The Impact of Non-alcoholic Steatohepatitis on Brain Vasculature

by

Alara Su Bilgez

A Dissertation Submitted to the
Graduate School of Health Sciences
in Partial Fulfillment of the Requirements for
the Degree of

Master of Science

in

Neuroscience

Thesis reference number: 10686496

October 4, 2024

The Impact of Non-alcoholic Steatohepatitis on Brain Vasculature

Koç University

Graduate School of Health Sciences

This is to certify that I have examined this copy of a master's thesis by

Alara Su Bilgez

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Prof. Yasemin Gürsoy Özdemir (Advisor)

Prof. Mehmet Kaya (Member)

Prof. Ertuğrul Kılıç (Member)

Date: October 4, 2024

This thesis is dedicated to my family, whose unwavering support, encouragement, and love have been my constant source of strength throughout this journey. I am forever grateful for everything you have done to help me reach this milestone.

ABSTRACT

The Impact of Non-alcoholic Steatohepatitis on Brain Vasculature

Alara Su Bilgez

Master of Science in Neuroscience

October X, 2024

Non-alcoholic fatty liver disease (NAFLD) and its more severe form, non-alcoholic steatohepatitis (NASH), are increasingly recognized as multi-system conditions with implications beyond the liver, including potential impacts on the brain. This study explores the relationship between NASH and cerebral vasculature using two different models in mice: the methionine- and choline-deficient (MCD) diet and carbon tetrachloride (CCl_4) injections. This thesis aimed to assess the effect of NASH on the blood-brain barrier (BBB) and perivascular fibrosis. Important differences were observed in the expression of CD13 and KDR in the MCD diet group, suggesting that NASH contributes to BBB disruption. Elevated LDL-H and LDL-C3 levels further indicated metabolic changes affecting brain health. However, liver enzyme markers like ALT and AST showed no significant changes, possibly due to the early NASH stage.. Additionally, increased perivascular pericyte activation, collagen IV, and fibronectin levels were noted, pointing to early BBB breakdown and fibrosis.This study highlights the complex relationship between NASH and brain health, emphasizing the need for further research to explore the mechanisms linking liver pathology to cerebral dysfunction.

ÖZETÇE

Alkole Bağlı Olmayan Karaciğer Yağlanmasının Beyin Damarları Üzerindeki Etkisi

Alara Su Bilgez

Nörobilim, Yüksek Lisans

4 Ekim 2024

Non-alkolik yağlı karaciğer hastalığı (NAFLD) ve non-alkolik steatohepatit (NASH), yalnızca karaciğeri değil, aynı zamanda beyin sağlığını da etkileyen çoklu sistem hastalıkları olarak giderek daha fazla tanınmaktadır. Bu çalışmada, NASH ile beyin damar yapısı arasındaki ilişkiyi incelemek amacıyla farelerde iki farklı model (metiyonin ve kolin eksikliği diyeti (MCD) ve karbon tetraklorür (CCl_4) enjeksiyonları) kullanılmıştır. Çalışmanın temel amacı, NASH'in kan-beyin bariyeri (BBB) ve perivasküler fibrozis üzerindeki etkilerini değerlendirmektir. MCD diyeti grubunda, CD13 ve KDR ekspresyonunda önemli değişiklikler gözlenmiş olup, bu durum NASH'in BBB bozulmasına katkıda bulunduğunu göstermektedir. Artan LDL-H ve LDL-C3 seviyeleri, metabolik değişikliklerin beyin sağlığı üzerindeki etkilerini işaret etmektedir. Bununla birlikte, ALT ve AST gibi karaciğer enzimlerinde anlamlı bir değişiklik tespit edilmemiştir; bunun, hayvanların NASH'in erken evresinde olmasından kaynaklanıyor olabileceği düşünülmektedir. Ayrıca, artan perivasküler perisit aktivasyonu, kolajen IV ve fibronektin seviyeleri, erken dönemde BBB bozulması ve fibrozise işaret etmektedir. Bu çalışma, NASH ile beyin sağlığı arasındaki karmaşık ilişkiye dikkat çekmekte ve karaciğer patolojisinin serebral disfonksiyon ile bağlantısının daha derinlemesine incelenmesi gerektiğini vurgulamaktadır.

AKNOWLEDGEMENTS

This thesis would not have been possible without the guidance, support, and encouragement of many individuals, to whom I owe my deepest gratitude.

First and foremost, I would like to thank my advisor, Prof. Yasemin Gürsoy Özdemir, for their invaluable guidance, constructive feedback, and unwavering patience throughout this research. Your expertise and dedication have been instrumental in shaping this work.

I am also deeply grateful to Dr. Esra Özkan for her valuable ideas, insightful suggestions, and guidance that shaped this research. Special thanks to the faculty and staff at Koç University Research Center for Translational Medicine (KUTTAM), who provided the resources and support that made this journey possible.

Above all, I am profoundly thankful to my family, whose unconditional love, encouragement, and belief in my abilities have been my greatest source of strength. I would not be here without my mother, İpek Meriç, my grandfather, Fahrettin Meriç, and my grandmother, Gönül Meriç. Thank you for always believing in me and supporting my dreams. Lastly, my heartfelt thanks go to Berfe Uzak, whose constant support, unwavering presence, and enduring love have been a source of strength throughout every high and low of this journey.

This accomplishment is not mine alone but shared with all of you. Thank you.

TABLE OF CONTENTS

1. List of Tables

2. List of Figures

3. ABBREVIATIONS

3DISCO	Three-dimensional imaging solvent-cleared organs
α-SMA	Alpha-smooth muscle actin
μL	Microliter
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
BBB	Blood-brain barrier
CCL2	Chemokine (C–C motif) ligand 2
CCl4	Carbon tetrachloride
Col1	Collagen 1
COL1A1	Collagen type I alpha I
COL4A	Collagen type IV alpha I
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
HSC	Hepatic stellate cells
IF	Immunofluorescence
KDR	Kinase insert domain receptor
MCD	Methionine-choline deficient
MMP9	Matrix metallo proteinase 9
NAFL	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NVU	Neurovascular unit
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PFA	Paraformaldehyde
RAGE	Receptor for advanced glycation endproducts
TIMP	Tissue inhibitors of metalloproteinases
VWF	Von Willebrand factor

CHAPTER 1:
INTRODUCTION

1.1 The Gut-Brain Axis and Liver

The gut-liver-brain axis consists of gastrointestinal tract, liver, and brain; and it is related to neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and stroke. When the gut barrier is disrupted, bacteria number and their metabolites increase and make their way into the liver. The peptides or hormones produced by the intestines access the bloodstream, affect the vagus nerve, spinal afferent neurons and consequently the brain. The liver vagal parasympathetic nerves receive feedback following this action, and innervate the gut and paracrine (Yan et al., 2023).

Some liver conditions, such as cirrhosis and hepatic encephalopathy (HE), and the brain are connected to each other through the gut-liver-brain axis. Hence, it is more likely for patients with liver diseases to experience cognitive dysfunctions. Mild cognitive impairment can be observed in patients with non-alcoholic fatty liver disease (NAFLD), chronic hepatitis C, and liver transplantation (Sun et al., 2023). Studies revealed that memory loss and forgetfulness are seen in approximately 70% of NAFLD patients. Cognitive impairments manifest themselves in various ways such as general cognition and mental speed and attention. Notably, a connection between these cognitive impairments and plasma liver enzymes was found in recent studies (Sun et al., 2023).

It is necessary to uncover the connections and the possible link between inflammatory liver diseases and the brain to provide new therapeutic approaches that might help relieve patients' symptoms (D'Melo & Swain, 2014).

1.2 Microcirculation of the Brain

The brain is protected from toxins and pathogens in a more specialized way than other organs and tissues of the body. The strict separation of blood and the brain and two-way substance transfer between them are carried out by the blood-brain barrier (BBB). The first person to describe this barrier was Paul Ehrlich, after observing that

the water-soluble dye that he injected to the experimental animals appeared in the peripheral organs, but not in the central nervous system (Gürsoy-Özdemir & Tas, 2017).

The components of BBB include endothelial cells, pericytes, astrocytes, and basal lamina. An important part that takes part in material transfer between the brain and vasculature is called the neurovascular unit. (Gürsoy-Özdemir & Tas, 2017). The neurovascular unit (NVU) consists of neurons, astrocytes, endothelial cells of BBB, pericytes, myocytes and components of the extracellular matrix. These cells are closely connected both anatomically and chemically with each other. This close connection allows them to sense neuronal demands and initiate appropriate responses, such as vasodilation or vasoconstriction, to meet the brain's supply needs (Muoio et al., 2014).

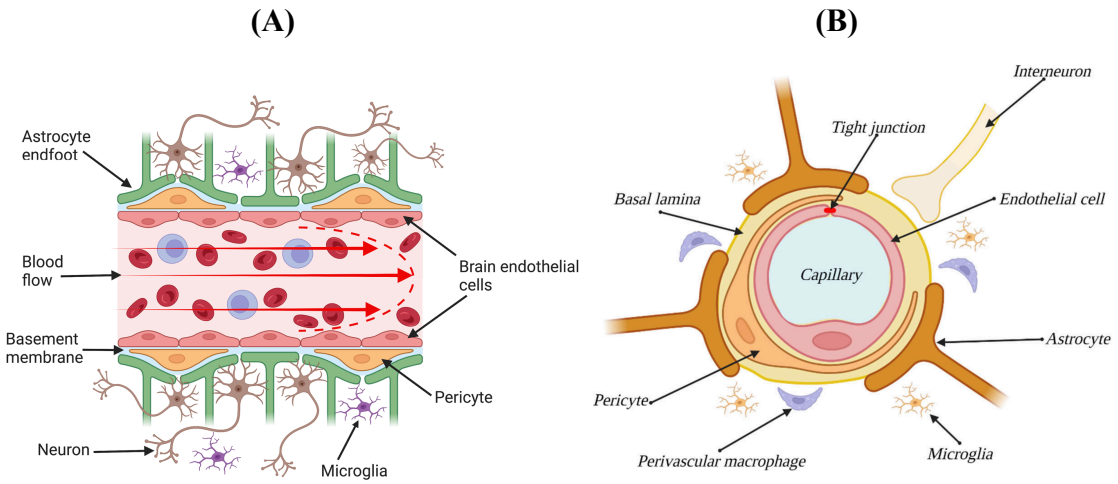


FIGURE 1. (A) Elements of the blood-brain barrier (BBB) (Patabendige & Janigro, 2023). (B) Structure of the BBB as seen in the cross-section of the capillary (Łach et al., 2023).

BBB forming bain capillary endothelial cells are attached to each other via intercellular tight junctions. In addition to its low permeability characteristic, astrocytes that surround the brain capillaries decrease its permeability further (Frank et al., 2011).

Pericytes are essential in preserving the integrity of the BBB and controlling cerebral blood flow. They are located on the capillary walls and work closely with endothelial cells to ensure that blood flow aligns with the neurons' metabolic needs. Pericytes regulate BBB permeability, allowing essential nutrients into the brain while blocking harmful substances. Additionally, they have functions in moderating the

immune response, especially during injury or inflammation, contributing to the brain's protection and recovery processes (Eltanahy et al., 2021).

The brain's microcirculation is highly flexible, adapting to fluctuations in neuronal activity through processes like neurovascular coupling. When neurons become more active, nearby blood vessels dilate to increase blood flow, providing additional oxygen and glucose to overcome the increased need. This dynamic regulation of blood supply is crucial for maintaining healthy brain function. Disruptions to this system have been associated with neurological conditions such as stroke, Alzheimer’s disease, and vascular dementia (Dalkara, 2015).

1.3 Neurological and Psychiatric Disorders Associated with Liver Diseases

1.3.1 Alzheimer’s Disease and NASH

NAFLD and Alzheimer’s disease share some genes and pathological mechanisms involved in the emergence of these diseases. This led the researchers to suggest a potential relationship between NAFLD and Alzheimer’s disease in recent years. NAFLD and dementia, including Alzheimer's disease, share several common risk factors such as older age, insulin resistance, and hypertension. In addition to the shared risk factors, there are some potential pathophysiological mechanisms that they share, such as chronic inflammation, vascular dysfunction, and oxidative stress (Hadjihambi, 2022).

Kim et al.'s study on transgenic mice showed that a modest increase in dietary lipids led to NAFLD and acute hepatic inflammation, and then a chronic low-grade inflammatory state. The mice showed increased neuro-inflammation, microgliosis, astrogliosis, and signs of Alzheimer's disease, as well as neuronal loss and neurodegenerative signs. However, changing the diet of mice from a high fat reversed NAFLD symptoms and decreased neuroinflammation. Chronic NAFLD also lead to AD signs, neurodegeneration, and decreased brain expression of Aβ clearing protein; low-density lipoprotein receptor-related protein-1. These findings clearly imply that NAFLD-associated inflammation and aging may induce neurodegeneration and fasten the process of AD even without genetic predisposition. Another study pointed out the relation between diet-induced NAFLD and imbalances of brain cholesterol and fatty acid metabolism, which are linked with neuro-inflammation, senescence, and oxidative

stress. The authors concluded that NAFLD could be a player in the progression of brain diseases like AD in mice leading to cognitive decline and impaired vasculature through dysregulated plasma and brain fatty acid metabolism hence supports the liver-brain axis concept (Hadjihambi, 2022).

Overproduction of β-amyloid is a hallmark of Alzheimer's disease pathophysiology. It is believed that cholesterol participates in amyloid precursor protein (APP) processing and β-amyloid overproduction (Wang et al., 2022). In a study where they modeled AD pathophysiology and cognitive impairment by using APP-PSEN1ΔE9 mice, a fibric acid called gemfibrozil decreased amyloid pathology and memory deficits (Luo et al., 2019, as cited in Wang et al., 2022). Gemfibrozil is normally used for the treatment of hyperlipidemias in clinical settings, and the result of this study could potentially lead to new methods for detecting AD pathophysiology since the fluctuations of lipoprotein in the blood can be identified before the emergence of cognitive decline (Wang et al., 2022).

1.3.2 Parkinson's Disease and NASH

Recent research indicates a possible link between Parkinson's disease (PD) and non-alcoholic steatohepatitis (NASH), given that both disorders exhibit shared characteristics, including metabolic imbalances, inflammation, and mitochondrial dysfunction. NASH, characterized by liver inflammation and fibrosis, is also linked to systemic inflammation, which may worsen the neurodegenerative processes that occur in PD (Bahitham et al., 2024). Additionally, disruptions in the gut-liver-brain axis, a pathway linking gut microbiota, liver health, and brain function, have been implicated in both NASH and PD (Lizardi-Cervera & Aguilar-Zapata, 2009).

Mitochondrial dysfunction is another shared mechanism between NASH and PD. In NASH, mitochondrial damage in liver cells leads to oxidative stress and inflammation, similarly, mitochondrial defects contribute to neuronal loss in PD. There are reports that patients with NASH are at a higher risk of neurodegenerative diseases like PD due to these overlapping metabolic and inflammatory pathways (Bahitham et al., 2024).

1.3.3 Psychiatric Disorders and NASH

Mitochondria, inflammation, and oxidative stress are significant factors in the development of psychiatric disorders, particularly mood disorders (Soto-Angona et al., 2020). NASH (Non-Alcoholic Steatohepatitis) is known to trigger the release of pro-inflammatory cytokines, such as TNF-α and IL-6, which can have systemic effects, including on brain function (Colognesi et al., 2020). Proinflammatory cytokines have been found to impact hormone release by directly interacting with receptors in the hypothalamic-pituitary-adrenal (HPA) axis, influencing the body's stress response system (Allison & Ditor, 2014).

Hormonal dysregulation, particularly involving cortisol and leptin, also plays a significant role; elevated cortisol levels associated with metabolic changes can lead to anxiety and depressive symptoms. Moreover, oxidative stress linked to NASH can disrupt neurotransmitter systems and influence individuals' mood further. Changes in gut microbiota related to NASH can also have an impact on mental health as a result of intestinal permeability allowing harmful substances to affect brain function (Sharma et al., 2022).

It is important to keep in mind that in addition to physiological impacts of NASH, such as systemic inflammation and metabolic dysfunction, the psychological strain of managing a chronic illness contributes to the increased prevalence of depression, anxiety, and stress in NASH patients (Shea et al., 2024).

1.4 Neuroinflammation & Learning and Memory

It is important to consider the possible effects of NASH on cognitive performance given that NASH is characterized by chronic inflammation that can result in neuroinflammation. Konsman (2022) highlights the effects of neuroinflammation on learning and memory by investigating interleukin expressions, a biomarker of inflammation. Studies show that IL-1β expression increases during long-term potentiation (LTP) in the hippocampus. Inhibiting IL-1 receptor activity impairs LTP maintenance. Similarly, IL-6 expression in the hippocampus also increases during LTP, and preventing its action can improve memory in specific tasks.

Other studies show that IL-4 and IL-13 are expressed more via Morris water maze training in the meninges. In addition to, if a genetic deficiency of either IL-4 or

IL-13 is present, cognitive performance during the Morris water maze gets affected by it (Konsman, 2022).

Researchers in another study found that IL-1α expression in the hippocampus increases during fear-motivated context-dependent training, and inhibiting IL-1 receptors improves retention performance in these tasks. Additionally, IL-1β expression in the hippocampus is increased during contextual fear conditioning, while its antagonist reduces freezing responses. An interesting finding is that low doses of IL-1β improve freezing responses, whereas higher doses impair them, suggesting a dose-dependent effect on fear conditioning and hippocampal function (Konsman, 2022).

1.5 Non-alcoholic Steatohepatitis (NASH)

Non-alcoholic steatohepatitis (NASH) is a subtype of non-alcoholic fatty liver disease (NAFLD), and it is characterized by hepatic steatosis (excess lipid accumulation in the liver), inflammation, and hepatocellular injury (hepatocyte ballooning). NASH is histologically defined and is diagnosed through liver biopsy in order to identify the presence of steatosis along with hepatocyte damage and liver inflammation. It is observed in a subgroup of individuals with nonalcoholic fatty liver disease (NAFLD), a clinically recognized condition comprised of simple hepatic steatosis, NASH, NASH with fibrosis, and NASH-related cirrhosis (Hashimoto, 2013). Simple steatosis is generally considered a non-progressive and benign clinical condition, whereas NASH has the potential to advance to cirrhosis or even hepatocellular carcinoma (Hashimoto, 2013).

Despite its clinical importance, NASH remains a challenging condition to diagnose and manage due to the often-asymptomatic nature of early stages and the lack of specific biomarkers. It can go unnoticed unless it progresses to other diseases such as cirrhosis and end-stage liver disease (Rinella, 2015; Younossi, 2016).

NASH is a significant public health problem with the potential for severe hepatic complications. Its prevalence continues to rise simultaneously with obesity and diabetes (Rinella, 2015; Younossi, 2016). It is predicted that the increase in the prevalence of the disease will result in a growing economic burden. The need for liver transplants is also thought to be on the rise due to the rising number of patients with cirrhosis and end-stage liver disease (Friedman, 2018).

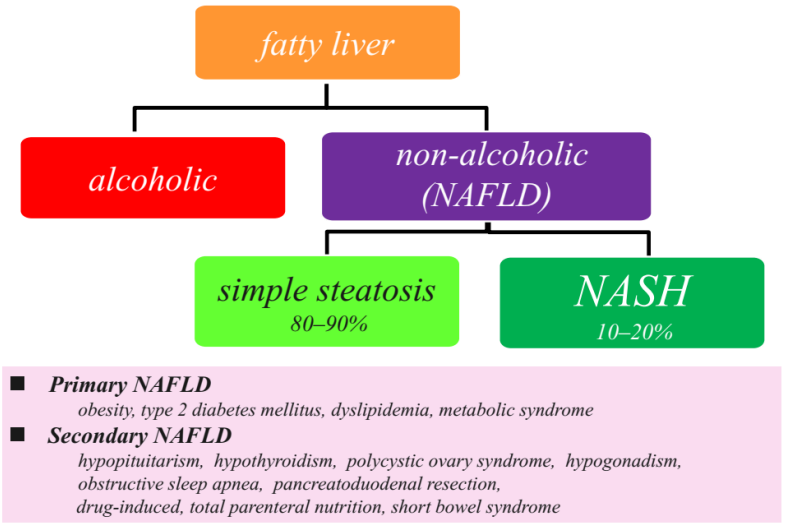


FIGURE 2 Accumulation of fat in the liver can stem from alcohol consumption or causes other than alcohol intake. (NAFLD can be divided into two categories. NAFLD/NASH is categorized into two types based on the causes: primary and secondary (Hashimoto, 2013).

1.5.1 Diagnosis of NASH

The identification of NAFLD relies on three key criteria: non-alcohol related, the steatosis detection with imaging or histology, and the careful exclusion of other liver disorders. NASH diagnosis is established through the identification of steatohepatitis via liver biopsy. Since definitive markers for NAFLD diagnosis are still lacking, it is crucial to rule out other liver conditions (Hashimoto, 2013).

Diagnosis of NASH has been challenging due to the low certainty of plasma biomarkers, which has a sensitivity of approximately 62-66% and specificity of 78-82%. No biomarker can accurately forcast the severity of NASH, and the stages of NASH and fibrosis.

Alanine aminotransferase (ALT) levels are found to be correlated with disease progression; however, according to the currently accepted threshold levels, around 30% to 60% of individuals diagnosed with biopsy-confirmed non-alcoholic steatohepatitis exhibit normal ALT levels. Thus, it is not possible to confidently diagnose patients based on increased levels of ALT (Rinella, 2015).

Body mass index (BMI) is another typically utilized predictor of NASH and fibrosis; however, its reliability diminishes in cases of obesity and morbid obesity, and this leads to false positives (Angelini et.al, 2022).

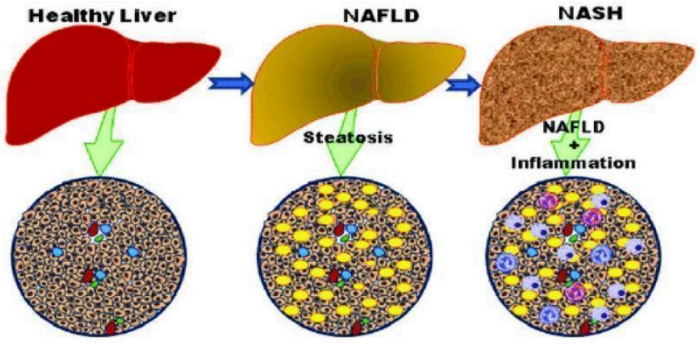


FIGURE 3 Progression from Healthy Liver to Non-Alcoholic Steatohepatitis (NASH). Nonalcoholic fatty liver disease (NAFLD) occurs when excess fat accumulates in the liver, a condition known as steatosis. This can progress to nonalcoholic steatohepatitis (NASH), a more severe liver disorder marked by inflammation and the formation of scar tissue (NCI Staff, 2022).

1.5.2 Inflammation and Immune Response

Tissue injury and infection triggers an inflammatory response in the body and initiates the release of inflammatory mediators such as chemokines, cytokines, and eicosanoids in order to repair the injured tissue. In the case of an inflammatory response turning into chronic inflammation, an abnormal wound healing response occurs. This process supports the transition of the disease to NASH in NAFLD patients (Schuster et al., 2018).

Chronic liver inflammation is an important factor that leads to the development of NASH and liver fibrosis. The disease progression from NAFLD to NASH is largely affected by the hepatic inflammatory response, which supports the continuity of hepatic fibrogenesis, and in the final stages leads to cirrhosis. In addition to the lipotoxicity and immune response and cell death pathways in the liver, the origin of the liver inflammation can also be outside of the liver. Adipose tissue and gut are some of the outside sources where the inflammation can begin. In studies done with mice and primates, it was shown that fructose has an effect of damaging the integrity of the

mucosal membrane of the gut, and in turn activating the macrophages in the liver and Kuppfer cells as a result of bacterial overgrowth and imbalance of the gut microbiome (dysbiosis) (Schuster et al., 2018). In many studies, it was found that fructose plays a role in disrupting metabolic activity by upregulating liver inflammation genes and decreasing mitochondrial β-oxidation and ATP levels (Song et al., 2023).

One of the liver-resident macrophages called hepatic Kupffer cells can transition into an inflammatory state, release inflammatory cytokines, and trigger the activation of other immune cells, respectively. In the next stages, macrophages derived from monocytes progressively replace Kupffer cells. Other immune cells such as lymphocytes are responsible for the degree of inflammation and fibrosis (Wiering & Tacke, 2022).

1.5.3 Hepatic Fibrosis

Hepatic parenchyma is made up of epithelial cells (hepatocytes), endothelial cells, and resident nonparenchymal cells such as hepatic stellate cells (HSCs) and Kupffer cells (KCs). In the case of an injury in the liver, extracellular matrix build-up occurs to heal the injury or the wound which is called hepatic fibrosis. Although this action is not permanent, if the injury is sustained, the scar tissue replaces the normal liver parenchyma which results in cirrhosis (Hernandez-Gea & Friedman, 2011).

All injuries to the hepatic cells trigger the activation of fibrogenic pathways. In the case of hepatic injury, changes in both composition and density of the extracellular matrix (ECM) occur. It is likely to observe an influx of ECM proteins and replacement of type IV collagen (non-fibrogenic) with type I and type II collagen (fibrogenic) during this incident. When fibrogenesis is initiated continuously, type I collagen gets produced more and degraded less, resulting in the deposition around the hepatic lobules (small divisions of the liver). As a result of this, ECM becomes more rigid than usual which leaves the blood vessels in the liver under pressure, causing intrahepatic vasoconstriction and increased vascular resistance within the liver (Acharya et al., 2021).

Neuroinflammation arises from the overactivation of microglia and astrocytes, which can disrupt the neurovascular unit and contribute to neuronal loss. Furthermore, blood vessels are also key sites of inflammation in the central nervous system,

characterized by the activation of endothelial cells. In vascular inflammation of the brain, activated endothelial cells show changes in their endophenotype and a decrease in tight junction proteins. This disruption impairs the blood-brain barrier's function, allowing the passage of peripheral immune cells and harmful or inflammatory plasma products to the brain tissue (Guo & Zhao, 2022).

Perivascular cells, including mural cells (such as pericytes and vascular smooth muscle cells), perivascular macrophages, and perivascular fibroblasts, are involved in a different CNS functions, like the formation of fibrotic scars (Dorrier et al., 2021). In the brain, perivascular fibrosis affects the BBB and can contribute to neurovascular dysfunction by altering vascular permeability and promoting inflammation. Perivascular fibroblasts (PVFs), located in the PVS, play a central role in this fibrotic process by producing ECM proteins and facilitating the structural changes associated with fibrosis (Sosa et al., 2023; Bonney et al., 2021).

1.6 Methionine and Choline Deficiency

Choline is an important nutrient that plays a role in phospholipid synthesis, lipid metabolism and transportation, and cell membrane signaling. In addition, choline acts as a precursor for acetylcholine production, which is a crucial neurotransmitter for muscle control and cognitive functions like memory. Sufficient amount of choline helps with maintaining the brain functions, liver functions, and lipid metabolism (Wallace et al., 2018; Zeisel & da Costa, 2009).

Choline deprivation is shown to be a disruptive factor in production and secretion of very-low-density lipoprotein (VLDL) in the liver. VLDLs play a crucial role in maintaining the lipid homeostasis in the body, and when this homeostasis is disrupted it leads to the emergence of multi-organ diseases (Casso & Farzam, 2022). In a study by Rinella et al. (2008), where the MCD diet was applied, hepatic fatty acid uptake was significantly increased while the VLDL secretion was decreased. Another important function of choline is maintaining the integrity of mitochondrial membrane, thus choline deficiency leads to disturbance of mitochondrial bioenergetics and fatty acid β-oxidation (Li et al., 2017).

Methionine is a valuable amino acid that contains sulfur. Its functions include methylation of DNA, metabolism of hepatic lipids, polyamines, creatine, and

phosphatidylcholine. Methionine is catabolized and recycled during the methionine cycle which is an important process for DNA, RNA, and histone methylation. This cycle results in the production of S-adenosylmethionine (SAM), the universal methyl donor (Aissa et al., 2022).

Methionine is known to reduce oxidative damage to the liver by decreasing the number of mitochondrial oxygen radicals (Séité et al., 2018). The amount of methionine metabolites are negatively correlated with prevalence of NAFLD, as low levels of methionine aggravate fat accumulation and inflammation in the liver (Tang et al., 2022).

1.7 Methionine-Choline Deficient Diet

MCD diet is a very common diet that is consumed as a model of nonalcoholic steatohepatitis (NASH) in animal experiments. Macrovesicular steatosis, hepatocellular death, inflammation, oxidative stress, and fibrosis are the characteristic features of NASH.

MCD diet is comprised of a high sucrose (40%) and high fat (10%) regimen, but it lacks methionine and choline (Takahashi & Fukusato, 2016). Choline has various functions in the human body, one of them being an acetylcholine (ACh) precursor. Methionine also plays a role in multiple biological processes, like redox maintenance, polyamine generation, protein synthesis and methylation of DNA and histones for epigenetic regulation of gene expression (Harada et.a., 2023). Methyl-donor deficiency is not only associated with liver disease but also with neurodegenerative diseases (Abu Ahmad et al., 2019).

Feeding mice with Western diet mimics metabolic disruptions frequently observed with mild non-alcoholic steatohepatitis in individuals. However, MCD diet more accurately emulates the underlying pathobiological processes that drive NAFLD to advanced NASH progression.

1.8 Methionine-Choline and Brain

The effects of the MCD diet on brain functions have not been widely explored compared to other subjects. However, high levels of homocysteine (tHcy) in the blood, often seen in older adults, are linked to increased risks of stroke, cognitive decline, dementia and Alzheimer's disease. These elevated levels are also associated with liver

disease and brain atrophy. Moreover, low choline levels increase the risk of dementia due to reduced availability of products from choline- and methionine-dependent methylation pathways. Animal studies show that a lack of choline impairs memory, while choline supplementation can improve cognitive aging in rodents (Ahmad et al., 2019).

Furthermore, a study by Vučević et al. (2016) shows that methionine-choline deprivation increases acetylcholinesterase (AChE) activity in the hypothalamus, hippocampus, cerebral cortex, and striatum. In the hypothalamus, this increase is observed by the fourth week, while in other brain regions, it becomes apparent around the sixth week. Although it was expected that choline deficiency would decrease AChE activity, their findings, consistent with other studies, show an increase, especially with prolonged choline deprivation. This suggests a complex relationship between choline availability and AChE activity in the brain (Vučević et al., 2016).

1.9 Carbon Tetrachloride (CCl_4)

Carbon tetrachloride (CCl_4) is an organic compound that is known for its toxicity to various organs such as kidneys, brains, and lungs. It is commonly used in animal experiments to induce hepatotoxicity. In addition to that, it has been used as an agent to model chemical hepatitis, renal failure, hepatocellular carcinoma, liver injury, hepatic fibrosis/cirrhosis, and nephrotoxicity (Unsal et al., 2020).

CCl_4 formerly had a commercial utilization as a dry cleaning solvent, refrigerant, and fire extinguisher; however, its industrial application has been abandoned after studies revealed its toxicity for humans and multiple reported cases (Manautou et.al, 2010).

It is also a chemical compound that has been commonly used in experimental models to induce liver injury, providing insights into hepatic pathophysiology. CCl_4 is a potent hepatotoxin known for its ability to induce acute and chronic liver damage in animal models through the formation of reactive intermediates, particularly trichloromethyl radicals (CCl_3) (Recknagel et al., 1989). These radicals can initiate lipid peroxidation and oxidative stress, leading to hepatocellular injury and inflammation.

In experimental studies, CCl_4 administration has been employed to simulate inflammation, hepatocellular necrosis and fibrosis. The hepatotoxic effects of CCl_4 are

well-documented, making it a good model for investigating the molecular mechanisms of liver injury and for testing potential therapeutic interventions. Studies utilizing CCl_4-induced liver injury models have provided data for our understanding of oxidative stress, inflammation, and fibrogenesis, providing crucial insights into liver diseases such as NAFLD, NASH, and liver fibrosis.

It's worth remembering that while CCl_4 has been extensively used in laboratory settings to model liver injury, caution must be exercised in translating findings to human pathology due to differences in metabolism and detoxification mechanisms between species (Sipes et al., 1997).

1.10 Carbon Tetrachloride Injection Model

Carbon tetrachloride (CCl4) is used to model liver fibrosis in C57Bl/6 mice since it can cause hepatocyte and liver fibrosis. CCl4 injection can be combined with high-fat diets in order to induce histopathological symptoms of NASH and increase serum alanine aminotransferase (ALT) and liver hydroxyproline levels. Although it is possible to model some characteristics of NASH, C57Bl/6 strain cannot fully show the features of diabetes associated with NASH in humans (Zhang et al., 2020).

The CCl_4 injection model is generally used to model liver injury and to study pathogenesis of NASH. CCl_4 is a potent hepatotoxin and it induces hepatocellular injury mainly through the formation of trichloromethyl radicals (CCl_3) upon metabolism. Researchers usually prefer this model when investigating the molecular and cellular aspects of liver inflammation, oxidative stress, and fibrosis, which are key features of NASH (Unsal et al., 2020).

CCl_4 is usually administered intraperitoneally or subcutaneously to rodents and it leads to the generation of free radicals that induce lipid peroxidation, hepatocyte necrosis, and as a result, inflammation (Friedman, 2008). The results are similar to the histopathological features of NASH in humans. Thus, it is considered a useful method to study the underlying mechanisms and potential therapeutic targets (Zhang et al., 2020).

1.11 Carbon Tetrachloride and Brain

The brain, due to its high lipid content, is particularly vulnerable to damage from reactive oxygen species (ROS). Mitochondria, a major site of ROS production, are especially susceptible to attack by neurotoxins like CCl_4 (Alam, 2018). This neurotoxin easily crosses the BBB, generating free radicals that trigger oxidative stress and contribute to brain damage, neurological diseases, such as AD, Parkinson's disease and amyotrophic lateral sclerosis, as well ischemia and excitotoxicity (Alam, 2018; Altinoz et al., 2018).

A study conducted by Oluwafemi et al. (2016), have shown that CCl_4 induces oxidative stress in rat brains. Similarly, De Souza et al. (2015) reported increased levels of pro-inflammatory cytokines in the brain due to CCl_4 exposure (Alam, 2018). Furthermore, as observed by Estevao et al. (2021), CCl_4 have a significant impact on junctional proteins and blood-brain barrier function, indicating its role in altering central nervous system (CNS) vascular permeability and promoting neuroinflammation (Estevao et al., 2021).

Abnormal chemokine levels, including CCL4, are also found in multiple sclerosis (MS) patients, and it has a correlation with the disease progression. CCL4 and other chemokines have been identified in brain sections and active MS lesions, indicating pathogenetic role in neuroinflammatory diseases (Estevao et al., 2021).

Clinically, both major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) are associated with increased inflammation, dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, and reduced neurogenesis. Interestingly, individuals suffering from depression, especially those who have attempted suicide, exhibit lower levels of the CCL4 chemokine compared to healthy individuals. Similarly, a meta-analysis showed that PTSD patients also had reduced levels of CCL4 compared to healthy individuals. On the other hand, elevated CCL4 levels have been associated with schizophrenia and bipolar disorder, with bipolar patients showing higher CCL4 levels than unipolar patients (Estevao et al., 2021).

1.12 Aim and Hypothesis

The primary objective of this thesis is to examine the potential impact of non-alcoholic steatosis on the brain. Given the interconnected nature of physiological

systems within the body, coupled with existing literature indicating various associations between non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) with the brain, our research aims to specifically investigate how the pathophysiology leading to fatty liver disease and liver fibrosis impacts the brain using two different methods to induce NASH. This study was focused on investigating the impact of NASH on the blood-brain barrier (BBB) and the brain vasculature, and the possible connection between NASH and perivascular fibrosis.

CHAPTER 2:
MATERIALS AND METHODS

2.1 Animal Maintenance

All planned methodologies have received approval from the Institutional Animal Care and Use Committee of Koç University. This study employed male C57B1/6 mice that are older than 8 weeks. The male C57B1/6 mice (n=36) were obtained from the Koç University Animal Research Facility and were randomly distributed into either the control group (n=3), MCD diet group (n= 18) or the CCl4 group (n=15). All animals were housed in Plexiglas cages within a room set at a temperature of 20±3 °C and under a 12-hour light/dark cycle. Animals in the control group were provided with unrestricted access to standard laboratory rat chow and the animals in both experimental groups were fed a high fat and high sucrose diet.

2.2 Methionine-Choline Deficient (MCD) Diet Model

Animals in the MCD diet group were fed a high-fat and high-sucrose diet by providing them the appropriate food and sucrose-induced water. Three animals were assigned to each designated week for the study. After one week of experimentation, the animals designated for “Week 1” were sacrificed. Following a similar sequential pattern, the animals assigned to “Week 2” were sacrificed at the end of the following week, and this process was repeated until the end of the sixth week. Animals were sacrificed with a dose of ketamine proportional to their body weights and then cervical dislocation.

2.3 Carbon Tetrachloride (CCl4) Model

Mouse models of liver fibrosis were induced by administering intraperitoneal injections of CCl4 (10µl/g) in a 10% mineral oil solution for two consecutive days per week. After one week of experimentation, “Week 1” animals were sacrificed. Similarly, the animals assigned to “Week 2” were sacrificed at the end of the following week, and this process was repeated until the end of the sixth week. Cervical dislocation was applied following determining and injecting the appropriate dose of ketamine depending on the body weights of the animals.

2.4 Biochemical Assays

Blood samples were collected via cardiac puncture and transferred to testing tubes. After clotting occurred, they were centrifuged at 3000 rpm for 15 min in order to separate the serum. Then they were sent to the Pathology Laboratory at Koç University Hospital for analysis.

2.5 Tissue Collection

After collecting blood, the animals were transcardially perfused with an ice-cold heparin-saline solution (10 IU/L), followed by 4% paraformaldehyde (PFA). The brains were then stored in 4% PFA overnight at 4°C for immunofluorescence preparation. The tissues were subsequently dehydrated in a gradient of sucrose (10%, 20%, and 30%) in 0.1 M phosphate buffer at 4°C. Finally, the brain tissues were embedded in Cryomatrix resin, sectioned at 40 μm thickness for further analysis. The brain tissues were kept in two different conditions depending on the procedure and imaging technique we wanted to utilize. The brain tissues for real-time PCR were kept in saline and the tissues for immunofluorescence were kept in paraformaldehyde (PFA).

2.6 Real-time PCR

Fifty milligrams of brain tissue were utilized to extract total RNA, following the guidelines provided by the manufacturer for Tri Reagent (MilliporeSigma). The Nanodrop spectrophotometer ND-1000 from Thermo Fisher Scientific system was utilized to evaluate concentration and purity of the RNA. The specific gene primers employed included CD13, kinase insert domain receptor (KDR), alpha-smooth muscle actin (αSMA), carbon tetrachloride (CCl2), cluster of differentiation 31 (CD31), collagen type I (Col1), collagen type I alpha 1 (Col1a1), collagen type I alpha 1 (Col4a1), fibronectin, matrix metalloproteinase-9 (MMP9), Notch3, platelet-derived growth factor receptor beta 1 (Pdgfrβ-1), platelet-derived growth factor receptor beta 2 (Pdgfrβ-2), receptor for advanced glycation end products (RAGE), TGFβ-1, and tissue inhibitors of metalloproteinases (TIMP). The data were collected, and the relative gene expression was computed using the $2^{-\Delta\Delta Ct}$ method with the reference genes. Statistical

analysis was carried out using GraphPad Prism 10, and significance was established at $p < 0.05$. All experiments were performed in duplicate.

2.7 Immunofluorescence

Brain sections were labelled with immunofluorescence (IF) staining as explained below, except for the clear tissues. Cleared tissues were incubated in primary and secondary antibodies for 48 h, and washing steps were 3 h. After completing the clearing protocol, 1 mm thick brain slides were embedded in glycerol and imaged with the confocal microscope (Leica system TCS SP8 microscopy).

For immunofluorescent labelling, 40 μm thick brain sections were incubated in methanol for 5 min and washed with Dulbecco's phosphate-buffered saline (DPBS, Gibco, #14190-094). Super Block (Thermo Fisher Scientific™, PI37535) was used for blocking background stain and incubated for 1 h at room temperature. Primary antibodies were diluted in Super Block and sections were incubated for 1.5 h at 37 °C. After washing, sections were incubated with suitable secondary antibodies. Then slides were washed and mounted with 4, 6-diamidino-2-phenylindole (DAPI, Abcam, Ab104139). Isolectin GS-IB4-Alexa Fluor 488 conjugate was used to label vasculature (Thermo Fisher Scientific™, I21411) as 1:100 dilution. Anti-Albumin (Thermo Fisher Scientific™, A90-234 A, 1/50) antibody used for demonstrating BBB leakage; anti-PDGFRβ (Abcam, Ab32570, 1/200) for pericytes; anti-Collagen I (Abcam, Ab34710, 1/100), anti-Collagen IV (Abcam, Ab6586, 1/200), anti-Fibronectin (Sigma-Aldrich, SAB4200760, 1/200), and anti-elastin (Abcam, Ab21610, 1/200) for labeling ECM.

2.8 Tissue Clearing

3DISCO method was used to image and analyse the microvasculature. Following the 3DISCO procedure, as described by Ertürk et al. (2012), animals underwent transcardial perfusion with an ice-cold heparin-saline solution (50IU/L), 4% PFA, and 2% gelatin-albumin-FITC solution. The brain tissues were collected and subsequently preserved in 4% PFA overnight at 4 °C, sliced as 1 mm thick sections, and immersed in an increasing gradient tetrahydrofuran (THF) solution. Cleared tissues embedded in the DBE solution were imaged using confocal microscopy.

For the CLARITY method, detailed in another source (17), animals underwent perfusion with ice-cold PBS and a hydrogel monomer solution. The brains were removed and stored at +4 °C in the hydrogel monomer solution for three days. Following a 3-hour incubation in a shaking water bath at 37 °C, the tissues were cleared in a 4% sodium dodecyl sulfate (SDS) solution.

Upon completion of the protocol, 1 mm thick brain slides immersed in glycerol solution were imaged with confocal microscope (Leica TCS SP8 microscopy).

2.9 Biochemical Assays

Blood samples were collected via cardiac puncture and transferred to testing tubes. After clotting occurred, they were centrifuged at 3000 rpm for 15 min in order to separate the serum. Then they were sent to the Pathology Laboratory at Koç University Hospital for analysis.

2.10 Image Analysis

Fluorescence microscopy images of 40 μm thick frozen sections (z sections per 2 μm) were exported as TIF files and the LAS X program (Leica, Wetzlar, Germany) was used to assess the amount of collagen and/or smooth muscle alpha-actin (αSMA) buildup on the blood vessels.

2.11 Data Analysis

Statistical analysis of the real-time PCR results for both the MCD diet and the CCl4 experiments were performed by using GraphPad Prism 10 (San Diego, USA). Kruskal-Wallis test was selected for these analyses. Dunn's *post hoc* test was used to compare the groups that showed statistical significance in the Kruskal-Wallis test. A p-value below 0.05 was deemed statistically significant. The analysis of the liver enzymes was done by calculating the means and their standard deviations. Fluorescence microscopy images were selected using the LAS X software (Leica, Germany) and exported as Tiff files. Following that, ImageJ was used to assess the amount of collagen and/or smooth muscle alpha-actin (αSMA) buildup on the blood vessels by calculating the area (%). The graphs were drawn by using GraphPad Prism 10 and Microsoft Excel.

CHAPTER 3:
RESULTS

3.1 Real-time PCR Results

3.1.1 MCD Diet

For MCD diet αSMA, CCL2, CD13, CD31, COL1, COL1A1, COL4A1, fibronectin, KDR, MMP9, Notch3, PDGRFβ-1, PDGFRβ-2, RAGE, TGFβ-1, TIMP, and VWF were studied. There was a significant difference between experiment and control animals when we performed q-PCR for CD13 (p=.016) and KDR (p=.031).

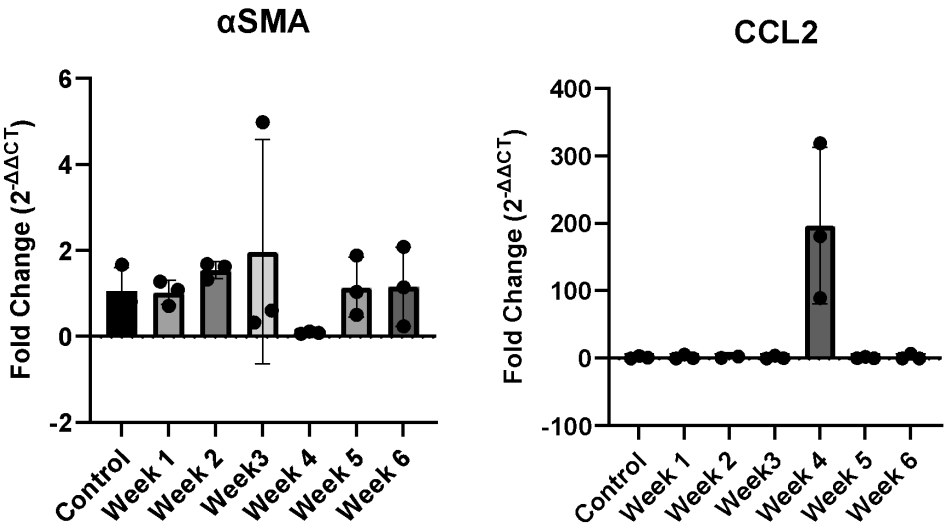
CD13 (Aminopeptidase N) is an ectoenzyme that falls under the category of "moonlight proteins" which are proteins that have more than one function. This multifunctionality of CD13 is one of the reasons why the effects of its inhibition of enzymatic activity or ligation might not be easy to interpret (Mina-Osorio, 2008). CD13 is expressed on various types of cells, such as myeloid cells, pericytes, fibroblasts, epithelial and endothelial cells, tumor cells, and stem cells (Nguyen et al., 2023).

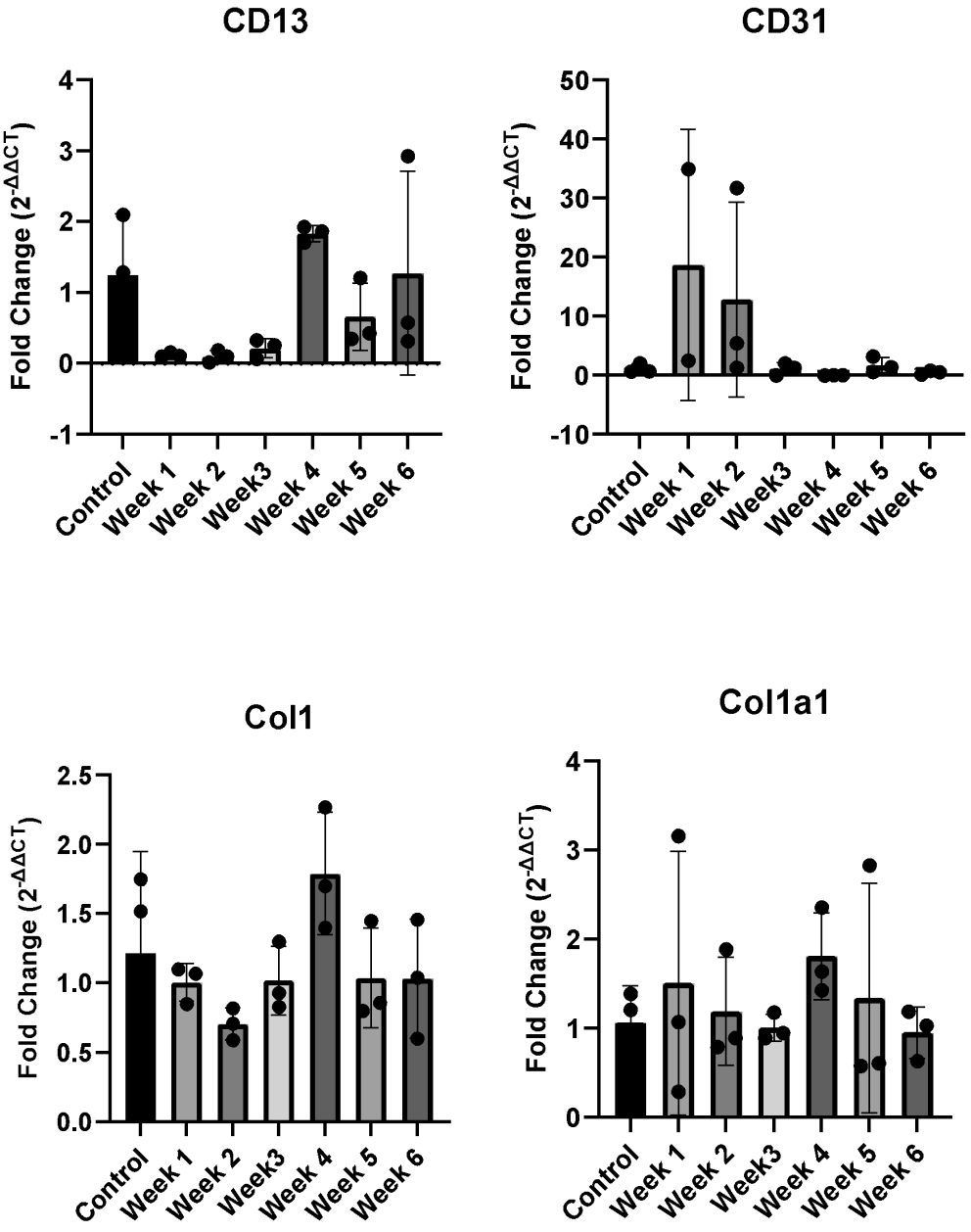
The results for CD13 revealed changes in the experimental groups dependent on time. One of the significant differences that we detected was between the control group and the animals from the Week 3 (p=.009) and Week 6 (p=.027) groups. These results suggest a temporal effect of MCD diet on mice. Furthermore, a significant difference was detected between Week 1 mice and Week 3-5-6 mice, and similarly between Week 2 and Week 3 mice. These results might indicate a potential underlying mechanism or response in addition to the effect of time.

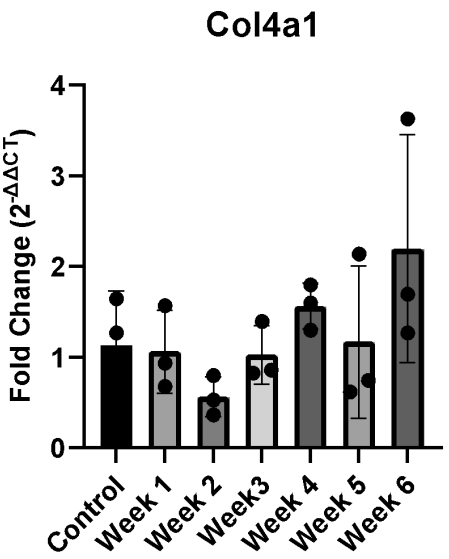
KDR (Kinase Insert Domain Receptor) is a gene that codes vascular endothelial growth factor receptors in humans. Following the execution of q-PCR analysis targeting KDR, our investigation revealed noteworthy differences among the experimental

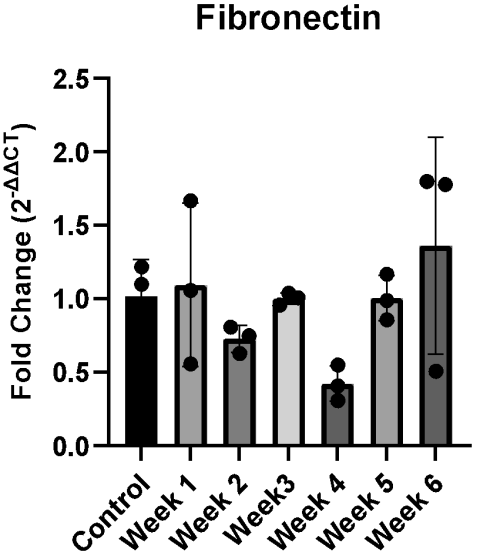
groups. Notably, we observed a statistically significant divergence between the control group and the animals assessed during the 6th week of our study, underscoring a substantial alteration in KDR expression levels over time. Furthermore, our results highlighted significant differences between the animals examined at the 1st week and those at the 2nd, 5th, and 6th weeks, as well as between the animals assessed at the 3rd week and those at the 5th and 6th weeks.

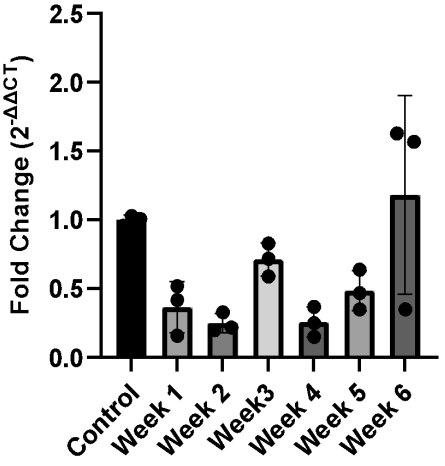
These findings illuminate the dynamic nature of KDR expression throughout the duration of our study, suggesting potential regulatory mechanisms or responses that evolve as the study progresses.

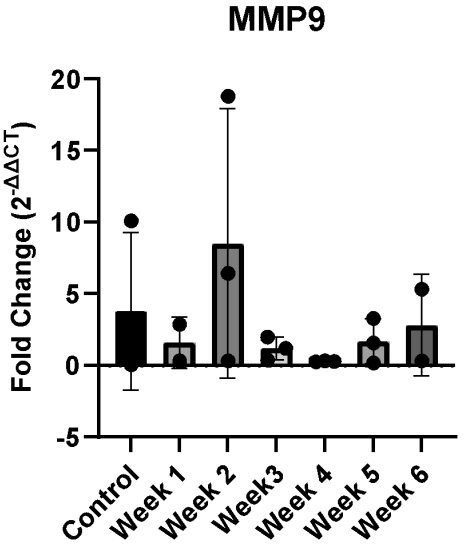


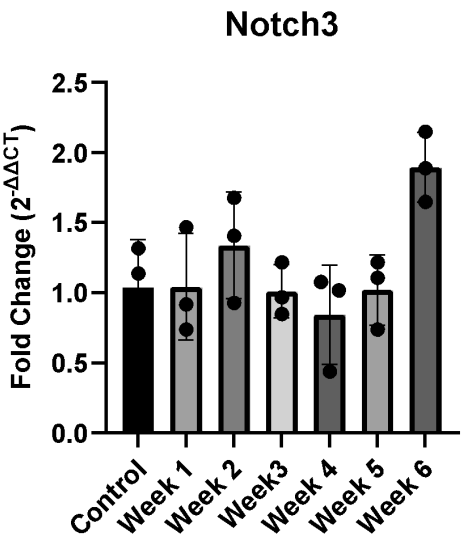

CD13
Fold Change ($2^{-\Delta\Delta CT}$)
4
3
2
1
0
-1
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6
CD31
Fold Change ($2^{-\Delta\Delta CT}$)
50
40
30
20
10
0
-10
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6
Col1
Fold Change ($2^{-\Delta\Delta CT}$)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6
Col1a1
Fold Change ($2^{-\Delta\Delta CT}$)
4
3
2
1
0
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6

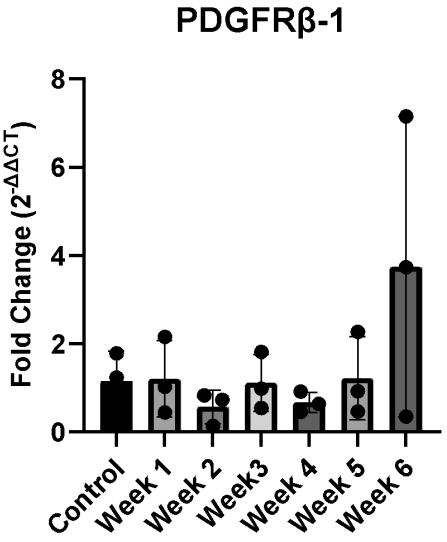

Col4a1
Fold Change ($2^{-\Delta\Delta CT}$)
0
1
2
3
4
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6

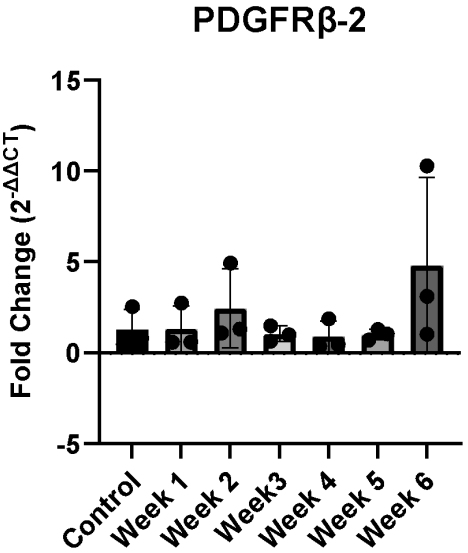

Fibronectin
Fold Change ($2^{-\Delta\Delta CT}$)
0.0
0.5
1.0
1.5
2.0
2.5
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6

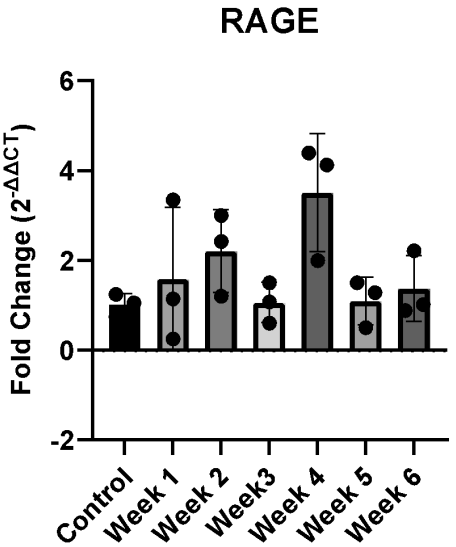

KDR
Fold Change ($2^{-\Delta\Delta CT}$)
0.0
0.5
1.0
1.5
2.0
2.5
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6

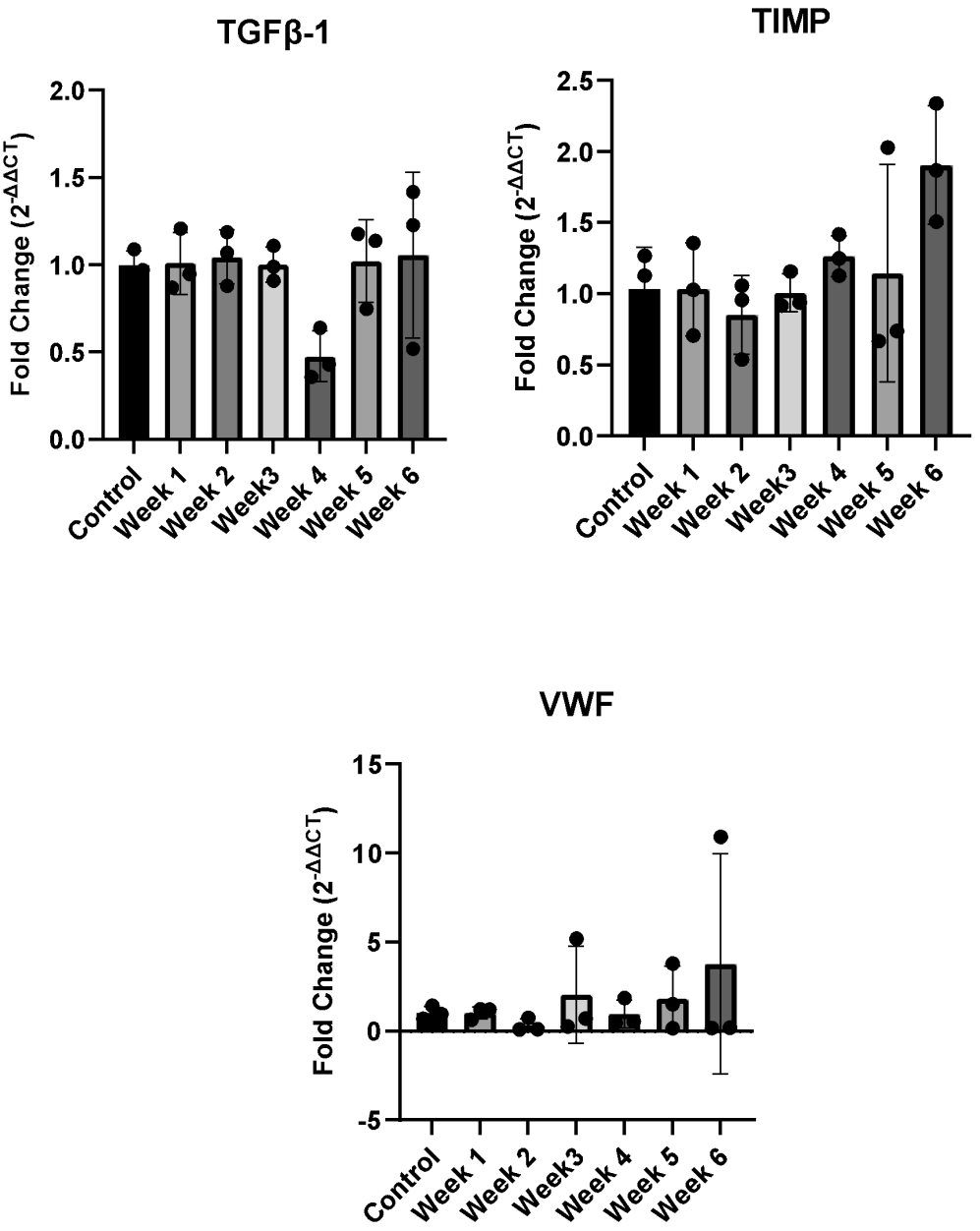

MMP9
Fold Change ($2^{-\Delta\Delta CT}$)
-5
0
5
10
15
20
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6


Notch3
Fold Change ($2^{-\Delta\Delta CT}$)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6


PDGFRβ-1
Fold Change ($2^{-\Delta\Delta CT}$)
8
6
4
2
0
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6


PDGFRβ-2
Fold Change ($2^{-\Delta\Delta CT}$)
15
10
5
0
-5
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6


RAGE
Fold Change ($2^{-\Delta\Delta CT}$)
6
4
2
0
-2
Control
Week 1
Week 2
Week3
Week 4
Week 5
Week 6

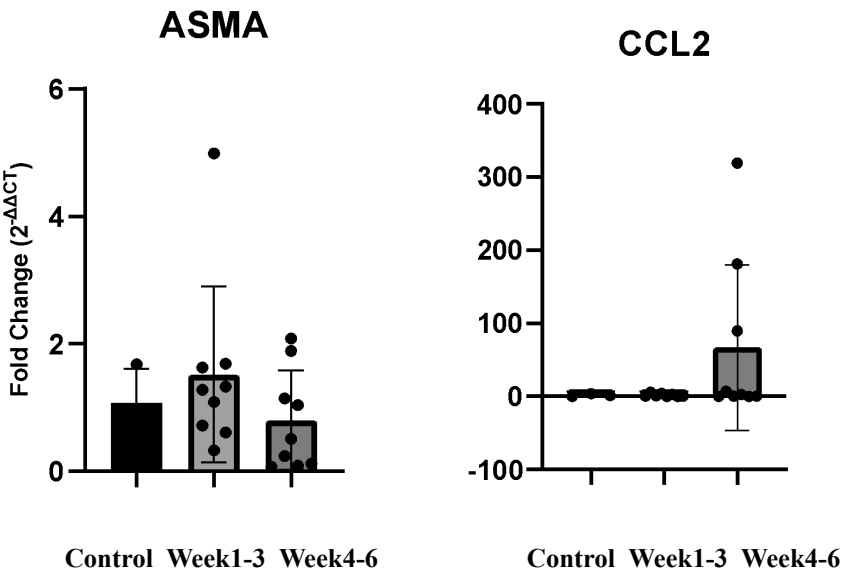


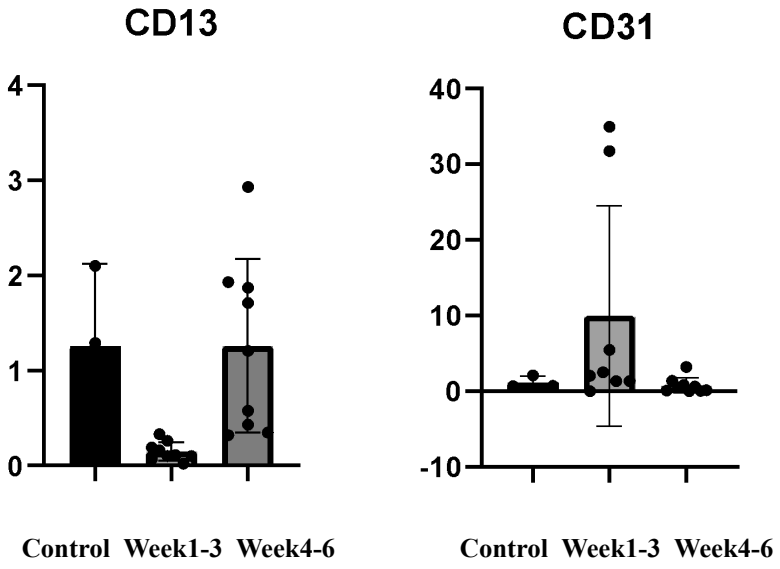
3 **FIGURE 4:** Real-time PCR results for the animals that were fed an MCD diet. αSMA, CCL2, CD13, CD31, COL1, COL1A1, COL4A1, fibronectin, KDR, MMP9, Notch3, PDGRFβ-1, PDGFRβ-2, RAGE, TGFβ-1, TIMP, and VWF were studied. The X axis shows the time period, and the Y axis shows the fold change calculated as $2^{(-\Delta\Delta CT)}$.

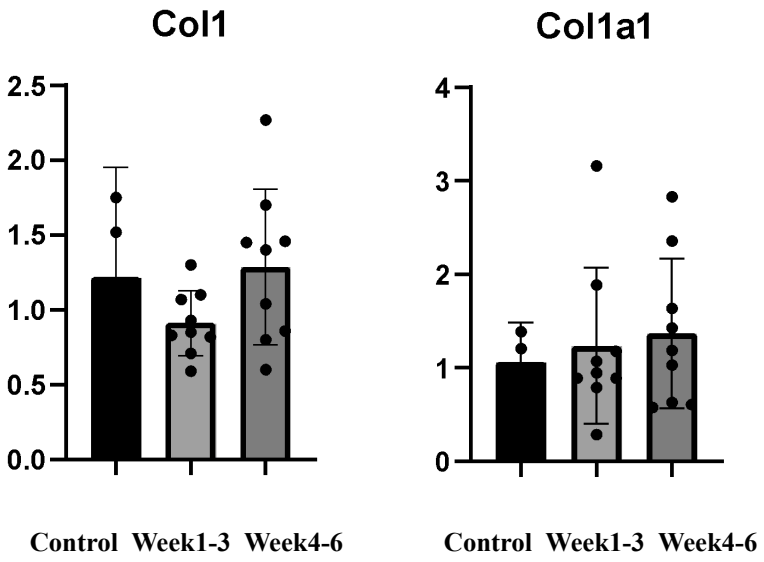
3.1.1.1 Grouping Weeks

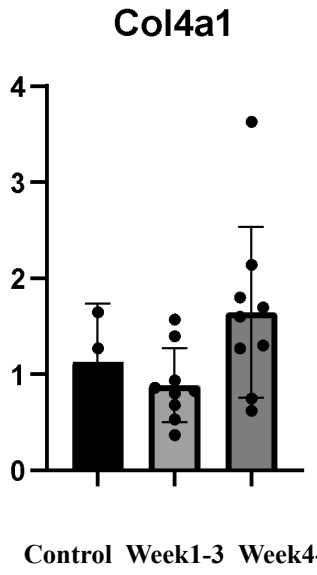
The animals were grouped and analyzed in order to examine a potential meaningful difference. The first group consisted of all the animals from the Week 1-2-3 groups, whereas the second group consisted of all the animals from the Week 4-5-6 groups. A comprehensive statistical analysis was conducted on these two groups and the control group.

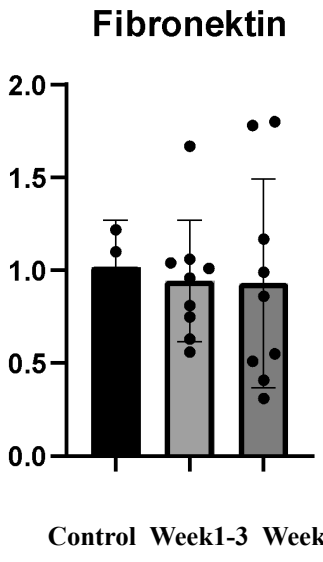
A significant difference was observed only for the CD13 marker as a result of this analysis, with a p-value of less than 0.001.

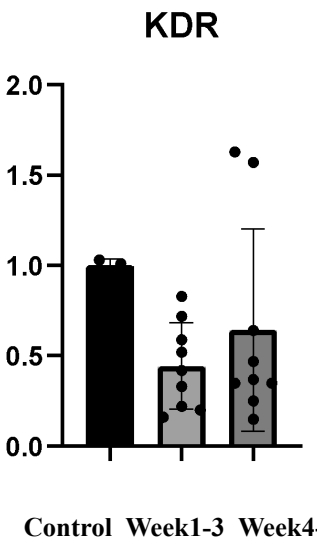


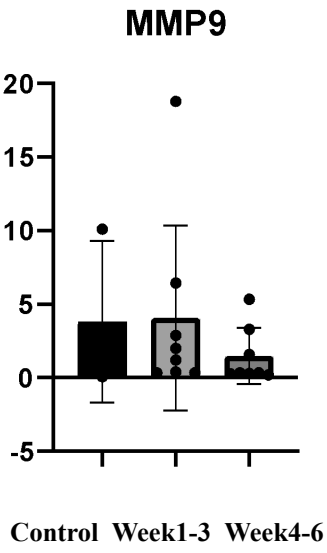

CD13
4
3
2
1
0
Control Week1-3 Week4-6
CD31
40
30
20
10
0
-10
Control Week1-3 Week4-6

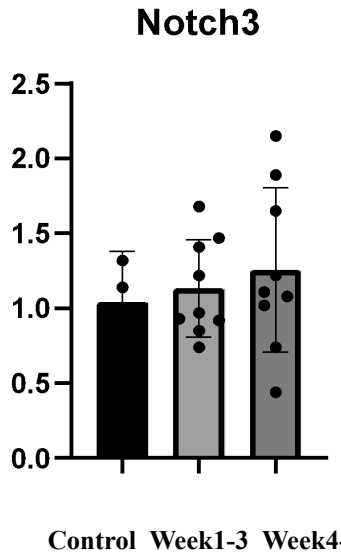

Col1
2.5
2.0
1.5
1.0
0.5
0.0
Control Week1-3 Week4-6
Col1a1
4
3
2
1
0
Control Week1-3 Week4-6

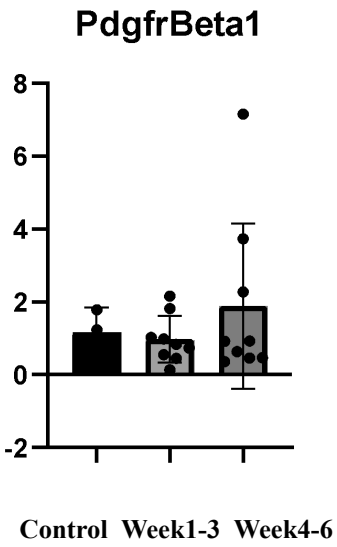

Col4a1
4
3
2
1
0
Control
Week1-3
Week4-6

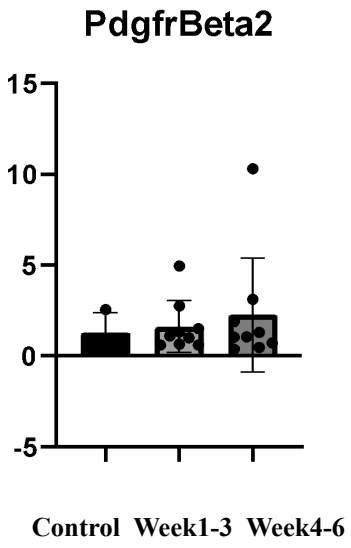

Fibronektin
2.0
1.5
1.0
0.5
0.0
Control
Week1-3
Week4-6

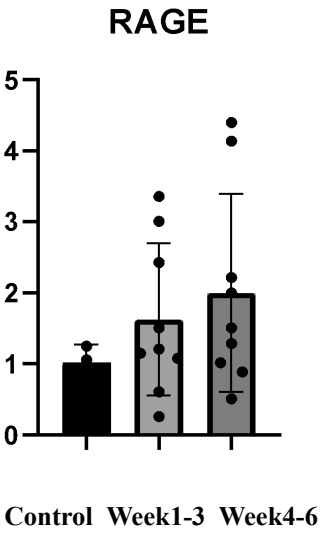

KDR
2.0
1.5
1.0
0.5
0.0
Control
Week1-3
Week4-6


MMP9
20
15
10
5
0
-5
Control
Week1-3
Week4-6


Notch3
2.5
2.0
1.5
1.0
0.5
0.0
Control
Week1-3
Week4-6


PdgfrBeta1
8
6
4
2
0
-2
Control
Week1-3
Week4-6


PdgfrBeta2
15
10
5
0
-5
Control
Week1-3
Week4-6


RAGE
5
4
3
2
1
0
Control
Week1-3
Week4-6

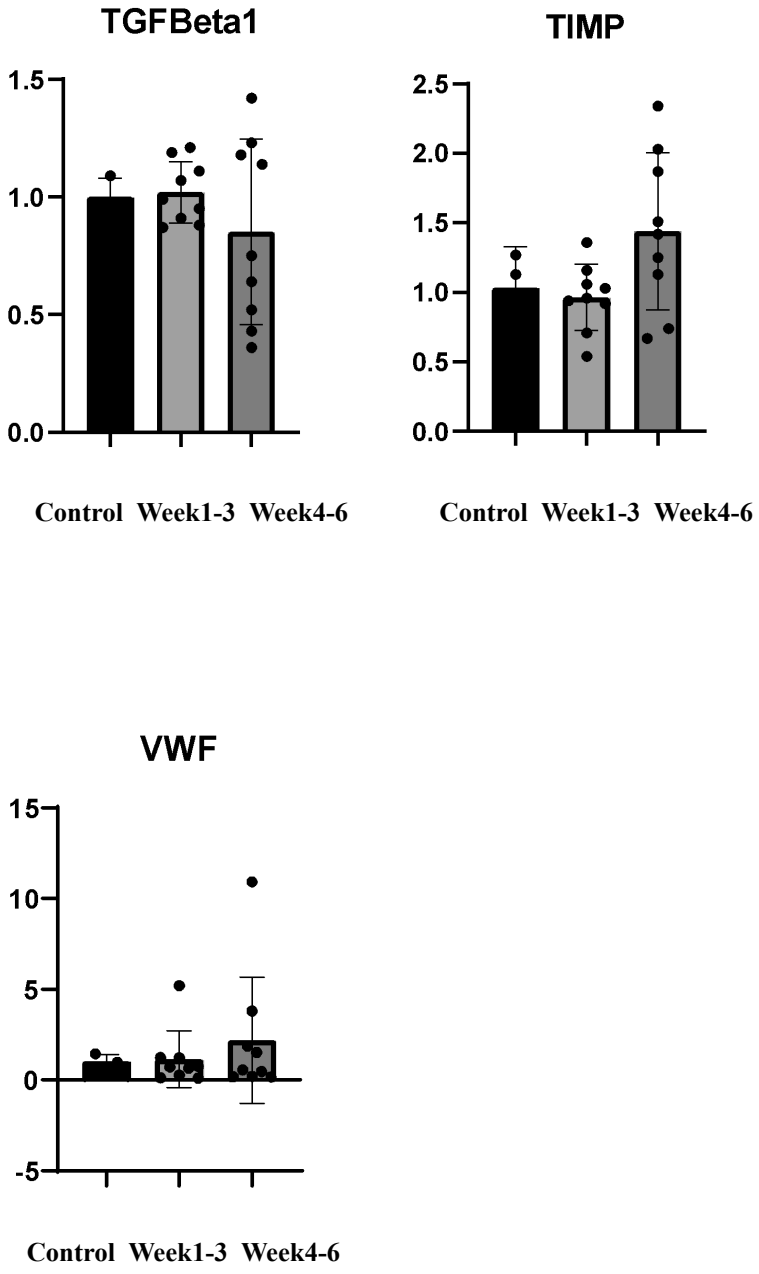
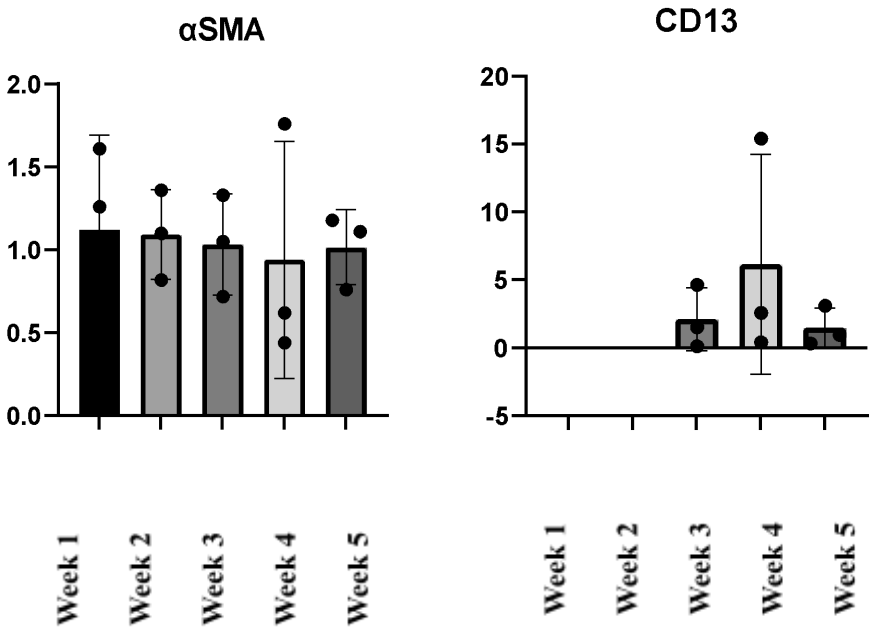
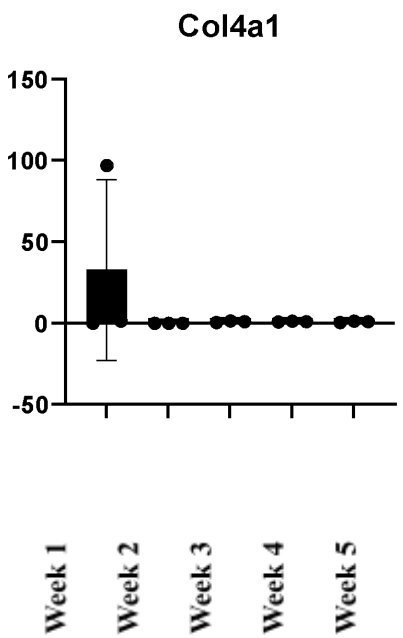


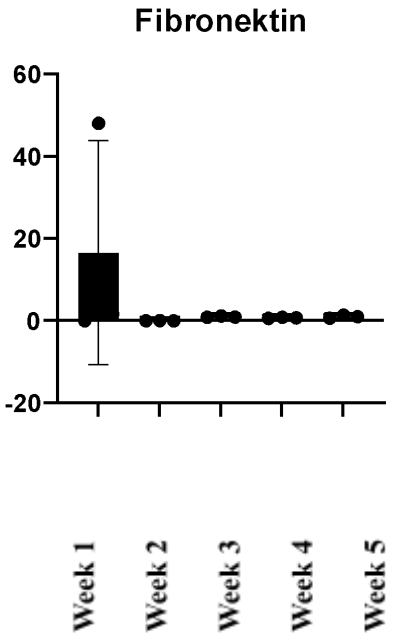
FIGURE 5: Real-time PCR results for animals subjected to the MCD diet, categorized into three groups based on the duration of their exposure: the control group, animals exposed for 1-3 weeks, and animals exposed for 4-6 weeks. αSMA, CCL2, CD13, CD31, COL1, COL1A1, COL4A1, fibronectin, KDR, MMP9, Notch3, PDGRFβ-1, PDGFRβ-2, RAGE, TGFβ-1, TIMP, and VWF were studied. The X axis shows the time period, and the Y axis shows the fold change calculated as 2 (-ΔΔCT) .

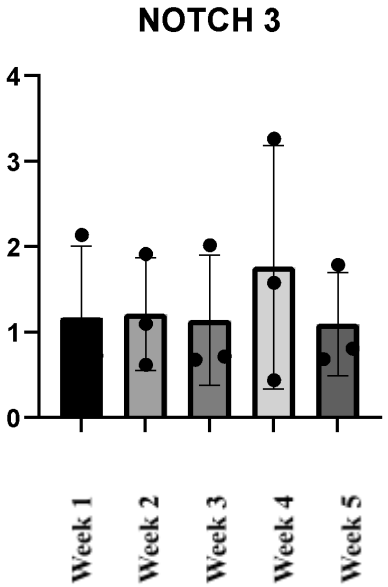
3.1.2 CCl4

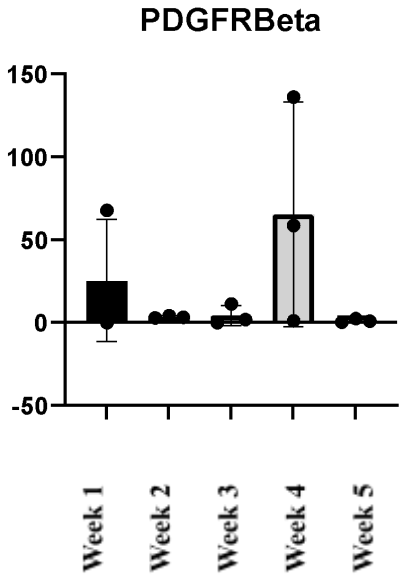
No statistically significant differences were observed on animals subjected to CCl4 treatment of quantitative polymerase chain reaction (q-PCR) analyses ($p<0.05$).




Col4a1
150
100
50
0
-50
Week 1
Week 2
Week 3
Week 4
Week 5


Fibronektin
60
40
20
0
-20
Week 1
Week 2
Week 3
Week 4
Week 5


NOTCH 3
4
3
2
1
0
Week 1
Week 2
Week 3
Week 4
Week 5


PDGFRBeta
150
100
50
0
-50
Week 1
Week 2
Week 3
Week 4
Week 5

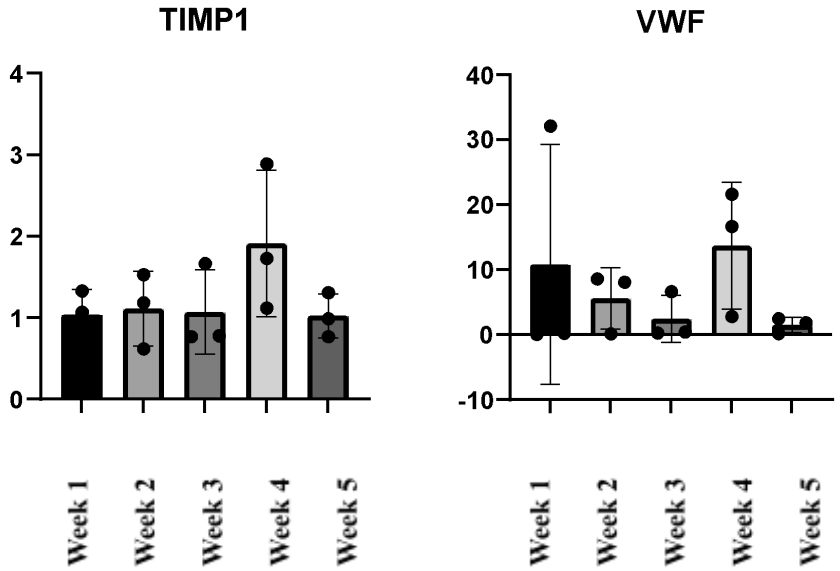


FIGURE 6: Real-time PCR results for animals that received CCl4 injections. αSMA, CD13, COL4A1, fibronectin, Notch3, PDGRFβ, TIMP, and VWF were studied. The X axis shows the time period, and the Y axis shows the fold change calculated as $2^{(-\Delta\Delta CT)}$.

3.2 Immunofluorescence Staining of Brain Sections

Brain tissue sections from animals fed the MCD diet were stained to identify brain vessels, using markers for alpha-SMA and collagen. The images revealed that in week one, the brain vessels remained intact with strong staining. However, by weeks three and five, a noticeable reduction in staining intensity was observed.

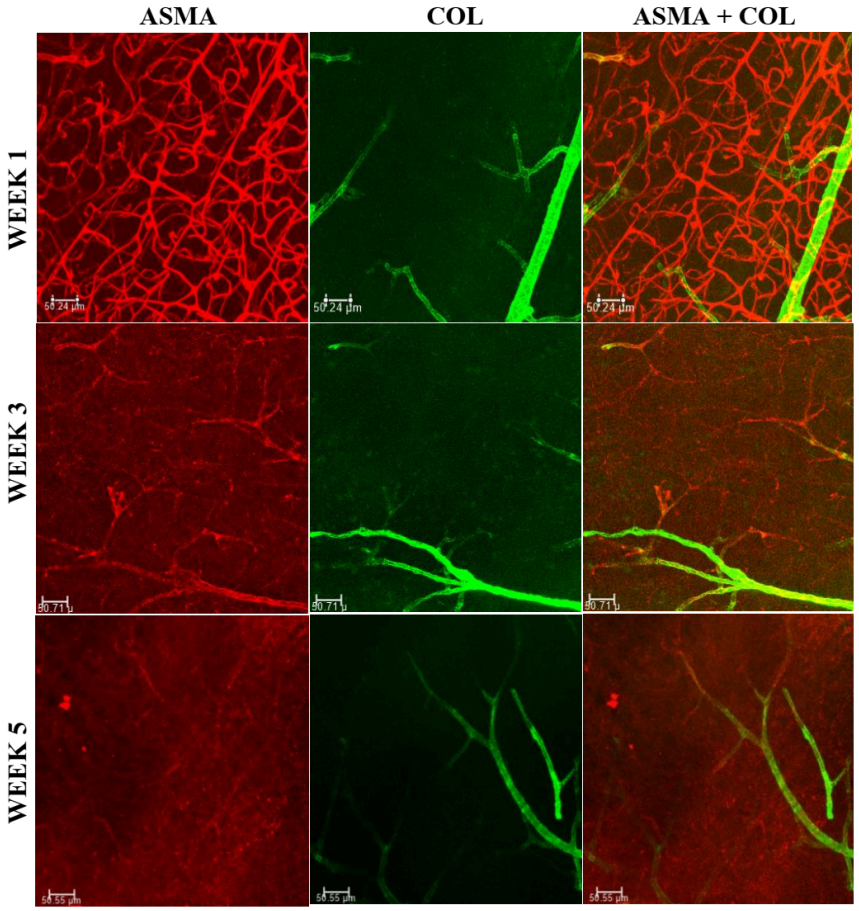


FIGURE 7 Brain tissue sections of the animals fed the MCD diet dyed using markers for alpha-SMA and collagen to identify brain vessels and their possible dysfunctions.

3.3 Analysis of Liver Enzymes

The liver enzyme levels, specifically ALT, AST, LDL C3, TRIGL, LDL-H, and VLDL levels were analyzed and listed in the table below.

TABLE 1 Results of the liver enzyme analyses.

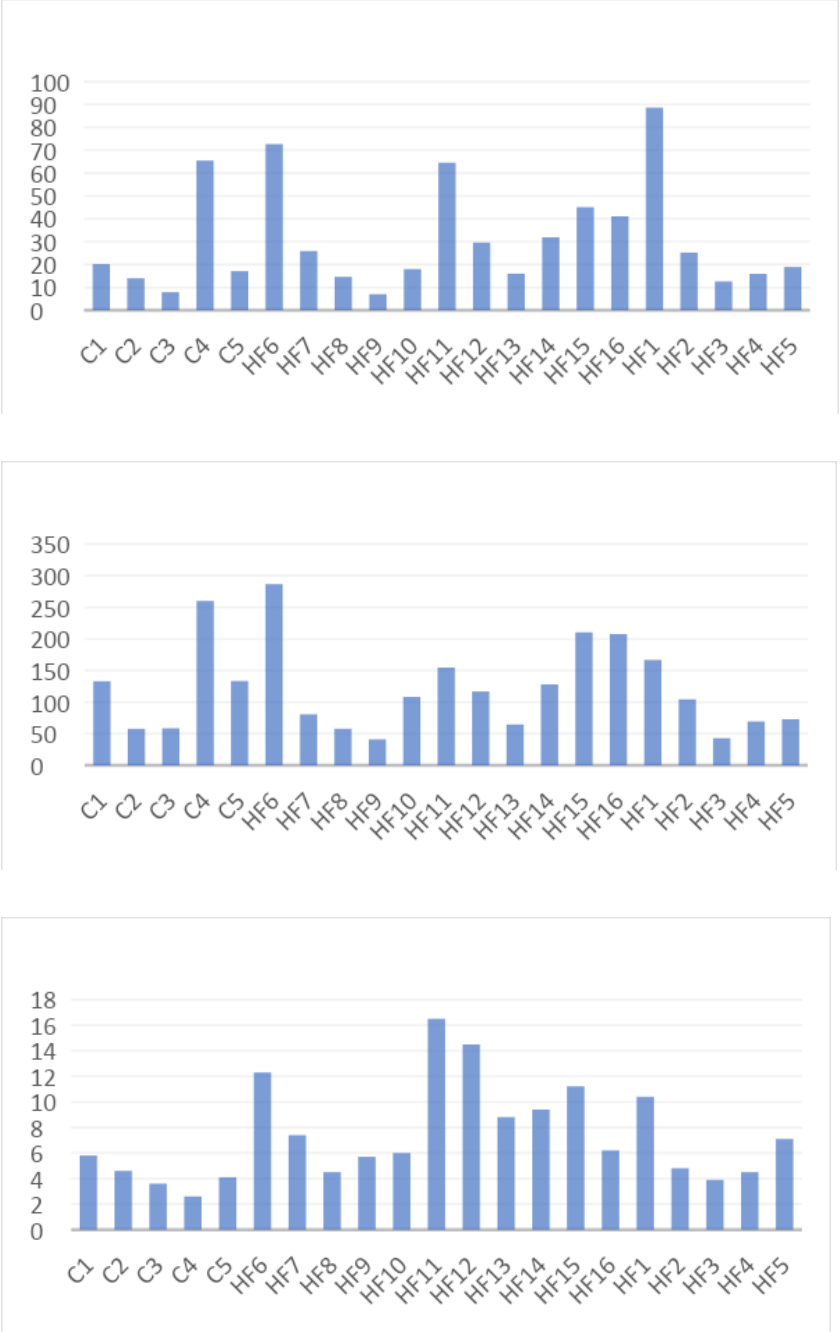
Before the experiment

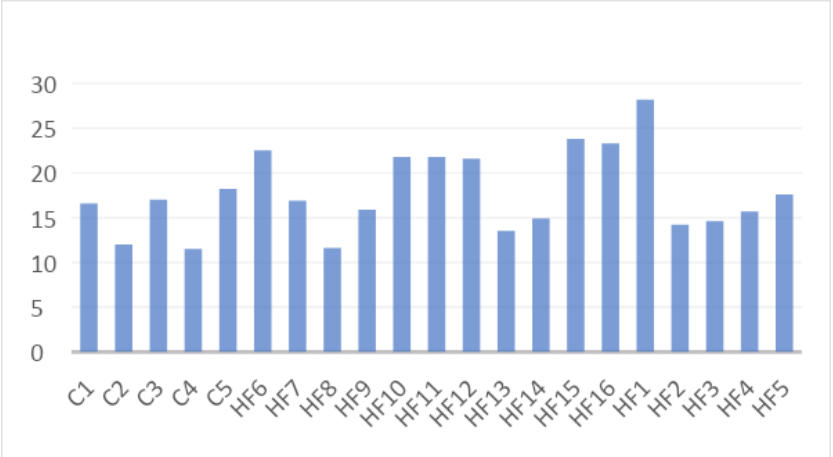
Sample No	ALT	AST	LDL C3	TRIGL	LDL-H	VLDL
x	3.7	23.2	2.0	8.4	1	2

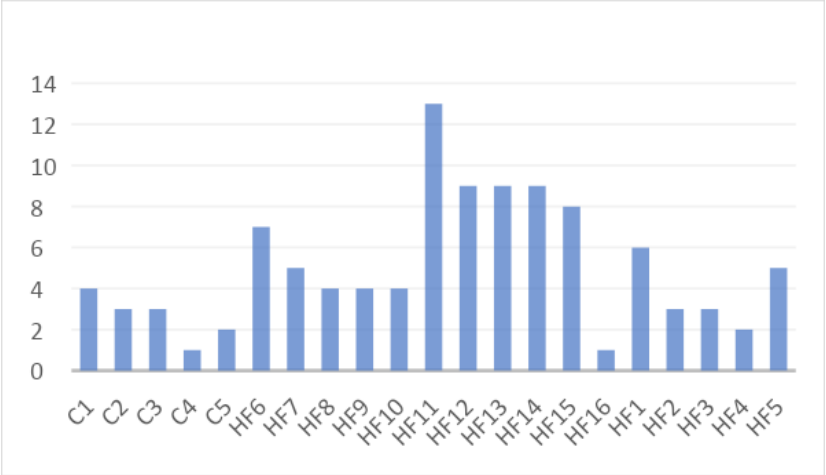
After the experiment

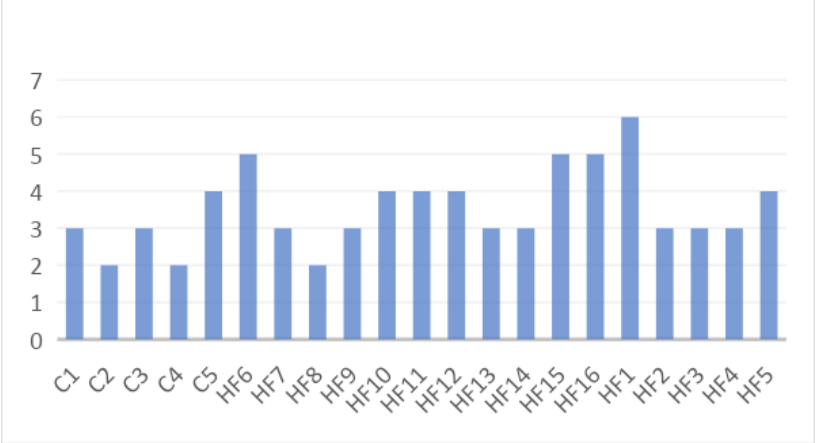
Sample No	ALT	AST	LDL C3	TRIGL	LDL-H	VLDL
C1	20.2	133	5.8	16.6	4	3
C2	14	57.8	4.6	12	3	2
C3	7.9	58.6	3.6	17	3	3
C4	65.5	259.9	2.6	11.5	1	2
C5	17.1	133.4	4.1	18.2	2	4
HF6	72.6	286.5	12.3	22.5	7	5
HF7	25.9	80.7	7.4	16.9	5	3
HF8	14.6	57.9	4.5	11.6	4	2
HF9	7	41.1	5.7	15.9	4	3
HF10	18	108.2	6	21.8	4	4
HF11	64.5	154.5	16.5	21.8	13	4
HF12	29.6	116.8	14.5	21.6	9	4
HF13	16	64.6	8.8	13.5	9	3
HF14	31.9	128	9.4	14.9	9	3
HF15	45.1	210.4	11.2	23.8	8	5
HF16	41.1	207.8	6.2	23.3	1	5
HF1	88.6	166.5	10.4	28.2	6	6
HF2	25.2	104.4	4.8	14.2	3	3
HF3	12.6	42.9	3.9	14.6	3	3
HF4	15.9	69.4	4.5	15.7	2	3
HF5	18.9	73	7.1	17.6	5	4
MEAN	32.97	119.54	8.33	18.62	5.75	3.75
CONTROL MEAN	24.94	128.54	4.14	15.06	2.6	2.8
STD. DEV.	22.95	67.46	3.67	4.54	3.09	1.03

FIGURE 8 The results of the liver enzyme analyses at the end of the experiment (end of Week 6). The studied enzymes were ALT, AST, LDL C3, TRIGL, LDL-H, and VLDL. The x-axis shows the animals from the control group (C1-C5) and animals from the treatment group (HF1-16), while the y-axis represents the level of enzymes (U/L).

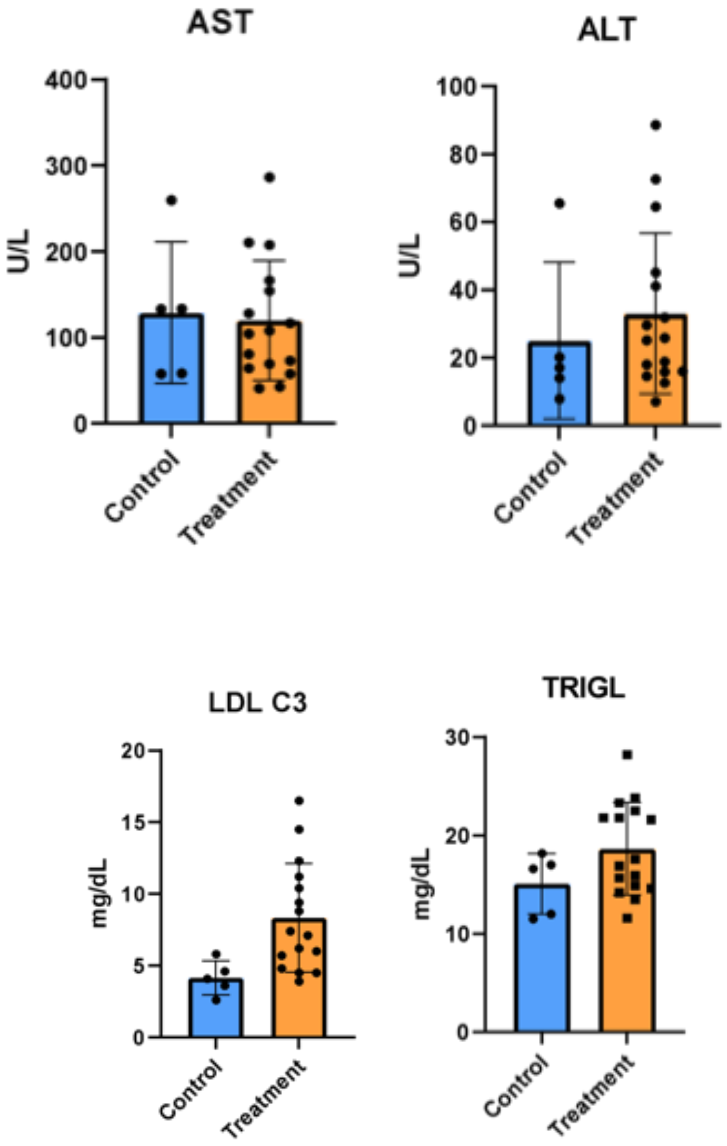



30
25
20
15
10
5
0
C1 C2 C3 C4 C5 HF6 HF7 HF8 HF9 HF10 HF11 HF12 HF13 HF14 HF15 HF16 HF1 HF2 HF3 HF4 HF5


14
12
10
8
6
4
2
0
C1 C2 C3 C4 C5 HF6 HF7 HF8 HF9 HF10 HF11 HF12 HF13 HF14 HF15 HF16 HF1 HF2 HF3 HF4 HF5


7
6
5
4
3
2
1
0
C1 C2 C3 C4 C5 HF6 HF7 HF8 HF9 HF10 HF11 HF12 HF13 HF14 HF15 HF16 HF1 HF2 HF3 HF4 HF5

By combining the levels of each liver enzyme from the treatment group, a comprehensive comparison between the treatment and control groups was conducted. This aimed to gain a clearer understanding of the differences between the groups and assess whether the treatment had a significant impact on the animals. Our results demonstrated significantly increased levels of LDL-C3 ($p<.0011$) and LDL-H ($p<.0037$) in the treatment group compared to the control group, while no significant changes were detected in the other enzyme levels.



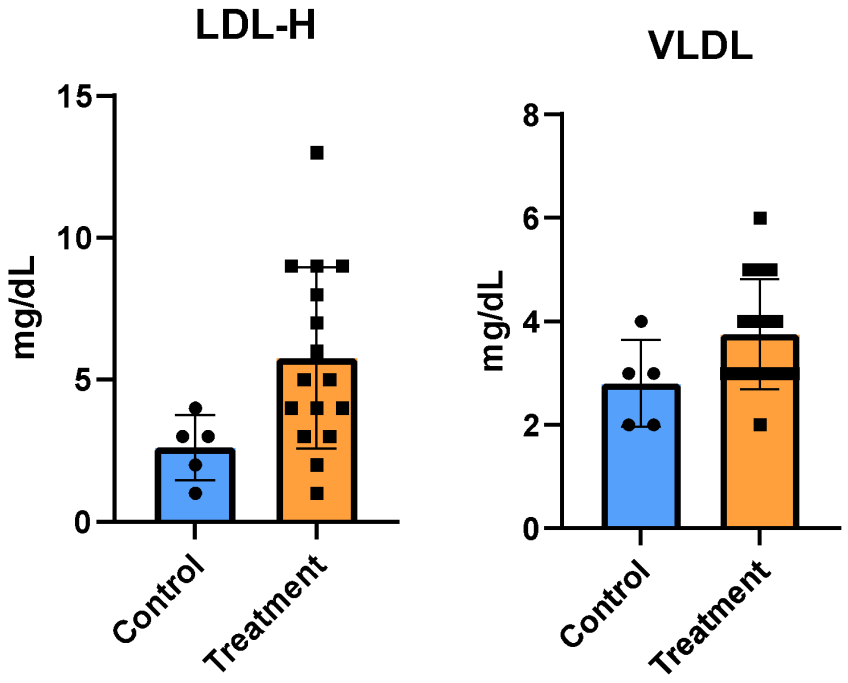


FIGURE 9: Comparison of liver enzyme analysis results between the control and treatment groups at the end of the experiment (Week 6). The studied enzymes were ALT, AST, LDL C3, TRIGL, LDL-H, and VLDL. The x-axis represents the control group and the treatment group, while the y-axis represents the level of enzymes (U/L).

CHAPTER 4:
DISCUSSION

NAFLD is a multisystem disease that also affects other organs in addition to the liver. NAFLD patients have higher risk to develop additional chronic conditions, such as cardiovascular diseases and diabetes (Hadjihambi, 2022). Diabetes, in addition to being linked to the development of NASH, is a disease characterized by high LDL levels. Another important characteristic of diabetes is that it increases the risk of stroke, as well as the severity of its outcomes. Individuals with diabetes have a higher risk of having another stroke, poorer recovery of the neurological outcomes, and greater mortality rates (Lui & Tan, 2020).

Methionine is an essential amino acid and its deficiency causes significant effects on the brain. It is known that S-adenosylmethionine (SAM) participates in the synthesis of neurotransmitters such as dopamine, serotonin, and norepinephrine. As methionine is a precursor to SAM, studies show that low levels of methionine disrupts the production of neurotransmitters and may lead to mood disorders, cognitive impairments, and other neurological problems (Tang et al., 2022; Mou et al., 2024). In addition to its function in neurotransmitter synthesis, methionine also participates in the antioxidant system.

Studies show a number of relationships between liver enzyme levels and brain function (Seo et al., 2016; Nho et al., 2019). Results of our biochemical assays revealed a significant increase in LDL-H and LDL-C3 levels. Increased levels of LDL-H is one of the indicators of dyslipidemia, which is strongly associated with NAFLD. Dyslipidemia is one of the hallmarks of metabolic syndrome (MetS) along with obesity and insulin resistance (Zhang & Lu, 2015). On the other hand, decreased activity levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and glutamyl transferase (GGT) signal oxidative stress. Low levels of these antioxidant enzymes lead to the tissue cells, specifically parenchymal cells in the liver and renal cells in the kidney, to be open to attacks by the products of oxidative stress (Song et al., 2021). However, our study did not reveal a significant change in ALT and AST levels, which could be attributed to the mice being in the early stages of NASH, where significant alterations in enzyme levels may not yet be present. Alternatively, it is possible that the mice had already reached the desired stage of

NASH, but the liver enzyme levels did not reflect this, as there is not always a direct correlation between enzyme elevation and NAFLD progression, particularly in cases such as cryptogenic or silent cirrhosis (Kumar et al., 2019). This underscores the complexity of NAFLD diagnosis, where liver enzyme levels alone may not provide a comprehensive indication of disease severity.

The main finding of our experiment was the difference in CD13 expression in animals that were exposed to the MCD diet. CD13 is one of the markers for pericytes. As stated in a study by Şekerdağ-Kılıç et al. (2023), pericytes have the ability to detach from the microcirculation and can migrate, or proliferate into various phenotypes within the perivascular area (Şekerdağ-Kılıç et al., 2023). When exposed to neurotransmitters, neuro-hormones, or inflammatory mediators, they can transform into myofibroblasts, macrophages, fibroblasts, smooth muscle cells, microglia, and stromal cells. Myofibroblasts are activated fibroblast-like cells, expressing αSMA, and pericytes are thought to contribute to fibrosis by differentiating into myofibroblasts and leading to increased extracellular matrix (ECM) components production. In different disease models, including spinal cord injury, dermal scarring, and liver, lung, and kidney fibrosis, pericytes transdifferentiate into fibrogenic phenotypes and produce collagen at the site of injury (Şekerdağ-Kılıç et al., 2023).

In the immunofluorescence staining of our brain sections **(Fig. 7)** , a gradual increase of αSMA was observed from the 1st week to the 5th week. The leakage can be explained by the damage to the integrity of the vessels in that area. There are various reasons and pathways that could explain the reason behind this damage. The integrity of the BBB is maintained by endothelial cells, pericytes, astrocytes, microglia, tight junctions, and the extracellular basement membrane. These components create a complex and dynamic structure, meaning that BBB impairment involves multiple elements (Sun et al., 2021). Pericytes play a role in regulating capillary structure and diameter (Şekerdağ-Kılıç et al., 2023). Pericyte coverage and quantity are linked to the permeability of BBB; reduced coverage increases permeability (Şekerdağ-Kılıç et al., 2023), leading to BBB dysfunction and the accumulation of neurotoxic molecules (Sun et al., 2021). These molecules can then access the brain parenchyma through endothelial

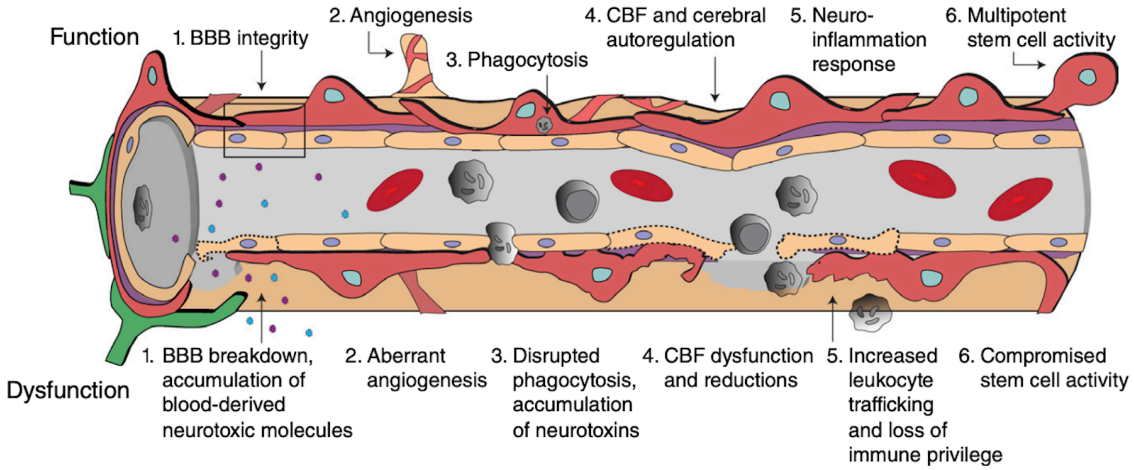


FIGURE 10 Role of pericytes in maintaining blood-brain barrier integrity and the consequences of pericyte dysfunction. The top row shows the functions of pericyte under physiological conditions, and the bottom row shows the result of pericyte dysfunction (Sweeney, 2016).

endocytosis. This dysfunction of the BBB initiates neuroinflammation and other pathological processes (Sun et al., 2021). Recent research has highlighted the crucial role of pericytes in maintaining the integrity of the BBB and how their loss or dysfunction can contribute to neurological disorders. Sweeney et al. (2016) stated that the loss or dysfunction of pericytes can result in the breakdown of the BBB, leading to increased permeability and the accumulation of neurotoxic molecules in the brain, which contributes to neurodegenerative conditions such as Alzheimer's disease (Sweeney et al., 2016). Similarly, Montagne et al. (2020) found that pericyte loss in patients with mild cognitive impairment was directly linked to BBB disruption, supporting the role of pericyte deficiency in the development and progression of neurodegenerative diseases (Montagne et al., 2020). This research supports the idea that pericyte deficiency plays a significant role in the pathogenesis of neurodegenerative diseases, linking vascular health to cognitive decline.

Pericytes express several antigen markers, including PDGFR-β, neural/glial antigen 2 (NG2), α-SMA, desmin, and aminopeptidase N (CD13). However, these markers are not exclusive to pericytes and are also found in other cell types, such as oligodendrocyte precursor cells and vascular smooth muscle cells (VSMCs). To accurately identify pericytes, their markers are often co-localized with vascular markers

like fluorescently conjugated lectins (e.g., FITC-tomato lectin) or endothelial cell markers (e.g., CD31) to confirm their proximity to capillaries (Brown et al., 2019). In our study, an increasing trend was observed in PDGFβ-1 levels, but it did not reach statistical significance. This is thought to be related to the increase in the number of pericytes. The increase in perivascular pericytes may be leading to vascular fibrosis. Although it did not reach statistical significance in the MCD diet, the trend of increasing col4a1 levels supports this.

Moreover, in animals subjected to the MCD diet, an increase in MMP-9 levels was observed in the second week, while a decrease in collagen and fibronectin was noted. The second week was identified as the peak period for BBB breakdown. It was followed by elevated levels of collagen 4 and fibronectin, which could be the result of increased perivascular pericyte activation and fibrosis. MMP-9 is important in neurology since it can be activated via interaction with the immune system, affects BBB function, regulates glutamate receptors, triggers glial cell activation, and influences synaptic plasticity. Elevated levels of MMP-9 have also been linked to increased morbidity and mortality rates in cardiopulmonary, vascular, and neoplastic disorders (Dickerson et al., 2023). The second week was identified as the peak period for BBB breakdown, followed by increased perivascular pericyte activation and fibrosis, along with elevated levels of collagen 4 and fibronectin. Additionally, fibrotic scars result from the abnormal accumulation of extracellular matrix (ECM) rich in Collagen I (COL1). Yang et. al. suggested that human perivascular fibroblasts are the primary producers of COL1 in the brain, indicating they likely play a key role in forming fibrotic scars following brain injury (Yang et al., 2022). The basement membrane primarily consists of four key proteins: collagen IV, laminin, nidogen, and perlecan (Knox et al., 2022). Collagens are the most abundant proteins in the extracellular matrix (ECM), providing mechanical strength and facilitating cellular signal transmission. Their tensile strength is determined by the cross-link density of collagen fibers. During tissue development, complex mechanisms create networks of collagen fibrils, each with unique structures and properties. Disruption in these processes can lead to mechanical damage and, in some cases, fibrosis. Collagen IV, in particular, is crucial for the basement membrane's role in maintaining structural integrity and regulating the exchange of substances between epithelial cells and their surroundings (Warzecha et al., 2022). Nonetheless, our results are not sufficient to draw precise conclusions.

When CCL4 and the MCD diet were compared, it was observed that the MCD diet had a greater impact on the BBB and perivascular space, while no significant findings were detected with CCL4. The reason behind this could be explained by the PCR results. The liver enzyme data was obtained from the PCR by analyzing the enzymes ALT, AST, LDL C3, TRIGL, LDL-H, and VLDL. The results revealed a significant change only in LDL C3 and LDL-H levels, whereas the other enzymes did not reach a statistically significant difference. Thus, we suggest that the lack of significant findings for CCl4 might be due to the incomplete development of the CCL4 model.

Distinctive Features of the Study

Although there are studies in the literature examining the effects of fatty liver diseases on the brain, very few focus specifically on their impact on cerebral vessels. This study aims to investigate the effects of fatty liver diseases on cerebral vessels and contribute to the literature as a resource for researching neurological and cognitive impairments, as well as related diseases (AD, PD, etc.), that result from damage to cerebral vessels caused by fatty liver diseases. Most of the existing research on this subject is specifically focused on discovering the relationship between liver pathologies and Alzheimer's disease (Kim et al., 2016; Bassendine et al., 2020; Estrada et al., 2019). Another notable feature of this study is the use of two different models (MCD diet and CCl4 injection models) to induce non-alcoholic fatty liver disease in animals. By using two different models to achieve the same outcome, it is hoped to provide researchers with guidance in selecting the model for their future studies. Additionally, in this study, the effects of NASH on cerebral vessels were examined both histochemically and biochemically. This approach enabled us to analyze the results of our experiments from a broader perspective.

Limitations

We aimed to compare two different models to induce NASH in our experimental animals; however, we were not able to detect significant changes in the CCl4 injection model. This led us to the idea that we might not have successfully established the CCl4 model. Thus, replication of our study is needed in order to test the reliability of our results and draw more accurate conclusions. Additionally, in future studies, it would be

beneficial to increase the sample size in order to enhance the statistical power of the study.

Furthermore, the length of the current study, which is 6 weeks, might be considered as a limitation in some aspects. The MCD diet can induce hepatic steatosis, inflammation, and early-stage fibrosis in rodents within a few weeks; thus, a 6-week duration typically sufficient to model early to intermediate NASH. Similarly, CCl_4 injections are capable of causing liver fibrosis in a similar timeframe. However, studying NASH's effects on other organs, such as the brain, might require longer periods to be able to observe more chronic impacts. While a 6-week study can identify acute and early molecular changes in brain vasculature, including elevated CD13 and KDR expression, long-term effects, such as neurovascular remodeling and potential neurodegeneration, may need longer observation. Long-term studies could better reveal the full extent of NASH's effects on the blood-brain barrier (BBB) and overall neurovascular health.

CHAPTER 5:
CONCLUSIONS

This study explored the impact of non-alcoholic steatohepatitis (NASH) on cerebral vessels using two distinct models: the methionine- and choline-deficient (MCD) diet and CCl_4 injections. Our findings indicated significant alterations in markers associated with vascular health, particularly in animals subjected to the MCD diet. Elevated levels of LDL-H and LDL-C3 and changes in pericyte markers, such as CD13, suggest that NASH contributes to blood-brain barrier (BBB) dysfunction and subsequent neurovascular complications. However, the lack of significant changes in liver enzyme levels like ALT and AST highlights the complexity of NAFLD diagnosis, indicating that enzyme levels alone may not fully capture the disease's progression.

The study also emphasized the role of pericytes in maintaining BBB integrity, with pericyte loss leading to increased permeability and neuroinflammation. The observed elevation in MMP-9 levels, alongside changes in collagen and fibronectin, further supports the connection between NASH and vascular fibrosis, potentially contributing to the development of neurological disorders. Although our results did not reveal significant effects of the CCl_4 model, this finding underscores the need for further research to optimize NASH models for studying its impacts on brain vasculature.

In summary, our research provides insight into the effects of NASH on cerebral vessels, emphasizing the importance of studying vascular health in the context of liver disease. Future studies with a larger sample size, longer duration, and refined models are necessary to fully elucidate the mechanisms linking NASH to neurovascular dysfunction and to identify potential therapeutic targets.

BIBLIOGRAPHY

Abu Ahmad, N., Raizman, M., Weizmann, N., Wasek, B., Arning, E., Bottiglieri, T., Tirosh, O., & Troen, A. M. (2019). Betaine attenuates pathology by stimulating lipid oxidation in liver and regulating phospholipid metabolism in brain of methionine‐choline–deficient rats. The FASEB Journal, 33(8), 9334–9349. https://doi.org/10.1096/fj.201802683r

Acharya, P., Chouhan, K., Weiskirchen, S., & Weiskirchen, R. (2021). Cellular mechanisms of liver fibrosis. Frontiers in Pharmacology, 12. https://doi.org/10.3389/fphar.2021.671640

Aissa, A. F., Tryndyak, V. P., de Conti, A., Rita Thomazela Machado, A., Tuttis, K., da Silva Machado, C., Hernandes, L. C., Wellington da Silva Santos, P., Mara Serpeloni, J., P Pogribny, I., & Antunes, L. M. (2022). Epigenetic changes induced in mice liver by methionine-supplemented and methionine-deficient diets. Food and Chemical Toxicology, 163, 112938. https://doi.org/10.1016/j.fct.2022.112938

Alam, M. F. (2018). Neuroprotective effects of zingerone against carbon tetrachloride (CCL4) induced brain mitochondrial toxicity in Swiss albino mice. Journal of Applied and Natural Science, 10(2), 548–552. https://doi.org/10.31018/jans.v10i2.1734

Allison, D. J., & Ditor, D. S. (2014). The common inflammatory etiology of depression and cognitive impairment: A therapeutic target. Journal of Neuroinflammation, 11(1). https://doi.org/10.1186/s12974-014-0151-1

Altinoz, E., Erdemli, M., Gul, M., Aksungur, Z., Gul, S., Bag, H., Kaya, G., & Turkoz, Y. (2018). Neuroprotection against CCL4 induced brain damage with crocin in wistar rats. Biotechnic & Histochemistry, 93(8), 623–631. https://doi.org/10.1080/10520295.2018.1519725

Angelini, G., Panunzi, S., Castagneto-Gissey, L., Pellicanò, F., De Gaetano, A., Pompili, M., Riccardi, L., Garcovich, M., Raffaelli, M., Ciccoritti, L., Verrastro, O., Russo, M. F., Vecchio, F. M., Casella, G., Casella-Mariolo, J., Papa, L., Marini, P. L., Rubino, F., le Roux, C. W., … Mingrone, G. (2022). Accurate liquid biopsy for the diagnosis of non-alcoholic steatohepatitis and liver fibrosis. *Gut*, 72(2), 392–403. https://doi.org/10.1136/gutjnl-2022-327498

Aoyama T, Peters JM, Iritani N, Nakajima T, Furihata K, Hashimoto T, et al. Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha). J Biol Chem. 1998 Apr 3;273(10):5678-84

Arkan, M. C., Hevener, A. L., Greten, F. R., Maeda, S., Li, Z. W., Long, J. M., ... & Karin, M. (2005). IKK-β links inflammation to obesity-induced insulin resistance. Nature Medicine, 11(2), 191-198.

Arrese, M., Cabrera, D., Kalergis, A. M., & Feldstein, A. E. (2014). Innate immunity and inflammation in NAFLD/NASH. Digestive Diseases and Sciences, 59(6), 1303-1313.

Arthur, M. J. (2000). Reversibility of liver fibrosis and cirrhosis following treatment for hepatitis C. Gastroenterology, 118(2), 306-308.

Bahitham, W., Alghamdi, S., Omer, I., Alsudais, A., Hakeem, I., Alghamdi, A., Abualnaja, R., Sanai, F. M., Rosado, A. S., & Sergi, C. M. (2024). Double trouble: How microbiome dysbiosis and mitochondrial dysfunction drive non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Biomedicines, 12(3), 550. https://doi.org/10.3390/biomedicines12030550

Bassendine, M. F., Taylor-Robinson, S. D., Fertleman, M., Khan, M., & Neely, D. (2020). Is Alzheimer's disease a liver disease of the brain? Journal of Alzheimer’s Disease, 75(1), 1–14. https://doi.org/10.3233/jad-190848

Bonney, S. K., Sullivan, L. T., Cherry, T. J., Daneman, R., & Shih, A. Y. (2021). Distinct features of brain perivascular fibroblasts and mural cells revealed by in vivo two-photon imaging. Journal of Cerebral Blood Flow & Metabolism, 42(6), 966–978. https://doi.org/10.1177/0271678x211068528

Brown, L. S., Foster, C. G., Courtney, J.-M., King, N. E., Howells, D. W., & Sutherland, B. A. (2019). Pericytes and neurovascular function in the healthy and diseased brain. Frontiers in Cellular Neuroscience, 13. https://doi.org/10.3389/fncel.2019.00282

Brunt, E. M., Wong, V. W., Nobili, V., Day, C. P., Sookoian, S., Maher, J. J., ... & Rinella, M. E. (2015). Nonalcoholic fatty liver disease. Nature Reviews Disease Primers, 1, 15080.

Casso, F. M. J., & Farzam, K. (2022, December 4). Biochemistry, very low density lipoprotein. StatPearls [Internet]. https://www.ncbi.nlm.nih.gov/books/NBK587400/

Chalasani, N., Younossi, Z., Lavine, J. E., Charlton, M., Cusi, K., Rinella, M., ... & Sanyal, A. J. (2018). The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology, 67(1), 328-357.

Colognesi, M., Gabbia, D., & De Martin, S. (2020). Depression and cognitive impairment—extrahepatic manifestations of NAFLD and Nash. Biomedicines, 8(7), 229. https://doi.org/10.3390/biomedicines8070229

Cusi K. Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. Gastroenterology. 2012 May;142(4):711-25.e6.

Dickerson, F., Vaidya, D., Liu, Y., & Yolken, R. (2023). Levels of matrix metalloproteinase 9 are elevated in persons with schizophrenia or bipolar disorder: The role of modifiable factors. Biological Psychiatry Global Open Science, 3(4), 766–772. https://doi.org/10.1016/j.bpsgos.2023.02.007

D'Mello, C., & Swain, M. G. (2014). Liver–brain interactions in inflammatory liver diseases: Implications for fatigue and mood disorders. Brain, Behavior, and Immunity, 35, 9–20. https://doi.org/10.1016/j.bbi.2013.10.009

Dooley, S., & Dijke, P. (2012). TGF-β in progression of liver disease. Cell and Tissue Research, 347(1), 245-256.

Dorrier, C. E., Jones, H. E., Pintarić, L., Siegenthaler, J. A., & Daneman, R. (2021). Emerging roles for CNS fibroblasts in health, injury and disease. Nature Reviews Neuroscience, 23(1), 23–34. https://doi.org/10.1038/s41583-021-00525-w

Eltanahy, A. M., Koluib, Y. A., & Gonzales, A. (2021). Pericytes: Intrinsic transportation engineers of the CNS microcirculation. Frontiers in Physiology, 12. https://doi.org/10.3389/fphys.2021.719701

Estevao, C., Bowers, C. E., Luo, D., Sarker, M., Hoeh, A. E., Frudd, K., Turowski, P., & Greenwood, J. (2021). CCL4 induces inflammatory signalling and barrier disruption in the neurovascular endothelium. Brain, Behavior, & Immunity - Health, 18, 100370. https://doi.org/10.1016/j.bbih.2021.100370

Estrada, L. D., Ahumada, P., Cabrera, D., & Arab, J. P. (2019). Liver dysfunction as a novel player in Alzheimer's progression: Looking outside the brain. Frontiers in Aging Neuroscience, 11. https://doi.org/10.3389/fnagi.2019.00174

Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology. 2010 Jun;51(2):679-89.

Frank, R. T., Aboody, K. S., & Najbauer, J. (2011). Strategies for enhancing antibody delivery to the brain. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1816(2), 191–198. https://doi.org/10.1016/j.bbcan.2011.07.002

Friedman, S. L. (2008). Mechanisms of hepatic fibrogenesis. Gastroenterology, 134(6), 1655–1669. https://doi.org/10.1053/j.gastro.2008.03.003

Friedman, S. L., Neuschwander-Tetri, B. A., Rinella, M., & Sanyal, A. J. (2018). Mechanisms of NAFLD development and therapeutic strategies. *Nature Medicine*, 24(7), 908–922. https://doi.org/10.1038/s41591-018-0104-9

Friedman, S. L. (2008). Hepatic stellate cells: Protean, multifunctional, and enigmatic cells of the liver. Physiological Reviews, 88(1), 125-172.

Friedman, S. L. (2010). Evolving challenges in hepatic fibrosis. Nature Reviews Gastroenterology & Hepatology, 7(8), 425-436.

Friedman, S. L., Ratziu, V., Harrison, S. A., Abdelmalek, M. F., Aithal, G. P., Caballeria, J., ... & Newsome, P. N. (2021). A randomized, placebo-controlled trial of cenicriviroc for treatment of nonalcoholic steatohepatitis with fibrosis. Hepatology, 67(5), 1754-1767.

Gong J, Tu W, Liu J, Tian D. Hepatocytes: A key role in liver inflammation. Front Immunol. 2023 Jan 18;13:1083780. https://doi.org/10.3389/fimmu.2022.1083780

Guo, X., & Zhao, Z. (2022). Vascular inflammation in the central nervous system. Neural Regeneration Research, 17(8), 1728. https://doi.org/10.4103/1673-5374.332140

Gürsoy-Özdemir, Y., & Tas, Y. C. (2017). Anatomy and physiology of the blood–brain barrier. Nanotechnology Methods for Neurological Diseases and Brain Tumors, 3–13. https://doi.org/10.1016/b978-0-12-803796-6.00001-0

Hadjihambi, A. (2022). Cerebrovascular alterations in NAFLD: Is it increasing our risk of Alzheimer's disease? Analytical Biochemistry, 636, 114387. https://doi.org/10.1016/j.ab.2021.114387

Harrison, S. A., Ratziu, V., Boursier, J., Francque, S., Bedossa, P., Majd, Z., ... & Razavi, H. (2020). A randomized, placebo-controlled trial of emricasan in patients with NASH and F1-F3 fibrosis. Journal of Hepatology, 72(5), 816-827.

Hashimoto, E., Taniai, M., & Tokushige, K. (2013). Characteristics and diagnosis of NAFLD/NASH. Journal of Gastroenterology and Hepatology, 28(S4), 64–70. https://doi.org/10.1111/jgh.12271

Hernandez-Gea, V. & Friedman, S. L. (2011). Pathogenesis of liver fibrosis. Annual Review of Pathology: Mechanisms of Disease, 6(1), 425–456. https://doi.org/10.1146/annurev-pathol-011110-130246

Huby, T., & Gautier, E. L. (2021). Immune cell-mediated features of non-alcoholic steatohepatitis. Nature Reviews Immunology, 22(7), 429–443. https://doi.org/10.1038/s41577-021-00639-3

Johannesen, C. D., Mortensen, M. B., Langsted, A., & Nordestgaard, B. G. (2022). ApoB and Non-HDL Cholesterol Versus LDL Cholesterol for Ischemic Stroke Risk. Annals of Neurology, 92(3), 379–389. https://doi.org/10.1002/ana.26425

Kim, J. W., Lee, Y.-S., Park, Y. S., Kim, B.-H., Lee, S. Y., Yeon, J. E., & Lee, C. H. (2020). Multiparametric MR index for the diagnosis of non-alcoholic steatohepatitis in patients with non-alcoholic fatty liver disease. *Scientific Reports*, 10(1). https://doi.org/10.1038/s41598-020-59601-3

Kim, D.-G., Krenz, A., Toussaint, L. E., Maurer, K. J., Robinson, S.-A., Yan, A., Torres, L., & Bynoe, M. S. (2016). Non-alcoholic fatty liver disease induces signs of Alzheimer's disease (AD) in wild-type mice and accelerates pathological signs of AD in an ad model. Journal of Neuroinflammation, 13(1). https://doi.org/10.1186/s12974-015-0467-5

Kisseleva, T. & Brenner, D. (2020). Molecular and cellular mechanisms of liver fibrosis and its regression. Nature Reviews Gastroenterology & Hepatology, 18(3), 151–166. https://doi.org/10.1038/s41575-020-00372-7

Knox, E. G., Aburto, M. R., Clarke, G., Cryan, J. F., & O'Driscoll, C. M. (2022). The blood-brain barrier in aging and neurodegeneration. Molecular Psychiatry, 27(6), 2659–2673. https://doi.org/10.1038/s41380-022-01511-z

Konsman, J. P. (2022). Cytokines in the brain and neuroinflammation: We didn't starve the fire! Pharmaceuticals, 15(2), 140. https://doi.org/10.3390/ph15020140

Kumar, R., Priyadarshi, R. N., & Anand, U. (2019). Non-alcoholic fatty liver disease: Growing burden, adverse outcomes and Associations. Journal of Clinical and Translational Hepatology, X(X), 1–11. https://doi.org/10.14218/jcth.2019.00051

Łach, A., Wnuk, A., & Wójtowicz, A. K. (2023). Experimental models to study the functions of the blood–brain barrier. Bioengineering, 10(5), 519. https://doi.org/10.3390/bioengineering10050519

Li, H., Toth, E., & Cherrington, N. J. (2017). Asking the right questions with animal models: Methionine- and choline-deficient model in predicting adverse drug reactions in human Nash. Toxicological Sciences, 161(1), 23–33. https://doi.org/10.1093/toxsci/kfx253

Lizardi-Cervera, J., & Aguilar-Zapata, D. (2009). Nonalcoholic fatty liver disease and its association with cardiovascular disease. Annals of Hepatology, 8. https://doi.org/10.1016/s1665-2681(19)31825-3

Lui, D. T., & Tan, K. C. (2020). Low‐density lipoprotein cholesterol and stroke: How low should we go? Journal of Diabetes Investigation, 11(6), 1379–1381. https://doi.org/10.1093/toxsci/kfx253

Manautou, J.E., Campion, S.N., Aleksunes, L.M. (2010). Regulation of hepatobiliary transporters during liver injury. Comprehensive Toxicology, 9(2), 175-220. Doi: 10.1016/B978-0-08-046884-6.01008-3.

Mato, J.M., Corrales, F.J., Lu, S.C., and Avila, M.A. S-adenosylmethionine: a control switch that regulates liver function. FASEB J. 2002 Aug;16(10):15.

Montagne, A., Nation, D. A., Sagare, A. P., Barisano, G., Sweeney, M. D., Chakhoyan, A., Pachicano, M., Joe, E., Nelson, A. R., D'Orazio, L. M., Buennagel, D. P., Harrington, M. G., Benzinger, T. L., Fagan, A. M., Ringman, J. M., Schneider, L. S., Morris, J. C., Reiman, E. M., Caselli, R. J., … Zlokovic, B. V. (2020). APOE4 leads to blood–brain barrier dysfunction predicting cognitive decline. Nature, 581(7806), 71–76. https://doi.org/10.1038/s41586-020-2247-3

Muoio, V., Persson, P. B., & Sendeski, M. M. (2014). The neurovascular unit - concept review. Acta Physiologica, 210(4), 790–798. https://doi.org/10.1111/apha.12250

NCI Staff. (2022, February 4). Study identifies target to prevent some liver cancers. National Cancer Institute. https://www.cancer.gov/news-events/cancer-currents-blog/2022/spectrin-fatty-liver-disease-liver-cancer

Nho, K., Kueider-Paisley, A., Ahmad, S., MahmoudianDehkordi, S., Arnold, M., Risacher, S. L., Louie, G., Blach, C., Baillie, R., Han, X., Kastenmüller, G., Trojanowski, J. Q., Shaw, L. M., Weiner, M. W., Doraiswamy, P. M., van Duijn, C., Saykin, A. J., & Kaddurah-Daouk, R. (2019). Association of altered liver enzymes with alzheimer disease diagnosis, cognition, neuroimaging measures, and cerebrospinal fluid biomarkers. JAMA Network Open, 2(7). https://doi.org/10.1001/jamanetworkopen.2019.7978

Parola, M. & Pinzani, M. (2019). Liver fibrosis: Pathophysiology, pathogenetic targets and clinical issues. Molecular Aspects of Medicine, 65, 37–55. https://doi.org/10.1016/j.mam.2018.09.002

Patabendige, A., & Janigro, D. (2023). The role of the blood–brain barrier during neurological disease and Infection. Biochemical Society Transactions, 51(2), 613–626. https://doi.org/10.1042/bst20220830

Promrat, K., Kleiner, D. E., Niemeier, H. M., Jackvony, E., Kearns, M., Wands, J. R., & Fava, J. L. (2010). Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. Hepatology, 51(1), 121-129.

Ratziu, V., Sheikh, M. Y., Sanyal, A. J., Lim, J. K., Conjeevaram, H., Chalasani, N., ... & Younossi, Z. (2016). A phase 2, randomized, double-blind, placebo-controlled study of GS-9450 in subjects with nonalcoholic steatohepatitis. Hepatology, 55(2), 419-428.

Recknagel, R. O., Glende, E. A., & Dolak, J. A. (1989). Mechanisms of carbon tetrachloride toxicity. Pharmacology & Therapeutics, 43(1), 139-154.

Rinella, M. E. (2015). Nonalcoholic fatty liver disease: a systematic review. *JAMA*, *313*(22), 2263. https://doi.org/10.1001/jama.2015.5370

Sanyal, A. J., Chalasani, N., Kowdley, K. V., McCullough, A., Diehl, A. M., Bass, N. M., ... & Lavine, J. E. (2010). Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. New England Journal of Medicine, 362(18), 1675-1685

Schuster, S., Cabrera, D., Arrese, M., & Feldstein, A. E. (2018a). Triggering and resolution of inflammation in NASH. Nature Reviews Gastroenterology & Hepatology, 15(6), 349–364. https://doi.org/10.1038/s41575-018-0009-6

Séité, S., Mourier, A., Camougrand, N., Salin, B., Figueiredo-Silva, A. C., Fontagné-Dicharry, S., Panserat, S., & Seiliez, I. (2018). Dietary methionine deficiency affects oxidative status, mitochondrial integrity and mitophagy in the

liver of rainbow trout (Oncorhynchus mykiss). Scientific Reports, 8(1). https://doi.org/10.1038/s41598-018-28559-8

Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. Hepatology. 2015 Jun;61(3):1066-79.

Seo, S. W., Gottesman, R. F., Clark, J. M., Hernaez, R., Chang, Y., Kim, C., Ha, K. H., Guallar, E., & Lazo, M. (2016). Nonalcoholic fatty liver disease is associated with cognitive function in adults. Neurology, 86(12), 1136–1142. https://doi.org/10.1212/wnl.0000000000002498

Sharma, A., Albhaisi, S., & Sanyal, A. J. (2022). Behavioral health disorders related to nonalcoholic steatohepatitis. Clinical Liver Disease, 20(2), 43–47. https://doi.org/10.1002/cld.1211

Shea, S., Lionis, C., Kite, C., Lagojda, L., Uthman, O. A., Dallaway, A., Atkinson, L., Chaggar, S. S., Randeva, H. S., & Kyrou, I. (2024). Non-alcoholic fatty liver disease and coexisting depression, anxiety and/or stress in adults: A systematic review and meta-analysis. Frontiers in Endocrinology, 15. https://doi.org/10.3389/fendo.2024.1357664

Sipes, I. G., Gandolfi, A. J., Popp, J. A., & Carter, D. E. (1997). Toxicology and biological monitoring of metals in humans (pp. 665-696). CRC Press.

Stower, H. Therapy for NASH. Nat Med 27, 21 (2021). https://doi.org/10.1038/s41591-020-01219-z

Song, B., Fu, M., He, F., Zhao, H., Wang, Y., Nie, Q., & Wu, B. (2021). Methionine deficiency affects liver and kidney health, oxidative stress, and ileum mucosal immunity in broilers. Frontiers in Veterinary Science, 8. https://doi.org/10.3389/fvets.2021.722567

Sosa, M. J., Shih, A. Y., & Bonney, S. K. (2023). The elusive brain perivascular fibroblast: A potential role in vascular stability and homeostasis. Frontiers in Cardiovascular Medicine, 10. https://doi.org/10.3389/fcvm.2023.1283434

Soto-Angona, Ó., Anmella, G., Valdés-Florido, M. J., De Uribe-Viloria, N., Carvalho, A. F., Penninx, B. W., & Berk, M. (2020). Non-alcoholic fatty liver disease (NAFLD) as a neglected metabolic companion of psychiatric disorders: Common pathways and future approaches. BMC Medicine, 18(1). https://doi.org/10.1186/s12916-020-01713-8

Sun, Z., Gao, C., Gao, D., Sun, R., Li, W., Wang, F., Wang, Y., Cao, H., Zhou, G., Zhang, J., & Shang, J. (2021). Reduction in pericyte coverage leads to blood–brain barrier dysfunction via endothelial transcytosis following chronic cerebral hypoperfusion. Fluids and Barriers of the CNS, 18(1). https://doi.org/10.1186/s12987-021-00255-2

Sun, T., Feng, M., Manyande, A., Xiang, H., Xiong, J., & He, Z. (2023). Regulation of mild cognitive impairment associated with liver disease by humoral factors derived from the gastrointestinal tract and MRI research progress: A literature review. Frontiers in Neuroscience, 17. https://doi.org/10.3389/fnins.2023.1206417

Suzuki, A. & Diehl, A. M. (2017). Nonalcoholic steatohepatitis. *Annual Review of Medicine*, 68(1), 85–98. https://doi.org/10.1146/annurev-med-051215-031109

Sweeney, M. D., Ayyadurai, S., & Zlokovic, B. V. (2016). Pericytes of the neurovascular unit: Key functions and signaling pathways. Nature Neuroscience, 19(6), 771–783. https://doi.org/10.1038/nn.4288

Şekerdağ-Kılıç, E., Ulusoy, C., Atak, D., Özkan, E., Gökyüzü, A. B., Seyaj, S., Deniz, G., Uçar, E. A., Budan, A. S., Zeybel, M., Öztop-Çakmak, Ö., Vural, A., Tuncer, A., Karabudak, R., Kücükali, C. I., Tüzün, E., & Gürsoy-Özdemir, Y. (2023). PERIVASCULAR PDGFRB+ cells accompany lesion formation and clinical evolution differentially in two different EAE models. Multiple Sclerosis and Related Disorders, 69, 104428. https://doi.org/10.1016/j.msard.2022.104428

Tilg, H., & Moschen, A. R. (2010). Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis. Hepatology, 52(5), 1836-1846.

Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology. 2010 Apr;52(5):1836-46.

Tang, Y., Chen, X., Chen, Q., Xiao, J., Mi, J., Liu, Q., You, Y., Chen, Y., & Ling, W. (2022). Association of serum methionine metabolites with non-alcoholic fatty liver disease: A cross-sectional study. Nutrition & Metabolism, 19(1). https://doi.org/10.1186/s12986-022-00647-7

Torres, D. M., & Harrison, S. A. (2008). Diagnosis and therapy of nonalcoholic steatohepatitis. Gastroenterology, 134(6), 1682–1698. https://doi.org/10.1053/j.gastro.2008.02.077

Unsal, V., Cicek, M., & Sabancilar, I. (2020). Toxicity of carbon tetrachloride, free radicals and role of Antioxidants. Reviews on Environmental Health, 36(2), 279–295. https://doi.org/10.1515/reveh-2020-0048

Vilar-Gomez, E., Martinez-Perez, Y., Calzadilla-Bertot, L., Torres-Gonzalez, A., Gra-Oramas, B., Gonzalez-Fabian, L., ... & Romero-Gomez, M. (2015). Weight loss through lifestyle modification significantly reduces features of nonalcoholic steatohepatitis. Gastroenterology, 149(2), 367-378.e5.

Vučević, D. B., Cerović, I. B., Mladenović, D. R., Vesković, M. N., Stevanović, I., Jorgačević, B. Z., Vukićević, R. J., & Radosavljević, T. S. (2016). Methionine-choline deprivation alters liver and brain acetylcholinesterase activity in c57bl6 mice. General Physiology and Biophysics, 35(03), 363–370. https://doi.org/10.4149/gpb_2015052

Wallace, T. C., Blusztajn, J. K., Caudill, M. A., Klatt, K. C., Natker, E., Zeisel, S. H., & Zelman, K. M. (2018). Choline. Nutrition Today, 53(6), 240–253. https://doi.org/10.1097/nt.0000000000000302

Wang, Q., Zang, F., He, C., Zhang, Z., & Xie, C. (2022). Dyslipidemia induced large-scale network connectivity abnormality facilitates cognitive decline in the Alzheimer's disease. Journal of Translational Medicine, 20(1). https://doi.org/10.1186/s12967-022-03786-w

Warzecha, D., Załęcka, J., Mańka, G., Kiecka, M., Lipa, M., Spaczyński, R., Piekarski, P., Banaszewska, B., Jakimiuk, A., Issat, T., Rokita, W., Młodawski, J., Szubert, M., Sieroszewski, P., Raba, G., Szczupak, K., Kluz, T., Kluza, M., Wielgoś, M., … Laudański, P. (2022). Plasma and peritoneal fluid fibronectin and collagen IV levels as potential biomarkers of endometriosis. International Journal of Molecular Sciences, 23(24), 15669. https://doi.org/10.3390/ijms232415669

Wiering, L., & Tacke, F. (2022). Treating inflammation to combat non-alcoholic fatty liver disease. Journal of Endocrinology, 256(1). https://doi.org/10.1530/joe-22-0194

Yan, C., Li, Y., & Wang, W. (2019). An overview of hepatic fibrosis research status in preclinical models. Animal Models and Experimental Medicine, 2(1), 1-10.

Yang, A. C., Vest, R. T., Kern, F., Lee, D. P., Agam, M., Maat, C. A., Losada, P. M., Chen, M. B., Schaum, N., Khoury, N., Toland, A., Calcuttawala, K., Shin, H., Pálovics, R., Shin, A., Wang, E. Y., Luo, J., Gate, D., Schulz-Schaeffer, W. J., …

Wyss-Coray, T. (2022). A human brain vascular Atlas reveals diverse mediators of Alzheimer's risk. Nature, 603(7903), 885–892. https://doi.org/10.1038/s41586-021-04369-3

Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L., & Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease—Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology, 64(1), 73-84.

Zeisel, S. H., & da Costa, K.-A. (2009). Choline: An essential nutrient for public health. Nutrition Reviews, 67(11), 615–623. https://doi.org/10.1111/j.1753-4887.2009.00246.x

Zhang, Q.-Q., & Lu, L.-G. (2015). Nonalcoholic fatty liver disease: Dyslipidemia, risk for cardiovascular complications, and treatment strategy. Journal of Clinical and Translational Hepatology, 3(1), 78–84. https://doi.org/10.14218/jcth.2014.00037

Zhang, G., Wang, X., Chung, T.-Y., Ye, W., Hodge, L., Zhang, L., Chng, K., Xiao, Y.-F., & Wang, Y. (2020). Carbon Tetrachloride (CCL4) Accelerated Development of Non-Alcoholic Fatty Liver Disease (Nafld)/Steatohepatitis (NASH) in MS-Nash Mice Fed Western Diet Supplemented with Fructose (WDF). https://doi.org/10.21203/rs.3.rs-30324/v1

Zlokovic, B. V. (2008b). The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron, 57(2), 178–201. https://doi.org/10.1016/j.neuron.2008.01.003

Zlokovic, B. V. (2008b). The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron, 57(2), 178–201. https://doi.org/10.1016/j.neuron.2008.01.003