ccm2, Pdcd10*) have a crucial role in angiogenesis; however, a reduction in expression and activity of VEGFR2 was also observed upon deletion of *Pdcd10* (94). Furthermore, loss of PDCD10 leads to earlier death and failure of embryonic growth, compared to the deletion of KRIT1 or CCM2, due to its essential role in endothelial cell ROCK activity, involving the STRIPAK signalling complex and increasing white matter permeability (68, 69).

As the CCM disease inheritance pattern in humans is autosomal dominant, heterozygosity of one of the CCM causative genes has emerged also in mice models. Heterozygous mice lacking one of the alleles of CCM genes, especially *Ccm3*, result in a low burden of CCM lesions (50, 68). Sensitised CCM mouse models were developed for enhancing the lesion burden by crossing animals heterozygous for one of the CCM genes with genetically unstable mouse lines. *Trp53*-/- or *Msh2*-/- mice carry deletions in these oncogenes, and are more likely to develop a second (somatic) loss-of-function mutation in the single preserved CCM gene allele, which promotes lesion development, in accordance with the two-hit hypothesis (28). Crossing either of these mouse lines with *Ccm1*+/- and *Ccm2*+/- mice resulted in CCM lesion formation and development in offspring aged 4-5 months (28, 98). However, offspring from *Ccm3*+/- mice crossed to either *Trp53*-/- or *Msh2*-/- mice manifested lesion formation at merely five weeks old (99). CCM lesions in these sensitised mice models are similar to human CCM lesions in terms of endothelial cell proliferation, capillary dilation and increased activity of ROCK (28, 98). However, the tendency of these mice lines to have confounding factors such as tumour development, due to the background of genetic instability, is a serious issue. Msh2-/- mice can develop lymphomas already by two months old, and Trp53-/- mice have the potential to establish neoplasms by six-month-old age (100-102). Difficulties with breeding and low chance of obtaining the correct genotype in offspring are major challenges of using sensitised CCM mice models (103).

Conditional deletion of CCM genes has revealed that CCM protein expression is a requirement for normal development in majority of cell types. Although specific endothelial deletion of *Ccm2* is embryonically lethal, loss of CCM2 in smooth muscle, neuronal or neuroepithelial tissue gives rises to viable mutant mice (69, 94, 95, 97, 104). Moreover, the deletion of *Ccm3* in neuronal and astrocytic cell types using cell-type specific Cre recombinases Gfap-Cre, Emx1-Cre and Sm22a-Cre, each result in CCM lesion formation in mice (105, 106). Lesions form within the forebrain in *Emx1*-Cre.*Ccm3*-flox mice when they get to seven months old. On the other hand, *Gfap*-Cre.*Ccm3*-flox mice show lesions across the whole brain and spinal cord at merely three weeks of age (106).

Another approach to model human CCM disease in mice is via the inducible deletion of CCM genes in animals after birth. Tamoxifen-inducible Cre recombinase (*CreERT2*) technology has

allowed the creation of CCM gene-deleted mice under inducible conditions with cell-type specificity, such as *Cdh5-CreERT2* or *Pdgfb-CreERT2* mice lines for deletion only in endothelial cells and *Mfsd2a-CreERT2* or *Slco1c1(BAC)-CreERT2* mice lines for targeting brain endothelial cells (22, 69, 107, 108). In these mouse models, tamoxifen injection to induce Cre recombinase expression, which results in the deletion of CCM genes in target cells, must be performed between P1 to P4 in newborn pups to generate CCM lesions in the adult (69, 108). Tamoxifen injection to pups after P8 does not lead to the formation of any CCM lesions in *Ccm2+/-; Cdh5-CreERT2* mice (108). In such a context, inducible deletion of *Krit1* and *Ccm2* only in endothelial cells causes the formation of lesions limited to the hindbrain and retina in mice with average 17 days survival time (57, 108). These inducible CCM mice models accurately recapitulate human CCM lesions by characteristics of endothelial-specific loss of CCM genes, mononuclear cell infiltration, hemosiderin deposits and lack of pericytes - astrocytic crosstalk (69). However, the localisation of lesions in human CCM, mainly around the brainstem, differs from the inducible CCM mouse model wherein lesions are primarily located in the hindbrain or retina (39, 57, 103, 108). Nonetheless, these inducible mouse models have been used to understand the key mechanisms behind CCM lesion formation such as MEKK3 pathway. In alternative studies, mouse models have been generated by crossing them with reporter mouse lines, such as R26R-Confetti mice. Through the utilization of fluorescent protein markers such as nuclear GFP, cytoplasmic YFP, cytoplasmic RFP, and membrane-bound CFP, recombinant cells can be labelled. These experimental approaches facilitate the examination of the cell-intrinsic functions of CCM proteins or the involvement of CCM-mutant cells within the tissue architecture, offering valuable insights. (109, 110). The reporter mice line in the CCM mice model revealed endothelial cells with loss of CCM3 could engage adjacent endothelial cells that are CCM3 sufficient to form larger CCM lesions, "cavernomas" (39, 40). In the field of CCM disease, the inducible CCM mice model is the most applicable and widely used animal model and elucidates CCM pathogenesis and main cellular signalling pathways involved in lesion formation (34, 57). The inducible mice model of CCM is a reliable animal model option for these investigations since CCM lesions formation occurs reliably and consistently (57, 108) and in this study we used the inducible mice model CCM that is described thoroughly in section 2.3 (page 22).

1.8. CCM and a third-hit?

The clinical manifestations of CCM exhibit significant variability, even among individuals sharing the same specific mutation and familial background. The exact drivers of lesion expansion are not fully identified but some studies suggest that inflammation, oxidative stress and epigenetic factors like the gut microbiome can play a role (21, 22, 111). According to a recent comprehensive genetic study, genetic variation in some other genes involved in inflammation and immune

response, such as IL-6R, MSR1, CD14, TGFBR2, IGH, and TLR4, correlated with CCM lesion burden and haemorrhage incidence (39). Among these genes, a study on polymorphism in TLR4 is notable because of playing a pivotal role in CCM lesion formation signalling (21). This observed divergence in the expression of clinical symptoms implies the involvement of non-genetic factors in determining the resulting CCM phenotype. The third-hit hypothesis suggests that the development of CCM requires the accumulation of multiple genetic and environmental factors, and the occurrence of a third hit is the key factor that ultimately leads to the formation of the characteristic CCM lesions in the brain. The first hit is usually a germline mutation, meaning it is inherited from one or both parents (112). However, inheriting a single mutation is not enough to cause the development of CCM. The second hit is thought to be a somatic mutation, which occurs spontaneously during a person's lifetime and affects the other copy of the CCM gene that was not affected by the first germline mutation. Solitary lesions are seen in sporadic cases because there is a lower probability of two somatic mutations occurring in the same gene compared to familial cases, where there is one inherited mutation on an allele in epithelial cells. The third hit is believed to result from a secondary insult to the blood vessels, leading to the formation of a CCM lesion. The three genetic or non-genetic "hits" have a cumulative effect on the genes, proteins, and cells responsible for maintaining the integrity of blood vessels in the brain: this third hit creates the final trigger that causes CCM disease (113). Environmental factors such as inflammation, radiation exposure, hypoxia and oxidative stress can all interact with the genetic mutations that predispose individuals to CCM, ultimately facilitating the development of brain lesions. However, it is important to note that not all individuals with CCM have a clear environmental trigger for their condition, and some cases may be caused solely by genetic factors.

A recent study reported that the gut microbiome in CCM patients had altered levels of certain bacterial species compared to healthy controls, suggesting a potential link between the gut microbiome and CCM (114). It is possible that the gut microbiome could act as a third hit in CCM by influencing the immune system and inflammatory processes in the body, which are known to play a role in CCM development. However, the role of the gut microbiome in CCM is an emerging area of research that requires further investigation to fully understand its potential impact on the development and progression of this condition.

1.9. The gut microbiome and dietary impacts

The gut microbiome refers to the assemblage of microorganisms, encompassing bacteria, viruses, fungi, and protozoa, which inhabit the gastrointestinal tract of humans and other animal species. The gut microbiome plays a vital role in digestion, immunity, and overall health. Diet significantly impacts the composition and function of the gut microbiome, and changes in the gut microbiome

can affect how the body processes food and nutrients. The communities of microorganisms living in the gut can produce a range of metabolites that fundamentally affect human health and disease conditions, including systemic manifestations of inflammatory and autoimmune disease (115, 116). The gut microbiome is the whole genome composition of all microorganisms living in the gastrointestinal tract (GI). Although most of the gut-resident microbes are unable to be cultured outside of the body, and are hard to isolate, emerging novel sequencing methods such as metagenomics have enabled us to identify microbial communities with greater precision from highly diverse species samples such as stool, allowing inferences on their functional impact. A study on twins' gut microbiome profile by metagenomics analysis revealed that environmental influences such as diet and living conditions could have a major impact on gut microbiome diversity and abundance (117).

Fibre is one of the most important dietary factors influencing the gut microbiome. Fibre consists of complex carbohydrates that are not digestible by human enzymes and require digestion in the gut by the gut microbiome. This process produces short-chain fatty acids (SCFAs) as breakdown products, that provide energy to the cells lining the intestine. SCFAs can also reduce inflammation and regulate immune function. Some of the anaerobic bacteria in the gut, mainly from *Firmicutes* and *Bacteroidetes* phyla, can hydrolyse host-indigestible carbohydrates by producing specific enzymes. For example, *Bifidobacterium* and *Lactobacillus* bacteria can digest galactooligosaccharides (GOS), fructooligosaccharides (FOS), and inulin (118). Fermentation of these complex oligosaccharides produces SCFAs such as acetate, propionate, and butyrate which are crucial for human health and immune system function (119). SCFAs are considered endogenous agonists of G-protein coupled receptors (GPCRs) such as free fatty acid receptor 3 (FFAR3), niacin receptor 1 (GPR109A) and GPR41 that are located on the surface of gut epithelial cells and adipocytes (120). This binding causes a significant effect on cell homeostasis and physiological procedures regardless of providing energy for the host as a carbon source (121-123). A diet high in fibre has been shown to increase the diversity of the gut microbiome and improve gut health. People adhering to a dietary regimen abundant in vegetables and high-fibre content exhibit microbial communities characterized by an increased prevalence of *Prevotella*, a genus within the *Bacteroidetes* phylum. In contrast, those following diets rich in saturated fats and animal proteins demonstrate a higher abundance of the genus *Bacteroides*. (124). *Prevotella* can degrade complex plant fibre via special enzymes this species can produce (125).

The balance of macronutrients (carbohydrates, fats, and proteins) in the diet can also affect the gut microbiome. Diets high in processed foods, typically low in fibre and high in added sugars and fats, can negatively influence the gut microbiome. This is because processed foods often lack the nutrients that feed beneficial bacteria and can promote the growth of harmful bacteria. For instance, a high intake of carbohydrates in food and drinks such as fizzy drinks, beer, white bread,

and savoury snacks leads to increased *bifidobacteria* and decreased *Lactobacillus, Streptococcus*, and *Roseburia* genera. On the other hand, fruits, coffee, vegetables and red wine increase gut microbiome diversity (126). High fat diets, for example, have been shown to reduce the diversity of the gut microbiome and alter the types of bacteria present. Some studies showed that a long-term "Western diet" (high in fat, sugar and salt) remarkably alter the abundance and reduce diversity of gut microbiome composition (127, 128). In contrast, one study on a mice model of a high fat diet showed increased gut flora diversity (129). Furthermore, diets high in protein can promote the growth of certain bacterial species that produce by-products that can be harmful or deleterious to gut health. In one study, the researchers found that a high fat diet, which was also high in protein, promoted the growth of a type of bacteria called *Bilophila wadsworthia*. These bacteria produce hydrogen sulphide, which has been shown to contribute to developing colitis (inflammation of the colon) in mice (130).

Fermented foods, such as yogurt, kefir, sauerkraut, and kimchi, are enriched with live bacteria capable of establishing residence in the gut, leading to enhanced diversity and functionality of the gut microbiome as well as improvements in the immune system (131). Research has demonstrated that the consumption of probiotics derived from fermented food can mitigate inflammation and enhance gastrointestinal well-being in both individuals with non-alcoholic fatty liver disease and those who are generally healthy (132, 133).

1.10. Gut microbiome and the brain

The interaction and communication of cells within the gastrointestinal (GI) tract with the CNS via a complex association of nerve cells, neurotransmitters, hormones, and immune system molecules are known as the "Gut-Brain Axis". Recent research has shown that the gut microbiome can produce neurotransmitters and other chemicals affecting the brain and behaviour, both in close proximity via nerves of the gut, and via systemic signalling through the bloodstream (134, 135). Gut microbiome dysbiosis has been observed in patients with brain disease and cognitive disorders. For instance, gastrointestinal complications such as constipation have been frequently noted in children with autism spectrum disorder (ASD). A metagenomics study on the gut microbiome profile of ASD children showed an increased ratio of *Firmicutes/Bacteroidetes* and more abundance of facultative anaerobic bacteria like *Escherichia, Shigella, and Candida* fungi (136, 137). These results correlate childhood autism with alteration of gut microbiome composition and their metabolites, suggestive of changes in neuroimmune and neuroendocrine systems in these individuals (138). Another important piece of evidence that the gut microbiome can affect CNS is via gamma-aminobutyric acid (GABA) production by the gut microbiome. GABA is one of the main neurotransmitters in the brain that slows down brain cell

communications, which reduces the symptoms associated with some neurological disorders such as anxiety, epilepsy and Alzheimer's disease (AD) (139). Metagenomics analysis also revealed that *Faecalibacterium* and *Coprococcus*, known as butyrate producing bacteria, associated with beneficial effect on mental health in people suffering from depression. *Dialister* and *Coprococcus* genera were found to be absent in gut microbiome samples taken from cases of severe depression compared to healthy controls (140).

Dietary interventions could also affect neurological conditions in the brain and CNS by changing the gut microbiome. One case in which there is clear evidence of the effect of diet and microbiome on human health is atherosclerosis in brain and heart vessels and dietary lifestyle. Atherosclerosis is a pathological state characterized by the accumulation of plaque within arterial walls. This gradual plaque deposition results in arterial narrowing, impeding the smooth flow of blood. Consequently, this vascular impairment can give rise to severe medical complications, including heart attacks, strokes, and peripheral artery disease (141). Diets rich in animal protein and lipids have a high amount of specific lipids called phosphatidylcholine (PC) that are hydrolysed by the gut microbiome and converted to trimethylamine (TMA) gas. After entering into the bloodstream and liver, TMA is metabolised to trimethylamine N-oxide (TMAO). Mice with high dietary PC have higher TMAO in the blood serum and are more susceptible to forming vascular plaques (142). Understanding the direct relationship between high animal fat/protein in the diet and increased TMAO and risk of heart attack and stroke indicates the prominent role that the gut microbiome can play in determining cardiovascular and cerebrovascular health (143). The ketogenic diet (KD), which is high in plant-based fat and protein and low in carbohydrates, has long been used as a therapeutic intervention for epilepsy in children, significantly reducing seizures (144, 145). During KD intervention, the body uses ketone as an energy source due to shortage of carbohydrates and glucose, also known as the ketosis condition. The effect of KD on different neurological diseases such as Alzheimer's disease AD and Parkinson's disease and has been investigated, which provides further evidence for a positive impact of KD; however, the mechanisms remain unclear (146, 147). A study on mice with KD intervention showed an increased abundance of *Akkermansia* and *Parabacteroides* genera in the gut and a significant increase of GABA in the hippocampus of KD-treated mice (148). Moreover, this study treated cohorts of germ-free mice and conventionally housed mice with KD, and found that there was no anti-seizure effect of KD in germ-free mice, whereas conventional mice had reduced seizures on KD diet. These findings confirm the effect of dietary intervention on seizure risk is via the gut microbiome (148). Alongside dietary changes, supplementation in the form of probiotics and prebiotics can change the gut microbiome and improve CNS conditions. "Psychobiotics" refers to probiotics and prebiotics that preclinical studies showed that they could affect neurological

diseases and decrease cognitive symptoms related to anxiety, depression, epilepsy and AD (149-152).

To date, no research has been conducted on the connection between diet and CCM disease in human patients. One investigation of a mouse CCM model using 16s RNA quantification provided evidence that changes in gut microbiome composition may be significant in the progression of CCM disease (22). Gut microbiome communities of KRIT1 and CCM2 knock-out mice that developed lesions within the brain (susceptible) were significantly different from knock-out mice with no evident CCM phenotype (lesion-resistant). *Bacteroides s24-7* were found in greater proportions in susceptible mice, and the relative abundance of *Ruminococcus* genera differs in KRIT1 and CCM2 knock-out mice versus WT controls. These results suggest that the gut microbiome may play a role during developing CCM disease and give rise to the hypothesis that modulating the types of bacteria in the gut microbiome could influence the development of CCM (22).

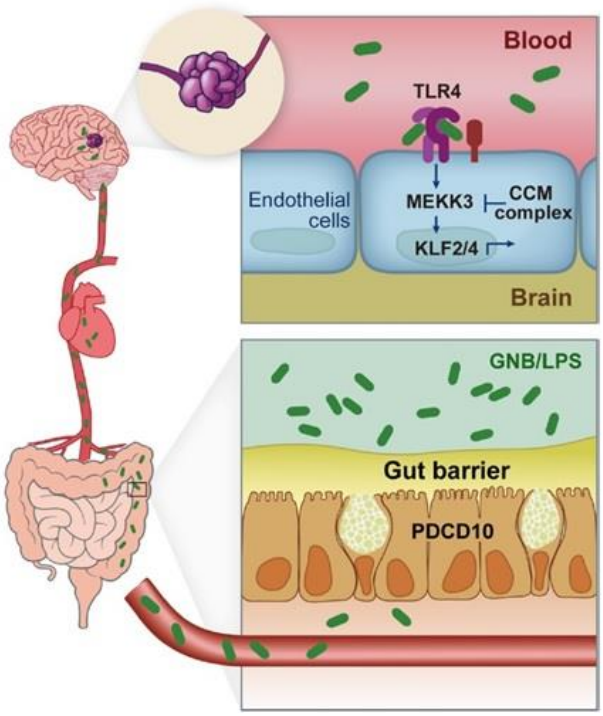


Figure 1.7 Gut-brain axis and potential avenues to influence CCM *- Lipopolysaccharides (LPS) are prominent constituents of the outer membrane in gram-negative bacteria. They possess the ability to traverse the barrier between the intestinal tract and the bloodstream, either as intact bacteria or through outer-membrane vesicles. Upon entering the bloodstream, LPS can activate Toll-like receptor 4 (TLR4) present on the surface of brain endothelial cells. TLR4 activation*

triggers increased expression of MEKK3, a pathway which has been implicated in the onset of CCM lesion development. extracted from (22).

1.11. Faecal microbiota transplant (FMT) to modify the gut microbiome

FMT is a medical procedure in which faecal matter is collected from a healthy donor and transplanted into a patient's gastrointestinal tract. This procedure aims to introduce a beneficial bacterial population into a patient's gut to overcome an imbalance or dysbiosis within the recipients gut microbiome. The procedure is typically performed by taking a stool sample from a healthy donor, screening it for infectious diseases, and preparing it for transplantation. The faecal matter can be transplanted in several ways, usually through a colonoscopy, endoscopy or enema (153). FMT is primarily employed as a therapeutic approach for managing recurrent *Clostridium difficile* infection (CDI), a bacterial infection characterized by debilitating symptoms such as severe diarrhoea, abdominal pain, and fever. While conventional treatment for CDI involves antibiotic administration, certain cases exhibit persistent infection despite multiple antibiotic courses. FMT has demonstrated remarkable efficacy in addressing recurrent CDI, exhibiting success rates exceeding 90% (154, 155). The application of FMT as a non-aggressive treatment is growing, and studies have been performed to investigate FMT as a potential therapeutic option for various diseases such as Parkinson's disease and diabetes (156, 157). In one study, eight weeks of treatment with FMT on children with autism resulted in cognitive and behavioural improvement and a significant reduction of constipation and abdominal pain, which is prevalent in autistic children (158). Microbiome composition analyses have shown high diversity of gut bacteria and an increased abundance of *Bifidobacterium* and *Prevotella* after FMT treatment in autistic children (158).

1.12. Omics analysis

The broad study of biological systems at the molecular level, capturing the diversity of molecular pathways, is referred to by the general term of omics analysis. These analyses include a range of high-throughput experimental methods and computational tools that enable extensive studies on biological molecules: genes, proteins, metabolites, lipids, and other biomolecules (159, 160). Metagenomics, transcriptomics, proteomics, and metabolomics are just a few of the fields that comprise omics analysis, each focusing on a distinct biomolecule class (161). To extract useful information from sizable datasets, omics analysis combines experimental approaches like DNA sequencing, mass spectrometry, and microarray analysis with computational techniques like machine learning and network analysis (161, 162).

1.12.1 Metagenomics

Metagenomics is a field of molecular biology that involves studying genetic material recovered directly from environmental and biological samples, such as soil, water, stool, sputum and bronchoalveolar lavage fluid (BALF), without the need for cultivation of individual organisms. Metagenomics has revolutionised how we study microbial communities, enabling the investigation of previously unculturable and unknown organisms, their functional potential, and their interactions with other members of their ecosystem (163). Metagenomics analysis involves the extraction of total DNA from a sample, followed by sequencing and computational analyses (bioinformatics) to identify and classify the microbial community and its functions (163, 164). Several sequencing platforms, including Illumina, PacBio, and Nanopore, are currently available for metagenomics analysis, each with advantages and limitations (165-167). The applications of metagenomics in biology are numerous and diverse. Metagenomics has been used to study the microbial diversity and ecology of various environments, such as soil, water, and the human gut (164, 168). Metagenomics has also been used to identify and characterise new microbial species and to understand their roles in biogeochemical cycling, disease, and biotechnology. For example, there is a growing comprehension pertaining to the biosynthetic pathway, which encompasses the intricate interplay of type I polyketide synthases. These enzymatic entities play a crucial role in the synthesis of antibiotics such as erythromycin, rapamycin, and epothilone (169).

1.12.2 Metabolomics

Metabolomics studies metabolites in biological systems and their changes in response to various stimuli, including diseases, drugs, and environmental factors. It involves identifying and quantifying metabolites in biological samples, such as blood, urine, and tissue, using analytical techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and chromatography. Metabolomics has become an increasingly important field of study in biology due to its ability to provide a comprehensive view of the metabolic state of an organism (170). It allows researchers to identify the metabolic pathways, networks and alterations involved in various biological processes, including energy production, cellular signalling, and disease development (171). By analysing changes in levels of different metabolites in biological samples, researchers can also identify biomarkers for disease diagnosis and monitor the effectiveness of treatments. Metabolomics has been used to identify biomarkers for various diseases, including cancer, diabetes, and cardiovascular disease. By measuring changes in metabolite levels, researchers can diagnose diseases at an early stage, monitor disease progression, and assess the effectiveness of treatments (171). Metabolomics can also be used to study the effects of diet on metabolic pathways and identify biomarkers for nutritional status. For instance, use of untargeted metabolomics analysis of healthy and tumour Xenograft Mouse with KD intervention to elucidate

molecular effects on disease (172). This information can then be used to develop personalised nutrition plans and improve overall health. Another application for metabolomics is the study of the metabolic pathways of microbes and their interactions with host organisms (173, 174). This information can be used to define new therapeutic targets for infectious diseases and improve our understanding of the human microbiome.

1.12.3 Lipidomics

Lipidomics is the comprehensive analysis of lipid molecules in biological systems. It involves identifying and quantifying lipid molecules, their molecular species, and their distribution in cells, tissues, and organs. Lipidomics is a rapidly growing field of study with significant implications for understanding cellular metabolism and the pathogenesis of lipid-related disorders such as cardiovascular disease, diabetes, and cancer (175, 176). Lipidomics has broad applications in biology, including the study of membrane structure and function, lipid metabolism, lipid signalling, and the regulation of cellular processes (177, 178). Lipidomics has been used to investigate the role of lipids in various biological processes such as inflammation, cell differentiation, and apoptosis (179). Several methods and technologies are used in lipidomics, including mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and chromatography. MS-based lipidomics is the most widely used approach, which allows for identifying and quantifying individual lipid molecules and their molecular species (175, 177, 180, 181).

1.13. Study rationale

CCM are abnormal clusters of small blood vessels in the brain, also known as cavernous hemangiomas or cavernomas. These clusters of blood vessels can range in size from a few millimetres to several centimetres and can cause neurological symptoms such as seizures, headaches, or even bleeding in the brain (5). There is an urgent unmet need to identify non-surgical therapeutic approaches for managing CCM (182). In mouse models of CCM disease, the development of brain lesions in genetically predisposed animals is associated with gut microbiome composition. A study conducted on a mouse model of CCM demonstrated that germ-free mice with *Krit1* knock-out did not exhibit the typical CCM phenotype or any lesions. Moreover, there is a correlation between the predisposition to CCM and particular gram-negative bacterial strains found in the gut (22). It has been proposed that Lipopolysaccharides (LPS) derived from GNB in the gut can translocate across the gut barrier and into the bloodstream,

subsequently activating Toll-like receptor 4 (TLR4) expressed on the surface of brain endothelial cells, which could contribute to or aggravate signalling pathways in leading to lesion formation.

We hypothesised that changes in the gut microbiome profile could affect the progression of CCM disease. We employed a mouse model of inducible *Krit1* knockout mice in endothelial cells, which enable us to quantify the lesion burden in the mice and mice are viable. Our study aimed to examine the effects of alternations in the gut microbiome on CCM disease progression by employing (i) dietary interventions and (ii) faecal microbiota transplantation (FMT) in this mouse model. Furthermore, to identify which metabolites, lipids and bacteria taxa increase or decrease before and after diet intervention via omics analysis

2. Chapter 2. Materials and Methods

2.1. Animal ethics and approval

This project is designed in line with the National Health and Medical Research Council of Australia code for the care and use of animals for scientific purposes issued. This project, as part of the leading project (Protocol No 2019/041 - "Finding safe and effective therapies targeting sex hormones and the microbiome for stroke in Cerebral Cavernous Malformation"), has been approved by The Sydney Local Health District (SLHD) animal welfare committee (AWC). All experiments were performed under the regulations of the Centenary Institute and SLHD. In accordance with the AWC approved protocol, all mice were checked for healthy movement and observation for signs of sickness at a four-day interval. Also, according to the ethics description, all mice were weighed weekly at a particular set time interval from the beginning of the diet modification and treatment.

2.2. Animal husbandry

Female and male Tg(*Cdh5-cre/ERT2*)1Rha (*Cdh5CreERT2*^{Tg}) and Krit1^{tm1Kwhi}/Krit1^{tm1Kwhi} (*Ccm1*$^{fl/fl}$) mice were used to establish a CCM experimental model (183, 184). The mice used in this study were bred and maintained in an inbred colony within a dedicated animal facility at the Centenary Institute in Sydney, Australia. Stringent quarantine measures were implemented to ensure the health and integrity of the colony. The mice were housed in cages that were routinely cleaned and replaced on a weekly basis. Their health condition was regularly monitored. To maintain optimal hygiene, the food, water (adjusted to a pH of 2.5-3.0), and bedding materials were sterilized using autoclaving prior to use. The cages were kept between 22-26 °C temperature and on 12-h light/dark cycles. 70% ethanol and TEGO 2000 (1:100 dilution of concentration, amphoteric-based disinfectant) were used for disinfecting surfaces and equipment. Each cage can hold no more than six mice of the same gender except for breeding pairs.

2.3. CCM Mouse model

2.3.1 Mice lines and breeding strategy

To generate mice with cell type specific and inducible *Ccm1* deletion, we crossed *Cdh5CreERT2*$^{Tg/+}$.*Ccm1*$^{fl/fl}$ (male or female) mice with *Ccm1*$^{fl/fl}$ to give rise to *Cdh5CreERT2* $^{Tg/+}$.*Ccm1*$^{fl/fl}$ and *Ccm1*$^{fl/fl}$ offspring (Fig 2.1) (185). *Cdh5CreERT2* $^{Tg/+}$*Ccm1*$^{fl/fl}$ pups do not exhibit any overt phenotype in the absence of tamoxifen administration (186). All breeding pairs were between two to ten months old for this study and were replaced with younger breeding pairs when they were approximately ten months old or after six litters.

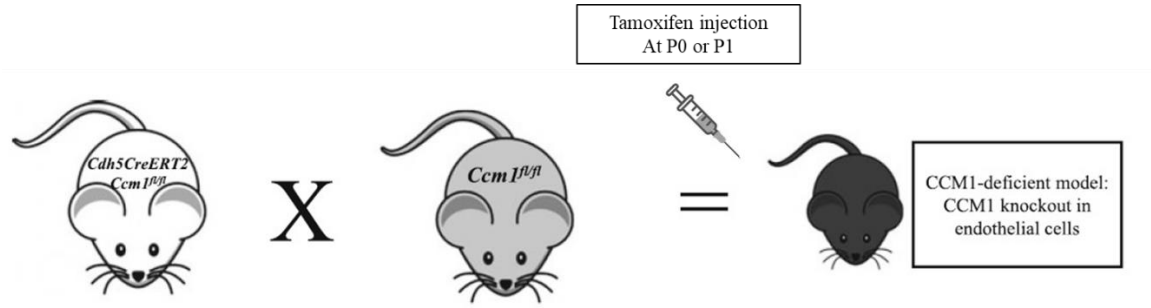


***Figure 2.1 Generation of offspring for experimental model of CCM**. - Breeding of Cdh5CreERT2*$^{Tg/+}$*. Ccm1*$^{fl/fl}$ *and Ccm1*$^{fl/fl}$ *parents resulted in newborn pups of both Cdh5CreERT2*$^{Tg/+}$*. Ccm1*$^{fl/fl}$ *and Ccm1*$^{fl/fl}$ *genotypes. All pups received tamoxifen at P0 or P1 intragastrically but only some get the lesions i.e. those carrying the Cre transgene.*

2.3.2 Induction of the CCM mouse model

Newborn pups received intragastric injection of ~220ug of 4-hydroxytamoxifen (4HT) (#H6278 Sigma Aldrich, United States) in 50 µl vehicle comprised of 45% ethanol (#E7023 Sigma Aldrich, United States) and 55% corn oil (#C8267 Sigma Aldrich, United States) as vehicle (185). The total injection volume was 50 µl per pup administrated by 0.5 ml Insulin syringe (#765914 BD Ultra-fine, New Jersey, United States). 4HT stocks were prepared by dissolution in 100% ethanol at 10mg/mL and stored at -80 degrees for up to one month. Working solutions were freshly prepared by diluting 5:6 in warm corn oil before injection. All the newborn pups were injected without knowledge of genotypes and gender.

The initiation of CCM lesions in pups carrying the *Cdh5CreERT2*$^{Tg/+}$.*Ccm1*$^{fl/fl}$ (CCM1i^{ECKO}) transgene is regularly observed around postnatal day 6 (P6), and maximum lesion numbers are established by week 2. From week 2 to week 6, there is a progressive increase in the volume of

all established lesions. At approximately six weeks of age, lesions are fully established both in terms of number and volume (103) (Figure 2.2).

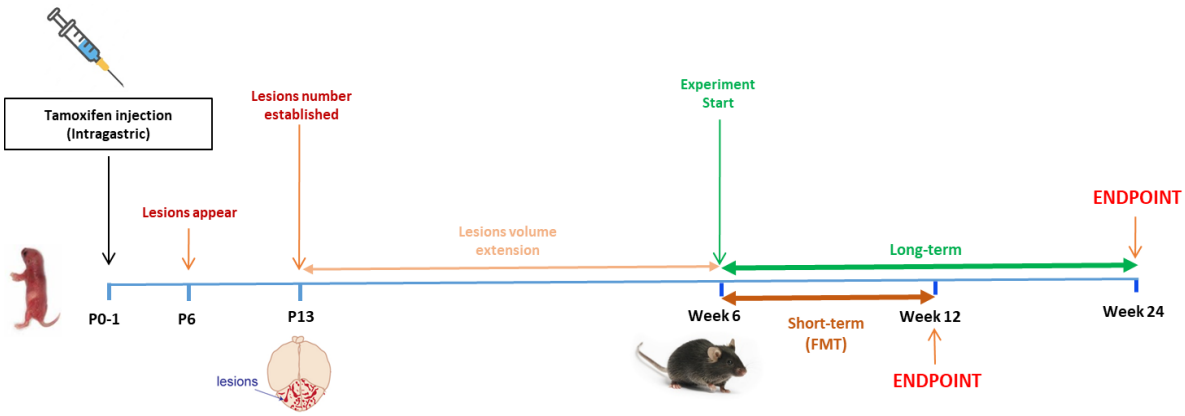


Figure 2.2 Mouse model of CCM disease and timeline of experimental interventions *- Neonatal mice are administered 4HT by intragastric injection. The initiation of CCM lesions in* $Cdh5CreERT2^{Tg/+}$. $Ccm1^{fl/fl}$ *mice was observed around postnatal day 6 (P6), and maximum lesion numbers were established by P13. From P13 to 6 weeks of age, there was a progressive increase in the volume of all established lesions. At approximately six weeks of age, lesions were fully established both in terms of number and volume, rendering it an appropriate time to commence experimental interventions. Diet modifications were initiated starting from week 6 and continued either for six weeks as a short-term intervention (brown, endpoint at week 12) or twelve weeks as a long-term intervention (green, endpoint at week 24). At the designated endpoint, the brains were harvested to analyse the burden of lesions.*

2.3.3 Dissection and sample collection (endpoint)

All experimental mice were euthanised via exposure to carbon dioxide (CO_2) for > 5 mins. All mice euthanised at 6 weeks or 12 weeks old were subjected to surgical dissection. Blood collection was performed by cardiac puncture, and centrifuged at 10,000 x g for 10 mins at 4°C to separate the serum and cells. Serum was collected and stored at -80 °C freezer. Intra-cardiac perfusion was performed with 5 ml of PBS solution (# 21600010, Life Technologies, United States). The brains were carefully dissected after cutting the skull gently and cutting the surrounding nerve cords. Images of fresh brain samples were taken immediately after dissection using a stereomicroscope (Leica M205FA) at standard settings of 7.82x magnification and 1x gain. Then, brain samples were kept overnight in formalin (#HT501320 Sigma Aldrich, United States) at 4°C. Other tissues such as testes /ovaries, ileum and colon, cecum content and femur were collected and snap frozen in liquid nitrogen, then stored at -80°C. For gut sample collection, the GI was sliced into three different sections: colon, cecum and ileum. Each unit was first flattened and after removing the intestinal content by washing with PBS and then rolled into a

cylinder with the mucosal layer facing inward. Finally, the coil was secured with a needle, fixed in formalin solution overnight, and then kept in 70% Ethanol for downstream histology processing.

2.3.4 Genotyping

Pups were genotyped prior to experimental intervention, at approximately 5 weeks of age, via ear samples. Tissue samples were lysed by adding 75 µl lysis buffer pH 11 (25mM NaOH, 0.2mM EDTA) and heating at 95°C for 30mins using a Thermoblock. Afterwards, 75 µl Neutralisation buffer pH 5 (40mM Tris-HCl) was added to each sample to balance pH. We used 10 µl GoTaq® Green Master Mix (Promega, M7122, Wisconsin, United States), 0.6 µl forward and reverse primers 10 µM (Cre and CCM1 Table 2.1), 4.8 Nuclease-free water UltraPure™ DNase/RNase-free distilled water (#10977023, Thermo Fischer Scientific, United States) and 4 µl DNA sample so that the total volume should be 20 µl per well. The PCR cycles were adjusted according to Table 2.2. The amplicons were separated by electrophoresis with 2% agarose gel (#1613101 Bio-Rad Laboratories, California, United States) run at 110V for 45 mins. In the Cre primer PCR analysis, it was observed that the *Cdh5CreERT2Ccm1fl/fl* samples displayed a single PCR band with an approximate size of 500 base pairs (bps), while no PCR band was detected in the *Ccm1fl/fl* samples. On the other hand, in the CCM1 primer PCR, the $Ccm1^{fl/+}$ samples exhibited two distinct bands of varying sizes, approximately 150 bps and 300 bps. In contrast, the $Ccm1^{fl/fl}$ 1 samples demonstrated a single band of 300 bps, while the *ccm1*+/+ or wild type samples exhibited only one band with an approximate size of 150 bps. We used a 100 bp DNA Ladder for the quantification of bands (Fig 2.3).

Primers	Sequences
Cre-Forward	GAACCTGATGGACATGTTCAGGGA
Cre-Reverse	ATTCTCCCACCGTCAGTACG
CCM1-Forward	TGTCTCCATTCCCTCCTCCCTAC
CCM1-Reverse	AAACCAGCAGTCTCAACTAATCGG

Table 2-1 ***Cre and CCM1 primers***

Temperature °C	Time	Cycles
94	2 min	1
94 61 72	30 s 30 s 30 s	32
72	2 min	1

Table 2-2 ***PCR cycle setting***

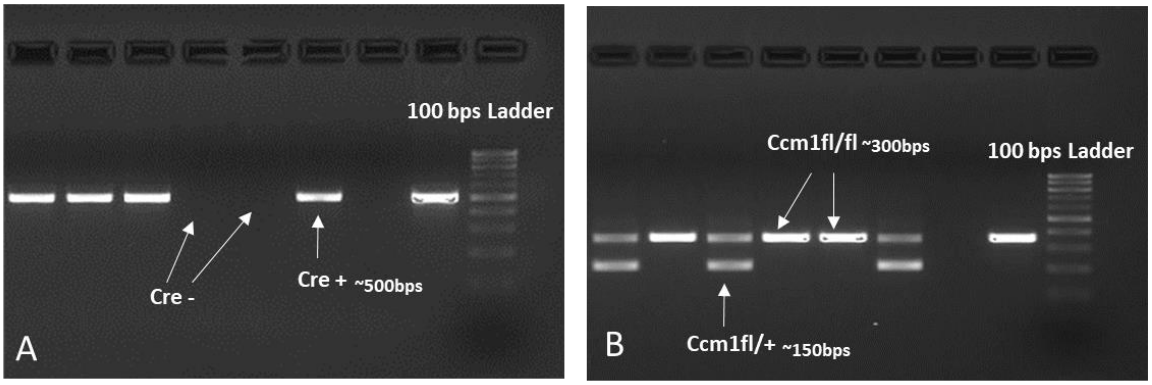


Figure 2.3 Gel electrophoresis separation for genotyping offspring mice - *(A) the PCR product is the presence of the Cdh5CreErt2Ccm1fl/fl genotype, (B) ccm1fl/+ showed two different size bands (~150bps, ~300bps), one PCR product results from the flexed allele and one PCR product results from the WT allele (left). Ccm1fl/fl showed only one band at 300bps and WT show only on 150bps.*

2.4. Micro-computed tomography (micro-CT)

2.4.1 Preparation of samples for Micro-CT

Dissected brain samples were submerged overnight in 10% neutral buffer formalin (#HT501320 Sigma Aldrich, United States) in PBS (# 21600010, Life Technologies, United States). After fixation, the hindbrain and forebrain were separated and incubated in aqueous Lugol's iodine solution (Sigma Chemical, L-6146, Steinheim, Germany) for 72 hours on a shaker. Then, soaked samples were removed gently, and excess Lugol's staining solution was discarded. The stained hindbrains and forebrains were placed into 1.5 ml Eppendorf tubes separately, which were sealed tightly with PARAFILM® M (#P7793, Sigma Aldrich, United States) to prohibit loss of volume of samples by evaporation and tissue shrinkage. (Fig 2.4)

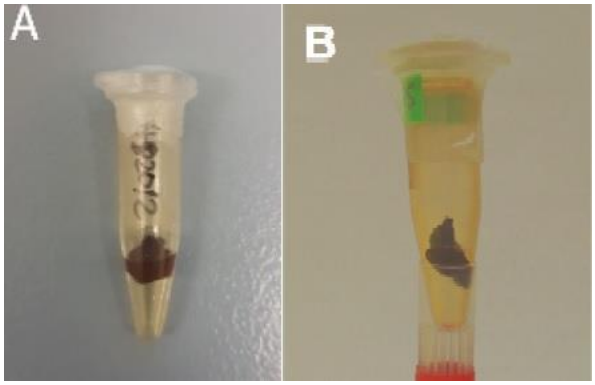


Figure 2.4 Brain samples packed in microcentrifuge tubes (A, B).

2.4.2 SkyScan 1272

In this project, we used one of the trustable Micro-CT devices, SkyScan 1272 (Skyscan, Bruker, Belgium), which performs a reliable resolution in vascular imaging in hard and soft tissues (59).

Figure 2.5 Skyscan 1272 with a sample changer in the top *– sample changer can set 16 samples in one run.*

2.4.3 Micro-CT scan of the mouse brain

We put the Eppendorf tubes containing hindbrains or forebrain on the sample holder (Rotation table. Fig 2.6), and used soft pressure-sensitive adhesive material (orthodontic wax) to fix tubes in place. After optimisation of the scanning parameters for brain samples to gain relevant scanning results, we thereafter used consistently 2.5 µm resolution with source conditions of 70 kV and 0.5 mm Aluminium filter in binning 1 to acquire an appropriate tomographic model. The scanning data are comprised of a series of axial image slices (TXM file, voxel size of 10×10×10 µm) that is imported to NRecon Reconstruction Software (SkyScan, Antwerp, Belgium) to convert to a 3D visualisation and topographic model, which permits the analysis of lesion burden within the brain.

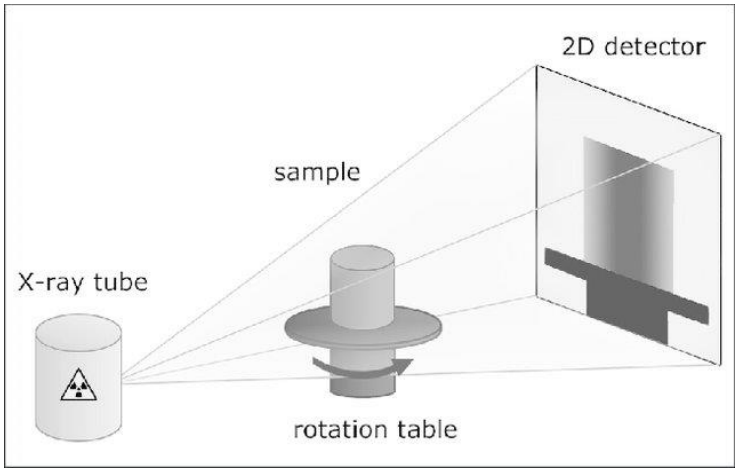


Figure 2.6 Schematic picture of how Micro-CT works (187)

2.4.4 Analysis of micro-CT tomography to determine CCM lesion burden

We used Aviso 3D visualisation and analysis software (Lite edition, FEI Visualization Sciences Group) to analyse micro-CT data. CCM lesions within brain tissue were labelled within each reconstructed topographic model according to an established protocol (186). Briefly, the 3D axial data were brought into Avizo 3D, and the image sequences were displayed in the XY, YZ, and XZ planes. To achieve a comparable view, digital rotation was applied to some of the reconstructed images as needed. The 3D reconstructions were divided into the desired planes through virtual sectioning, and 2D images were taken from these ortho-slices to show the distribution of lesions and the brain's structure. The raw image stacks from the scanned hindbrains were evaluated for identifying lesions. The lesions were recognised and marked using a threshold of greyscale intensity and the feature's shape. An included region-growing segmentation algorithm in the software called Magic Wand was utilised to label individual lesions through multiple cross-sections. After labelling the lesions, the hindbrain image stack was rendered as an isosurface volume, and the labelled lesions were superimposed in Avizo. The final step was to adjust the 3D rendered and segmented hindbrain model by cropping and rotating it within the program to get the desired significant spatial view, to effectively delineate the spatial relationship between the lesions and the hindbrain (Fig 2.7). Notably, these last visual modifications were made after lesions had been labelled and did not affect the detection or visual representation of the lesions. In each scanned brain sample, we determined the number of formed lesions and analysed all lesions by volume in cubic micrometres.

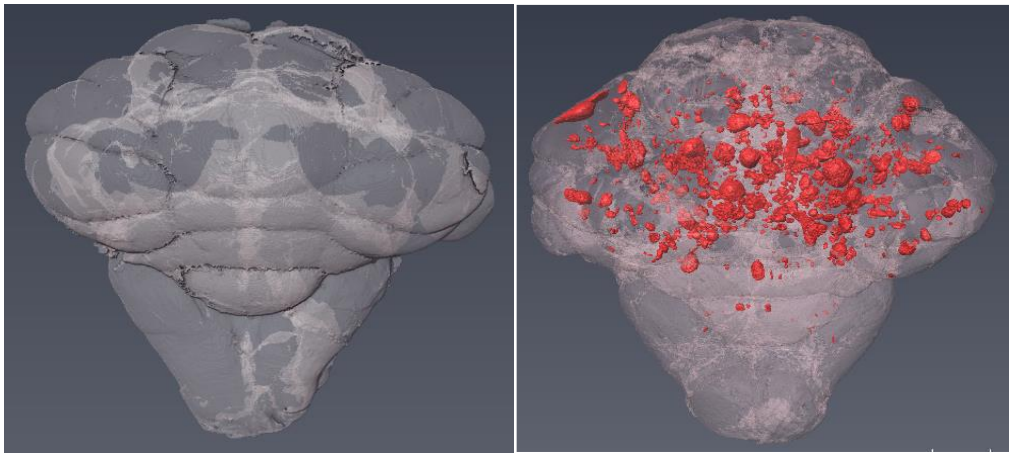


Figure 2.7 3D reconstructed topographic model of hindbrain before and after lesions marking *- the white transparent topographic shape represents brain tissue and the red dots represents lesions inside the brain.*

2.5. Histology

2.5.1 Sample processing for histological analysis

We performed Swiss Roll as gut histopathological sample collection according to the protocol described (188-190). GI including duodenum, jejunum, ileum, cecum and colon were extracted. We removed the ileum and colon, cut them longitudinally, and coiled them with direction from anus to cecum for the colon and cecum to jejunum for ileum in the mucosal layer inwards using a needle stick. Then, they were fixed in 10% neutral buffer formalin for 24 hours.

After fixation, tissue samples were replaced with 10% ethanol (#E7023, Sigma Aldrich, United States) in PBS (# 21600010, Life Technologies, United States), and samples were processed by (Histocore PEARL, Leica, Wetzlar, Germany) via dehydration steps of increasing concentration of ethanol from 50%, 60%, 70%, 80%, 90% and 100% followed by Xylene (#VWRC28975.325, Biostrategy, Australia). Subsequently, samples were embedded in parablast to make paraffin blocks (Fig 2.8). Blocks were sectioned using a microtome (# RM 2245 Leica, Wetzlar, Germany): the gut was sectioned in 4 μm slices (191)(Fig 2.9).

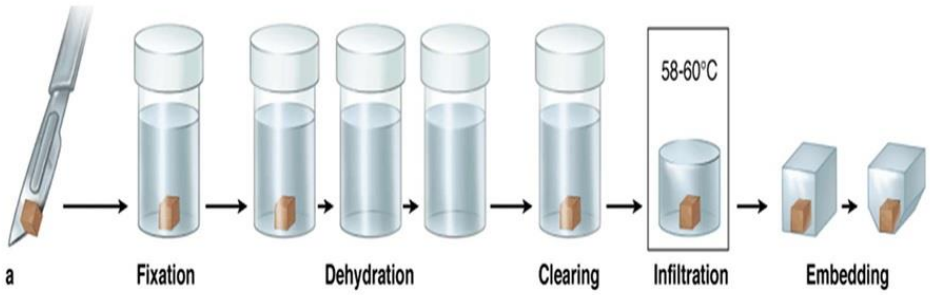


Figure 2.8 Diagram of tissue processing from collecting tissue to embedding (192)

2.5.2 Hematoxylin-Eosin stain (H & E)

The slides were submerged into Xylene for 10 mins twice for dewaxing sections. Thenceforward were put in ethanol from 100% to 50% for 5 mins, respectively. Then, they were rehydrated in PBS. All sections were washed with water 5 times after 10 mins incubation in hematoxylin nuclear stain (#H3136, Sigma Aldrich, United States). Then, they were stained with eosin as contrasting colour and rewashed for three 5 mins times (193). Eventually, all sides were mounted by media (#IM0225, ProSciTech, Australia), covered with coverslips (#CS24X50, Living Stone, Australia), and kept overnight for air-drying.

2.5.3 Periodic acid–Schiff (PAS)

PAS staining is commonly used to identify acidic and neutral mucins in tissue sections. This staining has different applications in diagnosis. To analyse the number of goblet cells in gut samples, we performed PAS (Periodic Acid Schiff) Staining on one of the sectioning slides (7). Firstly, the sections were treated with periodic acid (#395132, Sigma Aldrich, United States) for 5 minutes and then washed with distilled water. After, the slides were stained with Schiff's reagent (#3952016, Sigma Aldrich, United States) as counterstaining for 10 minutes. Finally, they washed with tap water to wipe off the excess stain. Finally, all slides were dehydrated and mounted and kept air-dry overnight.

2.5.4 Histological imaging

For imaging of histology slides, we used an Axio Imager microscope (Zeiss, Germany) equipped with a camera (Zeiss Axiocam ICm1, Carl Zeiss AG, Germany) using analysis software Zen Imaging (Zen 3.0 blue edition, Carl Zeiss Microscopy).

2.5.5 Analysis of gut samples (colon and ileum)

I. Quantification of the epithelial and muscle layer thickness and villus length of the ileum and colon

One H&E stained slide for each sample was imaged using Zen Imaging Software, once in 2.5X magnification for the whole section, as well as four images of random sections in 20X magnification (Fig 2.9). For the measurement of layers and villus length, we used ImageJ software: drawing a straight line along the diameter layers from the tip of the villus to the end of the crypt (194). The measurement was captured in pixels and converted to µm.

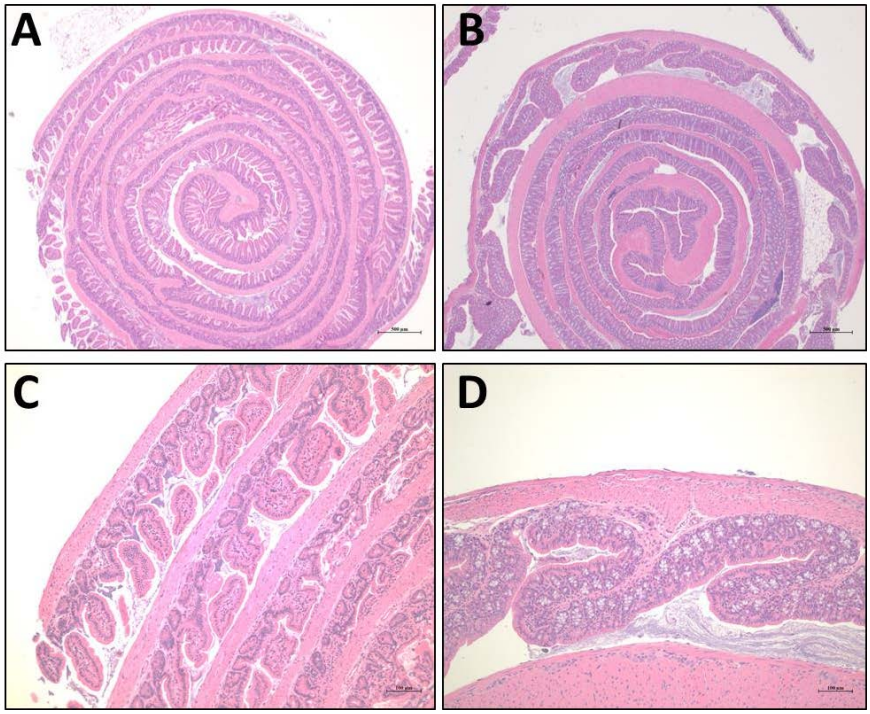


Figure 2.9 Hematoxylin and Eosin stained (H&E) *- image of entire section of ileum (A) and colon (B) sample and examples of the random area chosen for measurement of (C) ileum and (D) colon thickness.*

II. Quantification of goblet cells per villus in the ileum

Stained slides were imaged using Zen Imaging Software at 40X magnification. Images must contain the whole villi's length for accurate counting of goblet cells per villus. We used the point tab in ImageJ to mark goblet cells, and eventually, the number of villi to acquire an average (Fig 2.10) number of goblet cells per villus. The number of goblet cells of minimum three villi per sample were counted.

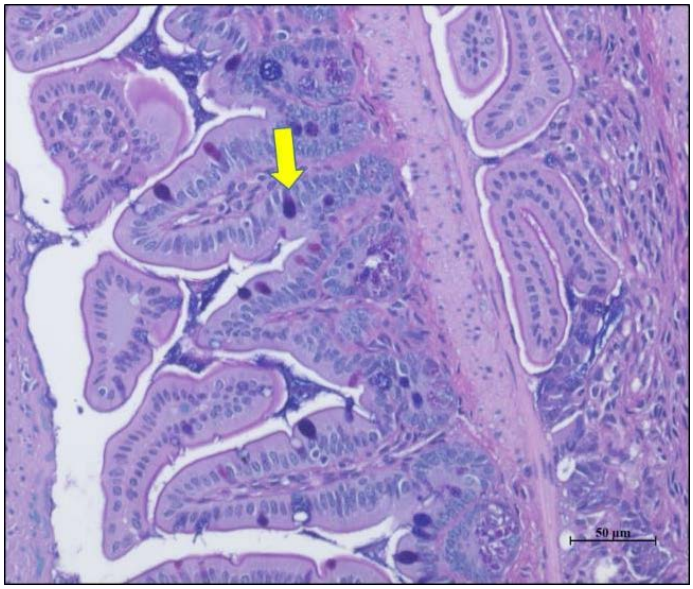


Figure 2.10 PAS staining of ileum for gablet cells counting *- Yellow arrow shows goblet cell*

2.6. LPS quantification

To measure lipopolysaccharide (LPS) endotoxin in serum samples, we used Pierce™ Chromogenic Endotoxin Quant Kit (#A39553, ThermoFisher Scientific, USA). This kit works based on The Limulus Amebocyte Lysate (LAL) assay method for quantifying LPS. The standard dilution was prepared for Low-standards ranging from 0.01 EU/mL to 0.1 EU/mL. Samples were pre-diluted 50-fold to be compatible and heated at 70°C for 15 minutes. Following the addition of 50 µL of standard solutions, blanks, and samples per well, a 50 µL volume of reconstituted Amebocyte Lysate Reagent was introduced into each well, followed by incubation at 37°C on a plate heater for 20 minutes. Subsequently, a 100 µL volume of Chromogenic substrate solution was added to each well and allowed to react for 6 minutes. To terminate the reaction, 50 µL of 25% acetic acid was added to each well, and the optical density (OD) at 405 nm was promptly measured upon completion of the assay. All LPS quantifications were performed using pyrogen-free tubes and pipette tips and within a biosafety cabinet hood to ensure no contamination.

2.7. Diet modification

2.7.1 Short-term and long-term dietary interventions

Pups of one litter of *CCM1i*^{ECKO} mice (*C57BL/6 background)* were weaned at approximately three weeks old, and after genotyping and gender identification, were separated at six weeks old. The pups that did not carry the transgene genotype (Cre-) were euthanized. Within each litter, pups were divided into two different cages: one supplied with standard mice chow (control diet), and another provided specific diets of interest (diet intervention). To account for the potential influence of different breeding pairs, age, and gender on CCM lesion progression, all pups from a single breeding pair were organised into both control and diet intervention groups based on gender. This ensures that each control and diet intervention group shares the same age and gender (either male or female) and originates from the same breeding pair. Moreover, to variation in CCM lesion burden induction, the Tamoxifen injection of newborn pups is consistently administered by the same person. Consequently, each diet intervention group is accompanied by its own set of control groups. The cage separation was maintained and continued the diet intervention until achieving a total of eight mice per group for both males and females. This meticulous approach aims to control for potential confounding variables and enhance the reliability of our study outcomes. All pups were weighed before cage separation, and we endeavoured to control for weight differences between control and diet intervention groups at onset by placing mice with approximately similar weights into different experimental cages. Dietary intervention experiments lasted either for six weeks (short-term, Fig 2.11) or twelve weeks (long-term, Fig 2.12). In this study, we deliberately selected both high-fibre and low-fibre diets to discern the specific impact of high-resistant starch content on our CCM mice model. Given the numerous existing studies indicating the detrimental effects of high fat diets on the cerebrovascular system, our focus on high-fibre diets aims to investigate a dietary modification that might offer potential benefits. Prior research has consistently demonstrated that dietary modifications, particularly the introduction of a high fat diet, can induce significant alterations in gut microbiome composition as early as two weeks from the initiation of dietary changes (195-197). However, for achieving a more stabilized and representative gut microbiome profile, an intervention period of 6 to 12 weeks is deemed ideal (198). Considering the high mortality observed in our CCM1iECKO mice as they age, we opted for a pragmatic approach, implementing a six-week duration for short-term interventions and extending it to 12 weeks for long-term dietary interventions. This duration selection aims to strike a balance between

achieving meaningful changes in gut microbiome composition and accommodating the lifespan limitations of the CCM1iECKO mice in our model.

Mice were weighed weekly at a set time to track weight gain or loss during the experiment. All diets were irradiated before import into the SPF facility, stored at -20°C, and were left to warm to room temperature two days before use. Food and mice cages were changed every two weeks to avoid any contamination and bacterial activities in the diet and mice cage that can have an effect on gut microbiome profile. Like the short-term diet intervention, the mice were separated into experimental cages at approximately six weeks old. The long-term model lasted for 12 weeks, and the weight and health of the mice were checked weekly at a particular time (Fig 2.12).

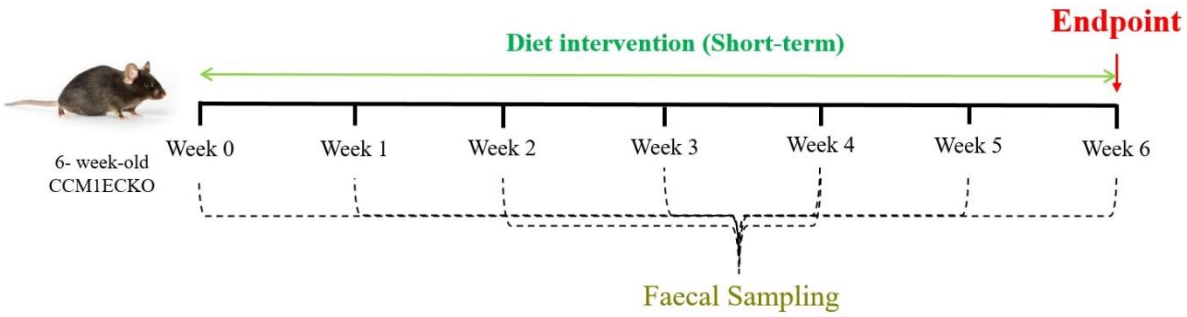


Figure 2.11 Timeline of CCM mice model for diet modifications (short-term) Six weeks of diet modification on experimental mouse model of CCM - *Throughout this period, faecal samples were collected once every week at a specific designated time.*

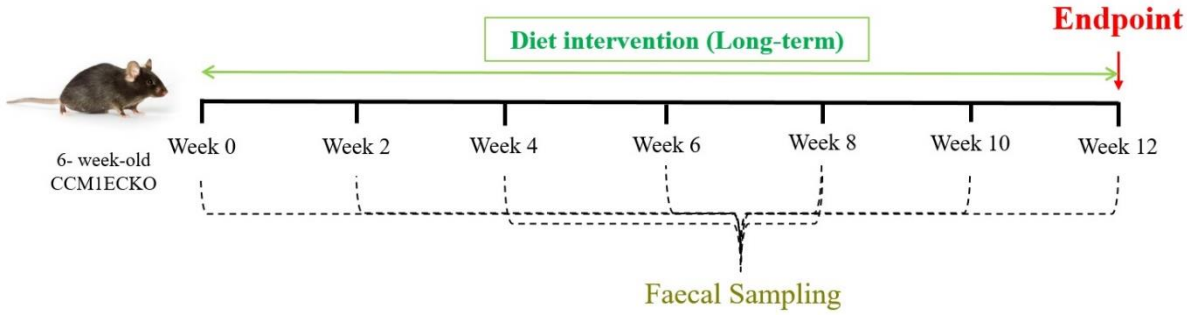


Figure 2.12 Timeline of CCM mice model for diet modifications (Long-term) twelve weeks of diet modification on experimental mouse model of CCM *- Throughout this period, faecal samples were collected once every two weeks at a specific designated time.*

2.7.2 The control diet (standard chow)

For control groups, except when controlling for the high-fibre diet (see below), we used normal standard chow (Irradiated Rat and Mouse Diet, Specialty Feeds Co, Glen Forrest, WA, Australia). The standard chow diet provides a fixed formulation of a diet fortified with vitamins and minerals to cover all requirements for breeding laboratory Rat and Mice animals. All nutritional parameters of this diet meet or exceed the NRC guidelines for Rats and Mice (Table 2.3). The total fat percentage of this diet is low, around 5%.

Ingredients	Standard Chow
Casein (Acid)	200 g/Kg
Sucrose	100 g/Kg
Canola Oil	70 g/kg
Cellulose	50 g/kg
L Methionine	3.0 g/kg
Calcium Carbonate	13.1 g/kg
Sodium Chloride	2.6 g/kg
AIN93 Trace Minerals	1.4 g/kg
Potassium Citrate	2.5 g/kg
Potassium Dihydrogen Phosphate	6.9 g/kg
Potassium Sulphate	1.6 g/kg
Choline Chloride (75%)	2.5 g/kg
AIN93 Vitamins	15 g/kg
Vitamin K Supplement	0.87 g/kg

Table 2-3 Ingredients of control (standard chow)

2.7.3 High-fibre diet

We employed Modified AIN93G Rodent Diet with Gel crisp starch (SF20-099, Specialty Feeds Co, Glen Forrest, WA, Australia) as a high-fibre diet. All carbohydrates in this diet are replaced with Gel Crisp/Crisp Film®, a type of high amylose resistant starch from maize (corn). The diets have been irradiated before import into the SPF facility to decrease the risk of unwanted contamination. All vitamin amounts increased as a side effect of the irradiation. As a control diet for this high-fibre diet modification, we used Standard AIN93G Rodent Diet (SF09-091, Specialty Feeds Co, Glen Forrest, WA, Australia), which contains wheat and dextrinised starch instead of Gel crisp starch (Table 2.4).

Ingredients	SF09-091 (Control)	SF20-099 (high Fibre diet)
Casein (Acid)	200 g/Kg	200 g/Kg
Gel Crisp Starch		636 g/Kg
Sucrose	100 g/Kg	
Canola Oil	70 g/Kg	70 g/Kg
Cellulose	50 g/Kg	50 g/Kg
Wheat Starch	399 g/Kg	
Dextrinised Starch	132 g/Kg	
L Methionine	3.0 g/Kg	3.0 g/Kg
Calcium Carbonate	13.1 g/Kg	13.1 g/Kg
Sodium Chloride	2.6 g/Kg	2.6 g/Kg
AIN93 Trace Minerals	1.4 g/Kg	1.4 g/Kg
Potassium Citrate	2.5 g/Kg	2.5 g/Kg
Potassium Dihydrogen Phosphate	6.9 g/Kg	6.9 g/Kg
Potassium Sulphate	1.6 g/Kg	1.6 g/Kg
Choline Chloride (75%)	2.5 g/Kg	2.5 g/Kg
AIN93 Vitamins	15 g/Kg	15 g/Kg
Vitamin K Supplement	0.87 g/Kg	0.087 g/Kg

Table 2-4 Ingredients of standard high-fibre diet and control

2.7.4 Low fibre diet

We used a semi-pure diet formulation for mice based on AIN93G Rodent Diet without adding fibre and starch (SF09-028, Specialty Feeds Co, Glen Forrest, WA, Australia) as low fibre diet in this study. This diet contains only dextrose instead of all fibre, starch and dextrinised starch (Table). 19.4% protein, 7% total fat and 0% crude fibre are nutritional parameters percentage of this diet.

Ingredients	SF09-028 (Low fibre)	Standard Chow (control)
Casein (Acid)	200 g/Kg	200 g/Kg
Sucrose		100 g/Kg
Dextrose Monohydrate	686 g/kg	
Canola Oil	70 g/Kg	70 g/kg
Cellulose		50 g/kg
L Methionine	3.0 g/Kg	3.0 g/kg
Calcium Carbonate	13.1 g/Kg	13.1 g/kg
Sodium Chloride	2.6 g/Kg	2.6 g/kg
AIN93 Trace Minerals	1.4 g/Kg	1.4 g/kg
Potassium Citrate	2.5 g/Kg	2.5 g/kg
Potassium Dihydrogen Phosphat	6.9 g/Kg	6.9 g/kg
Potassium Sulphate	1.6 g/Kg	1.6 g/kg
Choline Chloride (75%)	2.5 g/Kg	2.5 g/kg
AIN93 Vitamins	10 g/Kg	15 g/kg

Table 2-5 Ingredients of low fibre diet (SF09-028) and standard chow (Control)

2.7.5 High fat diet

Laboratory rat and mouse diet AIN-93G modified with very high fat (SF14-154, Specialty Feeds Co, Glen Forrest, WA, Australia) was utilised as a high fat diet in this project. This diet's total fat percentage has increased to 36%, providing 59.5% of total consumed energy from lipids. Sucrose is the only source of carbohydrates in this diet, and most of the starch and fibre is removed. The ratio of vitamins and minerals has been expanded, considering the reduction of food intake because of high energy density. 24% of Cocoa butter and 6% Hydrogenated Vegetable Oil are the fat sources in this diet (Table 2.6). In terms of nutritional parameters percentage, this diet comprises 19.5% protein, 36% total fat and 4.7% crude fibre.

Ingredients	SF14-154 (High fat diet)	Standard Chow (control)
Casein (Acid)	200 g/Kg	200 g/Kg
Sucrose	320 g/Kg	100 g/Kg
Canola Oil	60 g/Kg	70 g/kg
Cocoa Butter	240 g/Kg	
Hydrogenated Vegetable Oil	60 g/Kg	
Cellulose	50 g/Kg	50 g/kg
L Methionine	4.2 g/Kg	3.0 g/kg
Calcium Carbonate	18.3 g/Kg	13.1 g/kg
Sodium Chloride	3.6 g/Kg	2.6 g/kg
AIN93 Trace Minerals	2.0 g/Kg	1.4 g/kg
Potassium Citrate	3.5 g/Kg	2.5 g/kg
Potassium Dihydrogen Phosphat	9.7 g/Kg	6.9 g/kg
Potassium Sulphate	2.2 g/Kg	1.6 g/kg
Choline Chloride (75%)	3.5 g/Kg	2.5 g/kg
AIN93 Vitamins	21 g/Kg	15 g/kg
Vitamin K Supplement	1.7 g/Kg	0.87 g/kg
Antioxidant (Oxicap E2)	0.04 g/Kg	

Table 2-6 Ingredients of high fat diet (SF14-154) and standard chow (Control)

2.8. Faecal samples collection

All faecal samples were collected at the specified time of day between 11 am to 1 pm. Mice were handled and restrained merely by holding the tails to limit stress during sample collection. Faeces were collected from restrained mice directly into sterile, autoclaved 1.5 ml Safe-Lock Eppendorf Tubes (Catalogue No. 0030120086, Eppendorf, Hamburg, Germany). Faeces pellets were snap frozen in liquid nitrogen immediately and were kept at -80°C until further analysis. Faecal sample collection was performed every week on a specific day for each experiment, and a minimum of two pellets were collected from each mouse separately.

2.9. Faecal Matter transplant (FMT)

FMT from female mice receiving high fat diet to animals on standard chow and without dietary intervention was performed on CCM1i^{ECKO} mice to determine if FMT can recapitulate diet-induced changes in microbiome composition, and effects on CCM disease. In this model, we used real-time FMT, and all groups were approximately the same age at the start of the experiment (six weeks old). 6-week old CCM1i^{ECKO} female mice were divided into three groups: control (C), donor (D), recipient (R). To effectively control the experimental strategy and effects of FMT procedure alone, all groups were administered FMT by oral gavage twice per week at a set time for each experimental group at intervals of 3-4 days. Earlier studies have indicated that oral

gavage administration induces more pronounced alterations in the gut microbiome compared to other methods such as cage swapping (199, 200). Additionally, performing two or one faecal microbiota transplantation (FMT) per week results in a significant and drastic change in the microbiome profile (201). Given that our study involves real-time FMT from donor to recipient mice, we find that a maximum 4-day interval between FMT sessions is preferable. Control and donor group mice received self-FMT prepared from their own faeces. Recipient group mice were administrated HF-FMT (faeces from the donor group). FMT was performed for all groups twice weekly for a six-week course (Fig 2.13). Alongside, faecal samples were collected for all groups to track microbiome profile changes.

All faeces' samples were collected and prepared, and oral gavage was administered on the same day with a maximum of 2 hours' time duration from sample collection. We used only fresh samples. FMT sample preparation was performed under anaerobic conditions using an anaerobic chamber and anaerobic PBS with 15% glycerol (#G5516, Sigma Aldrich, United States) (PBS left inside the anaerobic chamber for two days). Faeces pellets were first weighed, then added to anaerobic PBS to produce a slurry at 0.1 g/ml concentration. Slurries were centrifuged on 10g briefly for one minute to separate supernatant from other debris in the faeces. Finally, 100 µl of supernatant was administrated to mice orally using dedicated oral gavage needles.

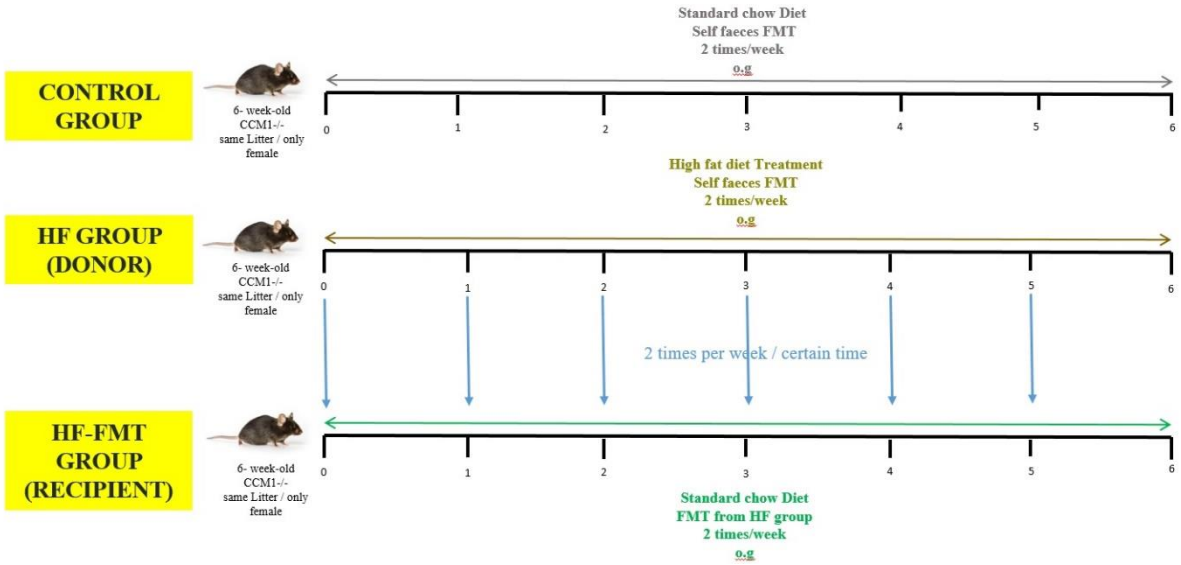


Figure 2.13 Faecal Matter Transplant (FMT) timeline *- This model involved the design of three distinct groups comprising female mice of identical age and originating from the same litter. The groups were categorized as control, donor, and recipient. The control and recipient groups were fed a standard chow diet, while the donor group received a high fat diet (HF) modification. Both the control and donor groups underwent FMT using their own faecal material, while the recipient group received FMT from donors of the same age. FMT was administered orally via gavage twice*

a week for a duration of six weeks, adhering to a specific time schedule. All faecal samples used in this model were fresh and processed within a maximum timeframe of two hours from collection to oral administration o.g. oral gavage.

2.10. Omics analysis

2.10.1 Metagenomics

Metagenomic sequencing was conducted at the Ramaciotti Centre. The preparation of libraries was accomplished utilizing the Nextera DNA Library Preparation Kit (Illumina, USA). Subsequently, the samples were sequenced on an Illumina NextSeq500 instrument, resulting in an average of 2 Gbp of 150 bp paired-end reads per sample. In order to remove any potential contamination from the host organism, the sequencing reads were aligned to the mouse genome (Mus musculus GRCm38.p5) using BWA v0.7.12. For a read to be classified as originating from the mouse, it had to exhibit a minimum alignment length of 30 bases and allow a maximum of 15 clipped bases. Raw reads devoid of host contamination obtained from each sample were subjected to assembly using Spades v3.12.0 (202) incorporating the --meta flag. The resulting assemblies were then utilised as references for read mapping using BamM v1.7.3 (available at https://github.com/ecogenomics/BamM). Subsequently, bins were generated from the mapped reads using Metabat v2.12.1 (203), MaxBin (204)and Concoct (205). Then Dastool was employed to combine the results obtained from the binning processes. To evaluate the level of contamination and completeness for the generated bins across all samples, CheckM v1.0.11 was utilized (206). Reads for each sample were mapped using Metaphlan 4.0.3 recovered MAGs for assessment of community composition. The relative abundance was determined by calculating the read counts as a proportion of the total non-host reads per sample, utilising the Metaplan diversity function. Alpha-diversity measure Shannon index are plotted in R studio using Vegan package. Lefse Analysis performed in Galaxy(version) Principal component analysis was conducted using the R package vegan v2.5-1 (207). PERMANOVA was performed with the adonis function within vegan using the Bray-Curtis dissimilarity index. Differential abundance between sample groups was determined using DESeq2 v1.20.0 (208) using read counts. Samples with missing scores were removed.

2.10.2 Metabolomics and lipidomics LC-MS analyses

Slurry cecum content samples from standard chow and high fat groups were processed for polar metabolites and lipids fractionation using the biphasic solvent-solvent fractionation procedure reported in the literature for further LC-MS analyses (209). LC-MS/MS analysis was conducted

using an Agilent 1290 High-Performance Liquid Chromatography (HPLC) system coupled with an Agilent 6560 triple quadrupole mass spectrometer, equipped with a dual AJS electrospray ion source (ESI). Data acquisition was performed using Agilent MassHunter Data Acquisition software (version B09.00). The samples were analysed in both positive and negative ion modes, employing data-dependent AutoMSMS acquisition method with specific parameter settings. Narrow isolation width MS/MS of ~1.3 amu, five maximum precursors per cycle with activated active exclusion after 1 spectrum for 0.5 min. Gas and sheath gas temperature was set at 300 °C, 5 L/min gas flow, and 12 L/min sheath gas flow, together with 3500 or 4500 V for capillary voltage in negative and positive modes, respectively with Agilent TOF reference mass solution kit (G1969-85001) parallel infused to calibrate masses.

For lipidomics, a 0.26 ml/min flow rate was implemented with 60% acetonitrile in water, and 90% isopropanol in acetonitrile were used as mobile phases A and B, respectively, with 10mM ammonium formate and 0.1% formic acid. The following gradient was used; 32% B was kept from 0-1.5 min, 45- 52% B from 4-5 min, 58-66% B at 8-11 min, 70-75% B at 14-18 and 97%B kept from 21-25 min followed by 5 min conditioning at 32%B. ACQUITY UPLC CSH Column (1.7 µm, 2.1 X 100 mm, Waters Corporation, Milford, USA) with a 2.1 X 5mm CSH VanGurd™ PreColumn (Waters Corporation, Milford, USA) was used, and the column temperature was kept at 60 °C. Raw data were processed with MSDIAL version 4.92 (210), and the internal lipidomics spectral library was utilised for lipid annotations where significant features (Absolute Log2 fold change ≥ 2 and FDR ≤ 0.001) among groups were retained. Metaboanalyst 5.0 was used for statistical testing and multivariate data visualisation and filtering (211).

The metabolomic analysis was performed on the same hardware setup with ACQUITY UPLC BEH Amide HILIC Column (1.7 µm, 2.1 X 100 mm, Waters Corporation, Milford, USA) kept at 25 °C at a flow rate of 0.25 ml/min. Water and 85 % acetonitrile were utilised as mobile phases A and B, respectively, with ten mM ammonium formate and either 0.1% formic acid or ammonia-adjusted PH at 8 for positive and negative modes. 100% B was kept for 5min, 12-16 min (70-60% B), 17-18 min (5%B), returned to 100%B at 19 min and kept for 1 min with 2 min post run equilibration. Acquired data were converted to MzML format using Proteowizard (MSConvertGUI) software and processed using Progenesis QI (PQI) software (Waters Corp., USA) for data processing as reported in the literature (212-216). In summary, features were deemed as reproducible if their coefficient of variation (CV) across the samples was less than 30%. Additionally, they were required to have ANOVA p-values and Q values (adjusted p-values calculated using an optimized False Discovery Rate [FDR]) of less than 0.01 when compared to blank samples. The Putative Query Ion (PQI) method was utilized to potentially identify metabolites of interest by comparing them with publicly available databases, including HMDB, MONA, LipidMaps, and the PQI-embedded METLIN v.2019 library.

2.11. Statistical analysis

The experimental findings are presented visually using the mean ± standard error (SEM or SD). ToN determine statistically significant differences between groups, an unpaired t-test was employed for two-group comparisons, while One-way or Two-way ANOVA was used for comparisons involving more than two experimental groups. Tukey's multiple comparisons post-test was performed to further analyse the significant differences and was conducted a normality test using D'Agostino-Pearson omnibus (K2) and Anderson-Darling test. All statistical analyses were conducted using GraphPad Prism version 9.0 for Windows (GraphPad Prism software, San Diego, California, United States, www.graphpad.com). Results were considered statistically significant when the p-value was equal to or less than 0.05.

3. Chapter 3: Defining the effect of dietary modifications on Cerebral cavernous malformation (CCM) using an experimental mouse model

3.1. Abstract

CCM is a vascular malformation primarily observed in the CNS. Recent investigations have indicated a possible association between lipopolysaccharide (LPS) derived from gram-negative bacteria (GNB) residing in the GI and the advancement of CCM disease. This interaction is proposed to occur via TLR4 stimulation, implicating a potential pathway in the disease progression. We aimed to determine the effects of changing gut microbiome composition on CCM disease, using an experimental mouse model of CCM. Diet plays a crucial role in determining gut microbiome composition: we therefore treated mice with 6-week dietary modifications, namely high-fibre, low-fibre or high fat compared to standard control diets. Although the high-fibre diet led to thickening of the muscle layer and an increase in goblet cells in the ileum and low-fibre diet was associated with thinning of the colon epithelium, no significant change was observed in CCM lesion burden in the brains of mice on high- or low-fibre diets compared to controls. In contrast, female mice placed on a high fat diet showed a reduction in CCM lesion volume and number within the brain. These results indicate that short-term dietary fibres do not affect CCM disease manifestations; whereas a high fat diet has a potentially beneficial effect, whether this be through alteration of the gut microbiome or other metabolic mechanisms.

3.2. Introduction

CCM or Cavernous hemangioma is a cerebrovascular malformation that forms mulberry shaped lesions predominantly in the brain (217). Moreover, the lesions can be created in other body parts like the skin, spinal cord and retina (218-220). Although CCM is considered a prevailing vascular malformation with 0.5% prevalence worldwide (15, 16), most patients will be asymptomatic for their whole life. Only 20% to 30% of patients will experience clinical symptoms, usually emerging in middle age (3). Surgery is the only treatment option for patients where a lesion becomes untenable owing to size and/or location. Therefore, we urgently need to find a non-surgical alternative therapy to hinder CCM lesion development and progression in patients (14).

In a recent study utilising a mouse model of CCM, it was proposed that lipopolysaccharide (LPS), a prominent constituent of the outer membrane in GNB residing in the GI, possesses the ability

to traverse the gut-blood barrier. Once in the bloodstream, LPS can activate TLR4 on the surface of brain endothelial cells. This activation pathway has been implicated in potentially exacerbating the burden of CCM lesions in the mouse brain (22). Moreover, these authors showed the increased expression of the *TLR4* gene as a result of genetic polymorphisms could accelerate the CCM lesion burden in patients (22). Several studies have evaluated alterations in gut microbiome composition by way of diet substitution or modification (221-223). In particular, increasing TAMO produced by gut bacteria in the context of a high protein diet was linked with exacerbation of atherosclerotic burden in a mouse model (224). Furthermore, some investigations have revealed the role of components of the gut microbiome, and their metabolites, in influencing cerebrovascular and nervous inflammatory diseases such as Stroke, Parkinson's disease, Alzheimer's disease and Vascular Dementia (225-230). No investigation has evaluated a diet modification approach to alter the gut microbiome in CCM disease. However, a recent study showed that the disruption of gut mucosal barrier by a dietary emulsifier (P80) caused increased CCM lesion burden in an experimental mouse model (21). If the gut microbiome is a significant contributor to CCM, manipulating the gut microbiome through dietary modifications could be a viable non-surgical therapeutic approach. Various diets have the potential to alter the composition of the gut microbiome in unique ways, which may result in distinct effects on the progression of CCM lesions. This chapter describes experiments conducted on a mouse model of CCM, where three common diet modifications were implemented. Short-term interventions involving high-fibre and low-fibre diets (six weeks) as well as short-term and long-term (12 weeks) high fat diets were employed to assess the impact of diet on CCM disease.

3.3. Results

3.3.1 High-fibre

a) 6-week high-fibre diet do not modify body weights.

To understand the effect of high-fibre diet on mice body weight, mice were weighed on a weekly basis. The mice's body weight was assessed weekly at a specified time, commencing from the pre-diet intervention period designated as week 0. This measurement continued until the end of the diet intervention, just before euthanasia (week 6). This assessment for both female and male mice showed that there is no significant difference in either absolute weight or percentage weight gain after six weeks high-fibre diet intervention, compared to controls (Fig 3.1).

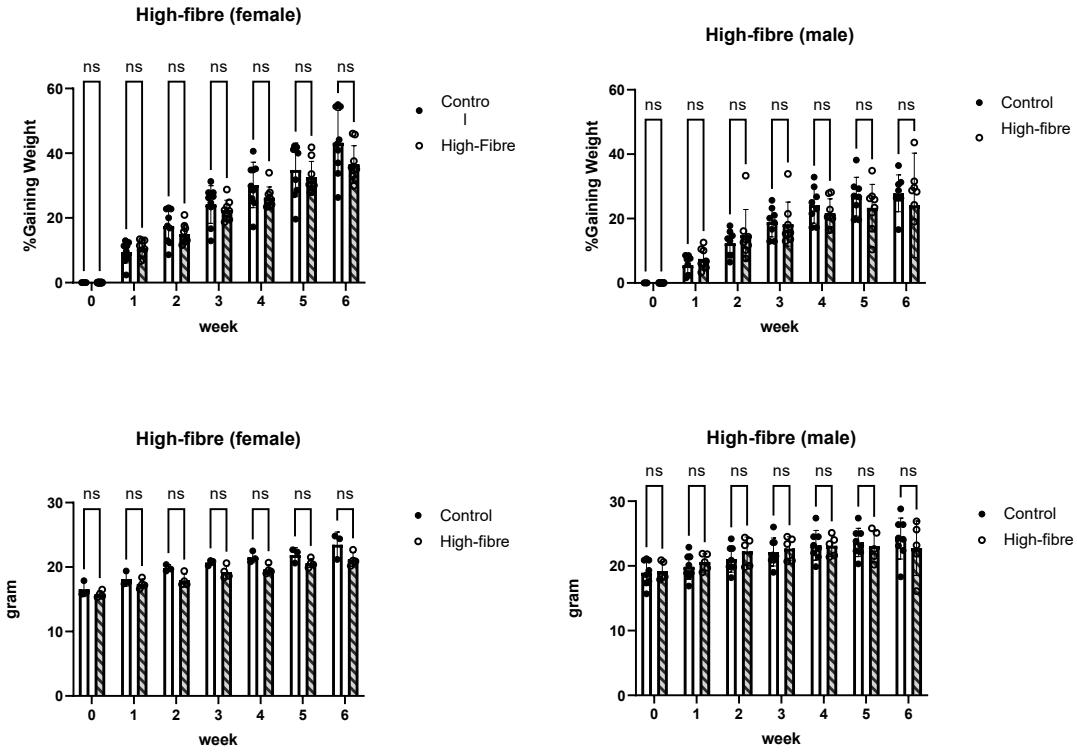


Figure 3.1 Weight analysis of High-fibre diet treated mice and control in female and male - *Weekly weight records showed there is no significant different between the groups from week 0 to week 6 in female and male mice. Error bars represent as mean SD and significance determined by Two-way ANOVA, Greenhouse–Geisser correction.*

b) 6-week high-fibre diet modification does not affect CCM lesion burden.

The mouse brains were harvested at week six after the experiment, submerged in formalin, and imaged with the Stereomicroscope (Fig 3.2). Following overnight fixation in formalin, hindbrains were scanned by Micro-CT, and lesion burden was quantified and analysed as previously described (see section 2.4 page 27). Hindbrains were analysed in terms of brain volume, total lesion volume and the number of lesions within the brain. The results showed no difference between controls or high fibre diet, for both males and females in the hindbrain volume. Furthermore, total CCM lesion volume and lesion number were comparable between control animals and those receiving high-fibre diet, across both males and females, indicating that 6-week high-fibre diet modification does not significantly affect CCM lesion progression in this mice model.

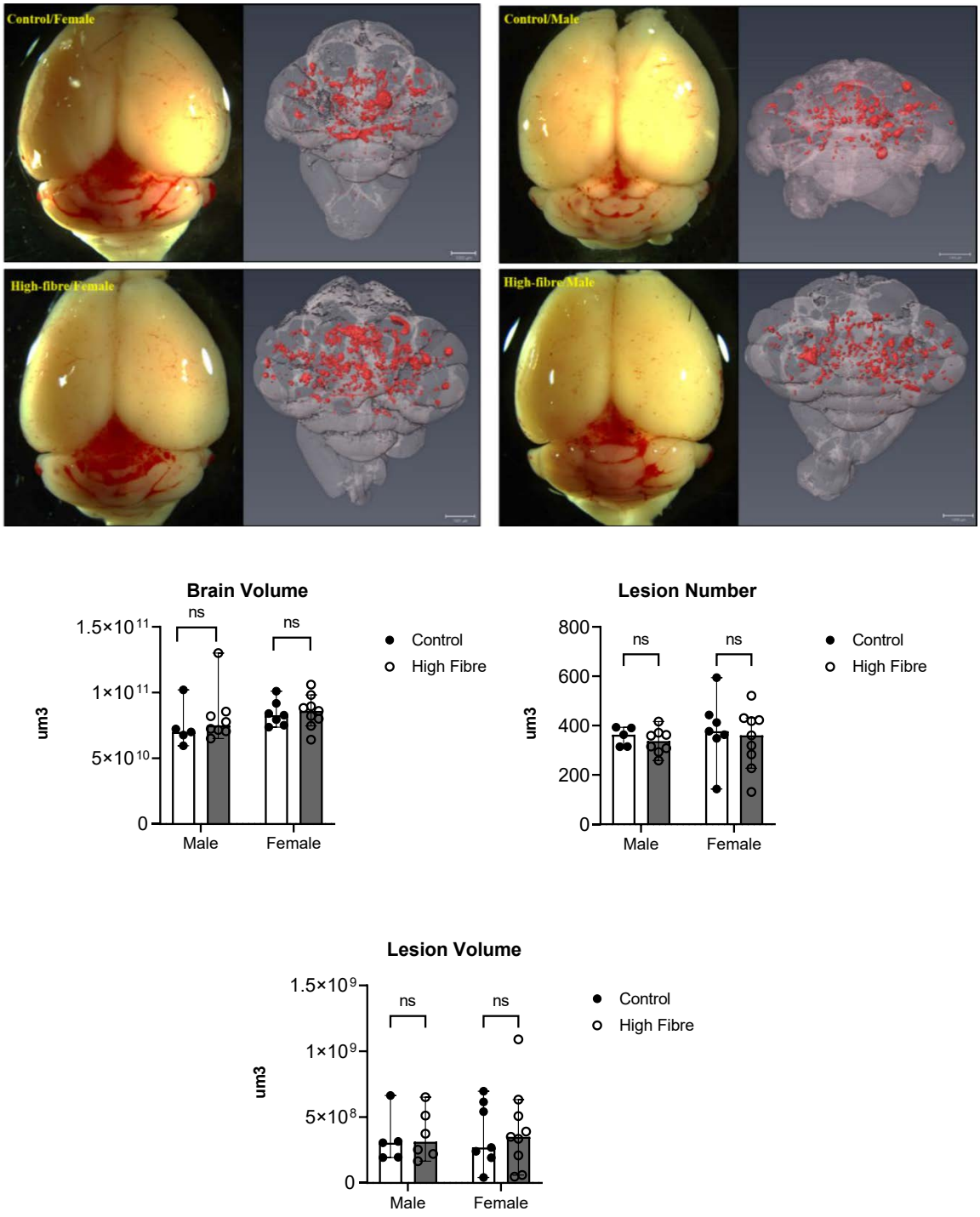


Figure 3.2 Stereomicroscope images and topographic pictures of marked lesions within the brain and analysis of brain volume, total lesion volume and lesion number of control female, high-fibre treated female mice, and control male mice and high-fibre treated male mice *- No significant difference between the male and female mice groups was observed. Error bars represent as mean with SD and significance determined by Two-way ANOVA, ordinary ANOVA test.*

c) 6-week high-fibre diet modification increased the number of goblet cells and ileum muscle layer thickness in female mice.

Ileum and colon were collected for gut morphology and integrity analysis during high-fibre diet modification. As previously described (section 2.5), all samples were processed and sectioned, and the slides were stained with hematoxylin and eosin, and PAS staining. Five criteria were chosen for measurement and analysis: The colon epithelial layer, the Colon muscle layer, the Ileum villus length, the Ileum muscle layer and the number of goblet cells per villus in the ileum. Our results elaborate that a 6-week high-fibre diet modification can affect the number of goblet cells per villus in female and male mice and the ileum muscle layer in female mice (Fig 3.4). The number of goblet cells per villus is significantly increased in female and male high-fibre-treated mice (Fig 3.5); however, a statistically significant thickness increase was observed only in female high-fibre-treated mice.

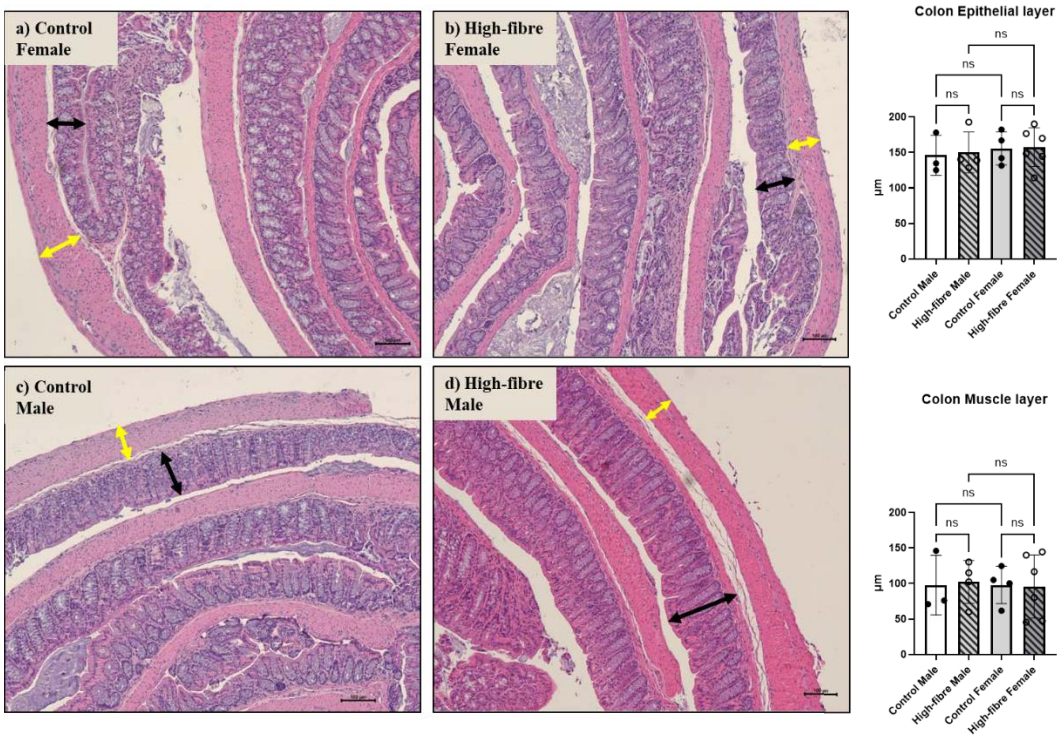


Figure 3.3 Colon epithelial layer (black arrow) and colon muscle layer (yellow arrow) in control and high-fibre treated male and female mice *- No significant difference detected between the groups in colon in both female and male mice. Arrows are hard to see, can make it bigger or change colour. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

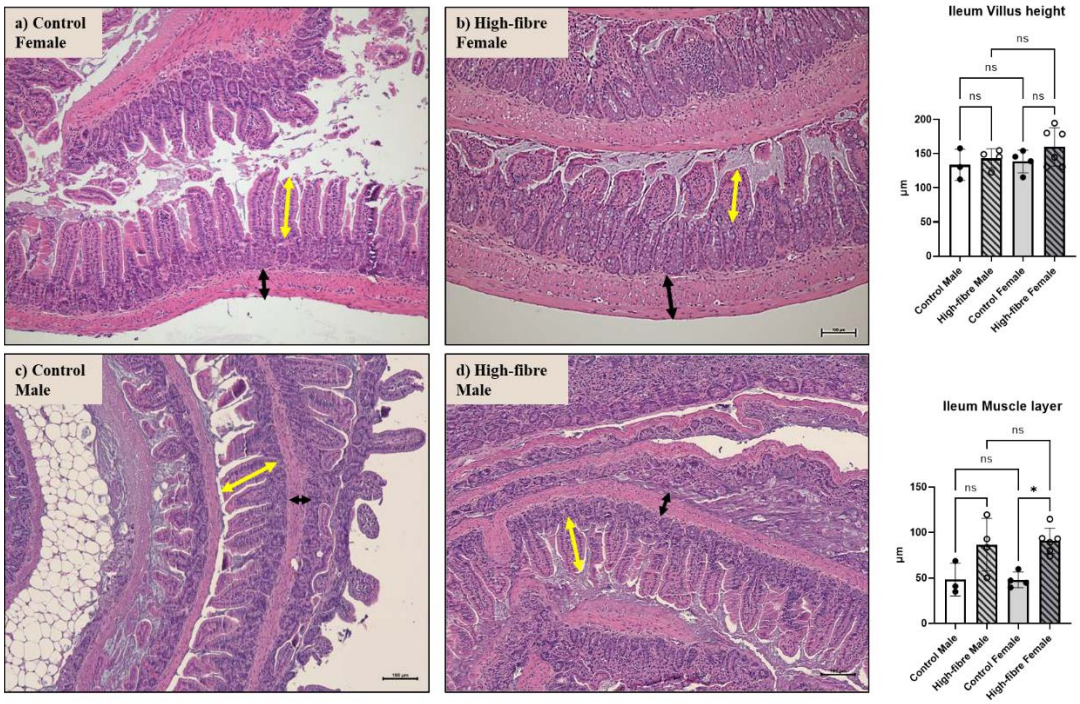


Figure 3.4 Ileum villus length (yellow arrow) and ileum muscle layer (black arrow) in control and high-fibre treated male and female mice *- The pictures illustrated an increase of thickness in ileum muscle layer in female mice but there is no significant change between groups in terms of ileum villus height. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

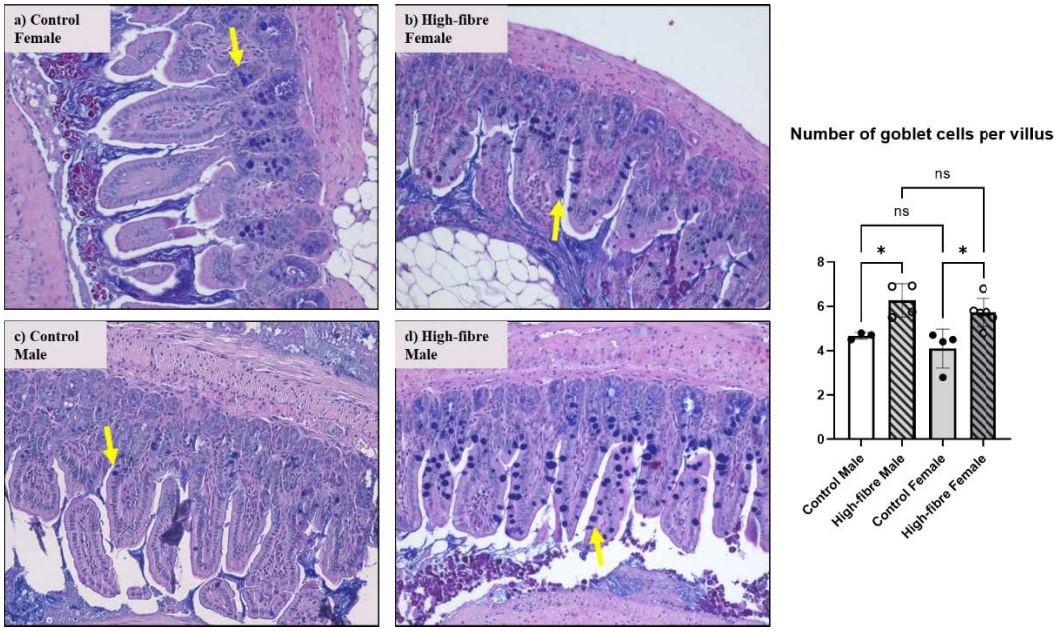


Figure 3.5 Ileum goblet cells in villus (yellow arrow) in control and high-fibre treated male and female mice *– The number of goblet cells in ileum of both male and female mice was increased after short-term high-fibre diet. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

d) High-fibre diet intervention for a duration of six weeks does not yield any significant alterations in the lipopolysaccharide (LPS) concentrations within the circulatory system.

The outcomes of study indicated that there was no notable difference in the levels of LPS in the blood serum between the mice treated with a high-fibre diet and the control group, for both male and female mice (Fig 3.6). Even though the high-fibre group showed slightly elevated levels of LPS in their serum, the difference was not statistically significant in either gender.

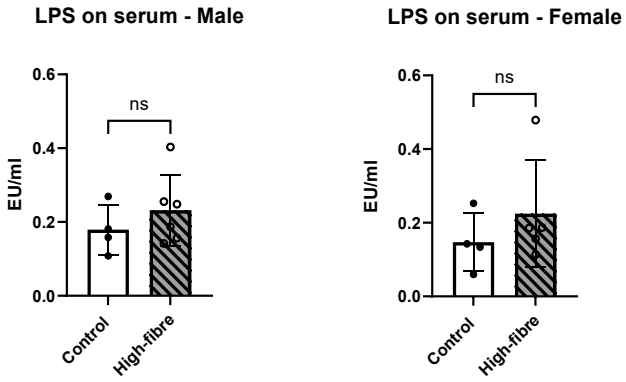


Figure 3.6 LPS levels in the blood serum of males and females from control and high-fibre diet groups *- The findings demonstrated that there was no significant difference in the levels of LPS in the blood across all the groups. Error bars represent as mean with SD and significance determined by Unpaired t-test, parametric test.*

3.3.2 Low-fibre diet

a) Six-week low-fibre diet does not cause changes in body weight compared to controls, in either female or male mice.

In order to examine the impact of a low-fibre diet on weight, all mice in the experimental group were weighed weekly. Like high-fibre diet modification, the control and low-fibre diets were weighed separately to prevent contamination and effects on the gut microbiome. The weight of mice were recorded weekly, starting from the beginning of the six-week experiment (week 0) until the end of the experiment (week 6). Analysis of the weight of both male and female mice revealed no significant difference in either absolute weight or percentage of weight gain between the two groups during the six-week period of the low-fibre diet intervention (Fig 3.7).

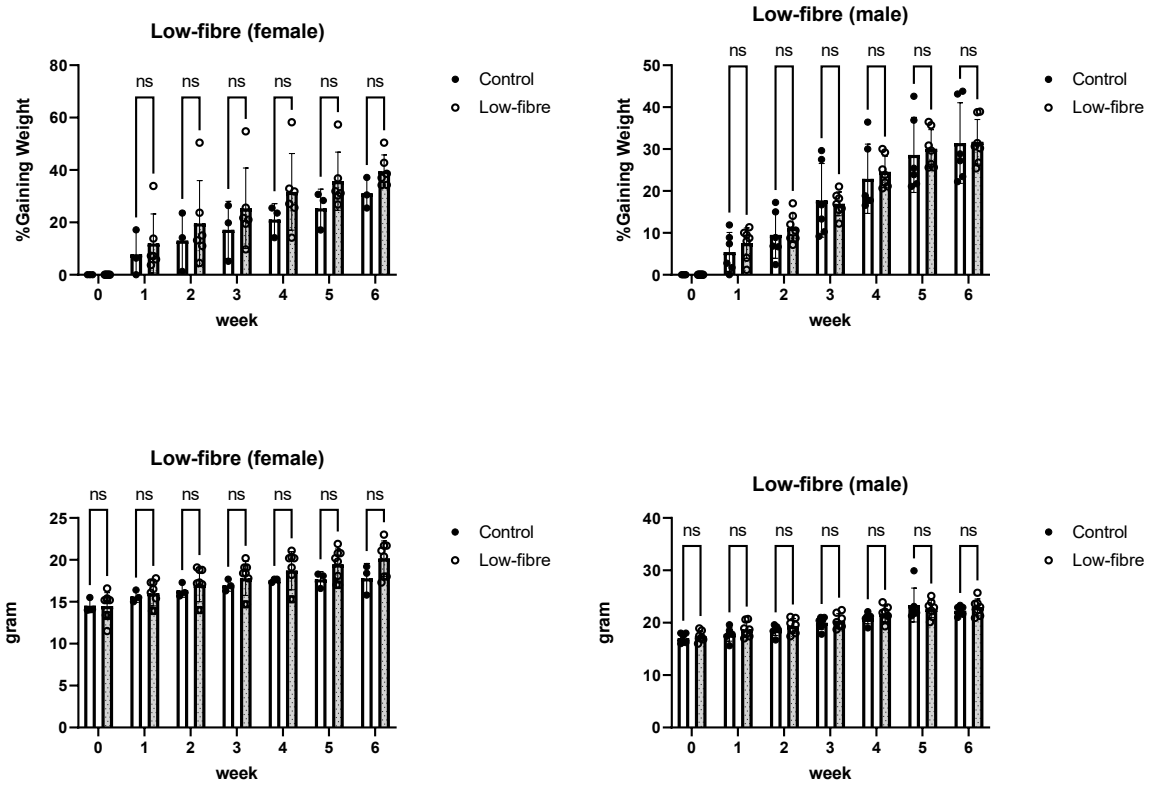


Figure 3.7 Body weight assessment of low-fibre diet treated female mice (left) and male mice (right) compared to controls *- Data are shown as % change from time 0 (upper panels) and absolute body weights (lower panels). No significant difference was observed between the groups. Error bars represent as SD and significance determined by Two-way ANOVA, Greenhouse–Geisser correction.*

b) No significant impact on the CCM lesion burden after six-week low-fibre diet modification.

Images of harvested brains of low-fibre treated mice and control by stereomicroscope and lesion burden analysis after lugol staining and scanning with Micro-CT showed no significant difference between the groups. Lesion burden of hindbrain analysis by Aviso software revealed that 6-week treatment with a low-fibre diet has no significant impact on CCM progression or regression. However, the results showed that the lesion volume in control female mice was significantly higher than in control male mice (Fig 3.8).. This trend was not observed in the lesion number analysis.

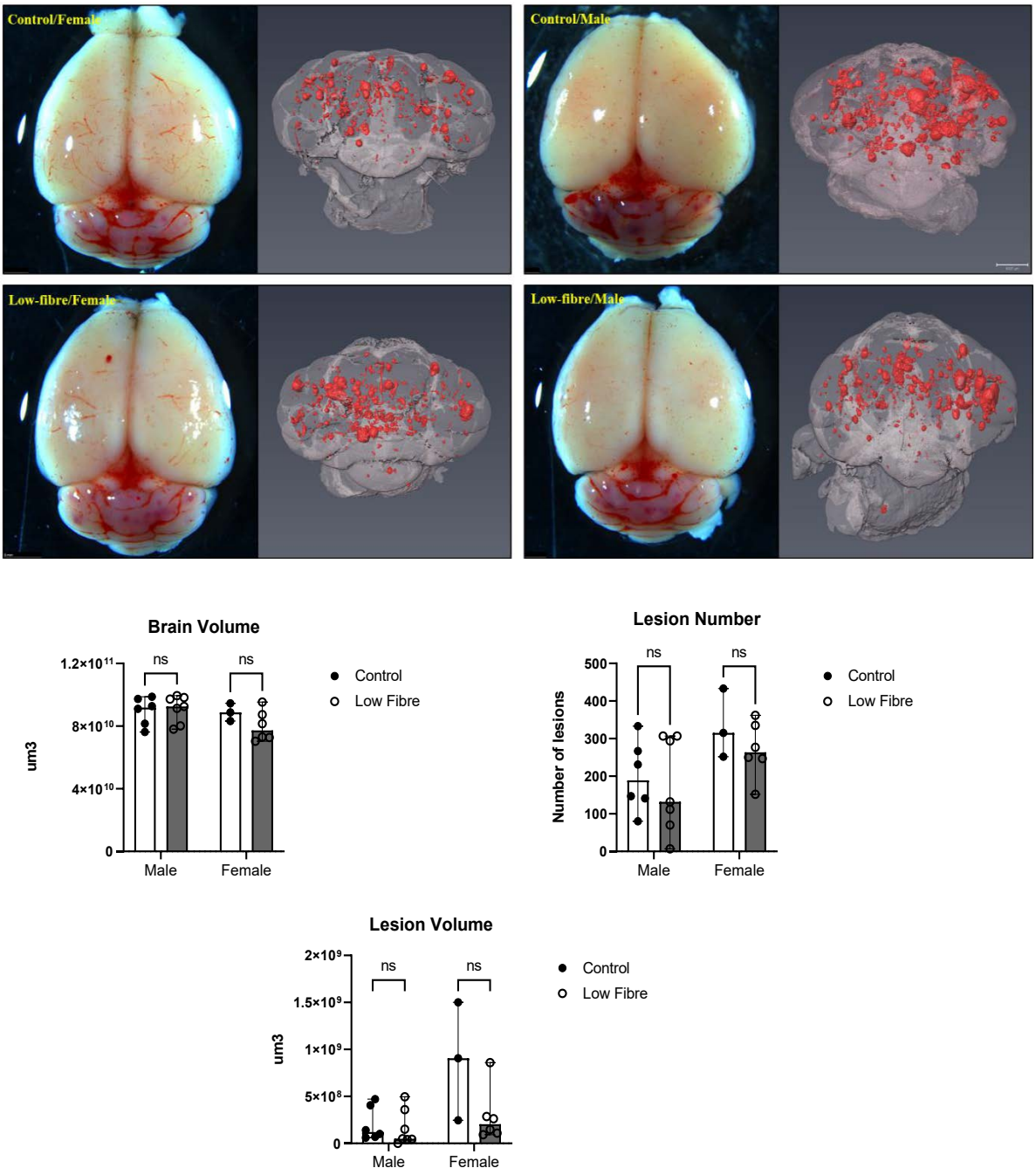


Figure 3.8 Stereomicroscope images and topographic marked brain lesion pictures and analysis of brain volume, total lesion volume and lesion number of low-fibre diet-treated mice and control in males and females *- Although a significant difference between control males and females was detected, no significant change was observed between control and treatment groups in both male and female mice. Error bars represent as mean with SD and significance determined by Two-way ANOVA, ordinary ANOVA test.*

c) Reduced thickness of the colon epithelial layer in male mice, but not females, after six–week treatment with a low-fibre diet.

After measuring five different criteria of gut and analysis of gut integrity, the results showed that a 6-week low-fibre diet modification could affect the colon epithelial layer and cause a significant reduction in thickness. However, this effect was only apparent in male mice. No significant changes were found between the groups regarding the colon muscle layer, the ileum muscle layer, the ileum villus length and the number of goblet cells per ileum in either male or female mice (Figures 3.9-11).

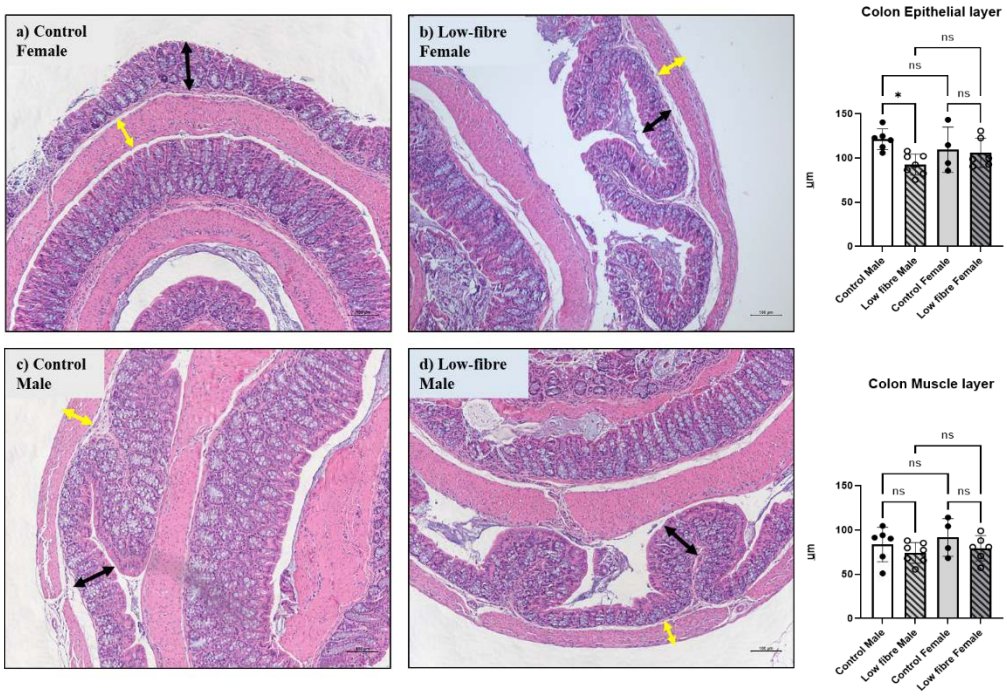


Figure 3.9 Colon epithelial layer (black arrow) and colon muscle layer (yellow arrow) in control and low-fibre treated male and female mice *- Colon epithelial layer thickness is significantly reduced in low-fibre treated male mice. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

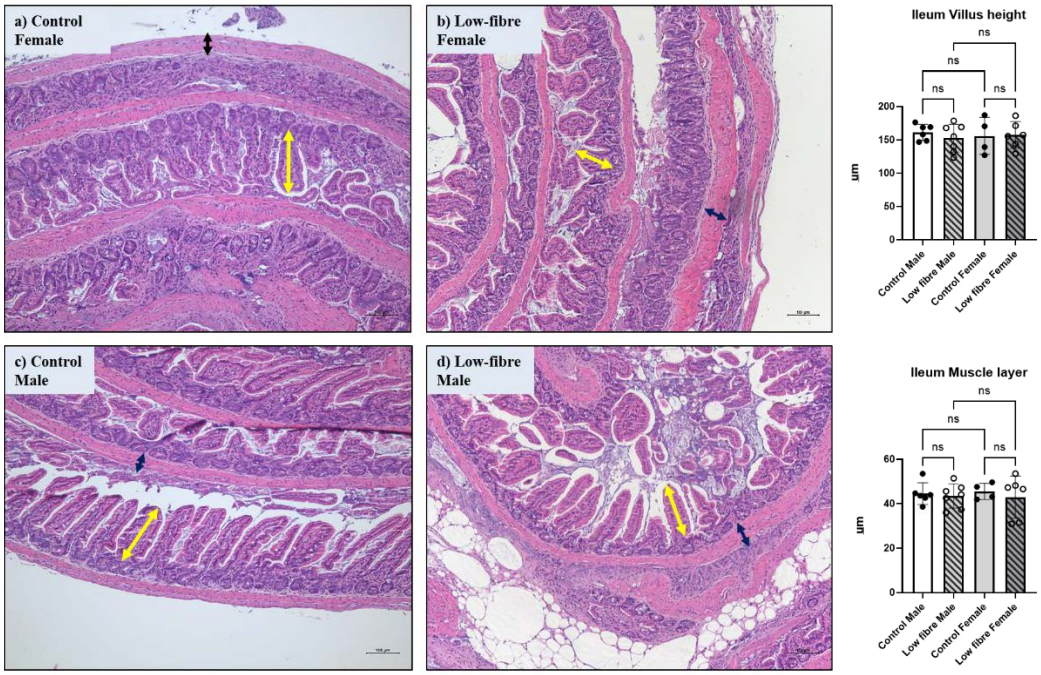


Figure 3.10 Ileum villus length (yellow arrow) and ileum muscle layer (black arrow) in control and low-fibre treated male and female mice *- No significant difference in the ileum was observed between the groups. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

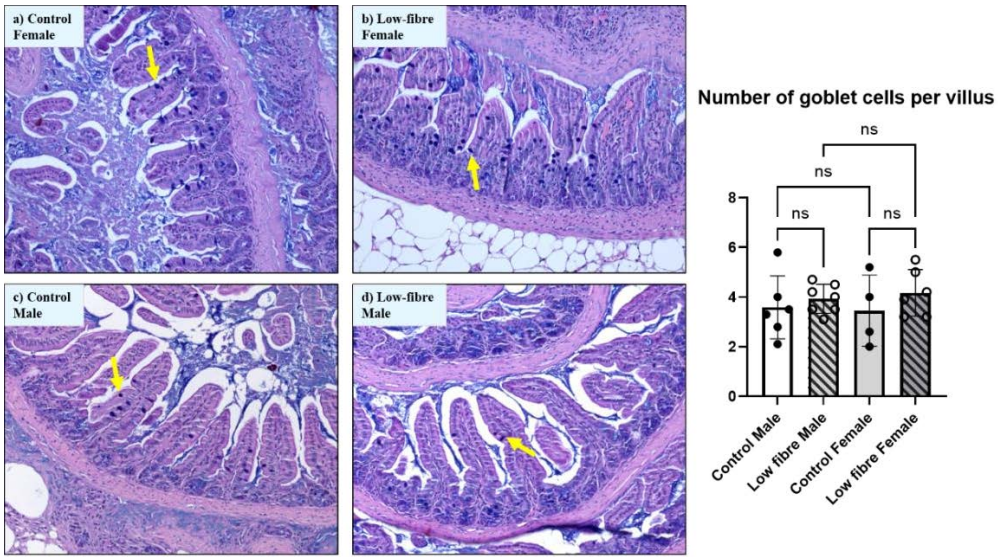


Figure 3.11 Ileum goblet cells in villus (yellow arrow) in control and low-fibre treated male and female mice *- No significant difference in terms of the number of goblets detected in all groups. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

d) Six weeks diet intervention with Low-fibre diet did not result in any notable changes in the levels of lipopolysaccharide (LPS) in the bloodstream.

Upon measuring the concentration of LPS in the blood serum, our findings indicated no significant distinction between the control and low-fibre groups in both male and female mice (Fig 3.12).

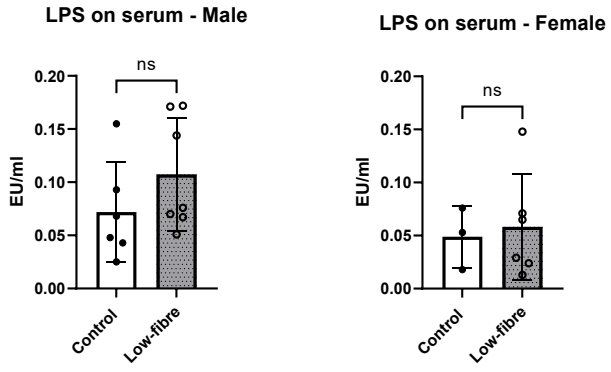


Figure 3.12 Analysing the quantity of LPS in the blood serum of male and female mice in both the control and low-fibre diet groups *- Results showed no significant change in level of LPS in blood in all the groups. Error bars represent as mean with SD and significance determined by Unpaired t-test, parametric test.*

3.3.3 High-fat diet

a) Mice body weight significantly increases after 6-week high fat diet modification in female and male mice.

The weekly records of the body weight of mice in all groups showed a significate difference between the control group and high fat diet treated group in female and male mice. High fat treated mice were considerably heavier than the controls. However, this gaining weight trend was different in females and males. In other words, the significant gaining weight percentage difference between the groups for female mice was observed from week 2 ($p=0.0003$), but this difference was detected in male mice from week 3 ($p=0.0091$) (Fig 3.13). Although the results showed both female and male mice gained weight after a 6-week high fat diet modification, the speed of gaining weight is not the same for both.

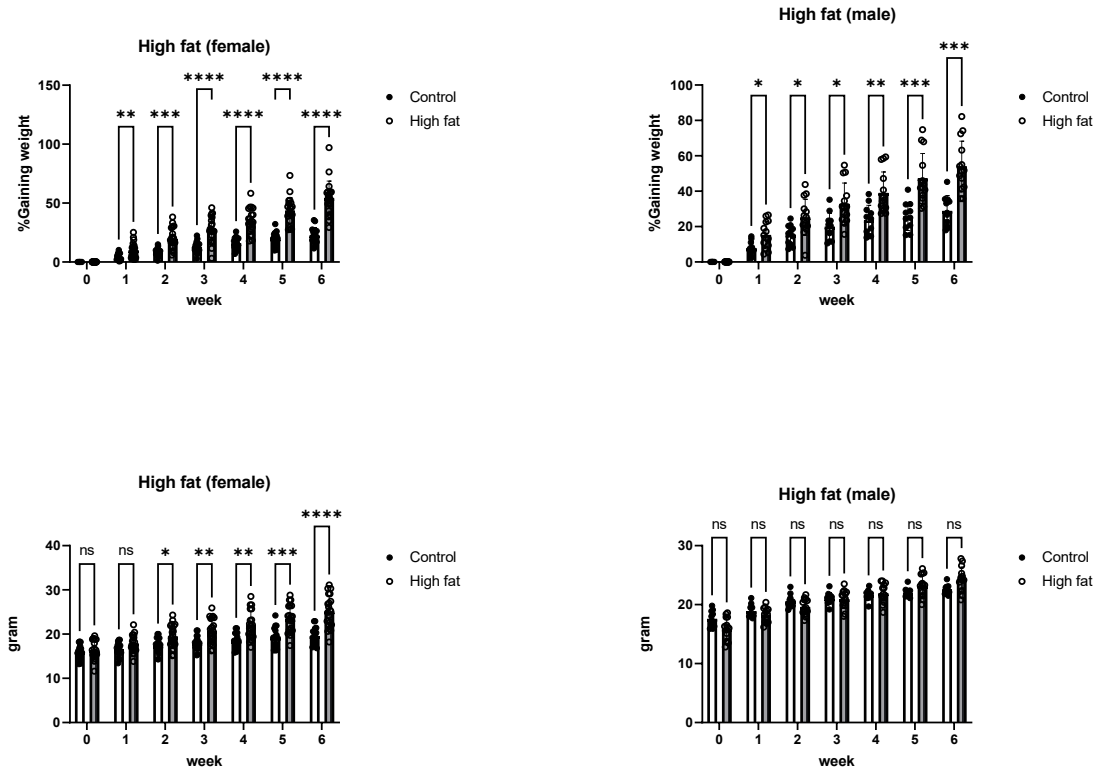


Figure 3.13 Weight analysis of high fat diet treated mice and control in males and females - *There are significant differences between the groups in female and male; however, the gaining significant weight difference in females are earlier than in males. Error bars represent as SD and significance determined by Two-way ANOVA, Greenhouse–Geisser correction.*

b) 6-week high fat diet modification causes a significant reduction in CCM lesion burden only in female mice.

After analysis of CCM lesion burden for control and high fat treated groups, the results revealed that 6-week high fat diet modification made a lesion regression in both lesion number and lesion volume, but only in female mice (Fig 3.14). No significant changes were observed between the groups in female and male mice regarding brain volume. Although there is no difference in the size and volume of the lesion in male mice, these results showed a significant difference between the control female and control male in the number of the lesion, and female mice showed a significantly higher number of the lesion compared to male mice.

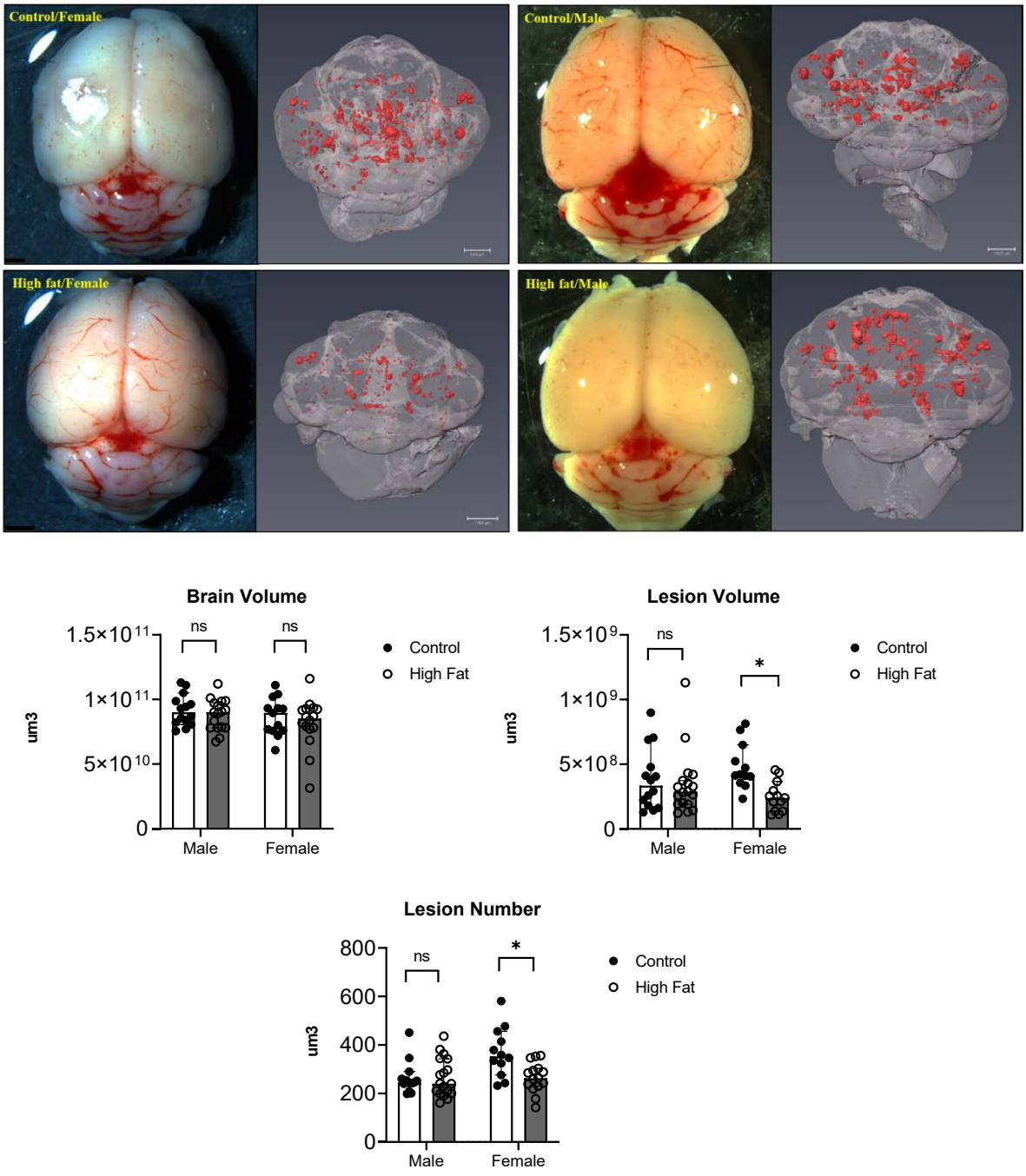


Figure 3.14 Stereomicroscope images and topographic pictures of marked lesions inside the brain and analysis of brain volume, total lesion volume and lesion number of high-fat diet-treated mice and control in males and females *- The high-fat-treated female mice group significantly reduced lesion volume and number. The lesion number is significantly higher in the female control group compared to the control male group. Error bars represent as mean with SD and significance determined by Two-way ANOVA, ordinary ANOVA test.*

c) Male mice that were subjected to a high fat diet for six weeks exhibited a significant decrease in the thickness of their colon epithelial layer.

Although no significant change was observed in colon muscle layer's thickness after measurement analysis in female and male mice, colon epithelial layer thickness showed a significant reduction after 6-week treatment with high fat diet in male mice (Fig 3.15). The analysis of other gut criteria such as the ileum muscle layer, the length of ileum villi, and the number of goblet cells per villus in ileum did not show any significant differences (Figures 3.16-17).

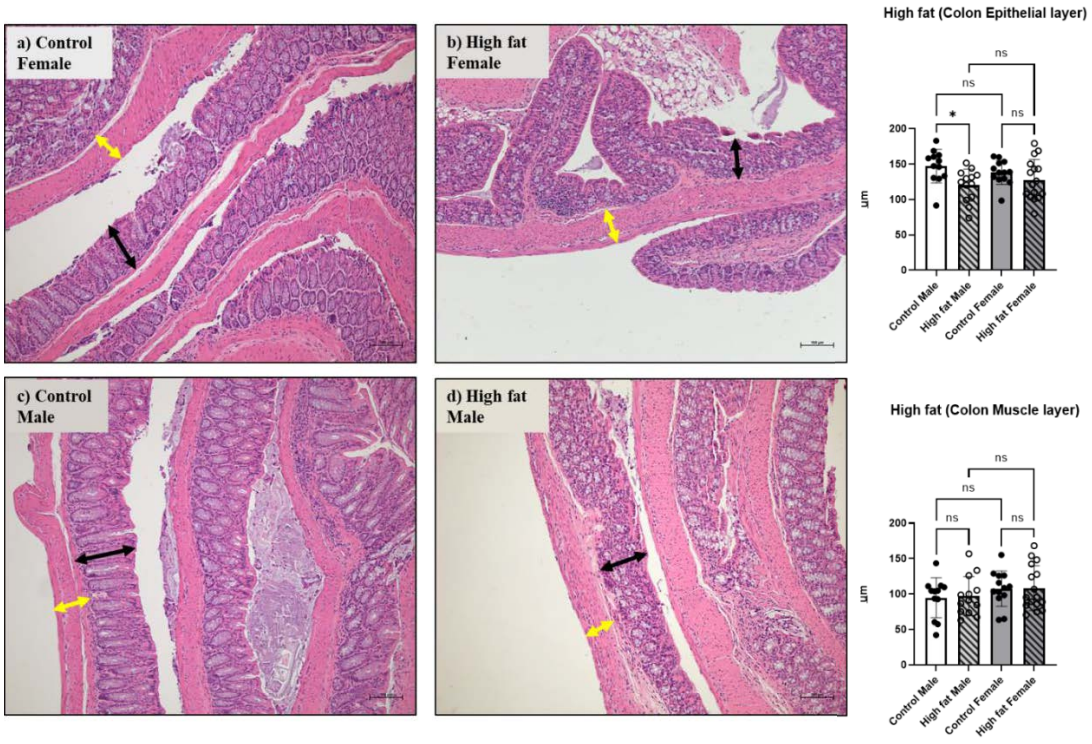


Figure 3.15 Colon epithelial layer (black arrow) and colon muscle layer (yellow arrow) in control and high fat treated male and female mice *- The thickness of colon epithelial layer is significantly decreased in male mice that were exposed to a high-fat diet. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

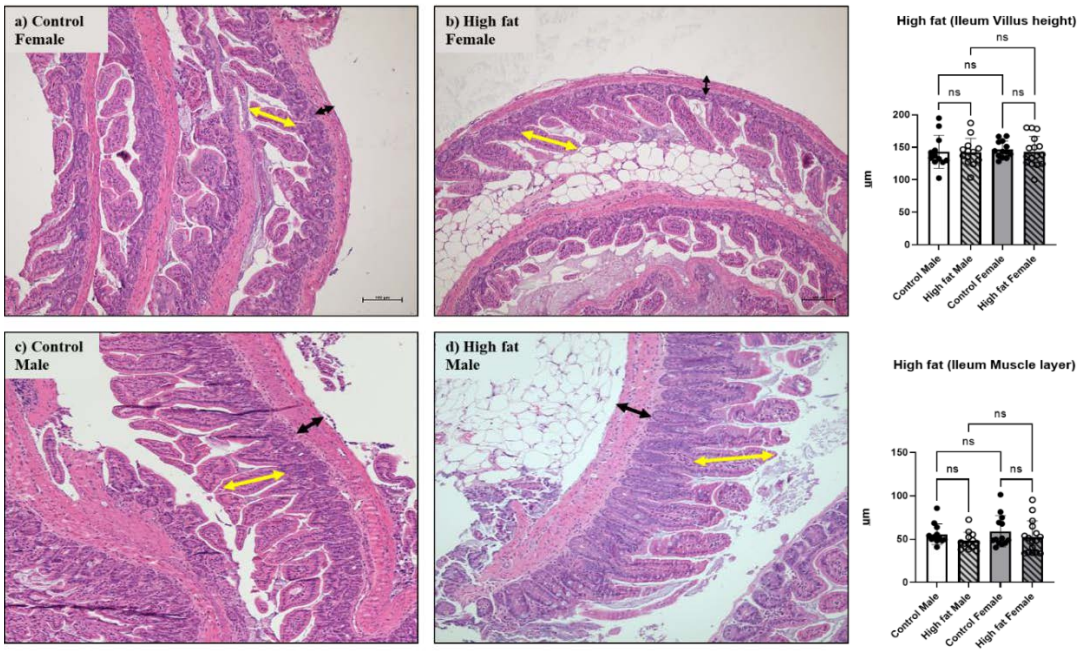


Figure 3.16 Ileum villus length (yellow arrow) and ileum muscle layer (black arrow) in control and high fat treated male and female mice *- There was no significant distinction detected in the ileum among the groups. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

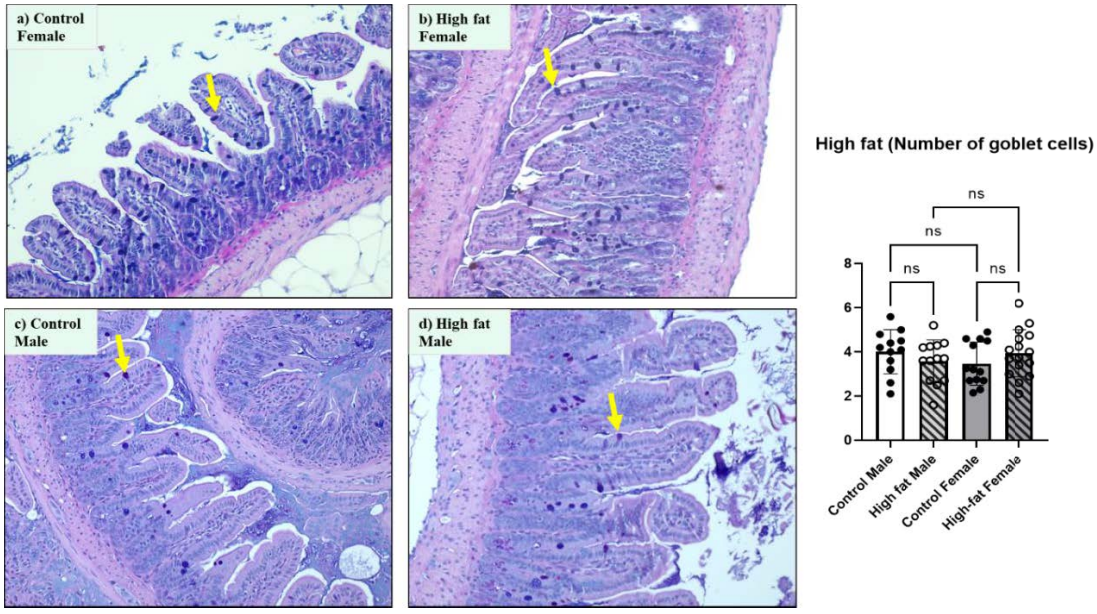


Figure 3.17 Ileum goblet cells in villus (yellow arrow) in control and high fat treated male and female mice *- No significant difference in terms of the number of goblets detected in all groups. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

d) ***A high fat diet intervention for 6 weeks does not result in any changes to the LPS levels in the bloodstream.***

The results showed no significant change between the control and high fat diet-treated mice regarding the level of LPS in the blood serum (Fig 3.18).

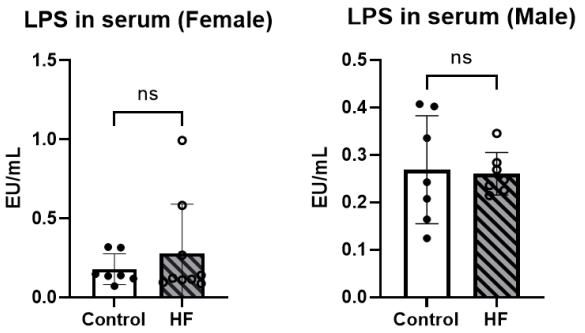


Figure 3.18 The amount of LPS in blood serum analysis in control diet and high fat diet groups in females and males *- Results showed no significant change in level of LPS in blood in all the groups. Error bars represent as mean with SD and significance determined by Unpaired t-test, parametric test.*

3.3.4 Long-term High fat diet

a) ***A significant increase in weight of the 12-week high fat diet modification group compared to the control group was observed in male mice.***

Similar to the 6-week high fat diet modification, male mice also show a significate difference in body weight in 12 weeks modification. The high fat diet-treated group is considerably heavier than the control group, and this difference is statistically significant from the sixth week of diet intervention (Fig 3.19). Although there are no female mice for long-term high fat diet, weight records of long-term high fat diet modification compared to 6-week records show that male mice are more resistant to gaining weight than female mice.

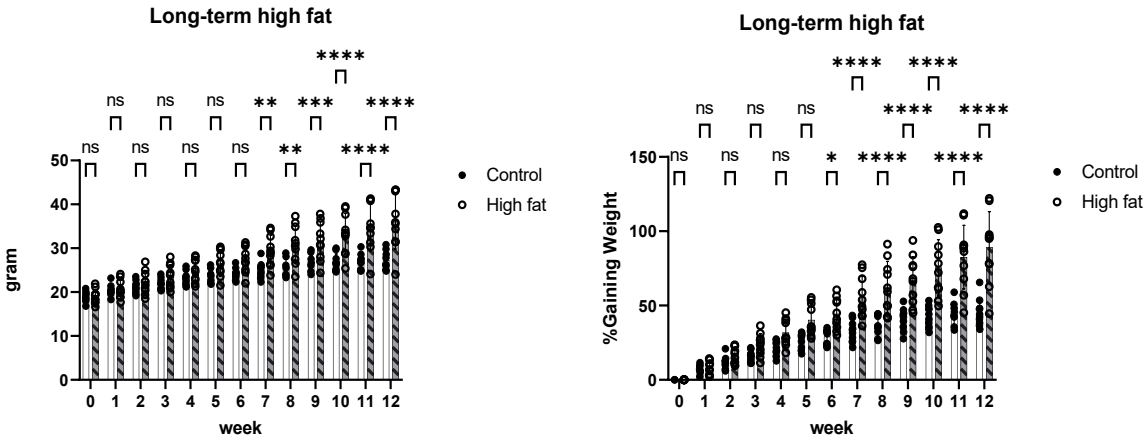


Figure 3.19 Weight analysis of 12-week high fat diet treated mice and control in males *- There is a significant difference between control males and high fat diet male mice, and the weight difference becomes statistically significant from week 6. Error bars represent as mean SD and significance determined by Two-way ANOVA, Greenhouse–Geisser correction.*

b) No statistically significant reduction of CCM lesion burden in males was distinguished after a 12-week high fat diet intervention.

Despite 12 weeks of intervention with a high fat diet, CCM lesion burden analysis results show no statistically significant difference in lesion volume and lesion burden. A reduction trend in lesion volume was detected in the high fat diet group; however, this trend is not statistically significant (Fig 3.20). Lesion number is lower in high fat mice but also not statistically significant. Similar to other diet modifications, no significant difference was identified between the groups regarding brain volume.

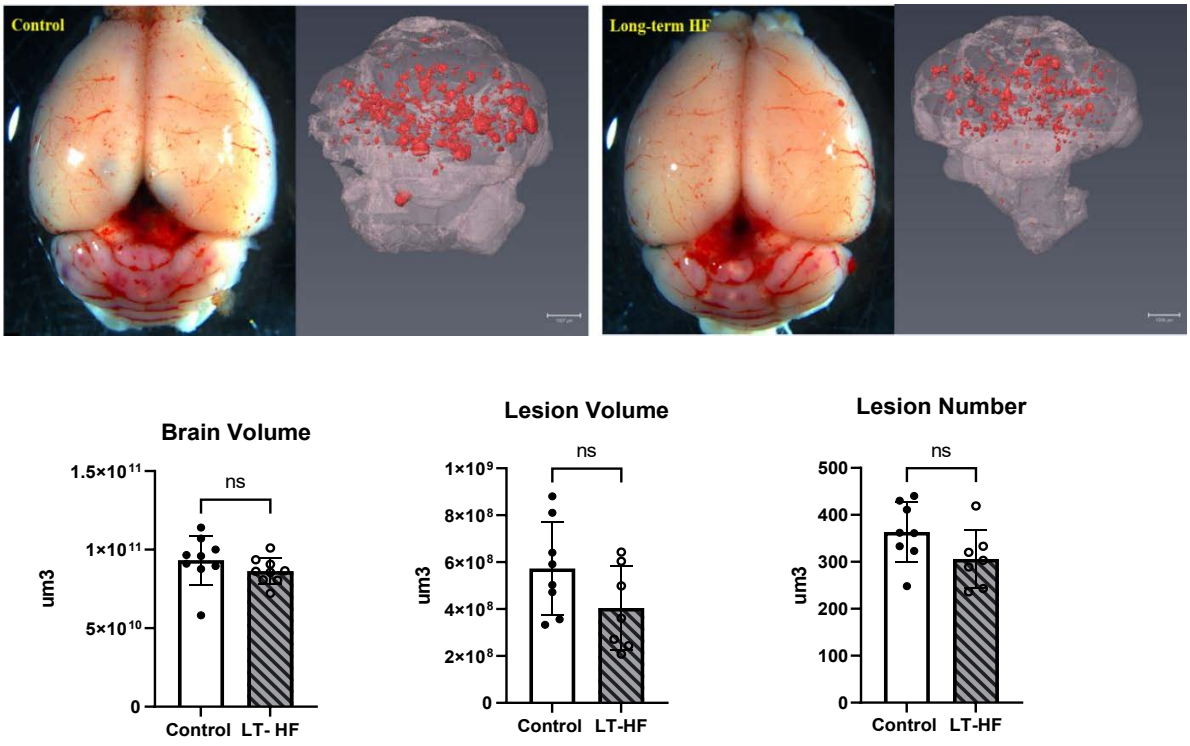


Figure 3.20 The stereomicroscope was utilised to capture images, which encompassed topographic representations of brain lesions. Subsequently, analysis was conducted to determine the brain volume, total lesion volume, and the number of lesions in both experimental groups *- There is no significant difference between the groups regarding lesion volume, number and brain size. Error bars represent as mean with SD and significance determined by Unpaired t-test, parametric test.*

c) 12-week high fat diet increased ileum villus height, but no significant change in the colon, ileum muscle layer and the number of goblet cells were observed in the males.

After morphology analysis of colon and ileum in both high fat diet intervention and control groups, no significant difference was observed in colon epithelial and muscle layer thickness (Fig 3.21). Although no statistically significant difference in ileum muscle layer thickness was detected, the length of villi in the ileum significantly increased in the high fat diet-treated group compared to controls (Fig 3.22). The groups have no significant difference in the number of goblet cells per villus (Fig 3.23).

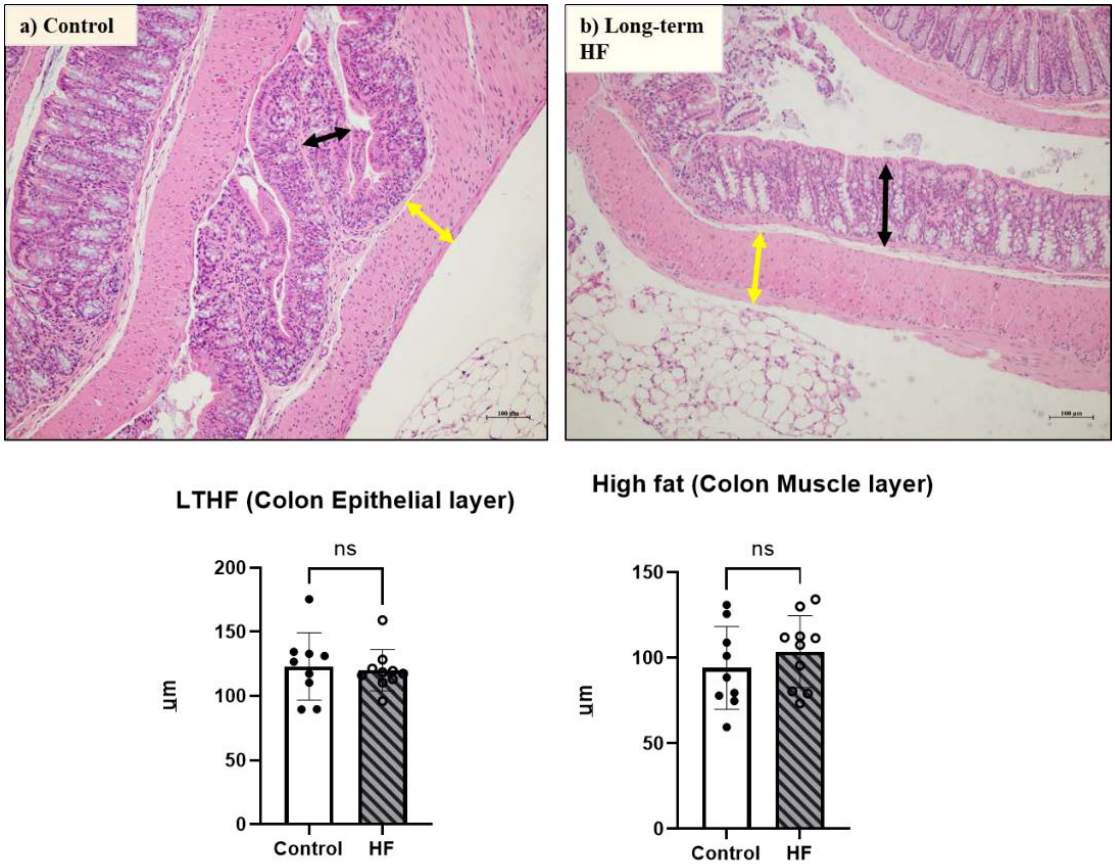


Figure 3.21 Colon epithelial layer (black arrow) and colon muscle layer (yellow arrow) in control and 12-week high fat treated male - *No significant difference was detected in both layers. Error bars represent as mean with SD and significance determined by Unpaired t-test, parametric test.*

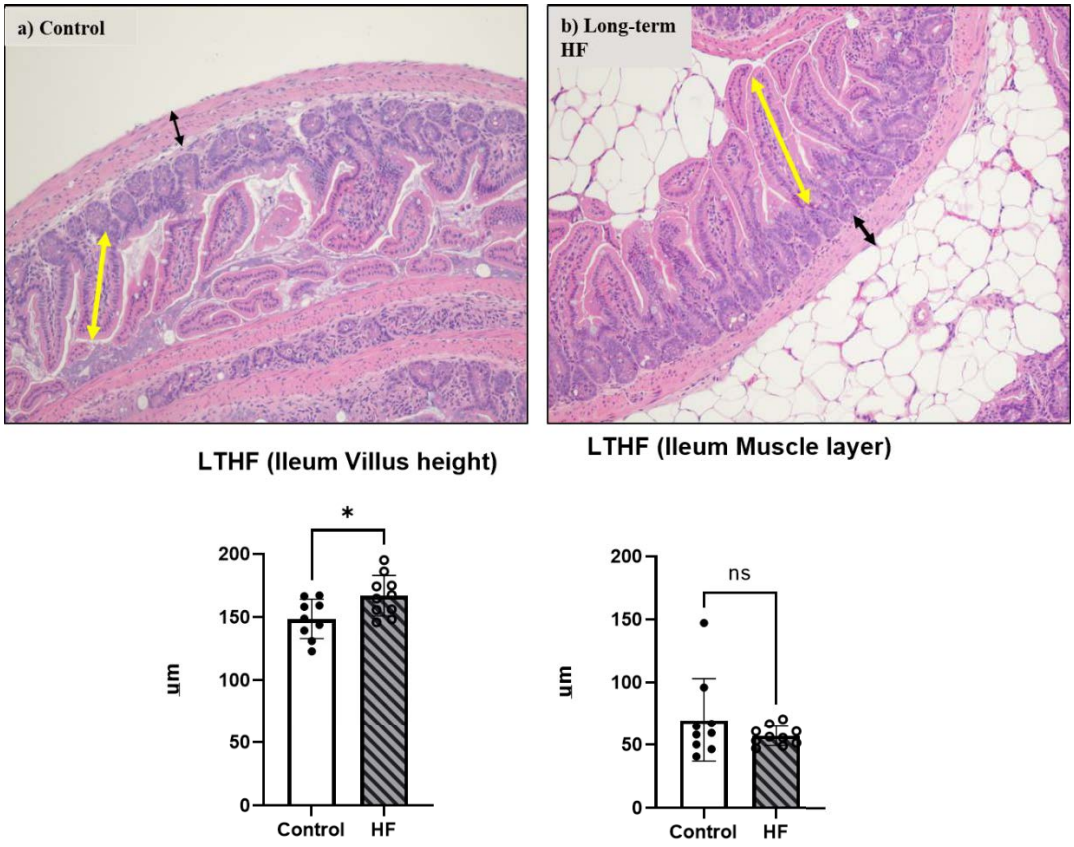


Figure 3.22 Ileum villus length (yellow arrow) and ileum muscle layer (black arrow) in control and 12-week high fat treated male mice *- The length of villi is significantly increased in the diet intervention group. Error bars represent as mean with SD and significance determined by Unpaired t-test, parametric test.*

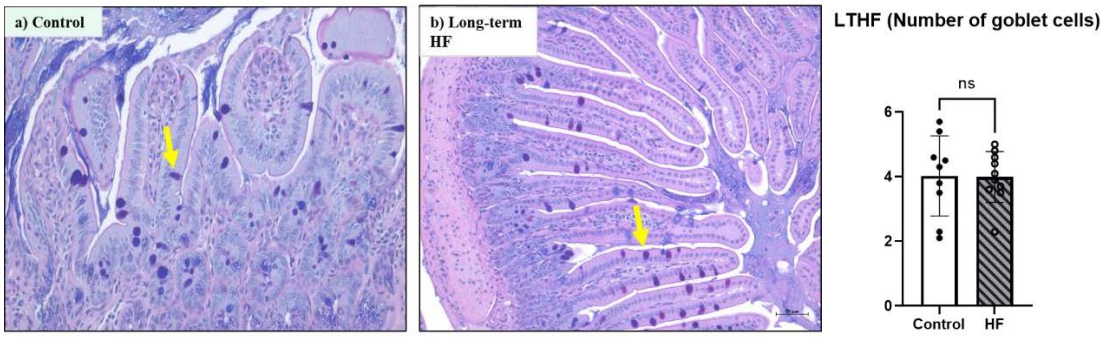


Figure 3.23 Ileum goblet cells in villus (yellow arrow) in control and 12-week high fat treated male mice (LTHF) *- No significant difference was observed between the groups. Error bars represent as mean with SD and significance determined by Unpaired t-test, parametric test.*

d) Blood circulating endotoxin (LPS) levels are not affected by 12-week high fat diet modification in male mice.

The quantification of LPS in blood serum results showed no significant difference between the diet intervention group and the control. However, an increased trend in LPS levels was observed in the high fat diet modification group (Fig 3.24).

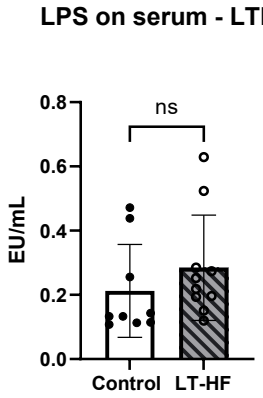


Figure 3.24 Analysis of the level of LPS in long-term high fat diet and control mice *- No difference was observed between the groups. Error bars represent as mean with SD and significance determined by Unpaired t-test, parametric test.*

3.4. Discussion and Conclusion

CCM is a common disease with a prevalence of approximately 0.5 per cent of the population. Most CCM patients do not have any clinical symptoms or exhibit mild symptoms like headache (1, 2, 231, 232). Different levels of severity of clinical symptoms were observed in patients with the same type of mutations in CCM causative genes and the same familiar history of CCM (233). Our understanding of CCM's molecular mechanism and genetic factors has increased over the years, yet we still do not know the cause of such variability between patient outcomes. However, the controversial debate is why people with similar genetic conditions show different clinical symptoms. Some patients will be asymptomatic throughout their lives; others experience a stroke during childhood. The compelling indications suggest that some factors outside of genetics trigger endothelial cells with mutations in CCM causative genes to initiate lesion formation. This non-genetic disease modifier is called a "third hit". That means preventing these triggers can be targeted as a non-surgical therapeutic option. A recent study on mice models of CCM revealed

that induced disruption of the gut barrier and reduced gut mucosal barrier could increase the CCM lesion burden within the mice brain (21). Moreover, an additional investigation utilising a mouse model for CCM demonstrated that administering lipopolysaccharides (LPS), a major part of the outer membrane of GNB, to mice initially resistant to CCM lesion formation resulted in the development of these lesions (22). Thus, potentially gut microbiome composition's dysbiosis and gut barrier defectiveness can be considered one of the third hit triggers.

As diet plays a crucial role in determining gut microbiome composition, in this study, we investigated the effect of three different diet modifications on two various time courses on the well-established and robust mice CCM model and disease development. Since gut integrity contributes significantly to CCM lesion progression, we investigated the effect of diet modifications on the major parts of the gut (colon and ileum) and the blood stream's circulation LPS level. This study results showed that diet modification with a high amount of resistant starch as dietary fibre and a diet without fibre for six weeks has no significant effect on CCM lesion formation in female and male CCM experimental mice. Although this study first investigates diet modification's impact on CCM, comprehensive studies showed that a high-fibre diet could regulate and prevent other vascular and neurodegenerative diseases (234-238). For instance, Liu et al. investigated the risk of stroke in individuals with different levels of whole-grain fibre consumption. Their results showed an inverse correlation between whole-grain fibre intake and the risk of ischemic stroke (239). Another study revealed that high-fibre consumption from fruits, vegetables and cereal sources could decrease the risk of cerebrovascular diseases, especially ischemic stroke (240). Also, diet modification with a high-fibre diet could reduce the risk of Alzheimer's disease (AD) through regulation of the activity of nuclear receptors such as histone deacetylases (HDAC) and peroxisome proliferator-activated receptors (PPAR), also can control the brain's cholesterol balance and the development of amyloidosis which have a significant effect on AD developing (238). Hypothetically, perhaps a 6-week high-fibre diet modification with high-resistant starch is not enough time to affect CCM lesion progression and a long-term time course needs to show regression on CCM lesion formation on mice model.

Potentially circulating LPS are considered as one of the proinflammatory factors for innate immune response through inducing TLR4, which is associated with some inflammatory diseases such as non-alcoholic fatty liver and Osteoarthritis (241, 242). A previous study showed that high-fibre diet modification could decrease lipopolysaccharide (LPS) levels by reducing gut barrier permeability and preventing bacteria or bacterial components from entering mice's bloodstream (243). However, the results of analysis of LPS level in blood circulation showed no significant difference after a 6-week diet intervention with high-fibre and low-fibre diets. Possible reasons for inconsistencies of this study results with previous studies could be variations in the duration

of dietary modification, the age of the mice, and the type of fibre utilised in the diet. Studies on the morphology of the ileum, the last part of small intestine, showed that high-fibre diet modification significantly impacts the number and length of villi in the ileum, escalating food absorption. Furthermore, a high-fibre diet could increase the thickness of ileum muscle layer and mucus production, reducing gut permeability and helping protect the gut from damage and inflammation (244, 245). Our project results about ileum morphology after a six-week high-fibre diet modification aligns with previous findings; however, no significant increase of ileum villi length was detected. The colon is the longest part of the large intestine and is crucial in water and electrolyte absorption. Also, the colon is the location of the vast majority of gut bacteria responsible for SCFAs production from dietary fibre fermentation. SCFAs benefit the gut and other organ health and decrease inflammation (246, 247). Previous studies showed that a high-fibre diet causes a significant increase in colonic crypts and increased mucosal layer thickness (248). Despite our results detecting that low-fibre diet modification could significantly decrease the colon epithelial layer responsible for mucus production, our high-fibre diet modification results do not show any significant effect in mice colon. The use of various species of mice, different sources of fibre and time courses may be reasons for these non-aligned results.

Studies on the mice model of stroke show that high fat diet modification can increase the risk of stroke and neuroinflammation (249). High fat diet modification can induce the formation of atherosclerotic plaques in arteries within the brain and lead to ischemic stroke by blocking blood flow to the brain, increasing the oxidative stress in vascular endothelial cells and increasing inflammatory markers in blood circulation (250-252). These findings prove the adverse effect of a high fat diet on vascular and cerebrovascular disease, but surprisingly our results of a 6-week high fat diet modification on mice model of CCM show a significant reduction of CCM lesions regarding size and volume in female mice. Although no significant reduction was found in male mice after six weeks of diet intervention, no increase in lesion burden was observed.

Our CCM lesion analysis results of twelve weeks of high fat diet treatment of male mice showed no significant change; however, a reduction trend in the high fat group was observed. These findings suggest that male mice are more resistant to high fat diet intervention and bear a more stable gut microbiome than females. Also, gaining weight analysis showed a different trend in males and females and a significant weight difference was observed in male later than female mice after six-week treatment with high fat diet. Previous study on the identification of the gut microbiome of different species of mice in males and females show sex differences in bacterial composition and non-identical effect of diet on gut bacterial communities in males and females (253).

Our results of the effect of a 6-week high fat diet on ileum and colon morphology showed no significant difference between the groups. On the other hand, long-term high fat diet modification (12 weeks) showed a significant increase in the length of villi in the ileum. This is in line with Sharifi, S. D., et al findings that indicate an increase in ileum villi length after high fat diet intervention (254). Also, another study revealed that the number of goblet cells in the ileum significantly decreased after high fat diet treatment. In contrast, our results show no significant difference between the groups in short-term and long-term diet interventions (255). The use of mature C57BL/6 mice and those aged five months, as opposed to younger mice aged six weeks, and the utilisation of a different percentage of fat (42%), could be contributing factors to the varying results observed in the goblet cells compared to our results.

Regarding the effect of high fat diet on colon morphology, some studies suggest that a high fat diet could increase the size of adipocytes (fat cells) in the colon of mice and change the expression of mucin on the surface of the colon epithelial layer (256, 257). However, no significant difference in colon morphology was detected after both short-term and long-term high fat diet intervention in our results. Previous studies showed increased circulation endotoxins (LPS) in mice treated with a high fat diet. High fat diet can change the gut microbiome composition and increase the ratio of gram-negative bacteria to gram-positive bacteria. Also, it can affect the gut barrier and increase the gut permeability, consequently leading to an increased level of LPS in the bloodstream (258-260). In contrast, our LPS quantification of blood serum of 6-week high fat treated mice showed no significant difference with the control group. Similarly, a long-term dietary intervention with a high fat diet for 12 weeks did not significantly change the circulation LPS concentration.

In conclusion, our study showed that a 6-week diet modification with fibre and lack of fibre has no significant effect on CCM lesion burden; however, it can increase the thickness of the ileum muscle layer and the number of goblet cells in the ileum. Probably we need to run long-term treatment with a high-fibre diet to observe a significant alteration in CCM lesion burden. Although high fat diet modification has a considerable adverse effect on most cerebrovascular diseases, our results showed a significant reduction of CCM lesion burden in female mice after a 6-week high fat diet modification. These findings align with a clinical study on CCM patients that revealed obese CCM patients carry significantly fewer total lesion numbers (47). We suppose this regression is due to changing gut microbiome composition and affecting CCM lesion initiation and extension via a different mechanism rather than stimulating TLR4 by LPS derived from GNB. Nonetheless, alternative mechanisms, distinct from gut microbiome modulation, may contribute to the regression of lesions through a high fat diet. A recent study suggested that a high fat diet has the potential to safeguard the blood-brain barrier (BBB) in the context of AD, subsequently

enhancing functionality in murine models. This effect is attributed to the diet's low carbohydrate content and elevated levels of monounsaturated fatty acids. Given that disruption of the BBB constitutes a hallmark of CCM lesions, it is conceivable that an elevation in circulating monounsaturated fatty acids may independently drive lesion regression, thereby circumventing the necessity for gut microbiome intervention.

4. Chapter 4: Identification of the gut microbiome composition and metabolites difference after a six-week high fat diet intervention via omics analysis.

4.1. Abstract:

Advancements in omics methodologies have facilitated high-throughput analysis of biological molecules or constituents in complex samples, such as stool, urine, and blood. By utilising these techniques, it becomes possible to identify changes in microbial communities within the gut and alterations in metabolite levels following short-term high fat diet interventions, providing insights into potential mechanisms underlying CCM lesion regression. To explore this, faecal samples were collected before the diet intervention and at the end of the dietary modification, allowing for the identification of alterations in gut microbiome composition through metagenomics analysis. Additionally, cecum contents were collected post-dissection to identify any changes in metabolites and lipids through metabolomics and lipidomics analysis after diet intervention. Our analysis showed a significant difference in gut microbiome composition between control and high fat-treated groups after diet intervention. Additionally, metabolites and lipids clusters significantly changed after high fat diet intervention. Our results suggest there is a potential link between specific bacterial genera like *Akkermansia muciniphila* on CCM lesion regression through sphingolipids pathways and metabolism.

4.2. Introduction:

Prior to the advent of next-generation sequencing technology, investigations into the gut microbiome relied on bacterial culturing and serotyping analysis as means of classifying microbial communities. However, it was observed that only a fraction, ranging from 10 to 30 percent, of the microbial population within the gut microbiome are culturable. Additionally, serotyping analysis was prone to a high risk of errors (261, 262). Recently, the rapid progress in next-generation sequencing techniques has facilitated the analysis and identification of a vast number of microorganisms within complex environments, including various human body sites such as the gut (263-266). This advancement has opened up new avenues for studying the diverse microbial communities present in these environments with unprecedented depth and accuracy. Metagenomics, initially introduced in 1998, emerged as a DNA sequencing technique with the

unique capability to analyse complex mixtures of microbial communities (267, 268). This methodology facilitates the identification and categorization of all genes present in microorganisms within a specific sample by employing the random sequencing of DNA obtained from said sample (269, 270). The extracted whole genome of the microbiome from faeces breaks down into scattered DNA fragments through a "shotgun" approach. According to phylogenetic markers sequences (16s rRNA), the sequenced fragments were analysed and identified species communities or genomic profiles according to the whole genome sequence (271, 272). The metagenomics methodology encompasses the filtration of shotgun sequence reads to extract high-quality sequences that significantly contribute to the comprehensive genomic profile. These filtered sequences are subsequently assembled based on sequence overlap, resulting in the generation of longer DNA sequence contigs. Through computational analysis, data mining, and database searching, the contig sequences are interpreted to identify specific genes (273). The utilisation of sequence-based and functional metagenomics provides a significantly enhanced comprehension of the structure and functionality of microbial communities, surpassing previous levels of understanding.

The interaction between the GI and the CNS has been observed in various brain-related diseases and disorders (274-276). The gut influences the brain through mechanisms such as the Vagus Nerve (VN), the production of neurotransmitters, immune responses, and inflammation (277, 278). For instance, a study conducted on animals demonstrated that both long-chain and short-chain fatty acids exert an influence on the stimulation of the VN and the innervation of the vagal pathway (279). Furthermore, research indicates that the gut microbiome can produce various metabolites that impact brain function and disease conditions (280-282). Notably, a compelling illustration of the gut microbiome's influence on the brain was observed following a flood in Walkerton, Canada, in 2000. During this incident, the city's drinking water became contaminated with *Escherichia coli* and *Campylobacter jejuni*. Among the 4,561 affected residents who consumed the contaminated water, the health status of 2,451 individuals was tracked for eight years. Among those infected, 1,166 individuals were diagnosed with irritable bowel syndrome (IBS), and a significant majority of them concurrently experienced anxiety and depression alongside IBS symptoms (283). These findings sparked increased scientific interest in understanding the gut microbiome and brain interaction. Although the precise mechanism of the gut-brain axis remains incompletely understood and necessitates further investigation for clarification, previous research has indicated that bidirectional communication between the gut and the brain occurs via the nervous, endocrine, immune, and metabolic systems (284-286). Furthermore, studies in this field have highlighted the gut microbiome's influence on the brain through interactions along the gut-brain axis (287, 288). Considering the high variability and independence of the gut microbiome from the gut itself, scientists have proposed the concept of

the gut microbiome-brain axis to underscore the critical role played by the microbiome in this intricate relationship.

Some studies showed that the gut microbiome could influence brain functions regardless of VN stimulation (289, 290). The gut microbiome has the capacity to affect the CNS by means of the neuroendocrine system, which regulates the synthesis and secretion of hormones from glands into the bloodstream (291, 292). In a particular study conducted on mice subjected to Griseofulvin (GF) treatment, which induced dysbiosis of the gut microbiome, it was observed that mild stress led to a significant increasing in the levels of corticosterone and adrenocorticotropic hormone (ACTH) in the bloodstream when compared to control mice. However, this increase in serum hormone levels could be reversed through FMT from control mice or the administration of *Bifidobacterium infantis* as a probiotic for a specific duration of time (293). Corticosterone and ACTH hormones play crucial roles in the stress response, with their levels increasing in response to acute and chronic stressors. Through various mechanisms, the gut microbiome can engage in interactions with the host, with Toll-like receptors (TLRs) on the surface of host cells playing a pivotal role in facilitating this interaction. TLRs are integral components of the innate immune system and are responsible for initiating the production of cytokines and triggering an inflammatory response. These receptors are abundantly distributed across the surfaces of neurons (294), allowing bacteria in the gut to induce and activate gut-associated neurons through TLR signalling. Additionally, a separate study conducted on germ-free mice demonstrated the significant contribution of the gut microbiome to the development of gut immunity, as germ-free mice exhibited an inactive immune system. However, the administration different diet interventions and increasing a specific bacteria, *Bacteroides thetaiotaomicron,* was found to restore the immune system's functionality, highlighting the crucial role of the gut microbiome in shaping immune responses (295-297).

Recently, thorough explorations employing omics analysis have been undertaken to unravel the intricate molecular mechanisms underlying the advancement of diverse brain diseases and disorders. These studies aim to enhance our comprehension of the fundamental molecular processes associated with these conditions. Following a stroke, cerebral tissue damage triggers intestinal dysfunction via the hypothalamus-pituitary-adrenal gland axis (HPA). This dysfunction manifests as an elevation in gut permeability, facilitating the translocation of gut bacteria and their metabolites into the bloodstream. Consequently, this heightened permeability poses an increased risk of sepsis development (298). A study conducted on individuals who have experienced stroke demonstrated significant differences in the faecal metabolites of stroke patients compared to healthy individuals. Furthermore, a robust correlation was observed between the gut metabolome and the metabolomes of urine and plasma ($P < 0.05$). The diverse array of metabolites present in faeces, as well as their subsequent presence in urine and plasma, exhibited

a significant association with the composition of the gut microbiome. Additionally, these metabolites were found to be linked to key bacterial species implicated in stroke. An illustrative example is the significant increase of phenylacetic acid, a precursor metabolite involved in the production of phenylacetylglutamine, which has been linked to cardiovascular disease (CVD). This particular metabolite exhibits a significant increase in the gut of individuals afflicted with ischemic stroke. (299). Furthermore, numerous investigations conducted on both human subjects and animal models of stroke have put forth evidence indicating that changes in the gut microbiome following a stroke, along with the subsequent translocation of gut bacteria and their metabolites to extra-intestinal organs, have the potential to influence post-stroke conditions and clinical symptoms (300-302).

Carotid atherosclerosis, characterized by the development of arterial plaque in the carotid arteries responsible for cerebral blood supply, remains an incompletely understood process. However, certain studies have put forth the hypothesis that the gut microbiome may play a contributory role in the initiation and advancement of carotid plaque formation (303, 304). The gut microbiome is produced TMAO due to the digestion of choline and carnitine, which are found in high-protein food like red meat and egg. The increasing blood level of TMAO is associated with an increased risk of atherosclerosis via the formation and development of artery plaques (305). In a clinical cohort study involving individuals diagnosed with Subclinical Carotid Atherosclerosis (SCA), notable dietary differences were observed compared to healthy controls. Specifically, SCA patients exhibited higher consumption of meat products and reduced intake of starchy vegetables, cereals, and bakery products. Furthermore, the gut microbiome composition analysis by metagenomics revealed a significant decrease in the abundance of *Faecalibacterium prausnitzii*, while *Escherichia coli* showed a significant increase in SCA patients compared to the healthy group (303). A pilot study on the gut microbiome composition of CCM mouse model using 16s rRNA sequencing has shed light on the pivotal role of the microbiome in CCM development. The results reveal significant difference in the gut microbiome communities between mice susceptible to brain lesions, resulting from knock-out of the KRIT1 and CCM2 genes, and those resistant to the CCM phenotype. Specifically, susceptible mice exhibit higher levels of *Bacteroides s24-7*, while the abundance of the *Ruminococcus* genera varies between KRIT1 and CCM2 knock-out mice (22). A recent investigation focusing on patients with CCM and their gut microbiome revealed substantial differences in gut microbiome composition in terms of alpha and beta-diversity when compared to healthy individuals. These findings suggest a notable shift in bacterial communities during the course of CCM disease. Furthermore, the abundance of gram-negative bacteria, specifically *Odoribacter splanchnicus*, was significantly higher in CCM patients, whereas gram-positive bacteria such as *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis* were significantly decreased in comparison to healthy individuals. The ratio of gram-

negative to gram-positive bacteria also displayed a significant increase in GNB among CCM patients. Additionally, the study findings indicated an upregulation of genes associated with lipopolysaccharide (LPS) synthesis, along with a significant decrease in the plasma levels of Lipopolysaccharide-binding protein (LBP) in CCM patients (114). By conducting ongoing metagenomics analysis, a comprehensive examination of metabolome disparities in plasma was performed between individuals diagnosed with CCM and healthy controls. This investigation revealed a noteworthy correlation between alterations in the gut microbiome and changes in plasma metabolites among CCM patients. Specifically, elevated levels of cholic acid and hypoxanthine in plasma were observed, which were associated with the upregulation of genes in *Bifidobacterium adolescentis*, *Faecalibacterium prausnitzii*, and *Odoribacter splanchnicus*. Pathway analysis further demonstrated the involvement of cholic acid and hypoxanthine in CCM disease-related signalling pathways, including PI3K-AKT, MAPK, NF-KB, and Rap (306). These findings suggest that alterations in the composition of the gut microbiome are linked to changes in blood metabolites in CCM patients when compared to healthy individuals (114, 306). These findings highlight the potential influence of specific microbial taxa on CCM pathogenesis. The aim of this study was to investigate and characterize changes in metabolite profiles and bacterial communities subsequent to a six-week intervention involving a high fat diet. The primary objective is to elucidate potential mechanisms that contribute to the regression of CCM lesions. Through comprehensive analysis, we aim to identify distinct changes in metabolites and bacterial composition that may contribute to the observed regression of CCM lesions, shedding light on the underlying mechanisms of this phenomenon.

4.3. Results:

4.3.1 Metagenomics Analysis

A statistically significant disparity was observed in the diversity and abundance of gut microbiome communities between the pre and post short-term high fat diet treatment groups in both male and female mice.

DNA was extracted from faecal samples collected at week 0 (prior to the commencement of high fat diet treatment) and at week 6 (following the completion of diet modification) for subsequent sequencing and metagenomics analysis. The resulting data were subjected to Principal Coordinate Analysis (PCoA) based on Bray-Curtis dissimilarity, which revealed distinct patterns of beta diversity in bacterial communities between the high fat and control groups, as well as noticeable dissimilarities between the two time points (week 0 and week 6) before and after the dietary

intervention (Figures 4.1-2). PCoA, is flexible, can accommodate non-linear relationships inherent in gut microbiome data, resulting in more accurate representations of sample relationships. PCoA is specifically designed for analysing beta diversity, which measures the variation in community composition among samples. These observations suggest substantial alterations in the composition and structure of the gut microbiome in response to the high fat diet treatment over the course of the study.

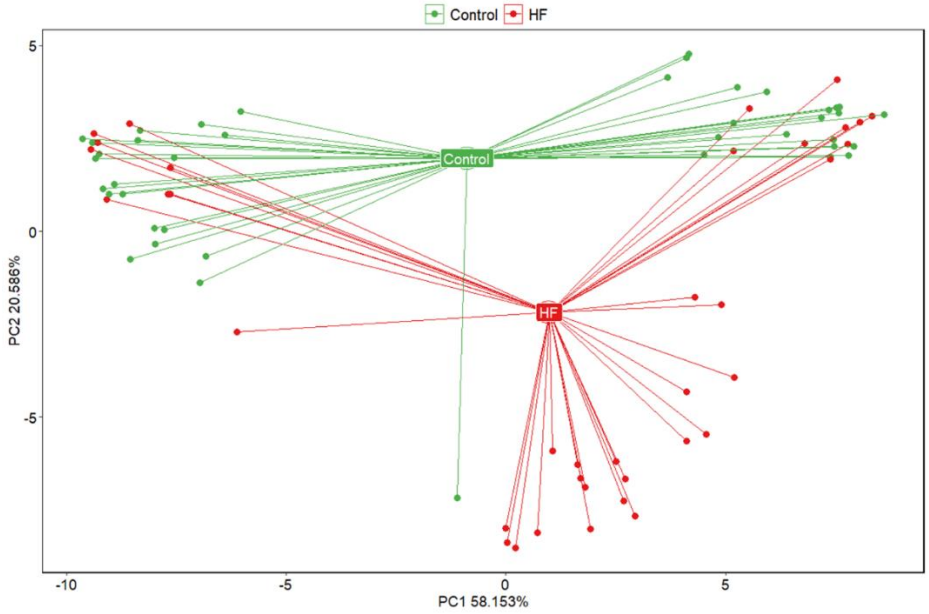


Figure 4.1 Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity for beta diversity between control and high fat group *- Beta diversity mapping analysis revealed a clear difference between the gut microbiome profiles of the control group and the high fat diet group. The analysis of the two groups indicated significant differences in the composition and structure of their gut microbiota.*

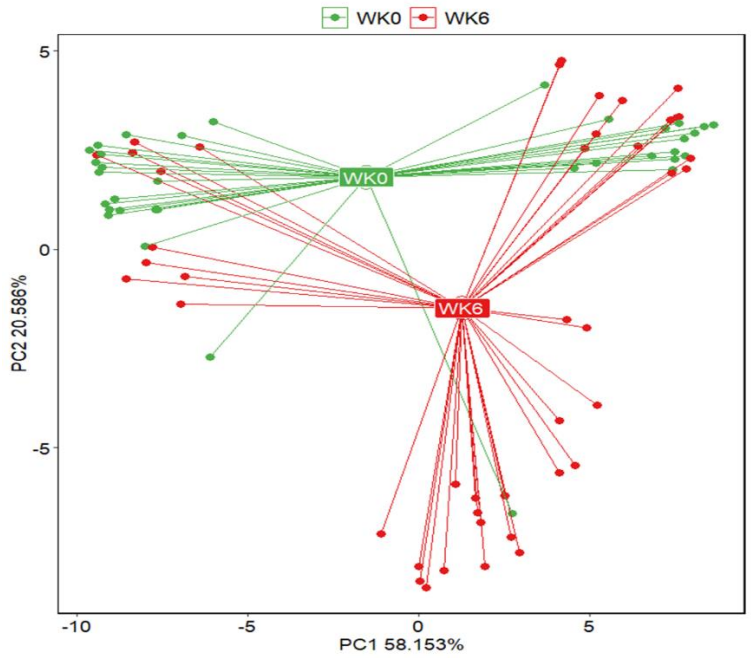


Figure 4.2 Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity for beta diversity before (week 0) and after (week6) high fat diet intervention *- The application of beta diversity mapping analysis unveiled a pronounced disparity in the gut microbiome profiles before and after the intervention of a high fat diet.*

Statistical analysis using Simpson's Diversity Index and Shannon Index was performed to assess the alpha diversity of gut microbiome composition in the different groups. In male mice receiving the standard chow diet (control group), no significant differences were observed in the microbiome composition between week 0 and week 6, as indicated by the alpha diversity metrics. Similarly, in female mice from the control group, there were no significant changes in alpha diversity between week 0 and week 6. However, in female mice subjected to the high fat diet, a significant difference in alpha diversity of gut microbiome communities was observed between week 0 and week 6 (Figures 4.3-4). Interestingly, this significant difference was not observed in males following the high fat diet intervention, suggesting a divergent influence of the high fat diet on the gut microbiome profile between male and female mice.

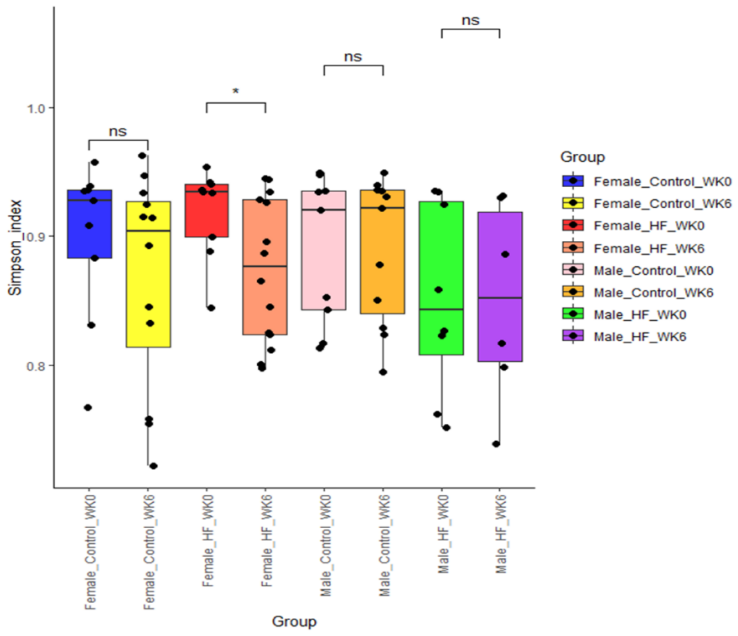


Figure 4.3 The Simpson index of alpha diversity between the high fat diet treated and control group in males and females before (week 0) and after (week 6) diet intervention - *The results showed a significant difference in gut microbiome diversity before and after high fat diet intervention in only female mice. No significant difference was observed in other groups. Simpson index: Lower values indicate higher diversity. It emphasizes the probability of encountering different species when drawing individuals randomly from the sample.*

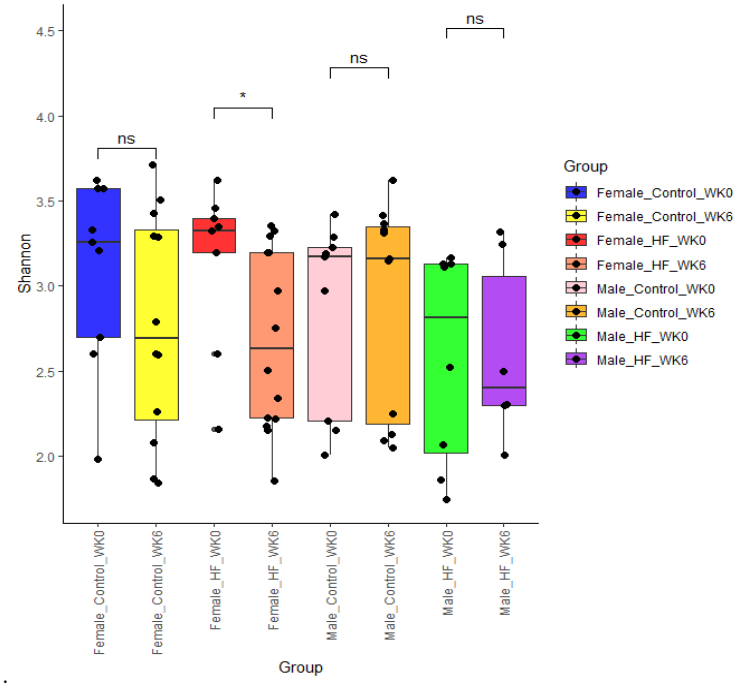


Figure 4.4 The alpha diversity, as measured by the Shannon index, was compared between the male and female groups subjected to high fat diet treatment and the control group both before (week 0) and after (week 6) the diet intervention *- The findings demonstrated a significant difference in gut microbiome diversity between the pre- and post-intervention stages of the high fat diet in female mice. However, no significant differences were observed in the gut microbiome diversity of the other experimental groups. Shannon index: Higher values indicate higher diversity. It considers both richness (number of species) and evenness (relative abundance of each species).*

Through metagenomics analysis of gut microbial communities, we investigated the abundance of bacterial species between the groups before and after diet intervention. In male mice, there was no statistically significant difference observed between the groups prior to the initiation of the high fat diet. However, in female mice, our findings revealed that *s_GGB28875_SGB41555* exhibited the highest abundance in the high fat diet group, while *s_Prevotella_sp_PCHR* was the most prevalent species in the control group at week 0 (Fig 4.5). It is worth noting that all mice were subjected to the same animal husbandry conditions and were littermates within their respective groups. The presence of *s_GGB28875_SGB41555* was not detected in control mice, and the prevalence of *s_Prevotella_sp_PCHR* was significantly higher in the control group.

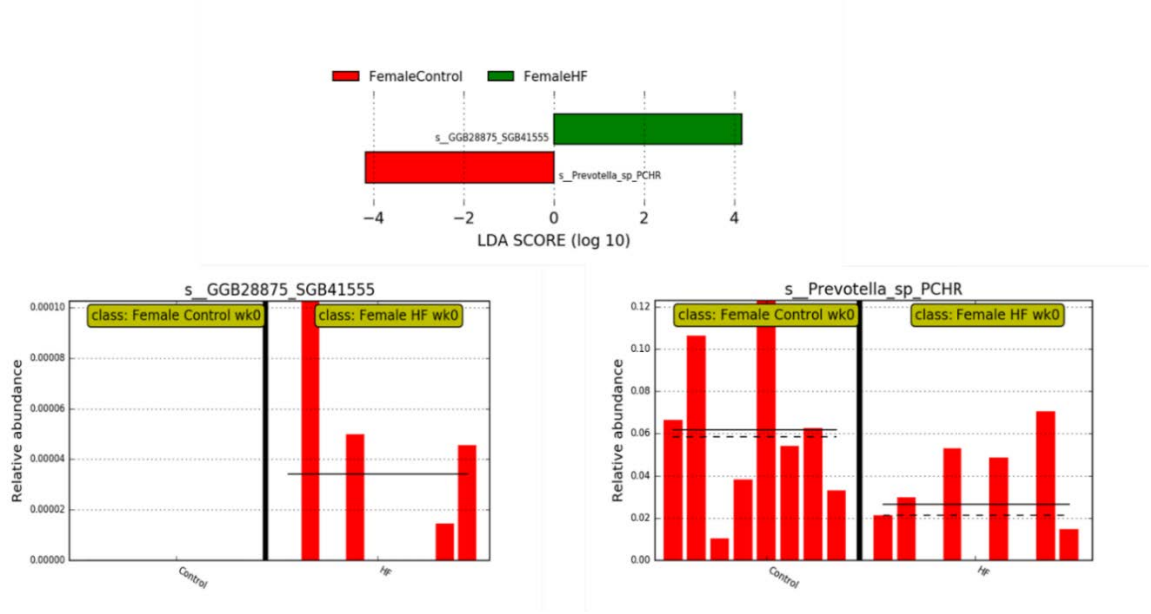


Figure 4.5 The Linear Discriminant Analysis (LDA) scores were calculated to assess the abundance of two distinct bacterial species, s_Prevotella_sp_PCHR and s_GGB28875_SGB41555, in female mice at week 0 *- These mice were divided into control and high fat diet groups prior to the initiation of the diet intervention. The LDA scores of the relative abundance of these bacterial species were evaluated to determine the discriminatory power between the control and high fat diet groups in female mice before diet intervention.*

Our metagenomics analysis of faecal samples revealed a significant difference in the abundance of bacterial species between the experimental groups, which consisted of both male and female subjects, at two different time points during the diet intervention. The differential abundance analysis, conducted using DESeq2, demonstrated statistically significant differences ($p<0.01$) in the abundance of various bacterial species between the groups (Fig 4.6). This suggests that the dietary intervention had a notable impact on the composition of the gut microbiome in both male and female subjects at the specified time points.

In male mice without diet intervention (control), our results revealed that *Prevotella sp_PCJ2, GGB27851_SGB40285, Bacteroidales bacterium, GGB14014_SGB21441, Muribaculum intestinale*, and *Duncaniella muris* were the most abundant bacterial species (Fig 4.7). However, in male mice subjected to a high fat diet, the most abundant species were *Parabacteroides goldsteinii, Enterorhabdus sp_P55, Bacteroides uniformis, Lachnospiraceae bacterium, and Adlercreutzia caecimuris*. In the female groups, the most abundant bacterial species in the control group without diet intervention were *Prevotella sp_PCHR, GGB28394_SGB40974, Sutterella sp_NM82*, and *Alistipes timonensis*. On the other hand, *Akkermansia muciniphila, Lachnospiraceae bacterium, GGB30466_SGBH3546, Faecalibaculum rodentium*, and

Limosilactobacillus reuteri were found to be the most abundant species in the female mice subjected to a high fat diet (Fig 4.8). These findings provide insights into the specific bacterial species that dominate the gut microbiome in each group and their potential associations with the respective dietary interventions. Our findings revealed a significant disparity in the abundance of bacterial species between female and male mice following the completion of a high fat diet intervention, despite both groups being subjected to the same dietary regimen for an identical duration. This discrepancy in bacterial species abundance highlights the influence of gender-specific factors on the gut microbiome response to high fat diet modifications. Further investigations are needed to elucidate the underlying mechanisms responsible for these observed gender-related differences and their potential implications for metabolic and physiological outcomes in response to high fat diet interventions.

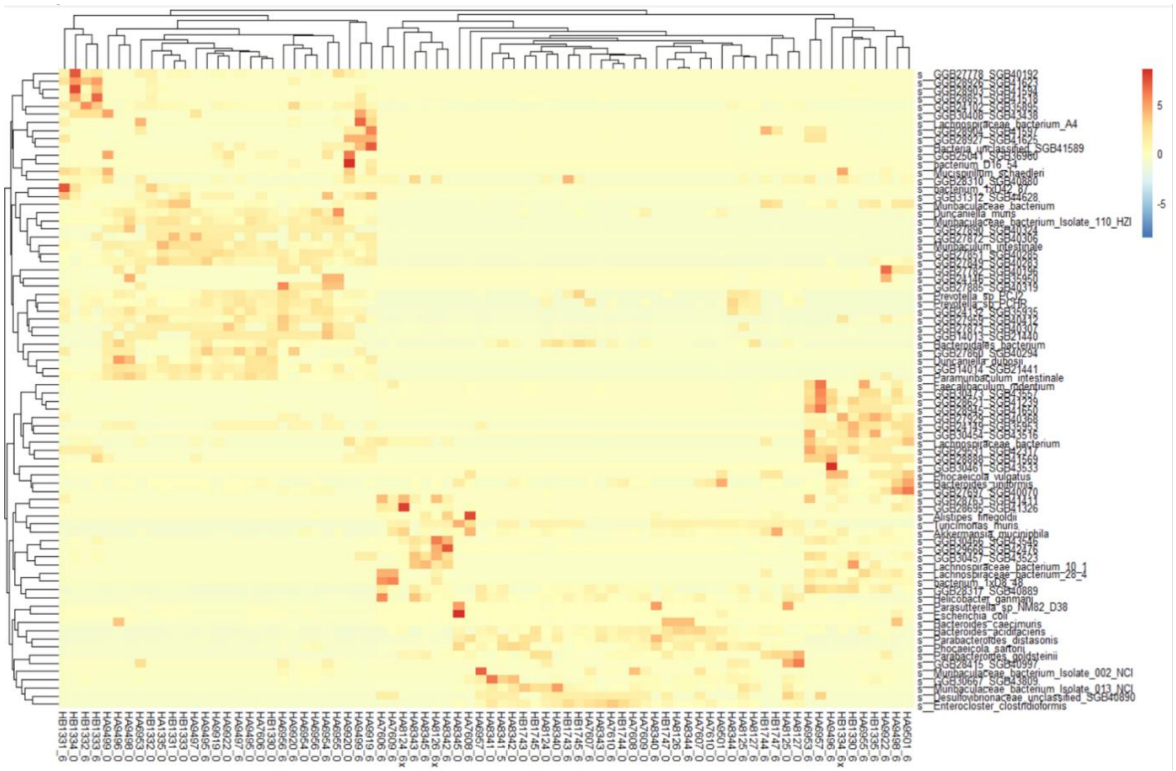


Figure 4.6 Differential Abundance of bacterial species between all samples (Male, Female) at different time points p<0.01 using DESeq2.

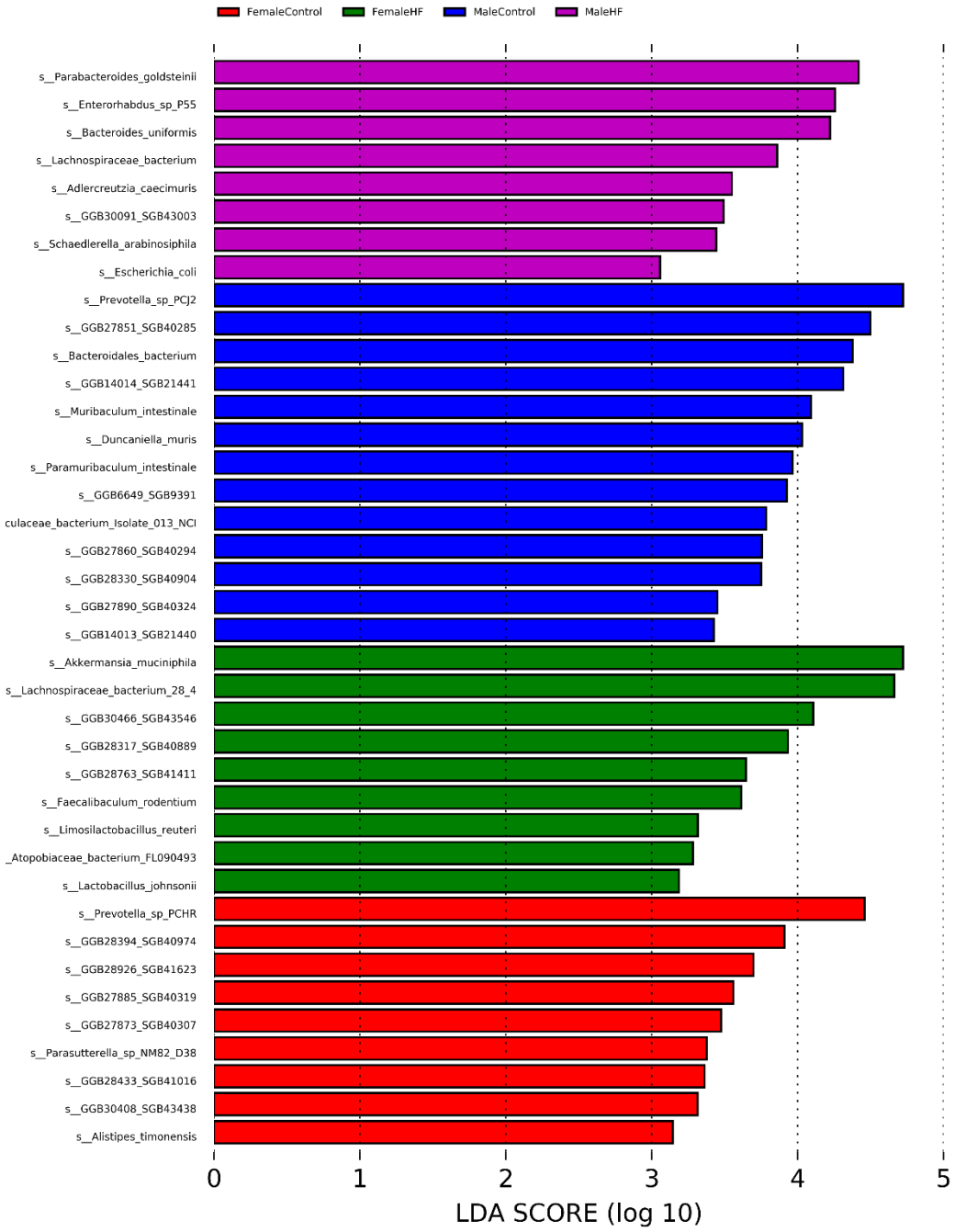


Figure 4.7 The Linear Discriminant Analysis (LDA) scores of the most abundance bacterial species between the experimental groups *- In both female and male controls, Prevotella sp. was found to be the most abundant bacterial species. However, in female mice subjected to a high fat diet intervention, Akkermansia muciniphila emerged as the most abundant bacterial species. Notably, this shift in abundance was observed specifically in the context of females with high fat diet intervention and its association with CCM lesion regression.*

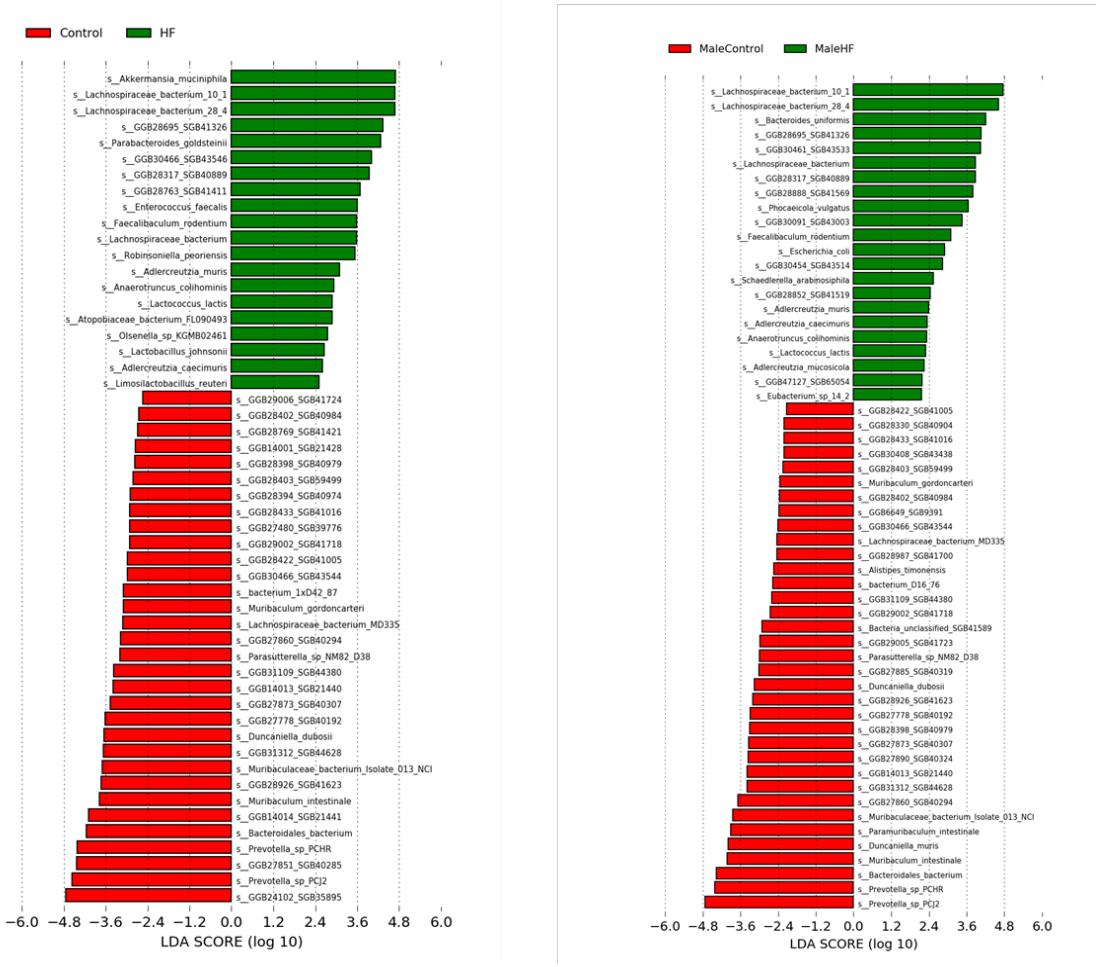


Figure 4.8 The Linear Discriminant Analysis (LDA) scores of bacterial species abundance of female and male mice after high fat diet intervention *- Our results showed that despite the same diet intervention and the same experiment duration, bacterial species abundance is significantly different between female and male mice at the endpoint of high fat diet modification.*

4.3.2 Metabolomics Analysis

The metabolome profile of the gut undergoes significant alterations following a short-term high fat diet modification in female mice, affecting various metabolic pathways.

To examine the alterations in metabolite profiles resulting from short-term high fat diet intervention, untargeted metabolomics analysis (LC-QToF MS) was conducted on the cecum content of five female mice subjected to the high fat diet and six female mice without diet intervention (control) at the end of the six-week intervention period. Metabolomics analysis of the cecum content revealed notable disparities in metabolite clusters between the high fat diet-

treated female mice and the control group, as indicated by the Principal component Analysis (PCA) score results (Fig 4.9). PCA can provide valuable insights into individual metabolites and overall trends, while PCoA helps visualize and understand relationships between samples based on their metabolic profiles. Combining these perspectives can lead to a more comprehensive understanding of your metabolomics data. These findings highlight the significant impact of the high fat diet on the metabolic composition of the gut.

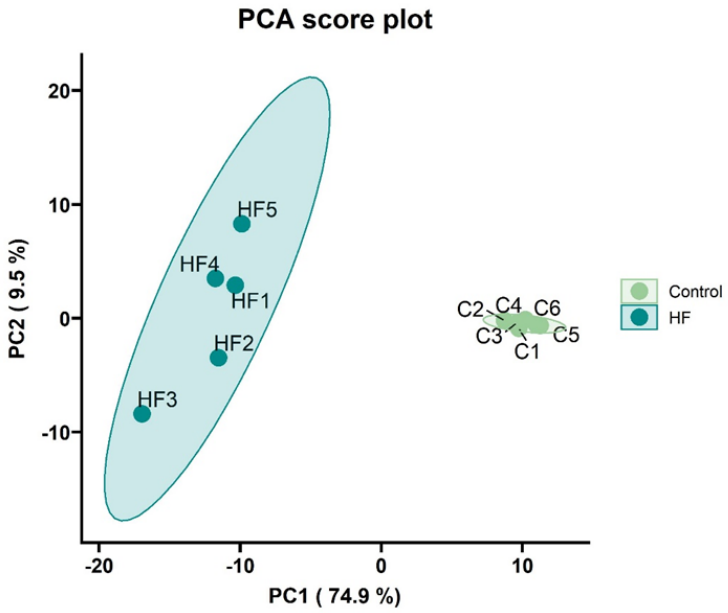


Figure 4.9 Principal component analysis (PCA) of metabolites of cecum content (gut) *- Our analysis showed two statistically different groups of metabolites between high fat diet intervention and control female mice at the end of diet modification (week 6).*

Our findings revealed significant alterations in the metabolite profiles following high fat diet modification (Fig 4.10). Several metabolites displayed a significant increase in abundance, including gamma Glutamylserine, Methyl linoleate, Riboflavin reduced, 5,24-dienolide 3-glucoside, and Histidinal. Conversely, certain metabolites exhibited a notable decrease, such as 2-Hydroxypyridine, 2-octene dioic acid, heptadeca-2,4-dien-6-one, and 2-methyldecane-1,3-dione, which were found to be more abundant in mice without dietary intervention (Fig 4.11). These observed changes highlight the impact of the high fat diet on the metabolic landscape and suggest potential alterations in specific metabolic pathways.

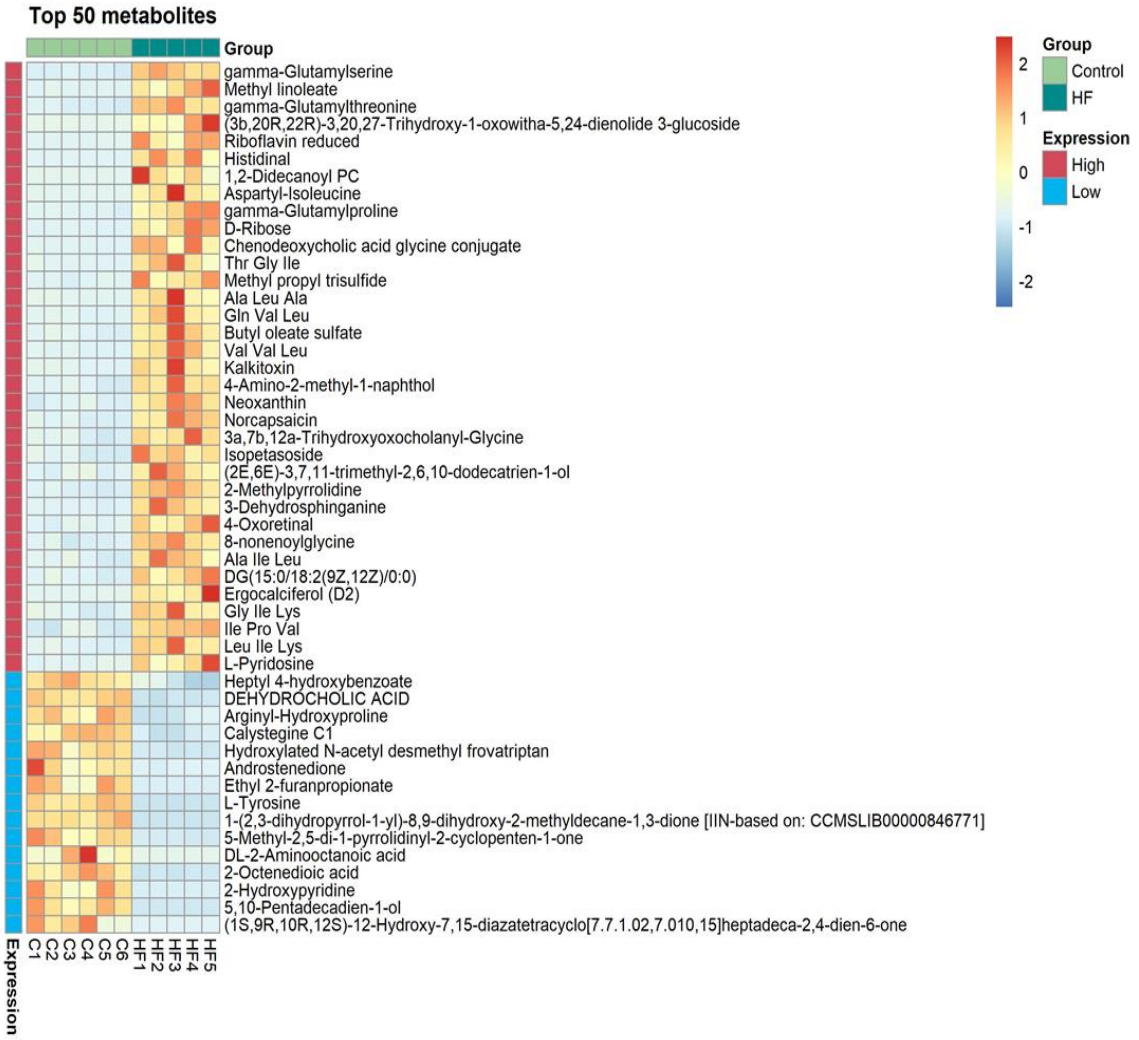
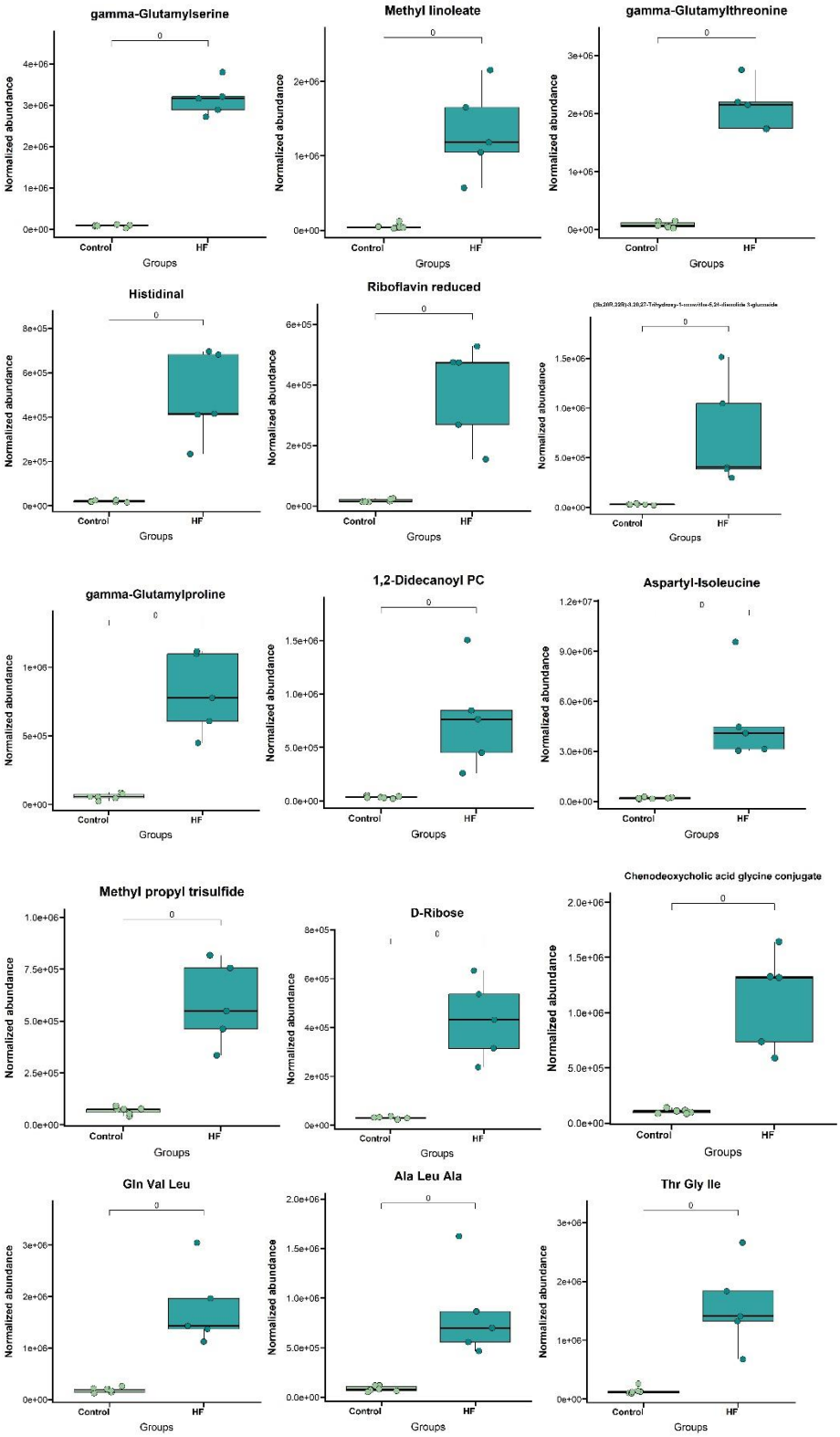


Figure 4.10 The analysis of metabolites revealed the top 50 metabolites that exhibited significant increases or decreases following the short-term high fat diet - *Among the top 50 metabolites that increased after the high fat diet, notable examples include gamma Glutamylserine, Methyl linoleate, Riboflavin reduced, 5,24-dienolide 3-glucoside, and Histidinal. Conversely, the top 50 metabolites that decreased after the high fat diet intervention included metabolites such as 2-Hydroxypyridine, 2-octene dioic acid, heptadeca-2,4-dien-6-one, and 2-methyldecane-1,3-dione.*


gamma-Glutamylserine
Methyl linoleate
gamma-Glutamylthreonine
Histidinal
Riboflavin reduced
gamma-Glutamylproline
1,2-Didecanoyl PC
Aspartyl-Isoleucine
Methyl propyl trisulfide
D-Ribose
Chenodeoxycholic acid glycine conjugate
Gln Val Leu
Ala Leu Ala
Thr Gly Ile
Normalized abundance
Groups
Control
HF

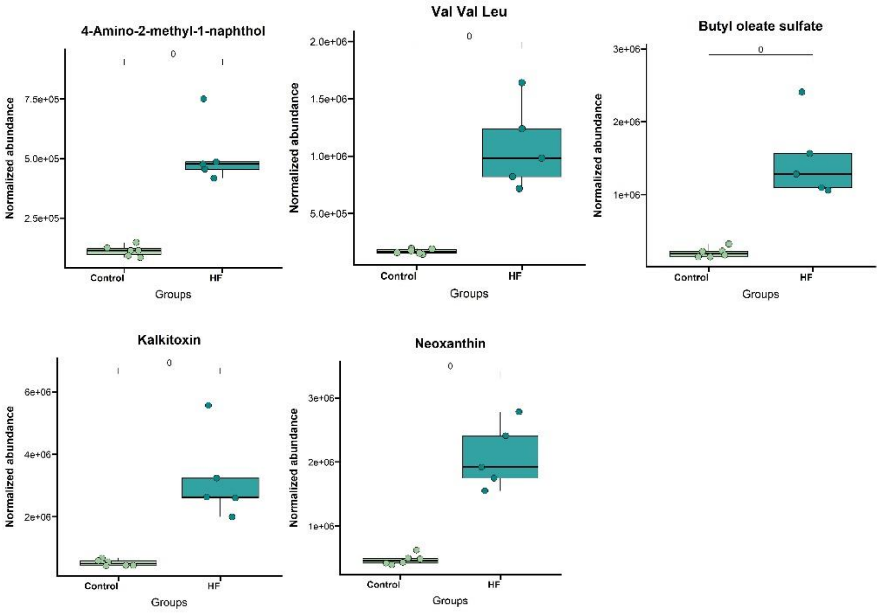
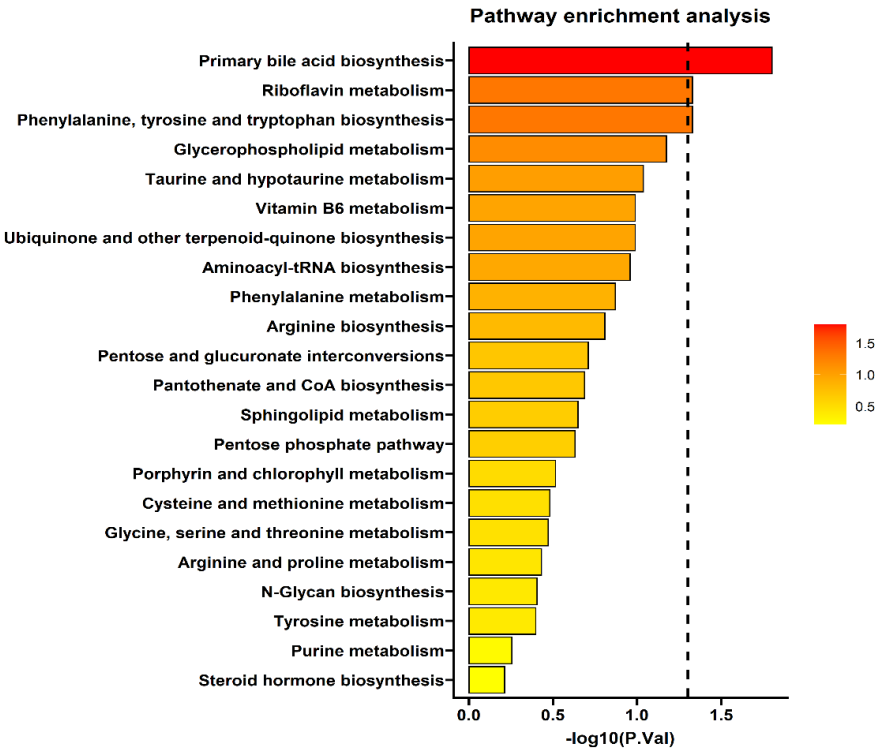


Figure 4.11 The normalised abundance of different metabolites between control and high fat diet treated groups *- Our metabolomics analysis of the cecum content from mice treated with high fat diet and control mice unveiled a significant increase in the abundance of several metabolites following a six-week high fat diet intervention.*

Following the identification of metabolome differences between the high fat diet intervention and control groups, all the identified metabolites were subjected to analysis using the "KEGG metabolic pathways" *via* the MetaboAnalyst software. Pathway enrichment analysis revealed that a majority of the metabolites that exhibited increased abundance following the short-term high fat diet intervention are involved in several metabolic pathways such as primary bile acid biosynthesis, riboflavin metabolism, amino acids (Phenylalanine, tyrosine, tryptophan, arginine, proline, cysteine and methionine) biosynthesis, glycerophospholipids metabolism, taurine and hypotaurine metabolism, vitamin B6 metabolism, ubiquinone biosynthesis, aminoacyl-tRNA biosynthesis, pentose glucoronate interconversions, panthotenate CoA biosynthesis, sphingolipid metabolism, pentose phosphate pathway, porphyrin and chlorophyll metabolism, N-glycan biosynthesis, purine metabolism and steroid hormones biosynthesis (Fig 4.12).



Column1	Total	Expected	Hits	Raw p	-log10(p)	Holm adjust	FDR	Impact
Primary bile acid biosynthesis	46	0.5498	3	0.015783	1.8018	1	1	0.0457
Riboflavin metabolism	4	0.047809	1	0.047004	1.3279	1	1	0
Phenylalanine, tyrosine and tryptophan biosynthesis	4	0.047809	1	0.047004	1.3279	1	1	0.5
Glycerophospholipid metabolism	36	0.43028	2	0.066888	1.1747	1	1	0.04318
Taurine and hypotaurine metabolism	8	0.095618	1	0.091917	1.0366	1	1	0
Ubiquinone and other terpenoid-quinone biosynthesis	9	0.10757	1	0.10283	0.98789	1	1	0
Vitamin B6 metabolism	9	0.10757	1	0.10283	0.98789	1	1	0.07843
Aminoacyl-tRNA biosynthesis	48	0.57371	2	0.11013	0.95811	1	1	0
Phenylalanine metabolism	12	0.14343	1	0.13482	0.87023	1	1	0
Arginine biosynthesis	14	0.16733	1	0.15555	0.80812	1	1	0.07614
Pentose and glucuronate interconversions	18	0.21514	1	0.19561	0.7086	1	1	0.14062
Pantothenate and CoA biosynthesis	19	0.22709	1	0.20534	0.68752	1	1	0.00714
Sphingolipid metabolism	21	0.251	1	0.22447	0.64884	1	1	0.07505
Pentose phosphate pathway	22	0.26295	1	0.23387	0.63102	1	1	0
Porphyrin and chlorophyll metabolism	30	0.35857	1	0.30529	0.51528	1	1	0.0902
Cysteine and methionine metabolism	33	0.39442	1	0.33042	0.48094	1	1	0
Glycine, serine and threonine metabolism	34	0.40637	1	0.3386	0.47031	1	1	0
Arginine and proline metabolism	38	0.45418	1	0.37039	0.43134	1	1	0.05786
N-Glycan biosynthesis	41	0.49004	1	0.39329	0.40529	1	1	0
Tyrosine metabolism	42	0.50199	1	0.40074	0.39714	1	1	0.13972
Purine metabolism	66	0.78884	1	0.55574	0.25513	1	1	0.02167
Steroid hormone biosynthesis	77	0.92032	1	0.61334	0.2123	1	1	0.12753

Figure 4.12 The pathway enrichment analysis of identified significantly increase metabolites after short-term high fat modification *- Gradient colour indicates the significance of pathways with the highest to lowest significance from red to yellow. Black horizontal dotted line indicates the cut-off of p.value =0.05. The identified metabolites which have increased after high fat diet, mainly involved in primary bile acid biosynthesis, Riboflavin metabolism, amino acids biosynthesis.*

4.3.3 Lipidomics Analysis

The diversity and abundance of lipid clusters in the gut of female mice underwent significant alterations following a short-term high fat diet intervention.

Following untargeted lipidomics analysis of cecum content from female mice subjected to a short-term high fat diet and those without any diet intervention, we observed a significant difference in lipid composition. Principal component Analysis (PCA) of the identified lipids revealed distinct lipid clusters between the control and high fat treated groups (Fig 4.13). These findings align with the observed differences in gut metabolites, indicating a significant impact of dietary intervention on gut metabolites, lipids, and microbiome composition. Our study revealed that the observed differences in lipid composition between the control and high fat diet groups were not limited to a few specific lipid species but encompassed a diverse range of lipid clusters. These lipid clusters exhibited distinct patterns and abundances, reflecting the substantial impact of short-term high fat diet intervention on the gut lipidome.

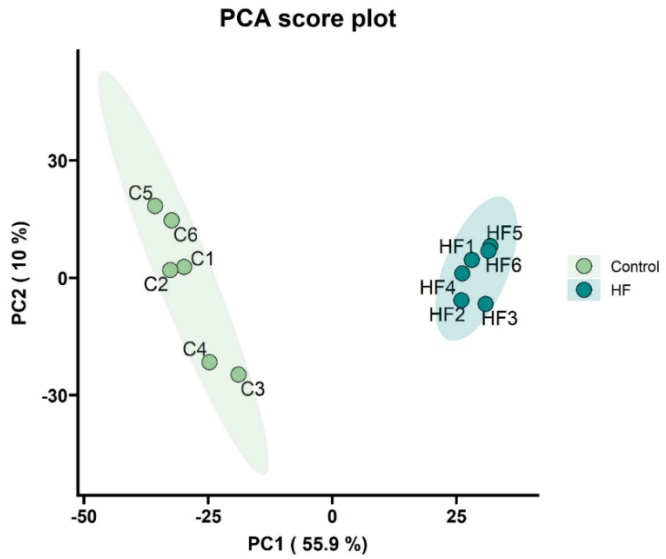
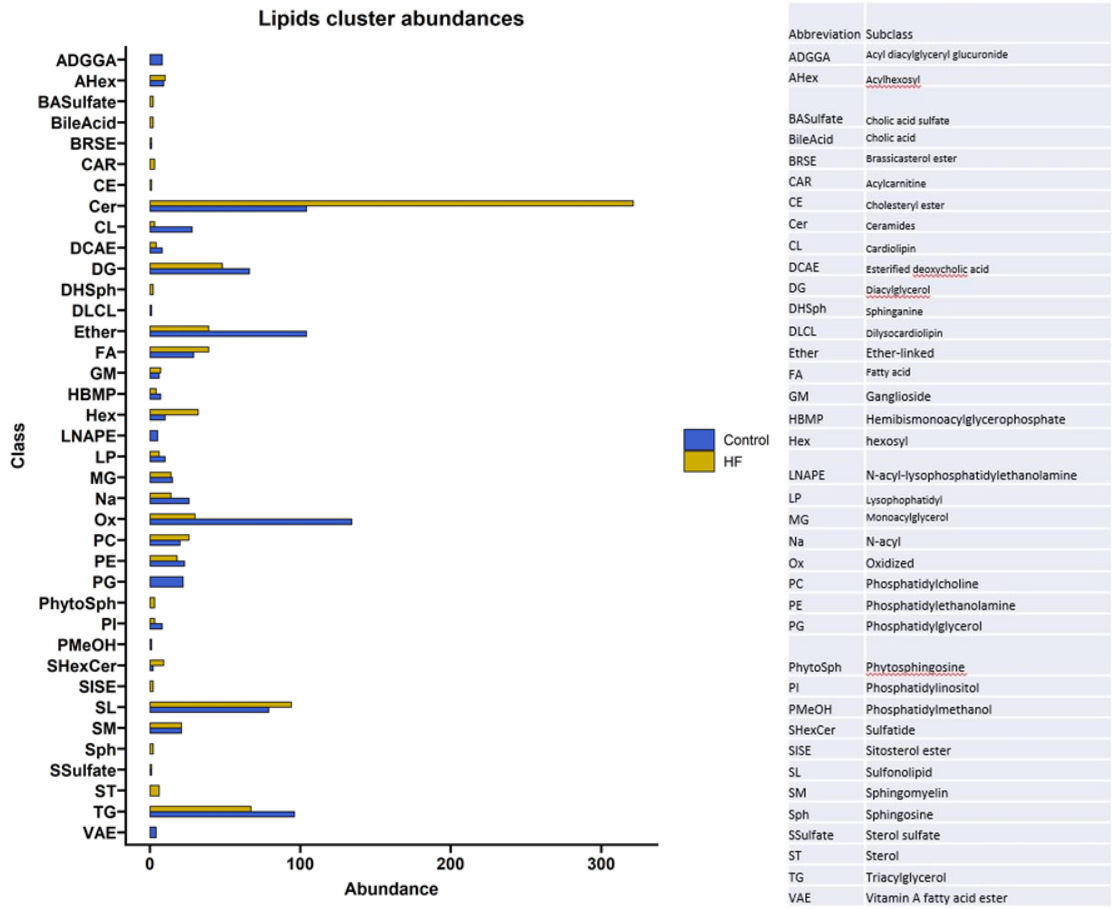


Figure 4.13 Principal component analysis (PCA) of lipidome of cecum content (gut) *- Our analysis unveiled distinct lipid profiles between female mice subjected to high fat diet intervention and those in the control group at end of diet intervention (week 6).*

Following the analysis of identified lipids, a thorough investigation of their cluster abundance was conducted in both the control and high fat diet groups. The results unveiled notable alterations in certain lipid clusters. Specifically, our findings revealed a significant increase in lipid clusters such as ceramides, sulfanolipids, and hexosyl following the short-term high fat diet intervention (Fig 4.14). Conversely, lipid clusters including cardiolipins, oxidized lipids, ether-linked lipids, diacylglycerol, and triacylglycerols displayed a significant decrease after the high fat diet

intervention. These results shed light on the distinct effects of the dietary intervention on specific lipid classes within the metabolic profile.



Abbreviation	Subclass
ADGGA	Acyl diacylglyceryl glucuronide
AHex	Acylhexosyl
BASulfate	Cholic acid sulfate
BileAcid	Cholic acid
BRSE	Brassicasterol ester
CAR	Acylcarnitine
CE	Cholesteryl ester
Cer	Ceramides
CL	Cardiolipin
DCAE	Esterified deoxycholic acid
DG	Diacylglycerol
DHSph	Sphinganine
DLCL	Dilysocardiolipin
Ether	Ether-linked
FA	Fatty acid
GM	Ganglioside
HBMP	Hemibismonoacylglycerophosphate
Hex	hexosyl
LNAPE	N-acyl-lysophosphatidylethanolamine
LP	Lysophophatidyl
MG	Monoacylglycerol
Na	N-acyl
Ox	Oxidized
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PhytoSph	Phytosphingosine
PI	Phosphatidylinositol
PMeOH	Phosphatidylmethanol
SHexCer	Sulfatide
SISE	Sitosterol ester
SL	Sulfonolipid
SM	Sphingomyelin
Sph	Sphingosine
SSulfate	Sterol sulfate
ST	Sterol
TG	Triacylglycerol
VAE	Vitamin A fatty acid ester

Figure 4.14 The lipid cluster abundance analysis of gut after short-term high fat diet intervention in female mice *- Our results showed a significant increase in the abundance of ceramides and sulfolipids and a decrease in cardiolipin and oxidised lipids after a six-week high fat intervention.*

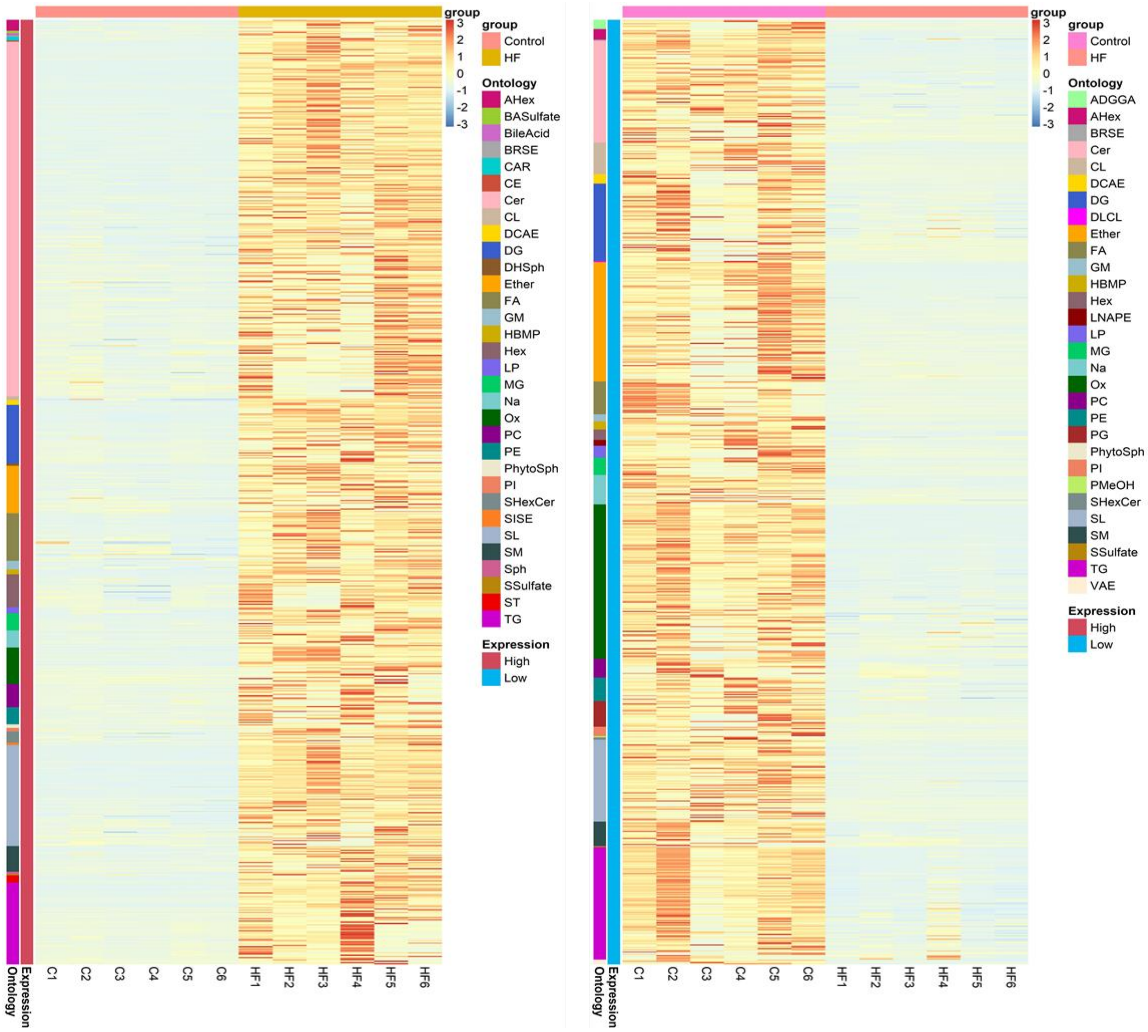
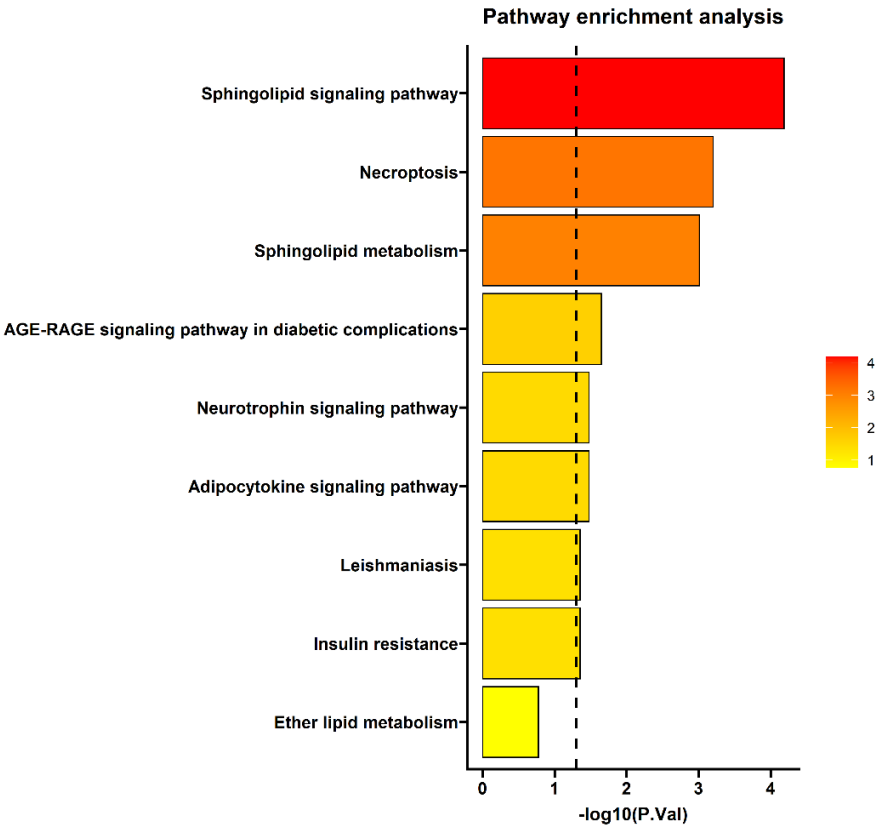


Figure 4.15 The lipid cluster abundance of each sample of high fat diet treated (HF) and control (C) female mice - *The results of our study revealed a substantial disparity in the abundance of lipid clusters between the control group and the group subjected to a six-week high-fat diet intervention.*

The identified lipids from our analysis were subjected to pathway analysis using the "KEGG metabolic pathways" approach implemented in the MetaboAnalyst software. The results of this analysis unveiled a range of metabolic pathways in which the identified lipids significantly participate. Notably, these pathways include sphingolipids signalling pathway, necroptosis, sphingolipids metabolism, AGE-RAGE signalling pathway, neurotrophins signalling pathway, adipocytokine signalling pathway, Leishmaniasis, Insulin resistance, and ether lipid metabolism (Fig 4.16). This comprehensive understanding of the involvement of lipids in various metabolic pathways provides valuable insights into the potential mechanisms underlying the effects of high fat diet intervention on CCM lesion regression in female mice.



Pathway name	Pathway lipids	Converted lipids (number)	Converted lipids (percentage)	Converted lipids (list)	p-value	Benjamini correction
Ether lipid metabolism	16	1	16.66667	C04475	0.167855	0.167855
Necroptosis	4	2	33.33333	C00195, C00550	0.000628	0.002828
Adipocytokine signalling pathway	3	1	16.66667	C00195	0.033455	0.049946
Insulin resistance	4	1	16.66667	C00195	0.044397	0.049946
Leishmaniasis	4	1	16.66667	C00195	0.044397	0.049946
Sphingolipid metabolism	21	3	50	C00195, C12126, C00550	0.000981	0.002943
AGE-RAGE signalling pathway in diabetic complications	2	1	16.66667	C00195	0.022408	0.049946
Sphingolipid signalling pathway	9	3	50	C00195, C12126, C00550	6.53E-05	0.000587
Neurotrophin signalling pathway	3	1	16.66667	C00195	0.033455	0.049946

Figure 4.16 The pathway enrichment analysis of the gut lipidome, which exhibited a significant increase subsequent to the high fat diet, indicates its involvement in diverse biological pathways *- The identified significantly increased lipids after short-term high fat diet exhibited playing a role in sphingolipid signalling pathway, necroptosis, sphingolipid metabolism.*

4.4. Discussion and Conclusion:

Prior research examining the composition of the gut microbiome has demonstrated significant distinctions between females and males in various treatments and disease states. Utilizing metagenomics analysis, we investigated the gut microbiome in our CCM mice model, specifically

focusing on the impact of diet intervention. Our findings indicated a significant alteration in the alpha-diversity of the gut microbiome composition in female mice following the modification to a high fat diet. However, there were no significant differences observed in the alpha-diversity between the control groups (males and females) and male mice subjected to the high fat diet intervention, when comparing the initial stage of the experiment (week 0) to the end of the experiment (week 6). In addition, our records of weight changes during the administration of short-term high fat diets in female and male mice revealed distinct patterns of weight gain. Despite being subjected to identical high fat diets, maintained under identical husbandry conditions, and originating from the same litter, female mice exhibited a significant divergence in weight earlier than their male counterparts following the intervention of a high fat diet. Intriguingly, only female mice displayed regression of CCM lesions subsequent to the short-term high fat diet intervention, while male mice did not exhibit a significant reduction in CCM lesions even after long-term exposure to a high fat diet. These observations imply that female mice exhibit heightened susceptibility to weight gain compared to males, and the gut microbiome of female mice manifests greater sensitivity to dietary interventions than male mice. Given that alterations in the gut microbiome induced by a high fat diet have been associated with CCM lesion regression in female mice, the more stable profile of the gut microbiome in male mice might hinder the observation of CCM lesion regression following high fat diet-induced gut microbiome modifications. Our results align with a prior study that examined the effects of a high fat diet on mice. Specifically, we noted a significant disparity in body weight gain, with female mice exhibiting a significantly greater increase compared to their male counterparts. However, male mice exhibited elevated levels of insulin resistance because of the high fat diet (307). Additionally, the effects of the high fat diet differed between females and males following pre-treatment with antibiotics. Females exhibited a significant reduction in insulin resistance, whereas males experienced increased blood glucose levels. Upon conducting 16S rRNA analysis of the gut microbiome in females and males, the bacterial genera such as *Parabacteroides, Lactobacillus, Bacteroides*, and *Bifidobacterium* were identified that were more abundant in female mice irrespective of the high fat diet intervention. Furthermore, the alterations observed in the gut microbiome due to antibiotic treatment and high fat diet intervention exhibited significant distinctions between females and males, thereby highlighting the influence of sex on the response to gut microbiome interventions (307).

In contrast, a separate study examining the impact of high fat diet and stress on the gut microbiome of mice found no significant disparity in weight gain between females and males. However, male mice exhibited a greater susceptibility to stress-induced effects and demonstrated heightened anxious behaviours compared to female mice. Interestingly, following modification to a high fat diet, these anxious behaviours in male mice exhibited a significant reduction, whereas no significant difference was observed in female mice. Furthermore, when analysing the Bray-Curtis

distances of 16S rRNA data pertaining to the gut microbiome of females and males, it was observed that stress induced a significant dissimilarity in the beta-diversity of the gut microbiome specifically in female mice, whereas no significant difference was detected in male mice subjected to high fat diet treatment (308). These findings from the study further support the notion that the effects of diet intervention on the gut microbiome exhibit divergence between female and male mice. Despite variations in the specific type of high fat diet utilised, the duration of diet modification, the mouse species employed, and the husbandry conditions implemented in our study compared to the mentioned research, we were able to observe a significant sex difference in the alteration of the gut microbiome in response to the short-term intervention of high fat diet. These findings suggest that, regardless of the specific experimental parameters, a consistent pattern of sex-related discrepancies exists in the gut microbiome response to dietary interventions involving high fat diets of varying compositions and durations.

In our study, short-term intervention with a high fat diet in female mice resulted in a significant reduction of CCM lesions in our mice model. Through metagenomics analysis of the gut microbiome, we gained further insights into the alterations occurring within the microbiome during the high fat diet intervention, both in female and male mice, and identified a potential association between the gut microbiome and lesion regression. Our metagenomics findings demonstrated a significant difference in the alpha-diversity of the gut microbiome in female mice before and after the high fat diet intervention. Moreover, the abundance profiles of bacterial genera exhibited distinct variations between the control and high fat diet groups for both females and males. Notably, *Prevotella spp.* emerged as the most prevalent bacterial taxa in the control groups, irrespective of sex. Previous research has highlighted the detrimental effects of *Prevotella* on gut inflammation and brain abscesses (309, 310). Certain species of *Prevotella*, such as *Prevotella intestinalis*, have been implicated in reducing the production of SCFAs by inducing alterations within the gut microbiome, subsequently leading to a decrease in the cytokine IL-18, which regulates immune responses (309). Therefore, the observed increase in *Prevotella* abundance, as observed in the control groups for both males and females, may contribute to CCM lesion formation by promoting gut inflammation. Metagenomics analysis of the gut microbiome showed that *Parabacteroides goldsteinii* was the most prevalent bacteria in male mice following a short-term high fat diet intervention. *Pa. goldsteinii* has been recognized for its potential beneficial effects on human health (311-313). A recent investigation employing a mouse model of Irritable Bowel Syndrome (IBS) demonstrated that the use of Red Ginseng (RG), a traditional herbal medicine known for its anti-inflammatory properties and brain function enhancement, led to an increased abundance of *Pa. goldsteinii* in the gut. This increase in *Pa. goldsteinii* abundance correlated with the downregulation of IL-1β and c-fos genes in both the gut and brain (314). Similarly, in another study utilising a mouse model of Autism Spectrum Disorder (ASD), the

administration of *Pa. goldsteinii* via oral gavage resulted in a reduction of anxiety-like behaviours, as well as decreased circulating LPS levels and mitigated intestinal inflammation (315). However, in our study, despite observing a significant increase in the abundance of *Pa. goldsteinii* in males subjected to the high fat diet, a significant reduction in CCM lesions, a decrease in blood LPS levels, or differences in gut morphology were not detected. These conflicting findings may be attributed to variations in the types of high fat diets employed, differences in mouse species utilised, or the presence of other bacterial species that could potentially influence the beneficial effects of *Pa. goldsteinii* in CCM disease.

The reduction of CCM lesions was specifically observed in female mice following a six-week treatment involving a high fat diet. Consequently, examining the prevailing bacterial taxa in the gut microbiome after the dietary intervention can provide valuable insights into the interaction between the gut microbiome and lesion reduction. Analysis of alpha-diversity in the gut microbiome of female mice revealed substantial differences in microbial communities before and after the high fat diet intervention. Examination of bacterial species abundance indicated that *Akkermansia muciniphila* and *Lachnospiraceae bacterium* were the most abundant bacteria in the gut following the diet intervention. Recent investigations have explored and identified the positive effects of *A. muciniphila* on human health (316-319). Metagenomics analysis of a mouse model of Alzheimer's disease (AD) demonstrated a decrease in *A. muciniphila* abundance in AD mice (320), while treatment with *A. muciniphila* via oral gavage for six months significantly reduced the accumulation of amyloid Aβ in the cerebral cortex (321). Additionally, *A. muciniphila* was found to have a protective effect against epilepsy in a mouse model by decreasing peripheral gamma-glutamyl (GG)-amino acids and increasing hippocampal GABA/glutamate ratios (322). Notably, *A. muciniphila* in the gut can regulate serotonin concentrations by upregulating genes involved in serotonin signalling and metabolism in the gut and brain, as observed in mice administered *A. muciniphila* via oral gavage (323). *Lachnospiraceae bacterium* possesses the capability to ferment various polysaccharides into SCFAs within the gut, and recent research has demonstrated the interaction between increasing metabolic health and the abundance of *Lachnospiraceae bacterium* (324). The effect of the short-term high fat diet on female and male mice resulted in distinct alterations in the gut microbiome composition, particularly in female mice, where the abundance of *Akkermansia muciniphila* and *Lachnospiraceae bacterium* was observed, lead to the regression of CCM lesions. These findings suggest a potential role of these bacteria in CCM disease.

To gain insight into the underlying mechanisms responsible for the reduction of CCM lesions in female mice following a short-term high fat diet, metabolomics analysis of the gut by examining cecum content, revealed two distinct groups of metabolites that exhibited significant differences between the control and high fat diet groups. Previous research comparing metabolite levels in

CCM patients with and without haemorrhage demonstrated that plasma concentrations of linoleic and arachidonic acids were significantly higher in patients with haemorrhage (306). However, a recent study demonstrated the positive effect of linoleic acid intake in reducing the risk of coronary heart disease (325). In our metabolomics results, any significant difference in the level of linoleic acid between the two groups was not observed. Interestingly, the level of kalkitoxin (KT) in the gut was significantly increased in the mice subjected to the high fat diet. KT has been suggested to possess anti-inflammatory properties and may impact bone diseases through the nuclear factor of activated T cells (NFATc1) and MAPK signalling pathways (326). Given that the MAPK pathway is involved in CCM, the elevated levels of KT in the gut may potentially contribute to the reduction of CCM lesions.

Riboflavin, also known as vitamin B2, and vitamin B3 have been acknowledged for their robust antioxidative properties within the human body. Riboflavin, in particular, assumes a pivotal role in mitigating oxidative stress within cells by actively scavenging reactive oxygen species (ROS) such as superoxide radicals and singlet oxygen. This scavenging activity serves to forestall potential damage to cellular constituents, including lipids and proteins (327). Moreover, riboflavin exhibits the capacity to upregulate the expression of genes implicated in antioxidant defence mechanisms (328). Acting as a coenzyme for essential enzymes such as glutathione reductase and glutathione peroxidase, riboflavin contributes significantly to cellular antioxidant processes. These enzymes, in turn, play a critical role in detoxifying ROS, thereby shielding cells from the deleterious effects of oxidative damage. The multifaceted antioxidative functions of riboflavin underscore its importance in maintaining cellular homeostasis and fortifying the body's defence mechanisms against oxidative stress (327-329). Undoubtedly, maintaining the appropriate concentration of reactive oxygen species (ROS) within cells is paramount for cellular homeostasis (330, 331). The integrity of essential macromolecules within cells, including DNA and proteins, is vulnerable to degradation or dysfunction when exposed to ROS. Moreover, the escalation of ROS levels is a primary contributor to oxidative stress, a leading cause of vascular remodelling and dysfunction in the Neurovascular Unit (NVU) associated with cerebrovascular diseases (332-334). The delicate balance in ROS concentrations is crucial for cellular health, as an aberrant increase can precipitate cascading effects that compromise the structural and functional integrity of vital cellular components, ultimately contributing to pathological processes in cerebrovascular contexts (332). In the context of Cerebral Cavernous Malformation (CCM), heightened oxidative stress within cells is implicated in a cascade of deleterious effects. Notably, the escalation of oxidative stress is associated with increased vascular permeability, disruption of endothelial cell-cell junctions, and heightened angiogenic activity. These consequences play a substantial role in driving the progression of CCM disease (335-337).Indeed, the CCM complex proteins play a crucial role in maintaining cellular homeostasis by actively mitigating oxidative

stress. Their absence or dysfunction may impair the cell's ability to counteract oxidative insults, contributing to an imbalance in redox homeostasis and fostering an environment conducive to heightened oxidative stress activity (338-340). The metabolomics results revealed an elevation in Riboflavin levels in the gut following a high fat diet. Additionally, pathway enrichment analysis of the identified metabolites indicated a substantial involvement in riboflavin metabolism. This suggests that a high fat diet enhances the presence of Riboflavin in the gut, potentially influencing its concentration in the bloodstream as well. The results of this study indicate that Riboflavin could potentially contribute to the regression of CCM lesions through dietary interventions. Furthermore, the analysis of plasma concentrations of cholic acid and hypoxanthine revealed significant difference between individuals with CCM and healthy control subjects (306). Previous studies have identified the role of these molecules in preserving blood-brain barrier integrity, reducing apoptosis, and inflammation (341). Our results demonstrated an increase in metabolites involved in primary bile acid biosynthesis, including cholic acid, as well as a significant reduction in dehydrocholic acid following short-term high fat diet intervention. Another pathway enrichment analysis of identified gut metabolites implicated steroid hormone biosynthesis. Previous studies have demonstrated that steroid hormones, specifically estrogens, have the capacity to influence the expression of endothelial nitric oxide synthase (eNOS) through interaction with estrogen receptors (ER) (342). Nitric oxide (NO) plays a crucial role in regulating cerebral blood flow by affecting smooth muscle cells and vascular endothelium (343-345). Therefore, alterations in steroid hormone levels resulting from modifications in the high fat diet may contribute to the potential mechanisms underlying CCM lesion regression.

Sphingolipids are a major constituent of the brain and play a critical role in maintaining the stability of the myelin sheath in the CNS (346). Perturbations in sphingolipid metabolism can have profound effects on the organisation of the plasma membrane (346). Through our metagenomics and lipidomics pathway enrichment analysis, the majority of identified metabolites and lipids that increased following a high fat diet intervention are discovered that are involved in the sphingolipid signalling pathway and metabolism. Furthermore, a significant elevation in lipid clusters such as ceramides and sulfanolipids, which are sub clusters of sphingolipids, in the gut after a short-term high fat diet was detected. Earlier in vitro investigations have proposed the involvement of ceramides in the stimulation and multiplication of smooth muscle cells via the MAPK signalling pathway (347). Notably, CCM lesions are characterized by the absence of elastin and a significant reduction in smooth muscle cells (348). Based on these findings, it is plausible to propose that the increased levels of sphingolipid clusters in the gut, along with their impact on vascular smooth muscle cells within CCM lesions, potentially through the MAPK pathway, could serve as mechanisms contributing to the reduction of CCM lesions following a high fat diet intervention.

Conversely, existing studies posit Ceramides as a potential tissue biomarker for Peripheral Arterial Disease (PAD), contributing to endothelial dysfunction (349). Furthermore, the upregulation of genes involved in Ceramide production and the subsequent increase in Ceramides have been shown to activate the Ceramide-induced NLRP3 inflammasome, resulting in pulmonary microvascular endothelial cell barrier dysfunction (350). The augmented production of Ceramides in the gut induced by high fat diet consumption may impact endothelial cells in the gastrointestinal tract, potentially leading to diminished gut integrity and increased permeability. This, in turn, could facilitate the entry of elevated Ceramides into the bloodstream, thereby exerting an influence on CCM lesions. Notably, despite these changes, no discernible alterations in the levels of lipopolysaccharides (LPS) were detected. An avenue worth exploring for a more comprehensive understanding of the role of Ceramides in CCM lesion reduction involves the investigation of gene expression related to ceramide metabolism before and after high fat diet intervention. Such an examination could provide valuable insights into the mechanistic underpinnings of Ceramides in the context of CCM pathology, shedding light on their potential impact on endothelial function and gut permeability.

In conclusion, our study delves into the intricate relationship between diet-induced alterations in the gut microbiome and the regression of CCM lesions in a murine model. Notably, the observed reduction of CCM lesions in female mice following a short-term high fat diet intervention highlights the potential therapeutic impact of dietary modifications. Metagenomics analysis revealed distinct changes in the alpha-diversity and abundance profiles of bacterial genera, particularly noting the prevalence of *Akkermansia muciniphila* and *Lachnospiraceae* bacterium in female mice, associated with CCM lesion regression. Metabolomics analysis uncovered significant variations in gut metabolites, including the elevation of Riboflavin, kalkitoxin, and alterations in steroid hormone levels, suggesting potential mechanisms contributing to lesion reduction. Additionally, lipidomics pathway enrichment analysis identified sphingolipid metabolism as a key player, with increased levels of ceramides possibly influencing smooth muscle cells within CCM lesions. While these findings underscore the intricate interplay between diet, gut microbiome, and molecular pathways in CCM, further investigations are warranted to elucidate the specific mechanisms driving these observed effects and their translational implications for CCM management.

5. Chapter 5: Study of the effect of gut microbiome alteration by faecal matter transplant (FMT) donated by 6-week high fat diet treated mice on experimental mouse model of Cerebral cavernous malformation (CCM)

5.1. Abstract:

CCM are aberrantly dilated blood vessels that form a non-malignant but potentially fatal condition causing a haemorrhagic stroke. Our previous diet intervention study showed a 6-week treatment with a high fat diet on the Tamoxifen inducible mouse model of CCM, resulted in a significant CCM lesion reduction in female mice. Furthermore, our investigation utilising metagenomics, metabolomics, and lipidomics analysis of the gut microbiome in mice subjected to a high fat treatment revealed the presence of two clearly discernible bacterial communities and distinct clusters of metabolites in comparison to the control group. To confirm the role of the microbiome, we designed the real-time faecal matter transplant from $CCM1^{KO}$ high fat diet-treated female mice to female mice without any dietary intervention for six weeks. Our results showed that FMT recipient mice without any dietary intervention showed the same lesion regression of high fat treated mice compared to controls, which have evidenced the crucial role of the gut microbiome in lesion regression derived by dietary intervention.

5.2. Introduction

Faecal matter transplant (FMT), also known as a faecal microbiota transplant, is a medical procedure in which faecal matter (stool) from a healthy donor is transplanted into the GI of a recipient to restore the balance of healthy bacteria in their gut. The goal of the procedure is to introduce a diverse population of beneficial bacteria, which can help treat various gastrointestinal disorders and improve overall gut health. Alteration of gut microbiome composition or intervention of gut microbiome can be result of changing daily diet, using antibiotics, consuming of probiotics and prebiotics and FMT. FMT has been shown to be an effective treatment for recurrent *Clostridioides difficile* infection, which is a bacterial infection that causes severe diarrhoea and can be life threatening (351). Previous studies on gut microbiome communities of

healthy individuals and some neurological disorders patients such as Parkinson's disease, multiple sclerosis, autism spectrum disorder, Alzheimer's disease, Rett syndrome, neuromyelitis optica, amyotrophic lateral sclerosis and epilepsy showed a significant difference in diversity and abundance of bacterial communities in the gut (352-360). Some patients with these diseases often demonstrate gastric problems, which can be sign of role of GI in disease pathogenicity (361, 362). In animal model of ASD and clinical studies on patients, gut microbiome intervention by using probiotics decrease neurological and gastrointestinal syndromes such as anxiety or lack of concentration and constipation or diarrhoea (363-365). FMT from ASD children donor to germ free mice showed ASD symptoms on the mice and their offspring. Furthermore, another study found a significant reduction in cerebral oxidative stress in ASD hamster model after FMT from normal hamster and this reduction fortified when *Lactobacillus paracaseii* administrated as probiotic (366, 367).

Gut microbial studies on patient with stroke are controversial. Some results of studies elicit a prominent difference of gut microbiome in individuals experience stroke compared to healthy people (368, 369). On the other side, some studies show any significance difference or only temporary alteration (300, 370). However, it is hard to confirm these results due to other influent factors such as age, obesity and diabetes. Gut microbiome composition alteration is highly related to severity of stoke. Stroke leads to decrease GI motility that causes exorbitance growth of some bacteria and changing diversity of microbiome. Consequently, disruption of gut barrier results in accumulation of proinflammatory immune cells in lymphoid glands and gut bacterial components or metabolites enter the bloodstream then the brain (371, 372). In one study on experimental mice model of stroke, the mice that treated with antibiotics after stroke had higher mortality rate compared to control group. When these mice got Specific pathogen-free (SPF) microbiota as FMT, the mice showed the same mortality rate and infarct volume to control group. These findings suggested that intervention of gut microbiome via antibiotics consumption could increase the rate of post stroke mortality (373). Furthermore, in another study on the diabetic mice with carotid occlusive disease that got suspension of *Clostridium butyricum* intragastrically show less neuronal injury (374).

Recent studies on AD patients revealed the distinct gut microbiome composition compared to healthy elderly individuals (356, 375-378). The increase of LPS level and number of bacteria were observed in AD patients (379, 380). LPS increase inflammation related to endotoxin that have amyloid neurotoxicity and neuroinflammation effect, which is supposed to play a crucial role in progression of AD (381, 382). Some bacteria can produce extracellular amyloid so called "curli fibers". The Toll-like receptor that identifies the Amyloid beta 42 (Aβ 42) peptide, which builds up in AD, is also capable of identifying certain bacteria that generate curli fibers (381, 383). Another example of gut bacterial effect is the production of neurotransmitters and alteration

of receptors that play a role in synaptic plasticity could be a factor in the development of AD (384, 385). Two studies performed gut microbiome intervention by FMT on AD mice model. In one study, when gut microbiome depleted via broad spectrum antibiotics on transgenic AD mice model, reduction in Aβ 42 accumulation and neuroinflammation were observed but only male mice. While Aβ-treated mice gained FMT from non- Aβ-treated mice this reduction partially restored (386). Another study on germ-free AD mice showed that FMT from AD mice and normal mice have different consequences in progression of AD on germ-free mice (387).

Although there is no study to show effect FMT and gut microbiome intervention on CCM disease, 16s rRNA analysis of gut microbiome composition of mice model of CCM revealed importance of microbiome in CCM. The findings indicate that there are notable distinctions in the gut microbiome communities of mice with brain lesions (susceptible) caused by KRIT1 and CCM2 knock-out and those without the CCM phenotype (resistant). The susceptible mice have elevated levels of *Bacteroides s24-7,* while the abundance of *Ruminococcus* genera differs in KRIT1 and CCM2 knock-out mice (22). To investigate the regression effect of high fat diet modification through microbiome, in this study FMT from High fat diet treated female mice performed on same age, sex, and litter mice without any diet intervention as control.

5.3. Results:

5.3.1 Weight Difference Analysis

High fat-treated mice (Donors) are significantly heavier than controls and recipients, and no significant weight difference was observed between the control and recipient groups.

The same age, litter, and female mice were separated into three groups according to diets and FMT administration. The control group used standard chow and self-faeces FMT; the Donor group used high fat diet intervention and self-faeces FMT, and the Recipient group used standard chow but real-time FMT from donor mice. Our weekly weight records analysis of control and donor and recipient groups show donor group (high fat) gained a significant weight difference from the third week of the experiment compared to recipient groups and from the fourth week compared to controls. Moreover, there is no significant weight difference between control and recipient mice (Fig 5.1).

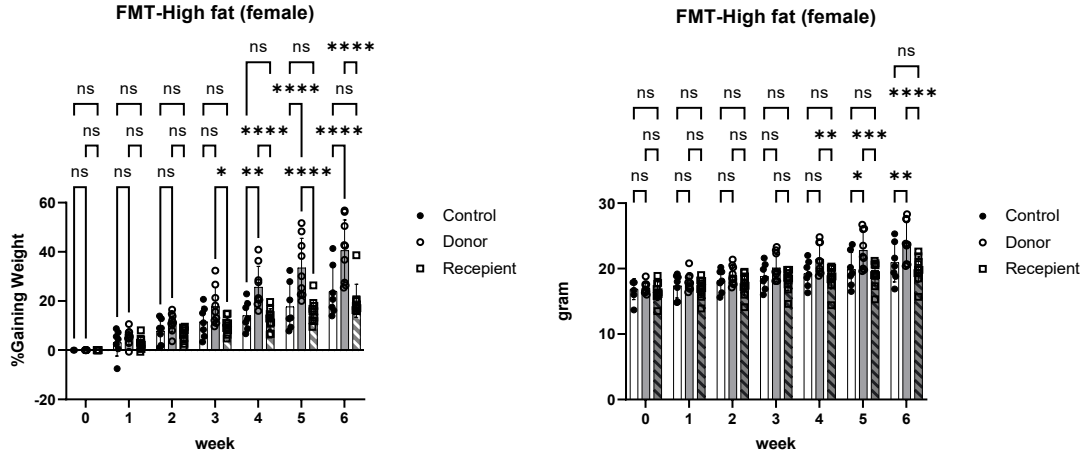


Figure 5.1 Weight analysis of control, donor (HF treated) and recipient (HF-FMT) female mice *- The results showed significant gaining weight of donor since week 3 compared to other groups. No significant weight difference was observed between controls and recipients. Error bars represent as mean SD and significance determined by Two-way ANOVA, Greenhouse–Geisser correction.*

5.3.2 CCM lesion Burden

Recipient mice showed a significant reduction in CCM lesion burden compared to controls after 6-week gaining FMT from HF-treated mice.

Analysing the brain volumes of control, donor and recipient groups using Micro-CT, the results showed that there were no noticeable differences between the groups. However, both donors and recipients showed significant reduction of CCM lesions in terms of their size and number, in comparison to the control group. Interestingly, there were no significant differences observed in the regression of lesions between the donors and recipients (Fig 5.2).

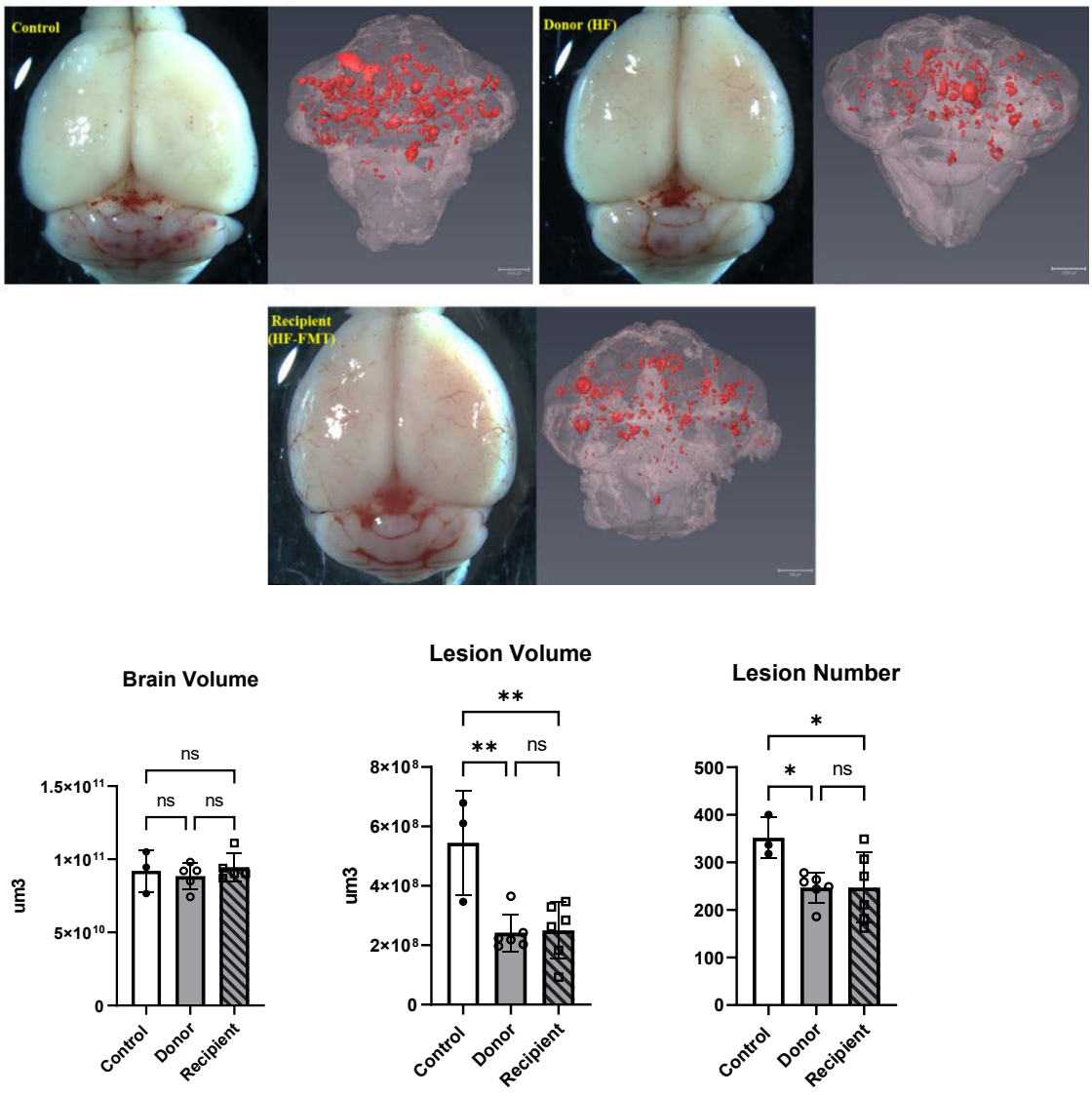


Figure 5.2 CCM lesion reduction was detected in donor and recipient mice after six weeks FMT administration *- The groups did not display any significant difference in brain volume. Donors and recipient showed approximately same CCM lesion burden. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

5.3.3 Gut Histology Analysis

The ileum muscle layer was significantly thinner in both the recipients and the donor groups, compared to the controls.

Regarding the morphology of gut, no statistically significant difference was detected in epithelial and muscle layer of colon between the groups (Fig 5.3). Furthermore, there is no significant difference in length of villi in ileum and number of the goblet cells (Fig 5.5). The ileum muscle layer of both the recipients and donors was found to be significantly thinner than that of the control group (Fig 5.4). Even though the thickness of the ileum muscle layer in the recipients is greater than that of the donors, the difference between them is not considered statistically significant.

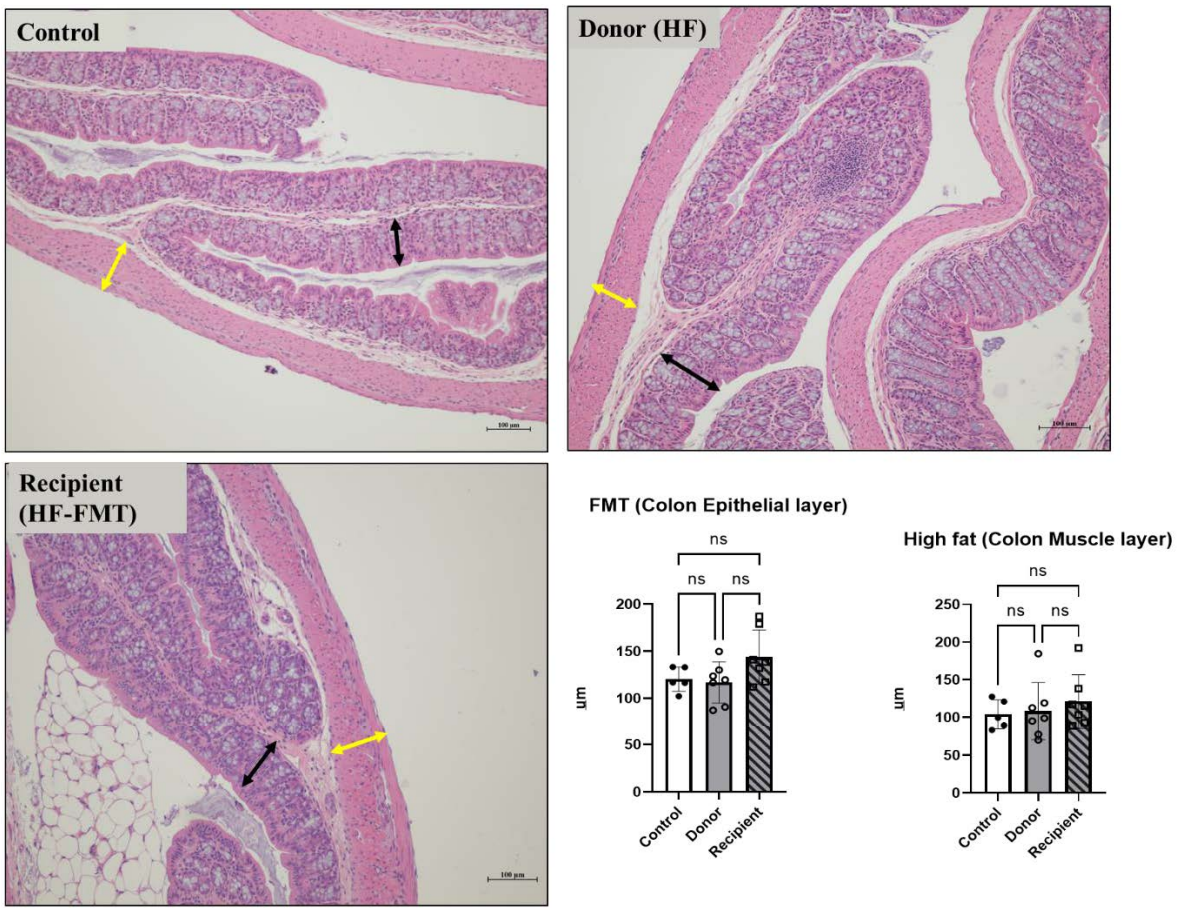


Figure 5.3 Colon epithelial layer (black arrow) and colon muscle layer (yellow arrow) in controls, donors (HF), recipients (HF-FMT) *- There was no significant difference found in either layer among the groups. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

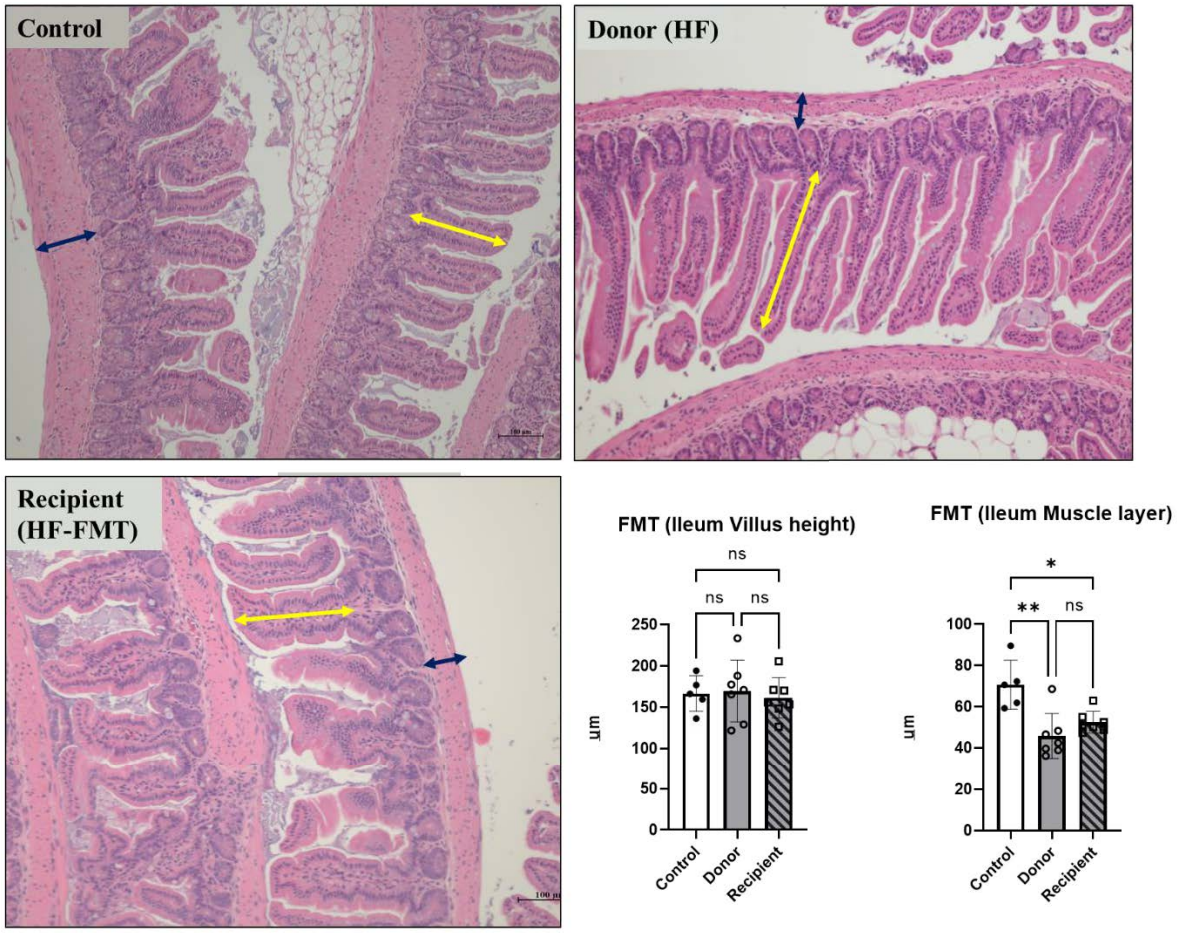


Figure 5.4 Ileum villus length (yellow arrow) and ileum muscle layer (black arrow) in controls, donors (HF), recipients (HF-FMT) *- Ileum muscle layer is significantly thinner in donors and recipients compared to controls. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

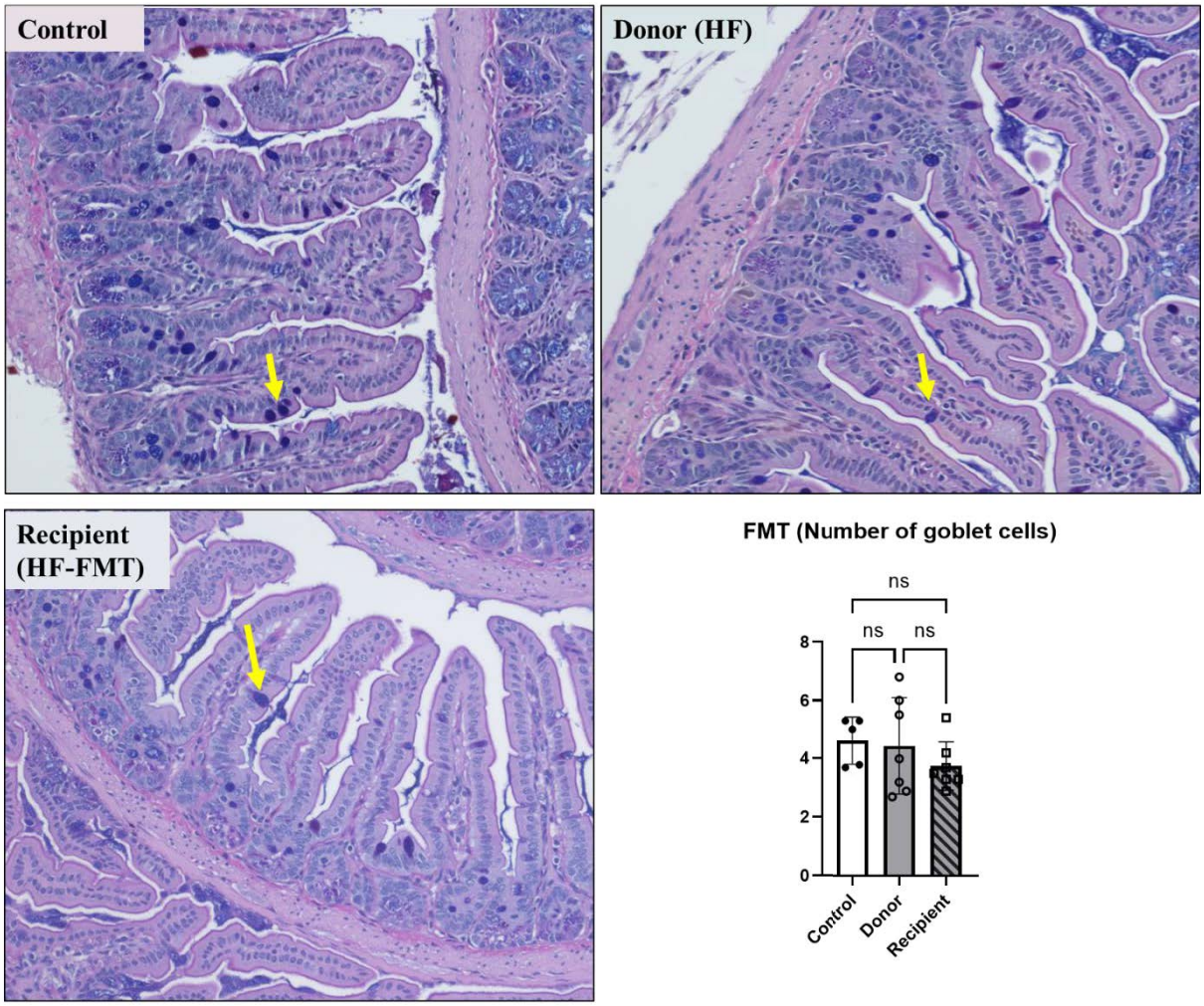


Figure 5.5 Ileum goblet cells in villus (yellow arrow) in controls, donors (HF), recipients (HF-FMT) *- There was no significant difference observed in the number of detected goblets among all the groups. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

5.3.4 Circulating LPS Level

No significant difference in the LPS level of blood was observed in the control, donor and recipient groups.

The analysis of blood serum LPS levels across all groups indicated no notable difference between the control, donor, and recipient groups. Specifically, there was no significant distinction in LPS levels between the control and recipient groups. However, the donor group displayed slightly elevated LPS levels compared to the other two groups. Nevertheless, the disparity between the donor group and the remaining groups did not reach statistical significance. Consequently, these

findings suggest that the transplantation procedure had minimal influence on LPS levels in the blood of the recipients (Fig 5.6).

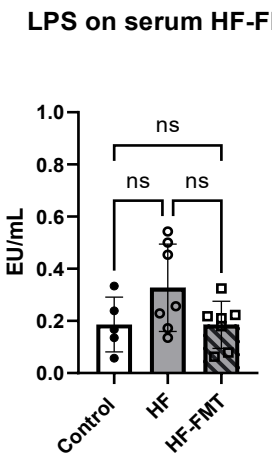


Figure 5.6 Level of circulating LPS in the controls, donors (HF) and recipients (HF-FMT) - *The results indicated that there was no considerable alteration in the blood LPS levels among all the groups. Error bars represent as mean with SD and significance determined by One-way ANOVA, ordinary ANOVA test.*

5.4. Discussion and conclusion:

Dysbiosis of the gut microbiome has been documented in numerous diseases. Nonetheless, the underlying mechanism of whether this dysbiosis is a consequence of disease progression or serves as an initiating factor in the development of the disease remains elusive. Despite the abundance of evidence highlighting the impact of the gut microbiome on human health and certain disease conditions, the use of FMT as a therapeutic option remains a topic of controversy. Among the various approaches for intervening in the gut microbiome, certain studies have indicated that the administration of FMT can result in more efficient and rapid alterations in gut microbiome communities, leading to increased bacterial diversity, in comparison to alternative interventions such as dietary modifications and probiotic supplementation (388-391). However, the effect of microbiome alteration on different diseases is diverse due to disease conditions, FMT administration and preparation methods.

In our FMT model, the weight measurements of the groups revealed that donors who were fed a high fat diet exhibited a significant increase in body weight, as anticipated, when compared to the control group and the recipient group. Conversely, the recipients who received FMT from the donors did not exhibit any statistically significant differences when compared to the control

group. This suggests that while there may be a similarity in gut microbiome profiles between recipients and donors following FMT the recipients did not experience weight gain. The observed weight gain was specifically attributed to the high fat diet intervention rather than alterations in the gut microbiome. In a separate animal investigation, the utilisation of FMT from control and lean mice to mice subjected to a high fat diet intervention demonstrated a significant reduction in weight gain among the recipient mice. Additionally, this intervention was observed to regulate glucose tolerance and modulate the expression of genes associated with obesity and high fat diets (392). These outcomes corroborate our own findings, indicating that microbiome manipulation via FMT not only has no impact on weight gain but also possesses the capacity to regulate and modify weight gain and other adverse consequences of a high fat diet in a mouse model (393). Another study investigating the effects of autologous FMT in conjunction with different diet interventions demonstrated varied impacts on weight. Specifically, autologous FMT derived from a healthy diet rich in vegetable and fruit fibre did not induce weight gain or loss following cessation of the diet intervention. Conversely, FMT derived from a green Mediterranean diet resulted in weight loss even after completion of the diet intervention (394). The diverse outcomes observed in weight following FMT are likely attributed to multiple factors, including the recipient's gut microbiome community diversity, the composition of the diet in terms of fat, fibre, protein, etc., the duration of both the diet intervention and FMT administration, as well as the specific strategies employed for FMT administration.

Based on prior research, it has been established that the composition of the gut microbiome can influence the structure of the colon and ileum, as well as modulate the permeability and integrity of the gut barrier (395-397). In our study, the analysis of gut morphology in both donor and recipient mice revealed a significant reduction in the thickness of the ileum muscle layer compared to the control group. However, in the context of our short-term high fat diet intervention experiment, there was no significant disparity observed in the thickness of the ileum muscle layer between females and males. This observation aligns with the findings of another study investigating the effects of a high fat diet on the ileum muscle layer, where no statistically significant impact was detected (255). The observed difference in the thickness of the ileum muscle layer is intriguing. While a six-week high fat diet did not elicit any notable effect on the ileum muscle layer, administration of FMT over the same duration resulted in a reduction of this layer. It is important to note that all groups (controls, donors, and recipients) received FMT using identical strategies and administration schedules. Therefore, this significant reduction in the ileum muscle layer cannot be attributed to the FMT procedure or the act of oral gavaging, as the control group did not exhibit any significant reduction in the size of the ileum muscle layer.

Prior studies investigating faecal transplantation in experimental animals have reported highly variable effects of gut microbiome intervention via FMT on gut morphology and integrity. For

instance, in one animal model study, FMT from angiotensin-converting enzyme 2 overexpressing mice (ACE2) led to a significant increase in the thickness of the ileum muscle layer and the number of goblet cells in wild-type mice (398). This finding aligns with another study on broilers, which demonstrated that FMT from healthy broilers resulted in an increased thickness of the ileum muscle layer and the mucosal layer in the recipients (399).

Conversely, FMT administration from complement 3 (C3) knockout mice to wild-type mice, following gut microbiome depletion through antibiotic consumption, exhibited a significant decline in the muscle layer of the gut. However, FMT from wild-type mice had the opposite effect, causing a significant increase in the muscle layer (400). The impact of FMT on gut morphology and the integrity of the gut-blood barrier is intricately linked to the diet and conditions of the donor. However, it is noteworthy that results from studies in this area exhibit considerable variability. The ileum, responsible for finalising the absorption process, particularly for specific nutrients and bile acids, also plays a robust role in immune function. Our metabolomics analysis has discerned a significant abundance of identified metabolites in the gut of mice treated with a high fat diet involved in the biosynthesis of bile acids. This prompts speculation that FMT from mice subjected to a high fat diet may potentially influence the ileum, potentially leading to a reduction in the muscle layer. Such alterations could be geared towards increasing the absorption capacity, specifically for the elevated bile acid products observed in the aftermath of FMT. Furthermore, our analysis demonstrated that the reduction in the thickness of the ileum muscle layer, which led to decreased gut permeability, did not result in an increase or exacerbation of CCM lesions in our mice model. On the contrary, significant reduction of lesions was observed in both recipients and donors.

Goblet cells play a crucial role in synthesizing and secreting high-molecular-weight glycoproteins called mucins, which are responsible for generating and maintaining the protective mucus layer. These cells contribute to the maintenance of stable gut barrier permeability and integrity (401, 402). Our previous investigation involving short-term high-fibre diet intervention demonstrated a significant increase in the quantity of goblet cells in the ileum. In contrast, both short-term and long-term modifications to a high fat diet did not exhibit any impact on goblet cell proliferation. Consistent with our FMT administration model, no significant differences were observed among the groups concerning the number of goblet cells. Despite the complete alteration of the gut microbiome composition in donors and recipients through diet intervention and FMT, this change did not significantly influence goblet cell proliferation in the ileum. This finding aligns with a study investigating FMT in a mouse model of ASD (Fmr1 KO mice), which demonstrated that FMT from wild-type mice to Fmr1 KO mice, while capable of ameliorating autistic-like behaviours and significantly increasing the abundance of *Akkermansia muciniphila* in the gut, did not have a significant effect on the number of goblet cells and the expression of the Muc2 (403).

Lipopolysaccharide (LPS) endotoxin, has been identified as a component that induces inflammation in humans through its Lipid A portion (404, 405). The impact of serum LPS, originating from GNB in the gut, has been recognized on brain microglia and the subsequent occurrence of neuroinflammation (406, 407). A clinical study conducted on individuals with Alzheimer's disease (AD) revealed a significant elevation in LPS levels within the hippocampus compared to age-matched healthy individuals. Furthermore, as the AD progresses, the concentration of LPS in the hippocampus can increase several-fold (408). Compelling evidence indicates a connection between Parkinson's disease (PD), a neuroinflammatory disorder, and inflammation induced by LPS (409, 410). In PD patients, a significant decrease in serum lipopolysaccharide-binding protein (LBP), responsible for suppressing LPS-induced cellular stimulation during the acute phase, has been observed. Additionally, PD patients exhibit elevated gut barrier permeability and activation of mononuclear cells (MNC) in response to LPS stimulation. These findings underscore the involvement of LPS in the progression of PD and the severity of clinical symptoms associated with the disease (411-413). In a recent study utilising a mouse model of CCM, it was discovered that the injection of LPS into normally lesion-resistant mice resulted in the formation of large CCM lesions in the brain. Similarly, intraperitoneal injection of live *Bacteroides fragilis* led to a significant increase in lesion formation. Conversely, Ccm1 knockout (KO) mice lacking the TLR4 receptor showed complete prevention of CCM lesion development. These findings suggest that LPS, acting through the TLR4 pathway, plays a role in the progression of CCM disease (22).

In our previous experiments involving diet interventions on the CCM mouse model, we did not observe a significant impact on circulating LPS levels after all diet interventions. However, short-term consumption of a high fat diet resulted in CCM lesion reduction. Additionally, when quantifying the level of LPS in the blood of controls, donors, and recipients in our FMT model, no statistically significant differences were found. Nevertheless, CCM lesion regression was observed in both the donors and recipients. These results indicate that while LPS does contribute to the exacerbation of CCM disease, the regression induced by a high fat diet and alterations in the gut microbiome operate through pathways distinct from LPS-TLR4 activation.

Although changing diet causes alteration in the gut microbiome composition, whether the lesion regression is due to diet-induced gut microbiome alteration or other effects of high fat diet on metabolism and different pathways regardless of the microbiome is still a question. According to our metagenomics analysis, gut microbiome profiles are significantly different before and after the diet intervention experiment, and gut microbiome communities are distinct between females and males at the end of high fat diet intervention. To address this question, A real-time FMT study was designed to involve three groups of female mice from the same litter and age. Recipients received FMT from donors of the same age, without any dietary intervention, to gradually modify

their gut microbiome over a six-week treatment period. In essence, the modification of the gut microbiome in recipients was carried out independently of any diet intervention, with the primary aim of investigating the isolated impact of gut microbiome alterations on the progression of CCM lesions. After six weeks of treatment with FMT, our results show that recipient mice exhibit significant regression of CCM lesion in volume and number, similar to donors with high fat diet modification compared to control mice. These findings suggest that the high fat diet-induced CCM regression was accomplished through FMT-induced gut microbiome changing.

Although our study was the first investigation of the gut microbiome intervention on CCM disease, prior studies suggested the role of the microbiome in CCM disease development. For instance, Tang, A.T et al. revealed that dextran sulphate sodium (DSS)-induced gut barrier disruption significantly increased CCM lesion burden in mice (21). Conversely, in our FMT model, an increase in gut permeability attributed to a reduction in the thickness of the ileum muscle layer was observed. However, rather than exacerbating the progression of CCM lesions, this increase in gut permeability failed to enhance the lesions and led to significant regression. These findings suggest whether increasing gut permeability leads to CCM lesion development or a regression depending on gut microbiome composition and bacterial communities. These results also could be linked to our short-term high-fibre diet intervention findings. Even though a high-fibre diet is known to decrease gut permeability by increasing the thickness of the ileum muscle and mucosal layer, it does not affect the burden of CCM lesions.

In conclusion, the findings indicate that FMT from high fat diet mice can lead to significant alterations in the gut microbiome without impacting weight gain and adverse consequences of diet interventions in the CCM mouse model. The reduction in the thickness of the ileum muscle layer associated with FMT, raises questions about its role in gut permeability and the progression of CCM lesions. Interestingly, the study results demonstrated that despite changes in gut permeability induced by alterations in the ileum muscle layer, there is no exacerbation of CCM lesions; rather, a significant regression is observed. This challenges previous notions about the relationship between gut permeability and CCM lesion development, suggesting that the outcome may be contingent on the specific composition and diversity of the gut microbiome. Moreover, short-term high fat diet-induced regression of CCM lesions is mediated through FMT-induced gut microbiome changes. This real-time FMT study, conducted independently of diet intervention, demonstrates significant regression in CCM lesions, paralleling the effects of donors subjected to a high fat diet. The study opens avenues for future research in understanding how specific components of the gut microbiome and/or their modulation through interventions like Probiotics may play a role in CCM disease progression and regression.

6. Chapter 6: General Discussion and Conclusion

6.1. Diet interventions, Gut permeability and CCM progression

A substantial body of evidence has elaborated the advantageous influence of a diet rich in dietary fibre on the progression of certain diseases and the mitigation of clinical symptoms (238, 414, 415). Ingested indigestible fibres undergo bacterial fermentation in the GI, resulting in the production of metabolites, including SCFAs, which exert favourable effects on human physiology (416).

On the contrary, using a fat-rich diet has been associated with deleterious health effects and a decline in the diversity of the gut microbiome (252, 417). Although there is no universally accepted definition for a high fat diet, it generally pertains to a dietary pattern where over 35% of daily caloric intake is derived from fat sources (418). Not all dietary fats are detrimental, as certain fats, such as monounsaturated and polyunsaturated fats found in nuts, seeds, avocados, and oily fish, confer health benefits when consumed in moderation. While dietary changes can induce modifications in the composition of the gut microbiome, another contributing factor to microbiome alteration is the permeability of the gut barrier. An increase in gut permeability facilitates the translocation of bacterial components or metabolites into the bloodstream. Different diets exert diverse effects on gut barrier permeability. For instance, in our study, short-term intervention with a high-fibre diet (high resistant starch) enhanced gut integrity by augmenting the muscular and mucosal layers of the ileum. However, the gut permeability reduction did not affect CCM lesions in mice. Consistent with previous research, our findings align with studies demonstrating that a high intake of inulin, a type of dietary fibre, induces the upregulation of genes associated with tight junction proteins and reduces gut permeability in mice (419). Additionally, another investigation revealed that consuming Cereal β-glucan, another form of dietary fibre, can increase the thickness of the mucosal layer and enhance the expression of occludin, a critical protein involved in tight junctions and gut permeability regulation (420). In contrast, the effects of fructooligosaccharides (FOS), a type of prebiotic, on the gut environment are influenced by other dietary components and the acidic conditions of the luminal contents. The intricate interplay between FOS and the gut ecosystem suggests that its impact on gut permeability and tight junction integrity may be modulated by various factors, highlighting the complexity of gut-microbiota interactions in response to different dietary components (421).

Consumption of a high fat diet has been found to have an impact on the integrity and permeability of the gut (422). Scientific research has demonstrated that the duration and type of high fat diet intervention are crucial factors in determining this effect (423). In our specific study involving a high fat diet intervention, no significant alterations were observed in the structure and layers of the gut. However, when a high fat diet is maintained over the long term, it leads to increased villi length in the ileum. Studies have indicated that a high fat diet can result in the downregulation of key genes involved in the tightness of tight junctions and the abnormal contraction of the prejunctional-actinomyosin ring (PAR) (424, 425). These changes negatively impact the integrity of the gut barrier. Additionally, a high fat diet can disrupt the mucosal layer of the gut by promoting bile acid production, which ultimately increases gut permeability (426). Our metabolomics analysis revealed a significant increase in metabolites associated with primary bile acid production. However, this elevation in bile acid levels did not affect the number of goblet cells in the ileum responsible for producing mucus.

The integrity of the gut barrier plays a crucial role in regulating the level of LPS in the bloodstream. As a major component of GNB in the gut, LPS can cross the gut barrier and enter the circulatory system. Previous research has indicated that dietary interventions can affect the circulating levels of LPS (241, 427). When quantifying LPS after implementing a high-fibre diet, a reduction in LPS levels was observed, while high fat diet intervention resulted in an increase. These findings align with the concept that gut barrier permeability decreases with high fibre intake and increases with high fat intake. In our study, however, no significant differences in LPS levels were observed across all dietary intervention groups. Nevertheless, we did detect a reduction in gut permeability due to an increased thickness of the muscle and mucosal layer in the ileum. Additionally, when performing a faecal microbiome transplant from mice treated with a high fat diet to mice without any dietary intervention, no significant difference in circulating LPS was observed between the groups. However, a significant decrease in the thickness of the ileum muscle layer was observed in both the donors and recipients. These results suggest that alterations in the gut microbiome and its metabolites resulting from faecal transplantation have a positive effect on the reduction of CCM lesions, despite an increase in gut barrier permeability. In summary, a high fat diet can impact gut integrity and permeability through various mechanisms, including changes in gene expression related to tight junctions and PAR contraction, as well as the disruption of the mucosal layer due to increased bile acid production. However, in our specific study, we did not observe significant changes in LPS levels across the different dietary interventions. Nonetheless, alterations in gut permeability were detected, indicating the complex interplay between diet, gut integrity, and circulating LPS levels.

In a previous investigation utilising a mouse model of CCM, it was demonstrated that the induction of colitis and disruption of the colon epithelial layer using dextran sulphate sodium

(DSS) resulted in a significant augmentation of CCM lesions in surviving mice at P21, indicating a significant relationship between increased gut permeability and CCM disease progression. Furthermore, a mutation in the CCM3 (PDCD10) gene, one of the causative genes for CCM, within gut epithelial cells led to an escalation in CCM lesions. However, this increase was not observed in the case of a Krit1 mutation within the gut epithelium. The involvement of CCM3 in the regulation of tight junction function suggests that the absence of CCM3 may contribute to enhanced gut permeability and a "leaky gut" condition, ultimately leading to an elevated burden of CCM lesions in the brain.

In summary, alterations in gut integrity and permeability have been implicated in the progression of CCM by elevating LPS levels in the bloodstream. However, it is noteworthy that interventions targeting the gut microbiome, such as dietary modifications or faecal transplantation, have the potential to mitigate the detrimental impact of increased gut permeability on the development of CCM lesions, regardless of changes in circulating LPS levels. Our findings indicate that the gut microbiome likely functions as a third-hit parameter influencing the progression of lesions and the severity of clinical symptoms in CCM disease. This suggests the potential for targeted manipulation and alteration of the gut microbiome as a non-surgical therapeutic option for CCM patients. Nevertheless, further research is required to uncover the underlying mechanisms and assess the therapeutic potential of manipulating the gut microbiome in the context of CCM.

6.2. Sex-Dependent Effects of High Fat Diet Intervention on the Gut Microbiome and CCM Disease Progression

The gut microbiome undergoes dynamic changes throughout an individual's lifespan influenced by various factors. Among these factors, age plays a significant role in shaping the composition of the gut microbiome. Diet, exercise, lifestyle, environmental factors, and sex hormones are key contributors to the age-associated alterations in the gut microbiome profile. Notably, metagenomics analyses have demonstrated a significantly higher bacteria-to-human cells ratio in females compared to males, suggesting a sex-based disparity in gut microbiota composition (428-432). Multiple studies have explored the disparity in gut microbiome between females and males (432, 433). In a mouse model study investigating the effects of a high fat diet, it was observed that the gut microbiome of male mice exhibited greater resistance to alterations induced by long-term high fat diet consumption. Conversely, the diversity and abundance of the gut microbiome in female mice underwent significant changes following high fat diet intervention (434). During childhood, the composition of the gut microbiome is more complex and unstable compared to adulthood (435). Notably, in the absence of sex hormones during childhood, no significant

differences in the gut microbiome between females and males are observed. However, studies in mice have shown that alterations in the gut microbiome driven by sex hormones occur during maturation. These sex hormone-mediated changes persist in females into adulthood, while shifts in the gut microbiome composition occur in male mice after reaching adulthood (436). Additionally, a study involving neutered male mice revealed that diminished testosterone levels resulted in the absence of sex-based differences in the gut microbiome during adulthood (436). Furthermore, a faecal microbiota transplantation (FMT) study demonstrated that transplanting caecal contents from adult male mice into female mouse pups led to an elevation in testosterone levels in female mice during adulthood (437).

These findings highlight the bidirectional relationship between sex hormone levels and gut microbiome composition. However, it remains uncertain whether the differential levels of sex hormones in males contribute to the stability of the gut microbiome in response to environmental factors, such as dietary changes. Our study corroborates this trend by demonstrating similar alpha diversity of the gut microbiome in female mice and statistically significant differences in composition before and after a six-week high fat diet intervention. Furthermore, our results reveal that reduction in CCM lesions solely occurred in female mice following high fat diet intervention, while males did not exhibit statistically significant reductions in CCM lesions even after a prolonged period of high fat diet modification (12 weeks). This suggests that significant alterations in gut microbiome diversity are associated with lesion regression, while the lack of diversity changes in the gut microbiome among males may explain the absence of lesion regression. Consequently, a longer duration of diet intervention may be required to induce gut microbiome alterations in males, potentially leading to lesion regression. These findings underscore the intricate relationship between sex hormones, gut microbiome composition, and the response to dietary interventions. Further investigations are needed to elucidate the mechanisms underlying the differential gut microbiome responses in males and females, as well as to explore prolonged diet interventions that may facilitate gut microbiome alterations and subsequent lesion regression in male mice.

An alternative explanation for the observed regression of CCM lesions in female mice following intervention with a high fat diet can be attributed to the interaction between estrogen production and the gut microbiome. Estrogen hormones, such as Estradiol, are synthesized in various organs and tissues, including the ovaries, adrenal glands, and adipose tissue (438). Estrogens are crucial regulators of numerous vital functions, including cell death and proliferation, lipid and glucose metabolism, homeostasis, cardiovascular functions, reproductive tract function, neuronal function and development, and neurodegeneration (439-442). The gut microbiome, through the activity of steroid receptors like estrogen receptor beta, can influence the metabolism of bacteria residing in the GI (443). Conjugated estrogens, which are inactive forms of estrogen present in the

bloodstream and produced by the liver during bile secretion, can be metabolized and deconjugated by the gut microbiome, resulting in their conversion to an active form. Deconjugated estrogens can then re-enter the bloodstream and exert their effects on the CNS and cardiovascular system via estrogen receptors (444, 445). While both males and females have circulating estrogen, the levels of estrogen hormones are considerably higher in females. Previous investigations have demonstrated a significant increase in estrogen levels in obese individuals, both male and female, due to the augmented adipose tissue and body fat (446, 447). In our study, obese female mice exhibited regression of CCM lesions following a high fat diet intervention, suggesting that the potential mechanism underlying this regression could be attributed to elevated estrogen levels resulting from the dietary intervention-induced alterations in the gut microbiome. However, further studies utilising estrogen receptor knockout mice or similar approaches are required to validate this hypothesis.

6.3. The Microbiome and Metabolites Alteration Induced by High Fat Diet Intervention and Their Impact on CCM Lesion Progression

In response to environmental factors such as diet, alterations in gut microbial communities lead to changes in the composition of metabolites produced by bacteria residing in the GI (448). These metabolites can arise from various processes, including the fermentation of indigestible fibres in the gastrointestinal lumen by gut bacteria, as well as the synthesis and breakdown of compounds derived from dietary intake. Advances in metabolomics analysis have facilitated the identification of a wide range of metabolites present in complex samples such as stool. Metabolites originating from gut microbial activity can significantly influence the functionality of the gut microbiome in both health and disease conditions (449). Moreover, these metabolites have the ability to traverse gut epithelial cells and enter the bloodstream (450). Consequently, the identification and characterization of gut-derived metabolites, along with their alterations during disease progression, hold significant promise in elucidating the underlying mechanisms involved.

One prominent class of gut bacterial metabolites known for their role as bacterial mediators are SCFAs, including acetate, propionate, butyrate, lactate, and succinate (451). During the process of digestion, the colon absorbs the majority of SCFAs, with more than 95% being taken up in this region. The production of SCFAs is influenced by the specific bacterial composition of the gut and the type of indigestible dietary fibre present; not all types of indigestible fibre can be converted into SCFAs (452, 453). SCFAs serve as a source of energy for the gut epithelial cells, contributing to overall energy intake (454). They exert their effects by interacting with G protein-coupled receptors (GPCRs) located on the surface of gut epithelial cells, including GPR41

(FFAR3), GPR43 (FFAR2), and GPR109A (455, 456). Recent research has highlighted the significance of GPR41 and GPR43 in maintaining the integrity of the gut barrier (457). Decreased expression of the GPR41 and GPR43 genes has been linked to elevated levels of circulating LPS, a marker of gut barrier dysfunction. Furthermore, knockout mice lacking GPR41 and GPR43 did not demonstrate the beneficial effects of a high-fibre diet in terms of reducing blood pressure and supporting cardiovascular health (457). These findings suggest that the production of SCFAs resulting from a high-fibre diet exerts an impact on hypertension and vascular health. Notably, downregulation of GPR41 and GPR43 gene expression has been observed in individuals with hypertension.

The PI3K/Akt signalling pathway assumes a complex and multifaceted role in the context of CCM. Although the precise mechanisms remain subjects of ongoing investigation, current research suggests that dysregulation of this pathway contributes significantly to the initiation and progression of CCM lesions. Notably, increased expression of vascular endothelial growth factor (VEGF), disruption of endothelial cell junctions and adhesion, and hyperactivation of Akt resulting from mutations in CCM genes are identified as factors impacting the PI3K/Akt signalling pathway (458). Additionally, the activation of GPCRs has been recognized as another modulator of the PI3K/Akt pathway through ligand binding with class IB PI3Ks (PI3K) (459). SCFAs, known activators of specific GPCRs, trigger downstream activation of PI3K/Akt. This pathway assumes a critical role in promoting cell growth and division, particularly within the gut epithelium, thereby contributing to the maintenance of intestinal health and barrier function (460). Furthermore, Akt activation by SCFAs enhances cell survival by inhibiting pro-apoptotic pathways, a particularly crucial protective mechanism in the gut where epithelial cells are consistently exposed to various stressors (461). While it is plausible that a high-fibre diet induces GPCR activation via SCFAs, thereby influencing the PI3K/Akt signalling pathway, our short-term high-fibre diet intervention in the CCM mice model did not yield significant changes in CCM lesions. Further investigation and perhaps a more extended duration of dietary intervention may be warranted to delineate the nuanced and time-dependent effects of a high-fibre diet on CCM lesion development through the modulation of the PI3K/Akt signalling pathway.

Our metagenomics analysis reveals that a short-term high fat diet intervention induces a significant increase in the abundance *of Akkermansia muciniphila* and *Lachnospiraceae bacterium* within the gut microbiome of female mice. *Akkermansia muciniphila* is an anaerobic gram-negative bacterium that utilises mucin as a carbon and nitrogen source, converting it into oligosaccharides and SCFAs such as acetate and propionate (462, 463). The degradation of mucins by *A. muciniphila* stimulates gut epithelial cells to enhance mucin production, resulting in an increased thickness of the mucin layer and improved gut barrier integrity. Propionate produced by *A. muciniphila* can bind to GPR43 (FFAR2) receptors and initiate signalling

cascades in epithelial cells, ultimately leading to the reduction of blood pressure and support for cardiovascular health (464, 465). While some studies have suggested a direct relationship between the abundance of *A. muciniphila* and the number of goblet cells (466, 467), In our study, there was no statistically significant variation observed in the abundance of goblet cells after a brief period of high fat diet intervention. However, we observed a notable increase in the abundance of goblet cells following a short-term intervention with a high-fibre diet. Unfortunately, no metagenomics analysis was conducted in our study to identify the abundance of *A. muciniphila* in the gut under these dietary conditions. The significant increase in the abundance of bacteria, such as *Akkermansia muciniphila* and *Lachnospiraceae bacterium*, known for their capacity to produce SCFAs, following a short-term high fat diet intervention, along with their effects on GPR41 and GPR43 receptors, may represent a potential mechanism underlying the regression of CCM lesions. Certain studies have demonstrated the agonistic role of SCFAs in activating G protein-coupled receptors (GPCRs), notably GPR41 and GPR43 (468). This activation has been associated with reduced inflammation, enhancement of cell survival, and improvements in blood flow, thereby conferring protective effects against stroke and cerebrovascular diseases (469). Modulating the composition of the gut microbiome to Favor an increased ratio of SCFA-producing bacteria holds the potential to augment GPCR activation and, consequently, can mitigate clinical symptoms associated with CCM disease. Furthermore, despite previous study indicating a reduction in goblet cell numbers following a high fat diet (255), the increased abundance of *A. muciniphila* and *Lachnospiraceae bacterium* then subsequent SCFA production could potentially counteract the adverse effects of the high fat diet on goblet cell numbers. Thus, our diet intervention study did not observe a significant difference in the number of goblet cells after the high fat diet intervention.

Extensive evidence supports the concept that oxidative stress plays a fundamental role in the vascular remodelling and dysfunction of the neurovascular unit (NVU) in cerebrovascular diseases, including CCM (470, 471). Oxidative stress has been suggested to play a role in numerous molecular and cellular modifications linked to cerebral CCM (111, 472). These alterations include the disruption of junctions between endothelial cells, heightened activation of β1 integrin, impaired maintenance of cellular quiescence, elevated vascular permeability, and intensified angiogenic activity. These findings strongly indicate that oxidative stress acts as a significant trigger in the initiation and progression of CCM. Notably, CCM proteins have been implicated in protecting cells against oxidative stress, suggesting that CCM lesions may arise from impaired defence mechanisms against oxidative stress in microvascular regions of genetically susceptible individuals (111). Our metabolomics analysis revealed a notable decrease in the levels of oxidized lipids following a short-term high fat diet. Furthermore, pathway enrichment analysis of the identified metabolites highlighted the involvement of Riboflavin

metabolism, which is renowned for its antioxidant properties. Considering the association between increased oxidative stress in epithelial cells and the progression of CCM, the significant reduction in oxidized lipids resulting from the upregulation of Riboflavin metabolism may contribute to the observed reduction of CCM lesions during high fat diet intervention.

Sphingolipids exert significant influence on the functionality and maturation of the nervous system, and they are present in various forms within brain cells and neurons (473, 474). However, the composition of different types of sphingolipids in the CNS is unstable, undergoing continuous modifications throughout different stages of life because of neural maintenance and differentiation processes (475-477). Among sphingolipids, glycosphingolipids (GSL) are particularly abundant on the neuronal cell membrane surface (478-480). Disruptions in sphingolipid metabolism can lead to impaired membrane organization, which has been observed in numerous neurological disorders (473, 481, 482). Ceramides, a class of bioactive sphingolipids, play a crucial role in the plasma membrane by providing structural integrity and serving as precursors for the synthesis of complex sphingolipids. These molecules are composed of a long-chain sphingoid base (LCB), either sphinganine or sphingosine, which is linked to a fatty acid through an amide bond at the C2 position (483, 484). Gangliosides are a specific class of sphingolipids that are attached to glycans. These molecules are predominantly found on the outer surface of the cell membrane as well as within microdomains within neural cells. Gangliosides play a crucial role in various cellular processes, including cellular recognition, adhesion, and signal transduction (485). In addition, numerous studies have indicated that sphingolipids and their enzymatic mediators interact with pivotal cellular processes such as proliferation, apoptosis, and cell migration (486, 487). Furthermore, investigations on Sphingosine-1-phosphate (S1P) have revealed that dysregulation of S1P is associated with the development of atherosclerosis through its effects on cell migration, proliferation of smooth muscle cells, and vascular tone. S1P also contributes to the elevation of pro-inflammatory cytokines by activating the NF-κB pathway (488). Furthermore, investigations into specific protein markers of smooth muscle cells within CCM lesions have revealed a significant reduction in SM1 and the absence of SM2 when compared to control samples (489). Notably, a parallel study exploring the SM-ceramide pathway has illuminated the mitogenic effects induced by specific concentrations of oxidized low-density lipoproteins (LDL). This effect is mediated through the activation of the SM-ceramide pathway, stimulating p44/42 MAPK in smooth muscle cells, and can be further potentiated by an increase in circulating Ceramides (347). Our lipidomics data, complemented by pathway enrichment analysis of identified lipids in the gut microbiome of mice subjected to a high fat diet, has unveiled a substantial augmentation in Ceramides and lipids associated with the sphingolipid signalling pathway. Importantly, the SM-ceramide pathway and the sphingolipid signalling pathway exhibit intricate interconnections and integration (490, 491). Metabolites generated in the SM-ceramide

pathway play a crucial role in fuelling the signalling events within the sphingolipid signalling pathway. Collectively, these findings propose that the high fat diet may contribute to the reduction of CCM lesions by fostering the proliferation of smooth muscle cells, potentially through the activation of the SM-ceramide pathway. This intricate network underscores the multifaceted impact of dietary interventions on cellular signalling pathways implicated in CCM pathogenesis.

Dysregulation of key genes involved in tight junctions and cell-cell adhesion, as well as the mesenchymal-to-epithelial transition and the absence of smooth muscle cells, are distinct characteristics of CCM lesions. Our comprehensive metabolomics analysis revealed significant alterations in the sphingolipid signalling pathway and metabolism following intervention with a high fat diet. Notably, the most significantly increased metabolites and lipids were found to be associated with sphingolipid pathways. Among these, lipid clusters such as ceramides and sulfonolipids, which represent clusters of sphingolipids, displayed a substantial increase in abundance within the gut of mice subjected to the dietary intervention. These observations strongly suggest that a short-term high fat diet exerts a considerable influence on sphingolipid pathways and the associated metabolites. Considering the prior studies highlighting the crucial role of sphingolipids in cell junctions and smooth muscle cells, the remarkable elevation of sphingolipid levels resulting from a short-term high fat diet may be a contributing factor to the regression of CCM lesions in female mice. It is plausible to propose that the mechanism underlying this regression involves the sphingolipid signalling pathway, whereby the dysregulation of sphingolipid metabolism and signalling influences the tight junctions, cell-cell adhesion, and smooth muscle cell presence, thereby contributing to the regression of CCM lesions. Nonetheless, further investigations are needed to elucidate the precise mechanisms by which sphingolipids and their signalling pathways are involved in CCM lesion regression. Future studies could explore the specific interactions between sphingolipids and key genes associated with tight junctions, cell-cell adhesion, and mesenchymal-to-epithelial transition. Additionally, in vivo experiments and longitudinal studies are needed to assess the long-term effects of high fat diets on sphingolipid metabolism and CCM lesion progression. These investigations will provide a deeper understanding of the complex relationship between sphingolipids, diet, and the pathogenesis of CCM, potentially leading to the development of targeted therapeutic strategies.

6.4. Modulating the Gut Microbiome to Mitigate CCM Disease by Preventing the Third-Hit Effect

The clinical manifestations of CCM are strongly influenced by the extent and location of the lesion within the brain. While a significant proportion of CCM patients remain asymptomatic

throughout their lives, certain clinical symptoms can pose life-threatening consequences and significantly impact the patient's quality of life (233, 492, 493). In cases where the CCM lesion is accessible and causes severe, life-altering symptoms, surgical resection represents the sole treatment option. Although certain symptoms such as headaches and seizures can be managed with pain relief and anti-seizure medications, these interventions do not address the underlying CCM lesions (494). Emerging clinical studies have demonstrated that there is considerable variability in clinical symptoms among individuals with the same genetic mutation and disease history, implying the involvement of non-genetic factors in the burden of CCM lesions and the severity of associated symptoms. This phenomenon is referred to as the "Third-Hit" effect, which encompasses external factors that modify and influence the progression of CCM disease throughout an individual's lifespan (495). Recent research has shed light on the potential impact of the gut microbiome and gut permeability on CCM disease progression and the formation of lesions within the brain. The interaction between the gut and the brain, known as the gut-brain axis, has been implicated in these processes using a CCM mouse model (21, 22). These findings suggest that factors beyond genetics, specifically related to the gut, play a role in the development and severity of CCM, highlighting the need for further investigation in this area.

The involvement of the gut microbiome in the pathogenesis and advancement of certain brain and vascular disorders has been identified (496, 497), and interventions targeting the gut microbiome are often recommended as non-invasive therapeutic approaches. Nevertheless, the precise relationship between alterations in the gut microbiome and the onset or predisposition to these diseases remains unclear. It is yet to be determined whether changes in the gut microbiome act as a causative factor in the development of diseases or if they are a consequence of disease initiation. In a recent investigation employing a mouse model of CCM, novel insights were gleaned regarding the involvement of LPS and its interaction with the TLR4 pathway in the pathogenesis of CCM lesions. Intriguingly, when LPS was administered via injection to mice that typically exhibit resistance to lesion formation, a noteworthy outcome was observed substantial CCM lesions developed within the brain. Furthermore, the intraperitoneal injection of live *Bacteroides fragilis*, induced a significant increase in the formation of these lesions. Conversely, the use of Ccm1 knockout (KO) mice, which lack the TLR4 receptor, yielded a striking outcome: complete prevention of CCM lesion development (22). These compelling findings underscore the involvement of LPS and its mediation through the TLR4 pathway in driving the progression of CCM disease.

Our research aims to investigate the impact of modifying the gut microbiome through dietary interventions and faecal transplantation on the burden of CCM lesions within the brains of mouse models. In brief, our dietary interventions encompassed short-term modifications involving both fibre-rich (high resistance starch) and non-fibre conditions, revealing no discernible effect on

CCM lesions. However, a short-term high fat diet elicited a significant reduction in CCM lesions specifically in female mice, whereas no significant reduction was observed in male mice following both short-term and long-term high fat diet modifications. Furthermore, a comprehensive analysis encompassing metagenomics and metabolomics of the gut microbiome in female mice displaying regression of CCM lesions following dietary intervention demonstrated significant alterations before and after the intervention. Notably, our findings diverged from previous studies, as we did not observe a significant difference in the levels of blood LPS across all dietary interventions. This suggests that while diet-induced alterations in the gut microbiome are evident, this modification does not appear to affect circulating LPS levels. Additionally, the regression of CCM lesion burden in female mice due to the high fat diet is unlikely to be associated with LPS-TLR4 stimulation. It is plausible that the gut microbiome exerts its effect on CCM lesion development within the brain via alternative pathways that are yet to be elucidated. Our study sheds light on the intricate relationship between the gut microbiome, diet interventions, and CCM lesions, providing valuable insights into potential mechanisms underlying lesion regression.

The effects of a high fat diet extend beyond changes in gut microbiome composition in mice, impacting various factors, including body weight, liver and adipose tissue function, as well as lipid and glucose levels in the bloodstream. In order to establish a causal relationship between gut microbiome alterations and lesion regression, we conducted faecal matter transplants (FMT) using faces collected from mice subjected to a high fat diet and transferred them to mice of the same age without any dietary intervention. Remarkably, our analysis of CCM lesions revealed consistent lesion regression in female mice receiving the FMT, despite not undergoing any dietary modifications. These intriguing findings strongly suggest that the observed regression of CCM lesions is indeed attributed to the alterations in the gut microbiome induced by short-term high fat diet interventions. By isolating the effect of gut microbiome alteration through faecal matter transplant, we were able to demonstrate the direct influence of the gut microbiome on CCM lesion regression, independent of other factors associated with high fat diets. The findings presented in this study offer strong evidence supporting the significant contribution of alterations in the gut microbiome to the regression of CCM lesions. These results emphasize the need for further investigations to gain a deeper understanding of the precise mechanisms and specific microbial species that are responsible for mediating this favourable effect. Such investigations hold promise for the development of potential therapeutic interventions targeting the gut microbiome as a viable approach to managing CCM and other related vascular disorders.

6.5. Other possible underlying mechanisms

While the precise mechanisms underpinning CCM remain elusive, investigations into genetics and molecular pathways have shed light on potential contributing factors to the emergence of CCM lesions. It has become apparent through genetic and molecular studies that the development of CCM lesions may stem from a complex interplay of various factors. Genetic mutations are recognized as a fundamental component in the initiation of lesions; however, their sole presence is insufficient for lesion formation. Additional non-genetic factors are believed to exert influence over the formation and severity of CCM disease. This suggests a multifaceted etiology where genetic predisposition interacts with environmental factors to culminate in the manifestation of CCM pathology. Findings from our study utilising a mouse model of CCM indicate that dietary factors, as part of the environmental milieu, may exert significant influence on the formation of CCM lesions via alterations in the gut microbiome. Intriguingly, our research underscores the critical importance of the specific composition of the diet in this context. Notably, dietary fibre appears to have no discernible impact on the development of CCM lesions. Conversely, diets rich in plant-based fats demonstrate a potential to mitigate both the incidence of CCM lesions and the associated clinical symptoms of the disease. Moreover, despite earlier investigations indicating a potential link between the gut microbiome and the severity of CCM via LPS derived from gram-negative bacteria, our study did not uncover any disparity in blood LPS levels between mice subjected to diet intervention and those without. This finding effectively discounts the involvement of LPS in the observed reduction of CCM lesions following dietary modification.

The pivotal question arising from this study pertains to whether changes in the gut microbiome resulting from dietary modifications contribute to the prevention of new CCM lesions or facilitate the regression and diminishment of existing lesions within the murine brain. To delve into this question, it is essential to consider the intricate interplay between the gut microbiome and systemic health. In CCM disease, understanding how dietary interventions influence the gut microbiome and subsequently impact lesion formation or regression is of paramount importance. Preclinical studies have shown that certain dietary components can modulate the gut microbiota composition, leading to alterations in immune responses and inflammatory pathways. These changes may, in turn, influence the susceptibility to CCM development or the ability of existing lesions to regress. While elucidating the precise influence of gut microbiome alterations on either the formation of new lesions or the regression of existing ones poses a considerable challenge, several potential mechanisms may underpin both scenarios.

As previously mentioned, the initiation of CCM lesions requires the presence of two mutations within one of the causative CCM genes, involving both germline and somatic mutations. Our research findings have shown an increase in Riboflavin levels, a compound intricately involved

in DNA repair mechanisms and various cellular processes, along with a decrease in oxidized lipids (498). These changes are hypothesized to indirectly reduce the occurrence of somatic mutations in endothelial cells, thus lowering the likelihood of new CCM lesions forming in the brain. Therefore, the observed rise in Riboflavin and decrease in oxidative stress within endothelial cells may impact the overall mutation rate within cells, potentially serving as a mechanism for preventing CCM lesions.

Previous research has provided compelling evidence of thrombosis occurring within CCM lesions (499). The abnormal blood vessels associated with CCM are thought to be inherently prone to inflammation and leakage, fostering an environment conducive to clot formation. These clots have the capacity to hinder blood flow, thereby exacerbating tissue damage surrounding the CCM lesion. This phenomenon is likely one of several factors contributing to the severity of clinical symptoms associated with CCM. Current evidence suggests that the influence of the gut microbiome on anticoagulant activity is likely indirect, mediated through mechanisms such as vitamin K production and modulation of inflammation (500). Specifically, certain bacteria in the gut microbiome have the ability to synthesize vitamin K2 (Menaquinone). Alterations in the gut microbiome profile resulting from dietary interventions may lead to an increase in Vitamin K2 levels, and since higher levels of Vitamin K are generally associated with a decreased risk of thrombosis, this could have a protective effect against clot formation (501). Furthermore, SCFAs produced by gut bacteria possess anti-inflammatory properties (502). Given that inflammation can contribute to blood clot formation, SCFAs may help reduce the risk of thrombosis by mitigating inflammation within the body. Additionally, SCFAs may enhance endothelial function, thereby reducing its susceptibility to activation and promoting smoother blood flow, potentially mitigating clot formation. The observed reduction in thrombosis associated with dietary intervention and gut microbiome alteration may represent one of the mechanisms underlying the regression of cerebral cavernous malformation (CCM) lesions induced by a high-fat diet. However, it's important to note that thrombosis within CCM lesions is a consequence of lesion formation rather than a determinant of lesion development.

The findings of this study revealed that alteration of the gut microbiome via a high fat diet led to a reduction in CCM lesions in a mouse model, but notably, this effect was observed exclusively in female mice. As comprehensively mentioned earlier, this raises the intriguing possibility that the observed reduction or regression of CCM lesions may be attributable to alterations in sex hormone levels induced by the high fat diet. However, it's important to note that results from FMT experiments suggest that this reduction in CCM lesions is likely mediated by changes in the gut microbiome composition rather than solely by the high fat diet itself. This underscores the complex interplay between dietary factors, sex hormones, and the gut microbiome in modulating CCM pathology. Indeed, one another plausible mechanism underlying the interaction between

sex hormones and gut microbiome alterations in the reduction of CCM lesions involves a synergistic effect between these two factors. Specifically, it's conceivable that the observed lesion reduction occurred as a result of the combined influence of sex hormones and changes in gut microbiome composition. For instance, the differential levels of sex hormones between female and male mice may have rendered the effects of the high fat diet more pronounced in female mice compared to their male counterparts. However, it's important to note that both female and male mice likely experienced similar alterations in gut microbiome composition due to the dietary intervention. Nevertheless, since the FMT experiment was exclusively conducted on female mice in this study, the specific impact of microbiome changes on male mice remains unclear. An alternative mechanism by which a high fat diet may impact CCM lesions through sex hormones involves modulation of estrogen receptor gene expression. Previous research has demonstrated that diet can influence gene expression, including that of estrogen receptors. It is plausible that dietary modifications could exert their effects on CCM lesion burden by altering the expression of estrogen receptor genes, rather than directly affecting sex hormone levels. Specifically, changes in the gut microbiome induced by a high fat diet in female mice may lead to an upregulation of estrogen receptor expression, ultimately resulting in a reduction in CCM lesion burden. However, it's important to consider that while this microbiome alteration in female mice may be sufficient to induce changes in estrogen receptor expression and subsequently impact CCM lesions, a similar effect may not be observed in male mice due to potential differences in the response to gut microbiome alterations. Further investigation into the specific mechanisms underlying the interaction between diet, gut microbiome dynamics, and estrogen receptor expression is necessary to fully elucidate the role of sex hormones in mediating the effects of dietary interventions on CCM pathology.

6.6. Limitations of the study

Delving into the research on CCM in human subjects presents several challenges that need careful consideration. The scarcity of CCM cases as a medical condition poses a substantial obstacle to recruiting a sufficiently large number of patients for thorough studies. This rarity not only makes assembling a diverse cohort challenging but also emphasizes the need for extensive efforts in data collection and analysis. Moreover, CCM exhibits genetic complexity, marked by the presence of multiple gene mutations associated with the disease. To comprehend the impact of these diverse genetic variations and their intricate interactions, large-scale genetic investigations involving diverse populations are necessary. This emphasizes the importance of a broad and inclusive approach to research, acknowledging the genetic diversity inherent in CCM. The procurement of brain tissue samples for detailed analysis and mechanistic studies faces hurdles due to the invasive

nature of the required procedures and the limited availability of suitable brain tissue specimens. Acquiring access to such crucial samples is vital for unravelling the complex cellular and molecular mechanisms that underlie CCM pathology. This underscores the importance of developing innovative and less invasive techniques for obtaining relevant brain tissue specimens. In addition to genetic and tissue-related challenges, conducting long-term follow-up studies is crucial for gaining insights into CCM, assessing disease progression, evaluating treatment effectiveness, and understanding overall outcomes. However, conducting longitudinal studies in this context proves demanding in terms of allocating resources, ensuring patient retention, fostering compliance, and accurately capturing the natural disease trajectory over extended periods.

Despite these formidable challenges, the exploration of CCM in human subjects is indispensable for making meaningful clinical observations, developing targeted therapeutic interventions, and facilitating the translation of findings derived from preclinical studies into practical applications. This multifaceted approach is essential for advancing our understanding of CCM and improving the prospects for effective diagnosis and treatment strategies.

- ***Personal challenges***

Throughout the course of my doctoral research, I encountered numerous difficulties and barriers that posed significant challenges. The initiation of my PhD coincided with the onset of the Covid pandemic, which had profound implications for my project. One particularly prominent hurdle involved limited access to crucial equipment, specifically Micro CT, which played a vital role in this project. However, due to the implementation of lockdown measures and adherence to social distancing protocols, I was unable to perform the necessary scans on my samples and carry out subsequent analyses. The unavailability of Micro CT scans had a substantial impact on my work, as I was restricted to modifying the diets of the mice in my study without having a comprehensive understanding of the effective or ineffective impacts on the CCM mice model. Consequently, I faced a significant time burden in terms of both scanning and analysing the accumulated brain samples.

One additional obstacle encountered during the course of my doctoral research pertained to the emergence of *Entamoeba* infection within a specific experimental facility at the Centenary Institute animal house. Given the focus of my project on the gut microbiome, the presence of *Entamoeba*, known to induce gut infections and exert a profound influence on the gut microbiome, necessitated the euthanisation of a substantial portion of the experimental mice population. Furthermore, the breeding pairs were relocated to an alternative, infection-free room. This circumstance imposed a considerable time constraint on my research progress, as I had to

patiently await the generation of new offspring from the breeding pairs in order to establish novel experimental models.

6.7. Future Direction

Targeted probiotics exhibit promising prospects as a therapeutic modality for diverse conditions, encompassing CCM and associated vascular disorders. While the conventional understanding of probiotics has primarily focused on gastrointestinal health, emerging research indicates their systemic influence, extending even to the brain. In the context of CCM, targeted probiotics pertain to specific strains or combinations of bacteria that are deliberately chosen due to their capacity to modulate the gut microbiome in a manner that may alleviate CCM lesion formation or progression. Selection of probiotic strains can be guided by their demonstrated efficacy in preclinical studies or by identifying bacterial species associated with a healthy gut microbiome in human populations. By selectively introducing beneficial bacteria based on our metagenomics findings, targeted probiotics aim to induce lesion regression without the need for dietary intervention or faecal transplantation. Certain probiotic strains possess the ability to fortify the intestinal barrier, thereby diminishing the translocation of harmful substances from the gut into the systemic circulation. This mechanism can indirectly impede CCM lesion development by impeding the entry of triggers or contributors to the disease into the brain. Targeted probiotics can also exert an impact on metabolism, including the metabolism of lipids and glucose. Through the modulation of these metabolic pathways, probiotics may indirectly influence the formation or regression of CCM lesions. Notably, our findings indicate an increase in the abundance of *Akkermansia muciniphila* in females displaying regression of CCM lesions following a high fat diet. *Akkermansia muciniphila* exhibits robust production of SCFAs and possesses anti-inflammatory properties, rendering it a strong candidate for targeted probiotic therapy in CCM disease. While the potential of targeted probiotics for CCM is captivating, further research is imperative to determine effective bacterial dosages and treatment durations that yield optimal therapeutic effects. Targeted probiotics represent a captivating avenue for therapeutic interventions in CCM and related vascular disorders. Their capacity to modulate the gut microbiome and confer beneficial effects on inflammation, gut barrier function, immune responses, and metabolism positions them as potential adjunctive therapies or preventive measures. Extensive research endeavours and clinical trials are indispensable for fully exploring the therapeutic potential of targeted probiotics in the management of CCM.

However, targeted probiotic therapy for CCM may encounter certain limitations. Interindividual variations in the gut microbiome composition, influenced by genetic factors, diet, lifestyle, and environmental factors, contribute to significant diversity. Such inherent variability in the gut

microbiome could impact the efficacy of targeted probiotics, as individual responses to specific bacterial strains or combinations may differ. The long-term effects and sustainability of targeted probiotic interventions in CCM remain uncertain, primarily due to the dynamic nature of the gut microbiome. Over time, the gut microbiome undergoes changes, necessitating evaluation of the prolonged stability and persistence of desired microbial alterations induced by targeted probiotics to ensure enduring therapeutic benefits. Notably, meticulous design and implementation of preclinical experiments utilizing appropriate mouse models will be pivotal in maximizing the complete therapeutic potential of targeted probiotics in the management of CCM.

Another prospective experiment aimed at elucidating the mechanism underlying the regression of CCM lesions following short-term high fat diet intervention involves the utilisation of sphingolipid knockout mice, such as sphingolipid activator protein (SAP knockout mice). In our study, we have observed a significant elevation in lipids and metabolites associated with the sphingolipid signalling pathway and metabolism within the gut of mice exhibiting lesion regression. By employing sphingolipid knockout mice, along with knockout of CCM causative genes, in female mice subjected to short-term high fat diet intervention, we can further investigate the intricate mechanisms at play. If no lesion regression is observed despite the short-term high fat diet intervention in these mice, it would provide insights into the role of sphingolipids and their associated signalling pathways in the regression of CCM lesions. This experimental approach offers an opportunity to expand our understanding of the underlying molecular processes and potential therapeutic targets involved in CCM lesion regression.

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