# THE INDUCTION OF INTESTINAL INFLAMMATION BY THE METHIONINE- AND CHOLINE-DEFICIENT DIET

A Thesis

by

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# ABSTRACT

Inflammatory digestive diseases, including non-alcoholic fatty liver disease (NAFLD), influence overall health. Substantial evidence supports the methionine- and choline-deficient (MCD) diet inducing non-alcoholic steatohepatitis (NASH) in mice, but little is known about the diet's effect on the intestine, where nutrients first encounter host cells. The abundance of macrophages in the intestine may also influence intestinal inflammation. Therefore, this study seeks to determine the signaling pathways and primary cell types responsible for MCD diet-induced intestinal inflammation well as the mechanisms for inhibiting this inflammation. In the in vivo experiment, wild-type C57BL/6J mice consumed either the chow or MCD diet for four weeks. For all in vitro experiments, cells proliferated in either control or MCD medium and each experimental group was also treated with lipopolysaccharide (LPS) to induce inflammation. In the original in vitro study, cultured intestinal epithelial cells (IECs) from a mouse cell line were treated and harvested. Western blotting revealed elevated levels of inflammation from animal and cell samples. In the final cell study, the macrophages responded most potently to the proinflammatory treatments. The findings elucidated by these studies highlight the intestine as a major contributor to NAFLD and NASH progression and a suitable target for digestive disease prevention.

# DEDICATION

I would like to dedicate this work to my mother, who is currently completing her degree and has always sacrificed her time and resources so that others may succeed. Now it is time for her to enjoy her own accomplishments!

This work is also dedicated to my entire family, who supported me in my academic endeavors, from high school to the completion of my master's degree. No matter the number of miles between us, whether a few hundred or thousands, I have always found a solid support system amongst my loved ones.

Lastly, I dedicate this thesis to my nieces, Laila and Kiana, and my nephew, Sterling. I am undeniably proud of these strong and capable young people. The future belongs to you!

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## Contributors

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# NOMENCLATURE

| | |
|---|---|
| NAFLD | Nonalcoholic Fatty Liver Disease |
| NASH | Nonalcoholic Steatohepatitis |
| MCD | Methionine- & Choline Deficient |
| MONW | Metabolically Obese Normal Weight |
| CETP | Cholesteryl Ester Transfer Protein |
| SREBP2 | Sterol Regulatory Element Binding Protein 2 |
| LPS | Lipopolysaccharide |
| MS | Metabolic Syndrome |
| CVD | Cardiovascular Disease |
| PFKFB3 or iPFK2 | 6-phosphofructo-2-kinase |
| (p)NFkB or (p)P65 | (phosphorylated) Nuclear factor kappa-light-chain-enhancer of Activated B Cells |
| (p)JNK, (p)P46 | (phosphorylated) c-Jun N-terminal Kinase |

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# 1. INTRODUCTION

Because NAFLD is such a complex disease state, it is not possible to associate its symptoms with just any one gene or nutrient. There are many factors ranging from overall digestive health to macronutrient balance within the diet or intensity of systemic inflammation. The existing body of literature has mainly focused upon effects of a high-fat diet on the liver but has not fully described the effects of an alternate proinflammatory diet [1, 2]. The methionine and choline-deficient (MCD) diet has also been examined in relation to initiating fatty liver disease, but its role has not been studied in the intestine, which may cause digestive dysregulation leading to non-alcoholic fatty liver disease (NAFLD) progression [Figure 1.1]. Prior studies have demonstrated that inflammation of the intestine may propel the progression of obesity and its comorbidities[3-6]. Ding et al. established that intestinal epithelial and immune cell types were involved in the progression of inflammation in response to the high fat diet [4]. Examining the intestinal response to the MCD diet is a key component in determining how the inflammatory response varies depending on diet exposure and organ in which it arises. It is possible for intestinal inflammation to also induce insulin resistance, which parallels systemic inflammation and NAFLD. NAFLD consists of varying stages of liver inflammation and injury, and it accounts for the highest prevalence of liver disease throughout Western countries and continues to increase [7-9].

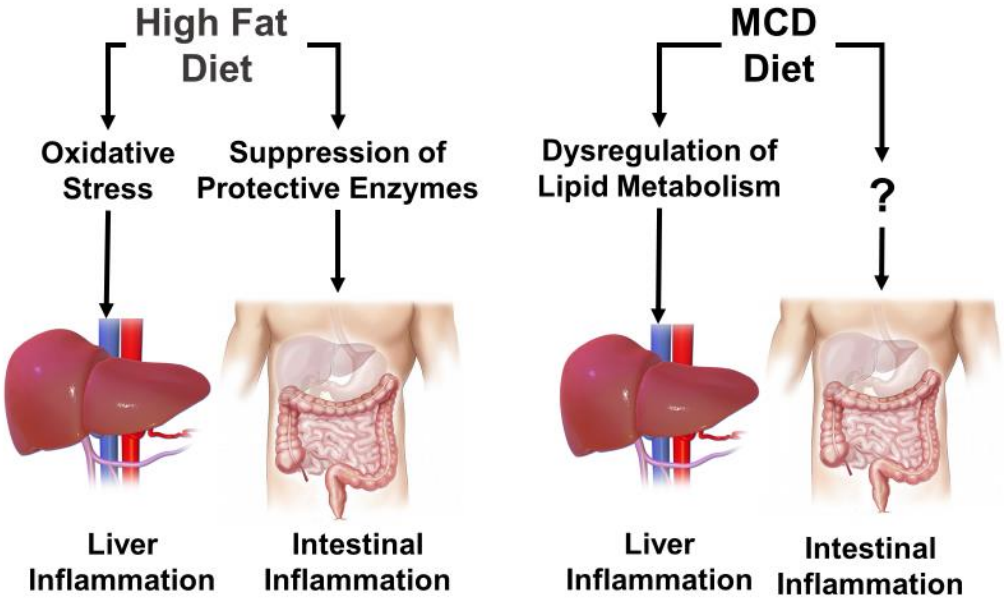


**Figure 1.1 Rationale for Study Aims**

The implications of the high-fat diet for digestive disease, including NAFLD and NASH are well understood. While it is clear that the MCD diet induces inflammation in the liver[8, 10], the presence of inflammation in the intestine has not been well-established and the mechanism by which this inflammation may occur, is yet to be uncovered. This conceptual illustration conveys the rationale for the gap in the literature filled by this research by juxtaposing the mechanisms involving inflammation of the liver and intestine[3, 11]. Images modified from [12, 13].

An animal model that accurately conveys the human disease pathology is crucial to better understanding human liver disease. Because obesity-related fatty liver disease is associated with overnutrition, the high-fat diet has been used to represent a calorically dense, Western-style diet and to cause NAFLD in animal models [1, 9, 14-16]. Mechanistically, diet-induced fat accumulation in the visceral organs results in oxidation, which can lead to cell damage and inflammation [10]. This inflammation and cellular damage to hepatocytes was originally only thought to occur in cases of obesity, inducing insulin resistance in the process. However, NAFLD occurs in the absence of obesity [17, 18], which supports that an animal model portraying only obesity-related NAFLD is not

sufficient. In contrast, the MCD diet often results in weight loss[10] and provides a different mechanism for NAFLD progression: Because methionine and choline serve as the major precursors of phosphatidylcholine, a phospholipid which comprises the cell membrane of lipoproteins, their absence is then associated with impaired lipid transport leading to fat accumulation[8]. The MCD diet fills a role that is greatly overlooked in existing NAFLD research by inducing NAFLD and NASH apart from obesity.

The MCD diet could be used to represent diet-induced inflammation that is observed in the significant proportion of non-obese individuals with NAFLD diagnoses[17, 18]. Current high fat and Western diets in animal models do not translate directly to this lower weight human phenotype. MCD diet-fed mice rapidly lose weight and develop non-alcoholic steatohepatitis (NASH), including the hallmark symptoms of lipid accumulation (which can result from malfunctioning lipoprotein secretion), fibrosis, and severe inflammation [8, 10, 19, 20]. The mechanism by which the MCD diet initiates fatty liver disease results in the expected symptoms associated with NASH, but it is also specific to a non-obese phenotype, which allows us to examine how the diet underlies these symptoms. The application of this diet to NAFLD as it pertains directly to the liver but also to intestinal health provides an opportunity to further understand why the MCD diet and gastrointestinal inflammation may induce systemic consequences.

When critical digestive organs are impacted, the disruption of lipid metabolism due to the calorically dense and MCD diets has widespread consequences in the host. The intestine may be a critical site in which impaired lipid transport affects the rest of the body. The liver and gut are connected by the portal circulation [4], and the small intestine is the first organ to be exposed to the individual nutrients in the diet. One can conclude that both

excessive fat intake and methionine and choline deficiency could impair the necessary transport of nutrients from the intestine to other sites of interest in the body, including the liver. Early signs of inflammation in the small intestine also induce changes within the intestine as well as other visceral organs and body tissues [3, 21]. Since obesity is often the starting point for fatty liver disease onset and MCD diet-fed mice are often underweight and glucose tolerant, the significance of the MCD diet has been called into question. However, given the impact of individual nutrients on intestinal health and the significant influence of the intestinal response on the host, these physiological characteristics in mice may actually be strengths of this disease model. Diet-induced inflammation in the intestine may even partly facilitate the onset of obesity [3].

Therefore, the juxtaposition of the disparate MCD and high-fat diets presents a key opportunity to examine the development of NAFLD. The comparison of the Western and MCD diets allows us to determine whether the inflammation originates in the digestive tract or if liver and intestine inflammation are brought on due to systemic inflammation. NAFLD pathophysiology could possibly be explained by an underlying inflammatory mechanism involving intestinal dysfunction which is initiated by the MCD and high fat diets independent of adiposity. Given the novelty of this hypothesis, our laboratory proposes that the intestine may be the starting point for fatty liver disease.

# 2. LITERATURE REVIEW

## 2.1. Obesity, Systemic Inflammation and Insulin Resistance

Unhealthy nutrition contributes to obesity and various related metabolic diseases, such as diabetes, fatty liver disease, and cardiovascular disease [22]. Obesity, especially in the case of central adiposity, has been linked to metabolic syndrome (MS) which features elevated triglycerides, cholesterol, blood pressure and blood glucose measures, all of which are also related to a higher risk of cardiovascular disease (CVD) and the onset of fatty liver disease [1, 22-24]. Given these consequences, metabolic syndrome is diagnosed using the following measures: waist circumference or waist-to-hip ratio, abnormal lipid profile and elevated blood pressure, and elevated fasting blood glucose parameters [25-27]. The onset of metabolic syndrome is attributed to insulin resistance, altered lipid metabolism, inflammation and oxidative stress [28].

Obesity is characterized by fat accumulation surrounding the visceral organs [29-31]. It is generally defined by the ratio of the height and weight, the body mass index (BMI) score, falling above 30 kg/m2, while a value between 25 and 30 $kg/m^2$ is considered overweight [24]. Additionally, other measures pertaining to fat distribution, including body fat percentage and fat mass index, play a role in the classification of obesity [24]. Accumulation of excessive visceral adipose tissue is associated with many metabolic complications and may directly affect glucose tolerance, lipoprotein metabolism and blood pressure, which the subsequent risk for developing MS following the onset of obesity [24]. Fat accumulation can also lead to inflammation within the adipose tissue and activates endothelial cells and causes fibrosis of blood vessels [32]. These processes contribute to

the development of atherosclerosis and coronary heart disease and provide another basis for the origin of metabolic syndrome in the host.

Adipose tissue induces a proinflammatory state because the cells can generate hormones such as angiotensinogen and cortisol and excessive amounts of inflammatory cytokines including TNF-alpha, IL-6, and IL-1β, which may also lead to MS and CVD[3, 4, 22]. These effects can be compounded as adipocytes also increase in size and number. Existing fat cells can store increasing amounts of lipids as well, and this often results in impairment of cell signaling events [22, 33, 34]. Tissue hypoxia within adipose tissue also results in altered cytokine production[35]. When the limit for fat storage within an adipocyte is reached, the fat cell must hypertrophy, or grow in size. This process is associated with hypoxia and the recruitment of macrophages [22, 36, 37].

In fact, the presence of macrophages may provide the basis for the overall inflammation induced within the fat and play a large role in the pathophysiology of obesity, especially through changes in glucose tolerance[33, 34, 38]. Inflammation resulting from the presence of macrophages occurs before insulin resistance. The accumulation of macrophages also results in liver fibrosis, and cardiovascular abnormalities[22, 32, 39].

Glucose intolerance is another feature of metabolic syndrome[28]. Insulin resistance[3], is often a consequence of poor fat metabolism and subsequently increased levels of inflammation. Two commonly studied pathways involving transcription factors which modulate obesity and insulin resistance include the NF-kB and c-Jun NH2-terminal kinase (JNK) pathways [22]. It is thought that TNF-alpha is elevated in obesity because it stimulates lipolysis and the apoptosis of adipocytes, and interleukin-6 may also initiate

insulin resistance [22]. The vicious cycle of obesity and systemic inflammation is perpetuated due to altered lipid metabolism within the adipose tissue. These symptoms and risk factors are associated with yet another consequence of obesity and insulin resistance: fatty liver disease.

**2.2. Nonalcoholic Fatty Liver Disease**

One third of adults in America have nonalcoholic fatty liver disease (NAFLD), and approximately one out of five NAFLD patients also have non-alcoholic steatohepatitis (NASH) [25]. NAFLD is mostly observed in obese populations with metabolic syndrome and is defined as fat accumulation in the liver with no history of excessive alcohol consumption[1, 23]. It is not merely one disease but actually a spectrum of diseases depending on the presence and severity of steatosis, inflammation and injury, which may also include liver cirrhosis [1, 7, 13, 40].

However, NAFLD does not only occur in overweight and obese individuals [18]. Yousef et al summarized recent international studies of nonobese NAFLD and revealed that NAFLD in normal weight patients is related to metabolic syndrome symptoms but less so compared to obese NAFLD [17]. They concluded that weight change plays an instrumental role in both obese and nonobese NAFLD. When examining NAFLD onset, triglyceride content in the liver is paramount to NAFLD progression. Disease progression may also be instigated by the presence of adipokines released by adipocytes. However, control of blood glucose and blood pressure do not seem to change nonobese NAFLD states. Furthermore, normal-weight NAFLD is unique from obese NAFLD due to the varying genetic predisposition in patients [17].

NAFLD in normal weight patients is associated with less inflammation and fibrosis compared to obese patients [18]. These patients are often considered to be metabolically obese but normal weight (MONW). However, these patients have been found to have a higher mortality than the obese patients with NAFLD. MONW patients may have started with marginal insulin resistance and only slight metabolic changes triggered the onset of their NAFLD. High cholesterol and fructose intake might account for some of these metabolic factors, even independent of caloric intake [18]. Participants were also found to have genetic variations in sequences controlling for the functions of proteins related to lipid metabolism such as CETP and SREBP2. The authors also concluded that weight loss may reverse nonobese NAFLD.

Currently, there are not many recommendations for nonobese NAFLD patients, apart from maintaining physical activity levels [18]. Thus, isolating a mechanism for the onset of non-obese NAFLD is necessary to understand NAFLD prevention in this population. Additionally, researching the effects on the entire digestive system provides a more complete understanding of the outcomes of lifestyle and dietary differences in these patients.

The liver is also intertwined with the immune system. Kupffer cells, which are resident macrophages within the liver, are demonstrated to initiate NASH due to their role in inducing inflammation. A study conducted by Tosello et al. sought to analyze the early effects of the MCD diet and found that macrophages release excessive amounts of TNF-alpha [41]. The same study confirmed that removing the Kupffer cells decreased liver injury, inflammation and fat accumulation. Interference with TNF-alpha expression mitigated liver injury and inflammation as well [41]. As aforementioned, macrophages also

infiltrate adipose tissues. These foundational discoveries allow us to conclude that Kupffer cells initiate NASH through release of TNF-alpha. These findings also substantiate the concept of crosstalk between the macrophages and lipogenesis [41].

### 2.3. Methionine & Choline Deficiency

The MCD diet is highly pro-inflammatory to visceral organs, including the liver and intestine. It is often used to induce a state of NAFLD or NASH, especially in animals prone to diabetes, because the diet altered glucose metabolism [8, 10]. The MCD-diet can also be extremely inflammatory to the liver and will provide another approach for studying markers of inflammation, potentially in other organ systems. Thus far, the diet's effects have not been studied extensively in the intestine [Figure 2.1]. The gut and liver are connected by the portal vein, through which the conferred metabolites impact the functioning of both organs[42], so potential initiation of metabolic changes would be of interest in future studies.

Considering the role of the diet prior to any metabolic abnormalities developing, methionine and choline are vital for normal functioning of the cells. Methionine and choline are major precursors of phosphatidylcholine, an abundant phospholipid found on lipoprotein surfaces. Choline is needed for cell growth and differentiation and composes most of the lipid layer that lines the gut. Another theory proports that the MCD diet limits triglyceride synthesis, so free fatty acids remain unbound in the tissues[8, 10]. S-adenosyl-methionine (SAM) is needed to transport fat from the liver, and its amount is reduced following the MCD diet. Rinella et al. also showed that fatty acid binding protein, which ordinarily binds free fatty acids for removal and storage, is downregulated in MCD-diet fed mice [8, 43]. If fat is allowed to accumulate, fatty acids may oxidize and subsequently

damage cell membranes [10]. Thus, the absence of methionine and choline is associated with impaired lipid transport [8]. The MCD diet may also be implicated in the reduction of bile acids [10]. Intriguingly, the MCD diet is associated with weight loss rather than weight gain [8], which is due to the highly inflammatory internal state of the animals [1]. Until recently, this diet was only examined in relation to liver function. The hypothesis of this study accounts for the effect that methionine and choline deficiency in the intestine may have on the rest of the body.

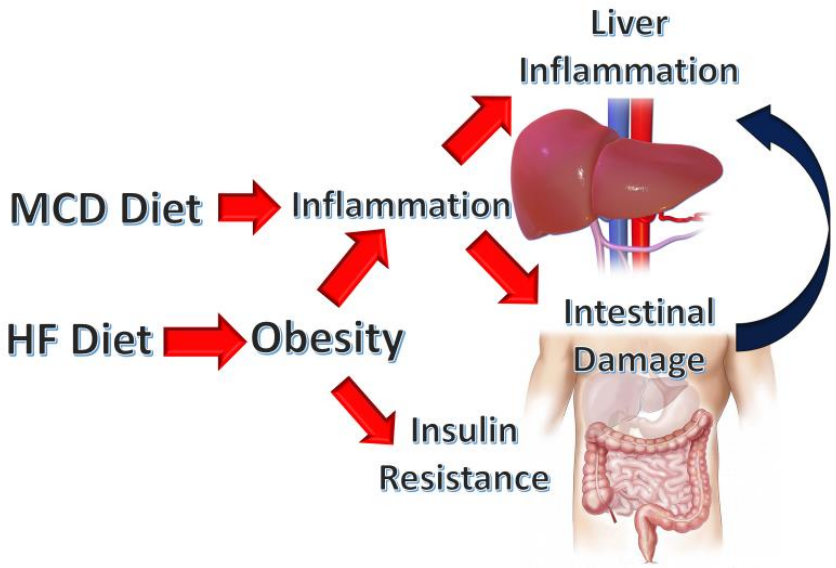


**Figure 2.1. Comparison of the Diet-Induced Mechanisms Affecting Digestive Organs Involved in NAFLD**
The HFD-induced NAFLD and MCD-diet induced fatty liver disease have been presented as two independent mechanisms[3, 4, 8, 11, 44]. The two diets may actually share underlying mechanisms, leading to inflammation in the digestive and accessory organs. Images modified from [12, 13].

### 2.4. Intestinal Inflammation

Every nutrient will enter the digestive system before any other organs in the body. This primacy of the intestine reveals that when individuals consume excess nutrients, the

gastrointestinal tract may also be the first organ system to incur the damages. Recent evidence has shown that inflammatory markers were upregulated in the ilea of obesity-prone rats after high-fat diet feeding; diet-induced inflammation is also associated with dysfunction of the intestinal microbiota (reviewed in Ding, 2011). Likewise, a high-fat diet can lead to increased activation of toll-like receptor 4 (TLR4), which initiates an inflammatory signaling cascade within the cells of the intestine [3]. A highly inflammatory diet, such as a high-fat (HF) diet, impairs normal intestinal function[4, 11]. Because intestinal dysfunction may result in consequential metabolic dysfunction in the host, determining the role of the MCD diet in the pathophysiology of intestinal inflammation and NAFLD is of great importance.

Defining the roles of individual macro- and micronutrients in gastrointestinal inflammation may be the key to understanding NAFLD. The high fat diet been utilized to cause inflammation and also tissue damage and fat accumulation. Since damage to the gastrointestinal tract cannot be isolated from these outcomes, further research must identify if intestinal inflammation can directly and independently trigger consequences such as obesity, insulin resistance and steatohepatitis. Evidence of diet-induced intestinal damage provides the basis for the hypothesis that specific dietary components may reduce intestinal function, which ultimately has detrimental outcomes for digestive health and overall health.

Recent research has demonstrated that the compromising of the gut barrier is linked to the onset of NASH: fructo-oligosaccharides (FOS) have been demonstrated to counteract liver inflammation and gut permeability [45]. Matsumoto et. al. found that microbiota composition will change in response to the MCD diet, and they also revealed

that plasma levels of enzymes associated with liver injury were lower in FOS-fed mice. These mice also possessed healthier livers as determined by histological measures. Histology of the intestine demonstrated that the intestine was longer in FOS-fed mice and MCD-fed mice than in the control [45].

Similarly, diets which are high in sugar and fat generally contribute to hyperphagia, obesity and systemic inflammation [6]. Botchlett et al. confirmed the effects of a high-fat diet on inflammation in the intestine [11]. They examined the role of PFKFB3, an influential gene in glycolysis regulation, which codes for the enzyme, inducible 6-phosphofructo-2-kinase (iPFK2). This enzyme is known to protect against diet-induced inflammation of the intestine [11]. The study demonstrated that, through this mechanism, high-fat diet in mice increased intestinal inflammation compared to a low-fat diet. Higher levels of inflammation were also detected in isolated IECs exposed to a high-glucose environment when compared to a low-glucose environment [Figure 3] [11, 21]. The overall result was a decrease in PFKFB3 expression and an increase in intestinal inflammation following a high-fat diet. However, PFKFB3 expression and inflammation increased in the high-glucose environment. In summation, PFKFB3—and consequently, 6-phosphofructo-2-kinase—responds uniquely to different macronutrients [11]. The gene and its corresponding enzyme illustrate how specific nutrients in the diet cause inflammation in the intestine, altering intestinal function. This study will build on this work, using a different dietary model.

Another study conducted by Guo et al. also emphasized the influential role of PFKFB3 in intestinal inflammation [21]. This study revealed that in wild-type mice, intestine PFKFB3 increased after high-fat diet (HFD) feeding when compared to low-fat

diet (LFD). For mice in whom PFKFB3/iPFK2 regulation was disrupted, intestinal proinflammatory markers were increased [21].

Similarly, Ding et al. discovered that a high-fat Western-style diet interacts with intestinal bacteria to increase inflammation [4]. Following this logic, intestinal inflammation may significantly contribute to insulin resistance and obesity. Intestinal inflammation may also coincide with the actions of cytokines released from adipocytes to result in obesity-related inflammation.

Additionally, recent evidence elucidates that the high-fat diet can promote excessive intake and overnutrition by suppressing the neurons of the intestine which normally communicate satiety to the brain [3, 6, 46, 47]. This supports the intriguing rationale that intestinal inflammation can directly impact obesity, without systemic inflammation and insulin resistance as mediators [Figure 2].

Not unlike the liver, the intestine contains resident macrophages that may be responsible for inducing inflammation [48]. However, resident macrophages are not solely responsible for inflaming the intestinal epithelium; intestinal macrophages are replenished by other monocytes from the circulation [48]. This may be another vantage point from which NAFLD is initiated. The role of the immune system within the intestinal tract provides yet another reason to consider that the inflammation related to the onset of NAFLD could begin in the digestive tract. This discovery would change the way that NAFLD is viewed in the context of digestive disease research.

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# 3. METHODS

This study hypothesized that intestinal inflammation will increase in the absence of methionine and choline. The objectives of this series of studies include the following: 1. Examine the induction of intestinal inflammation in the absence of methionine and choline; 2. Determine methods of reversing intestinal inflammation in the absence of methionine and choline; 3. Describe the inflammatory response of macrophages in a methionine and choline deficient environment.

## 3.1. MCD Diet Feeding in Preparation of Mice Experiments

Wild type male C57BL6/J mice at 6-8 weeks of age were fed a standard chow diet [N=15] (Lab Diets, St. Louis, MO) or methionine and choline deficient diet [N=20] (Research Diets, Inc., New Brunswick, NJ) for four weeks [Table 3.1].

## 3.2. Blood Glucose Tolerance Test in Mice

All mice were fasted for 4 hours prior to the initial intraperitoneal glucose injection at T=0. Blood samples were then collected from the tail veins of the mice every 30 minutes using capillary tubes until the last timepoint, T=120 minutes. Finally, samples were centrifuged at 14,000 revolutions per minute for 5-10 minutes and the plasma was isolated. A Glucose (HK) Assay kit (Sigma, St. Louis, MO) was then used to determine the concentration of glucose remaining in the samples at the various timepoints by spectrophotometry at a wavelength of 340 nm.

**Table 3.1 Comparison of Diet Composition.**

| | CHOW | | MCD | |
|---|---|---|---|---|
| **MACRONUTRIENTS** | **gm%** | **kCal%** | **gm%** | **kCal%** |
| **Protein** | 24.1 | 28.7 | 17 | 16 |
| **Carbohydrates** | 57.6 | 57.9 | 66 | 63 |
| **Fat** | 11.4 | 13.4 | 10 | 21 |
| **kCal/ gram** | *3.36* | | | *4.21* |
| | | | | |
| **INGREDIENTS** | **gm%** | **kCal%** | **gm%** | **kCal%** |
| **Casein** | 20.00 | 20.71 | 17.0 | 16% |
| **L-methionine** | 0.30 | 0.31 | 0 | 0 |
| **Choline Bitrartate** | 2250 ppm* | 0 | 0 | 0 |
| **Corn Starch** | 15 | 15.54 | 15 | 14 |
| **Sucrose** | 50.00 | 51.79 | 45.53 | 42.48 |
| **Maltodextrin** | 0 | 0 | 5 | 5 |
| **Cellulose** | 5 | 0 | 3 | 0 |
| **Corn Oil** | 5 | 11.65 | 10 | 21.19 |

LabDiets**[49]**; *ResearchDiets,Inc.**[50].**

### 3.3. Body Composition Measurement in Mice

A body composition measurement was also performed in the Kleberg Center Animal Facility using the EchoMRI machine to detect fat mass and lean mass percentages in the mice.

### 3.4. Harvesting of Tissue Samples from Mice

During the harvesting process, the liver, adipose tissue, small and large intestines were collected and weighed. The internal organs were harvested from the mice, homogenized in lysis buffer and stored in -80 degrees Celsius conditions. The intestinal samples were then purified and protein concentrations were measured using the BCA kit and the plate reader. Finally, samples were prepared at a consistent protein concentration for the Western blot.

### 3.5. Cell Culture & Treatment

In the MCD study, intestinal epithelial cells from a rectal carcinoma cell line (CMT-93) were used. For the macrophage study, a macrophage cell line (RAW) was used. The cells were originally grown in IMDM glucose medium in petri dishes placed in a humidified incubator with 5% CO2 at 37 degrees Celsius. The medium was exchanged as needed until the cells grew to confluence, which takes 24-48 hours.

The medium was then removed before washing the cells twice in 2 mL of pH 7.4 PBS (4 degrees Celsius) and detaching them with trypsin. The cells were transferred to six-well plates and treated with either complete 1:1 DMEM/Ham's F-12 medium or 1:1 DMEM/Ham's F-12 medium that was manufactured without methionine and choline. Following treatment, the plates were returned to the incubator for 24 hours.

Thirty minutes prior to harvesting, 20 uL of lipopolysaccharide (LPS) (100 ng/mL) was added to one of two plates from each treatment group. Cells were then returned to the incubator for the remaining several minutes, removed, and washed twice with 2 mL of PBS (4 degrees Celsius) and stored on ice. The cells were detached from the plates using cell scrapers and 250 uL of lysis buffer (20 mM Hepes, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% NP-40, 0.1% SDS, 2 mM EDTA, 2 mM Na Vanadate, pH 7.4). The cell lysate was then frozen at -80 degrees Celsius.

### 3.6. Sample Preparation

The cell lysate was thawed within 1-3 days to analyze the protein concentration. The samples were centrifuged for 15 minutes at 14,000 rpm and 4 degrees Celsius in order to separate the protein from the cell debris. The Micro BCA Protein Assay kit (Thermo scientific, USA) was used to create a standard curve and determine the protein concentration. The absorbance of each sample was quantified using a ClarioStar plate reader (Tecan, Switzerland) at a wavelength of 562 nm. After measuring the concentration, samples were prepared at a fixed protein concentration using lysis buffer, loading buffer , and DTT before being heated to 95-100 degrees Celsius for 10 minutes and stored in the freezer at -80 degrees Celsius.

### 3.7. Western Blot Procedure

The protein marker and protein samples were loaded into two 15-well SDS-PAGE gels. The protein within the gel was transferred to nitrocellulose membranes using a semi-dry transfer method in a Trans-Blot machine set to 25 V for 30 minutes. Following the transfer procedure, the membranes were incubated for 2.5 hours in 5%

milk protein in TBS solution to block nonspecific binding. Blocking was followed by brief rinses in TBS three times for 5 minutes each round. After rinsing, membranes were then incubated in a 1:1000 primary antibody (Cell Signaling Technology; Danvers, MA) to TBS solution in 1% BSA. Antibodies for total protein incubated overnight while antibodies for phosphorylated proteins incubated for two nights.

After the incubation period, membranes were again rinsed with TBS for 5-10 minutes three times over and incubated for 2 hours with 1:10,000 goat anti-rabbit secondary antibody (Invitrogen; Rockford, IL) to TBS solution. The membranes were then washed in TBS-T three times for 10 minutes. The protein bands were visualized using a Chemidoc Touch Western Blot imaging system (Bio-Rad Laboratories; Hercules, California) and chemiluminescent reagents (Millicore Corp.; Billerica, MA). The signal strength of phosphorylated NFkB, phosphorylated JNK, phosphorylated P38, and their corresponding total proteins were determined. GAPDH was used as a control for total protein.

### 3.8. Quantification & Statistical Analysis

The protein band intensity was determined using ImageLab and ImageJ software (NIH). Statistical comparisons between treatment groups were analyzed using two-tailed Student's t-tests with a significance level of alpha = 0.05. Unless otherwise stated, significant differences of $p < 0.05$, $p < 0.01$, and $p < 0.005$ are shown by "*," "**" and "***," respectively.

# 4. RESULTS

## 4.1. The Effect of a Methionine & Choline-Deficient Diet on Body Weight Independent of Food Intake

To determine the effect of the MCD diet on the normal physiology and intestinal function of WT mice, mice were given the diet and their intestines were compared to mice who were fed a standard chow diet. As anticipated, the body weights of mice fed the MCD diet declined in comparison to those fed the control diet [Figure 4.1]. This change was due solely to the properties of the diet and not to the amount of food that the mice consumed because the mass of food consumed per mouse per day was essentially identical [Figure 4.1].

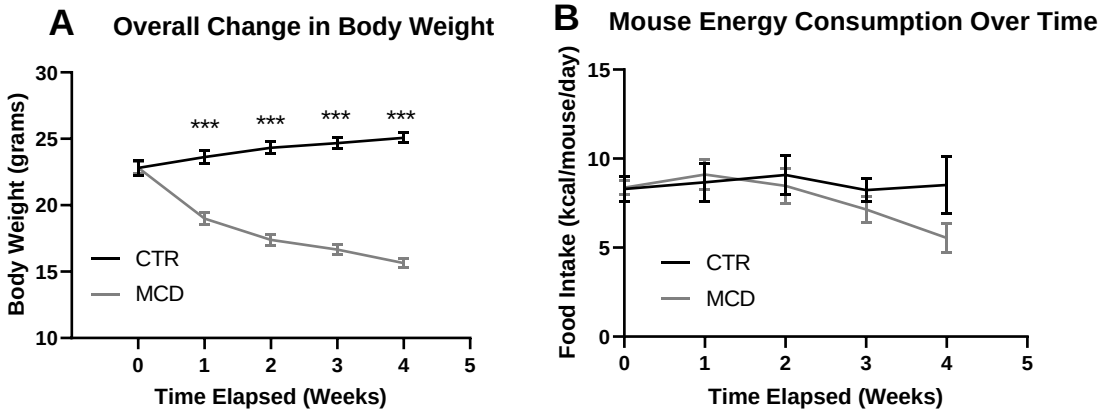


**Figure 4.1. Food Intake and Body Weight in MCD-diet-fed Mice versus Control-diet-fed Mice**

(A) Wild-type male C57BL6 mice were given a chow (CTR) or methionine/choline deficient (MCD) diet for 4 weeks. Body weight was measured weekly by investigative staff. Statistically significant differences of $p<0.005$ are noted by the asterisks "***". (B) Food intake was also measured weekly by investigative staff (in grams). Based on the diet composition, the kilocalories per day consumed were calculated for each mouse.

Comparing the caloric intake allows us to determine that the animals are consuming the same amount of energy. The food intake (kilocalories/mouse/day) was calculated by recording the total number of grams of food consumed per week in a given cage, and dividing by the number of mice in the cage, and dividing this ratio again by 7 days in a week. This ratio was then multiplied by the number of calories in the control diet (3.36 kcals per gram) and the MCD diet (4.1 kcals per gram) to reach the final value.

### 4.2. The Effect of the MCD Diet on Body Composition

There was a markedly lower percentage of total body fat and a comparably higher amount of lean mass for the mice that were fed the MCD diet [Figure 4.2]. This result is to be expected considering that the MCD diet resulted in overall weight loss. Additionally, the tissue weights for the MCD mice were significantly reduced. Again, because most of these tissue weights represent fat depots surrounding specific organs, the results are consistent with expectations. Furthermore, the liver weight is consistent with the overall size of the animals.

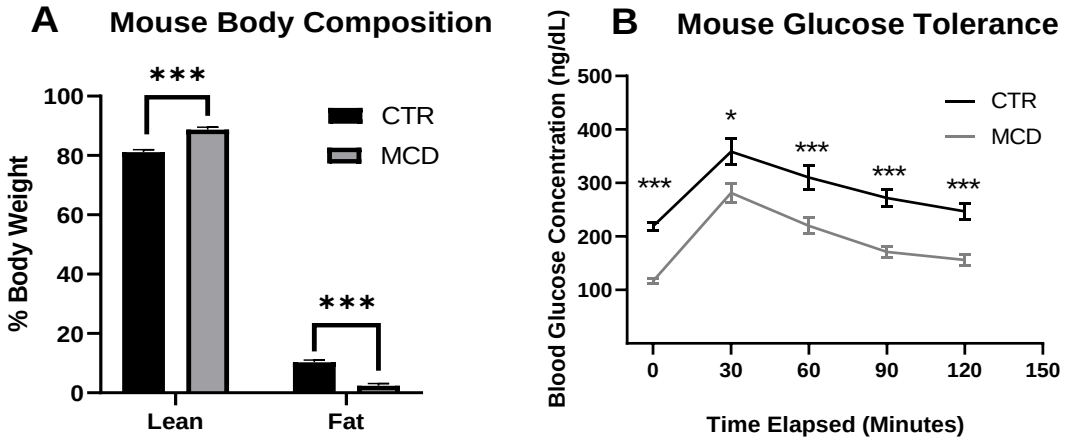


**Figure 4.2. The MCD Diet Alters Body Composition and Glucose Tolerance**
(A) All mice involved in the study underwent a body composition measurement one week prior to tissue harvest. (B) One week prior to tissue harvest, the mice were examined for glucose tolerance. A hexokinase (HK) assay was performed in order to measure the enzyme activity at various timepoints. Statistically significant differences are given by the asterisks: "*" = $p<0.05$; "**" = $p<0.01$, "***" = $p<0.005$. (CTR = control diet; MCD = methionine/choline deficient)

### 4.3. The Effect of the MCD Diet on Glucose Control

The glucose tolerance test demonstrated that MCD mice actually had lower plasma glucose concentrations at every time point [Figure 4.2]. MCD mice have a lower body weight and lower tolerance for insulin. The MCD diet has caused insulin resistance in mice, but insulin injections can lead to mortality in these mice due to their smaller size. Therefore, an insulin tolerance test (ITT) was not performed in this study.

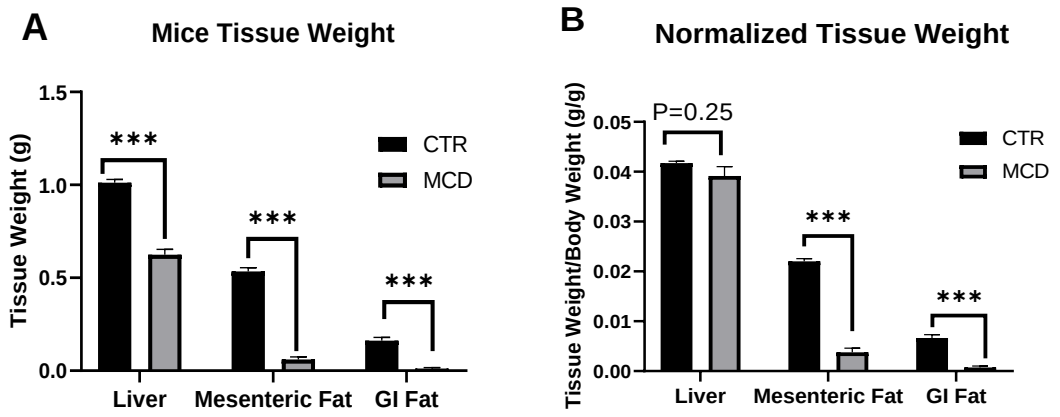


**Figure 4.3. The Effect of the MCD Diet on Tissue Weight and Fat Distribution**
(A) During the tissue harvest, the liver, mesenteric adipose tissue and adipose tissue affiliated with the gastrointestinal tract were weighed and collected. Statistically significant differences of $p<0.005$ are shown by the asterisks, "***". (B)These values were then normalized to account for differences in the body weights of the animals. (CTR: control; MCD: methionine/choline deficient diet; GI: gastrointestinal).

### 4.4. The Proinflammatory Effect of a Methionine & Choline Deficient Diet on Mouse Intestines In-Vivo

For mice that were fed the MCD diet, the phosphorylation levels of JNK increased compared to those that were fed the control diet [Figure 4.4], signifying higher levels of intestinal inflammation. This observation also occurred in the confirmation study [Figure 4.5]. Phosphorylation levels of NFkB appear to stay essentially the same, which demonstrates that methionine and choline may not impact the induction of this pathway [Figure 4.4; Figure 4.5]. P38 induction was also examined in the second study and was found to remain unchanged with MCD diet feeding [Figure 4.5]. Because this study describes the overall inflammation in the intestine, it was still necessary to analyze the inflammation at the cellular level for all of these pathways.

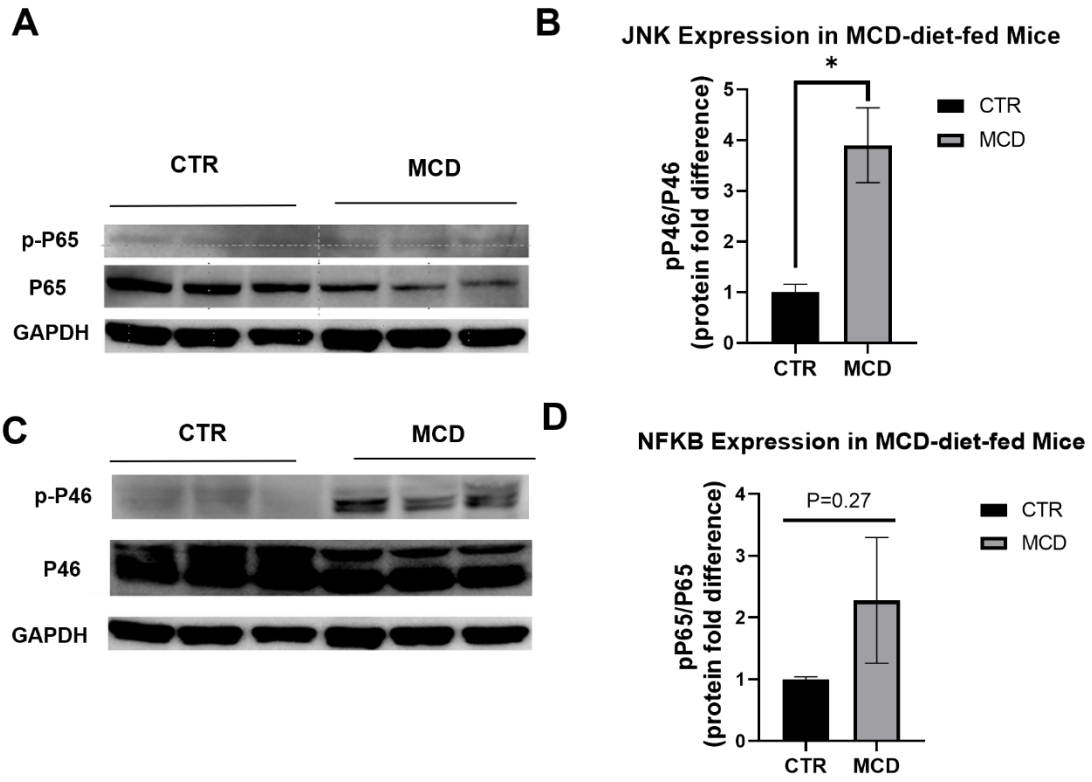


**Figure 4.4. MCD-diet-feeding Modulates Expression of Inflammatory Markers**
(A) Western blot analysis compares the expression of phosphorylated signaling protein, NFkB. (B) The quantification of the fold difference between the average pNFkB levels is given. (C) Western blot analysis compares the expression of JNK, another inflammatory signaling molecule. (D) The ratio of pJNK to JNK and the control protein, GAPDH, was calculated to yield the fold difference between experimental groups. Statistically significant differences are given by the asterisks: "*" = $p<0.05$; "**" = $p<0.01$, "***" = $p<0.005$. (CTR = control diet; MCD = methionine/choline deficient).

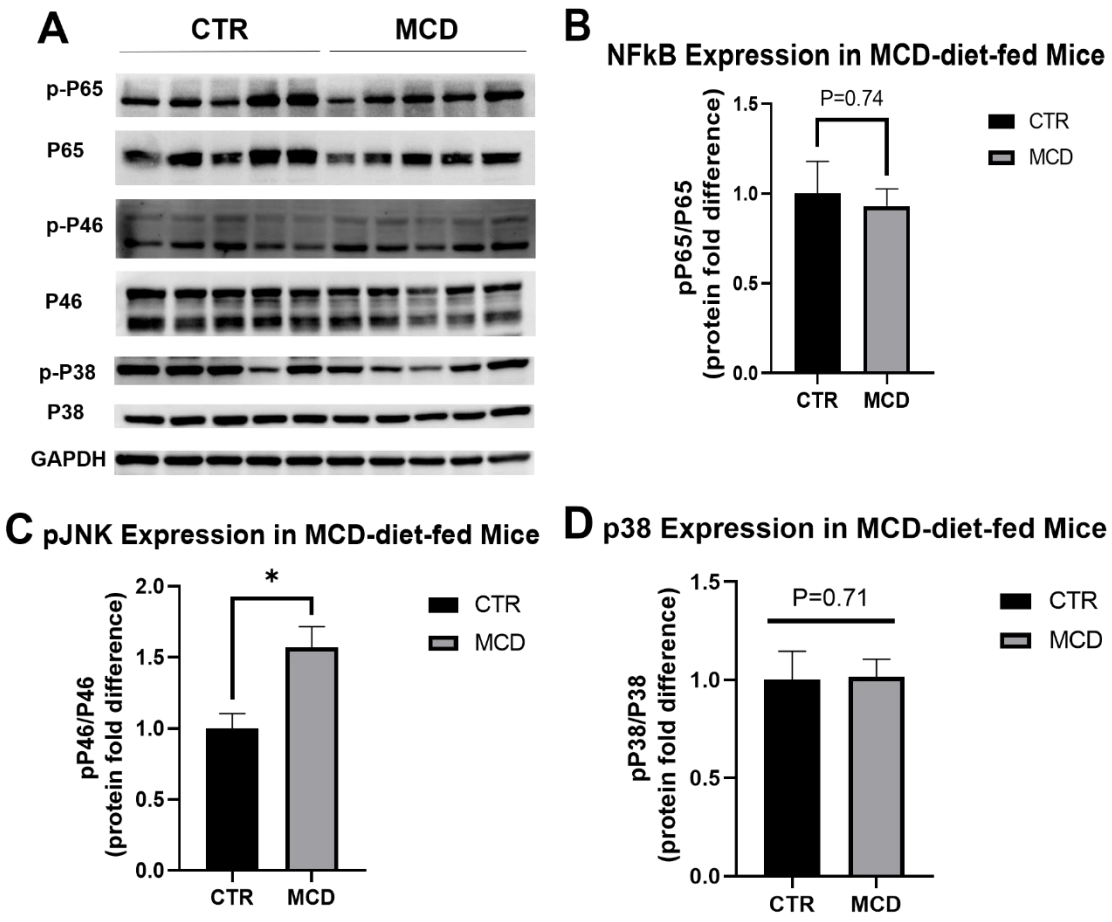


**Figure 4.5. Confirmation of Inflammatory Marker Concentration in MCD-diet-fed and Control-diet-fed Mice**

(A)Western blot analysis demonstrates the level of expression for specific phosphorylated signaling molecules, NFkB (p65) and JNK as well as the control protein, GAPDH. (B) The ratio of pNFkB (pp65) to NFkB (p65) control protein, GAPDH, was calculated to yield the fold difference between experimental groups. (C) The ratio of pJNK to JNK to GAPDH, was calculated to yield the fold difference between experimental groups. (D) The ratio of pP38 to P38 to the loading control, GAPDH, was calculated to yield the fold difference between experimental groups. Statistically significant differences are given by the asterisks: "*" = $p<0.05$; "**" = $p<0.01$, "***" = $p<0.005$. (CTR: control diet; MCD: methionine/choline deficient).

### 4.5. The Response of Intestinal Epithelial Cells to Methionine & Choline Deficiency

To confirm the increase of intestinal inflammation found in vivo, a series of in vitro studies were conducted. In the initial study, an intestinal epithelial cell line was exposed to a methionine and choline deficient cell medium.

Analysis of the inflammatory markers present in these samples revealed no significant change in the phosphorylation of pNFkB in MCD-treated cells [Figure 4.6]. The tendency towards an increase in pNFKB in the MCD-LPS group suggests that the MCD medium sensitizes the cells to LPS induction.

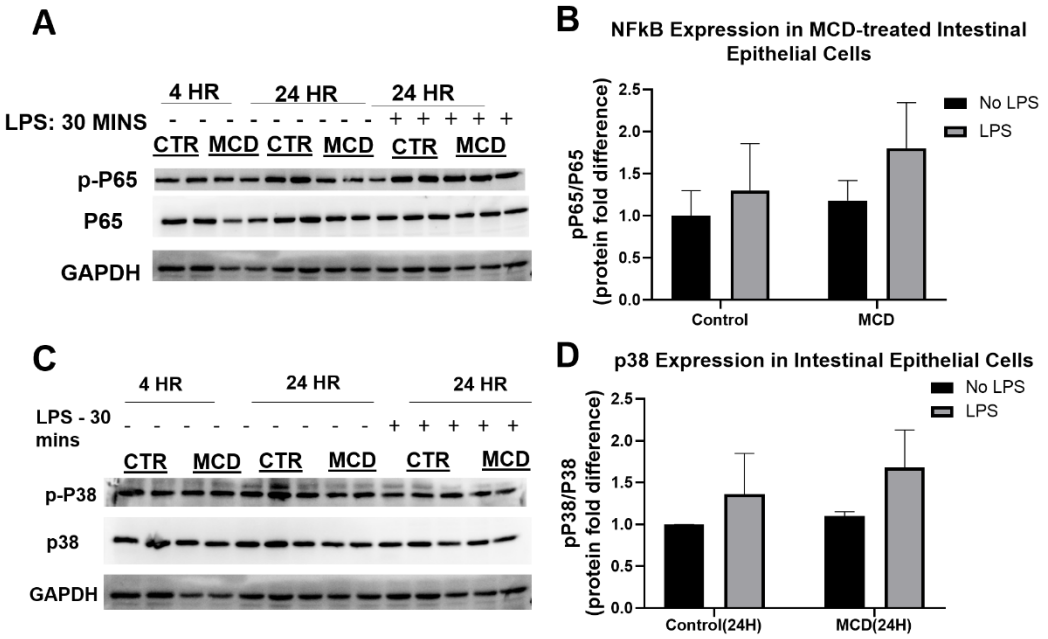


**Figure 4.6 The Response of Intestinal Epithelial Cell Inflammatory Markers to Methionine & Choline Deficiency**

(A) CMT-93 cells were treated with control (CTR) or methionine- and choline-deficient (MCD) medium and lipopolysaccharide (LPS). Cells were treated for 4 hours with no LPS and for 24 hours with and without LPS. The inflammatory response was measured for NFkB (P65) by Western blotting. (B) The quantification of the fold difference between the average pNFkB levels for the treatment groups is given. (C) Western blot analysis compares the expression of P38 MAPK. (D) The ratio of phosphorylated P38 to total P38 to GAPDH was calculated to yield the fold difference between experimental groups. Statistically significant differences are given by the asterisks: "*" = $p<0.05$; "**" = $p<0.01$, "***" = $p<0.005$.

### 4.6. The Response of Macrophages to Methionine & Choline Deficiency

Macrophages were treated with MCD medium to determine whether they play a role in the onset of inflammation in the intestine. The results demonstrate that macrophages respond intensely to inflammatory stimulation by both MCD medium and LPS [Figure 4.8].

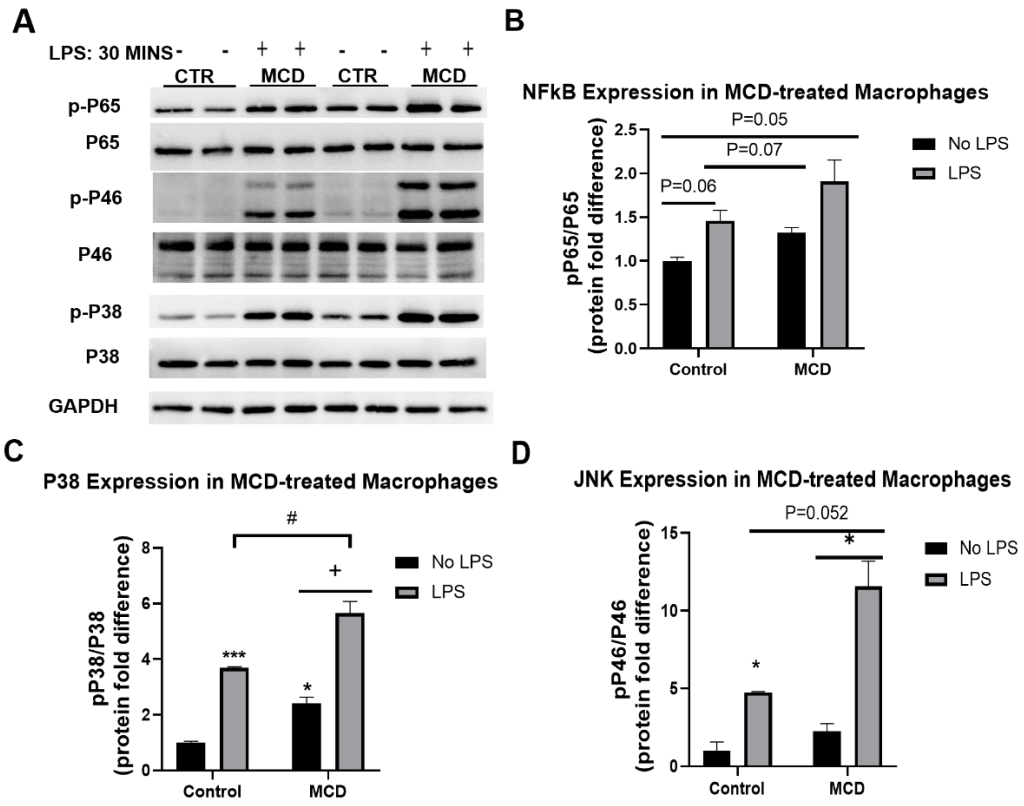


**Figure 4.7. The Response of Macrophages to a Methionine and Choline Deficient Environment and LPS Induction**

(A) RAW cells, from a macrophage cell line, were treated with control (CTR) and methionine/choline deficient (MCD) medium with or without LPS for 24 hours and Western blotting demonstrates levels of inflammatory proteins. (B) The quantification of the fold difference between the average pNFkB levels for the treatment groups is given. (C) The ratio of phosphorylated P38 to total P38 to GAPDH was calculated to yield the fold difference between experimental groups. (D) The ratio of phosphorylated JNK to total JNK to GAPDH was calculated to yield the fold difference between experimental groups. Statistically significant differences are given by the asterisks: "*" = $p<0.05$; "**" = $p<0.01$, "***" = $p<0.005$ with "+" representing within-group differences and # representing between-group differences.

Notably, the three inflammatory markers highlight varying trends. In the case of P38, all of the groups were significantly different, which indicates that the macrophage cells responded strongly to both the MCD medium (P=0.03, P=0.043;) and the LPS treatment (p=0.001, P=0.01) as well as the combination of the two treatments (P=0.004). The results for NFkB seem to support the opposite conclusion. None of the groups were significantly different from the control, though the results from all groups elucidated patterns similar to the earlier studies: signaling increased with MCD and LPS both independently and jointly. The difference between the control medium and MCD-treated groups still support the trend that MCD medium increases inflammation (p=0.07) and that LPS induces inflammation (p=0.06). Lastly, upon examining the JNK induction, only the groups treated with LPS were significantly different from those without LPS (P=0.023, P=0.016). The MCD-treated group was not different (p=0.82) from the control, but the combination group was significantly different from the control (P=0.016), and demonstrated a strong increase in JNK induction compared to the LPS only group, almost reaching statistical significance (p=0.052), indicating that there may be an interaction between the MCD environment and the LPS treatment and the MCD medium may make the cells more sensitive to induction of inflammation by LPS.

# 5. CONCLUSIONS

## 5.1. Summary

The objectives of this study were to achieve the following: 1. Examine the induction of intestinal inflammation in the absence of methionine and choline; 2. Describe the inflammatory response of macrophages, another potential source of inflammation within the intestine, in a methionine and choline deficient environment.

In the animal study, the MCD diet resulted in a higher degree of inflammation compared to the control diet, according to the level of phosphorylated JNK expression found in the original experiment. Similarly, in the confirmation study, the expression of phosphorylated NFkB was not significantly different between the two groups, but JNK expression increased. This trend demonstrates that methionine and choline may contribute to the body's functions which act in mitigating inflammation.

Similarly, in the original cell study, the time-course revealed that initial treatment of intestinal cells with MCD medium resulted in higher levels of inflammation compared to the control medium, which just bordered statistical significance. The heightened response of the epithelial cells to LPS in the presence of the MCD medium indicates that methionine and choline deficiency sensitize the intestinal epithelium to inflammatory insults.

Lastly, but perhaps most importantly, the macrophage study showed that the macrophage cell line responds even more intensely than the intestinal cells to MCD medium and LPS for all of the treatments. These results are disruptive in that they

challenge the original hypothesis that NAFLD originates in the intestinal epithelium and suggest that NAFLD may actually be a product of immune dysfunction.

It was originally hypothesized that intestinal inflammation directly influenced NAFLD onset. However, based upon these findings, the NAFLD narrative may surprisingly be centered on the immune response within each of the tissue types: liver, adipose and intestine. The intestine houses an abundance of macrophages which can contribute to the inflammatory response. This organ represents a central hub in a complex web involving nerve signaling, the microbiome and the immune system.

### 5.2. Strengths and Limitations

The strengths of this study are the inclusion of adequate controls, the use of established experimental diets, and appropriate statistical analysis. The use of an intestinal epithelial cells from a rectal carcinoma cell line (CMT-93) allows us to draw conclusions regarding how cancer cells may behave in a methionine- and choline-deficient environment. The comparison between the small intestinal tissue in the in vivo study and large intestinal tissue in the in vitro study also allow us to demonstrate how the small and large intestine may respond differently to nutrient deficiencies.

The limitations of this study include certain characteristics of the experimental treatments. In the animal study, the small intestine was collected as a whole. In the future, it may be useful to separate the intestine into anatomically relevant layers (mucosa, submucosa, etc.) for analysis, which would allow unique cell types to be isolated. The chow diet was also used for the animal study, which serves the purpose of this current project, but a matched diet would be required in the future in order to control

for specific nutrients in the diet, including micronutrients. Additionally, the cell study utilized CMT-93 cells, a rectal carcinoma cell line. This difference may or may not have contributed to the inflammatory response of the cells. It would be helpful in the future to repeat these studies utilizing physiologically normal small or large intestinal cells and compare the results to the colorectal cancer cells. This would reveal whether the cancer cells respond differently to the MCD treatment than normal intestinal cells. However, due to the limited availability of intestinal cell lines and the likelihood of epithelial cell death in methionine- and choline-deficient conditions, it may not be possible to carry out this latter study. If it is feasible, it may be useful to treat small intestinal epithelial cells with MCD medium in order to make a direct comparison to the in vivo study.

### 5.3. Discussion & Future Experiments

NAFLD is a widespread and complicated disease, initiating many deleterious systemic consequences to the organism. Better understanding of the pathophysiology of this disease requires substantiated cell and animal models. The objective of this research was to determine if intestinal inflammation induced by the MCD diet may play a central role in initiating the inflammation seen in NAFLD progression. The MCD diet is already established to induce fatty liver disease by disrupting the normal mechanisms of lipid metabolism in the accessory digestive organs[7-10]. Because the intestine and liver are closely interconnected within the gastrointestinal tract, it is likely that inflammation originating in the intestine will affect the liver's metabolic functions[3, 4, 11, 21]. This could then still lead to the classic symptoms of systemic insulin resistance and affect other insulin-sensitive tissues such as the adipose tissue[2, 9, 40, 51]. From prior work

and these additional experiments, we have found that, in addition to interacting with adipose tissue, NAFLD involves other organ systems including the intestine, brain and immune system[52, 53].

### 5.3.1. The Gut-Brain Axis & NAFLD

The gut-brain axis remains a larger part of the picture that was not introduced in these studies but remains pervasive in the current digestive disease literature. There also exists a brain-gut bidirectionality that could contribute to the onset of gastrointestinal disease [54]. Prolonged intake of high-fat diets can alter nutrient-sensing mechanisms in the intestine, therefore modifying signals sent to the brain [53]. However, these responses of the gut and brain to nutrients are not restricted to overweight subjects or high fat diet. Supporting these observations, Bruce-Keller et al. established that "obese-type" microbiota composition can influence susceptibility to neurological disorders independent of weight status[55]. This further justifies the need for an animal model that induces NAFLD and alters the intestinal function independent of obesity. Many disease states, including NAFLD, diabetes, neurological disorders and their comorbidities, affect non-obese as well as obese populations[17, 18, 56]. The MCD diet may perpetuate NAFLD symptoms and could also have other consequences in NAFLD patients if it were to modify the gut-brain axis following intestinal inflammation. For this reason, effects of the MCD diet on the gut-brain axis should be examined in the future. One critical contributor to intestinal and brain function which cannot be overlooked is the gut microbiota.

### 5.3.2. The Gut Microbiome & NAFLD

Intestinal dysfunction can often result from disruption of the microbial community within the intestine. An altered gut microbiome can also lead to systemic inflammation by modulating the gut's permeability, triggering adipogenesis in other tissues [52]. In fact, dysfunction of the intestinal barrier can result in obesity, insulin resistance and fat accumulation in the liver [53]. Fatty liver is often associated with a fat-rich diet, and high fat intake can result in bacteria entering the liver and adipose tissue due to a compromised gut barrier. A high fat diet may also raise blood endotoxin levels by increasing LPS, and if bacteria from the gut are able to enter the liver, the toxins released may initiate fibrosis [52].

Previous studies have illustrated the role of microbiota in establishing a leaky gut and promoting insulin resistance, thereby setting the conditions for NAFLD to develop[2, 3, 5, 47, 52, 57-59]. The MCD diet may also reduce gut permeability by modulating the gut ecosystem and allow bacteria or toxins to travel into the blood stream and other tissues. Additionally, because large amounts of choline are digested and used by the intestine, dysbiosis of the intestine can also cause defective choline metabolism, leading to NAFLD [52]. The MCD diet may mimic this loss of necessary choline. The diet's inflammatory and disruptive nature presents an opportunity to examine the role that this diet could play in modifying the gut microbiome and inducing NAFLD.

### 5.3.3. The Immune System & NAFLD

Absorption of nutrients is not the only action that influence the health of the digestive tract. Dysregulation of immune cells unleashes severe inflammation within the

host, and the intestine, is an integral home for immune cells [48]. Like the intestine, the liver is also a key player in immune function. It includes many white blood cell types, including lymphocytes as well as dendritic cells, and of course macrophages. The specific cell type of note is the Kupffer cell—the resident macrophage embedded in the hepatic tissue. The Kupffer cell features toll-like receptors (TLRs) which are engaged by LPS, activating numerous inflammatory pathways including Jun N-terminal kinase (JNK), p38 and NFkB. The increased inflammation contributes to the onset of NAFLD and NASH.

### 5.3.4. Future Experiments

The experiments conducted in this project support the conclusion that the digestive and immune systems seem to play a joint role in the onset of NAFLD and NASH as well as several other diseases. Therefore, studies examining the interactions between these systems are a point of future research. Experiments should be conducted in the future to demonstrate the effects of the MCD diet on the gut-brain axis to see if it will have the same impacts on signaling to the brain as the high-fat diet. Tight junction protein experiments should also be completed to demonstrate the specific effects of the MCD on gut permeability. The role of the immune system in NAFLD was not anticipated in the hypothesis of this study, but it is very promising. A macrophage and intestinal cell co-culture experiment would also be valuable in demonstrating the direct effects of immune cells on the intestine. The original experiments create a firm basis for the future research to reveal how all of these components interact to induce the symptoms of liver disease.

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