MICROBIOTA DERIVED METABOLITES AS MODULATORS OF INFLAMMATION IN NON-ALCOHOLIC FATTY LIVER DISEASE

A Dissertation

by

YUFANG DING

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Arul Jayaraman
Committee Members,	Mike McShane
	Akhilesh Gaharwar
	Victor Ugaz
	Robert Alaniz
Head of Department,	Mike McShane

May 2019

Major Subject: Biomedical Engineering

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in western countries and characterized by a spectrum of liver injuries ranging from simple steatosis to steatohepatitis (NASH) with or without fibrosis. Among the patients with NAFLD, about 10-20% of them have NASH and may eventually progress to fibrosis, cirrhosis and liver carcinoma. Accumulating evidences indicate gut microbiota plays an important role in NAFLD and different bacterial taxa have been correlated to disease. However, the specific metabolites through which the microbiota exert their effects and the target pathways in host cells are not fully understood. Our previous work demonstrated that intestinal microbiota are capable of producing bioactive molecules that engage host cellular pathway. The goal of this study is to investigate the hypothesis that gut microbiota derived metabolites could directly act on the liver macrophages to modulate inflammation and this affects the progression of NAFLD.

Utilizing liquid chromatography-mass spectrometry metabolomics we identify two tryptophan-derived metabolites – tryptamine (TA) and indole-3-acetate (I3A) – that require the microbiota and are depleted in high-fat diet mice compared to low fat diet mice. Both I3A and TA reduced palmitate and LPS induced production of pro-inflammatory cytokines (TNFα, IL-1β and MCP-1) in macrophages, as well as inhibited macrophage migration toward the chemokine MCP-1.

We identified that both palmitate and LPS reduced levels of p-AMPK and the p-AMPK activator AICAR reduced palmitate and LPS induced inflammatory cytokine production. The addition of I3A and TA reversed p-AMPK reduction induced by palmitate and LPS. siRNA

knockdown of AMPK in macrophages partially abrogated I3A and TA's anti-inflammatory effects suggests that I3A and TA's effect is dependent on AMPK signaling.

In summary, these studies demonstrated that gut microbiota derived metabolites can modulate inflammatory responses in macrophages by engaging the AMPK signaling pathway.

ACKNOWLEDGEMENTS

I deeply appreciate my advisor Dr. Arul Jayaraman for offering me this incredible opportunity to study in his lab, and for his invaluable input in every aspect of my research life in the past 7 years. Without him, I would not have been able to learn diverse areas ranging from microfluidics to metabolomics to cell biology. His guidance, encouragement and support helped me learned how to become an independent scientist. Personally, his hardworking and passionate attitude inspired me to pursue academia and dream to become a PI like him one day.

I also thank my committee members for their great support and feedback. I especially want to acknowledge Dr. Alaniz for providing significant help with *in vivo* studies as well as for sharing his tremendous experience in the field of immunology. I'm also grateful to Dr. Ugaz, Dr. McShane and Dr. Gaharwar for their insightful comments and advice in the past years. I am thankful to Dr. Kyongbum Lee and his lab members especially Dr. Smitha Krishnan at Tufts University for their hard work in our collaborated projects.

I want to thank the past and current lab members in the Jayaraman lab. Without all their support and help, I would not have become who I am today. Thank you for being my colleagues, my friends, and my family for the past 7 years.

Last, but not the least, my deepest gratitude goes to my family and friends. I thank my parents, my brother, and my in-laws for their love, endless support and continued patience. I am grateful to my dearest husband for his love, understanding and support every single day. Thanks for all the troubleshooting and encouragement when my life went down. My little girl, Jenny, and the upcoming boy, Jason, thank you for making my life brighter than ever. Love you all!

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a dissertation committee consisting of Professors Arul Jayaraman (advisor), Mike McShane, Akhilesh Gaharwar and Vitor Ugaz of the Department of Biomedical Engineering and Professor Robert Alaniz of the Department of Health Science Center.

The data for Chapter III was in part by Dr. Smitha Krishnan and her lab mates at Tufts University (Figure 3.1, Figure 3.2, Figure 3.3, Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8 B-D, Figure 3.9 and Figure 3.10) and were published in 2018 in an article listed in the References.

All other work conducted for the dissertation was completed by the student independently.

This work was made possible in part by National Science Foundation (NSF) grant 1264526 to Professors Arul Jayaraman and Kyongbum Lee (Tufts University) and NIH 1R01 AI110642-10 to Professors Arul Jayaraman, Robert Alaniz and Juergen Hahn (Rensselaer Polytechnic Institute).

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in Western countries and is characterized by a spectrum of liver injuries ranging from simple accumulation of fat in the liver to inflammation and scarring (1). Steatosis of the liver is the first step in NAFLD and its characteristic feature is the accumulation of lipids in the liver tissue (1). While simple steatosis is reversible, the progression of steatosis to steatohepatitis, or steatosis with a significant inflammatory component, is generally considered an irreversible progression of the disease. However, clinical studies have shown that among NAFLD patients, only 10-20% of patients with steatosis develop steatohepatitis (2). Apart from chronic inflammation, other triggers such as hypertension, weight gain and diabetes all can contribute to steatohepatitis, and eventually the development of fibrosis, cirrhosis and liver carcinoma (2). Although the pathogenesis of NAFLD has been extensively investigated, the factors underlying the initiation of steatosis and the irreversible progression of simple steatosis to steatohepatitis are poorly understood.

Several factors such as excessive levels of free fatty acids (FFAs) from dietary fat intake or lipolysis of adipose tissue, increased *de-novo* lipogenesis by liver, decreased fatty acid oxidation, and decreased secretion of hepatic very low density lipoprotein (3) have been proposed as contributors to hepatic steatosis. Of these, the supply of fatty acids from adipose tissue lipolysis is the primary contributor and has been shown to account for almost 60% of hepatic lipid accumulation (4). Triglycerides (TG) are the main lipids stored in the liver (5) and TG pathways elicit both negative and protective effects in NAFLD (5-9). Thus, it is hypothesized that TG in hepatocytes serves as a biomarker instead of leading to lipotoxicity directly.

A two-hit model is commonly used to investigate the pathogenesis of steatosis and NAFLD (10). In this model, steatosis or lipid accumulation represents the first hit that arises due to the increased accumulation of fatty acids and *de-novo* lipogenesis in the liver. Inflammation, increased expression of inflammatory cytokines and generation of reactive oxygen species (ROS), and decreased hepatic ATP production are second hits which result in the progression of steatosis to steatohepatitis (11). Although the two-hit model is well accepted and captures several aspects of NAFLD pathogenesis such as not all patients with steatosis develop hepatic inflammation (2) and in rodent models of NAFLD, steatosis can be induced by relatively short time high fat diet feeding (12) but extended diet feeding with additional stimuli such as fructose supplemental is necessary to induce NASH (13), many variations to the model have been proposed.

Jou et al. (9) proposed that hepatocyte death and lack of cell repair is a putative "third hit" in the progress of NASH. They suggest that as sustained inflammation (manifested as aberrant cytokine profiles and oxidative stress) persists, hepatocyte anti-inflammatory and anti-oxidative stress mechanisms are eventually overwhelmed, leading to increased rate of hepatocyte death (9). Since cell death can result in further activation of macrophages and production of chemokines leading to infiltration of other immune cells such as monocytes and neutrophils, the initial hepatocyte cell death may act in a feed-forward manner to amplify hepatic inflammation and eventually leads to the development of NASH.

In a different model, hepatic inflammation has been proposed to precede steatosis in NAFLD. This model is based on the observation that hepatic inflammatory stress can exacerbate lipid accumulation in hepatic cells and fatty liver in mice (14). Tilg et al. have also proposed a parallel-hit hypothesis in which multiple gut-derived and adipose tissue-derived factors induce hepatic inflammation and lead to liver damage (15). Together, these studies demonstrate that while

it is accepted that inflammation plays a key role in the progression of steatosis to steatohepatitis, there is little consensus on the causative role of inflammation in NAFLD. As a result, a detailed understanding of the molecular mechanisms are currently lacking.

In the last decades, a large body of evidences showed that the AMP-activated protein kinase is involved in hepatic lipid metabolism and increasing the activity of AMPK has been proposed as a therapeutic target for NAFLD.

The AMP-activated protein kinase (AMPK) is an energy sensing protein complex and essential in balancing cellular nutrition supply and energy demands through integrating hormonal signals and coordinating metabolic pathways (16).

The downstream substrates of AMPK are typically energy metabolic enzymes and proteins involved in transcription regulation, it is not surprising that deficiency of AMPK is related to the appearance of metabolic syndrome including obesity, diabetes, cardiovascular disease and NAFLD (17). Evidences showed that both two main pathological factors in NAFLD, lipid overload (18) and inflammatory signals (19) can inhibit AMPK activity. The deficiency of AMPK found in various cells with metabolic diseases make it possible that AMPK activation is important in counteract inflammation. Thus, AMPK might be a promising therapeutic target and activation of AMPK through pharmacological activator improves NAFLD (20).

Obesity is thought to be a major risk factor for NAFLD, as obesity is often observed in NAFLD patients (1). Since recent studies have identified the gut microbiota as an emerging factor contributing to obesity and related diseases (21), alterations in the microbiota have also been proposed to underlie the etiology of hepatic steatosis and steatohepatitis (22). The gut microbiota is involved in carrying out several functions that are essential to the host, including the metabolism of dietary compounds, synthesis of essential nutrients such as vitamins, defense against pathogen

colonization (23, 24), development of the immune system (25, 26) as well as conditioning and maintenance of homeostasis in the GI tract (26, 27). The impact of the gut microbiota is not limited to the GI tract, as recent studies have identified correlations or causal roles for the microbiota in peripheral organs as well. The gut-liver (28), gut-lung (29), and gut-brain (30) axis that have been proposed clearly highlight the importance of the GI tract microbiota in host physiology.

Several mechanisms have been proposed to explain the contribution of the microbiota to the pathogenesis of NAFLD. One of the simplest mechanisms through which the microbiota can contribute to the development of steatohepatitis is through increased exposure to lipopolysaccharide (LPS). Dysbiosis of the microbiota, or the alteration of the community that is characterized by a reduction in the diversity of the community (31, 32), leads to increased abundance of Gram-negative bacteria such as *Bacteroidetes* (33) that in turn leads to an increase in the levels of endotoxins LPS load. Microbiota dysbiosis also decreases the barrier integrity of the intestine, and leads to an increase in the translocation of bacterial endotoxins from the lumen. The increase in circulating levels of endotoxins like LPS triggers the initiation of pro-inflammatory cytokine production in several mesenteric organs including the liver and adipose tissue depots (22).

Recently, Le Roy et al. demonstrated the potential causal involvement of microbiota in the progress of NASH using fecal transplantation experiments in mice (34). When high fat diet (HFD) fed germ-free (GF) mice were inoculated with bacteria isolated from feces of HFD-fed hyperglycemic mice, they developed steatohepatitis. On the other hand, HFD-fed GF mice inoculated with bacteria from HFD-fed normoglycemic mice only showed mild steatosis. This study suggests that the intestinal microbiota and their metabolic products may have a causal role in determining whether simple steatosis can progress to steatohepatitis.

Henao-Mejis et al. demonstrated that inflammasome deficiency leads to an altered gut microbiota composition, which in turn exacerbates hepatic steatosis and inflammation (35). Another potential possibility is that dybiosis leads to increased generation of toxic metabolic byproducts which could result in an elevated chemical burden on the liver. The association between the intestinal microbiota and fatty liver development under choline deficiency has also been reported in a clinical study (36). Taken together, these studies strongly suggest a causal link between microbiota dysbiosis and steatohepatitis. However, the identities of microbiota species, target cells in the host, and signaling pathways that are modulated are only partially known.

Recent findings suggest that bioactive metabolites produced by the intestinal microbiota could be the mediators through which the microbiota modulate host phenotypes. Studies have shown that microbiota metabolites such as short chain fatty acids (e.g., butyrate) directly affect the development of gut immune system (37), inflammation (38) and defense against pathogens (38). In addition, recent work from our lab and others have proposed that metabolites produced by the microbiota reactions from dietary tryptophan (e.g., indole, indole-3-aldehyde) attenuate indicators of inflammation in multiple cell types and in mouse models of disease (39-42). Moreover, the detection of microbiota-derived metabolites in circulation (43) further supports the hypothesis that the microbiota, through the molecules that it produces, can module host cell signaling and phenotypes at distant sites such as the liver and suggest communication between the gut and the liver through microbiota-derived metabolites.

The central hypothesis of this dissertation is that alterations in the microbiota that are manifested through changes in the levels of its metabolites plays a causative role in the progression of steatosis to steatohepatitis. We further propose that these metabolites enter circulation and exert their immunomodulatory activities on different host cells. Specifically, we focus on the resident

macrophages (Kupffer cells) in the liver actions as they are well established to be involved in the liver inflammatory response. We hypothesize that the metabolites produced by intestinal microbiota can directly act on resident macrophages to modulate inflammation, and this affects the progression of steatosis to steatohepatitis. This hypothesis will be tested through three specific aims.

Aim1: To identify microbiota derived metabolites which are altered in steatosis. We will utilize an untargeted metabolomics strategy to identify microbiota derived metabolites that are altered in a murine model of NAFLD. Mice fed a high fat diet (HFD) and normal chow control (LFD) will be used for these studies. Metabolites will be extracted from different luminal sites (caecal contents, fecal pellets), liver tissue, and serum. Different LC-MS approaches will be used for separating and identifying metabolites that are differentially altered. We will initially focus on tryptophan-derived metabolites as our prior work has shown that tryptophan-derived metabolites elicit anti-inflammatory effects in different cell types, including macrophages. The expected outcome is the identification of metabolites that are increased in HFD as potentially mediators of inflammation while those that are decreased in HFD mice would be putative beneficial metabolites produced by the microbiota.

Aim2: To characterize the effects of microbiota metabolites on macrophage inflammation. We will test the hypothesis that microbiota metabolites modulate the inflammation component in NAFLD by regulating inflammation in macrophages. Our working model is that metabolites can modulate cytokine signaling and macrophage migration to modulate inflammation. We will treat macrophages with FFAs (e.g., palmitic acid) followed by LPS to mimic the two-hit *in vitro* model of NAFLD. The production of inflammatory cytokines (TNFα, IL-1β and MCP-1) will be quantified using qRT-PCR and ELISA, and used as indicators of macrophage inflammation. In

addition, the ability of macrophages to migrate towards inflammatory stimuli in the presence of microbiota metabolites will be investigated using primary bone-marrow derived macrophages. The expected outcome is the elucidation of the role for microbiota metabolites in attenuating the inflammation component during NAFLD.

Aim3: To elucidate the mechanism underlying the effects of microbiota metabolites on macrophage inflammation. Since AMPK activation is inhibited by both lipid loading and LPS exposure, and the activation of AMPK improves NAFLD progress, we will test the hypothesis that microbiota metabolites modulate macrophage inflammation through upregulating AMPK levels. We will analyze changes in the levels of AMPK in macrophages treated with FFAs or LPS in the presence of microbiota metabolites. We will reduce AMPK levels in macrophages using siRNA, and use any changes in inflammatory cytokine levels to determine the role of AMPK in mediating the responses observed with microbiota metabolites.

1.1 Innovation

Although many hypotheses have been proposed, including on the role of the microbiota, to explain the pathophysiology of NAFLD, very few studies have identified specific molecules and their target pathways in the liver. The proposed work is significant as it will help understanding the role of specific microbiota-derived metabolites in NAFLD and can lead to the development of postbiotics for improving liver health and function. Second, the molecular pathways through which microbiota metabolites mediate their effects in host cells are poorly understood, and the proposed work will lead to a mechanistic understanding on these interactions. Third, identifying the pathways/molecular targets will also lead to a fundamental understanding on how non-canonical ligands such as microbiota metabolites are sensed in host cells.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the western countries (44). Globally, the prevalence of NAFLD is at around 25% with highest in South America and the Middle East. In the United States, the number of NAFLD patients was approximately 83.1 million in 2015, which is about 25% of the population. This number is projected to expand to more than 100 million by the year 2030 (45). Notably, the projected at-risk population is expected to have NASH, which is an advanced and more detrimental stage of NAFLD (45). Although NAFLD is typically accompanied by an increase in body weight and obesity in Western countries, there is a large portion of NAFLD patients in Asia who have normal body weight index (46). The increase in the prevalence of NAFLD is expected to lead to an increase in number of cirrhosis and hepatocellular carcinoma (HCC) patients (47, 48).

Although steatosis can be diagnosed based on imaging and lab tests, NASH can only be diagnosed by liver biopsy (49). NASH patients also have increased risks of both liver and non-liver related outcomes such as cirrhosis, liver cancer and cardiovascular diseases (50). A large fraction of liver cancers in NASH patients occur much earlier compared to HCCs in other liver diseases (51, 52), and these tumors also tend to be larger and resistant to curative therapies (53).

2.2 Risk factors in NAFLD

Although significant progress has been made on understanding the pathogenesis of NAFLD, key drivers such as factors driving steatosis to NASH and optimal animal models are poorly understood. Many risk factors such as diet, diabetes and genetics have been proposed to describe the progression of NAFLD from steatosis to NASH (**Figure 2.1**) (2); however, they do not

adequately capture the progression of disease. Metabolic syndrome is thought to be the strongest risk factor in NASH (54) and includes a wide variety of conditions including obesity, hyperglycemia, systemic hypertension as well as dyslipidemia (55). The relationship between metabolic syndrome and NAFLD is bidirectional, as metabolic syndrome increases the risk of NASH and NASH further enhances features of metabolic syndrome (56, 57).

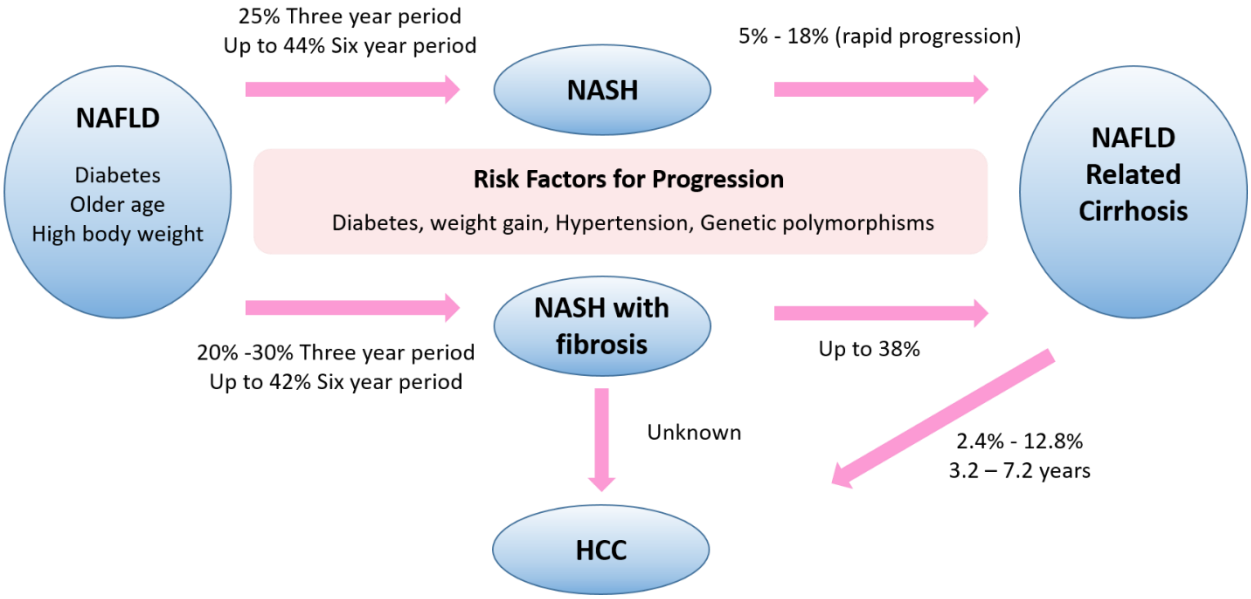

Figure 2.1 The progression of NAFLD to NASH with or without fibrosis and HCC (adapted from Ref. 2 (2)).

2.2.1 Diet

Dietary intake and composition affects metabolism and plays a significant role in the development of NAFLD. Saturated FAs (SFAs) and trans-FAs promote inflammation in the liver due to their lipotoxicity (58, 59). FAs also affect intestinal microbiota, impairing the intestinal barrier integrity,

and leading to endotoxemia (60). FAs and endotoxin synergistically promotes the production of ceramides in macrophages which further augments hepatic lipotoxicity (61). Conversely, poly-unsaturated fatty acids (PUFA) positively regulate hepatic lipogenesis and FA oxidation through modulation of genes such as the peroxisome proliferator-activated receptor alpha (PPARα) and sterol regulatory element-binding proteins (SREBP) (62, 63). PUFA are precursors of eicosanoids which play an important role in the modulation of inflammation. Among the PUFAs, n-3 PUFA has been associated with anti-inflammatory effects while n-6 PUFA is pro-inflammatory (64). In many chronic diseases including NAFLD, the ratio of n-6: n-3 PUFA is increased due to an imbalance in the diet (65).

Cholesterol intake also contributes to the development of steatosis and hepatic inflammation by altering lipid metabolism and mitochondria function in the liver (66). The dysregulation of cholesterol metabolism can promote oxidative stress and progression of steatosis to NASH, increased disease severity, and cardiovascular risk in some NAFLD patients (67, 68).

Dietary fructose leads to NASH even in the absence of obesity (69). Fructose increases hepatic *de novo* lipogenesis (DNL) and impairs FA oxidation; thereby, contributing to lipid accumulation (69). Moreover, fructose increases gluoconeogenesis and liver insulin resistance through upregulation of related kinase and glucose transporter expression, such as GLUT5, PI3K, PKC and MAPK (70-72). Fructose also promotes the development of NASH by increasing oxidative stress, production of inflammatory mediators such as TNFα and TGF-β, and affecting intestinal bacterial composition (70, 71).

2.2.2 Diabetes

The link between diabetes and NAFLD is thought to be the clearest compared to other risk factors (54). Nearly 75% of patients with type 2 diabetes (T2D) have NAFLD, and the prevalence of

NASH, fibrosis and other liver related complications is higher in patients with both diabetes and NAFLD (73-75). Both T2D and NAFLD are strongly associated with insulin resistance (76). As insulin resistance develops, liver inflammation develops due to the increase in FFAs and pro-inflammatory cytokines such as IL-6, TNFα and leptin, as well as a concomitant decrease in anti-inflammatory adiponectin from adipose tissue (77, 78). T2D has also been shown to be associated with HCC. As insulin resistance progresses, the increased concentration of insulin in circulation leads to higher production of insulin-like growth factor 1 (IGF-1) which is a hormone that stimulates cellular proliferation and inhibits apoptosis in the liver (79, 80). Insulin also stimulates insulin receptor substrate-1 (IRS-1) to promote HCC development (81). On the other hand, adiponectin has been shown to decrease angiogenesis and affect cellular apoptotic response thus has a protective role in HCC development (82). Thus, insulin resistance that is common in T2D leads to cellular proliferation and growth in the liver, and contributes to the development of HCC.

2.2.3 Hypertension

Approximately 50% of patients with hypertension (HTN) have NAFLD, and blood pressure is strongly associated with NAFLD in both normotensive and hypertensive individuals (83-85). The presence and severity of NAFLD is associated with HTN, pre-HTN, and arterial stiffness (86, 87). Therefore, the relationship between HTN and NAFLD is bi-directional, as increased blood pressure values predicts the progression of NAFLD and the diagnosis of NAFLD predicts the risk of HTN (88).

HTN has also been correlated with increased fibrosis in NAFLD (89, 90). It has been reported that the renin-angiotensin-aldosterone (RAA) system plays a role in the development of HTN and fibrosis in a rat HTN model (91). NASH patients express higher levels of genes encoding

angiotensinogen compared to healthy individuals (92) and certain nucleotide polymorphism of angiotensin receptor has been shown to relate to fibrosis in NASH (93).

Systemic insulin resistance and inflammation in NAFLD may activate the pro-inflammatory transcription factor NF-κB (94, 95) and sympathetic nervous system (96) to promote the development of HTN. Altered adipokine profile in NAFLD can also activate sympathetic nervous system and induce inflammation to lead to HTN (97-99). Moreover, the secretion of cytokines such as fibroblast growth factor-21, retinol-binding protein-4 and fetuin-A from inflamed liver are reported to play a pathogenic role in the development of HTN (100).

2.2.4 Genetics

Several genes that encode proteins involved in the regulation of lipid metabolisms have been strongly associated with the progression of NASH (101, 102). A single nucleotide polymorphism (SNP) at position 148 in the PNPLA3 gene is the most well characterized genetic variant linked to NASH (103-105). Wild type PNPLA3 protein has hydrolase activity for triglyceride and thus plays an important role in regulating lipolysis of lipid droplets in hepatocytes (106-108). The isoleucine to methionine substitution at position 148 in PNPLA3 causes loss of hydrolase activity, and leads to the entrapment of TG in hepatocytes and HSCs, contributing further to liver damage (104, 106-109). Experiments using animal models have shown that overexpression or knock-in of the I148M mutation also give rise to higher susceptibility to hepatic fat accumulation after excess carbohydrate feeding (110). The underlying mechanism has been speculated to be related to the accumulation of the I148M mutation in the lipid droplet, resulting in the reduced degradation by the proteasome and inhibition of other lipase activity in hepatocytes (110).

Other genes that have been found to associate with NASH development are: transmembrane 6 superfamily member 2 (TM6SF2), Membrane bound O-acyltransferase domain-

containing 7 (MBOAT7) and glucokinase regulator (GCKR) (102). TM6SF2 is involved in the pathway of very low density lipoprotein (VLDL) secretion from hepatocytes (102). A SNP at position 167 in TM6SF2 gene results in a loss of function thus induce higher contents of TG and lower amount of circulating lipoproteins (102). MBOAT7 is a protein that involved in the remodeling of phosphatidyllinostitol (111, 112). Genetic variant of the *MBOAT7* gene downregulate both mRNA and protein level of MBOAT7, is associated with the risk of NAFLD, inflammation and fibrosis (111, 112). GCKR regulates DNL and genetic variance induced loss of function mutation is associated with hepatic fat accumulation (113).

2.3 Pathogenesis of NAFLD

A "two-hit" model has been widely used for studying the pathogenesis of NAFLD. In this model, lipid accumulation in the liver is thought to be the first hit and additional stimuli such as increased levels of inflammatory cytokines, increased reactive oxygen species (ROS) and decreased hepatic ATP production are regarded as possible second hits that result in the progress of steatosis to NASH (11). Many signaling pathways have been identified to contribute to the development of NASH. However, the idea that NASH always progresses from steatosis is debatable as studies observed that hepatic inflammatory stress can exacerbate lipid accumulation in hepatocytes and fatty liver in mice (14), suggesting that hepatic inflammation precedes steatosis in NAFLD. On the other hand, pathogenic drivers for NASH are not likely to be the same with different animal models or all patients. Thus, the mechanisms underlying the pathogenesis of NASH are highly heterogeneous and a parallel hit model has been proposed, in which multiple gut-derived, adipose tissue-derived, as well as systemic factors are considered as drivers inducing hepatic inflammation during NASH(15, 55) (**Figure. 2.2**) (1).

2.3.1 Hepatic lipid metabolism

The liver tissue is the metabolic hub of the body and has a central role in the metabolism of glucose, lipid and proteins. Although there are many variations in the proposed models of NASH pathogenesis, it is well accepted that the liver's capacity to handle energy metabolism plays a significant role. When the liver's metabolic function is overwhelmed, it will lead to the accumulation of toxic metabolites which can induce hepatocellular inflammation, injury and cell death (114-117).

Triglycerides (TG) are synthesized in the liver by FA esterification and are the main form in which lipids are stored in the liver. FAs are primarily delivered into the liver from adipose tissue lipolysis following excessive food intake. A secondary source of FAs is through *de novo* lipogenesis (DNL), a complex process that synthesizes FAs from glucose and fructose derived from the adipose tissue and the liver (4, 118, 119). Upon intake, FAs are bound to fatty acid binding protein (FABP) and then metabolized through mitochondrial β-oxidation or esterification to form TG (54). Patients with NASH have impaired mitochondria function and impaired FA β-oxidation (120, 121). Although it has been proposed that TG formation is a protective response to excess supply of FAs that exceeds the liver's metabolic capacity, some studies also suggest that excessive TG may also contribute to metabolic abnormalities in NAFLD (122-124). Diacylglycerol acyltransferase 2 (DGAT2) is the enzyme that catalyzes the final step in hepatic TG synthesis (6). Using a model in which NASH is induced by maintaining mice on a methionine and choline deficient diet (MCD), Yamaguchi et al. demonstrated that inhibiting TG synthesis through DGAT2 inhibition resulted in amelioration of steatosis in obese mice but exacerbated liver damage and fibrosis (8). Monetti et al. showed that mice with overexpression of liver DGAT2 developed hepatic steatosis with increased TG accumulation in the liver but with normal in plasma glucose

and insulin levels. Insulin resistance, which is a common metabolic syndrome closely linked with NAFLD, was also not observed (7). The formed TGs can be exported to the circulation as very low-density lipoprotein (VLDL) or used to form lipid droplets in hepatocytes. These droplets may serve as the precursor to for FA synthesis in the liver which further worsens lipid metabolism (54).

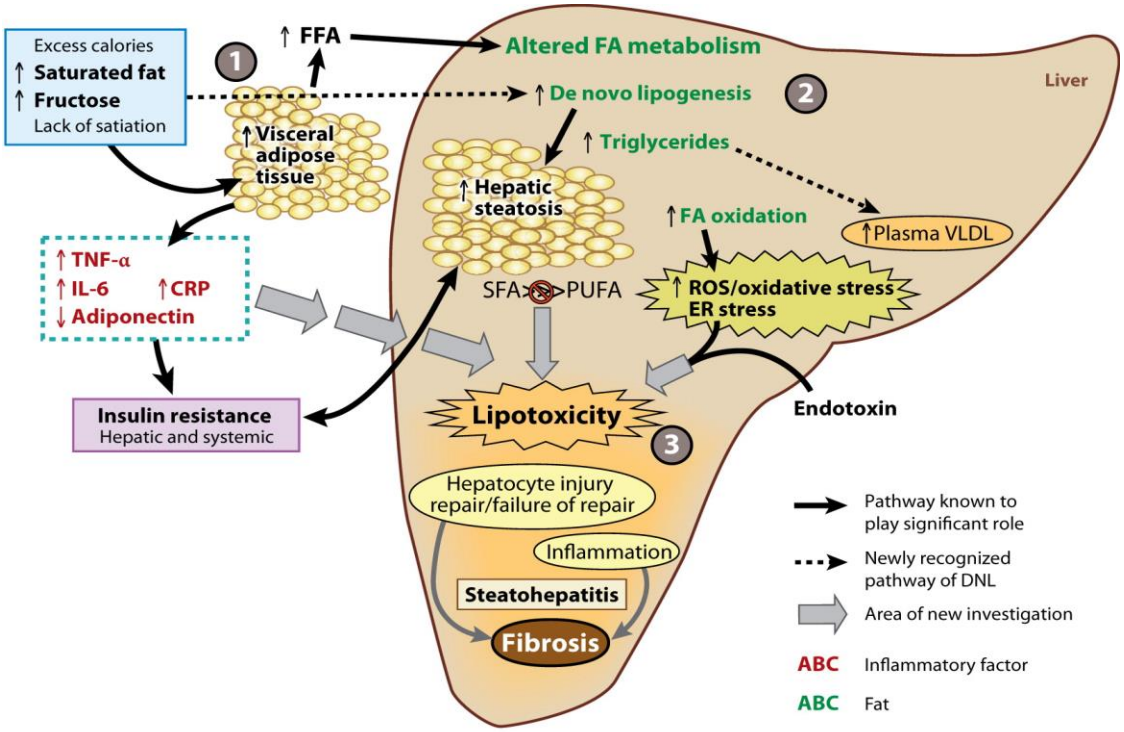

Figure 2.2 Current concepts in the pathways of steatosis and NASH, reprinted from ref. 1 (1). Three major intersecting components are highlighted in current concepts of NAFLD. 1. Increased VAT and insulin resistance. Excess calories intake especially with increased saturated fat and fructose consumption causes VAT increase and stimulates DNL. Increased VAT changes its cytokine profile that secreted to the circulation including increased pro-inflammatory cytokine (TNFα, IL-6) and decreased anti-inflammatory adiponectin to cause and further worse insulin resistance as a feed forward manner. 2. Altered FA metabolism in the liver. With increased DNL from the adipose tissue, FFAs that delivered to the liver is increased, results in an increased load of FA metabolism within the liver. DNL by the liver, esterification into TG as well as FA oxidation are all affected. 3. The effects of lipotoxicity. When the compensatory mechanisms for altered FA metabolisms failed, lipotoxicity happens. In some patients, lipotoxicity leads to NASH and fibrosis.

When the lipid metabolism is dysregulated, toxic lipids such as ceramide, diacylglycerols and lysophosphatidyl choline species are formed and accumulate in the liver, leading to lipotoxicity (125-128). The accumulation of toxic lipids can cause endoplasmic reticulum (ER) stress, oxidative stress, inflammasome activation, as well as activation of apoptotic pathways to cause hepatic injury (129-131). Since cell death can result in further activation of macrophages and production of chemokines leading to infiltration of other immune cells such as monocytes and neutrophils, the initial hepatocyte cell death may act in a feed-forward manner to amplify hepatic inflammation and promote the development of NASH (9). Thus, it has been proposed that hepatocyte death and liver injury is a putative "third hit" in the progress of NASH (9).

2.3.2 The role of adipose tissue in NAFLD

The adipose tissue is also a metabolic active organ that plays an essential role in maintaining energy balance. It can store fat as TG and release free FAs (FFAs) from fat depending on the energy demands (132). The two main sources of FFAs in adipose tissue are uptake from serum or from DNL, and through the activity of transport enzymes such as fatty acid transport protein (FATP) and fatty acid translocase (CD36) (133). In healthy individuals, insulin stimulates GLUT4-mediated glucose uptake in adipose tissue and promotes esterification of FFAs into TG (134). Simultaneously, the activity of lipoprotein lipase (LPL) which is the enzyme that contributes to lipid storage under fed state is increased (135). In obese individuals, the expression of adipose tissue LPL is increased resulting higher FFAs levels from adipose tissue (136, 137). Studies have found that both CD36 and FATP are also overexpressed in obese patients (138). Although the up-regulation of these transport proteins is a compensation mechanism for excessive FAs, over-

expression of CD36 in adipocytes and macrophages within adipose tissue causes inflammation and cell death (139).

Furthermore, with increased caloric intake, enzymes involved in TG synthesis are also up-regulated in order to store excess TG, leading to further enlargement of adipocytes (140). When adiposity is increased, adipocytes become dysfunctional through the onset of insulin resistance and the release of cytokines (140). Insulin resistance in adipose tissue leads to imbalanced FFA release that further results in systemic insulin resistance including the liver (141). Cytokines released from adipose tissue such as IL-6 and TNFα have been shown to have pro-inflammatory effects in the liver (142). Another cytokine that mainly produced by adipocytes and decreased during NAFLD (142) is adiponectin, and has protective effects such as suppression hepatic lipogenesis, suppression of hepatic glucose uptake, stimulation of FAs oxidation as well as inhibition of pro-inflammatory cytokines (143).

2.3.3 Insulin resistance

The liver tissue plays a central role in lipid and glucose metabolism and is sensitive to insulin action (144). NAFLD is associated with features of insulin resistance, and insulin resistance is thought to contribute to the pathogenesis of NAFLD (145). Lipid accumulation in the liver reduces its responsiveness to insulin which causes an increase in the levels of serum glucose and insulin, and results in chronic hyperinsulinemia (146). However, the causal relationship between hepatic fat deposition and insulin resistance is not fully understood (146). Hepatic insulin resistance is associated with FA metabolism and the production of FA metabolites, such as ceramides, DAG and fatty acyl-coA (147). In healthy individuals, insulin stimulates tyrosine kinase activity of the insulin receptor and activates downstream signaling (148). In NAFLD, toxic lipids such as DAG can activate protein kinase C (PKC) which inhibits the insulin receptor kinase, and results in

blocked insulin action. This also leads to reduced activity of phosphoinositol 3-kinase (PI3K) and protein kinase Akt2, which results in the release of glucose into the circulation (147, 149). On the other hand, saturated FAs can cause hepatic insulin resistance through activation of toll-like receptor 4 (TLR4) pathway, leading to NF-κB activation, *de novo* synthesis of ceramides. Ceramides can activate protein phosphatase 2A, which can directly inhibit Akt phosphorylation (150, 151).

Hepatic insulin resistance also increases glucose production and leads to hyperglycemia (152). Moreover, lipid metabolism in the liver is also affected by insulin resistance (153). In NAFLD, hepatic uptake of FAs is increased through the action of insulin on CD36 and increased lipolysis from adipose tissue (4). Hyperinsulinemia as a result of insulin resistance also increases FA synthesis via DNL and increases the production of malonyl-coA to impair FA beta-oxidation. Malonyl-coA inhibits carnitine palmitoyltransferase 1 (CPT1), which is the enzyme that regulates FAs β-oxidation in mitochondria (154, 155). Mitochondria dysfunction, which has been found in parts of NASH patients can also cause insulin resistance and toxic lipid metabolites production which can further impair insulin action (156).

2.3.4 Inflammation

Hepatic inflammation promotes liver fibrogenesis that can lead to cirrhosis and even HCC (157); thus, it has been proposed that inflammation is an important driving force in the development of NAFLD (157). Inflammation is the host response to infection or tissue injury that coordinates clearance of the infection agent, cellular defense, and tissue repair mechanisms via secretion of inflammatory mediators, such as chemokines and cytokines (158). However, persistent inflammation is also harmful as chronic inflammatory responses can exacerbate tissue injury and lead to the development of NASH and fibrosis in NAFLD (158). There are multiple triggers of

hepatic inflammation in NAFLD, including those produced in the liver as well as those derived from the adipose tissue and gut (158).

2.3.4.1 Dietary component and lipotoxicity

Diet plays an important role in the pathogenesis of NAFLD since some dietary components directly promote hepatic inflammation. Previous studies have indicated that toxic lipid metabolites, and not TG, are the primary triggers of liver inflammation and disease progression (114). These triggers include saturated fatty acids (SFAs) such as palmitate and stearate which are the most abundant SFAs in high-fat diet and can cause lipotoxicity directly. SFAs activate JUN N-terminal kinase (JNK) (159-161), enhance ER stress and promote inflammasome activation to induce hepatocyte apoptosis (162). On the other hand, dysregulated lipid metabolism as a result of increased FFAs influx into the liver generates toxic lipid metabolites such as ceramides, lysophosphatidycholine (LPC), diacylglycerols and oxidized fatty acids that can act as ROS (128). Moreover, palmitate-mediated LPC production can also activate pro-inflammatory responses in macrophages through stimulation of pro-apoptotic signaling in hepatocytes (163-165).

Cholesterol has also been shown to cause hepatotoxicity and is associated with the progression of NASH (166). NASH patients have higher levels of cholesterol in the liver compared to healthy individuals (167). The accumulation of cholesterol has been observed in the liver of both mice and human NASH subjects (166), and omitting cholesterol from the high-fat, high-cholesterol diet in mice prevented the formation of foam macrophages and hepatic inflammation (168). The accumulation of free cholesterol in most liver cells such as hepatocytes, macrophages and hepatic stellate cells (HSCs) causes oxidative stress, ATP depletion, mitochondrial dysfunction and apoptosis via disrupting membrane fluidity (169). Cholesterol crystals that present in the lipid droplet of hepatocytes in both human and mouse NASH leads to activation and

cholesterol loading of macrophages (170). Furthermore, cholesterol crystals also activates the NLRP3 inflammasome and caspase 1 in macrophages, leading to TNF and IL-1β secretion (170-172). In summary, the accumulation of cholesterol in macrophages and HSCs activates signaling pathways that might contribute to hepatic inflammation in NAFLD (166, 168, 170, 171, 173).

Another dietary risk factor for the development of metabolic syndrome and NAFLD is fructose (174-176). Fructose reduces mitochondrial β-oxidation and ATP production (177, 178) and upregulates the expression of inflammatory genes in the liver (179, 180). In rats and mice, high fructose intake is associated with hepatocellular ballooning, inflammation, impaired insulin signaling, increased liver fibrosis and dyslipidemia (181-184). Moreover, fructose also disrupts intestinal membrane integrity and causes microbiota dysbiosis that activate liver macrophages to trigger inflammation (185-187). In mice and primates, high fructose feeding has been shown to cause increased TNF and LPS serum levels, and monkeys that fed with fructose showed rapid liver damage in the absence of weight gain and hepatic steatosis (187).

2.3.4.2 Adipose tissue inflammation

It is well accepted that the adipose tissue (AT) dysfunction plays a central role in the development of NASH. Macrophages in visceral AT (VAT) produces cytokines and chemokines, such as TNFα and MCP-1, which correlates with hepatic inflammation and fibrosis in human NAFLD (188). The causal role of VAT inflammation in NAFLD is supported by mouse studies that showed up-regulation of macrophage inflammatory gene expression in VAT preceded gene expression changes in the liver (189). Another hallmark of AT dysfunction is the infiltration and accumulation of macrophages in VAT. The infiltrated macrophages surround dead adipocytes to form "crown-like" structures and are associated with the secretion of pro-inflammatory cytokines, such as TNFα, IL-6 and IL-1β (190).

AT secretes a variety of cytokines, hormones, complements and growth factors that are often referred as adipokines, and their expression is altered in NAFLD. For example, increased levels of leptin and decreased levels of adiponectin are observed in NAFLD, and correlates with disease severity (191). Adiponectin reduces insulin resistance and liver steatosis, and also has anti-inflammatory and anti-apoptotic properties (192, 193). Conversely, TNF and IL-6 promote insulin resistance and their levels are elevated in the serum of NAFLD patients and correlate with NASH severity and fibrosis stage (193).

2.3.4.3 Gut-liver axis

Recent studies have shown that dysbiosis of the microbial community correlates with NASH in both animal models and human studies (194, 195). The underlying mechanism that has been proposed is that dysbiosis causes intestinal barrier dysfunction, leading to bacterial translocation across the intestinal barrier, and eventually to liver inflammation (158). It has been shown that the circulating levels of endotoxin are higher in NASH patients compared to healthy individuals (196). Endotoxin can activate inflammatory cells such as macrophages through the TLR4 pathway and trigger inflammation. Not surprisingly, the absence of TLR4 abrogates hepatic inflammation and liver injury in a mouse model of NASH (197). On the other hand, deletion of the flagellin receptor TLR5 in hepatocytes exacerbates liver disease in mice fed with methionine and choline deficient diet (MCD) and high fat diet (HFD) due to the impaired ability to eliminate circulating bacteria (198). Mice deficient in junction adhesion molecule A (JAMA), a tight junction protein, show increased gut permeability and bacterial translocation, which drives the progression of NASH (199). Patients with NASH also have lower expression of JAMA in the colonic mucosa thus have increased mucosal inflammation (199). Therefore, a reduction in the integrity of intestinal barrier might drive liver inflammation and the progression of NASH.

Bile acids (BAs) are important metabolic molecules that are involved in the pathophysiology of NASH. The synthesis and secretion of BAs require both the liver and the gut microbiota (200, 201). BAs have essential regulatory effects on carbohydrates and lipid metabolism through the farnesoid X-activated receptor (FXR) and G protein-coupled BA receptor 1 (TGR5) (200, 201). Dysregulation of FXR and TGR5 leads to altered lipid and glucose homeostasis and inflammation and fibrosis (200, 201). Furthermore, BAs can shape the intestinal microbiome via their antimicrobial effect and FXR-induced production of antimicrobial peptides (202). The gut microbiota can also modify the composition of BA pool through defined enzymatic activities which potentially promote NASH progression (202). It has been shown in an experimental model of NASH that the transport of BAs in the liver is decreased leading to increased exposure of hepatocytes to BAs and liver injury (203, 204). Puri et al. also reported that conjugated primary BAs were higher in NASH patients and changes in BAs composition were associated with the histological features of NASH (205). Thus, alternations in BAs metabolism might play a role in hepatic inflammation and fibrosis progression.

2.3.4.4 Innate immunity

The innate immune system has been proposed to act as a metabolic sensor against metabolic-related stress such as dysregulated lipid metabolism (158). It eliminates immunogenic molecules through pattern recognition pattern receptors (PRR) that recognize and binds to pathogen associated molecular patterns (PAMP) expressed on bacterial pathogens, and damage associated molecular pattern (DAMP) present in damaged tissue and dead cells (206). As with all immune responses, an overactive innate immune response becomes pathogenic and leads to the progression of NASH (158). It has been shown that PRRs play an essential role in the pathophysiology underlying NASH (207). Toll-like receptors (TLRs) are a well-known PRRs family found in liver

cells (208). Activation of TLR signaling leads to transcriptional activation of inflammatory cytokine and chemokine gene expression (208). TLR2, TLR4 and TLR9 are the most studied TLRs in liver disease especially in NASH and are all found to be overexpressed in NASH livers (208, 209). Genetic ablation of TLR2, TLR4 and TLR9 attenuate liver inflammation in animal models of NASH (209, 210).

Besides TLRs, NOD-like receptors (NLRs) are also found to be associated in NASH. NLRs are intracellular PRRs that recognize both PAMPs and DAMPs (158). The NLRP3 inflammasome is the most studied NLR and found to be related to inflammation and the progression of NASH (158). The expression of NLRP3 is increased in both mice and human NASH, indicating their role in contributing inflammation and fibrosis (211). NRLP3 components are expressed not only on macrophages in the liver but also on non-immune cells such as hepatocytes and HSCs. Kupffer cells (KCs) isolated from NASH mice express higher levels of NRLP3 inflammasome components (212) and activation of NRLP3 inflammasome in HSCs leads to the transdifferentiation of HSCs into collagen-producing myofibroblasts that might contribute to firbrosis (213).

As the most abundant immune cells, KCs are the liver resident macrophages, and play a central role in controlling inflammation. Upon activation, KCs expand rapidly and secrete cytokines and chemokines, such as IL-1β, TNFα and MCP-1 that contribute to protective and apoptotic signaling in hepatocytes and recruitment of other immune cells (214, 215). It has been reported that the infiltrated monocytes in response to MCP-1 signaling is a crucial pathogenic event that promotes NASH and subsequent fibrosis (216, 217). Depletion of KCs in early stage of NASH ameliorated liver damage and abrogated monocytes infiltration (218). Therefore, the secretion of cytokines and chemokines by KCs is thought to be important in the initiation of

NASH, and monocyte infiltration in response to secreted chemokines further augments and amplifies hepatic inflammation (218).

Besides macrophages, bone marrow derived neutrophils can also be activated by PAMPs and DAMPs (219). Like monocytes, neutrophils also respond to chemokines produced by KCs, and after migration to the site of inflammation, induce liver injury through production of ROS, inflammatory mediators and release of granule proteins (220). Once inflammation is resolved, neutrophils undergo apoptosis and induce signaling for repair of damages tissue (220). When neutrophil function is dysregulated, the ability of the organism to resolve inflammation is affected and may contribute to the propagation of inflammation in NASH (220).

2.4 The role of macrophages in NAFLD

As the most abundant immune cells in the liver, macrophages play a central role in restricting and initiating hepatic inflammation (221) (**Figure. 2.3** (222)).

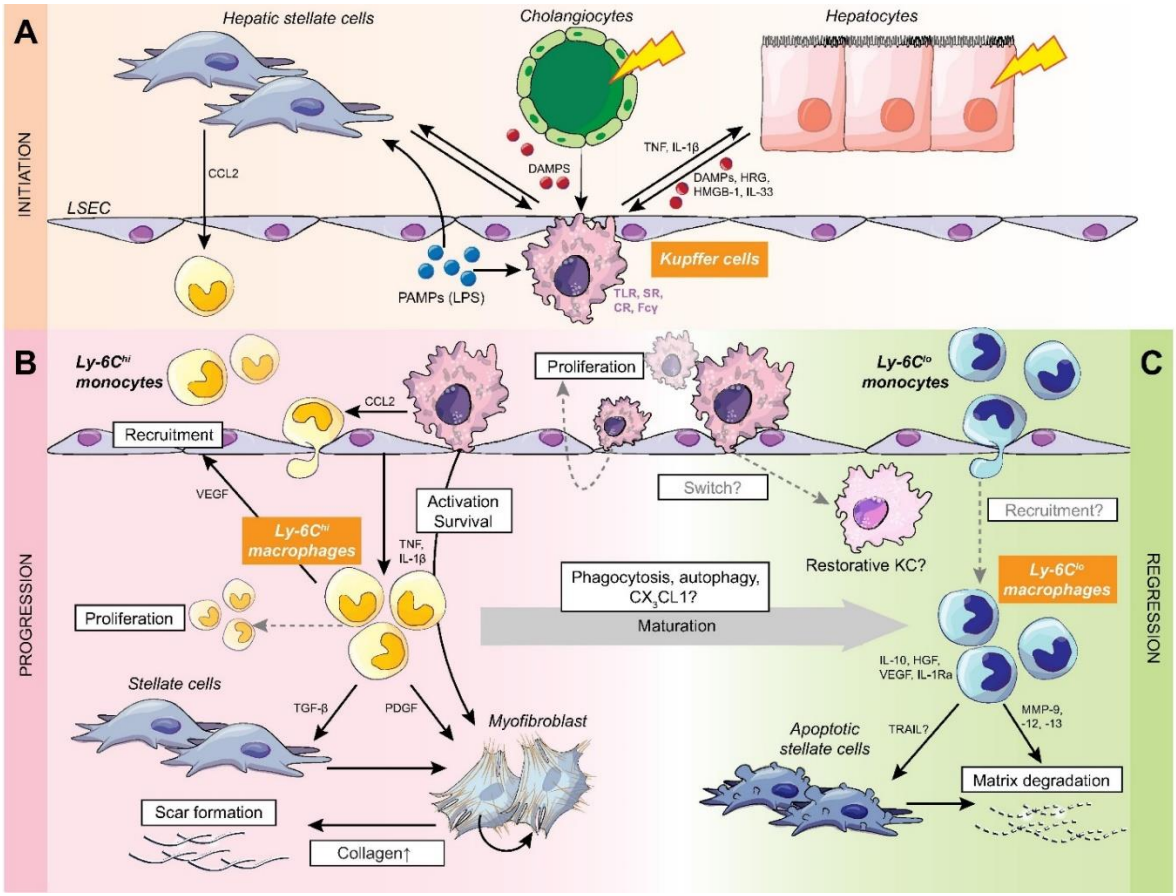

Figure 2.3 Role of macrophages in the initiation, progression and regression of liver diseases, reprinted from ref. 222 (222). A. Role of macrophages in the initiation of liver disease. KCs can be activated by DAMPs that released by hepatocytes or HSCs during liver injury. They also can be activated through translocated PAMPs such as LPS. Activated KCs secretes cytokines such as TNF, IL-1β to further contribute to liver injury. B. Macrophages in the progression of liver injury. Activated KCs also secretes chemokines such as CCL2 to recruit circulated monocytes infiltration. The recruited Ly-6C^{hi} monocytes will differentiate into inflammatory macrophages in the liver to further amplify liver damage. During chronic injury, these Ly-6C^{hi} macrophages activate HSCs to become collagen-producing myofibroblasts to contribute to fibrosis. C. the regression of liver injury. When liver injury cease, Ly-6C^{hi} macrophages mature into anti-inflammatory Ly-6C^{low} macrophages to promote resolution of the tissue injury.

2.4.1 Subtypes of hepatic macrophages

Hepatic macrophages are highly heterogeneous with different functions in the progression of liver diseases (223). There are main two types of liver macrophages: Kupffer cells and monocytes derived macrophages. Kupffer cells (KCs) are liver resident, non-migrating and self-renewing phagocytes that originate from yolk sac-derived progenitor cells during embryogenesis (224-226). KCs are effective phagocytic cells that can recognize, process and degrade foreign material, pathogens and cellular debris to maintain liver homeostasis (227). In healthy individuals, KCs interact with regulatory T cells to maintain immune tolerance (228). However, KCs express several receptors such as pattern recognition receptors (TLRs), scavenger receptors and complement receptors (229). When activated through TLRs upon infection, KCs can also induce immunogenic T-cells responses (228). The expression of scavenger receptors ensure KC function in the clearance of apoptotic cell debris and maintenance of iron homeostasis (230).

The hepatic macrophage pool can be rapidly expanded through recruitment of blood-derived monocytes. In mice, there are two major population of circulating monocytes: Ly-6C^{high} and Ly-6C^{low} monocytes (222). Ly-6C^{high} monocytes expresses PRRs and chemokine receptors such as CCR2 and are thought to differentiate into pro-inflammatory macrophages after infiltration into the liver (231, 232). Ly-6C^{low} monocytes express more scavenging receptors and elicit "patrolling behavior" in the liver (228, 233, 234). It was indicated that Ly-6C^{high} monocytes primarily migrate from bone marrow (235) while the spleen is the major source of Ly-6C^{low} monocytes (236). Under liver injury or hepatic inflammation, KCs and other liver cells secrete chemokines such as MCP-1 to recruit Ly-6C^{high} monocytes to the injured liver for rapidly expanding the macrophage pool (232, 237, 238). Moreover, when KCs are reduced due to liver injury or experimentally depleted, monocytes can replace KCs and generate an indistinguishable

phenotype as KCs (233, 239, 240). In summary, both KCs and monocyte derived macrophages are highly plastic and can exhibit different phenotypes based on the signals in the hepatic microenvironment (241, 242).

Traditionally, macrophages have been described as two functional subgroups: M1 and M2 subtypes (243, 244). M1 macrophages are inflammatory macrophages that secrete pro-inflammatory cytokines such as IL-6, IL-12, TNFα and IL-1β when treated with interferon-γ (IFN-γ), TNFα and LPS *in vitro* (245). M2 macrophages or alternatively activated macrophages are anti-inflammatory cells that are involved in tissue repair and are differentiated in response to IL-4, IL-10 and IL-13 (245). While this model captures some functional aspects of macrophage function, it is too simplistic to describe the polarization of hepatic macrophages. Macrophages are highly plastic and heterogenic and their response are shaped by integration of multiple signals (246). Human monocytes derived macrophages display a broad spectrum of transcriptomic macrophage activation states when exposure to diverse stimuli (247). Macrophages often express inflammatory and anti-inflammatory markers simultaneously in liver injury (232, 242). Therefore, instead of the simple M1/M2 paradigm, a specific macrophage polarization should be described with the source of macrophages, the specific stimuli and markers that used to define macrophages activation (244).

2.4.2 The role of macrophages in liver tissue inflammation

The activation of KCs is a key step in the initiation and progression of liver inflammation in experimental models (248, 249). Ablation of KCs results in significant reduction of hepatic insulin resistance and inflammation in diet-induced steatosis (250, 251). Along the same lines, depletion of KCs prevented pro-inflammatory cytokines production and alleviated liver damage (252). Thus, KCs play an essential role in the pathogenesis of NAFLD, especially the progression from steatosis to NASH. Hepatic macrophages in NASH are pro-inflammatory as a result of the

excess lipid and FFAs (253), signals derived from the surrounding steatotic hepatocytes (254) and DAMP induced KCs activation (255). The accumulation and inflammatory polarization of macrophages in the liver is considered as a hallmark feature of progressive disease in NASH patients (249, 256). Generally, signals that lead to KC activation converge on two main downstream pathways: JNK and NF-κB (257). The JNK pathway can be activated by SFAs, cholesterol crystallization as well as ROS in the set of liver disease (257, 258). NF-κB is a transcription factor that can be activated by diverse stimuli, such as TNFα, IL-1β and TLRs (259). Once activated, NF-κB can modulate inflammatory and cell death signaling through transcriptional regulation (259). Once activated, the inflammatory macrophages can promote the progression of disease in NAFLD through various mechanisms, such as attracting inflammatory immune cells (260), influencing hepatocyte lipid metabolism (261) and promoting liver fibrosis (262).

Inflammatory KCs are capable of secreting a variety of cytokines, such as IL-1β and TNFα to promote inflammation and fibrosis. IL-1β is mainly produced by TLR-activated macrophages and has potent inflammatory effects in the liver (263). It decreases the transactivating activity of PPARα and inhibits FA oxidation, thus promoting triglyceride synthesis in hepatocytes (264). Lack of IL-1β inhibits the progression of steatosis to NASH and liver fibrosis in a mouse model (263). TNFα is also crucial in the progression of NASH as it contributes to the increase of monocyte recruitment and hepatocyte apoptosis (265). Moreover, the engulfment of apoptotic bodies by KCs stimulates TNFα secretion in a feed-forward manner (266). Experimentally, the depletion of KCs or inhibition of hepatocyte apoptosis, attenuated phagocytosis of apoptotic bodies and TNFα secretion. As a result, immune cell infiltration and HSCs activation are attenuated, confirming the role of KCs secreted TNFα in hepatic inflammation and fibrosis (266).

Activated KCs also secrete chemokines such as MCP-1 to recruit circulating monocytes for infiltrating in the liver. It has been demonstrated in mouse models of NASH that the infiltration of Ly-6C^{+} monocytes is a central event promoting NASH and fibrosis (216, 217, 267, 268). In a diet-induced mouse model of NASH, the expression of hepatic CCR2 and MCP-1 are up-regulated; and depletion of KCs improved NASH outcomes with reduced MCP-1 expression and monocyte infiltration (268). Moreover, macrophage migration inhibitory factor-knockout mice showed higher degree of liver inflammation and macrophage inflammation, which further confirms the importance of macrophage recruitment in NASH (269).

2.4.3 Macrophages in liver fibrosis

Macrophages are also essential in the progression of NASH to liver fibrosis. However, both pro- and anti-fibrotic roles for macrophages have been proposed, which may be due to the presence of different macrophage subtypes (259, 270). In response to liver injury, Ly6C^{high} monocytes are recruited into the liver and differentiated into inflammatory macrophages that release cytokines to stimulate HSCs, resulting in fibrogenesis (216, 238, 271). Under homeostasis, HSCs are quiescent cells (270); however, cytokines secreted from inflammatory macrophages such as TGF-β and PDGF-bb can stimulate HSCs to differentiate into activated myofibroblast-like cells (271, 272). Activated HSCs secrete extracellular matrix (ECM) and tissue inhibitors of metalloproteinases (TIMPs) to alter the balance between ECM synthesis and degradation thus leads to fibrosis (270, 271). Furthermore, the secretion of TNFα and IL-1β from inflammatory macrophages can promote HSCs survival by inducing NF-κB signaling (270, 271). As a result, depletion of KCs attenuates the progression of liver fibrosis in mice models (270).

On the other hand, macrophages can switch their phenotype from fibrogenic to "restorative" to promote the resolution of fibrosis (242). These macrophages are characterized by

Ly-6C^{low}, high expression of matrix degrading metalloproteinses, such as MMP-9 and MMP-12 and anti-inflammatory mediators such as IL-10 and HGF in mice (242, 273). The mechanisms that underlying the phenotypic switch are not fully understood yet. It was suggested that reduction in the expression of DAMPs and phagocytosis of cell debris as a consequence of liver injury cessation may play a role (242, 274). Moreover, the activation of autophagy in macrophages was thought to favor the maturation of restorative macrophages in NASH (275, 276).

2.5 The role of microbiota in NAFLD

The human body is colonized by more than 3 x 10^{13} microbes, with a majority of them resident in gastrointestinal (GI) tract (277-279). Next-generation sequencing studies have significantly expanded our knowledge of the intestinal microbiota under both health and disease states (280). Although a majority of the microbiota remains uncharacterized, several studies have identified key members and the composition of microbiota under specific conditions such as intestinal inflammation and metabolic syndrome (281). These studies have clearly demonstrated that the microbiota have a variety of functions in the GI tract including defense against pathogens (38), modulation of inflammation (38) and development of immune system (37). Alternation of the microbiota has also been found to have profound effect on the host (282-285). Beyond the GI tract, gut-brain (30), gut-liver axis (28) and the association of gut microbiota in the development of obesity, diabetes as well as NAFLD (286-288) have been identified.

The relationship between gut and the liver was observed as early as 1950's when researchers noticed that non-absorbable antibiotics prevent cirrhosis in an animal model of NASH (289). More recently, studies have shown that germ-free mice are resistant to high-fat diet induced hepatic steatosis and obesity (290). Furthermore, fecal transplantation experiments in mice models have shown that obesity and NASH are transmissible by microbial components in the feces (35,

285). These data showed more direct evidence for the contribution of gut microbiota in the progression of liver diseases. The gut microbiota compositional changes that observed in both animal models and human patients have been proposed to contribute to NAFLD. The underlying mechanisms included affecting metabolism and energy harvesting, dysbiosis-induced intestinal inflammation and gut barrier dysfunction as well as interactions with the systemic immune system (291, 292) (**Figure 2.4** (293)).

2.5.1 Intestinal microbial composition and NAFLD

The microbiota in the human intestine is dominated by four main phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Actinobacteria*, (294) with *Bacteroidetes* and *Firmicutes* being the most abundant ones and accounting for 70% of the community (295). Altered microbiome composition (such as ratios of *Bacteroidetes* to *Firmicutes*), abundance of specific taxa (i.e., microbiota dysbiosis) have been correlated with NAFLD in both human and experimental models (31, 32, 296). Furthermore, the changes in microbiota composition has been shown to correlate with disease severity, and specific bacteria such as *Bacteroides* have been associated with disease phenotypes in NAFLD development (297-300).

Profiling the intestinal microbiome of NAFLD patients and healthy controls led to the identification of phyla, genera and families that differed in their abundance between patients and healthy individuals (301). Wang et al. reported that Gram-negative bacteria were significantly enriched and Gram-positive bacteria were decreased in diagnosed NAFLD patients compared to lean controls (33). At the phylum level, the authors found that the *Bacteroidetes* were increased and *Firmicutes* were decreased in NAFLD patients (33). Another study reported that, two genera, *Bateroides* and *Prevotella* were significantly different from healthy controls in fecal samples from NASH patients (195). The abundance of *Bacteroides* was higher while that of *Prevotella* was lower

in NASH patients (195). Since plant material-rich diet favors *Prevotella* and HFD favors *Bacteriodes*, it has been proposed that changes in the diet can cause imbalance between the two genera (302). The increased abundance of *Bacteroides* has been shown to correlate with increased levels of the bile acid deoxycholic acid and oligosaccharides, as well as decreased levels of SCFAs and amino acids (303). Deoxycholic acid also induces hepatic apoptosis in rat (304) and was found to at higher levels in human livers with NASH (204). Boursier et al. reported that the abundance of *Ruminococcus* was associated with fibrosis severity (195). However, the underlying mechanism is not yet clear due to the heterogeneity of the *Ruminococcus* genus (194).

Besides compositional changes in the gut microbiota, NAFLD patients are also been found with bacterial overgrowth in the small intestine (32). The prevalence of small intestine bacteria overgrowth (SIBO) is increased from 21% in healthy individuals to 56% in NAFLD patients (305). However, the correlation between SIBO and the presence of NASH, hepatic inflammation and severity of fibrosis is not significant (305). Since the endotoxin levels in patients with SIBO is significantly higher (306-308), it has been proposed that the impaired intestinal epithelial barrier as a result of SIBO may contribute to the development of NAFLD (309).

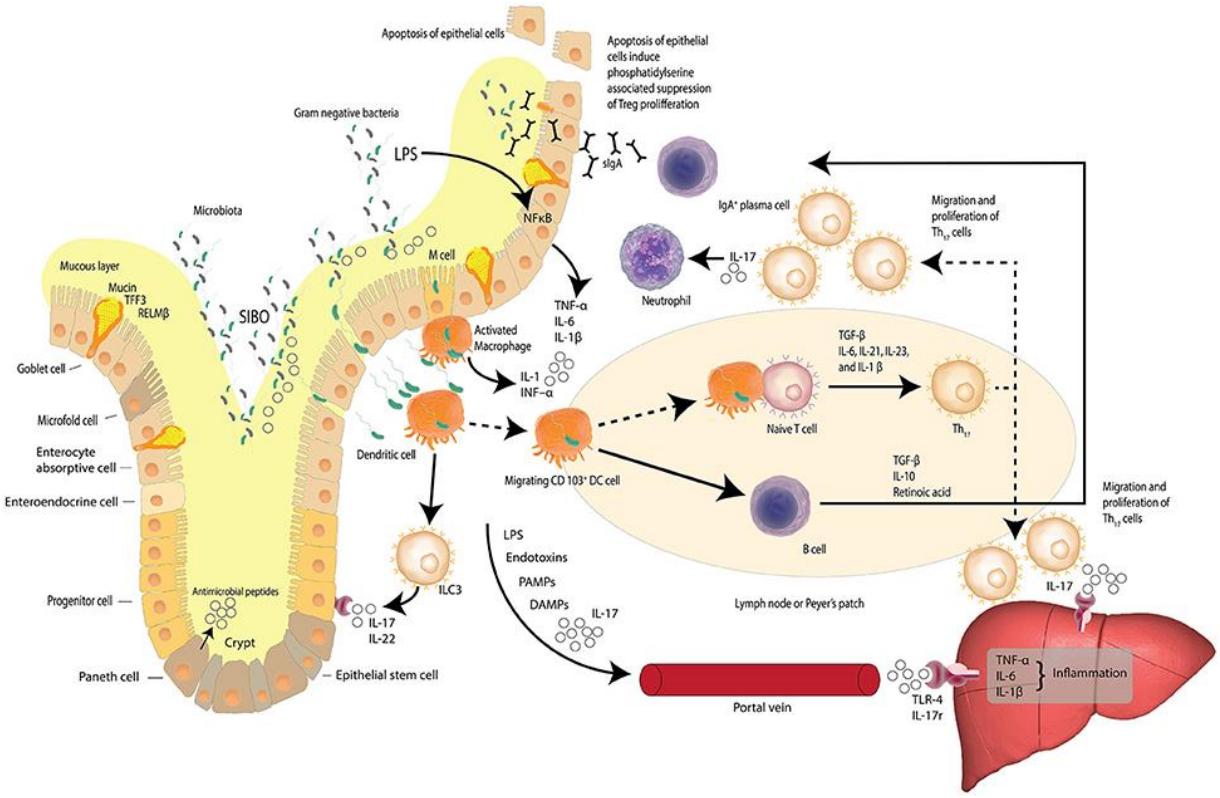

Figure 2.4 Dysbiosis and liver diseases, reprinted from ref.293 (293). Imparied intestinal barrier integrity, small intestine bacterial overgrowth and elevated serum endotoxin such as LPS are found in animal models and patients with NASH. Intestinal microbiota dysbiosis cause increased intestinal permeability thus leads to increased bacterial translocation. The translocated bacterial and bacterial products will activate macrophages and dendritic cells to secrete pro-inflammatory cytokines sch as TNFα, IL-1β and IL-6. Activated macrophages and DCs promote the differentiate of naïve T cells into Th17 cells to promote intestinal inflammation. Pro-inflammatory IL-17 secreted by Th17 cells will stimulate monocytes, KCs and HSCs to secrete pro-inflammatory cytokines to promote liver inflammation. On the other hand, endotoxin such as LPS, PAMPs and DAMPs can reach the liver through protal vein to activate liver macrophages to lead to hepatic inflammation.

2.5.2 Intestinal inflammation and intestinal barrier dysfunction

Intestinal barrier dysfunction has been associated with hepatic inflammation due to increased bacterial translocation (196). It has been proposed that the compositional alternation and dysbiosis in the gut microbiota adversely affects the gut epithelial barrier and exacerbate epithelial cells dysfunction (293). Intestinal macrophages can be affected by dysregulated epithelial cells to act as a critical effector in the pathogenesis of metabolic diseases including NAFLD (310). Intestinal epithelial cells form a barrier to the external environment through the tight junction protein (TJP) network which allows selective permeability and limits bacterial translocation. However, the TJP network is impaired in NAFLD patients compared to healthy controls (311, 312), suggesting that compromised barrier integrity results in increased intestinal permeability and bacterial translocation (312). This is supported by the observation that the serum levels of tight junction proteins is higher in NAFLD patients than lean control (311), suggesting that intestinal barrier dysfunction is a contributing factor to the NAFLD pathogenesis.

As a consequence of impaired intestinal barrier, endotoxin levels in NASH patients are significantly higher and the translocation of endotoxin from gut microbiota to the liver can activate immune cells in the liver to induce inflammation (194). Consistent with this, TLR4 and MD2 deficient mice were protected from diet induced steatosis and liver inflammation (197).

Another proposed mechanism through which gut microbiota could affect the physiological function of other organs such as the liver is by increasing pro-inflammatory activity in the intestinal mucosa and indirectly affecting inflammation in the liver (293). Many bacterial pathogens and their products can cross the epithelial barrier through M cell junction to activate intestinal macrophages (313). Increased levels of inflammatory cytokines might result in increased epithelial barrier permeability and further exacerbate inflammation in the liver (314, 315). It has been

reported that the gut immune cells show a pro-inflammatory shift upon HFD feeding in mice, as well as in obese patients (316). Treatment with a gut anti-inflammatory agent 5-aminosalicylic acid (5-ASA) reduced intestinal inflammation and insulin resistance (316). Further investigation found that, the intestinal permeability and liver steatosis were improved upon 5-ASA treatment indicated the association between intestinal inflammation, gut permeability change and progression of liver inflammation (316).

2.5.3 Microbiota derived metabolites

In addition to the gut microbiota compositional change, the metabolites produced by the microbiota are now increasingly proposed as important signaling molecules that contribute to improper immune cell activation and impaired epithelial barrier function (317) (**Figure 2.5** (317)).

Ethanol is a common metabolite produced by a variety of bacteria in the gut, and endogenous ethanol production has been implicated as a pro-inflammatory stimuli in pathogenesis of NAFLD (318, 319). It has been reported that ethanol-producing bacteria are enriched in patients with NAFLD and contributes to the progression of the disease (32, 311, 320). The underlying mechanism might be that, ethanol increases intestinal epithelial permeability to cause hepatic inflammation (311, 321).

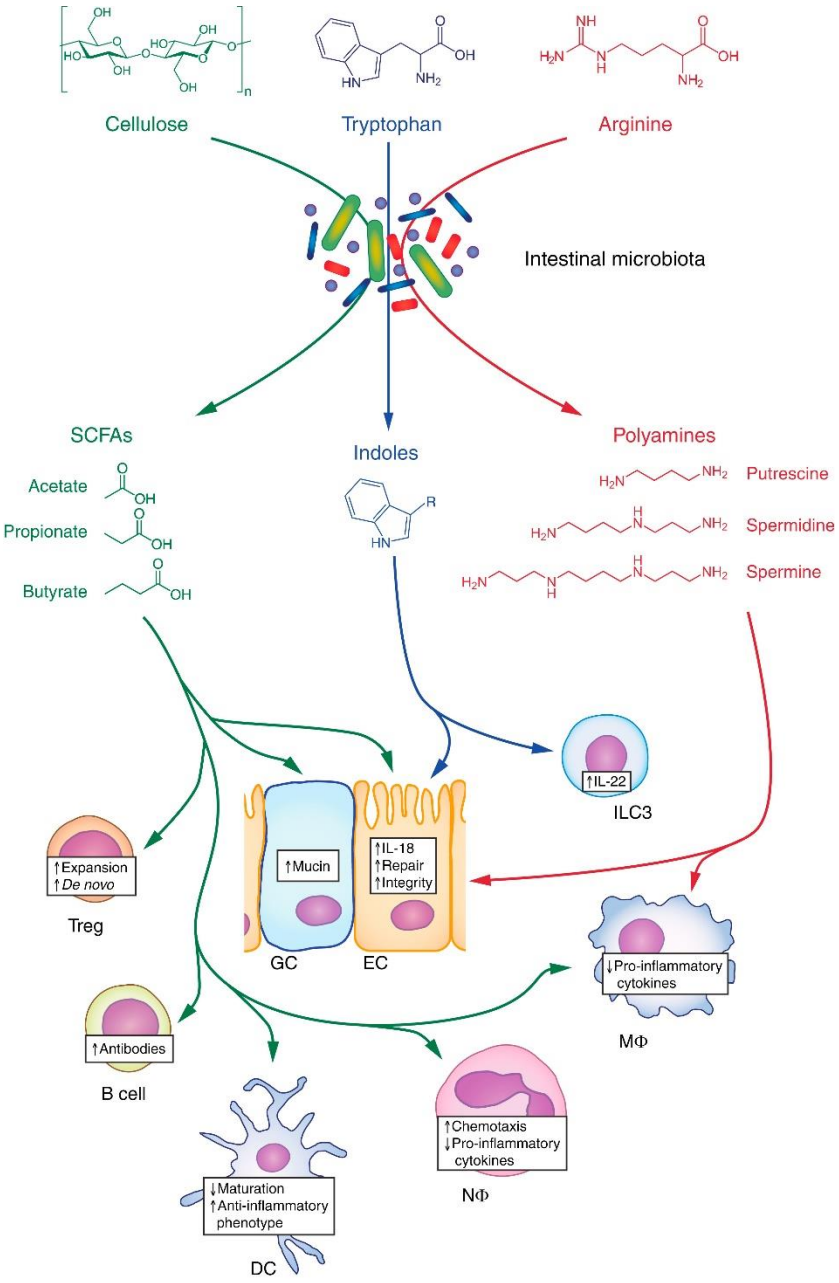

Figure 2.5 Immunomodulatory metabolites produced by intestinal microbiota from dietary nutrients, reprinted from ref.317 (317). SCFAs that metabolized by intestinal microbiota from dietary fiber such as cellulose has a variety of anti-inflammatory effects. They can enhancing epithelial barrier function through goblet cells and epithelial cells, promote expansion and de novo differentiation of regulatory T cells, promote an anti-inflammatory phenotype of innate immune cells. Indole derivatives produced by intestinal microbiota from dietary amino acids also promote epithelia barrier function through their effects on epithelial cells as well as supporting the maintenance of type 3 innate lymphoid cells.

Intestinal microbiota can produce SCFAs through fermentation of plant-derived polysaccharides that cannot be digested by host enzymes (322). The most abundant intestinal SCFAs are butyrate, acetate and propionate (323). Some of the SCFAs produced in the intestine are readily taken up by the host to reach different organs. While butyrate is primarily metabolized within the intestinal epithelial layer and serves as a major energy source for colonocytes (324, 325), the remaining butyrate is metabolized in the liver (326, 327). Most of the acetate and propionate can reach the liver, and while acetate is released into the circulation, a portion of propionate is metabolized in the liver (323, 326). SCFAs have been shown to have anti-inflammatory effects in the intestine (328) and have a protective role in animal models of IBD (329-331). Moreover, patients with IBD have been shown to have decreased abundance of SCFA-producing bacteria in the gut, and increased SCFA production through dietary changes has been shown to ameliorate IBD symptoms (332-335).

The anti-inflammatory effects of SCFAs are also observed in different cell types of the immune system. SCFAs can promote epithelial barrier integrity by activating NLRP3 inflammasome in intestinal epithelial cells to induce the secretion of IL-18 which is important in epithelial repair (336-338). Beside IL-18 secretion, SCFAs also can promote the production of mucin by goblet cells (339, 340). SCFAs reduces the production of pro-inflammatory cytokines from LPS-activated neutrophils and macrophages (341-343). Studies have also shown that SCFAs can promote the production of antibodies by modulating the energy metabolism of B cells (344). Lastly, the most potent anti-inflammatory function of SCFAs is that they can promote the expansion and *de novo* differentiation of regulatory T cells (Tregs) (329, 331, 345, 346).

While the beneficial effects of SCFAs are well established, recent studies by our lab and other groups have identified that metabolites derived from aromatic amino acids also have

pronounced anti-inflammatory effects on host (39, 40). For example, Bansal *et al*. showed that indole, produced from dietary tryptophan by the bacterial enzyme Trytophanase A, attenuated TNFα-mediated NF-κB activation and expression of IL-8, while promoting anti-inflammatory IL-10 production and increasing tight junction resistance in HCT-8 intestinal epithelial cells (39). Shimada *et al*. (40) extended these observations to an *in vivo* model by showing that indole increases the expression of genes that promote intestinal barrier function and attenuated indicators of inflammation in a mouse model of colitis. Wikoff *et al.* showed that indole-containing molecules were depleted in germ-free mice and their levels in normal mice was dependent on the presence of an intact bacterial community (43).

Kynurenine produced from tryptophan by the host through Tryptophan dioxygenase activity has been shown to play an important role in maintaining host homeostasis through the activation of aryl hydrocarbon receptor (AhR) (347). Indole-3-aldehyde was shown to promote the transcription of IL-22 to contribute to antifungal resistance in mouse (41). Alexeev *et al*. reported that indole and indole-derived metabolites were depleted in human and murine colitis (348), and of these, indole-3-propionic acid (IPA) was diminished in the serum of patients with active colitis (348). Oral administration of IPA significantly ameliorated disease in a mouse model of colitis and it has been proposed that indole and indole derivatives can up-regulate the expression of IL-10 receptor in host cells to promote anti-inflammatory functions (348). Lamas *et al*. found that mice deficient for *Card9* which is a gene that associated with human IBD susceptibility have lower amounts of indole derivatives produced in the murine gut (349). This observation is similar to that made with IBD patients and gives compelling evidence on the significance and relevance of indole derivatives as mediators of inflammation through their AhR-ligand activity.

In addition to converting dietary substrates, the gut microbiota can also metabolize substrates that secreted into the intestinal lumen by the host. Primary bile acids are synthesized and conjugated to taurine or glycine in the liver (350). The primary bile acids is transported from the liver to the intestine through the bile duct and converted to secondary bile acids through deconjugation and dehydroxylation by gut microbiota (351). It has been reported that, free taurine generated by deconjugation of primary bile acids can increase the production of IL-18 by intestinal epithelium to maintain epithelial barrier function (352). The gut microbiota plays an important role in regulating bile acids composition. This is supported by the observation that the composition of bile acids varies dramatically between control and germ-free mice in the liver, feces and plasma (353-355).

Bile acids have been reported to act as signaling molecules in several tissues. For example, bile acids reduce the expression of pro-inflammatory cytokines in macrophages, dendritic cells and Kupffer cells (356). These effects are mainly mediated by two receptors: the bile acid receptor FXR, and the G protein- coupled bile acid receptor TGR5 (357-359). Studies have found that the activation of FXR in LPS-activated macrophages by synthetic agonists induced FXR-dependent gene expression and inhibited NF-κB activated gene expression; thus, resulting in a significant reduction of pro-inflammatory cytokine expression (360, 361). In a murine models of colitis, treatment of FXR agonists has protective effects and FXR deficient (*Nrlh4*$^{-/-}$) mice exhibit exacerbated colitis (361, 362). Moreover, *Nrlh4*$^{-/-}$ also develop hepatic inflammation and HCCs spontaneously (363, 364) and the inflammatory effect of FXR deficiency in the liver may be mediated by Kupffer cells as well as hepatocytes and NKT cells (365, 366). Similar to FXR, activation of TGR5 on macrophages and Kupffer cells by agonists also inhibited LPS induced pro-inflammatory cytokines expression by inhibiting NF-κB dependent transcription (367-369).

2.6 AMP-activated protein kinase (AMPK)

As a ubiquitously expressed protein kinase, AMPK is a central energy regulator that may be important for the treatment of metabolic diseases, such as obesity, T2D and NAFLD (16, 17). When the energy status is changed in the cell, the ratio of ATP to ADP or ATP to AMP is altered, and cellular AMPK is activated (phosphorylated) through an allosteric mechanism (370, 371). Once activated, AMPK is capable of phosphorylating key proteins in multiple pathways to change the cellular metabolism towards increased catabolism and decreased anabolism(372). Moreover, AMPK can regulate cellular metabolism via targeting transcription regulators involved in metabolism regulation (373, 374).

2.6.1 AMPK structure and activation

AMPK is a heterotrimeric complex comprised with a catalytic (α) subunit and two regulatory (β and γ) subunits. Each subunit has multiple isoforms that is encoded by different genes. In humans, there are two α-subunits (α1 and α2) that encoded by the genes *Prkaa1* and *Prkaa2*(375), two β-subunits (β1 and β2) (376), three γ-subunits (γ1, γ2 and γ3) (377). Each AMPK complex contains one α-subunit, one β-subunit and one γ-subunit. Since all combinations of different isoforms are possible, there are potentially 12 distinct AMPK complexes (378). Different combinations may elicit different function with different cellular localization. For example, only the α1β2γ1, α2β2γ1, α2β2γ3 are detected in muscle and only the α2β2γ3 complex is activated following exercise (379).

AMPK α-subunit contains a kinase domain on its N terminus and binding domains for β and γ subunits on its C terminus (380). The AMPK β-subunit acts as a scaffold to maintain the AMPK heterotrimer structure and is critical to control the enzymatic activity upon activation (381-383). The γ-subunit is the regulatory subunit capable of binding adenine nucleotides such as AMP or ATP to allosteric modulate AMPK (384). Regulation of AMPK activity is complex and involves

several aspects: allosteric regulation by AMP and ATP, phosphorylation by upstream kinase and dephosphorylation by phosphatases. The binding of AMP to the γ-subunit has been proposed to stimulate the activity of upstream kinase to phosphorylate Thr172, inhibit the dephosphorylation of Thr172 by phosphatase and cause allosteric activation of AMPK that already phosphorylated on Thr172 (385).

AMPK can be activated by phosphorylation of Thr172 on the α-subunit by an upstream kinase, and three such AMPK upstream kinases (AMPKK): LKB1 (386, 387), $Ca2^{+}$/calmodulin-dependent protein kinase kinase β (CaMKKβ) (388, 389) and TGF-β- activated kinase 1 (TAK1) (390) have been identified to-date. LKB1 is a tumor suppressor and responsible for the majority of AMPK activation in most examined mammalian tissues including the liver and muscle (386, 387, 391, 392). AMPK also can be activated in response to calcium flux by CaMKKβ (388, 389, 393). CaMKKβ can be activated by increased intracellular calcium level and leads to the phosphorylation of Thr172 independent of LKB1 and AMP levels (388, 389). TAK1 kinase is upstream of LKB1 and AMPK (390) but studies in yeast also suggest that it can directly phosphorylate AMPK (394). A variety of physiological conditions, such as nutrient starvation, exercise and mitochondria inhibition has been shown to lead to the activation of AMPK *in vivo* (372).

2.6.2 The role of AMPK in metabolism and inflammation

As an energy sensor, AMPK can directly phosphorylate factors in multiple pathways to regulate energy balance. The role of AMPK in energy metabolism has two aspects: inhibits anabolism to minimize ATP consumption and enhances catabolism to increase ATP production (372) (**Figure 2.6** (395)).

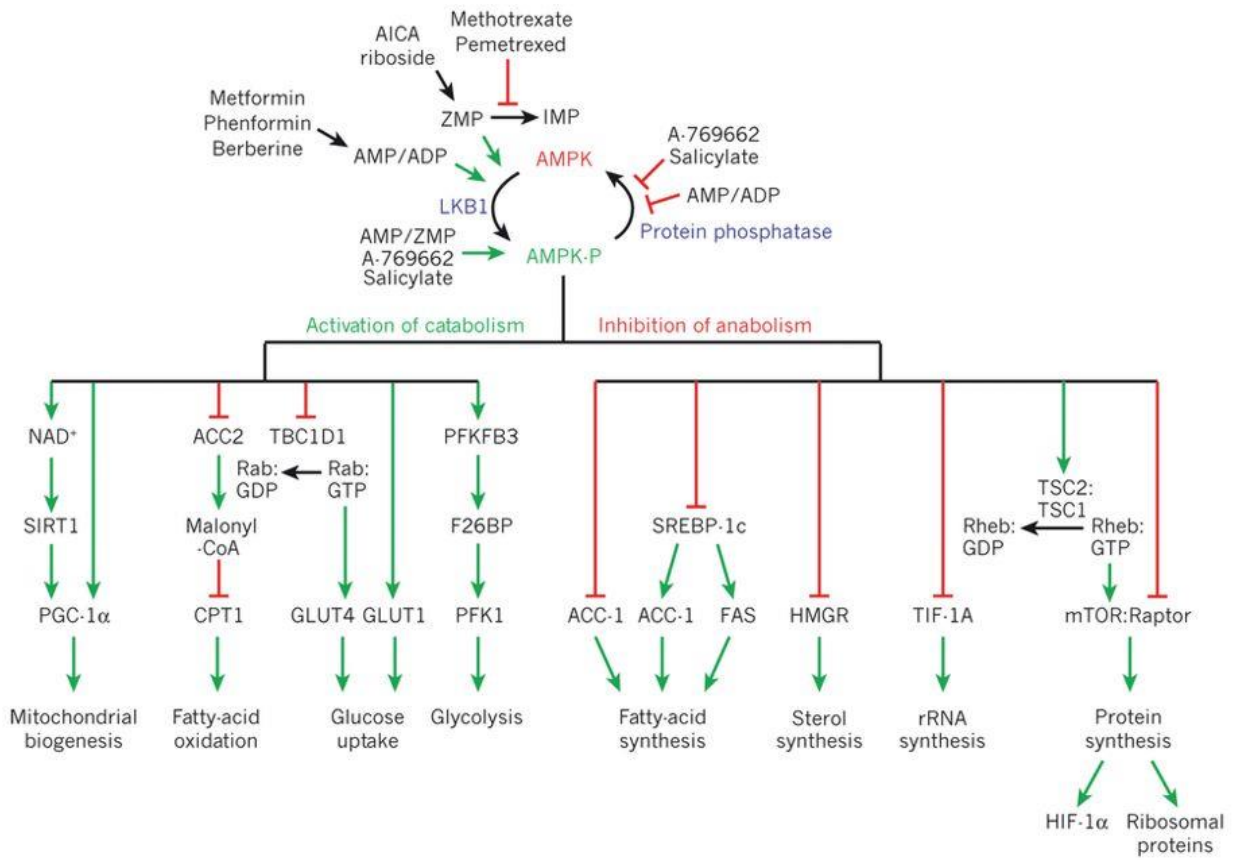

Figure 2.6 Metabolic pathways regulated by AMPK, reprinted from ref.395 (395). AMPK can be activated by upstream kinase such as LKB1 and deactivated by protein phosphatase. The activation of AMPK signaling will activate cellular catabolism and inhibit anabolism. One mechanism that underlying AMPK's anti-inflammatory effects would be its inhibition of glycolysis through mTOR and promotion of fatty-acid oxidation and mitochondrial biogenesis.

AMPK can inhibit lipid synthesis by phosphorylating acetyl-CoA carboxylases (ACC) 1 and ACC2, which are the rate-limiting enzymes in DNL (396, 397). The inhibition of sterol synthesis by AMPK is through inhibitory phosphorylation of the HMG-CoA reductase (HMGCR), which is an important enzyme in the synthesis of cholesterol (396, 397). Due to its inhibitory effect in lipid synthesis, liver specific expression of activated AMPK has been shown to reverse steatosis

(398). AMPK also regulates metabolism at the transcriptional level. It can regulate the transcription of key regulators of lipid and glucose metabolism, such as sterol regulatory element-binding protein 1 (SREBP-1) (399), carbohydrate-responsive element-binding protein (ChREBP) (400) and hepatocyte nuclear factor 4α (HNF4α) (401). AMPK can also regulate cell growth and protein translation through mTOR (372). Activation of AMPK can inhibit mTOR through two different mechanisms: inhibition of the mTOR subunit RAPTOR (402) and activation of TSC2 which is a negative mTOR regulator (403). Under energy stress, the inhibition of mTOR by AMPK results in decreased protein synthesis and cell growth that slow the consumption of ATP (372, 402, 403).

AMPK also stimulate catabolism to produce ATP through stimulating pathways, such as mobilization of lipid stores, utilization of glucose and autophagy. AMPK promotes glucose uptake into the cells through activate proteins involved in the trafficking of glucose transporter (404, 405). AMPK also promotes lipolysis by activating lipase to release fatty acids from triglyceride pool in the liver and adipose tissue (406). The released FFAs are then transported into mitochondria for β-oxidation. Interestingly, AMPK activation can modulate the activity of acyl transferase CPT1, which is an important enzyme that required for the transportation of FFAs into mitochondria. ACC1 and ACC2 also generate a potent CPT1 inhibitor, malonyl-CoA, and the inhibition of ACC1 and ACC2 by AMPK decreases the production of malonyl-CoA, results in increased β-oxidation and decreased lipid synthesis (407, 408).

Chronic low-grade inflammation is a critical characteristic of metabolic diseases including NAFLD, and AMPK activity is reduced in response to LPS, TNFα, and HFD-induced obesity in multiple tissues, such as adipose tissue (409-411), muscle (412), and the liver (409, 413). A decrease in AMPK activity is also observed in adipose tissue of obese patients (414), and the

activity of AMPK is reversed following diet-induced weight loss (415). HFD and inflammatory stimuli have been shown to decrease the expression and activity of AMPKα1 in mouse adipose tissue and macrophages, and AMPK activator AICAR treatment reversed diet and LPS induced inflammation (410, 416). On the other hand, anti-inflammatory cytokines, such as IL-10 and TGF-β stimulated activation of AMPK in both mouse and human macrophages (416). The underlying mechanism is largely unknown, but increased protein phosphatase 2A (18) and 2C (412), inhibitory phosphorylation in the α-subunit by protein kinase D (417), Akt (418-421) and S6 kinase (422) have been proposed.

AMPK also inhibits acute inflammatory responses in macrophages. It has been shown that reduced expression of AMPKα1 attenuated pro-inflammatory cytokine production and NF-κB signaling (19, 410), while activation of AMPK by pharmacological activator or constitutively activated AMPK elicited the opposite effect (19, 410). Yang *et al.* utilized a macrophage-adipocyte co-culture model and found that inactivation of AMPK in macrophages inhibited adipocyte insulin signaling and glucose uptake (410). Consistent with *in vitro* studies, myeloid-deficient AMPKα1 knockout mice (423) and mice lacking AMPKβ1 in hematopoietic cells (424) showed an increased accumulation of inflammatory macrophages in adipose tissue and the liver, and when fed with HFD, these mice showed accelerated development of insulin resistance.

The mechanisms underlying AMPK's anti-inflammatory effect in macrophages is still unclear, but reduced FAO has been proposed by multiple studies. As mentioned above, the inhibitory phosphorylation of ACC1 and ACC2 by AMPK leads to reduced production of malonyl-CoA thus stimulating FAO and inhibiting lipogenesis (424). Mice lacking ACC activity in T-cells showed decreased Th-17 cells and increased Treg polarization which is in consistent with the important role of AMPK mediated ACC inhibition in lowering inflammation (425). In addition to

the inhibition of ACC, the reduction of AMPK activity is also shown to be accompanied by decreased mitochondrial function (424, 426) and increased lipogenesis (427) which all could contribute to decreased FAO. Moreover, the increased FAO increases the expression of retinaldehyde dehydrogenase type 2 (RALDH2) (428). Retinoic acid synthesized by RALDH2 has anti-inflammatory effects in macrophages and is essential for recruiting Tregs (428).

AMPK also can regulate key proteins involved in inflammatory pathways. AMPK inhibits JAK1 (429), NF-κB (410, 430, 431) and CHOP (432, 433) while increases the activity of p38 (434), MAP kinase phosphatase-1 which is a JNK inhibitor (434) and PIAS1 (435), a STAT1 inhibitor. Although AMPK has a variety of direct phosphorylation targets, the inhibitory effect on NF-κB signaling is indirect and through downstream mediators, such as SIRT1 (410), peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) (431) and Forkhead box O (FoxO) family (430). SIRT1 is a deacetylase that could deacetylate p65 subunit of the NF-κB complex to inhibit NF-κB signaling (436). Moreover, the deacetylation of p65 could trigger the ubiquitination and degradation of p65 (437). AMPK can activate SIRT1 via increasing cellular NAD^+ levels (438). Conversely, SIRT1 can activate LKB1 to further activate AMPK in a feedforward manner to enhance anti-inflammatory effect and cell survival (439).

PGC-1α is an important regulator of energy metabolism and has been shown to bind to the NF-κB p65 subunit in human cardiac cells and mouse heart (431). On the other hand, the activation of NF-κB signaling increases the interaction between PGC-1α and p65, results in reduced expression of PGC-1α (431). In hepatocytes, Morari *et al*. found that PGC-1α favors association with the p50 component of NF-κB when stimulated with fatty acids (440). PGC-1α and p50 can bind to the IL-10 promoter to induce IL-10 expression (440). Studies also showed that PGC-1α and NF-κB are reciprocally-regulated. Increased expression of PGC-1α could inhibit NF-κB

activity (441) and NF-κB signaling can decrease the expression of PGC-1α (442). AMPK can phosphorylate PGC-1α protein to trigger SIRT1-mediated deacetylation and activation of PGC-1α (431).

The activation of AMPK can also attenuate oxidative stress (443-446). AMPK signaling reduces the production of superoxide radicals in endothelial cells through upregulating the expression of mitochondrial uncouple protein-2 (UCP-2) (445). Another study also observed that AMPK can suppress ROS production through NADH oxidase (443). Moreover, AMPK activation can inhibit inflammasome activity (447) and ER stress (448-450) that are increased in many metabolic diseases.

2.6.3 The role of AMPK in NAFLD

It has been shown that hepatic AMPK activity is reduced during NAFLD (451) despite the lower ATP levels (452). Inflammatory factors that are elevated with NAFLD, such as LPS, TNFα and FFAs are proposed to be responsible for the reduction of AMPK activity (412, 424). Activation of AMPK could affect the progress of NAFLD through three mechanisms: increasing hepatic fatty acid oxidation, inhibiting hepatic DNL and promotion of mitochondrial function and integrity (20). AMPK β1-knockout hepatocytes show reduced FAO and increased lipogenesis due to the reduction in ACC inhibition by AMPK (453). On the other hand, overexpression of AMPK in hepatocytes leads to reduced triglyceride levels and lipid accumulation *in vitro* (454). Overexpression of AMPK in the liver reduced hepatic steatosis, the expression of lipogenic genes in type 2 diabetes rat *in vivo* (455). Consistently, mice with constitutive activated AMPK in the liver showed lower rate of lipogenesis and when fed with a high-fructose diet, mice were protected from NAFLD and insulin resistance (398). These effects of AMPK on promoting FAO and inhibiting DNL are likely mediated through the inhibitory phosphorylation of ACC. This is

supported by the observation that hepatocytes that lack the AMPK phosphorylation site on ACC1 and ACC2 have reduced FAO and increase fatty acid synthesis (456). A recent study by Harriman et al. showed that inhibition of ACC by a pharmacological inhibitor also showed increased FAO and reduced lipogenesis in isolated hepatocytes, and the ACC inhibition resulted in alleviated NAFLD in an animal model (457). In summary, AMPK could positively affect NAFLD through phosphorylation of ACC.

AMPK also regulates hepatic fatty acid metabolism via the regulation of mitochondrial biogenesis (373), autophagy (458) and fission (459, 460). The downstream mediators of this effect might be PGC-1α, histone deacetylases (HDAC) (373), mitochondria fission factor (MFF) (459, 460) and unc-51-like autophagy activating kinase 1 (ULK1) (458). Studies have shown that dysregulation of these pathways leads to NAFLD progression in both human and mice (461-463). Mice that have liver specific AMPKα1 and α2 depletion displayed impaired mitochondrial content and oxidative capacity, indicating that AMPK is essential for the maintenance of hepatic mitochondria function (464). Moreover, recent work has shown that AMPK could trigger intracellular lipid degradation by lipophagy, and defects in lipophagy have been shown to contribute to NAFLD (465). In summary, AMPK is important in regulating hepatic lipid metabolism and maintenance of mitochondrial function. Therefore, AMPK activation is expected to improve NAFLD and represents a promising therapeutic target against NAFLD.

CHAPTER III

GUT MICROBIOTA-DERIVED TRYPTOPHAN METABOLITES MODULATE INFLAMMATORY RESPONSE IN HEPATOCYTES AND MACROPHAGES*

3.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in Western countries, and its incidence correlates strongly with obesity and the metabolic syndrome (44). It encompasses a spectrum of disorders from simple steatosis, by itself a benign condition, to non-alcoholic steatohepatitis (NASH), characterized by intrahepatic inflammation, hepatocellular ballooning and progressive fibrosis (46). Sustained steatohepatitis can lead to cirrhosis and liver cancer (466).

The mechanisms driving NAFLD progression are not well understood, although it is generally agreed that there are at least two components. First, cellular lipid deposits accumulate in the liver. This is then followed by activation of immune cells and production of pro-inflammatory cytokines (467). Nutritional, genetic, and epigenetic factors each play a role in determining whether an individual with steatosis develops the more severe pathologies of fatty liver disease (102). Another emerging factor contributing to NAFLD pathogenesis is dysbiosis of the intestinal microbiota. Germ-free (GF) mice fed a high-fat diet (HFD) and inoculated with bacterial isolates from feces of hyperglycemic mice developed steatohepatitis, whereas GF mice fed the same HFD but inoculated with bacteria from normoglycemic mice only showed mild

* This chapter is reprinted with permission from "Gut Microbiota-Derived Tryptophan Metabolites Modulate Inflammatory Response in Hepatocytes and Macrophages" by Smitha Krishnan, Yufang Ding, Nima Saedi, Maria Choi, Gautham V. Sridharan, David H. Sherr, Martin L. Yarmush, Robert C. Alaniz, Arul Jayaraman, and Kyongbum Lee. ***Cell Reports*** *23, 1099–1111, April 24, 2018*

steatosis (34). A study comparing intestinal microbiomes of children found subtle, yet significant differences between children with obesity and NASH (32).

Several hypotheses have been put forth regarding the role of dysbiosis (468). One possibility is that dysbiosis disrupts production of microbial metabolites that are utilized by the intestinal epithelial cells for maintaining barrier integrity, which could elevate bacterial endotoxins in circulation, and trigger a pro-inflammatory cytokine cascade in the liver. Another hypothesis is that dysbiosis leads to increased generation of toxic metabolic byproducts in the intestine and elevates the chemical burden on the liver. A third hypothesis is that dysbiosis leads to aberrant metabolism of dietary residues (e.g. choline) and/or endogenous metabolites (e.g. bile acids), which impairs export of lipids or elevates lipogenesis in the liver. A common denominator of these hypotheses is that progression towards steatohepatitis is linked to alterations in the metabolic outputs of the intestinal microbiota. Recent findings have shown that the microbiota produces bioactive metabolites that engage host cellular pathways to modulate immune cell fate and function (469) and lipid absorption in the intestine (470). Microbial metabolites have been detected in circulation (43) and the liver (471), and several of these metabolites (e.g., indoxyl sulfate) are ligands for host receptors such as the aryl hydrocarbon receptor (AhR). Studies in transgenic animal models suggest that the AhR plays a significant role in regulating lipid and fatty acid metabolism (472). In mice, AhR activation negatively regulates several lipogenesis enzymes, including fatty acid synthase (Fas), and the cholesterol metabolism regulator sterol regulatory element-binding protein-1c (SREBP-1c) (473). In humans, exposure to the AhR ligand TCDD disrupts lipid and fatty acid metabolism (474).

The present study investigates the hypothesis that gut microbiota dysbiosis perturbs the balance of immunomodulatory microbiota metabolites, which increases the susceptibility of the

liver to inflammation. We utilize a metabolomics approach to identify microbiota-dependent metabolites that activate the AhR and characterize their effects on liver inflammatory responses and metabolic function.

3.2 Material and method

3.2.1Reagents

RAW 264.7, AML12, and HepG2 cells were purchased from ATCC (Manassas, MA). Dulbecco's Modified Eagle Medium (DMEM), alpha minimal essential medium (αMEM), RPMI-1640, penicillin/streptomycin, phosphate-buffered saline (PBS), TRIzol, RNAse-free, DNase-free water, LPS (from *Salmonella minnesota*), and fetal bovine serum (FBS) for culture of HepG2 and AML12 cells were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) for culture of RAW 264.7 was purchased from Atlanta Biologicals (Flowery Branch, GA). Insulin-transferrin-selenium (ITS) was purchased from Thermo Fisher Scientific (Waltham, MA). The AhR inhibitor CH-223191 was developed in the Sherr lab at Boston University. H1G1.1c3 reporter cells were obtained from the Denison lab at UC Davis and utilized in the Sherr lab. Unless otherwise noted, all other reagents were purchased from Sigma Aldrich (St. Louis, MO).

3.2.2 Comparison of germ-free and conventionally raised mice

To screen for intestinal metabolites that depend on the microbiota, we performed untargeted and targeted analysis of cecal contents and fecal material collected from conventionally raised (CONV-R) and germ-free (GF) female C57BL/6N mice. The mice were purchased from Taconic (Albany, NY) at 6 weeks of age, and immediately sacrificed by euthanasia upon receipt at Texas A&M Health Science Center. Cecal contents and fecal material were collected as described previously (469) and stored at -80 °C until further analysis.

3.2.3 Sample collection from mice fed a low- or high-fat diet

Two groups of male C57BL/6J mice 14 weeks of age were purchased from Jackson labs. One group (n = 5) was initially raised on a LFD consisting of 10% calories from fat (D12450B, Research Diets, New Brunswick, NJ) until 6 weeks of age, after which the mice were fed a HFD consisting of 60% calories from fat (D12492, Research Diets), while the other group (n = 5) was continued on LFD. Mice from both groups had access to the respective diets during transit and prior to sacrifice (within 16 h of receipt). Mice were sacrificed by euthanasia, and blood was collected via heart puncture and centrifuged at 4,000*g* for 30 min to obtain serum. The liver was quickly excised, rinsed with 10x volume of ice-cold PBS, flash-frozen in liquid nitrogen, and weighed. Cecal contents were gently squeezed out of the excised cecum into cold tubes and flash frozen. Serum, liver, and cecal content samples were stored at -80 °C until further analysis. All animals were handled in accordance with the Texas A&M University Health Sciences Center Institutional Animal Care and Use Committee guidelines under an approved animal use protocol (2015-0346).

3.2.4 HepG2 and AML12 hepatocyte culture

HepG2 cells were seeded into 12-well plates at a density of ~$1.25x10^5$ cells/cm^2 and cultured in a humidified incubator at 37°C and 5% CO_2. The culture medium was low-glucose DMEM supplemented with 10% (v/v) heat inactivated FBS, penicillin (200 U/mL) and streptomycin (200 μg/mL). The medium was changed every 24 h for three days after seeding. AML12 cells were seeded into 12-well plates at a density of ~$1.25x10^5$ cells/cm^2 and cultured in a humidified incubator at 37°C and 5% CO_2. The culture medium was a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 40 ng/mL dexamethasone, 10% (v/v) heat inactivated FBS, 1.2 g/L sodium bicarbonate, penicillin

(200 U/mL), and streptomycin (200 μg/mL). The medium was changed every 24 h until the culture reached ~80% confluence. For a 'one-hit' inflammation model, the cells were treated for 24 h with dimethylsulfoxide (DMSO) or 10, 100, or 500 μM I3A or TA, and then treated with either DMSO or 25 ng/mL TNFα for another 24 h. For a 'two-hit' steatosis and inflammation model, the cells were grown for 48 h in medium supplemented with 0.5 mM palmitic acid and 0.5 mM oleic acid, then treated for 24 h with DMSO or 500 μM I3A, and finally treated for 24 h with vehicle or 25 ng/mL TNFα. The AhR inhibitor CH-223191 was prepared as a stock solution of 20 mM in DMSO. AML12 cultures were dosed with either 1 μL of inhibitor solution or 0.1% DMSO 10 min prior to I3A treatment and again 10 min prior to TNFα treatment.

3.2.5 Murine macrophage culture

Raw 264.7 murine macrophages were seeded into 24-well plates at a density of $4x10^5$ cells/ml and cultured in a humidified incubator at 37°C and 5% CO_2. The culture medium was DMEM supplemented with 10% heat inactivated FBS, penicillin (200 U/mL) and streptomycin (200 μg/mL). Cells were pretreated with different concentrations of I3A or TA for 4 h, followed by addition of 300 μM palmitate complexed with BSA. After 18 h, 10 ng/mL LPS was added to the culture medium. The cells were then incubated for an additional 6 h. Bone marrow derived macrophages (BMDMs) were isolated from the femur of wild type C57/BL6 mice as previously described (475). The isolated progenitor cells were re-suspended in RPMI-1640 medium supplemented with 10 ng/mL M-CSF (Invitrogen), seeded in polystyrene dishes, and incubated for 3 days at 37°C and 5% CO_2 in a humidified incubator. The culture medium was replenished on day 3, and the cells were incubated for an additional 4 days. At the end of the 7-day culture period, >95% of the cells were positive for macrophages markers F4/80 and CD11b.

3.2.6 Metabolite extraction

Metabolites were extracted from tissue samples using a solvent-based method. Liver tissue samples were weighed and placed into a pre-cooled garnet bead beating tube followed by adding 500 μL ice cold methanol and 250 μL ice cold chloroform. After homogenization (6500 rpm for 30 s), the sample tube was centrifuged under refrigeration at 700*g* for 10 min. The supernatant was then transferred to a new tube through a (70 μm) cell strainer, and 1 mL ice-cold water was added and the sample centrifuged at 3700*g* for 10 min. The upper and lower phases were each separately transferred into new tubes while taking care not to disturb the interface. The liver tissue pellet was extracted again with ice cold methanol (500 μL). The polar phases from both extractions were combined and concentrated using a centrifugal evaporator (Vacufuge, Eppendorf, Hauppauge, NY). Metabolites from cecal content samples were extracted as described above without the homogenization step. Serum metabolites were extracted as previously described (43). Extracted samples were concentrated using the Vacufuge and stored at -80 °C until analysis. Prior to metabolite analysis, the samples were reconstituted in 100 μL methanol/water (50% v/v).

3.2.7 Untargeted metabolomics

The extracted samples were analyzed for global metabolite profiles using IDA experiments (**Table 3.1 and Table 3.5**) performed on a triple-quadrupole time-of-flight (TOF) instrument (AB Sciex 5600+). Raw data were processed in MarkerView (v. 1.2, AB Sciex) to determine the ion peaks. The peaks were aligned based on accurate mass (m/z) and retention time (RT) (30 ppm and 2.5 min tolerance, respectively), and then filtered based on intensity (100 cps threshold) to eliminate low quality peaks. An additional filter was applied to retain only monoisotopic ions. The remaining ions were organized into a feature table, with each feature specified by m/z and

RT. In the case a precursor ion detected by the TOF survey scan triggered an MS/MS scan, the corresponding MS/MS spectrum was extracted from the product ion scan data and added to the feature table. Each feature was searched against spectral libraries in METLIN (476) and HMDB (477) and analyzed using *in silico* fragmentation tools MetFrag (478) and CFM-ID (479). In some cases, the database search or *in silico* analysis returned more than one matching compound for a given feature. In these cases, the highest ranked match was selected as the search result. The search results from all four sources were used together to assign a putative identity (ID) to the features. An ID was categorized as a confident assignment, if the search results for the feature were consistent across at least one experimental database and one other data source.

3.2.8 Targeted analysis of bile acids and fatty acids

The extracted samples were analyzed for specific metabolites (I3A, TA, bile acids, and fatty acids) using product ion experiments performed on the TripleTOF instrument (**Table 3.1–Table 3.4**). Metabolite-specific parameters for product ion scans were determined from runs using high-purity chemical standards.

3.2.9 Metabolite quantitation

For each metabolite with a confirmed or putative identity, the corresponding peak in the ion chromatogram was integrated using MultiQuant (version 2.1, AB SCIEX) to determine the area-under-the-curve (AUC). For comparisons of metabolite levels between samples, fold-changes were calculated based on the AUC values normalized to the corresponding tissue weight (liver and cecum samples) or DNA content (*in vitro* cell culture samples). Cellular DNA content was measured using Hoechst dye.

3.2.10 RNA extraction and qRT-PCR

Cells were washed with PBS, and stored at -80 °C until RNA extraction. Total RNA was extracted from RAW 264.7 cells using the EZNA Total RNA kit (Omega Bio-Tek, Norcross, GA). RTPCR analysis was carried out using the qScript One-Step PCR kit (Quanta Biosciences, Gaithersburg, MD) on a LightCycler 96 System (Roche, Indianapolis, IN). For hepatocytes and AML12 cells, RNA was extracted using TRIzol (Invitrogen). Total RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). Quantification of RNA was performed using Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies) on a Stratagene Mx3000P qPCR System (Agilent Technologies). Fold-change values were calculated using the $2^{-\Delta\Delta Ct}$ method, with β-actin and GAPDH as the housekeeping genes for macrophages and hepatocytes, respectively. The primer sequences are listed in **Table 3.6**.

3.2.11 Proteomics

Proteins were extracted from cell lysates, purified, digested, and analyzed using LC-MS experiments as described previously (480). An IDA scan was used to generate an ion library in ProteinPilot (v. 5.1, AB Sciex) of all proteins and their corresponding peptides in the sample, and a data-independent acquisition (DIA) scan was used to obtain high-quality MS/MS data for quantification.

3.2.12 Cytokine quantification

Raw 264.7 cells were cultured in the presence of LPS for 24 h. Culture supernatants were centrifuged at 5,000 x *g* for 15 min and secreted cytokines were quantified using a multiplex kit (Mouse Inflammation Panel, BioLegend, San Diego, CA) using the manufacturer's suggested protocol.

3.2.13 Macrophage migration assay

Bone marrow derived macrophage (BMDM) migration was investigated in transwell cell culture chambers with polycarbonate membranes (8 μm pore size, Corning Costar, Corning, NY). Cells were added to the upper chamber and either vehicle or different concentrations of I3A or TA was added to both the upper and lower chambers. After 4 h, 20 ng/mL MCP-1 (Invitrogen) was added to the bottom chamber. After 24 h, cells remaining on the upper side of the membrane were scraped off with a cotton swab. The migrated cells in the bottom chamber were fixed with methanol for 15 min, stained with 0.1% crystal violet for 30 min, and counted under a microscope (Axiovert 200M, Zeiss). Three replicate wells were analyzed in each experiment, with cells counted in 15 randomly chosen fields of view per well.

3.2.14 AhR ligand activation assay

A previously described AhR reporter assay (481) using murine H1G1.1c3 hepatoma cells was adapted to test ligand activation of the AhR by I3A and TA. These experiments were performed at the Sherr lab. Indoxyl sulfate (IS) and 6-formylindolo(3,2-b)carbazole (FICZ) were used as positive controls. Fluorescence readings were recorded at 24 and 48 h using a plate reader (SpectraFluor Plus, Tecan, Männedorf, Switzerland). Relative fluorescence as a measure of induced AhR activity was calculated by subtracting the average background fluorescence of untreated cells from the experimental values.

3.2.15 CARS microscopy

Lipid accumulation in hepatocytes grown in fatty acid supplemented medium was visualized using Coherent Anti-stokes Raman Scattering (CARS) microscopy. Images were recorded every 24 h by tuning the Stokes (1064 nm) and pump (817 nm) beams to excite the Raman peak at 2845 cm^{-1} (663 nm) corresponding to the CH_2 stretch in lipid molecules. The microscopy system

was configured to detect both forward- and epi-CARS signals. All images were averaged over 64 frames. Overlays of forward- (green) and epi-CARS (red) signals were generated using raw images (LAS X Software v3.0.2, Leica Microsystems).

3.2.16 Statistical analysis

Metabolite level comparisons from *in vivo* data were performed using the Wilcoxon rank-sum test. Statistical significance of *in vitro* data was calculated using one-way ANOVA for single treatment comparisons and two-way ANOVA for multiple treatment comparisons. A *p*-value of less than 0.05 was considered statistically significant. All heat maps were generated using pareto-scaled data. The similarity of the metabolite profiles was assessed based on group centroid distances using PLS-DA. Ellipses in PLS-DA plots show 95% confidence regions for each treatment group.

3.3 Results

3.3.1 Microbiota-dependent metabolites in the intestine include tryptophan-derived AhR ligands

Cecal contents and fecal material from GF and conventionally-raised (CONV-R) mice were analyzed using both targeted and untargeted LC-MS experiments. Hierarchical clustering of detected features showed qualitative differences between the two groups (**Figure 3.1A**). The majority of the differentially present features (60% of total) were reduced in GF samples, suggesting that these features include products that depend on microbiota metabolic activity (**Figure 3.1B**). In the fecal material, a majority of differentially present features (36% of total) were elevated in GF samples (**Figure 3.1C**), suggesting that these features include dietary resides that could have been catabolized by the intestinal microbiota or host intestinal enzymes activated by the microbiota.

Of the 1,444 LC-MS features depleted in both cecal contents and fecal material from GF mice, we annotated 156 features with putative identities. The majority of these metabolites are associated with phytochemical and lipid metabolism pathways (**Figures 3.1D-E**). Other major categories are amino acid derived compounds, nucleotides, vitamins, and cofactors. Notably, these depleted metabolites include bioactive molecules such as indole-3-acetate (I3A) and tryptamine (TA) (**Figures 3.1F-G**), which we previously found (469) to activate the AhR in a breast cancer cell line.

3.3.2 HFD alters the levels of microbiota-dependent metabolites in cecum, serum, and liver

We next investigated whether administering a HFD, which can induce steatosis in mice following a prolonged feeding period (482), alters the levels of microbiota-dependent metabolites. The duration of HFD feeding was kept relatively short in order to avoid substantially altering host physiology. Using qRT-PCR, we confirmed that an 8-week HFD significantly perturbed the microbiota. The ratio of Firmicutes to Bacteroidetes was significantly higher in fecal material from HFD-fed mice (8.62) compared to mice fed a low-fat diet (LFD) (0.82) (**Figure 3.8A**). No significant differences in liver triglyceride levels were observed (not shown).

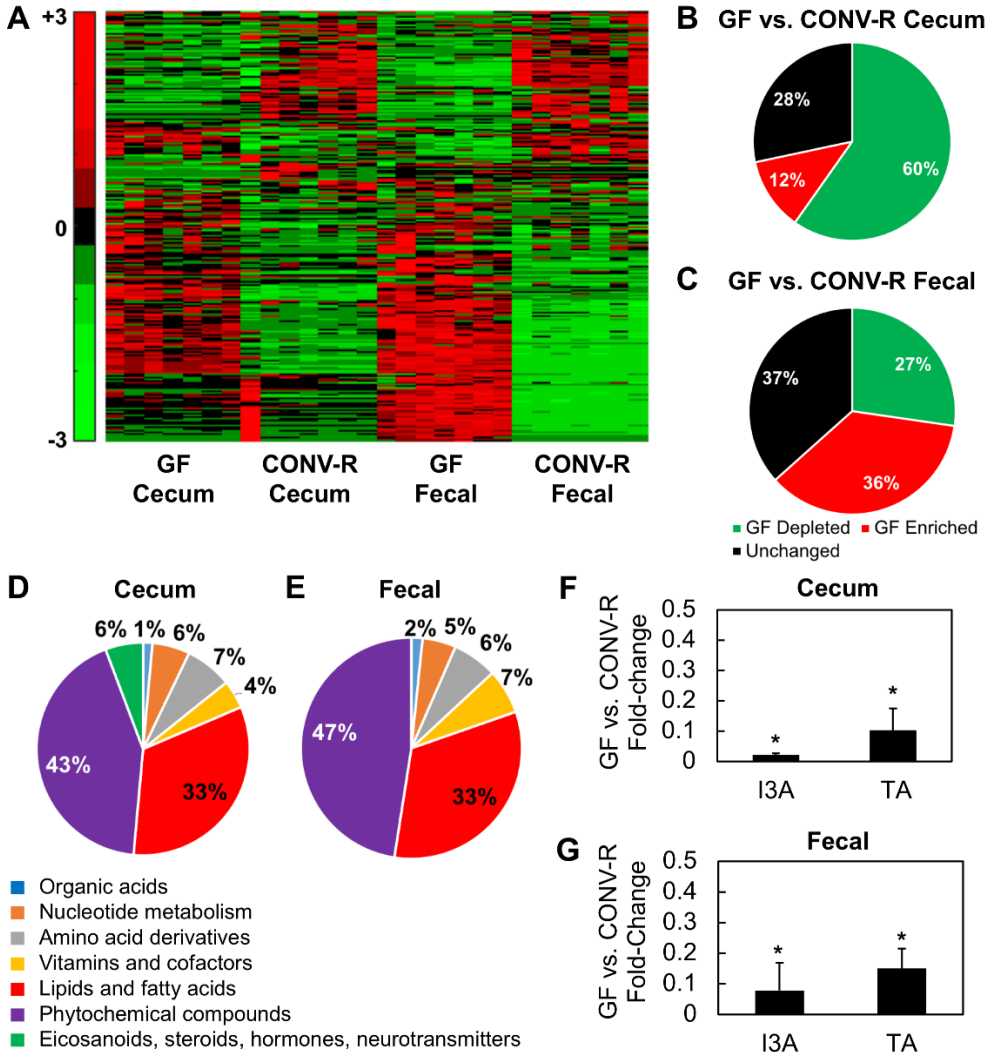

Figure 3.1 Results from untargeted analysis of samples from GF and CONV-R (female C57BL/6N) mice. Heat maps of hierarchically clustered LC-MS features (A). Features from fecal material and cecal contents were separately scaled prior to clustering using the Pareto method. Fraction of detected features that are depleted, elevated, or not significantly different in cecal contents (B) and fecal material (C) between GF and CONV-R mice. Putatively identified metabolites that depend on the gut microbiota (D-G). Features depleted in cecal contents (D) and fecal material (E) from GF mice were annotated and mapped to metabolic pathways in KEGG. Statistical tests on fold-changes identified several metabolites that are consistently depleted in both fecal material and cecal contents from GF mice. I3A and TA are both significantly reduced in cecal contents (F) and fecal material (G) from GF mice. Data shown are averages of n = 7 mice. Error bars represent one standard deviation. *: $p<0.05$ using Wilcoxon rank-sum test.

Untargeted analysis of metabolites in the cecum, serum, and liver (**Figure 3.8B**) did not show any obvious qualitative differences between HFD- and LFD-fed mice. However, a more detailed examination of the LC-MS data using hypothesis testing for equal medians (Wilcoxon rank-sum test) followed by feature annotation revealed significant differences in several classes of metabolites (**Figure 3.2A**). The major classes are phytochemicals, lipids, and amino acid-derived compounds (**Figure 3.8C**). Intersecting the list of metabolites differentially present in HFD and LFD mice with the list of metabolites differentially present in GF and CONV-R mice identified 19 microbiota-dependent metabolites that are consistently altered by the HFD in at least two of the three tissue compartments (**Figure 3.2C**). This panel of 19 metabolites was further narrowed to eliminate small effect sizes (fold-change <2 between HFD and LFD mice). Finally, searching the narrowed list for potential AhR ligands identified three tryptophan metabolites: I3A, TA, and xanthurenic acid (XA).

Targeted LC-MS analysis for I3A and TA confirmed significant depletion of both metabolites in the liver and cecum of HFD mice compared to LFD mice (**Figure 3.2B**). The decrease in TA varied from two- (cecum) to ten-fold (liver). Similarly, I3A decreased between three- (liver) and ten-fold (cecum). The concentrations of TA and I3A in the LFD liver are 2 and 0.03 µmoles/g tissue, respectively.

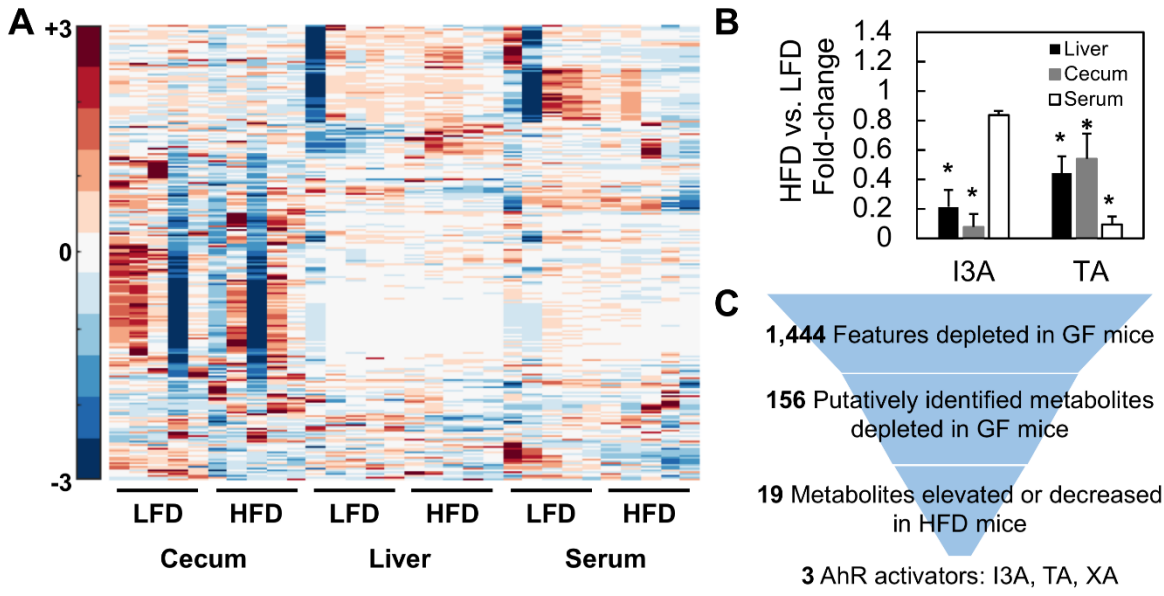

Figure 3.2 Liver, cecum, and serum samples from LFD and HFD fed CONV-R mice (male C57BL/6J) were analyzed using untargeted LC/MS experiments. Significantly different features were Pareto scaled and clustered along with features of interest based on annotation of GF and CONV-R samples (A). TA and I3A were quantified using targeted analysis to confirm depletion of these metabolites in HFD samples. Metabolite levels were normalized to sample weights and plotted as fold-changes comparing HFD to LFD (B). Data shown are averages of n = 5 mice. Error bars represent one standard deviation. *: $p<0.05$. Metabolite selection process based on features differentially present in GF vs. CONV-R samples and HFD vs. LFD samples (C). Final selections are suspected AhR ligands.

3.3.3 HFD increases levels of free fatty acids and alters composition of primary bile acid pool

Targeted analysis for major free fatty acid (FFA) species confirmed significant increases in both saturated and unsaturated fatty acids in liver, cecum, and serum of HFD mice compared to LFD mice (**Figure 3.3**). In the liver, palmitic and stearic acids were increased 4-fold. In the cecum, palmitic acid was increased 10-fold, while stearic acid was unchanged (**Figure 3.3B**). In serum,

feeding with the HFD increased almost every major FFA species (**Figure 3.3C**). The increases in serum FFAs largely correlated with the composition of the HFD. The quantitatively dominant FFAs in the diet are palmitic, oleic, linoleic, and stearic acids (**Figure 3.3D**), which are also the four most abundant FFAs in all three tissue compartments examined in this study.

In addition to increasing the FFA levels, HFD also altered the composition of the primary bile acid pools in the liver, cecum, and serum. Targeted analysis for cholic acid (CA) and chenodeoxycholic acid (CDCA) found a significant ($p = 0.013$) decrease in the relative pool size of CA in the liver of HFD mice (**Figure 3.8D**).

3.3.4 I3A and TA attenuate pro-inflammatory cytokine expression in macrophages

In a previous study (39), we found that the tryptophan-derived bacterial metabolite indole attenuates indicators of inflammation in epithelial cells. As inflammation is a key secondary insult driving NASH, we investigated whether the tryptophan metabolites decreased in HFD could attenuate inflammation. Cultured macrophages were first exposed to palmitic acid and then lipopolysaccharide (LPS) to mimic the sequence of two key insults in NAFLD. Changes in the expression of tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and monocyte chemoattractant protein-1 (MCP-1) in the presence of I3A and TA were determined (**Figure 3.4A-B**). Pretreating macrophages with I3A or TA prior to palmitate and LPS exposure significantly attenuated the increase in the mRNA levels of TNFα, IL-1β, and MCP-1. Of the two metabolites, I3A exhibited greater potency, reducing the expression of all three cytokines in a dose dependent manner (**Figure 3.4A**). While TA pretreatment resulted in similar trends, only IL-1β expression was significantly attenuated (**Figure 3.4B**). Moreover, significant toxicity was observed at TA concentrations greater than 500 μM. The trends for cytokine secretion were largely consistent with gene expression (**Figure 3.4C**).

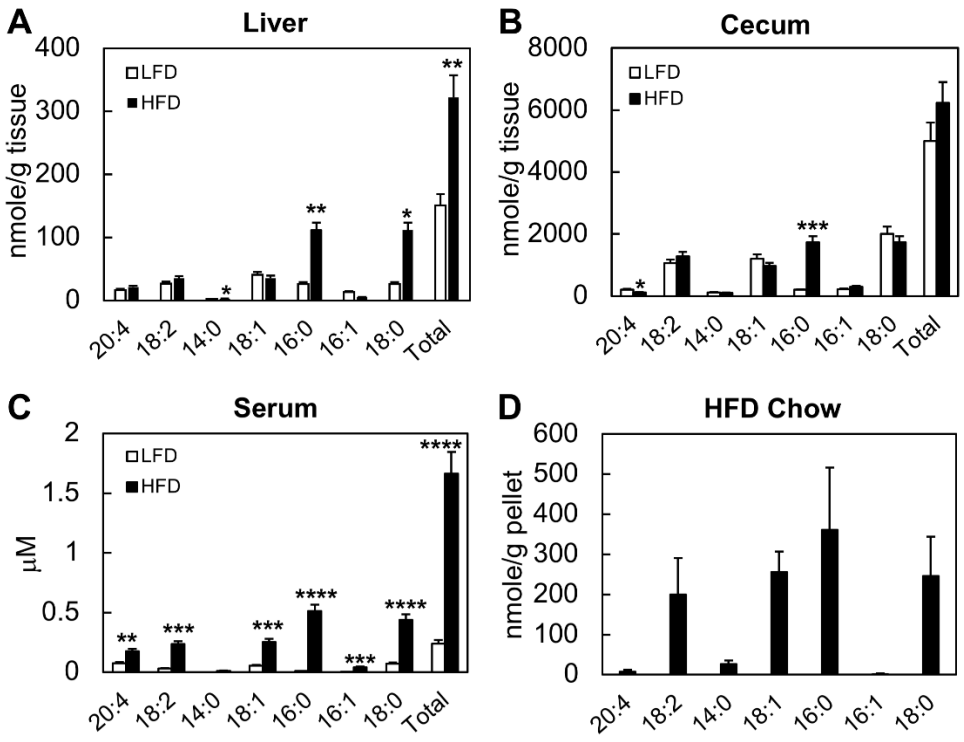

Figure 3.3 Comparison of free fatty acid (FFA) profiles in HFD and LFD mice. Major FFA species were quantified in liver (A), cecal luminal content (B), and serum (C) samples from HFD- and LFD-fed mice. The same FFAs were quantified in the HFD pellets (D). Data shown are averages of n = 5 mice. Error bars represent one standard deviation. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ***: $p<0.0005$.

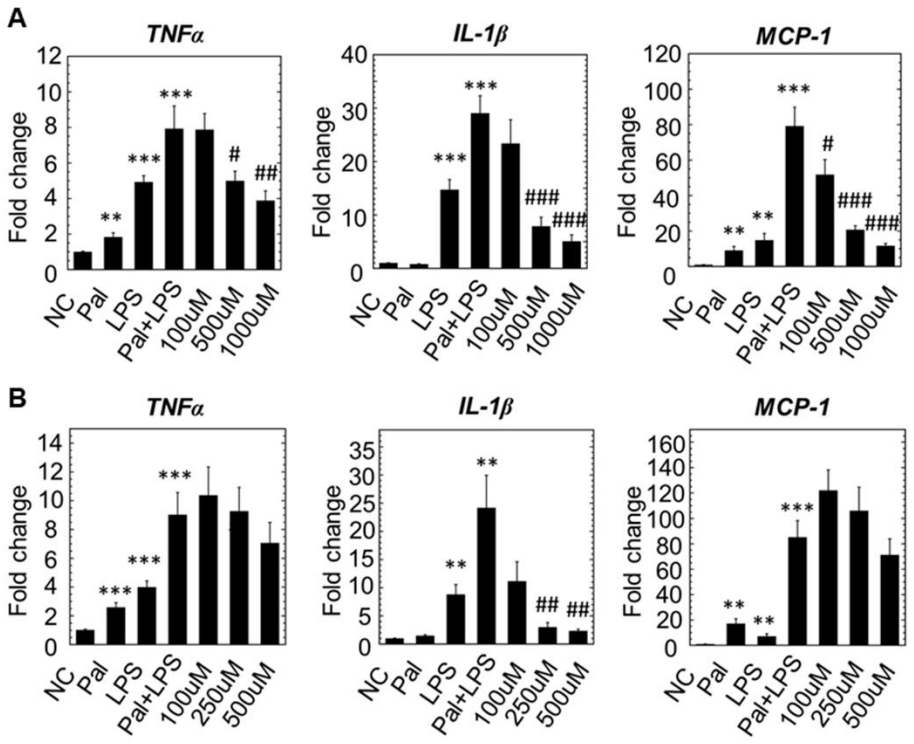

Figure 3.4 I3A and TA reduce pro-inflammatory cytokine production at the mRNA and protein level, and chemotactic migration in macrophages exposed to palmitate and LPS. (A-C). RAW 264.7 cells were stimulated with palmitic acid (Pal) followed by LPS, with or without adding varying doses of (A) I3A or (B) TA. The negative control (NC) group was only treated with the vehicle, dimethylformamide (DMF). Changes in the expression of TNFα, IL-1β, and MCP-1were determined using qRT-PCR. (C) Culture supernatants were analyzed for secreted cytokines using a bead-based assay. Data shown are averages of 3 independent experiments with 3 biological replicates. Error bars represent one standard error of the mean. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ compared to NC; and #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$ compared to Pal+LPS group. (D) I3A and TA inhibit BMDM migration toward MCP-1. BMDMs were incubated with MCP-1 in a transwell with vehicle (DMF) or different doses of I3A or TA. The chemotactic index (CI) for a treatment condition was calculated as the ratio of average number of migrated cells in the treatment group relative to the control group (incubated in medium only). Data shown are averages of 3 independent experiments with 3 biological replicates. Error bars represent one standard error of the mean. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

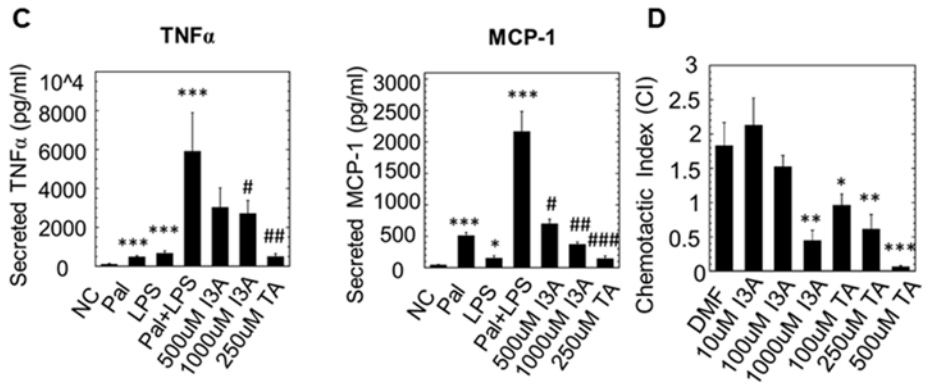

Figure 3.4 Continued

3.3.5 I3A and TA inhibit macrophage migration to MCP-1

In addition to activation of resident macrophages, progression of steatohepatitis is marked by increased infiltration of macrophages into the liver. Macrophage infiltration is promoted by MCP-1, a chemokine that is increased in serum and liver of NASH patients (483). Thus, we investigated if I3A or TA alters migration of macrophages towards MCP-1. Pretreatment of bone marrow derived macrophages (BMDMs) with I3A or TA prior to stimulation with MCP-1 significantly decreased migration in a dose dependent manner. At the highest concentrations tested in this study, I3A (1 mM) and TA (500 μM) both completely abolished BMDM migration toward MCP-1 (**Figure 3.4D**).

3.3.6 I3A attenuates effects of TNFα and fatty acids in hepatocytes

Based on the above observation that I3A significantly attenuated the production of inflammatory mediators in macrophages, we examined whether I3A also modulated the metabolic response of hepatocytes. We first confirmed that culturing AML12 cells in medium supplemented with palmitic and oleic acids at a high, but sub-toxic (484) dose (500 μM) led to accumulation of

visible lipid droplets within 24 to 48 h (**Figures 3.5A-C**). A similar trend was observed in HepG2 cells, a human liver cell line (**Figure 3.9A-C**), which accumulated noticeably larger lipid droplets. We followed the fatty acid supplementation with TNFα treatment, taking this cytokine as a representative pro-inflammatory signal produced by macrophages in the liver.

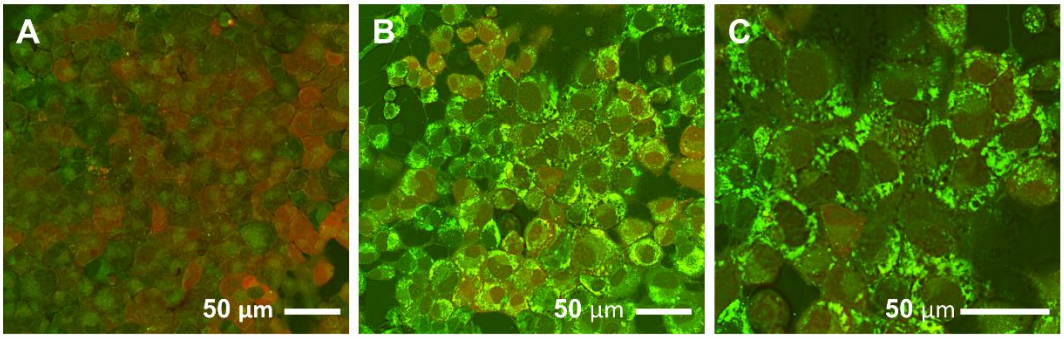

Figure 3.5 Micrographs of AML12 hepatocytes obtained using CARS microscopy. Micrographs were recorded after the cells had been treated with either (A) vehicle or (B) FFAs (500 µM palmitate + 500 µM oleate) for 48 h. All images are composites of forward- (green) and epi-CARS (red) signals. Lipid inclusion bodies are primarily detected by the stronger forward signal.

Targeted LC-MS analysis of major fatty acid species showed that the AML12 cells cultured in FFA-supplemented medium contained 5-fold greater levels of intracellular palmitic and oleic acids (**Figures 3.6A-C**), suggesting that not all of the FFAs taken up by the cells were stored into droplets. A similar trend was observed for the HepG2 cells (**Figure 3.9D-F**). In both AML12 and HepG2 cells, exposure to TNFα increased the intracellular levels of both palmitic and oleic acids, even without fatty acid preconditioning. Combining the treatments did not lead to an

additive effect. Pretreating the cells with I3A prior to TNFα exposure significantly decreased the levels of palmitic and oleic acids. Similarly, I3A treatment attenuated the effects of fatty acid preconditioning, even when it was combined with TNFα.

Since a hallmark of liver inflammation in fatty liver disease is dysregulation of bile acid metabolism (201), we examined the effect of I3A on bile acid production. Targeted LC-MS analysis showed that fatty acid preconditioning and TNFα exposure independently and together increased the fraction of CDCA by more than 30% in AML12 cells (**Figure 3.6D**). A similar trend was observed for the HepG2 cells (**Figure 3.9G**). By itself, I3A also increased the CDCA fraction, but when combined with the other treatments, I3A reduced the CDCA fraction by 20% compared to the conditions where the cells were exposed to either FFAs or TNFα. However, I3A treatment had no significant impact on cultures exposed to both TNFα with FFA. Similar trends were observed in HepG2 cell cultures (**Figure 3.9G**); however, CA accounted for a larger share of the bile acid pool in the control cultures and TNFα exposure increased the CDCA fraction by 40%. In HepG2 cells, I3A treatment had a significant impact on bile acid levels even when both FFA and TNFα were present.

3.3.7 I3A attenuates hepatocyte response to TNFα

Based on the observation that I3A attenuates the both palmitic acid- and TNFα-stimulated fatty acid level increase, we more broadly profiled the effects of I3A on hepatocyte metabolite levels using untargeted LC-MS analysis. Hierarchical clustering (**Figure 3.10A**) suggested that I3A pretreatment was more effective in reversing the metabolic alterations due to TNFα exposure compared to fatty acid preconditioning. A similar trend was observed when the metabolite profiles were compared using partial least squares discriminant analysis (PLS-DA)

(**Figure 3.10B-C**). The profiles of cells cultured in fatty acid supplemented medium formed a distinct cluster independent of I3A treatment, whereas the cells exposed to TNFα following I3A pretreatment clustered closely with vehicle control.

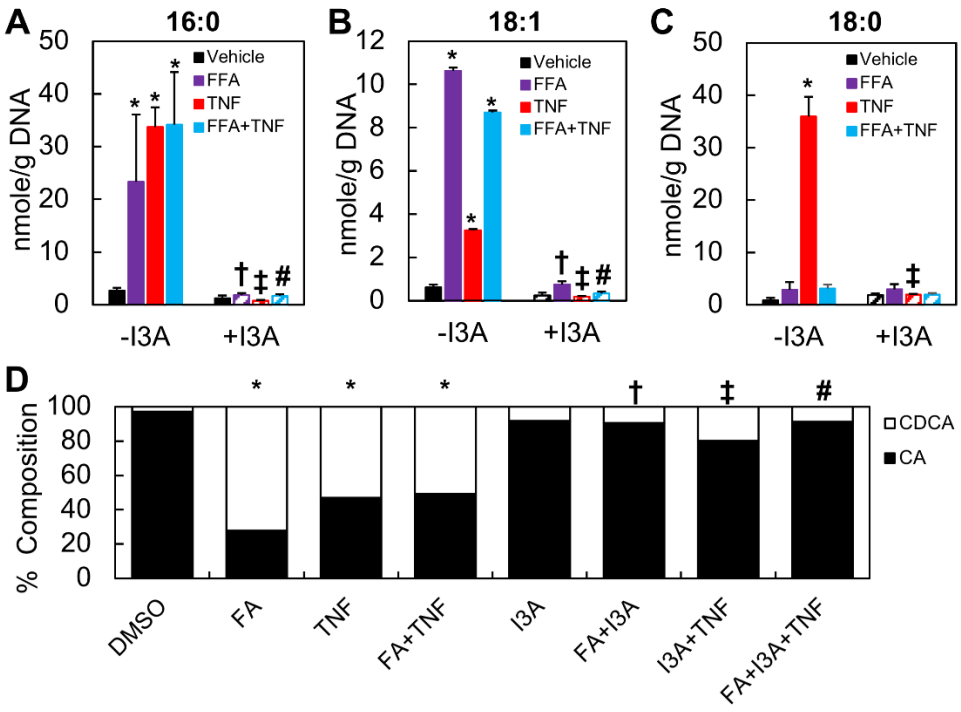

Figure 3.6 I3A attenuates the effects of TNFα in cultured AML12 cells preconditioned with FFAs. Palmitic (A), oleic (B), and stearic acids (C) were quantified using targeted LC-MS experiments. CA and CDCA were quantified and represented as % contribution to the combined pool of primary bile acids (D). Data shown are averages of 4 independent experiments with 6 biological replicates. Error bars represent one standard deviation. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ compared to cells in the negative control group exposed to vehicle (DMSO) only; †: $p<0.05$ compared to the FFA group that was not treated with TNFα; ‡: $p<0.05$ compared to the TNF group that was not preconditioned with FFAs; #: $p<0.05$ compared to the FFA+TNF group that was preconditioned with FFAs and treated with TNFα.

3.3.8 I3A modulates the expression of Fas and SREBP-1c via activation of the AhR

To investigate the impact of I3A on lipid and fatty acid pathways, we measured the expression of a key lipogenesis enzyme, Fas, and a transcription factor regulating cholesterol/bile acid biosynthesis, SREBP-1c. By itself, I3A treatment significantly reduced the expression of Fas in AML12 cells. This effect was also observed when I3A treatment was combined with fatty acid preconditioning and/or TNFα exposure (**Figure 3.7A**). The effect of I3A on Fas at the protein level was consistent with the gene expression data (**Figure 3.7B**). Treatment with TNFα independently or in conjunction with FFA increased Fas protein levels by four-fold while independent treatment with FFA did not significantly alter Fas. Treatment with I3A significantly attenuated the effects of TNFα on Fas by two-fold. While fatty acid supplementation increased SREBP-1c gene expression, TNFα exerted a weaker effect (**Figure 3.7C**). Combining both treatments did not have a significant effect compared to vehicle control.

Treatment with I3A again attenuated the effect of TNFα or fatty acid supplementation. We also tested the effects of I3A on AhR gene expression and activation, which negatively regulates both Fas and SREBP-1c. Fatty acid supplementation and TNFα exposure, either individually or in combination, significantly reduced AhR gene expression compared to vehicle control. Treatment with I3A attenuated the TNFα-induced reduction in AhR expression, regardless of fatty acid supplementation (**Figure 3.7D**). Treatment with I3A by itself had no significant impact on AhR gene expression, suggesting that the effects of I3A on AhR target genes are mediated through ligand activation of the nuclear receptor. Activation of AhR by I3A was confirmed in a H4IIE murine liver cell line expressing a stable enhanced green fluorescent protein (EGFP) reporter regulated by a minimal promoter containing xenobiotic response

elements. At 10 μM or higher doses, I3A increased EGFP expression by over 5-fold compared to vehicle control (**Figure 3.7E**). To determine if the observed effects of I3A are AhR-dependent, AML12 cells were incubated with CH-223191, a potent antagonist of AhR (485), prior to I3A treatment. In the presence of the inhibitor, I3A only weakly attenuated the TNFα- or fatty acid supplementation-induced increases in palmitic (**Figure 3.7F**) and oleic acids (**Figure 3.7G**). In the case of oleic acid, incubation with CH-223191 completely abrogated the effects of I3A.

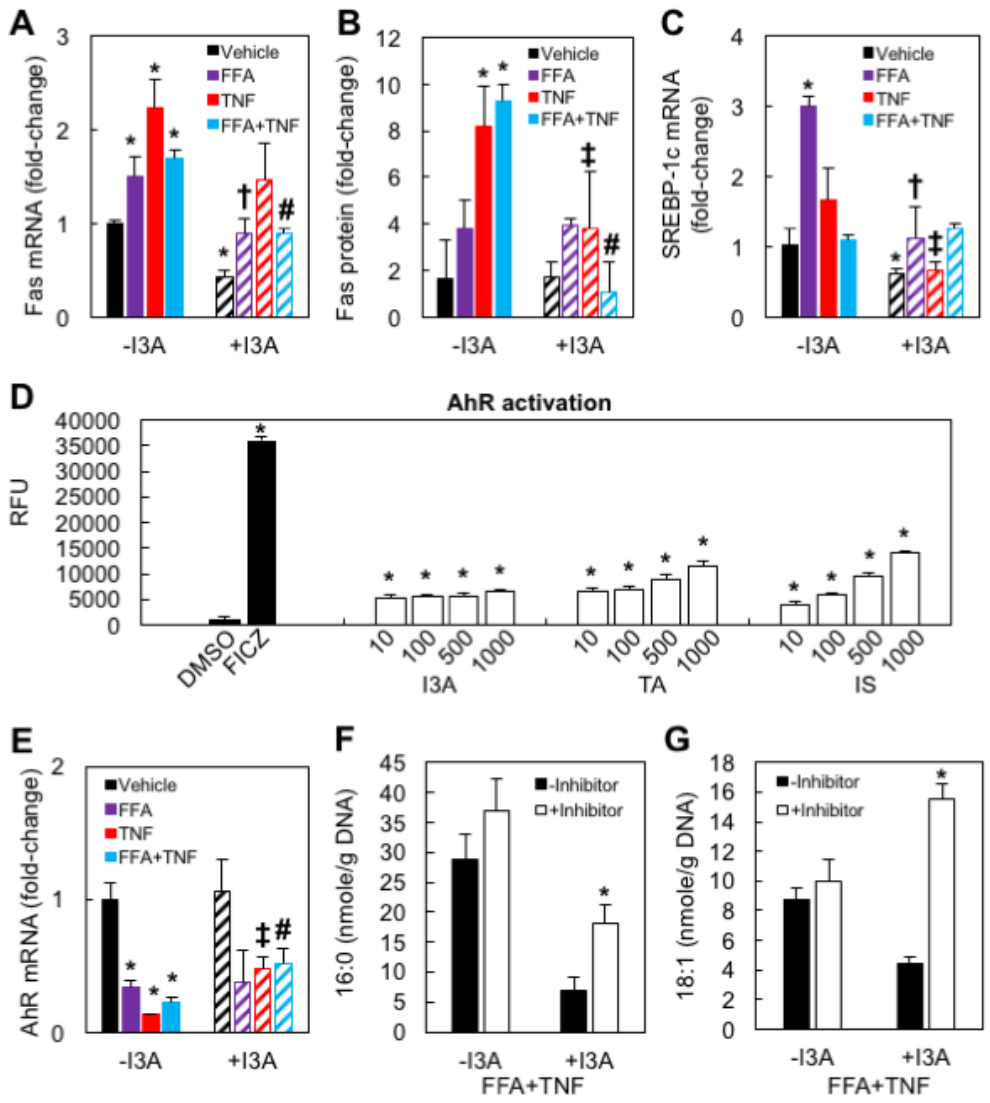

Figure 3.7 I3A attenuates the effects of TNFα on AhR and its target genes in cultured AML12 cells preconditioned with FAs. Gene expression (A) and relative abundance of Fas protein (B). Gene expression of SREBP-1c (C). AhR activation by different doses of I3A, TA, and IS (D). FICZ and DMSO were used as positive and negative controls (NC), respectively. ***: p<0.005, ****: p<0.001 compared to NC. Gene expression of AhR (E). Data shown are averages of 4 independent experiments with 6 biological replicates. Error bars represent one standard deviation. *: p<0.05, **: p<0.01, ***: p<0.001 compared to NC; †: p<0.05 compared to FA group, ‡: p<0.05 compared to TNF group; #: p<0.05 compared to FA+TNF group. Palmitic (F) and oleic acids (G) in cultures treated with AhR antagonist CH-223191. *: p<0.05 compared to corresponding culture without CH-223191.

3.4 Discussion

A number of studies have shown that the gut microbiota composition is altered in a HFD regimen, suggesting that this dysbiosis could drive various phenotypic changes and progression of HFD-associated diseases, including fatty liver disease (12). By co-analyzing the differences in the metabolite profiles between HFD- and LFD-fed mice alongside the differences between CONV-R and GF mice, we identified metabolites that depend on the gut microbiota and are significantly depleted under HFD. The metabolite profile comparisons were only performed within the same sub-strain of mice (C57BL/6J or C57BL/6N) to examine the effect of a single factor, either diet or colonization.

The untargeted LC-MS experiments detected a large number of features differentially present in GF and CONV-R mice (**Figure 3.1A-C**). A subset of these features (26 %) is consistently depleted in both cecal luminal contents and fecal material from GF mice, and thus could be products of gut microbiota metabolism. A common practice for annotating untargeted LC-MS data is to match the MS/MS spectra of the detected features to spectral libraries of reference databases. Unfortunately, the coverage of bacterial metabolites in these databases remains incomplete. Moreover, different databases often return conflicting results. To address these issues, we searched multiple databases and supplemented these searches with *in silico* fragmentation analyses. A putative identity was assigned only if two or more of the data sources agreed. The identification rate achieved in the present study (156/1,444) is comparable to other global profiling studies, with the identified LC-MS features broadly representing known categories of microbiota metabolites (**Figure 3.2D-E**).

In order to determine which of the microbiota-dependent intestinal metabolites could be altered in fatty liver disease, we compared the metabolite profiles of liver, cecum, and serum

from HFD-fed mice against corresponding profiles from LFD-mice. The HFD model has been used extensively to study phenotypes associated with fatty liver disease in mice (12). It is generally accepted that HFD typically leads to simple steatosis, and that steatohepatitis and fibrosis require additional challenges such as fructose supplementation (13). We limited the HFD to 8 weeks based on previous reports (486) showing that this duration is sufficient to significantly modify the gut microbiome, while also elevating circulating levels of inflammatory cytokines, without producing overt liver dysfunction. Targeted LC-MS experiments confirmed the impact of the HFD on cecum, serum and liver FFA profiles, in good agreement with previous reports on similar HFD models (486, 487).

Of the 156 putatively identified metabolites depleted in GF mice, 19 were also depleted by the HFD in at least two of the three tissue compartments. Querying this panel of metabolites against the literature for previously reported bioactivity, specifically ligand activation of the AhR, further narrowed the panel to 3 metabolites (**Figure 3.2C**): XA, TA, and I3A. Enzymes for producing TA (488) and I3A (469) from tryptophan have been characterized in the gut microbiota, whereas such enzymes have not yet been identified for XA. Therefore, we focused on TA and I3A as likely microbiota metabolites. The doses of TA and I3A were based on concentrations measured in the liver, estimated to be 2 and 0.03 mM, respectively, assuming a tissue density of 1 g/ml.

Multiple triggers can activate liver resident macrophages and induce hepatic inflammation. One well-known trigger is exposure to LPS. Increased bacterial translocation from the digestive tract has been observed in NASH patients (305). Another potential trigger is activation of toll-like receptors (e.g., TLR4) by elevated FFAs (489). Treating the cells with both palmitate and LPS synergistically induced the production of several pro-inflammatory cytokines.

This finding is consistent with an earlier study reporting that palmitate amplifies the production of inflammatory cytokines (IL-6 and IL-8) upon LPS stimulation (490) of in human THP-1 monocytes. A similar observation was reported by Wen et al., who showed palmitate induction of IL-1β secretion in LPS primed macrophages (491). The synergistic effects of LPS and palmitate could involve production of ceramides (61), which strongly induce lipotoxicity (492), or inflammasome activation through AMP-activated protein kinase (491). Both palmitate and LPS have been shown to activate macrophages through TLR4 and nuclear factor-κB (NF-κB) signaling (489), suggesting that signaling from both stimuli could converge onto this classical inflammation pathway. The addition of I3A or TA to the culture medium significantly decreased palmitate and LPS induced cytokine production in a dose dependent manner (**Figure 3.4A-C**). Interestingly, TA shows greater potency than I3A in decreasing TNFα and MCP-1 secretion, but is less potent in decreasing their gene expression. One possible explanation for this observation is that I3A and TA affect cytokine production at different points.

Another source of hepatic inflammation in NASH is increased infiltration of monocytes to the liver. Activated Kupffer cells produce chemokines, including MCP-1, which recruit multiple immune cells including monocytes (223). Accumulation of monocytes in the liver depends on signaling between C-C chemokine receptor type 2 (CCR2) and MCP-1 (223), and blocking this axis improves NASH in a murine model (216). Our results show that I3A and TA both inhibit BMDM migration toward MCP-1 in a dose dependent manner; however, the mechanism for this inhibition remains to be elucidated. While both I3A and TA exhibit the ability to attenuate inflammation in macrophages, only I3A significantly attenuated both gene expression and secretion of cytokines (**Figure 3.4D**). Moreover, TA exhibited toxicity at higher doses. Based on these observations, we focused on I3A for further *in vitro* testing in hepatocytes.

Fatty acid supplementation has been used to mimic diet-derived lipid accumulation in hepatocytes (493), although these previous studies did not report visual evidence for lipid droplets that characterizes steatosis. Here, we used an imaging technique to confirm the presence of lipid droplets in both murine (AML12) and human (HepG2) liver cell lines. The HepG2 cell line was used to confirm that the fatty acid supplementation and other treatments performed in the study elicit a similar response in murine and human cells, since the latter is in some cases not appropriately modeled by the former. Targeted LC-MS experiments confirmed significant uptake of both palmitic and oleic acids in both cell types, although at least a fraction these pools remained non-esterified as FFAs.

While elevation of serum FFAs is an indicator of the metabolic syndrome (494), hepatic lipid accumulation more specifically indicates NAFLD. Targeted analysis for major FFAs confirmed that palmitic, stearic, and myristic acids were significantly elevated in livers of HFD-fed mice. The most abundant unsaturated fatty acid measured in our study is oleate, although its level in the liver was not significantly altered by the HFD. Palmitic acid can act as an inflammatory signaling molecule, whereas oleic acid is a quantitatively important substrate for esterification and promotes lipid accumulation through modulation of SREBP-1c activity (495). We supplemented the culture medium of hepatocytes with both fatty acids to induce lipid loading and present an inflammatory stimulus.

Two common denominators of NAFLD are lipid accumulation and elevation of pro-inflammatory factors in the liver. Lipid accumulation occurs primarily in hepatocytes, whereas pro-inflammatory cytokines are produced by resident and/or infiltrating macrophages (496). We found that FFA exposure along with LPS synergistically promotes TNFα, IL-1β, and MCP-1 production and secretion by macrophages. We selected TNFα as a representative cytokine to

mimic the effects of local macrophage activation on hepatocytes. In both AML12 and HepG2 cells, TNFα significantly increased the levels of intracellular FFAs. Similar observations were reported *in vivo* by Endo et. al. (497) who found that induction of TNFα expression through LPS injection in mice significantly enhances the expression of SREBP-1c and Fas, and elevates liver steatosis. We show that the effects of TNFα are nearly abolished by treating the cells with I3A. Treatment with I3A also attenuated the effects of TNFα on bile acid metabolism. Clinical studies (201, 204) have suggested that an alteration in both serum and liver bile acid compositions could be a biomarker for NAFLD; in particular, CDCA was found to be higher in NASH patients compared to healthy subjects. In our two-hit *in vitro* model, FFA and cytokine treatments independently and together increased the ratio of CDCA to CA, which was partially reversed by I3A treatment, again suggesting a normalization of lipid metabolism.

While the mechanism whereby I3A counters the effects of TNFα on lipid metabolism remains to be fully elucidated, our results, together with previously reported findings, point to activation of the AhR. We, and others (469, 498, 499), have shown that I3A is an agonist for the AhR in several cell types, including hepatocytes (**Figure 3.7D**). The present study shows that I3A also modulates the expression of two AhR regulated genes, SREBP-1c and Fas, in a manner consistent with ligand activation of AhR. Rats administered the AhR agonist TCDD exhibited decreased fatty acid synthesis in the liver (500). Liver-specific knockout (KO) of AhR exacerbated the effects of a HFD on liver steatosis, inflammation, and lipotoxicity (501). The same study also found increased *de novo* lipogenesis in AhR KO mice. Conversely, TNFα appears to interfere with transcriptional regulation by the AhR (502). However, the mechanism

remains to be elucidated, and conflicting findings have been reported in other cell types (e.g., MCF7 cells) (503)).

The protective role of the AhR in NAFLD likely depends on the degree of activation and the cellular context. Gorski et. al (504) reported that TCDD increased fatty acid synthesis in rat livers when administered at a very high dose (ten-fold higher than Lakshman et al.). We observed a similar dose dependence in both murine and human hepatocytes. At the highest dose (500 μM), we found that I3A by itself increased the levels of both palmitic and oleic acids compared to vehicle control, an effect we did not observe at lower doses (data not shown). On the other hand, I3A consistently lowered intracellular palmitic and oleic acids when presented together with FFAs, TNFα, or both. One possible explanation for these different findings is that negative regulation of lipogenesis by the AhR depends on whether the nuclear receptor is activated in a naïve setting or under conditions of lipid loading or heightened state of inflammation, when expression of AhR is suppressed.

Of the two "hits" applied in the present study, I3A more strongly attenuated the effects of TNFα. Hierarchical clustering (**Figure 3.10A**) and PLS-DA of untargeted LC-MS data indicate that the global metabolite profiles of I3A and TNFα treated cells (**Figure 3.10B**) more closely resemble the vehicle control compared to I3A and FFA treated cells (**Figure 3.10C**). Interestingly, treatment with I3A alone led to a metabolite profile that is distinct from the vehicle control, suggesting that this metabolite has broad effects on cellular metabolism. While our results indicate that these effects are at least partially mediated by the AhR, the involvement of other regulatory pathways cannot be ruled out. Furthermore, the stronger attenuation of TNFα

effects could reflect the timing of I3A treatment, which preceded TNFα exposure and followed FFA supplementation.

In conclusion, we have shown that the gut microbiota-dependent metabolite I3A can directly modulate inflammatory responses of hepatocytes and macrophages. Our results suggest that I3A could modulate liver inflammatory responses in at least two ways. Acting on the macrophages, I3A could attenuate the release of pro-inflammatory cytokines that induce the liver to synthesize FFAs, which in turn stimulate the macrophages. Acting on the hepatocytes, I3A could attenuate the cytokine-mediated upregulation in lipogenesis. These actions of I3A on the hepatocytes are AhR-dependent, as inhibition of AhR by a specific antagonist suppressed the effects of I3A. The AhR is likely but one of several host cellular receptors mediating host-microbiota crosstalk. In this regard, the approach presented in this paper could serve as a useful template to characterize other microbiota-dependent ligands and their effects on specific host cell types and pathways. Finally, further studies are warranted in animal models and human subjects to determine whether I3A or other microbiota metabolites can effectively intervene in the pathogenesis of NAFLD.

3.5 Supplemental information

Supplemental figures and tables are listed in the following pages.

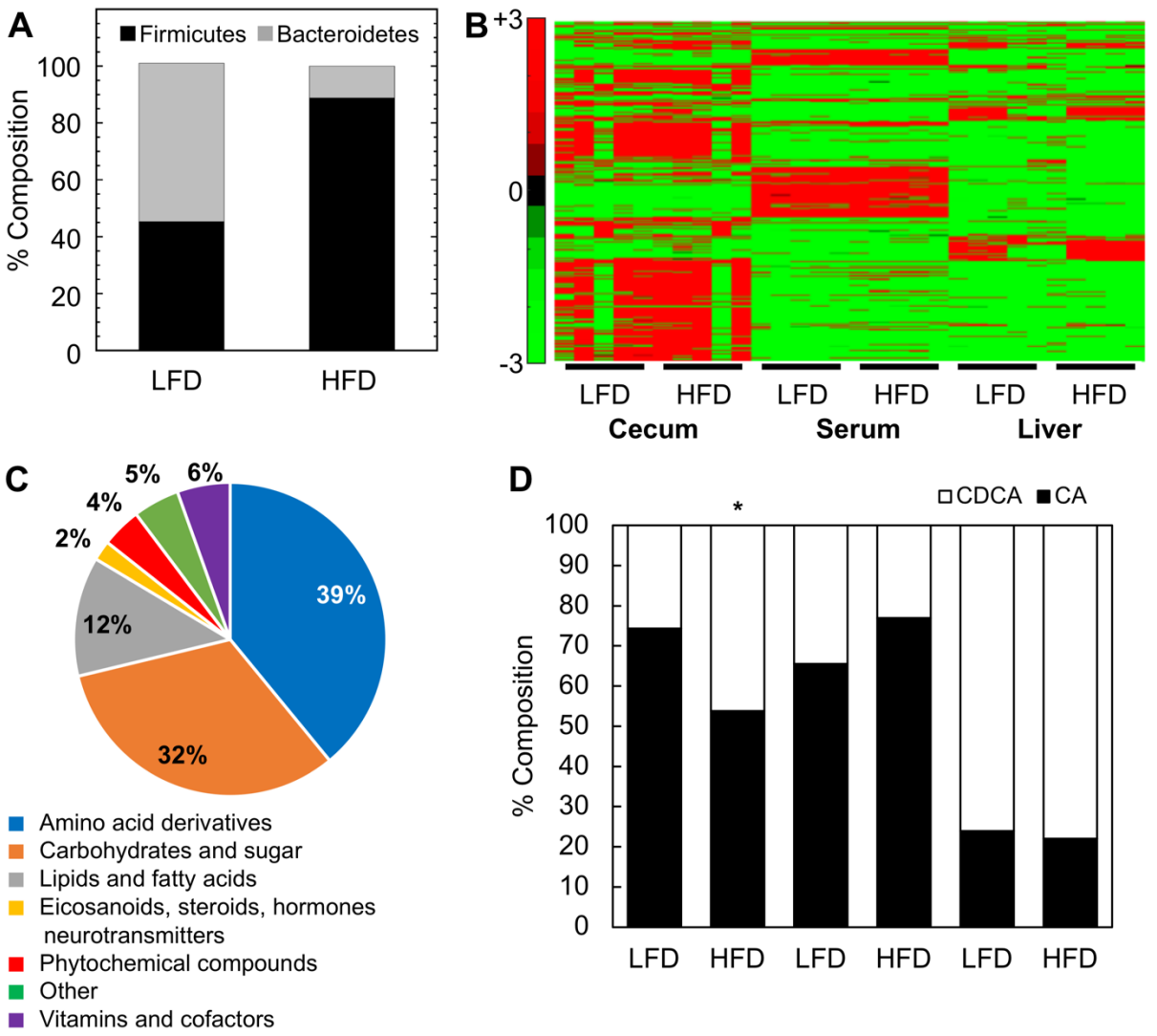

Figure 3.8 Effect of diet on microbiome and metabolite profile. Fecal material from mice fed a high-fat diet (HFD) or low-fat diet (LFD) were assayed for Firmicutes and Bacteroidetes using qRT-PCR (A). Ratios between the two phyla were determined from copy numbers calculated based on a DNA standard curve. Data shown are averages of n = 5 mice. Error bars represent one standard deviation. *: $p<0.05$ compared to LFD using Wilcoxon rank-sum test. Heat map shows hierarchically clustered LC-MS features (i.e., metabolites) detected in cecum, liver, and serum samples (B). Features from cecum, serum, and liver samples were separately scaled prior to clustering using the Pareto method. Red or green color denotes a feature that is elevated or depleted, respectively, relative to the mean value for the feature in the tissue compartment. Features significantly elevated or depleted were annotated and mapped to pathways cataloged in KEGG (C). Primary bile acid composition showing contribution from cholic acid (CA) and chenodeoxycholic acid (CDCA) (D).

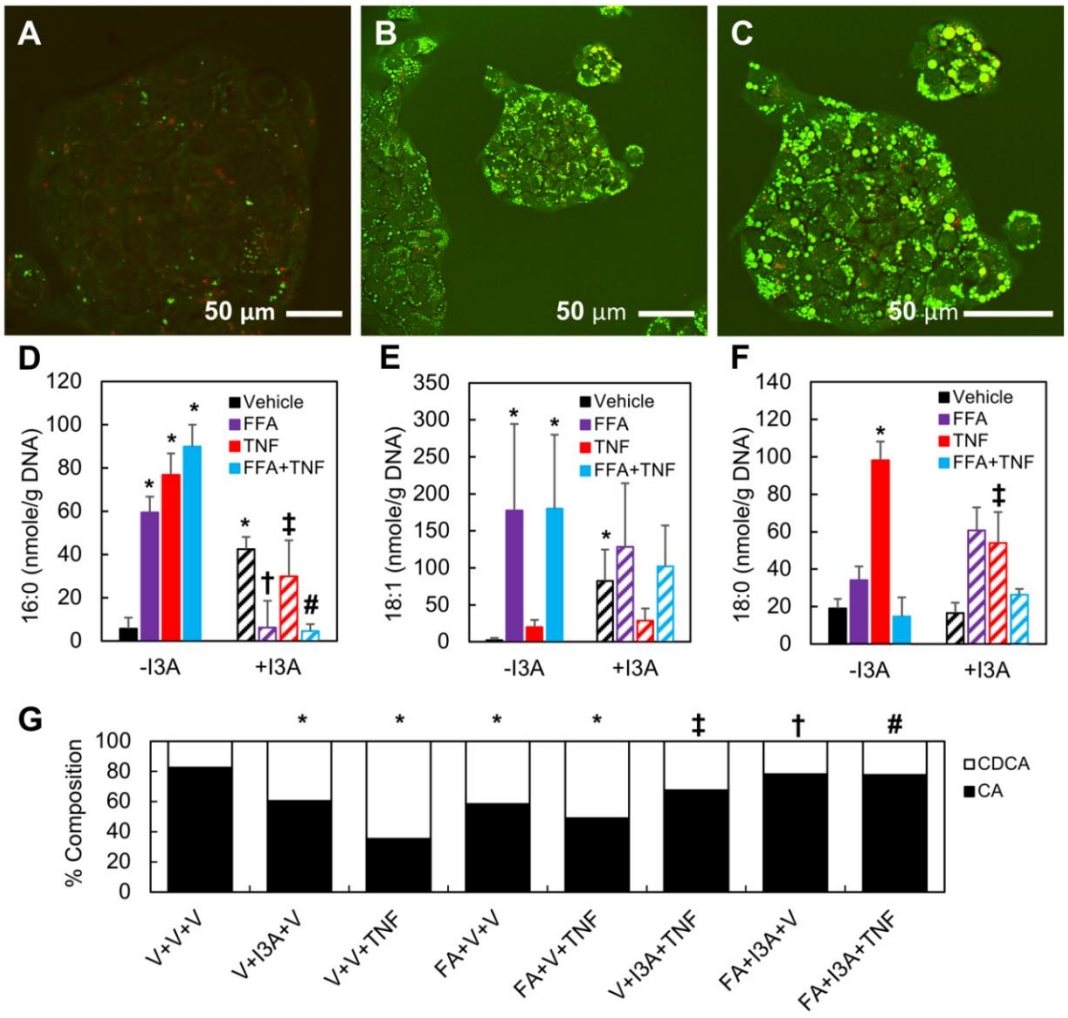

Figure 3.9 I3A attenuates TNFα-induced alterations in free fatty acid (FA) and bile acid (BA) metabolism in cultured HepG2 cells. Micrographs of HepG2 cells obtained using Coherent anti-Stokes Raman Scattering (CARS) microscopy (A-C). The cells were treated with either (A) vehicle or (B) a combination of palmitic (500 µM) and oleic acids (500 µM) for 48 hours. The microscope was tuned to detected aliphatic C-H vibrations. Lipid inclusion bodies show as green circles. Metabolites were extracted from cell cultures using a solvent-based method and analyzed using a product ion scan experiment for FFAs and BAs. The three most abundant FFAs were palmitic (D), oleic (E), and stearic (F) acids. Composition of primary BAs shown as a ratio of cholic acid (CA) to chenodeoxycholic acid (CDCA) (G). Data shown are averages of 4 independent experiments, each with 6 biological replicates. Treatment group means were compared using two-tailed Student *t*-test. *: $p<0.05$ compared to negative vehicle control (V+V+V), † : $p<0.05$ compared to the corresponding FFA supplementation group (FA+V+V), ‡: $p<0.05$ compared to the corresponding TNFα treatment group (V+V+TNF), #: $p<0.05$ compared to combined FFA supplementation and TNFα treatment (FA+V+TNF).

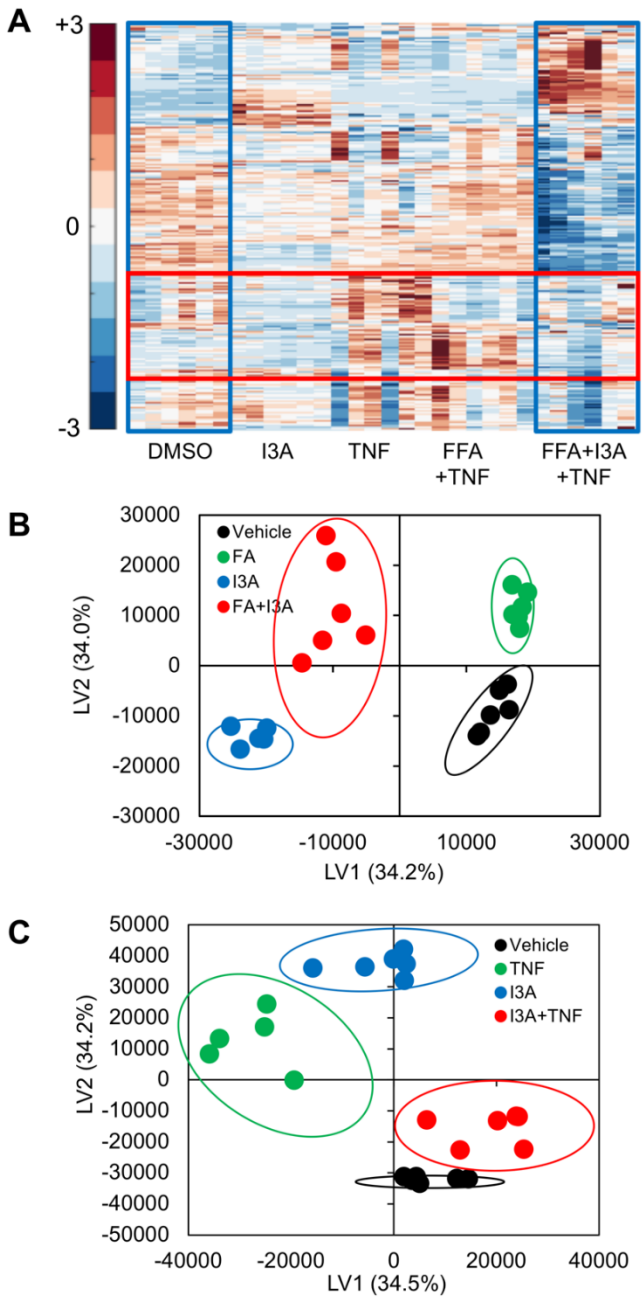

Figure 3.10 Multivariate analysis of untargeted metabolomics data from AML12 cultures. LC-MS data features were pareto-scaled and hierarchically clustered (A) for qualitative comparisons between the effects of TA and I3A on global metabolite profile. The similarity of the metabolite profiles was assessed based on group centroid distances using PLS-DA (B-C). Ellipses show 95% confidence intervals for each group. Numbers in parentheses show % of variance in the data explained by the latent variable (LV).

Table 3.1 Parameters for product ion scan and IDA experiments

	Bile Acid Method	**Free Fatty Acid Method**	**Hydrophilic Interaction Liquid Chromatography (HILIC)**
Column	Kinetex® 5 µm EVO C18 100 Å, 50 x 2.1 mm	Luna® 5 µm C8(2) 100 Å, 150 x 4.6 mm	Luna® 5 µm NH2 100 Å, 250 x 2 mm
Solvent A	50:50 v/v water:methanol with 0.1% w/v NH_4OH and 10 mM ammonium acetate	97:3 v/v water:methanol with 10 mM tributylamine and 15 mM acetic acid	95:5 v/v water:acetonitrile with 20 mM ammonium acetate and 20 mM NH_4OH
Solvent B	Methanol with 0.1% w/v NH_4OH and 10 mM ammonium acetate	Methanol	Acetonitrile
pH Solvent A	9	4.5	9.45
pH Solvent B	9	N/A	N/A
Column Temperature (°C)	50	25	25
Injection volume (µL); Draw speed (µL/min); Flowrate (µL/min)	10; 200; 200	10; 300; 300	10; 300; 300
MS Method	Product Ion Scan	Product Ion Scan	IDA

Table 3.1 Continued

Source (Ionization Mode)	ESI (-)	ESI (-)	ESI (+)
Calibration Solution	1 μM cholate, chenodeoxycholate, taurocholate, and glycocholate in 50:50 v/v water:methanol	0.01 mg/mL palmitic, oleic, stearic, palmitoleic, linoleic, and myristic acid in 0.01% v/v chloroform in methanol	Pos PPG Solution (AB Sciex)
Source Gas 1	40	60	35
Source Gas 2	35	60	45
Curtain Gas	25	25	25
Source Temperature (°C)	450	550	450
TOF MS Collison Energy (V)	-10	-5	+5
Declustering Potential (V)	-100	-100	80

Table 3.2 Collision energies (CE) and expected retention times (RT) for target product ions

Metabolite	LC/MS Method	Collision Energy (V)	Expected RT (min)
Cholic acid	Bile Acid	-30	0.6
Chenodeoxycholic acid	Bile Acid	-30	0.41
Palmitic Acid	Free Fatty Acid	-25	25.2
Oleic Acid	Free Fatty Acid	-25	26.02
Stearic Acid	Free Fatty Acid	-25	27.74
Palmitoleic Acid	Free Fatty Acid	-25	23.44
Indole-3-acetate	HILIC	+25 (IDA)	16.7
Tryptamine	HILIC	+25 (IDA)	3.4

Table 3.3 LC method for bile acid assay

Time (min)	Flowrate (uL/min)	%A	%B
0.50	500	100	0
4.50	500	50	50
5.50	500	0	100
6.50	500	100	0

Table 3.4 LC method for free fatty acid assay

Time (min)	Flowrate (uL/min)	%A	%B
0	300	20	80
20	300	1	99
40	300	1	99
41	300	20	80
50	300	20	80

Table 3.5 LC method for IDA experiments

Time (min)	Flowrate (uL/min)	%A	%B
0	300	15	85
15	300	100	0
28	300	100	0
30	300	15	85
60	300	15	85

Table 3.6 Primer sequences for qRT-PCR analysis

Gene	Forward Primer Sequence	Reverse Primer Sequence
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
TNFα	TCTCATGCACCACCATCAAGGACT	TGACCACTCTCCCTTTGCAGAACT
MCP-1	CTCTCTTCCTCCACCACCAT	ACTGCATCTGGCTGAGCCA
IL-1β	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
Fas	TACCAGTGCCACAGGAGTCTCA	TAAACACCTCGTCGATTTCGTTC
SREBP-1c	CTCCAAGGTTTCGTCTGACG	TCCAGTGGCAAAGAAACACC
Ahr	CGCGGGCACCATGAGCAG	GAGACTCAGCTCCTGGATGG
Cyp7a1	CCTTGGACGTTTTCTCGCT	GCGCTCTTTGATTTAGGAAG
Cyp27a1	TGCCTGGGTCGGAGGAT	GAGCCAGGGCAATCTCATACTT
GAPDH	GAAGGTCGGTGTGAACGGATTTGGC	TGTTGGGGGCCGAGTTGGGATA
Bacteroidetes	ACGCTAGCTACAGGCTTAACA	ACGCTACTTGGCTGGTTCA
Firmicutes	GCGTGAGTGAAGAAGT	CTACGCTCCCTTTACAC

CHAPTER IV

MECHANISMS UNDERLYING INHIBITION OF MACROPHAGE INFLAMMATION BY INDOLE-3-ACETATE AND TRYPTAMINE

4.1 Introduction

NAFLD is the most prevalent chronic liver disease in the western countries (44). It is a multi-stage disease, that starts as steatosis, which is a benign and reversible condition, and subsequently progresses to NASH, which is characterized as liver inflammation, and eventually progresses to fibrosis (1). In a subset of NASH patients, fibrosis further progresses to cirrhosis and eventually to hepatocellular carcinoma (HCC) (2).

Although significant progress has been made on understanding the pathogenesis of NAFLD, key drivers such as factors leading to the progression from steatosis to NASH are poorly understood. A "two-hit" model has been widely used for studying the pathogenesis of NAFLD. In this model, lipid accumulation in the liver is thought to be the first hit, and additional stimuli such as increased levels of inflammatory cytokines, increased reactive oxygen species (ROS) and decreased hepatic ATP production, are regarded as possible second hits that result in the progression of steatosis to NASH (11). However, the mechanisms underlying the pathogenesis of NASH are highly heterogeneous and a "parallel hit" model has been proposed, in which multiple gut-derived, adipose tissue-derived, as well as systemic factors are considered as drivers inducing hepatic inflammation during NASH (15, 55).

Hepatic inflammation is a hallmark feature of NASH and is an important driver in the development of liver fibrosis, cirrhosis, and even HCC (157). The activation of Kupffer cells (KCs), the liver resident macrophages, has been established in experimental models as a key step in the initiation and progression of liver inflammation (248, 249). Hepatic macrophages in NASH

produce pro-inflammatory cytokines and chemokines as a result of excess lipid loading and FFAs (253), signals derived from the surrounding steatotic hepatocytes (254), and damage associated molecular pattern (DAMP) induced activation of KCs (255). The accumulation and polarization of macrophages to a pro-inflammatory phenotype in the liver is considered as a hallmark feature of progressive disease in NASH patients (249, 256). Once activated, inflammatory macrophages are capable of secreting a broad range of cytokines, such as IL-1β and TNFα to further increase inflammation leading to fibrosis. Activated KCs also secrete chemokines such as MCP-1 to recruit monocytes from circulation to infiltrate the liver and further exacerbate inflammation (216, 223).

A large body of evidence has shown that the AMP-activated protein kinase (AMPK) is involved in hepatic lipid metabolism, and increasing the activity of AMPK has been proposed as a therapeutic target for NAFLD (17, 20, 372, 385). Decreased AMPK activity has been linked to obesity, diabetes, cardiovascular disease and NAFLD (17). Dysregulation of AMPK activity due to either impaired AMPK phosphorylation or AMPK protein expression in the liver has been observed in high fat diet fed mice that show increased lipid loading (18, 19, 505). In addition, stimulation of mouse bone marrow derived macrophages with LPS also leads to down-regulation of AMPK activity, and inhibition of AMPK by RNA interference increases LPS-induced TNFα expression (19). Thus, the two main pathological factors in NAFLD - lipid overloading (18) and pro-inflammatory signals (19) - can inhibit AMPK activity. The deficiency of AMPK found in various cells with metabolic diseases strongly suggest that restoring AMPK activity could be important in attenuating inflammation.

In Chapter 3, we identified microbiota derived metabolites that are depleted in a HFD-fed mouse model of NAFLD. Among them, two metabolite I3A and TA reduced the expression of inflammatory cytokines (TNFα, IL-1β and MCP-1) in macrophages exposed to palmitate and LPS.

While this established the anti-inflammatory phenotype elicited by these metabolites, the signaling pathways and underlying mechanisms involved are not understood. Although gut microbiota derived metabolites such as indole derivatives have been previously identified as aryl hydrocarbon receptor (AhR) ligands (43, 469) and shown to be important in maintaining intestinal homeostasis (506, 507), the expression level of AhR in macrophages is low compared to other cell types (508). Given the strong anti-inflammatory effect elicited by I3A and TA, it is possible that these metabolites engage signaling pathway(s) other than the AhR for their anti-inflammatory activity. The present study investigates the hypothesis that the anti-inflammatory effect of I3A and TA on macrophages is mediated through AMPK signaling.

4.2 Materials and Methods

4.2.1 Reagents

RAW 264.7 cells were purchased from ATCC (Manassas, MA). Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, LPS (from *Salmonella minnesota*) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) was purchased Sigma Aldrich (St. Louis, MO). Unless otherwise noted, all other reagents were purchased from Sigma Aldrich (St. Louis, MO).

4.2.2 Murine macrophage culture

Raw 264.7 murine macrophages were cultured in a humidified incubator at 37°C and 5% CO_2 with DMEM supplemented with 10% heat inactivated FBS, penicillin (200 U/mL) and streptomycin (200 μg/mL). Cells were passaged every 2-3 days and used within 10 passages after thawing.

4.2.3 RNA extraction and qRT-PCR

RAW 264.7 cells were seeded into 24-well plate as density of ~4 x 10^5 cells/ml. When the cell density reached ~ 80% confluence, cells were pre-treated with 1 mM I3A, 250 μM TA or DMF (solvent control) for 4 hours, followed by addition of 300 μM palmitate complexed with BSA. After 18 h, 10 ng/mL LPS was added to the cells for an additional 6 h. At the end of the exposure period, cells were washed twice with PBS, and the cell pellets were stored at -80 °C until RNA extraction. Total RNA was extracted from RAW 264.7 cell pellets using the EZNA Total RNA kit (Omega Bio-Tek, Norcross, GA). Purity of isolated RNA was confirmed by A260/A280 ratio. RTPCR analysis was carried out using the qScript One-Step PCR kit (Quanta Biosciences, Gaithersburg, MD) on a LightCycler 96 System (Roche, Indianapolis, IN). Fold-change values were calculated using the $2^{-\Delta\Delta Ct}$ method, with β-actin as the housekeeping genes. The primer sequences used are listed in **Table 4.1**.

4.2.4 Cytokine quantification

RAW 264.7 cells were treated with metabolites for 4 hours, followed by 300 μM Palmitate for 18 hours and 10ng/ml LPS for 24 hours. Culture supernatants were centrifuged at 5,000 x *g* for 15 min at 4^{o}C and cytokines (TNFα and MCP-1) in the supernatant were quantified using commercially available ELISA kits (BioLegend, San Diego, CA) using the manufacturer's suggested protocol.

4.2.5 Protein isolation immunoblotting

RAW 264.7 cells were treated as described in each experiment. Cells were washed twice with PBS and stored at -80 °C until protein isolation. Cell pellets were lysed with modified RIPA buffer (50 mM Tris-HCl, PH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and 0.5% Sodium deoxycholate) supplemented with a protease inhibitor cocktail (Sigma), 10mM NaF, and 1mM

Na_3VO_4. The protein concentration was determined using the BCA protein assay kit (Pierce). Protein samples were denatured with SDS and equal amounts (~ 20 ug) were loaded on polyacrylamide 10% SDS-PAGE gels. Proteins were initially resolved at 60V for 30 min, followed by 120 min at 90V.

Proteins were transferred to a PVDF membrane (Thermo Scientific, Waltham, MA) by wet-transfer electrophoresis following the Bio-Rad standard protocol. A blocking solution of 5% non-fat milk in TBST were used to block non-specific binding sites. The blots were then probed with appropriate primary antibodies (p-AMPK: 2535, total AMPK: 2603, β-actin: 12620, Cell Signaling Technology) and secondary antibody (Anti-rabbit horseradish peroxidase-conjugated secondary antibody, 7074, Cell Signaling Technology). Proteins were visualized based on chemoluminescence by incubating the blot with Clarity and Clarity Max Western ECL Blotting Substrate (Bio-Rad, Hercules, CA) and imaged on a ChemiDoc gel imaging system (Bio-Rad, Hercules, CA). Protein quantification was carried out by normalizing the intensity of the protein band of interest to the intensity of the β-actin band in the same lane using the Imagelab software (Bio-Rad, Hercules, CA).

4.2.6 Small interfering RNA (siRNA) transfection

RAW 264.7 cells were seeded into 6-well or 24-well plates at ~ 30% confluence and cultured for 24 hours prior to transfection. Cells were transfected with ON-TARGETplus Mouse prkaa1 siRNA (Dharmacon, Lafayette, CO) or ON-TARGETplus non-targeting pool (negative control, Dharmacon, Lafayette, CO) using the GenMute siRNA transfection reagent (SignaGen Laboratories, Rockville, MD) according to manufacturer's instruction. After 24 h, the medium was replaced with siRNA-free growth medium and incubated for an additional 24 – 72 h. The

transfection efficiency was determined by monitoring the change in AMPK mRNA and protein levels using qRT-PCR and Western blot, respectively.

4.3 Results

4.3.1 I3A and TA inhibit macrophage inflammatory cytokine production induced by FFA and LPS

In Chapter 3, we utilized an *in-vitro* two-hit model of macrophage inflammation to investigate the anti-inflammatory potential of I3A and TA. In this model, palmitate is the first-hit that increases lipid loading, and LPS is the second hit that induces inflammation. We observed that both I3A and TA inhibited pro-inflammatory cytokine production in macrophages in a dose-dependent manner.

To investigate the underlying mechanisms, we first tested whether I3A and TA's effects specifically targeted palmitate- or LPS-induced inflammation in RAW 264.7 macrophages. Cells stimulated with either palmitate, LPS, or palmitate followed by LPS, were exposed to I3A or TA as described in Materials and Methods. Changes in the expression of three pro-inflammatory cytokines, TNFα, IL-1β and MCP-1 were quantified at the mRNA level by qRT-PCR. **Figure 4.1 (A)** shows that exposure to I3A attenuated the increase in the mRNA levels of MCP-1 in all three groups. The attenuation only showed in palmitate and palmitate followed by LPS for TNFα levels, and LPS and palmitate followed by LPS groups for IL-1β. **Figure 4.1 (B)** shows a similar decrease in the levels of secreted TNFα upon exposure to the metabolites relative to the DMF control.

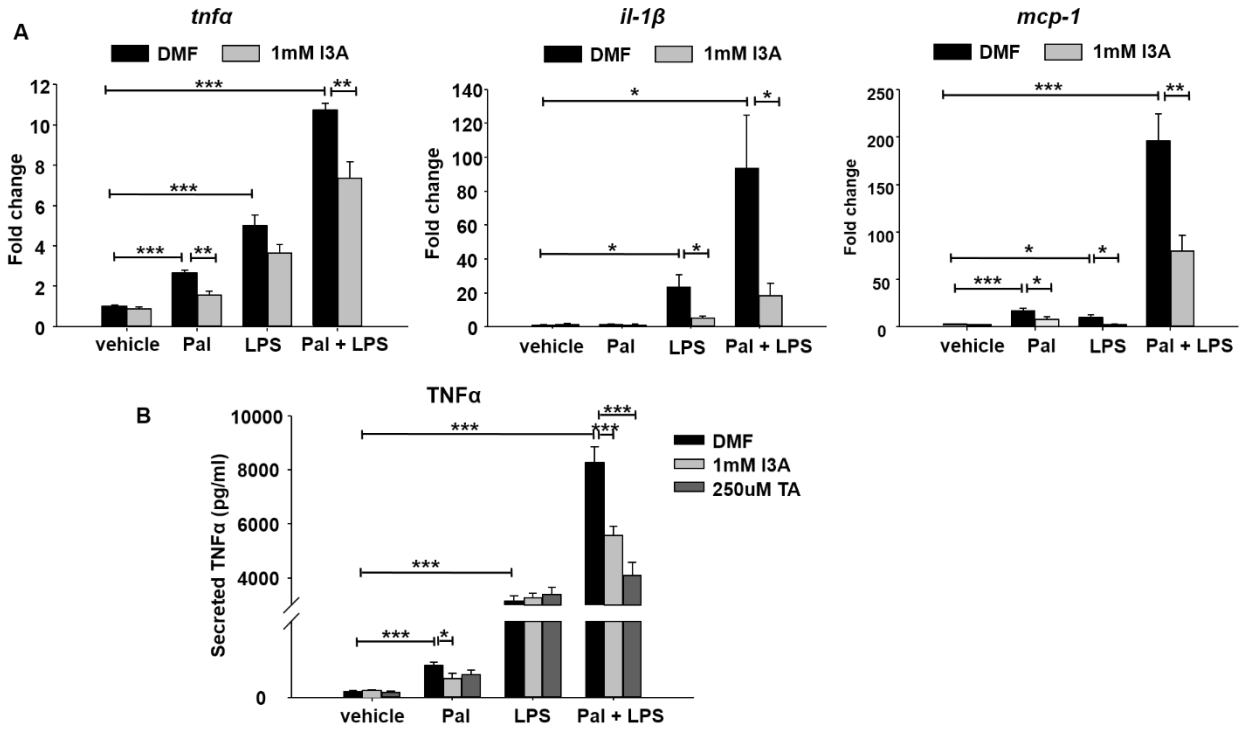

Figure 4.1 I3A and TA reduce both palmitate and LPS induced inflammation in macrophages. RAW264.7 cells were pre-treated with DMF (solvent control), 1 mM I3A or 250 μM TA for 4 hours, stimulated with 300 μM palmitate or vehicle control for 18 hours, followed by stimulation with 10 ng/ml LPS or media control for another 6 hours. (A). Total RNA was isolated and gene expression of inflammatory cytokines (TNFα, IL-1β and MCP-1) were analyzed with qRT-PCR. (B). Cells were kept in culture for another 24 hours after LPS stimulation and cell culture supernatants were harvested for secreted TNFα ELISA. Data shown are mean +/- standard error from three independent experiments, and three biological replicates in each experiment.*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ indicate statistical significance assessed by the Student's *t*-test.

4.3.2 Activation of AMPK inhibits inflammatory cytokine expression induced by palmitate and LPS

Since I3A and TA inhibit inflammatory cytokine production induced by both palmitate and LPS, we tested the hypothesis that I3A and TA target common pathway(s) that are induced by both palmitate and LPS in macrophages. It has been reported that palmitate activates NF-κB in different

cell types, including macrophages, through the TLR2 and TLR4 receptors (509-514). As a classic TLR4 agonist, LPS activates NF-κB to initiate pro-inflammatory signaling and cytokine production in different cell types, including macrophages (515-518). However, a recent report by Lancaster et al. (519) found that although TLR4 is required for palmitate-induced inflammation, palmitate is not a TLR4 agonist and cannot activate NF-κB signaling like LPS. On the other hand, a large body of evidence show that AMPK is involved in hepatic lipid metabolism, and both lipid overloading and inflammatory signals can inhibit AMPK activity (18, 19). Therefore, we investigated the effect of AMPK in the attenuation of palmitate- and LPS-induced inflammation by I3A and TA.

We first tested if treatment of palmitate and LPS affect p-AMPK levels in macrophages. Western blot analysis of p-AMPK and total AMPK levels in macrophages indicated that both palmitate and LPS reduced p-AMPK levels compared to controls (**Figure 4.3 A and B**, bands marked with red box), which was consistent with previous reports (18, 19). We then tested if increasing AMPK activation attenuated palmitate- and LPS-mediated inflammation in RAW 264.7 macrophages. Cells were incubated with the AMPK activator AICAR (1 mM) along with palmitate and LPS, and the expression of TNFα, IL-1β and MCP-1 were analyzed by qRT-PCR. As shown in **Figure 4.2**, activation of AMPK by AICAR significantly inhibited palmitate and LPS induced increase in the levels of the three cytokines, compared to the PBS control.

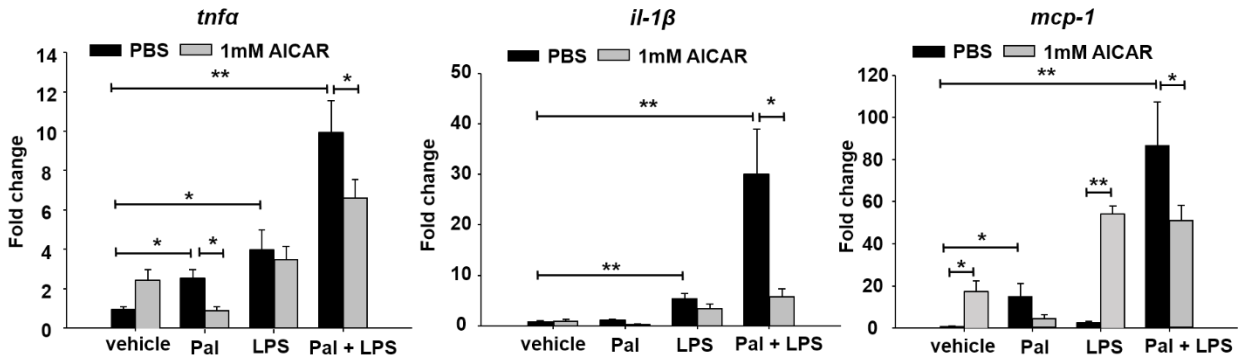

Figure 4.2 Activation of AMPK reduces palmitate and LPS induced macrophage inflammation. RAW264.7 cells were stimulated with 300 μM Palmitate or vehicle control for 18 hours, followed by 10 ng/ml LPS or media control for another 6 hours in the presence of 1mM AICAR or PBS. Total RNA was isolated and inflammatory cytokine expression analyzed by qRT-PCR. Data shown are mean +/- standard error from two independent experiments, with three biological replicates in each experiment. *: $p<0.05$, **: $p<0.01$ indicate statistical significance assessed by the Student's *t*-test.

4.3.3 I3A and TA reversed palmitate and LPS induced p-AMPK reduction

We also investigated the effect of palmitate and LPS on AMPK phosphorylation in RAW 264.7 macrophages. Both palmitate and LPS reduced p-AMPK levels (**Figure 4.3 A, B**), and activation of AMPK by AICAR inhibited palmitate and LPS induced macrophages inflammation (**Fig 4.2**). Since I3A and TA also inhibited palmitate and LPS induced inflammation, we hypothesized that I3A and TA inhibited palmitate and LPS induced inflammation through increasing AMPK activity. To test this hypothesis, we first investigated if I3A and TA altered p-AMPK levels in palmitate and LPS treated macrophages. When cells were exposure to I3A and TA, the levels of p-AMPK were significantly increased (4-fold increase for LPS treatment and 2-fold for palmitate treatment) compared to vehicle control (**Figure 4.3**). These results demonstrate that I3A and TA can reverse palmitate and LPS-induced reduction in AMPK activity in macrophages.

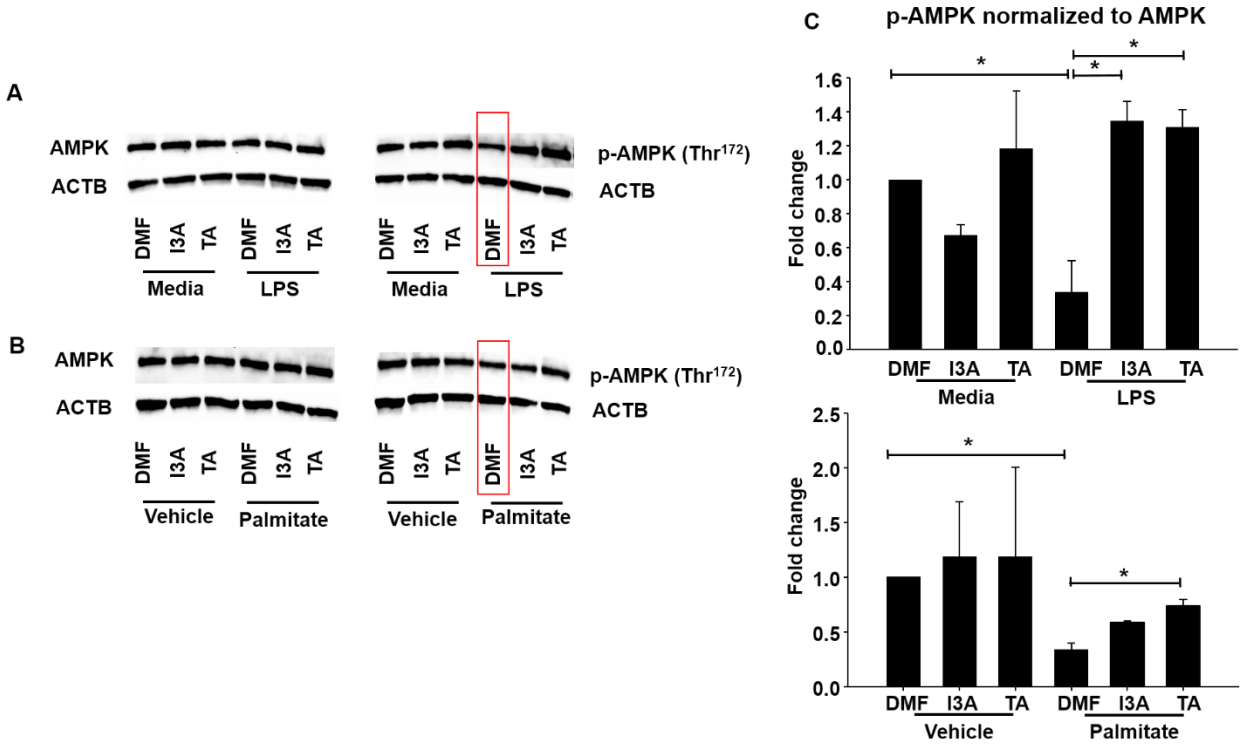

Figure 4.3 I3A and TA reverse palmitate- and LPS-induced decrease in p-AMPK. Raw 264.7 cells were pre-treated with 1 mM I3A, 250 µM TA or DMF control for 24h, and then stimulated with 300 µM Palmitate or 10 ng/ml LPS for 4 hours. Total protein (20 µg/lane) was used for Western blot analysis of p-AMPK and total AMPK. (A) and (B). Representative blots of p-AMPK and total AMPK with LPS and palmitate stimulation, respectively. (C). Ratio of quantified p-AMPK levels normalized to total AMPK levels. Both p-AMPK and AMPK levels were normalized to ACTB first and then the ratio of p-AMPK/AMPK calculated for either metabolite treatment or solvent controls. The fold-change of p-AMPK normalized to AMPK for I3A or TA relative to DMF controls is plotted. Data shown are mean +/- standard error from two independent experiments. *: $p<0.05$ indicates statistical significance assessed by the Student's *t*-test.

4.3.4 Knock down of AMPK by siRNA significantly reduces I3A and TA's anti-inflammatory effect

Based on data showing that activation of p-AMPK reduces inflammation (**Figure 4.2**) and I3A and TA reverse palmitate- and LPS-induced decrease in p-AMPK levels in macrophages

(**Figure 4.3**), we tested the effect of reduced AMPK activity on the anti-inflammatory effect of I3A and TA. Since AMPKα1 is the main form of AMPKα in murine macrophages (410), and AMPKα plays an important role in NAFLD and inflammation (19, 410, 416, 423), we used siRNA against AMPKα1 to reduce AMPK levels in RAW 264.7 macrophages. Significant knockdown of AMPK mRNA (50%), as well as AMPK (40% to 50%) and p-AMPK (40% to 60%) protein, was observed with prkaa1 siRNA compared to non-targeted siRNA control, for up to 96 hours (**Figure 4.4**).

We tested if I3A and TA attenuated inflammation to a lesser-degree in AMPK siRNA-treated macrophages. RAW 264.7 cells were transfected with prkaa1 or non-targeted siRNA for 24 hours, change to siRNA free media for 48h. Cells were then treated with I3A, TA or DMF control, followed by palmitate and LPS. Pro-inflammatory cytokines production were measured by qRT-PCR and ELISA. As shown by **Figure 4.5**, knockdown of AMPK by prkaa1 siRNA significantly reduced I3A and TA's ability to reduce palmitate- and LPS-induced production of TNFα, IL-1β and MCP-1. Specifically, while I3A addition to the non-targeted control resulted in a 30% reduction in TNFα, no decrease in the levels of TNFα was observed with prkaa1-treated cells. Similarly, a 50% reduction in IL-1β was observed with the non-targeted control which decreased to 30% in prkaa1-treated cells, while a 60% reduction for MCP-1 was observed with the non-targeted control compared to 45% in prkaa1 siRNA treated cells (**Fig. 4.5 C**). Consistent with the change in mRNA levels, AMPK knockdown resulted in less decrease in MCP-1 secretion with I3A and TA upon palmitate- and LPS-induced inflammation (~ 2-fold less decrease compared to untargeted siRNA) (**Figure 4.6**). In summary, these data suggest that I3A and TA alter AMPK signaling to modulate palmitate- and LPS-induced macrophage inflammation.

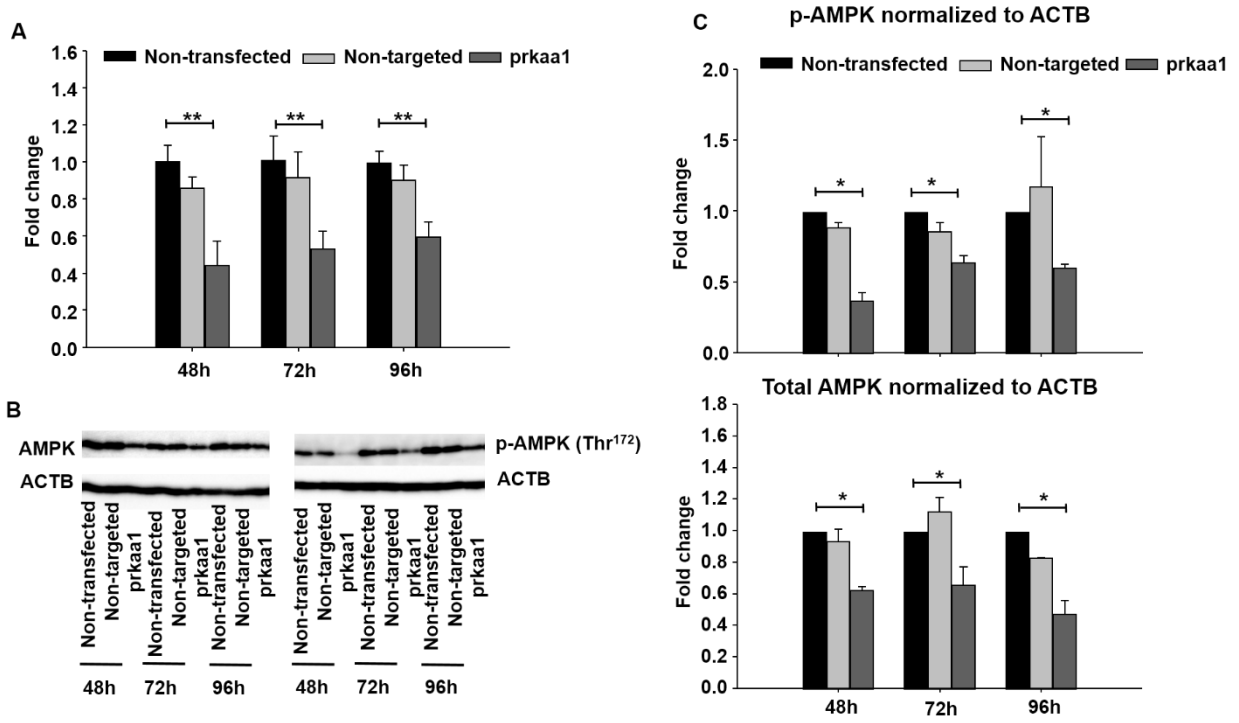

Figure 4.4 Prkaa1 siRNA reduces both p-AMPK and AMPK levels in macrophages. (A). RAW 264.7 cells were transfected with non-targeted or prkaa1 siRNA as described in Materials & Methods, and AMPKα mRNA levels were analyzed by qRT-PCR. Data shown are mean +/- standard error from two independent experiments, with three biological replicates in each experiment. (B), Representative Western blot for p-AMPK and AMPK detection. (C). Normalized p-AMPK or AMPK levels. Both p-AMPK and AMPK levels were normalized to ACTB first and the fold-change of p-AMPK or AMPK for prkaa1 siRNA relative to non-transfected controls is plotted. Data showed are mean +/- standard error from four individual experiments. *: $p<0.05$, **: $p<0.01$ indicate statistical significance assessed by the Student's *t*-test.

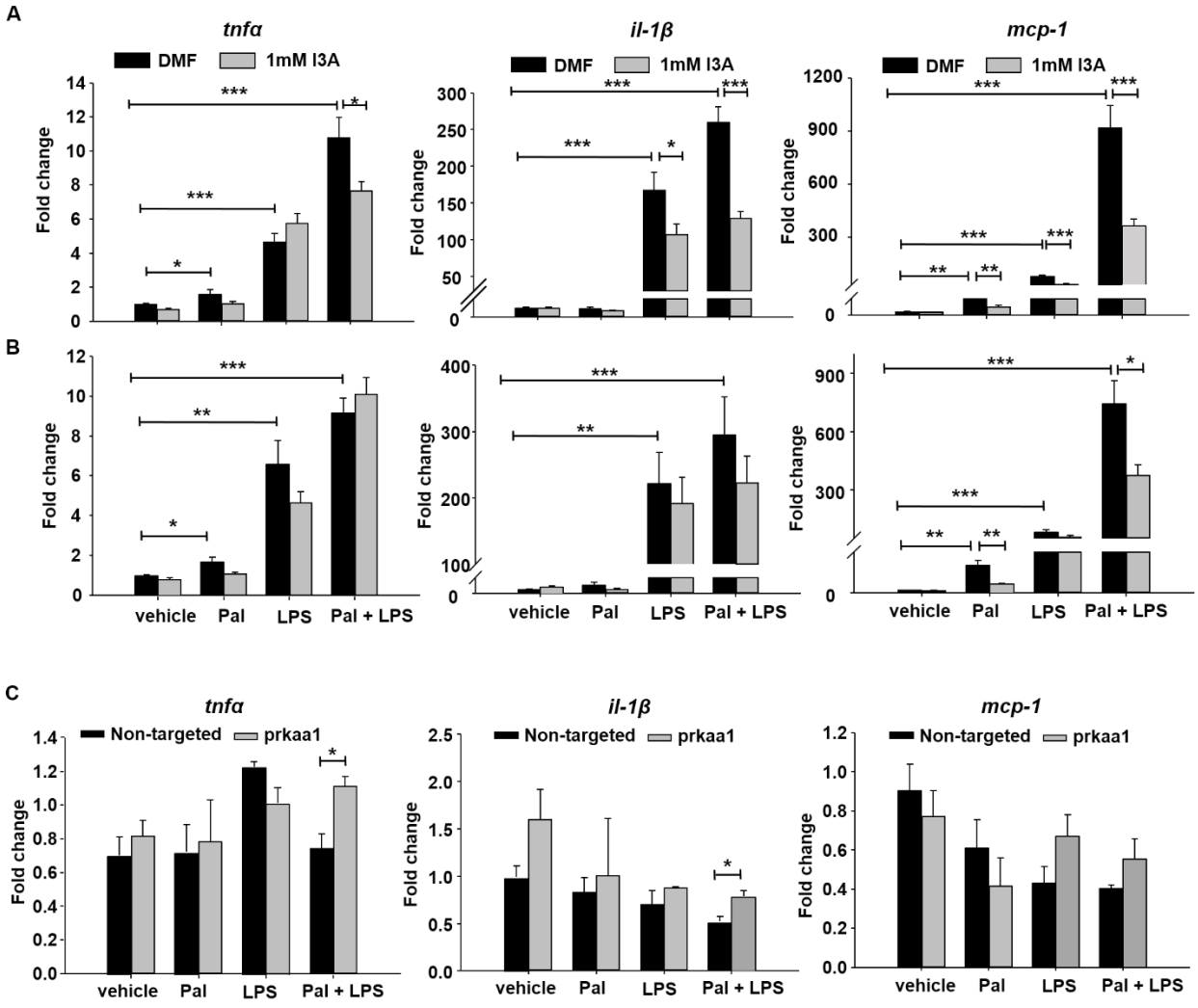

Figure 4.5 Knockdown of AMPK by prkaa1 siRNA significantly reduces I3A's ability to decrease pro-inflammatory cytokine gene expression. RAW264.7 cells were transfected with (A) non-targeted siRNA or (B) Prkka1 siRNA as described in Materials & Methods. Cells were treated with 1 mM I3A or DMF control for 4 hours, stimulated with 300 µM palmitate or vehicle control for 18 hours, followed by 10ng/ml LPS for 6 hours. Total RNA was isolated and inflammatory cytokine mRNA levels were analyzed by qRT-PCR. (C).The ratio of inflammatory genes expression level with I3A treatment relative to DMF is shown. Data are mean +/- standard error from three individual experiments with three biological replicates in each experiment. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ indicate statistical significance assessed by the Student's *t*-test.

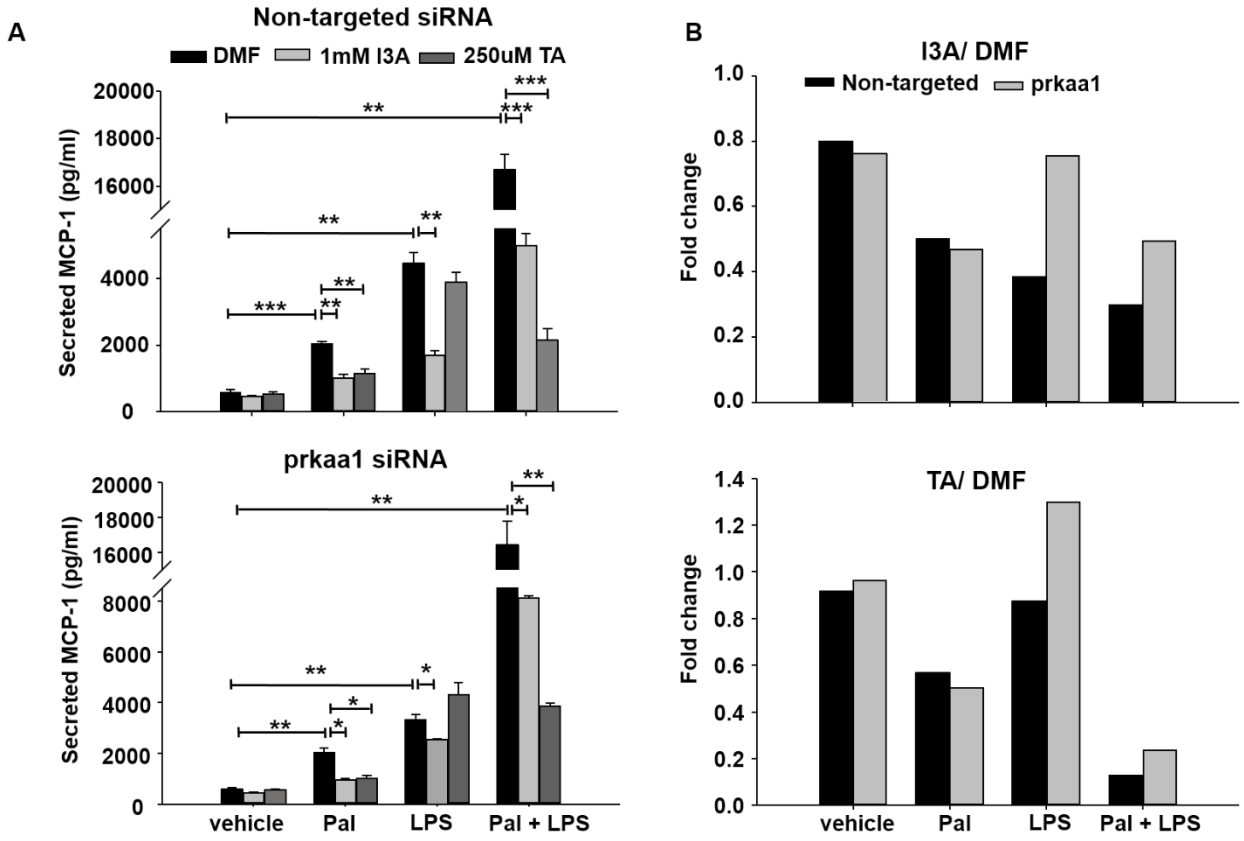

Figure 4.6 Knockdown of AMPK by prkaa1 siRNA significantly reduces I3A and TA's ability to decrease MCP-1 secretion. RAW264.7 cells were transfected with (A, top panel) non-targeted siRNA or (A, bottom panel) prkka1 siRNA as described in Materials & Methods. Cells were treated with 1 mM I3A, 250 µM TA or DMF control for 4 hours, stimulated with 300 µM palmitate or vehicle control for 18 hours, followed by 10ng/ml LPS for 6 hours. Cell culture supernatants were harvested for ELISA. (B).The ratio of secreted MCP-1 with (top panel) I3A or (bottom panel) TA treatment relative to DMF is shown. Data shown are mean +/- standard error from one experiment with 3 biological repeats. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ indicate statistical significance assessed by the Student's *t*-test.

4.4 Discussion

An increasing number of studies have shown that diet is an important risk factor in metabolic syndrome including NAFLD. High fat diet or western diets that are most commonly used in rodent models of metabolic syndrome alter the composition of the gut microbiota (12), which is associated with a series of physiological conditional change including fatty liver disease (195, 520). Besides the compositional change itself, the levels of microbiota metabolites that are altered with dysbiosis have been linked to multiple diseases including NAFLD (317). In Chapter 3, we co-analyzed the differences in metabolites profiles between HFD- and LFD fed mice alongside the differences between CONV-R and GF mice to identify metabolites that depend on gut microbiota, and are significantly depleted in HFD mice. By using a two-hit model of hepatic inflammation where saturated fatty acid palmitate is the first hit and LPS is the second hit, we demonstrated that both I3A and TA inhibited the production of TNFα, IL-1β, and MCP-1 in macrophages in a dose-dependent manner. Here, we report on the identification of one of the signaling pathways that are activated by these metabolites to attenuate inflammation.

Based on gene expression and protein data showed I3A and TA could inhibit macrophage pro-inflammatory cytokine production when exposed to palmitate alone (TNFα, MCP-1), LPS alone (IL-1β, MCP-1), or palmitate followed by LPS (TNFα, IL-1β and MCP-1) (**Fig 4.1**), we investigated the possibility that I3A and TA modulate a macrophage signaling pathway that is common to both palmitate- and LPS-induced inflammation. The activation of NF-κB signaling through TLR2 and TLR4 by saturated fatty acids is thought to be the main mechanism that SFAs such as palmitate induce inflammation in multiple tissues during metabolic disease (509-514). However, a recent study by Lancaster et al. suggests that palmitate is not a TLR4 agonist, based on molecular modeling simulation showed that palmitate cannot bind to TLR4 like LPS and

experimentally evidence that palmitate cannot induce dimerization and endocytosis of TLR4 or activate NF-κB signaling up to 6 h in both mouse and human macrophages (519). Instead, the authors proposed that BSA that used to solubilize palmitate could alter macrophage metabolism and consequently the cellular lipidome to prime cells through TLR4 signaling, and palmitate then acts as the second hit to induce inflammation in primed macrophages.

As an energy sensor, AMPK activity is reduced in response to LPS, TNFα, and HFD-induced obesity in multiple tissues, such as adipose tissue (409-411), muscle (412), and the liver (409, 413). HFD and inflammatory stimuli decrease the expression of AMPK and activity of AMPKα1 in mouse adipose tissue and macrophages; treatment with the AMPK activator AICAR reverses diet- and LPS-induced inflammation (410, 416). On the other hand, anti-inflammatory cytokines, such as IL-10 and TGF-β activate AMPK in both mouse and human macrophages (416). AMPK also inhibits acute inflammatory responses in macrophages, and reduced expression of AMPKα1 augments pro-inflammatory cytokine production and NF-κB signaling (19, 410), while activation of AMPK by AICAR or constitutively active AMPK elicits the opposite effect (19, 410). Myeloid-deficient AMPKα1 knockout mice (423) and mice lacking AMPKβ1 in hematopoietic cells (424) show increased accumulation of inflammatory macrophages in adipose tissue and the liver, and when fed with HFD, mice demonstrate accelerated development of insulin resistance. Thus, AMPK signaling could be a target candidate mediating the anti-inflammatory effects of I3A and TA.

Activation of AMPK signaling by the pharmacological activator AICAR significantly reduced palmitate- and LPS-induced inflammatory cytokine expression (**Figure 4.2**), which suggest that AMPK signaling is important in regulating macrophage inflammation. Compared to AICAR, I3A inhibited TNFα expression to a similar level, but was more potent in inhibiting the

expression of IL-1β induced by both LPS, and palmitate followed by LPS. While I3A inhibited the expression of MCP-1 stimulated by palmitate, LPS, and palmitate followed LPS, AICAR inhibited the expression of MCP-1 stimulated by palmitate followed LPS but increased the expression of MCP-1 in LPS-stimulated cells.

While both I3A and TA's effect on reducing inflammatory cytokine production was significantly reduced when AMPK levels were knocked down by siRNA (**Figures 4.4 - 4.5**), complete abolishment of the anti-inflammatory effect was not observed because only 50% knockdown in AMPK levels was achieved. It is possible that the residual AMPK is sufficient for partially propagating the effects of I3A and TA on inflammatory cytokine production. It is also possible that additional signaling pathways are involved in mediating the effects of I3A and TA. Complete knockdown of AMPK using CRISPR/Cas9 would help elucidate the role of AMPK in the observed response.

Recent studies have demonstrated that tryptophan derived metabolites are AhR agonists in several cell types (469, 498, 499). The activation of AhR by tryptophan derived metabolites in intestinal epithelial cells plays an important role in maintaining intestinal homeostasis (39, 41, 348). AhR is also expressed by different intestinal immune cells such as intraepithelial lymphocytes (IELs) (521), T cells (522), innate lymphoid cells (ILCs) (523), DCs (508, 524) and neutrophils (525). Deficiency of AhR in mice results in the loss of IELs (521) and altered production and function of group 3 ILC (ILC3) (526). The lack of IL-22 secretion by ILC3 due to AhR knockout has been correlated to infections and metabolic disorders (527). Kimura et al. showed that peritoneal macrophages derived from mice lacking AhR produce more pro-inflammatory cytokines when stimulated by LPS compared to wild type mice, and suggesting the importance of AhR in the modulation of inflammation in peritoneal macrophages (528). However,

unpublished data from our lab (Klemashevich, Cheng, and Jayaraman) shows that the tryptophan metabolite indole attenuates LPS-induced TNFα production and chemotactic migration towards MCP-1 in BMDMs derived from both $AhR^{-/-}$ mice and wild type mice. Since there is no known intersection between the AhR and AMPK signaling pathways, our results strongly suggest that tryptophan metabolites can engage a signaling pathway other than the AhR for eliciting anti-inflammatory effects.

Our observations on the involvement of a signaling pathway other than the AhR is consistent with observations on the differences in the levels of AhR expression in different cell types. Frericks et al. showed that the expression level of AhR is different in a range of tissue and cell types, with BMDMs at the lower end of the AhR expression level spectrum (508). Moreover, the canonical AhR agonist TCDD does not induce the expression of the AhR target gene Cyp1a1 in both BMDMs and RAW264.7 cells (Cheng and Jayaraman, unpublished). Furthermore, in hepatocytes where AhR is highly expressed, I3A modulates lipid metabolism in hepatocytes through AhR signaling (529). Thus it is possible that the signaling pathway engaged by tryptophan metabolites could depend on the relative abundance of the AhR in the cell type of interest.

In conclusion, we have shown that gut microbiota derived metabolites I3A and TA can directly modulate inflammatory responses in macrophages. Mechanistically, we identified that both palmitate and LPS reduced levels of p-AMPK and the p-AMPK activator AICAR reduced palmitate and LPS induced inflammatory cytokine production. The addition of I3A and TA reversed p-AMPK reduction induced by palmitate and LPS. siRNA knockdown of AMPK in macrophages reduced I3A and TA's anti-inflammatory effects suggests that I3A and TA's effect is dependent on AMPK signaling.

Table 4.1 Primer sequences for qRT-PCR analysis of inflammation markers in macrophages

Gene	Forward Primer Sequence	Reverse Primer Sequence
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
TNFα	TCTCATGCACCACCATCAAGGACT	TGACCACTCTCCCTTTGCAGAACT
MCP-1	CTCTCTTCCTCCACCACCAT	ACTGCATCTGGCTGAGCCA
IL-1β	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
prkaa1	GTCAAAGCCGACCCAATGATA	CGTACACGCAAATAATAGGGGTT

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

The gut microbiota has been identified as a factor that contributes to the progression of NAFLD, and compositional changes of the microbiota have been associated with NAFLD progression in both animal models and patients (31, 32, 296). Mechanistically, the gut microbiota has been proposed to affect the progression of NAFLD through altering gut barrier integrity, and affecting intestinal inflammation to indirectly modulate inflammation in peripheral tissues such as the adipose tissue and the liver (292, 293). It has also been proposed that microbiota metabolites could engage host cellular signaling pathways to modulate inflammation in the intestine (317). While these microbiota-produced bio-active molecules have been detected in circulation (43, 469), few have been identified as being associated with disease. Moreover, the signaling pathways that are engaged by these molecules are also poorly understood. This work tested the hypothesis that gut microbiota derived metabolites could modulate progression of NAFLD. Our findings strongly support the possibility that gut microbiota derived metabolites can directly modulate host cell signaling and phenotypes in distal organs such as the liver.

We used LC-MS metabolomics and compared metabolites in cecum, serum and the liver between HFD- and LFD-fed mice. Three tryptophan-derived metabolites were significantly depleted in HFD-fed mice compared to LFD-mice in all three locations, and two metabolites (I3A and TA) were selected for subsequent characterization. *In vitro* experiments using a two-hit model of macrophage inflammation showed that both I3A and TA attenuated palmitate- and LPS-induced inflammatory cytokine production and inhibited macrophage migration toward the chemokine MCP-1. I3A also attenuated lipid loading induced inflammatory response and cytokine mediated upregulation in lipogenesis in hepatocytes through AhR signaling. We further identified that I3A

and TA reversed palmitate- and LPS induced p-AMPK reduction, and p-AMPK activation by AICAR significantly reduced palmitate- and LPS-induced production of TNFα, IL-1β and MCP-1. The role of AMPK signaling in mediating the effects of I3A and TA was confirmed by siRNA-knockdown of AMPK, which led to partial abrogation of the anti-inflammatory effect elicited by I3A and TA in macrophages. Together, our results strongly suggest that the microbiota-derived metabolites I3A and TA can function in peripheral tissue through activation of AMPK signaling.

Direct extensions of this work include comprehensive characterization of the role of AMPK and evaluating the translational potential of I3A and TA as therapeutic options against NAFLD. While the effect of I3A and TA on palmitate and LPS induced macrophage inflammation is reduced upon AMPK knockdown, it's not completely abolished. Since the knockdown level achieved in this study is about 50%, one possibility is that the residual AMPK activity is sufficient for I3A and TA to modulate macrophage inflammation. Another possibility that we have not yet ruled out is that I3A and TA could engage other pathway(s) to modulate macrophage inflammation, and their contribution to the observed decrease in inflammatory cytokine. Thus, experiments with cells that lack AMPK activity are important for elucidating the role of AMPK in I3A and TA mediated anti-inflammatory effect in macrophages. This can be achieved either by generating a AMPK knockout macrophage cell line using CRISPR/CAS9 or be repeating the above experiments using bone marrow-derived macrophages isolated from $AMPK^{-/-}$ mice.

Furthermore, while AMPK signaling was identified as one of the mediators by which I3A and TA elicit their anti-inflammatory effects, the receptor(s) that are engaged by I3A and TA to lead to AMPK activation are not known. Future studies aimed to elucidate the receptor(s) and mediators that are engaged by I3A and TA will further our understanding of how microbiota metabolites such as I3A and TA modulate host responses under NAFLD.

Although our study showed I3A and TA can modulate macrophage inflammation *in vitro*, future *in vivo* studies are needed to determine if I3A and TA can be effectively used to modulate NAFLD pathogenesis. An *in vivo* model of NAFLD (13) can be used to investigate the effectiveness of I3A and TA to inhibit the progression of steatosis to steatohepatitis (i.e., the first irreversible step in disease progression) through modulation of inflammation. Both prophylactic (i.e., administration of metabolites prior to inflammation) and therapeutic (metabolites administered after onset of inflammation) approaches need to be explored to fully determine the translational potential of these metabolites.

Our study showed that I3A and TA can inhibit the migration of BMDM toward MCP-1 in a dose-dependent manner. However, mature BMDM cells might not be a good model for chemotactic migration. A preliminary study using a human monocyte cell line (THP-1) showed that I3A did not inhibit MCP-1 mediated THP-1 cell migration while TA inhibited both baseline migration as well as MCP-1 mediated THP-1 cells migration. The limitation of the transwell experimental setup used for these chemotaxis studies is that the migration of the cells cannot be observed in real-time or dynamic information cannot be determined. To resolve this, microfluidic chemotaxis models (530, 531) can be utilized to monitor the migration of macrophages/ monocytes in response to chemoattractant and metabolites in real-time. The ability to collect dynamics data will help us better understand the mechanisms underlying the effectiveness of TA, but not I3A, in THP-1 cells.

In addition to the above studies, new directions of research have emerged from this work. First, a systems biology investigation of microbiota metabolites and their ability to attenuate macrophage inflammation needs to be carried out. Lipid-loading has been shown to induce inflammatory responses through different signaling pathways in different cell types and the

specific signaling pathways through which palmitate induces inflammation in macrophages are still poorly understood. A mathematical model can be used to predict FFA-induced activation of inflammatory signaling through AMPK signaling, and the dynamics interactions between different components in FFA-mediated AMPK and inflammatory signaling can be investigated. Model predictions, coupled with experimental validation, will guide experimental design for *in vitro* studies such as duration of exposure to metabolites and administration of different inflammatory stimuli. It can also provide information on the mechanisms underlying the anti-inflammatory role of I3A and TA in macrophages.

While our results strongly support the potential of I3A and TA in attenuating macrophage inflammation in the liver, it is also possible that these (or other microbiota metabolites) are functional in other peripheral sites such as adipose tissue in the context of NAFLD. Thus, a second line of investigation should be to test if these metabolites are depleted in adipose tissue. Preliminary studies from our lab have identified that I3A is indeed depleted in mesenteric adipose tissue from HFD-fed mice, which supports the possibility that I3A is functional in multiple tissues important in obesity and associated diseases such as insulin resistance. This is especially important given that recruitment of monocytes to adipose tissue is thought to be the key step in the initiation of adipose tissue inflammation.

A third potential direction of work is to mine the acquired metabolomic data for additional molecules that may have beneficial effects. For example, while this work focused on I3A and TA, a third metabolite (xanthenuric acid) was also identified as depleted in HFD-fed mice but not investigated. As metabolite databases are constantly updated with new compound information, it would be worthwhile to redo the bioinformatic analysis carried out in Chapter 3 and determine if any of the previously unidentified metabolites that are associated with HFD administration, have

since been identified. Thus, the data sets generated in Chapter 3 are expected to be a rich source of information that can drive future studies.

REFERENCES

1. Tiniakos DG, Vos MB, Brunt EM. Nonalcoholic fatty liver disease: pathology and pathogenesis. Annual review of pathology. 2010;5:145-71. doi: 10.1146/annurev-pathol-121808-102132. PubMed PMID: 20078219.

2. Calzadilla Bertot L, Adams LA. The Natural Course of Non-Alcoholic Fatty Liver Disease. International journal of molecular sciences. 2016;17(5). doi: 10.3390/ijms17050774. PubMed PMID: 27213358; PMCID: 4881593.

3. Fabbrini E, Mohammed BS, Magkos F, Korenblat KM, Patterson BW, Klein S. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. Gastroenterology. 2008;134(2):424-31. doi: 10.1053/j.gastro.2007.11.038. PubMed PMID: 18242210; PMCID: 2705923.

4. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. The Journal of clinical investigation. 2005;115(5):1343-51. doi: 10.1172/JCI23621. PubMed PMID: 15864352; PMCID: 1087172.

5. Triglyceride Coronary Disease Genetics C, Emerging Risk Factors C, Sarwar N, Sandhu MS, Ricketts SL, Butterworth AS, Di Angelantonio E, Boekholdt SM, Ouwehand W, Watkins H, Samani NJ, Saleheen D, Lawlor D, Reilly MP, Hingorani AD, Talmud PJ, Danesh J. Triglyceride-mediated pathways and coronary disease: collaborative analysis of 101 studies. Lancet. 2010;375(9726):1634-9. doi: 10.1016/S0140-6736(10)60545-4. PubMed PMID: 20452521; PMCID: 2867029.

6. Stone SJ, Myers HM, Watkins SM, Brown BE, Feingold KR, Elias PM, Farese RV, Jr. Lipopenia and skin barrier abnormalities in DGAT2-deficient mice. The Journal of biological chemistry. 2004;279(12):11767-76. doi: 10.1074/jbc.M311000200. PubMed PMID: 14668353.

7. Monetti M, Levin MC, Watt MJ, Sajan MP, Marmor S, Hubbard BK, Stevens RD, Bain JR, Newgard CB, Farese RV, Sr., Hevener AL, Farese RV, Jr. Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. Cell metabolism. 2007;6(1):69-78. doi: 10.1016/j.cmet.2007.05.005. PubMed PMID: 17618857.

8. Yamaguchi K, Yang L, McCall S, Huang J, Yu XX, Pandey SK, Bhanot S, Monia BP, Li YX, Diehl AM. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. Hepatology. 2007;45(6):1366-74. doi: 10.1002/hep.21655. PubMed PMID: 17476695.

9. Jou J, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. Seminars in liver disease. 2008;28(4):370-9. doi: 10.1055/s-0028-1091981. PubMed PMID: 18956293.

10. Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology. 1998;114(4):842-5. PubMed PMID: 9547102.

11. Day CP, James OF. Hepatic steatosis: innocent bystander or guilty party? Hepatology. 1998;27(6):1463-6. doi: 10.1002/hep.510270601. PubMed PMID: 9620314.

12. Murphy EA, Velazquez KT, Herbert KM. Influence of high-fat diet on gut microbiota: a driving force for chronic disease risk. Current opinion in clinical nutrition and metabolic care. 2015;18(5):515-20. doi: 10.1097/MCO.0000000000000209. PubMed PMID: 26154278; PMCID: 4578152.

13. Asgharpour A, Cazanave SC, Pacana T, Seneshaw M, Vincent R, Banini BA, Kumar DP, Daita K, Min HK, Mirshahi F, Bedossa P, Sun X, Hoshida Y, Koduru SV, Contaifer D, Jr., Warncke UO, Wijesinghe DS, Sanyal AJ. A diet-induced animal model of non-alcoholic fatty liver disease and hepatocellular cancer. Journal of hepatology. 2016;65(3):579-88. doi: 10.1016/j.jhep.2016.05.005. PubMed PMID: 27261415; PMCID: 5012902.

14. Ma KL, Ruan XZ, Powis SH, Chen Y, Moorhead JF, Varghese Z. Inflammatory stress exacerbates lipid accumulation in hepatic cells and fatty livers of apolipoprotein E knockout mice. Hepatology. 2008;48(3):770-81. doi: 10.1002/hep.22423. PubMed PMID: 18752326.

15. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology. 2010;52(5):1836-46. doi: 10.1002/hep.24001. PubMed PMID: 21038418.

16. Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell metabolism. 2005;1(1):15-25. doi: 10.1016/j.cmet.2004.12.003. PubMed PMID: 16054041.

17. Viollet B, Horman S, Leclerc J, Lantier L, Foretz M, Billaud M, Giri S, Andreelli F. AMPK inhibition in health and disease. Critical reviews in biochemistry and molecular biology. 2010;45(4):276-95. doi: 10.3109/10409238.2010.488215. PubMed PMID: 20522000; PMCID: 3132561.

18. Wu Y, Song P, Xu J, Zhang M, Zou MH. Activation of protein phosphatase 2A by palmitate inhibits AMP-activated protein kinase. The Journal of biological chemistry. 2007;282(13):9777-88. doi: 10.1074/jbc.M608310200. PubMed PMID: 17255104.

19. Sag D, Carling D, Stout RD, Suttles J. Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. Journal of immunology. 2008;181(12):8633-41. PubMed PMID: 19050283; PMCID: 2756051.

20. Smith BK, Marcinko K, Desjardins EM, Lally JS, Ford RJ, Steinberg GR. Treatment of nonalcoholic fatty liver disease: role of AMPK. American journal of physiology Endocrinology and metabolism. 2016;311(4):E730-E40. doi: 10.1152/ajpendo.00225.2016. PubMed PMID: 27577854.

21. Duseja A, Chawla YK. Obesity and NAFLD: the role of bacteria and microbiota. Clinics in liver disease. 2014;18(1):59-71. doi: 10.1016/j.cld.2013.09.002. PubMed PMID: 24274865.

22. Moschen AR, Kaser S, Tilg H. Non-alcoholic steatohepatitis: a microbiota-driven disease. Trends in endocrinology and metabolism: TEM. 2013;24(11):537-45. doi: 10.1016/j.tem.2013.05.009. PubMed PMID: 23827477.

23. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;307(5717):1915-20. doi: 10.1126/science.1104816. PubMed PMID: 15790844.

24. Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. Science. 2001;292(5519):1115-8. PubMed PMID: 11352068.

25. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nature reviews Immunology. 2004;4(6):478-85. doi: 10.1038/nri1373. PubMed PMID: 15173836.

26. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nature reviews Immunology. 2009;9(5):313-23. doi: 10.1038/nri2515. PubMed PMID: 19343057; PMCID: 4095778.

27. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nature reviews Immunology. 2008;8(6):411-20. doi: 10.1038/nri2316. PubMed PMID: 18469830.

28. Compare D, Coccoli P, Rocco A, Nardone OM, De Maria S, Carteni M, Nardone G. Gut--liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. Nutr Metab Cardiovasc Dis. 2012;22(6):471-6. Epub 2012/05/02. doi: 10.1016/j.numecd.2012.02.007. PubMed PMID: 22546554.

29. Budden KF, Gellatly SL, Wood DLA, Cooper MA, Morrison M, Hugenholtz P, Hansbro PM. Emerging pathogenic links between microbiota and the gut-lung axis. Nat Rev Microbiol. 2017;15(1):55-63. doi: 10.1038/nrmicro.2016.142. PubMed PMID: WOS:000391345200008.

30. Cryan JF, O'Mahony SM. The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol Motil. 2011;23(3):187-92. Epub 2011/02/10. doi: 10.1111/j.1365-2982.2010.01664.x. PubMed PMID: 21303428.

31. Mouzaki M, Comelli EM, Arendt BM, Bonengel J, Fung SK, Fischer SE, McGilvray ID, Allard JP. Intestinal microbiota in patients with nonalcoholic fatty liver disease. Hepatology. 2013;58(1):120-7. Epub 2013/02/13. doi: 10.1002/hep.26319. PubMed PMID: 23401313.

32. Zhu LX, Baker SS, Gill C, Liu WS, Alkhouri R, Baker RD, Gill SR. Characterization of Gut Microbiomes in Nonalcoholic Steatohepatitis (NASH) Patients: A Connection Between Endogenous Alcohol and NASH. Hepatology. 2013;57(2):601-9. doi: 10.1002/hep.26093. PubMed PMID: WOS:000315643400020.

33. Wang B, Jiang X, Cao M, Ge J, Bao Q, Tang L, Chen Y, Li L. Altered Fecal Microbiota Correlates with Liver Biochemistry in Nonobese Patients with Non-alcoholic Fatty Liver

Disease. Scientific reports. 2016;6:32002. Epub 2016/08/24. doi: 10.1038/srep32002. PubMed PMID: 27550547; PMCID: PMC4994089.

34. Le Roy T, Llopis M, Lepage P, Bruneau A, Rabot S, Bevilacqua C, Martin P, Philippe C, Walker F, Bado A, Perlemuter G, Cassard-Doulcier AM, Gerard P. Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice. Gut. 2013;62(12):1787-94. doi: 10.1136/gutjnl-2012-303816. PubMed PMID: 23197411.

35. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature. 2012;482(7384):179-85. doi: 10.1038/nature10809. PubMed PMID: 22297845; PMCID: 3276682.

36. Spencer MD, Hamp TJ, Reid RW, Fischer LM, Zeisel SH, Fodor AA. Association between composition of the human gastrointestinal microbiome and development of fatty liver with choline deficiency. Gastroenterology. 2011;140(3):976-86. doi: 10.1053/j.gastro.2010.11.049. PubMed PMID: 21129376; PMCID: 3049827.

37. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. Science. 2012;336(6086):1268-73. doi: 10.1126/science.1223490. PubMed PMID: 22674334.

38. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. Nature immunology. 2013;14(7):685-90. doi: 10.1038/ni.2608. PubMed PMID: 23778796; PMCID: 4083503.

39. Bansal T, Alaniz RC, Wood TK, Jayaraman A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. Proceedings of

the National Academy of Sciences of the United States of America. 2010;107(1):228-33. doi: 10.1073/pnas.0906112107. PubMed PMID: 19966295; PMCID: 2806735.

40. Shimada Y, Kinoshita M, Harada K, Mizutani M, Masahata K, Kayama H, Takeda K. Commensal bacteria-dependent indole production enhances epithelial barrier function in the colon. PloS one. 2013;8(11):e80604. doi: 10.1371/journal.pone.0080604. PubMed PMID: 24278294; PMCID: 3835565.

41. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, Zecchi R, D'Angelo C, Massi-Benedetti C, Fallarino F, Carvalho A, Puccetti P, Romani L. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity. 2013;39(2):372-85. doi: 10.1016/j.immuni.2013.08.003. PubMed PMID: 23973224.

42. Schiering C, Wincent E, Metidji A, Iseppon A, Li Y, Potocnik AJ, Omenetti S, Henderson CJ, Wolf CR, Nebert DW, Stockinger B. Feedback control of AHR signalling regulates intestinal immunity. Nature. 2017;542(7640):242-5. doi: 10.1038/nature21080. PubMed PMID: 28146477; PMCID: 5302159.

43. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(10):3698-703. doi: 10.1073/pnas.0812874106. PubMed PMID: 19234110; PMCID: 2656143.

44. Day CP. Non-alcoholic fatty liver disease: a massive problem. Clinical medicine. 2011;11(2):176-8. PubMed PMID: 21526706.

45. Estes C, Razavi H, Loomba R, Younossi Z, Sanyal AJ. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. Hepatology. 2018;67(1):123-33. doi: 10.1002/hep.29466. PubMed PMID: 28802062; PMCID: 5767767.

46. Loomba R, Sanyal AJ. The global NAFLD epidemic. Nature reviews Gastroenterology & hepatology. 2013;10(11):686-90. doi: 10.1038/nrgastro.2013.171. PubMed PMID: 24042449.

47. Goldberg D, Ditah IC, Saeian K, Lalehzari M, Aronsohn A, Gorospe EC, Charlton M. Changes in the Prevalence of Hepatitis C Virus Infection, Nonalcoholic Steatohepatitis, and Alcoholic Liver Disease Among Patients With Cirrhosis or Liver Failure on the Waitlist for Liver Transplantation. Gastroenterology. 2017;152(5):1090-9 e1. doi: 10.1053/j.gastro.2017.01.003. PubMed PMID: 28088461; PMCID: 5367965.

48. Wong RJ, Aguilar M, Cheung R, Perumpail RB, Harrison SA, Younossi ZM, Ahmed A. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. Gastroenterology. 2015;148(3):547-55. doi: 10.1053/j.gastro.2014.11.039. PubMed PMID: 25461851.

49. Siddiqui MS, Harrison SA, Abdelmalek MF, Anstee QM, Bedossa P, Castera L, Dimick-Santos L, Friedman SL, Greene K, Kleiner DE, Megnien S, Neuschwander-Tetri BA, Ratziu V, Schabel E, Miller V, Sanyal AJ, Liver Forum Case Definitions Working G. Case definitions for inclusion and analysis of endpoints in clinical trials for nonalcoholic steatohepatitis through the lens of regulatory science. Hepatology. 2018;67(5):2001-12. doi: 10.1002/hep.29607. PubMed PMID: 29059456; PMCID: 5906171.

50. Rinella ME, Sanyal AJ. Management of NAFLD: a stage-based approach. Nature reviews Gastroenterology & hepatology. 2016;13(4):196-205. doi: 10.1038/nrgastro.2016.3. PubMed PMID: 26907882.

51. Mittal S, El-Serag HB, Sada YH, Kanwal F, Duan Z, Temple S, May SB, Kramer JR, Richardson PA, Davila JA. Hepatocellular Carcinoma in the Absence of Cirrhosis in United States Veterans is Associated With Nonalcoholic Fatty Liver Disease. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2016;14(1):124-31 e1. doi: 10.1016/j.cgh.2015.07.019. PubMed PMID: 26196445; PMCID: 4690789.

52. Dyson J, Jaques B, Chattopadyhay D, Lochan R, Graham J, Das D, Aslam T, Patanwala I, Gaggar S, Cole M, Sumpter K, Stewart S, Rose J, Hudson M, Manas D, Reeves HL. Hepatocellular cancer: the impact of obesity, type 2 diabetes and a multidisciplinary team. Journal of hepatology. 2014;60(1):110-7. doi: 10.1016/j.jhep.2013.08.011. PubMed PMID: 23978719.

53. Piscaglia F, Svegliati-Baroni G, Barchetti A, Pecorelli A, Marinelli S, Tiribelli C, Bellentani S, Group H-NIS. Clinical patterns of hepatocellular carcinoma in nonalcoholic fatty liver disease: A multicenter prospective study. Hepatology. 2016;63(3):827-38. doi: 10.1002/hep.28368. PubMed PMID: 26599351.

54. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Nature medicine. 2018;24(7):908-22. doi: 10.1038/s41591-018-0104-9. PubMed PMID: 29967350.

55. Huang PL. A comprehensive definition for metabolic syndrome. Disease models & mechanisms. 2009;2(5-6):231-7. doi: 10.1242/dmm.001180. PubMed PMID: 19407331; PMCID: 2675814.

56. Karajamaki AJ, Bloigu R, Kauma H, Kesaniemi YA, Koivurova OP, Perkiomaki J, Huikuri H, Ukkola O. Non-alcoholic fatty liver disease with and without metabolic syndrome: Different long-term outcomes. Metabolism: clinical and experimental. 2017;66:55-63. Epub 2016/07/18. doi: 10.1016/j.metabol.2016.06.009. PubMed PMID: 27423871.

57. Allen AM, Therneau TM, Larson JJ, Coward A, Somers VK, Kamath PS. Nonalcoholic fatty liver disease incidence and impact on metabolic burden and death: A 20 year-community study. Hepatology. 2018;67(5):1726-36. Epub 2017/09/25. doi: 10.1002/hep.29546. PubMed PMID: 28941364; PMCID: PMC5866219.

58. Leamy AK, Egnatchik RA, Young JD. Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease. Prog Lipid Res. 2013;52(1):165-74. Epub 2012/11/28. doi: 10.1016/j.plipres.2012.10.004. PubMed PMID: 23178552; PMCID: PMC3868987.

59. Alisi A, Bedogni G, De Vito R, Comparcola D, Manco M, Nobili V. Relationship between portal chronic inflammation and disease severity in paediatric non-alcoholic fatty liver disease. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver. 2011;43(2):143-6. Epub 2010/06/29. doi: 10.1016/j.dld.2010.05.007. PubMed PMID: 20580331.

60. Estadella D, da Penha Oller do Nascimento CM, Oyama LM, Ribeiro EB, Damaso AR, de Piano A. Lipotoxicity: effects of dietary saturated and transfatty acids. Mediators of

inflammation. 2013;2013:137579. Epub 2013/03/20. doi: 10.1155/2013/137579. PubMed PMID: 23509418; PMCID: PMC3572653.

61. Schilling JD, Machkovech HM, He L, Sidhu R, Fujiwara H, Weber K, Ory DS, Schaffer JE. Palmitate and lipopolysaccharide trigger synergistic ceramide production in primary macrophages. The Journal of biological chemistry. 2013;288(5):2923-32. doi: 10.1074/jbc.M112.419978. PubMed PMID: 23250746; PMCID: 3561515.

62. Clarke SD, Jump DB. Polyunsaturated fatty acid regulation of hepatic gene transcription. Lipids. 1996;31 Suppl:S7-11. Epub 1996/03/01. PubMed PMID: 8729087.

63. Kim HJ, Takahashi M, Ezaki O. Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. The Journal of biological chemistry. 1999;274(36):25892-8. Epub 1999/08/28. PubMed PMID: 10464332.

64. Patterson E, Wall R, Fitzgerald GF, Ross RP, Stanton C. Health implications of high dietary omega-6 polyunsaturated Fatty acids. J Nutr Metab. 2012;2012:539426. Epub 2012/05/10. doi: 10.1155/2012/539426. PubMed PMID: 22570770; PMCID: PMC3335257.

65. Scorletti E, Bhatia L, McCormick KG, Clough GF, Nash K, Hodson L, Moyses HE, Calder PC, Byrne CD, Study W. Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. Hepatology. 2014;60(4):1211-21. Epub 2014/07/22. doi: 10.1002/hep.27289. PubMed PMID: 25043514.

66. Vinaixa M, Rodriguez MA, Rull A, Beltran R, Blade C, Brezmes J, Canellas N, Joven J, Correig X. Metabolomic assessment of the effect of dietary cholesterol in the progressive development of fatty liver disease. J Proteome Res. 2010;9(5):2527-38. Epub 2010/04/21. doi: 10.1021/pr901203w. PubMed PMID: 20402505.

67. Kerr TA, Davidson NO. Cholesterol and nonalcoholic fatty liver disease: renewed focus on an old villain. Hepatology. 2012;56(5):1995-8. Epub 2012/11/02. doi: 10.1002/hep.26088. PubMed PMID: 23115010; PMCID: PMC3627394.

68. Min HK, Kapoor A, Fuchs M, Mirshahi F, Zhou H, Maher J, Kellum J, Warnick R, Contos MJ, Sanyal AJ. Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. Cell metabolism. 2012;15(5):665-74. Epub 2012/05/09. doi: 10.1016/j.cmet.2012.04.004. PubMed PMID: 22560219; PMCID: PMC3361911.

69. Schultz A, Neil D, Aguila MB, Mandarim-de-Lacerda CA. Hepatic adverse effects of fructose consumption independent of overweight/obesity. International journal of molecular sciences. 2013;14(11):21873-86. Epub 2013/11/08. doi: 10.3390/ijms141121873. PubMed PMID: 24196354; PMCID: PMC3856040.

70. Tappy L, Le KA. Does fructose consumption contribute to non-alcoholic fatty liver disease? Clin Res Hepatol Gastroenterol. 2012;36(6):554-60. Epub 2012/07/17. doi: 10.1016/j.clinre.2012.06.005. PubMed PMID: 22795319.

71. Spruss A, Bergheim I. Dietary fructose and intestinal barrier: potential risk factor in the pathogenesis of nonalcoholic fatty liver disease. J Nutr Biochem. 2009;20(9):657-62. Epub 2009/08/15. doi: 10.1016/j.jnutbio.2009.05.006. PubMed PMID: 19679262.

72. Basciano H, Federico L, Adeli K. Fructose, insulin resistance, and metabolic dyslipidemia. Nutr Metab (Lond). 2005;2(1):5. Epub 2005/02/23. doi: 10.1186/1743-7075-2-5. PubMed PMID: 15723702; PMCID: PMC552336.

73. Bazick J, Donithan M, Neuschwander-Tetri BA, Kleiner D, Brunt EM, Wilson L, Doo E, Lavine J, Tonascia J, Loomba R. Clinical Model for NASH and Advanced Fibrosis in Adult

Patients With Diabetes and NAFLD: Guidelines for Referral in NAFLD. Diabetes care. 2015;38(7):1347-55. Epub 2015/04/19. doi: 10.2337/dc14-1239. PubMed PMID: 25887357; PMCID: PMC4477334.

74. Portillo-Sanchez P, Bril F, Maximos M, Lomonaco R, Biernacki D, Orsak B, Subbarayan S, Webb A, Hecht J, Cusi K. High Prevalence of Nonalcoholic Fatty Liver Disease in Patients With Type 2 Diabetes Mellitus and Normal Plasma Aminotransferase Levels. J Clin Endocrinol Metab. 2015;100(6):2231-8. Epub 2015/04/18. doi: 10.1210/jc.2015-1966. PubMed PMID: 25885947.

75. Kwok R, Choi KC, Wong GL, Zhang Y, Chan HL, Luk AO, Shu SS, Chan AW, Yeung MW, Chan JC, Kong AP, Wong VW. Screening diabetic patients for non-alcoholic fatty liver disease with controlled attenuation parameter and liver stiffness measurements: a prospective cohort study. Gut. 2016;65(8):1359-68. Epub 2015/04/16. doi: 10.1136/gutjnl-2015-309265. PubMed PMID: 25873639.

76. Choudhury J, Sanyal AJ. Insulin resistance and the pathogenesis of nonalcoholic fatty liver disease. Clinics in liver disease. 2004;8(3):575-94, ix. Epub 2004/08/28. doi: 10.1016/j.cld.2004.04.006. PubMed PMID: 15331065.

77. Jung UJ, Choi MS. Obesity and its metabolic complications: the role of adipokines and the relationship between obesity, inflammation, insulin resistance, dyslipidemia and nonalcoholic fatty liver disease. International journal of molecular sciences. 2014;15(4):6184-223. Epub 2014/04/16. doi: 10.3390/ijms15046184. PubMed PMID: 24733068; PMCID: PMC4013623.

78. Younossi ZM, Otgonsuren M, Henry L, Venkatesan C, Mishra A, Erario M, Hunt S. Association of nonalcoholic fatty liver disease (NAFLD) with hepatocellular carcinoma (HCC)

in the United States from 2004 to 2009. Hepatology. 2015;62(6):1723-30. Epub 2015/08/15. doi: 10.1002/hep.28123. PubMed PMID: 26274335.

79. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer. 2004;4(8):579-91. Epub 2004/08/03. doi: 10.1038/nrc1408. PubMed PMID: 15286738.

80. Ish-Shalom D, Christoffersen CT, Vorwerk P, Sacerdoti-Sierra N, Shymko RM, Naor D, De Meyts P. Mitogenic properties of insulin and insulin analogues mediated by the insulin receptor. Diabetologia. 1997;40 Suppl 2:S25-31. Epub 1997/07/01. PubMed PMID: 9248698.

81. Rhee SG, Bae YS, Lee SR, Kwon J. Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. Sci STKE. 2000;2000(53):pe1. Epub 2001/12/26. doi: 10.1126/stke.2000.53.pe1. PubMed PMID: 11752613.

82. Brakenhielm E, Veitonmaki N, Cao R, Kihara S, Matsuzawa Y, Zhivotovsky B, Funahashi T, Cao Y. Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(8):2476-81. Epub 2004/02/26. PubMed PMID: 14983034; PMCID: PMC356975.

83. Lorbeer R, Bayerl C, Auweter S, Rospleszcz S, Lieb W, Meisinger C, Heier M, Peters A, Bamberg F, Hetterich H. Association between MRI-derived hepatic fat fraction and blood pressure in participants without history of cardiovascular disease. J Hypertens. 2017;35(4):737-44. Epub 2017/03/03. doi: 10.1097/HJH.0000000000001245. PubMed PMID: 28253218.

84. Lopez-Suarez A, Guerrero JM, Elvira-Gonzalez J, Beltran-Robles M, Canas-Hormigo F, Bascunana-Quirell A. Nonalcoholic fatty liver disease is associated with blood pressure in hypertensive and nonhypertensive individuals from the general population with normal levels of

alanine aminotransferase. European journal of gastroenterology & hepatology. 2011;23(11):1011-7. Epub 2011/09/15. doi: 10.1097/MEG.0b013e32834b8d52. PubMed PMID: 21915061.

85. Qian LY, Tu JF, Ding YH, Pang J, Che XD, Zou H, Huang DS. Association of blood pressure level with nonalcoholic fatty liver disease in nonhypertensive population: Normal is not the new normal. Medicine (Baltimore). 2016;95(29):e4293. Epub 2016/07/22. doi: 10.1097/MD.0000000000004293. PubMed PMID: 27442673; PMCID: PMC5265790.

86. Long MT, Wang N, Larson MG, Mitchell GF, Palmisano J, Vasan RS, Hoffmann U, Speliotes EK, Vita JA, Benjamin EJ, Fox CS, Hamburg NM. Nonalcoholic fatty liver disease and vascular function: cross-sectional analysis in the Framingham heart study. Arteriosclerosis, thrombosis, and vascular biology. 2015;35(5):1284-91. Epub 2015/03/07. doi: 10.1161/ATVBAHA.114.305200. PubMed PMID: 25745056; PMCID: PMC4520415.

87. Aneni EC, Oni ET, Martin SS, Blaha MJ, Agatston AS, Feldman T, Veledar E, Conceicao RD, Carvalho JA, Santos RD, Nasir K. Blood pressure is associated with the presence and severity of nonalcoholic fatty liver disease across the spectrum of cardiometabolic risk. J Hypertens. 2015;33(6):1207-14. Epub 2015/02/19. doi: 10.1097/HJH.0000000000000532. PubMed PMID: 25693058.

88. Ma J, Hwang SJ, Pedley A, Massaro JM, Hoffmann U, Chung RT, Benjamin EJ, Levy D, Fox CS, Long MT. Bi-directional analysis between fatty liver and cardiovascular disease risk factors. Journal of hepatology. 2017;66(2):390-7. Epub 2016/10/13. doi: 10.1016/j.jhep.2016.09.022. PubMed PMID: 27729222; PMCID: PMC5250546.

89. Miyaaki H, Ichikawa T, Nakao K, Yatsuhashi H, Furukawa R, Ohba K, Omagari K, Kusumoto Y, Yanagi K, Inoue O, Kinoshita N, Ishibashi H, Yano M, Eguchi K.

Clinicopathological study of nonalcoholic fatty liver disease in Japan: the risk factors for fibrosis. Liver international : official journal of the International Association for the Study of the Liver. 2008;28(4):519-24. Epub 2007/11/03. doi: 10.1111/j.1478-3231.2007.01614.x. PubMed PMID: 17976158.

90. Hossain N, Afendy A, Stepanova M, Nader F, Srishord M, Rafiq N, Goodman Z, Younossi Z. Independent predictors of fibrosis in patients with nonalcoholic fatty liver disease. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association. 2009;7(11):1224-9, 9 e1-2. Epub 2009/06/30. doi: 10.1016/j.cgh.2009.06.007. PubMed PMID: 19559819.

91. Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, Tsujinoue H, Fukui H. Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. Hepatology. 2001;34(4 Pt 1):745-50. Epub 2001/10/05. doi: 10.1053/jhep.2001.28231. PubMed PMID: 11584371.

92. Ono M, Ochi T, Munekage K, Ogasawara M, Hirose A, Nozaki Y, Takahashi M, Okamoto N, Saibara T. Angiotensinogen gene haplotype is associated with the prevalence of Japanese non-alcoholic steatohepatitis. Hepatol Res. 2011;41(12):1223-9. Epub 2011/10/13. doi: 10.1111/j.1872-034X.2011.00883.x. PubMed PMID: 21988197.

93. Yoneda M, Hotta K, Nozaki Y, Endo H, Uchiyama T, Mawatari H, Iida H, Kato S, Fujita K, Takahashi H, Kirikoshi H, Kobayashi N, Inamori M, Abe Y, Kubota K, Saito S, Maeyama S, Wada K, Nakajima A. Association between angiotensin II type 1 receptor polymorphisms and the occurrence of nonalcoholic fatty liver disease. Liver international : official journal of the International Association for the Study of the Liver. 2009;29(7):1078-85. Epub 2009/03/24. doi: 10.1111/j.1478-3231.2009.01988.x. PubMed PMID: 19302184.

94. Fan JG, Li F, Cai XB, Peng YD, Ao QH, Gao Y. Effects of nonalcoholic fatty liver disease on the development of metabolic disorders. Journal of gastroenterology and hepatology. 2007;22(7):1086-91. Epub 2007/07/05. doi: 10.1111/j.1440-1746.2006.04781.x. PubMed PMID: 17608855.

95. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nature medicine. 2005;11(2):183-90. Epub 2005/02/03. doi: 10.1038/nm1166. PubMed PMID: 15685173; PMCID: PMC1440292.

96. Bhagat B, Burke WJ, Dhalla NS. Insulin-induced enhancement of uptake of noradrenaline in atrial strips. Br J Pharmacol. 1981;74(2):325-32. Epub 1981/10/01. PubMed PMID: 6274461; PMCID: PMC2071738.

97. Aizawa-Abe M, Ogawa Y, Masuzaki H, Ebihara K, Satoh N, Iwai H, Matsuoka N, Hayashi T, Hosoda K, Inoue G, Yoshimasa Y, Nakao K. Pathophysiological role of leptin in obesity-related hypertension. The Journal of clinical investigation. 2000;105(9):1243-52. Epub 2000/05/03. doi: 10.1172/JCI8341. PubMed PMID: 10791999; PMCID: PMC315441.

98. Beltowski J. Role of leptin in blood pressure regulation and arterial hypertension. J Hypertens. 2006;24(5):789-801. Epub 2006/04/14. doi: 10.1097/01.hjh.0000222743.06584.66. PubMed PMID: 16612235.

99. Rojas E, Rodriguez-Molina D, Bolli P, Israili ZH, Faria J, Fidilio E, Bermudez V, Velasco M. The role of adiponectin in endothelial dysfunction and hypertension. Curr Hypertens Rep. 2014;16(8):463. Epub 2014/06/14. doi: 10.1007/s11906-014-0463-7. PubMed PMID: 24924994.

100. Yoo HJ, Choi KM. Hepatokines as a Link between Obesity and Cardiovascular Diseases. Diabetes Metab J. 2015;39(1):10-5. Epub 2015/03/03. doi: 10.4093/dmj.2015.39.1.10. PubMed PMID: 25729707; PMCID: PMC4342531.

101. Sookoian S, Pirola CJ. Genetic predisposition in nonalcoholic fatty liver disease. Clin Mol Hepatol. 2017;23(1):1-12. Epub 2017/03/09. doi: 10.3350/cmh.2016.0109. PubMed PMID: 28268262; PMCID: PMC5381829.

102. Eslam M, Valenti L, Romeo S. Genetics and epigenetics of NAFLD and NASH: Clinical impact. Journal of hepatology. 2018;68(2):268-79. doi: 10.1016/j.jhep.2017.09.003. PubMed PMID: 29122391.

103. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet. 2008;40(12):1461-5. Epub 2008/09/30. doi: 10.1038/ng.257. PubMed PMID: 18820647; PMCID: PMC2597056.

104. Dongiovanni P, Donati B, Fares R, Lombardi R, Mancina RM, Romeo S, Valenti L. PNPLA3 I148M polymorphism and progressive liver disease. World J Gastroenterol. 2013;19(41):6969-78. Epub 2013/11/14. doi: 10.3748/wjg.v19.i41.6969. PubMed PMID: 24222941; PMCID: PMC3819533.

105. Singal AG, Manjunath H, Yopp AC, Beg MS, Marrero JA, Gopal P, Waljee AK. The effect of PNPLA3 on fibrosis progression and development of hepatocellular carcinoma: a meta-analysis. Am J Gastroenterol. 2014;109(3):325-34. Epub 2014/01/22. doi: 10.1038/ajg.2013.476. PubMed PMID: 24445574; PMCID: PMC5610907.

106. Huang Y, Cohen JC, Hobbs HH. Expression and characterization of a PNPLA3 protein isoform (I148M) associated with nonalcoholic fatty liver disease. The Journal of biological

chemistry. 2011;286(43):37085-93. Epub 2011/09/01. doi: 10.1074/jbc.M111.290114. PubMed PMID: 21878620; PMCID: PMC3199456.

107. Pingitore P, Pirazzi C, Mancina RM, Motta BM, Indiveri C, Pujia A, Montalcini T, Hedfalk K, Romeo S. Recombinant PNPLA3 protein shows triglyceride hydrolase activity and its I148M mutation results in loss of function. Biochimica et biophysica acta. 2014;1841(4):574-80. Epub 2013/12/27. doi: 10.1016/j.bbalip.2013.12.006. PubMed PMID: 24369119.

108. Pirazzi C, Valenti L, Motta BM, Pingitore P, Hedfalk K, Mancina RM, Burza MA, Indiveri C, Ferro Y, Montalcini T, Maglio C, Dongiovanni P, Fargion S, Rametta R, Pujia A, Andersson L, Ghosal S, Levin M, Wiklund O, Iacovino M, Boren J, Romeo S. PNPLA3 has retinyl-palmitate lipase activity in human hepatic stellate cells. Hum Mol Genet. 2014;23(15):4077-85. Epub 2014/03/29. doi: 10.1093/hmg/ddu121. PubMed PMID: 24670599; PMCID: PMC4082369.

109. Pingitore P, Dongiovanni P, Motta BM, Meroni M, Lepore SM, Mancina RM, Pelusi S, Russo C, Caddeo A, Rossi G, Montalcini T, Pujia A, Wiklund O, Valenti L, Romeo S. PNPLA3 overexpression results in reduction of proteins predisposing to fibrosis. Hum Mol Genet. 2016;25(23):5212-22. Epub 2016/10/16. doi: 10.1093/hmg/ddw341. PubMed PMID: 27742777; PMCID: PMC5886043.

110. BasuRay S, Smagris E, Cohen JC, Hobbs HH. The PNPLA3 variant associated with fatty liver disease (I148M) accumulates on lipid droplets by evading ubiquitylation. Hepatology. 2017;66(4):1111-24. Epub 2017/05/19. doi: 10.1002/hep.29273. PubMed PMID: 28520213; PMCID: PMC5605398.

111. Mancina RM, Dongiovanni P, Petta S, Pingitore P, Meroni M, Rametta R, Boren J, Montalcini T, Pujia A, Wiklund O, Hindy G, Spagnuolo R, Motta BM, Pipitone RM, Craxi A,

Fargion S, Nobili V, Kakela P, Karja V, Mannisto V, Pihlajamaki J, Reilly DF, Castro-Perez J, Kozlitina J, Valenti L, Romeo S. The MBOAT7-TMC4 Variant rs641738 Increases Risk of Nonalcoholic Fatty Liver Disease in Individuals of European Descent. Gastroenterology. 2016;150(5):1219-30 e6. Epub 2016/02/07. doi: 10.1053/j.gastro.2016.01.032. PubMed PMID: 26850495; PMCID: PMC4844071.

112. Luukkonen PK, Zhou Y, Hyotylainen T, Leivonen M, Arola J, Orho-Melander M, Oresic M, Yki-Jarvinen H. The MBOAT7 variant rs641738 alters hepatic phosphatidylinositols and increases severity of non-alcoholic fatty liver disease in humans. Journal of hepatology. 2016;65(6):1263-5. Epub 2016/08/16. doi: 10.1016/j.jhep.2016.07.045. PubMed PMID: 27520876.

113. Beer NL, Tribble ND, McCulloch LJ, Roos C, Johnson PRV, Orho-Melander M, Gloyn AL. The P446L variant in GCKR associated with fasting plasma glucose and triglyceride levels exerts its effect through increased glucokinase activity in liver. Human Molecular Genetics. 2009;18(21):4081-8. doi: 10.1093/hmg/ddp357. PubMed PMID: WOS:000270708300007.

114. Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. Hepatology. 2010;52(2):774-88. doi: 10.1002/hep.23719. PubMed PMID: 20683968.

115. Cusi K. Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. Gastroenterology. 2012;142(4):711-25 e6. doi: 10.1053/j.gastro.2012.02.003. PubMed PMID: 22326434.

116. Hirsova P, Ibrahim SH, Gores GJ, Malhi H. Lipotoxic lethal and sublethal stress signaling in hepatocytes: relevance to NASH pathogenesis. Journal of lipid research. 2016;57(10):1758-70. doi: 10.1194/jlr.R066357. PubMed PMID: 27049024; PMCID: 5036373.

117. Mota M, Banini BA, Cazanave SC, Sanyal AJ. Molecular mechanisms of lipotoxicity and glucotoxicity in nonalcoholic fatty liver disease. Metabolism: clinical and experimental. 2016;65(8):1049-61. doi: 10.1016/j.metabol.2016.02.014. PubMed PMID: 26997538; PMCID: 4931958.

118. Kawano Y, Cohen DE. Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. Journal of gastroenterology. 2013;48(4):434-41. doi: 10.1007/s00535-013-0758-5. PubMed PMID: 23397118; PMCID: 3633701.

119. Ameer F, Scandiuzzi L, Hasnain S, Kalbacher H, Zaidi N. De novo lipogenesis in health and disease. Metabolism: clinical and experimental. 2014;63(7):895-902. doi: 10.1016/j.metabol.2014.04.003. PubMed PMID: 24814684.

120. Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Shiffman ML, Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology. 2001;120(5):1183-92. doi: 10.1053/gast.2001.23256. PubMed PMID: 11266382.

121. Pessayre D, Fromenty B. NASH: a mitochondrial disease. Journal of hepatology. 2005;42(6):928-40. doi: 10.1016/j.jhep.2005.03.004. PubMed PMID: 15885365.

122. Bril F, Barb D, Portillo-Sanchez P, Biernacki D, Lomonaco R, Suman A, Weber MH, Budd JT, Lupi ME, Cusi K. Metabolic and histological implications of intrahepatic triglyceride content in nonalcoholic fatty liver disease. Hepatology. 2017;65(4):1132-44. doi: 10.1002/hep.28985. PubMed PMID: 27981615.

123. Samuel VT, Shulman GI. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. The Journal of clinical investigation. 2016;126(1):12-22. doi: 10.1172/JCI77812. PubMed PMID: 26727229; PMCID: 4701542.

124. Yki-Jarvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. The lancet Diabetes & endocrinology. 2014;2(11):901-10. doi: 10.1016/S2213-8587(14)70032-4. PubMed PMID: 24731669.

125. Perry RJ, Samuel VT, Petersen KF, Shulman GI. The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. Nature. 2014;510(7503):84-91. doi: 10.1038/nature13478. PubMed PMID: 24899308; PMCID: 4489847.

126. Luukkonen PK, Zhou Y, Sadevirta S, Leivonen M, Arola J, Oresic M, Hyotylainen T, Yki-Jarvinen H. Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease. Journal of hepatology. 2016;64(5):1167-75. doi: 10.1016/j.jhep.2016.01.002. PubMed PMID: 26780287.

127. Mauer AS, Hirsova P, Maiers JL, Shah VH, Malhi H. Inhibition of sphingosine 1-phosphate signaling ameliorates murine nonalcoholic steatohepatitis. American journal of physiology Gastrointestinal and liver physiology. 2017;312(3):G300-G13. doi: 10.1152/ajpgi.00222.2016. PubMed PMID: 28039158; PMCID: 5401989.

128. Han MS, Park SY, Shinzawa K, Kim S, Chung KW, Lee JH, Kwon CH, Lee KW, Lee JH, Park CK, Chung WJ, Hwang JS, Yan JJ, Song DK, Tsujimoto Y, Lee MS. Lysophosphatidylcholine as a death effector in the lipoapoptosis of hepatocytes. Journal of lipid research. 2008;49(1):84-97. doi: 10.1194/jlr.M700184-JLR200. PubMed PMID: 17951222.

129. Han J, Kaufman RJ. The role of ER stress in lipid metabolism and lipotoxicity. Journal of lipid research. 2016;57(8):1329-38. doi: 10.1194/jlr.R067595. PubMed PMID: 27146479; PMCID: 4959874.

130. Puri P, Mirshahi F, Cheung O, Natarajan R, Maher JW, Kellum JM, Sanyal AJ. Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease.

Gastroenterology. 2008;134(2):568-76. doi: 10.1053/j.gastro.2007.10.039. PubMed PMID: 18082745.

131. Szabo G, Petrasek J. Inflammasome activation and function in liver disease. Nature reviews Gastroenterology & hepatology. 2015;12(7):387-400. doi: 10.1038/nrgastro.2015.94. PubMed PMID: 26055245.

132. Feng B, Zhang T, Xu H. Human adipose dynamics and metabolic health. Annals of the New York Academy of Sciences. 2013;1281:160-77. doi: 10.1111/nyas.12009. PubMed PMID: 23317303; PMCID: 3618577.

133. Reccia I, Kumar J, Akladios C, Virdis F, Pai M, Habib N, Spalding D. Non-alcoholic fatty liver disease: A sign of systemic disease. Metabolism: clinical and experimental. 2017;72:94-108. doi: 10.1016/j.metabol.2017.04.011. PubMed PMID: 28641788.

134. Denechaud PD, Girard J, Postic C. Carbohydrate responsive element binding protein and lipid homeostasis. Current opinion in lipidology. 2008;19(3):301-6. doi: 10.1097/MOL.0b013e3282ffafaa. PubMed PMID: 18460923.

135. Eckel RH. Lipoprotein lipase. A multifunctional enzyme relevant to common metabolic diseases. The New England journal of medicine. 1989;320(16):1060-8. doi: 10.1056/NEJM198904203201607. PubMed PMID: 2648155.

136. Boivin A, Deshaies Y. Contribution of hyperinsulinemia to modulation of lipoprotein lipase activity in the obese Zucker rat. Metabolism: clinical and experimental. 2000;49(1):134-40. PubMed PMID: 10647077.

137. Nilsson-Ehle P. Impaired regulation of adipose tissue lipoprotein lipase in obesity. International journal of obesity. 1981;5(6):695-9. PubMed PMID: 7033153.

138. Bower JF, Davis JM, Hao E, Barakat HA. Differences in transport of fatty acids and expression of fatty acid transporting proteins in adipose tissue of obese black and white women. American journal of physiology Endocrinology and metabolism. 2006;290(1):E87-E91. doi: 10.1152/ajpendo.00194.2005. PubMed PMID: 16339926.

139. Cai L, Wang Z, Ji A, Meyer JM, van der Westhuyzen DR. Scavenger receptor CD36 expression contributes to adipose tissue inflammation and cell death in diet-induced obesity. PloS one. 2012;7(5):e36785. doi: 10.1371/journal.pone.0036785. PubMed PMID: 22615812; PMCID: 3353961.

140. Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nature reviews Molecular cell biology. 2008;9(5):367-77. doi: 10.1038/nrm2391. PubMed PMID: 18401346; PMCID: 2886982.

141. Sabio G, Das M, Mora A, Zhang Z, Jun JY, Ko HJ, Barrett T, Kim JK, Davis RJ. A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. Science. 2008;322(5907):1539-43. doi: 10.1126/science.1160794. PubMed PMID: 19056984; PMCID: 2643026.

142. Tilg H. The role of cytokines in non-alcoholic fatty liver disease. Digestive diseases. 2010;28(1):179-85. doi: 10.1159/000282083. PubMed PMID: 20460908.

143. Marra F, Bertolani C. Adipokines in liver diseases. Hepatology. 2009;50(3):957-69. doi: 10.1002/hep.23046. PubMed PMID: 19585655.

144. Wiernsperger N. Hepatic function and the cardiometabolic syndrome. Diabetes, metabolic syndrome and obesity : targets and therapy. 2013;6:379-88. doi: 10.2147/DMSO.S51145. PubMed PMID: 24143116; PMCID: 3797612.

145. Loomba R, Abraham M, Unalp A, Wilson L, Lavine J, Doo E, Bass NM, Nonalcoholic Steatohepatitis Clinical Research N. Association between diabetes, family history of diabetes, and risk of nonalcoholic steatohepatitis and fibrosis. Hepatology. 2012;56(3):943-51. doi: 10.1002/hep.25772. PubMed PMID: 22505194; PMCID: 3407289.

146. Vanni E, Bugianesi E, Kotronen A, De Minicis S, Yki-Jarvinen H, Svegliati-Baroni G. From the metabolic syndrome to NAFLD or vice versa? Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver. 2010;42(5):320-30. doi: 10.1016/j.dld.2010.01.016. PubMed PMID: 20207596.

147. Nagle CA, Klett EL, Coleman RA. Hepatic triacylglycerol accumulation and insulin resistance. Journal of lipid research. 2009;50 Suppl:S74-9. doi: 10.1194/jlr.R800053-JLR200. PubMed PMID: 18997164; PMCID: 2674743.

148. Solow BT, Harada S, Goldstein BJ, Smith JA, White MF, Jarett L. Differential modulation of the tyrosine phosphorylation state of the insulin receptor by IRS (insulin receptor subunit) proteins. Molecular endocrinology. 1999;13(10):1784-98. doi: 10.1210/mend.13.10.0361. PubMed PMID: 10517679.

149. Samuel VT, Liu ZX, Qu X, Elder BD, Bilz S, Befroy D, Romanelli AJ, Shulman GI. Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. The Journal of biological chemistry. 2004;279(31):32345-53. doi: 10.1074/jbc.M313478200. PubMed PMID: 15166226.

150. Galbo T, Perry RJ, Jurczak MJ, Camporez JP, Alves TC, Kahn M, Guigni BA, Serr J, Zhang D, Bhanot S, Samuel VT, Shulman GI. Saturated and unsaturated fat induce hepatic insulin resistance independently of TLR-4 signaling and ceramide synthesis in vivo. Proceedings

of the National Academy of Sciences of the United States of America. 2013;110(31):12780-5. doi: 10.1073/pnas.1311176110. PubMed PMID: 23840067; PMCID: 3732992.

151. Holland WL, Bikman BT, Wang LP, Yuguang G, Sargent KM, Bulchand S, Knotts TA, Shui G, Clegg DJ, Wenk MR, Pagliassotti MJ, Scherer PE, Summers SA. Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. The Journal of clinical investigation. 2011;121(5):1858-70. doi: 10.1172/JCI43378. PubMed PMID: 21490391; PMCID: 3083776.

152. Ortiz-Lopez C, Lomonaco R, Orsak B, Finch J, Chang Z, Kochunov VG, Hardies J, Cusi K. Prevalence of prediabetes and diabetes and metabolic profile of patients with nonalcoholic fatty liver disease (NAFLD). Diabetes care. 2012;35(4):873-8. doi: 10.2337/dc11-1849. PubMed PMID: 22374640; PMCID: 3308295.

153. Meshkani R, Adeli K. Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. Clinical biochemistry. 2009;42(13-14):1331-46. doi: 10.1016/j.clinbiochem.2009.05.018. PubMed PMID: 19501581.

154. Bechmann LP, Hannivoort RA, Gerken G, Hotamisligil GS, Trauner M, Canbay A. The interaction of hepatic lipid and glucose metabolism in liver diseases. Journal of hepatology. 2012;56(4):952-64. doi: 10.1016/j.jhep.2011.08.025. PubMed PMID: 22173168.

155. Fon Tacer K, Rozman D. Nonalcoholic Fatty liver disease: focus on lipoprotein and lipid deregulation. Journal of lipids. 2011;2011:783976. doi: 10.1155/2011/783976. PubMed PMID: 21773052; PMCID: 3136146.

156. Nassir F, Ibdah JA. Role of mitochondria in nonalcoholic fatty liver disease. International journal of molecular sciences. 2014;15(5):8713-42. doi: 10.3390/ijms15058713. PubMed PMID: 24837835; PMCID: 4057755.

157. Koyama Y, Brenner DA. Liver inflammation and fibrosis. The Journal of clinical investigation. 2017;127(1):55-64. Epub 2017/01/04. doi: 10.1172/JCI88881. PubMed PMID: 28045404; PMCID: PMC5199698.

158. Schuster S, Cabrera D, Arrese M, Feldstein AE. Triggering and resolution of inflammation in NASH. Nature reviews Gastroenterology & hepatology. 2018;15(6):349-64. Epub 2018/05/10. doi: 10.1038/s41575-018-0009-6. PubMed PMID: 29740166.

159. Das S, Alhasson F, Dattaroy D, Pourhoseini S, Seth RK, Nagarkatti M, Nagarkatti PS, Michelotti GA, Diehl AM, Kalyanaraman B, Chatterjee S. NADPH Oxidase-Derived Peroxynitrite Drives Inflammation in Mice and Human Nonalcoholic Steatohepatitis via TLR4-Lipid Raft Recruitment. The American journal of pathology. 2015;185(7):1944-57. Epub 2015/05/20. doi: 10.1016/j.ajpath.2015.03.024. PubMed PMID: 25989356; PMCID: PMC4483465.

160. Da Silva Morais A, Lebrun V, Abarca-Quinones J, Brichard S, Hue L, Guigas B, Viollet B, Leclercq IA. Prevention of steatohepatitis by pioglitazone: implication of adiponectin-dependent inhibition of SREBP-1c and inflammation. Journal of hepatology. 2009;50(3):489-500. Epub 2009/01/22. doi: 10.1016/j.jhep.2008.10.027. PubMed PMID: 19155087.

161. Cynis H, Kehlen A, Haegele M, Hoffmann T, Heiser U, Fujii M, Shibazaki Y, Yoneyama H, Schilling S, Demuth HU. Inhibition of Glutaminyl Cyclases alleviates CCL2-mediated inflammation of non-alcoholic fatty liver disease in mice. International journal of experimental pathology. 2013;94(3):217-25. Epub 2013/04/09. doi: 10.1111/iep.12020. PubMed PMID: 23560443; PMCID: PMC3664967.

162. Chen HL, Tsai TC, Tsai YC, Liao JW, Yen CC, Chen CM. Kefir peptides prevent high-fructose corn syrup-induced non-alcoholic fatty liver disease in a murine model by modulation of

inflammation and the JAK2 signaling pathway. Nutr Diabetes. 2016;6(12):e237. Epub 2016/12/13. doi: 10.1038/nutd.2016.49. PubMed PMID: 27941940; PMCID: PMC5223135.

163. Hirsova P, Ibrahim SH, Krishnan A, Verma VK, Bronk SF, Werneburg NW, Charlton MR, Shah VH, Malhi H, Gores GJ. Lipid-Induced Signaling Causes Release of Inflammatory Extracellular Vesicles From Hepatocytes. Gastroenterology. 2016;150(4):956-67. Epub 2016/01/15. doi: 10.1053/j.gastro.2015.12.037. PubMed PMID: 26764184; PMCID: PMC4808464.

164. Kakazu E, Mauer AS, Yin M, Malhi H. Hepatocytes release ceramide-enriched pro-inflammatory extracellular vesicles in an IRE1alpha-dependent manner. Journal of lipid research. 2016;57(2):233-45. Epub 2015/12/02. doi: 10.1194/jlr.M063412. PubMed PMID: 26621917; PMCID: PMC4727419.

165. Cazanave SC, Mott JL, Bronk SF, Werneburg NW, Fingas CD, Meng XW, Finnberg N, El-Deiry WS, Kaufmann SH, Gores GJ. Death receptor 5 signaling promotes hepatocyte lipoapoptosis. The Journal of biological chemistry. 2011;286(45):39336-48. Epub 2011/09/24. doi: 10.1074/jbc.M111.280420. PubMed PMID: 21941003; PMCID: PMC3234758.

166. Arguello G, Balboa E, Arrese M, Zanlungo S. Recent insights on the role of cholesterol in non-alcoholic fatty liver disease. Biochimica et biophysica acta. 2015;1852(9):1765-78. Epub 2015/06/02. doi: 10.1016/j.bbadis.2015.05.015. PubMed PMID: 26027904.

167. Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, Sargeant C, Contos MJ, Sanyal AJ. A lipidomic analysis of nonalcoholic fatty liver disease. Hepatology. 2007;46(4):1081-90. Epub 2007/07/27. doi: 10.1002/hep.21763. PubMed PMID: 17654743.

168. Wouters K, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lutjohann D, Kerksiek A, van Kruchten R, Maeda N, Staels B, van Bilsen M, Shiri-Sverdlov R, Hofker MH. Dietary

cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. Hepatology. 2008;48(2):474-86. Epub 2008/07/31. doi: 10.1002/hep.22363. PubMed PMID: 18666236.

169. Andreozzi P, Viscogliosi G, Colella F, Subic M, Cipriani E, Marigliano B, Verrusio W, Servello A, Ettorre E, Marigliano V. [Predictors of liver fibrosis in patients with non-alcoholic fatty liver disease. The role of metabolic syndrome, insulin-resistance and inflammation]. Recenti Prog Med. 2012;103(12):570-4. Epub 2012/12/22. doi: 10.1701/1206.13358. PubMed PMID: 23258240.

170. Ioannou GN, Subramanian S, Chait A, Haigh WG, Yeh MM, Farrell GC, Lee SP, Savard C. Cholesterol crystallization within hepatocyte lipid droplets and its role in murine NASH. Journal of lipid research. 2017;58(6):1067-79. Epub 2017/04/14. doi: 10.1194/jlr.M072454. PubMed PMID: 28404639; PMCID: PMC5456359.

171. Rajamaki K, Lappalainen J, Oorni K, Valimaki E, Matikainen S, Kovanen PT, Eklund KK. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. PloS one. 2010;5(7):e11765. Epub 2010/07/30. doi: 10.1371/journal.pone.0011765. PubMed PMID: 20668705; PMCID: PMC2909263.

172. Wree A, McGeough MD, Inzaugarat ME, Eguchi A, Schuster S, Johnson CD, Pena CA, Geisler LJ, Papouchado BG, Hoffman HM, Feldstein AE. NLRP3 inflammasome driven liver injury and fibrosis: Roles of IL-17 and TNF in mice. Hepatology. 2017. Epub 2017/09/14. doi: 10.1002/hep.29523. PubMed PMID: 28902427; PMCID: PMC5849484.

173. Gan LT, Van Rooyen DM, Koina ME, McCuskey RS, Teoh NC, Farrell GC. Hepatocyte free cholesterol lipotoxicity results from JNK1-mediated mitochondrial injury and is HMGB1

and TLR4-dependent. Journal of hepatology. 2014;61(6):1376-84. Epub 2014/07/30. doi: 10.1016/j.jhep.2014.07.024. PubMed PMID: 25064435.

174. Bray GA, Nielsen SJ, Popkin BM. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. The American journal of clinical nutrition. 2004;79(4):537-43. Epub 2004/03/31. doi: 10.1093/ajcn/79.4.537. PubMed PMID: 15051594.

175. Barquera S, Hernandez-Barrera L, Tolentino ML, Espinosa J, Ng SW, Rivera JA, Popkin BM. Energy intake from beverages is increasing among Mexican adolescents and adults. J Nutr. 2008;138(12):2454-61. Epub 2008/11/22. doi: 10.3945/jn.108.092163. PubMed PMID: 19022972.

176. Duffey KJ, Popkin BM. Shifts in patterns and consumption of beverages between 1965 and 2002. Obesity (Silver Spring). 2007;15(11):2739-47. Epub 2007/12/12. doi: 10.1038/oby.2007.326. PubMed PMID: 18070765.

177. Crescenzo R, Bianco F, Falcone I, Coppola P, Liverini G, Iossa S. Increased hepatic de novo lipogenesis and mitochondrial efficiency in a model of obesity induced by diets rich in fructose. Eur J Nutr. 2013;52(2):537-45. Epub 2012/05/01. doi: 10.1007/s00394-012-0356-y. PubMed PMID: 22543624.

178. Rebollo A, Roglans N, Baena M, Sanchez RM, Merlos M, Alegret M, Laguna JC. Liquid fructose downregulates Sirt1 expression and activity and impairs the oxidation of fatty acids in rat and human liver cells. Biochimica et biophysica acta. 2014;1841(4):514-24. Epub 2014/01/18. doi: 10.1016/j.bbalip.2014.01.002. PubMed PMID: 24434080.

179. Yang ZH, Miyahara H, Takeo J, Katayama M. Diet high in fat and sucrose induces rapid onset of obesity-related metabolic syndrome partly through rapid response of genes involved in lipogenesis, insulin signalling and inflammation in mice. Diabetol Metab Syndr. 2012;4(1):32.

Epub 2012/07/06. doi: 10.1186/1758-5996-4-32. PubMed PMID: 22762794; PMCID: PMC3407732.

180. Liu J, Zhuang ZJ, Bian DX, Ma XJ, Xun YH, Yang WJ, Luo Y, Liu YL, Jia L, Wang Y, Zhu ML, Ye DW, Zhou G, Lou GQ, Shi JP. Toll-like receptor-4 signalling in the progression of non-alcoholic fatty liver disease induced by high-fat and high-fructose diet in mice. Clin Exp Pharmacol Physiol. 2014;41(7):482-8. Epub 2014/04/18. doi: 10.1111/1440-1681.12241. PubMed PMID: 24739055.

181. Charlton M, Krishnan A, Viker K, Sanderson S, Cazanave S, McConico A, Masuoko H, Gores G. Fast food diet mouse: novel small animal model of NASH with ballooning, progressive fibrosis, and high physiological fidelity to the human condition. American journal of physiology Gastrointestinal and liver physiology. 2011;301(5):G825-34. Epub 2011/08/13. doi: 10.1152/ajpgi.00145.2011. PubMed PMID: 21836057; PMCID: PMC3220319.

182. Dupas J, Goanvec C, Feray A, Guernec A, Alain C, Guerrero F, Mansourati J. Progressive Induction of Type 2 Diabetes: Effects of a Reality-Like Fructose Enriched Diet in Young Wistar Rats. PloS one. 2016;11(1):e0146821. Epub 2016/01/23. doi: 10.1371/journal.pone.0146821. PubMed PMID: 26799836; PMCID: PMC4723014.

183. Kawasaki T, Igarashi K, Koeda T, Sugimoto K, Nakagawa K, Hayashi S, Yamaji R, Inui H, Fukusato T, Yamanouchi T. Rats fed fructose-enriched diets have characteristics of nonalcoholic hepatic steatosis. J Nutr. 2009;139(11):2067-71. Epub 2009/09/25. doi: 10.3945/jn.109.105858. PubMed PMID: 19776184.

184. Ren LP, Chan SM, Zeng XY, Laybutt DR, Iseli TJ, Sun RQ, Kraegen EW, Cooney GJ, Turner N, Ye JM. Differing endoplasmic reticulum stress response to excess lipogenesis versus lipid oversupply in relation to hepatic steatosis and insulin resistance. PloS one.

2012;7(2):e30816. Epub 2012/02/23. doi: 10.1371/journal.pone.0030816. PubMed PMID: 22355328; PMCID: PMC3280252.

185. Spruss A, Kanuri G, Wagnerberger S, Haub S, Bischoff SC, Bergheim I. Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. Hepatology. 2009;50(4):1094-104. Epub 2009/07/29. doi: 10.1002/hep.23122. PubMed PMID: 19637282.

186. Rosas-Villegas A, Sanchez-Tapia M, Avila-Nava A, Ramirez V, Tovar AR, Torres N. Differential Effect of Sucrose and Fructose in Combination with a High Fat Diet on Intestinal Microbiota and Kidney Oxidative Stress. Nutrients. 2017;9(4). Epub 2017/04/20. doi: 10.3390/nu9040393. PubMed PMID: 28420148; PMCID: PMC5409732.

187. Kavanagh K, Wylie AT, Tucker KL, Hamp TJ, Gharaibeh RZ, Fodor AA, Cullen JM. Dietary fructose induces endotoxemia and hepatic injury in calorically controlled primates. The American journal of clinical nutrition. 2013;98(2):349-57. Epub 2013/06/21. doi: 10.3945/ajcn.112.057331. PubMed PMID: 23783298; PMCID: PMC3712547.

188. van der Poorten D, George J. Disease-specific mechanisms of fibrosis: hepatitis C virus and nonalcoholic steatohepatitis. Clinics in liver disease. 2008;12(4):805-24, ix. Epub 2008/11/06. doi: 10.1016/j.cld.2008.07.003. PubMed PMID: 18984468.

189. Stanton MC, Chen SC, Jackson JV, Rojas-Triana A, Kinsley D, Cui L, Fine JS, Greenfeder S, Bober LA, Jenh CH. Inflammatory Signals shift from adipose to liver during high fat feeding and influence the development of steatohepatitis in mice. Journal of inflammation. 2011;8:8. Epub 2011/03/18. doi: 10.1186/1476-9255-8-8. PubMed PMID: 21410952; PMCID: PMC3070617.

190. Cancello R, Tordjman J, Poitou C, Guilhem G, Bouillot JL, Hugol D, Coussieu C, Basdevant A, Bar Hen A, Bedossa P, Guerre-Millo M, Clement K. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. Diabetes. 2006;55(6):1554-61. Epub 2006/05/30. doi: 10.2337/db06-0133. PubMed PMID: 16731817.

191. Adolph TE, Grander C, Grabherr F, Tilg H. Adipokines and Non-Alcoholic Fatty Liver Disease: Multiple Interactions. International journal of molecular sciences. 2017;18(8). Epub 2017/08/02. doi: 10.3390/ijms18081649. PubMed PMID: 28758929; PMCID: PMC5578039.

192. Buechler C, Wanninger J, Neumeier M. Adiponectin, a key adipokine in obesity related liver diseases. World J Gastroenterol. 2011;17(23):2801-11. Epub 2011/07/08. doi: 10.3748/wjg.v17.i23.2801. PubMed PMID: 21734787; PMCID: PMC3120939.

193. Hui JM, Hodge A, Farrell GC, Kench JG, Kriketos A, George J. Beyond insulin resistance in NASH: TNF-alpha or adiponectin? Hepatology. 2004;40(1):46-54. Epub 2004/07/09. doi: 10.1002/hep.20280. PubMed PMID: 15239085.

194. Brandl K, Schnabl B. Intestinal microbiota and nonalcoholic steatohepatitis. Curr Opin Gastroenterol. 2017;33(3):128-33. Epub 2017/03/04. doi: 10.1097/MOG.0000000000000349. PubMed PMID: 28257306; PMCID: PMC5662009.

195. Boursier J, Mueller O, Barret M, Machado M, Fizanne L, Araujo-Perez F, Guy CD, Seed PC, Rawls JF, David LA, Hunault G, Oberti F, Cales P, Diehl AM. The Severity of Nonalcoholic Fatty Liver Disease Is Associated With Gut Dysbiosis and Shift in the Metabolic Function of the Gut Microbiota. Hepatology. 2016;63(3):764-75. doi: 10.1002/hep.28356. PubMed PMID: WOS:000374493200014.

196. Farhadi A, Gundlapalli S, Shaikh M, Frantzides C, Harrell L, Kwasny MM, Keshavarzian A. Susceptibility to gut leakiness: a possible mechanism for endotoxaemia in non-alcoholic steatohepatitis. Liver international : official journal of the International Association for the Study of the Liver. 2008;28(7):1026-33. Epub 2008/04/10. doi: 10.1111/j.1478-3231.2008.01723.x. PubMed PMID: 18397235; PMCID: PMC4303249.

197. Csak T, Velayudham A, Hritz I, Petrasek J, Levin I, Lippai D, Catalano D, Mandrekar P, Dolganiuc A, Kurt-Jones E, Szabo G. Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice. American journal of physiology Gastrointestinal and liver physiology. 2011;300(3):G433-41. Epub 2011/01/15. doi: 10.1152/ajpgi.00163.2009. PubMed PMID: 21233280; PMCID: PMC3302188.

198. Etienne-Mesmin L, Vijay-Kumar M, Gewirtz AT, Chassaing B. Hepatocyte Toll-Like Receptor 5 Promotes Bacterial Clearance and Protects Mice Against High-Fat Diet-Induced Liver Disease. Cell Mol Gastroenterol Hepatol. 2016;2(5):584-604. Epub 2017/01/17. doi: 10.1016/j.jcmgh.2016.04.007. PubMed PMID: 28090564; PMCID: PMC5042709.

199. Rahman K, Desai C, Iyer SS, Thorn NE, Kumar P, Liu Y, Smith T, Neish AS, Li H, Tan S, Wu P, Liu X, Yu Y, Farris AB, Nusrat A, Parkos CA, Anania FA. Loss of Junctional Adhesion Molecule A Promotes Severe Steatohepatitis in Mice on a Diet High in Saturated Fat, Fructose, and Cholesterol. Gastroenterology. 2016;151(4):733-46 e12. Epub 2016/06/28. doi: 10.1053/j.gastro.2016.06.022. PubMed PMID: 27342212; PMCID: PMC5037035.

200. Arab JP, Karpen SJ, Dawson PA, Arrese M, Trauner M. Bile acids and nonalcoholic fatty liver disease: Molecular insights and therapeutic perspectives. Hepatology. 2017;65(1):350-62. doi: 10.1002/hep.28709. PubMed PMID: 27358174; PMCID: 5191969.

201. Chow MD, Lee YH, Guo GL. The role of bile acids in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Molecular aspects of medicine. 2017;56:34-44. doi: 10.1016/j.mam.2017.04.004. PubMed PMID: 28442273.

202. Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. Bile acids and the gut microbiome. Curr Opin Gastroenterol. 2014;30(3):332-8. Epub 2014/03/15. doi: 10.1097/MOG.0000000000000057. PubMed PMID: 24625896; PMCID: PMC4215539.

203. Pizarro M, Balasubramaniyan N, Solis N, Solar A, Duarte I, Miquel JF, Suchy FJ, Trauner M, Accatino L, Ananthanarayanan M, Arrese M. Bile secretory function in the obese Zucker rat: evidence of cholestasis and altered canalicular transport function. Gut. 2004;53(12):1837-43. Epub 2004/11/16. doi: 10.1136/gut.2003.037689. PubMed PMID: 15542525; PMCID: PMC1774316.

204. Aranha MM, Cortez-Pinto H, Costa A, da Silva IB, Camilo ME, de Moura MC, Rodrigues CM. Bile acid levels are increased in the liver of patients with steatohepatitis. European journal of gastroenterology & hepatology. 2008;20(6):519-25. doi: 10.1097/MEG.0b013e3282f4710a. PubMed PMID: 18467911.

205. Puri P, Daita K, Joyce A, Mirshahi F, Santhekadur PK, Cazanave S, Luketic VA, Siddiqui MS, Boyett S, Min HK, Kumar DP, Kohli R, Zhou H, Hylemon PB, Contos MJ, Idowu M, Sanyal AJ. The presence and severity of nonalcoholic steatohepatitis is associated with specific changes in circulating bile acids. Hepatology. 2017. Epub 2017/07/12. doi: 10.1002/hep.29359. PubMed PMID: 28696585; PMCID: PMC5764808.

206. Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, Devera ME, Liang X, Tor M, Billiar T. The grateful dead: damage-associated molecular pattern

molecules and reduction/oxidation regulate immunity. Immunological reviews. 2007;220:60-81. Epub 2007/11/06. doi: 10.1111/j.1600-065X.2007.00579.x. PubMed PMID: 17979840.

207. Arrese M, Cabrera D, Kalergis AM, Feldstein AE. Innate Immunity and Inflammation in NAFLD/NASH. Dig Dis Sci. 2016;61(5):1294-303. Epub 2016/02/05. doi: 10.1007/s10620-016-4049-x. PubMed PMID: 26841783; PMCID: PMC4948286.

208. Kesar V, Odin JA. Toll-like receptors and liver disease. Liver international : official journal of the International Association for the Study of the Liver. 2014;34(2):184-96. Epub 2013/10/15. doi: 10.1111/liv.12315. PubMed PMID: 24118797.

209. Bieghs V, Trautwein C. Innate immune signaling and gut-liver interactions in non-alcoholic fatty liver disease. Hepatobiliary Surg Nutr. 2014;3(6):377-85. Epub 2015/01/09. doi: 10.3978/j.issn.2304-3881.2014.12.04. PubMed PMID: 25568861; PMCID: PMC4273113.

210. Miura K, Yang L, van Rooijen N, Brenner DA, Ohnishi H, Seki E. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice. Hepatology. 2013;57(2):577-89. Epub 2012/09/19. doi: 10.1002/hep.26081. PubMed PMID: 22987396; PMCID: PMC3566276.

211. Alegre F, Pelegrin P, Feldstein AE. Inflammasomes in Liver Fibrosis. Seminars in liver disease. 2017;37(2):119-27. Epub 2017/06/01. doi: 10.1055/s-0037-1601350. PubMed PMID: 28564720.

212. Cai C, Zhu X, Li P, Li J, Gong J, Shen W, He K. NLRP3 Deletion Inhibits the Non-alcoholic Steatohepatitis Development and Inflammation in Kupffer Cells Induced by Palmitic Acid. Inflammation. 2017;40(6):1875-83. Epub 2017/07/22. doi: 10.1007/s10753-017-0628-z. PubMed PMID: 28730512.

213. Watanabe A, Sohail MA, Gomes DA, Hashmi A, Nagata J, Sutterwala FS, Mahmood S, Jhandier MN, Shi Y, Flavell RA, Mehal WZ. Inflammasome-mediated regulation of hepatic stellate cells. American journal of physiology Gastrointestinal and liver physiology. 2009;296(6):G1248-57. Epub 2009/04/11. doi: 10.1152/ajpgi.90223.2008. PubMed PMID: 19359429; PMCID: PMC2697939.

214. Krenkel O, Tacke F. Liver macrophages in tissue homeostasis and disease. Nature reviews Immunology. 2017;17(5):306-21. Epub 2017/03/21. doi: 10.1038/nri.2017.11. PubMed PMID: 28317925.

215. Krenkel O, Tacke F. Macrophages in Nonalcoholic Fatty Liver Disease: A Role Model of Pathogenic Immunometabolism. Seminars in liver disease. 2017;37(3):189-97. Epub 2017/08/29. doi: 10.1055/s-0037-1604480. PubMed PMID: 28847030.

216. Baeck C, Wehr A, Karlmark KR, Heymann F, Vucur M, Gassler N, Huss S, Klussmann S, Eulberg D, Luedde T, Trautwein C, Tacke F. Pharmacological inhibition of the chemokine CCL2 (MCP-1) diminishes liver macrophage infiltration and steatohepatitis in chronic hepatic injury. Gut. 2012;61(3):416-26. doi: 10.1136/gutjnl-2011-300304. PubMed PMID: 21813474.

217. Reid DT, Reyes JL, McDonald BA, Vo T, Reimer RA, Eksteen B. Kupffer Cells Undergo Fundamental Changes during the Development of Experimental NASH and Are Critical in Initiating Liver Damage and Inflammation. PloS one. 2016;11(7):e0159524. Epub 2016/07/28. doi: 10.1371/journal.pone.0159524. PubMed PMID: 27454866; PMCID: PMC4959686.

218. Tosello-Trampont AC, Landes SG, Nguyen V, Novobrantseva TI, Hahn YS. Kuppfer cells trigger nonalcoholic steatohepatitis development in diet-induced mouse model through tumor necrosis factor-alpha production. The Journal of biological chemistry.

2012;287(48):40161-72. doi: 10.1074/jbc.M112.417014. PubMed PMID: 23066023; PMCID: 3504730.

219. Soehnlein O, Steffens S, Hidalgo A, Weber C. Neutrophils as protagonists and targets in chronic inflammation. Nature reviews Immunology. 2017;17(4):248-61. Epub 2017/03/14. doi: 10.1038/nri.2017.10. PubMed PMID: 28287106.

220. Xu R, Huang H, Zhang Z, Wang FS. The role of neutrophils in the development of liver diseases. Cell Mol Immunol. 2014;11(3):224-31. Epub 2014/03/19. doi: 10.1038/cmi.2014.2. PubMed PMID: 24633014; PMCID: PMC4085492.

221. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, Becker CD, See P, Price J, Lucas D, Greter M, Mortha A, Boyer SW, Forsberg EC, Tanaka M, van Rooijen N, Garcia-Sastre A, Stanley ER, Ginhoux F, Frenette PS, Merad M. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. Immunity. 2013;38(4):792-804. Epub 2013/04/23. doi: 10.1016/j.immuni.2013.04.004. PubMed PMID: 23601688; PMCID: PMC3853406.

222. Tacke F. Targeting hepatic macrophages to treat liver diseases. Journal of hepatology. 2017;66(6):1300-12. Epub 2017/03/08. doi: 10.1016/j.jhep.2017.02.026. PubMed PMID: 28267621.

223. Tacke F, Zimmermann HW. Macrophage heterogeneity in liver injury and fibrosis. Journal of hepatology. 2014;60(5):1090-6. doi: 10.1016/j.jhep.2013.12.025. PubMed PMID: 24412603.

224. Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, Garner H, Trouillet C, de Bruijn MF, Geissmann F, Rodewald HR. Tissue-resident macrophages originate

from yolk-sac-derived erythro-myeloid progenitors. Nature. 2015;518(7540):547-51. Epub 2014/12/04. doi: 10.1038/nature13989. PubMed PMID: 25470051; PMCID: PMC5997177.

225. Mass E, Ballesteros I, Farlik M, Halbritter F, Gunther P, Crozet L, Jacome-Galarza CE, Handler K, Klughammer J, Kobayashi Y, Gomez-Perdiguero E, Schultze JL, Beyer M, Bock C, Geissmann F. Specification of tissue-resident macrophages during organogenesis. Science. 2016;353(6304). Epub 2016/08/06. doi: 10.1126/science.aaf4238. PubMed PMID: 27492475; PMCID: PMC5066309.

226. Hoeffel G, Chen J, Lavin Y, Low D, Almeida FF, See P, Beaudin AE, Lum J, Low I, Forsberg EC, Poidinger M, Zolezzi F, Larbi A, Ng LG, Chan JK, Greter M, Becher B, Samokhvalov IM, Merad M, Ginhoux F. C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. Immunity. 2015;42(4):665-78. Epub 2015/04/23. doi: 10.1016/j.immuni.2015.03.011. PubMed PMID: 25902481; PMCID: PMC4545768.

227. Varol C, Mildner A, Jung S. Macrophages: development and tissue specialization. Annual review of immunology. 2015;33:643-75. Epub 2015/04/12. doi: 10.1146/annurev-immunol-032414-112220. PubMed PMID: 25861979.

228. Heymann F, Peusquens J, Ludwig-Portugall I, Kohlhepp M, Ergen C, Niemietz P, Martin C, van Rooijen N, Ochando JC, Randolph GJ, Luedde T, Ginhoux F, Kurts C, Trautwein C, Tacke F. Liver inflammation abrogates immunological tolerance induced by Kupffer cells. Hepatology. 2015;62(1):279-91. Epub 2015/03/27. doi: 10.1002/hep.27793. PubMed PMID: 25810240.

229. Heymann F, Tacke F. Immunology in the liver--from homeostasis to disease. Nature reviews Gastroenterology & hepatology. 2016;13(2):88-110. Epub 2016/01/14. doi: 10.1038/nrgastro.2015.200. PubMed PMID: 26758786.

230. Sieweke MH, Allen JE. Beyond stem cells: self-renewal of differentiated macrophages. Science. 2013;342(6161):1242974. Epub 2013/11/23. doi: 10.1126/science.1242974. PubMed PMID: 24264994.

231. Ingersoll MA, Spanbroek R, Lottaz C, Gautier EL, Frankenberger M, Hoffmann R, Lang R, Haniffa M, Collin M, Tacke F, Habenicht AJ, Ziegler-Heitbrock L, Randolph GJ. Comparison of gene expression profiles between human and mouse monocyte subsets. Blood. 2010;115(3):e10-9. Epub 2009/12/08. doi: 10.1182/blood-2009-07-235028. PubMed PMID: 19965649; PMCID: PMC2810986.

232. Mossanen JC, Krenkel O, Ergen C, Govaere O, Liepelt A, Puengel T, Heymann F, Kalthoff S, Lefebvre E, Eulberg D, Luedde T, Marx G, Strassburg CP, Roskams T, Trautwein C, Tacke F. Chemokine (C-C motif) receptor 2-positive monocytes aggravate the early phase of acetaminophen-induced acute liver injury. Hepatology. 2016;64(5):1667-82. Epub 2016/10/22. doi: 10.1002/hep.28682. PubMed PMID: 27302828.

233. David BA, Rezende RM, Antunes MM, Santos MM, Freitas Lopes MA, Diniz AB, Sousa Pereira RV, Marchesi SC, Alvarenga DM, Nakagaki BN, Araujo AM, Dos Reis DS, Rocha RM, Marques PE, Lee WY, Deniset J, Liew PX, Rubino S, Cox L, Pinho V, Cunha TM, Fernandes GR, Oliveira AG, Teixeira MM, Kubes P, Menezes GB. Combination of Mass Cytometry and Imaging Analysis Reveals Origin, Location, and Functional Repopulation of Liver Myeloid Cells in Mice. Gastroenterology. 2016;151(6):1176-91. Epub 2016/10/30. doi: 10.1053/j.gastro.2016.08.024. PubMed PMID: 27569723.

234. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, Sarnacki S, Cumano A, Lauvau G, Geissmann F. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science. 2007;317(5838):666-70. Epub 2007/08/04. doi: 10.1126/science.1142883. PubMed PMID: 17673663.

235. Serbina NV, Pamer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. Nature immunology. 2006;7(3):311-7. Epub 2006/02/08. doi: 10.1038/ni1309. PubMed PMID: 16462739.

236. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, Figueiredo JL, Kohler RH, Chudnovskiy A, Waterman P, Aikawa E, Mempel TR, Libby P, Weissleder R, Pittet MJ. Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. Science. 2009;325(5940):612-6. doi: 10.1126/science.1175202. PubMed PMID: WOS:000268493000051.

237. Dal-Secco D, Wang J, Zeng Z, Kolaczkowska E, Wong CH, Petri B, Ransohoff RM, Charo IF, Jenne CN, Kubes P. A dynamic spectrum of monocytes arising from the in situ reprogramming of CCR2+ monocytes at a site of sterile injury. The Journal of experimental medicine. 2015;212(4):447-56. Epub 2015/03/25. doi: 10.1084/jem.20141539. PubMed PMID: 25800956; PMCID: PMC4387291.

238. Karlmark KR, Weiskirchen R, Zimmermann HW, Gassler N, Ginhoux F, Weber C, Merad M, Luedde T, Trautwein C, Tacke F. Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology. 2009;50(1):261-74. doi: 10.1002/hep.22950. PubMed PMID: 19554540.

239. Scott CL, Zheng F, De Baetselier P, Martens L, Saeys Y, De Prijck S, Lippens S, Abels C, Schoonooghe S, Raes G, Devoogdt N, Lambrecht BN, Beschin A, Guilliams M. Bone

marrow-derived monocytes give rise to self-renewing and fully differentiated Kupffer cells. Nature communications. 2016;7:10321. Epub 2016/01/28. doi: 10.1038/ncomms10321. PubMed PMID: 26813785; PMCID: PMC4737801.

240. Klein I, Cornejo JC, Polakos NK, John B, Wuensch SA, Topham DJ, Pierce RH, Crispe IN. Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. Blood. 2007;110(12):4077-85. Epub 2007/08/11. doi: 10.1182/blood-2007-02-073841. PubMed PMID: 17690256; PMCID: PMC2190614.

241. Beattie L, Sawtell A, Mann J, Frame TCM, Teal B, de Labastida Rivera F, Brown N, Walwyn-Brown K, Moore JWJ, MacDonald S, Lim EK, Dalton JE, Engwerda CR, MacDonald KP, Kaye PM. Bone marrow-derived and resident liver macrophages display unique transcriptomic signatures but similar biological functions. Journal of hepatology. 2016;65(4):758-68. Epub 2016/06/06. doi: 10.1016/j.jhep.2016.05.037. PubMed PMID: 27262757; PMCID: PMC5028381.

242. Ramachandran P, Pellicoro A, Vernon MA, Boulter L, Aucott RL, Ali A, Hartland SN, Snowdon VK, Cappon A, Gordon-Walker TT, Williams MJ, Dunbar DR, Manning JR, van Rooijen N, Fallowfield JA, Forbes SJ, Iredale JP. Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(46):E3186-95. Epub 2012/10/27. doi: 10.1073/pnas.1119964109. PubMed PMID: 23100531; PMCID: PMC3503234.

243. Murray PJ. Macrophage Polarization. Annu Rev Physiol. 2017;79:541-66. Epub 2016/11/05. doi: 10.1146/annurev-physiol-022516-034339. PubMed PMID: 27813830.

244. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M, Mantovani A, Martinez FO, Mege JL, Mosser DM, Natoli G, Saeij JP, Schultze JL, Shirey KA, Sica A, Suttles J, Udalova I, van Ginderachter JA, Vogel SN, Wynn TA. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014;41(1):14-20. Epub 2014/07/19. doi: 10.1016/j.immuni.2014.06.008. PubMed PMID: 25035950; PMCID: PMC4123412.

245. Sica A, Invernizzi P, Mantovani A. Macrophage plasticity and polarization in liver homeostasis and pathology. Hepatology. 2014;59(5):2034-42. Epub 2013/10/12. doi: 10.1002/hep.26754. PubMed PMID: 24115204.

246. Lavin Y, Winter D, Blecher-Gonen R, David E, Keren-Shaul H, Merad M, Jung S, Amit I. Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. Cell. 2014;159(6):1312-26. Epub 2014/12/07. doi: 10.1016/j.cell.2014.11.018. PubMed PMID: 25480296; PMCID: PMC4437213.

247. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, De Nardo D, Gohel TD, Emde M, Schmidleithner L, Ganesan H, Nino-Castro A, Mallmann MR, Labzin L, Theis H, Kraut M, Beyer M, Latz E, Freeman TC, Ulas T, Schultze JL. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. Immunity. 2014;40(2):274-88. Epub 2014/02/18. doi: 10.1016/j.immuni.2014.01.006. PubMed PMID: 24530056; PMCID: PMC3991396.

248. Sakaguchi S, Takahashi S, Sasaki T, Kumagai T, Nagata K. Progression of alcoholic and non-alcoholic steatohepatitis: common metabolic aspects of innate immune system and oxidative stress. Drug Metab Pharmacokinet. 2011;26(1):30-46. Epub 2010/12/15. PubMed PMID: 21150132.

249. Wan J, Benkdane M, Teixeira-Clerc F, Bonnafous S, Louvet A, Lafdil F, Pecker F, Tran A, Gual P, Mallat A, Lotersztajn S, Pavoine C. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. Hepatology. 2014;59(1):130-42. Epub 2013/07/09. doi: 10.1002/hep.26607. PubMed PMID: 23832548.

250. Huang W, Metlakunta A, Dedousis N, Zhang P, Sipula I, Dube JJ, Scott DK, O'Doherty RM. Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. Diabetes. 2010;59(2):347-57. Epub 2009/11/26. doi: 10.2337/db09-0016. PubMed PMID: 19934001; PMCID: PMC2809951.

251. Lanthier N, Molendi-Coste O, Cani PD, van Rooijen N, Horsmans Y, Leclercq IA. Kupffer cell depletion prevents but has no therapeutic effect on metabolic and inflammatory changes induced by a high-fat diet. FASEB J. 2011;25(12):4301-11. Epub 2011/08/30. doi: 10.1096/fj.11-189472. PubMed PMID: 21873555.

252. Parlesak A, Schafer C, Schutz T, Bode JC, Bode C. Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease. Journal of hepatology. 2000;32(5):742-7. Epub 2000/06/14. PubMed PMID: 10845660.

253. Jindal A, Bruzzi S, Sutti S, Locatelli I, Bozzola C, Paternostro C, Parola M, Albano E. Fat-laden macrophages modulate lobular inflammation in nonalcoholic steatohepatitis (NASH). Exp Mol Pathol. 2015;99(1):155-62. Epub 2015/06/27. doi: 10.1016/j.yexmp.2015.06.015. PubMed PMID: 26112094.

254. Bartneck M, Fech V, Ehling J, Govaere O, Warzecha KT, Hittatiya K, Vucur M, Gautheron J, Luedde T, Trautwein C, Lammers T, Roskams T, Jahnen-Dechent W, Tacke F.

Histidine-rich glycoprotein promotes macrophage activation and inflammation in chronic liver disease. Hepatology. 2016;63(4):1310-24. Epub 2015/12/25. doi: 10.1002/hep.28418. PubMed PMID: 26699087.

255. Kamari Y, Shaish A, Vax E, Shemesh S, Kandel-Kfir M, Arbel Y, Olteanu S, Barshack I, Dotan S, Voronov E, Dinarello CA, Apte RN, Harats D. Lack of interleukin-1alpha or interleukin-1beta inhibits transformation of steatosis to steatohepatitis and liver fibrosis in hypercholesterolemic mice. Journal of hepatology. 2011;55(5):1086-94. Epub 2011/03/01. doi: 10.1016/j.jhep.2011.01.048. PubMed PMID: 21354232; PMCID: PMC3210940.

256. Gadd VL, Skoien R, Powell EE, Fagan KJ, Winterford C, Horsfall L, Irvine K, Clouston AD. The portal inflammatory infiltrate and ductular reaction in human nonalcoholic fatty liver disease. Hepatology. 2014;59(4):1393-405. Epub 2013/11/21. doi: 10.1002/hep.26937. PubMed PMID: 24254368.

257. Zhou D, Huang C, Lin Z, Zhan S, Kong L, Fang C, Li J. Macrophage polarization and function with emphasis on the evolving roles of coordinated regulation of cellular signaling pathways. Cell Signal. 2014;26(2):192-7. Epub 2013/11/14. doi: 10.1016/j.cellsig.2013.11.004. PubMed PMID: 24219909.

258. Van Rooyen DM, Gan LT, Yeh MM, Haigh WG, Larter CZ, Ioannou G, Teoh NC, Farrell GC. Pharmacological cholesterol lowering reverses fibrotic NASH in obese, diabetic mice with metabolic syndrome. Journal of hepatology. 2013;59(1):144-52. Epub 2013/03/19. doi: 10.1016/j.jhep.2013.02.024. PubMed PMID: 23500152.

259. Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. Hepatology. 2015;61(3):1066-79. Epub 2014/07/30. doi: 10.1002/hep.27332. PubMed PMID: 25066777; PMCID: PMC4306641.

260. Wehr A, Baeck C, Heymann F, Niemietz PM, Hammerich L, Martin C, Zimmermann HW, Pack O, Gassler N, Hittatiya K, Ludwig A, Luedde T, Trautwein C, Tacke F. Chemokine receptor CXCR6-dependent hepatic NK T Cell accumulation promotes inflammation and liver fibrosis. Journal of immunology. 2013;190(10):5226-36. Epub 2013/04/19. doi: 10.4049/jimmunol.1202909. PubMed PMID: 23596313.

261. Navarro LA, Wree A, Povero D, Berk MP, Eguchi A, Ghosh S, Papouchado BG, Erzurum SC, Feldstein AE. Arginase 2 deficiency results in spontaneous steatohepatitis: a novel link between innate immune activation and hepatic de novo lipogenesis. Journal of hepatology. 2015;62(2):412-20. Epub 2014/09/23. doi: 10.1016/j.jhep.2014.09.015. PubMed PMID: 25234945; PMCID: PMC4736721.

262. Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. Cell Mol Immunol. 2016;13(3):316-27. Epub 2016/02/26. doi: 10.1038/cmi.2015.104. PubMed PMID: 26908374; PMCID: PMC4856798.

263. Tilg H, Moschen AR. IL-1 cytokine family members and NAFLD: neglected in metabolic liver inflammation. Journal of hepatology. 2011;55(5):960-2. Epub 2011/07/12. doi: 10.1016/j.jhep.2011.04.007. PubMed PMID: 21742000.

264. Stienstra R, Saudale F, Duval C, Keshtkar S, Groener JE, van Rooijen N, Staels B, Kersten S, Muller M. Kupffer cells promote hepatic steatosis via interleukin-1beta-dependent suppression of peroxisome proliferator-activated receptor alpha activity. Hepatology. 2010;51(2):511-22. Epub 2010/01/08. doi: 10.1002/hep.23337. PubMed PMID: 20054868.

265. Eckert C, Klein N, Kornek M, Lukacs-Kornek V. The complex myeloid network of the liver with diverse functional capacity at steady state and in inflammation. Front Immunol.

2015;6:179. Epub 2015/05/06. doi: 10.3389/fimmu.2015.00179. PubMed PMID: 25941527; PMCID: PMC4403526.

266. Canbay A, Feldstein AE, Higuchi H, Werneburg N, Grambihler A, Bronk SF, Gores GJ. Kupffer cell engulfment of apoptotic bodies stimulates death ligand and cytokine expression. Hepatology. 2003;38(5):1188-98. Epub 2003/10/28. doi: 10.1053/jhep.2003.50472. PubMed PMID: 14578857.

267. Nakashima H, Nakashima M, Kinoshita M, Ikarashi M, Miyazaki H, Hanaka H, Imaki J, Seki S. Activation and increase of radio-sensitive CD11b+ recruited Kupffer cells/macrophages in diet-induced steatohepatitis in FGF5 deficient mice. Scientific reports. 2016;6:34466. Epub 2016/10/07. doi: 10.1038/srep34466. PubMed PMID: 27708340; PMCID: PMC5052649.

268. Miura K, Yang L, van Rooijen N, Ohnishi H, Seki E. Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. American journal of physiology Gastrointestinal and liver physiology. 2012;302(11):G1310-21. Epub 2012/03/24. doi: 10.1152/ajpgi.00365.2011. PubMed PMID: 22442158; PMCID: PMC3378163.

269. Heinrichs D, Berres ML, Coeuru M, Knauel M, Nellen A, Fischer P, Philippeit C, Bucala R, Trautwein C, Wasmuth HE, Bernhagen J. Protective role of macrophage migration inhibitory factor in nonalcoholic steatohepatitis. FASEB J. 2014;28(12):5136-47. Epub 2014/08/15. doi: 10.1096/fj.14-256776. PubMed PMID: 25122558; PMCID: PMC4232286.

270. Carpino G, Renzi A, Onori P, Gaudio E. Role of hepatic progenitor cells in nonalcoholic fatty liver disease development: cellular cross-talks and molecular networks. International journal of molecular sciences. 2013;14(10):20112-30. Epub 2013/10/12. doi: 10.3390/ijms141020112. PubMed PMID: 24113587; PMCID: PMC3821605.

271. Pradere JP, Kluwe J, De Minicis S, Jiao JJ, Gwak GY, Dapito DH, Jang MK, Guenther ND, Mederacke I, Friedman R, Dragomir AC, Aloman C, Schwabe RF. Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. Hepatology. 2013;58(4):1461-73. Epub 2013/04/05. doi: 10.1002/hep.26429. PubMed PMID: 23553591; PMCID: PMC3848418.

272. Carotti S, Morini S, Corradini SG, Burza MA, Molinaro A, Carpino G, Merli M, De Santis A, Muda AO, Rossi M, Attili AF, Gaudio E. Glial fibrillary acidic protein as an early marker of hepatic stellate cell activation in chronic and posttransplant recurrent hepatitis C. Liver Transpl. 2008;14(6):806-14. Epub 2008/05/30. doi: 10.1002/lt.21436. PubMed PMID: 18508359.

273. Baeck C, Wei X, Bartneck M, Fech V, Heymann F, Gassler N, Hittatiya K, Eulberg D, Luedde T, Trautwein C, Tacke F. Pharmacological inhibition of the chemokine C-C motif chemokine ligand 2 (monocyte chemoattractant protein 1) accelerates liver fibrosis regression by suppressing Ly-6C(+) macrophage infiltration in mice. Hepatology. 2014;59(3):1060-72. Epub 2014/02/01. doi: 10.1002/hep.26783. PubMed PMID: 24481979.

274. Wang M, You Q, Lor K, Chen F, Gao B, Ju C. Chronic alcohol ingestion modulates hepatic macrophage populations and functions in mice. Journal of leukocyte biology. 2014;96(4):657-65. Epub 2014/07/18. doi: 10.1189/jlb.6A0114-004RR. PubMed PMID: 25030420; PMCID: PMC4163632.

275. Lodder J, Denaes T, Chobert MN, Wan J, El-Benna J, Pawlotsky JM, Lotersztajn S, Teixeira-Clerc F. Macrophage autophagy protects against liver fibrosis in mice. Autophagy. 2015;11(8):1280-92. Epub 2015/06/11. doi: 10.1080/15548627.2015.1058473. PubMed PMID: 26061908; PMCID: PMC4590651.

276. Gual P, Gilgenkrantz H, Lotersztajn S. Autophagy in chronic liver diseases: the two faces of Janus. American journal of physiology Cell physiology. 2017;312(3):C263-C73. Epub 2016/12/03. doi: 10.1152/ajpcell.00295.2016. PubMed PMID: 27903585.

277. Bianconi E, Piovesan A, Facchin F, Beraudi A, Casadei R, Frabetti F, Vitale L, Pelleri MC, Tassani S, Piva F, Perez-Amodio S, Strippoli P, Canaider S. An estimation of the number of cells in the human body. Ann Hum Biol. 2013;40(6):463-71. Epub 2013/07/09. doi: 10.3109/03014460.2013.807878. PubMed PMID: 23829164.

278. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol. 2016;14(8):e1002533. Epub 2016/08/20. doi: 10.1371/journal.pbio.1002533. PubMed PMID: 27541692; PMCID: PMC4991899.

279. Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. Cell. 2016;164(3):337-40. Epub 2016/01/30. doi: 10.1016/j.cell.2016.01.013. PubMed PMID: 26824647.

280. Marchesi JR, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, Quraishi MN, Kinross J, Smidt H, Tuohy KM, Thomas LV, Zoetendal EG, Hart A. The gut microbiota and host health: a new clinical frontier. Gut. 2016;65(2):330-9. Epub 2015/09/05. doi: 10.1136/gutjnl-2015-309990. PubMed PMID: 26338727; PMCID: PMC4752653.

281. Marcy Y, Ouverney C, Bik EM, Losekann T, Ivanova N, Martin HG, Szeto E, Platt D, Hugenholtz P, Relman DA, Quake SR. Dissecting biological "dark matter" with single-cell genetic analysis of rare and uncultivated TM7 microbes from the human mouth. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(29):11889-94. Epub 2007/07/11. doi: 10.1073/pnas.0704662104. PubMed PMID: 17620602; PMCID: PMC1924555.

282. Liou AP, Paziuk M, Luevano JM, Jr., Machineni S, Turnbaugh PJ, Kaplan LM. Conserved shifts in the gut microbiota due to gastric bypass reduce host weight and adiposity. Science translational medicine. 2013;5(178):178ra41. Epub 2013/03/29. doi: 10.1126/scitranslmed.3005687. PubMed PMID: 23536013; PMCID: PMC3652229.

283. Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 2008;6(11):e280. Epub 2008/11/21. doi: 10.1371/journal.pbio.0060280. PubMed PMID: 19018661; PMCID: PMC2586385.

284. Turnbaugh PJ, Backhed F, Fulton L, Gordon JI. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell host & microbe. 2008;3(4):213-23. Epub 2008/04/15. doi: 10.1016/j.chom.2008.02.015. PubMed PMID: 18407065; PMCID: PMC3687783.

285. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 2006;444(7122):1027-31. Epub 2006/12/22. doi: 10.1038/nature05414. PubMed PMID: 17183312.

286. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nature Reviews Immunology. 2011;11(2):98-107. doi: 10.1038/nri2925. PubMed PMID: WOS:000286589300014.

287. Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes care. 2004;27(3):813-23. Epub 2004/02/28. PubMed PMID: 14988310.

288. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of

obesity-related insulin resistance. The Journal of clinical investigation. 2003;112(12):1821-30. Epub 2003/12/18. doi: 10.1172/JCI19451. PubMed PMID: 14679177; PMCID: PMC296998.

289. Rutenburg AM, Sonnenblick E, Koven I, Aprahamian HA, Reiner L, Fine J. The role of intestinal bacteria in the development of dietary cirrhosis in rats. The Journal of experimental medicine. 1957;106(1):1-14. Epub 1957/07/01. PubMed PMID: 13439110; PMCID: PMC2136738.

290. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(44):15718-23. Epub 2004/10/27. doi: 10.1073/pnas.0407076101. PubMed PMID: 15505215; PMCID: PMC524219.

291. Schnabl B, Brenner DA. Interactions between the intestinal microbiome and liver diseases. Gastroenterology. 2014;146(6):1513-24. Epub 2014/01/21. doi: 10.1053/j.gastro.2014.01.020. PubMed PMID: 24440671; PMCID: PMC3996054.

292. Bluemel S, Williams B, Knight R, Schnabl B. Precision medicine in alcoholic and nonalcoholic fatty liver disease via modulating the gut microbiota. American journal of physiology Gastrointestinal and liver physiology. 2016;311(6):G1018-G36. Epub 2016/10/01. doi: 10.1152/ajpgi.00245.2016. PubMed PMID: 27686615; PMCID: PMC5206291.

293. Saltzman ET, Palacios T, Thomsen M, Vitetta L. Intestinal Microbiome Shifts, Dysbiosis, Inflammation, and Non-alcoholic Fatty Liver Disease. Frontiers in microbiology. 2018;9:61. Epub 2018/02/15. doi: 10.3389/fmicb.2018.00061. PubMed PMID: 29441049; PMCID: PMC5797576.

294. Mokhtari Z, Gibson DL, Hekmatdoost A. Nonalcoholic Fatty Liver Disease, the Gut Microbiome, and Diet. Adv Nutr. 2017;8(2):240-52. Epub 2017/03/17. doi: 10.3945/an.116.013151. PubMed PMID: 28298269; PMCID: PMC5347097.

295. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. Science. 2005;308(5728):1635-8. Epub 2005/04/16. doi: 10.1126/science.1110591. PubMed PMID: 15831718; PMCID: PMC1395357.

296. Wigg AJ, Roberts-Thomson IC, Dymock RB, McCarthy PJ, Grose RH, Cummins AG. The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis. Gut. 2001;48(2):206-11. Epub 2001/01/13. PubMed PMID: 11156641; PMCID: PMC1728215.

297. Eslamparast T, Poustchi H, Zamani F, Sharafkhah M, Malekzadeh R, Hekmatdoost A. Synbiotic supplementation in nonalcoholic fatty liver disease: a randomized, double-blind, placebo-controlled pilot study. The American journal of clinical nutrition. 2014;99(3):535-42. Epub 2014/01/10. doi: 10.3945/ajcn.113.068890. PubMed PMID: 24401715.

298. Shavakhi A, Minakari M, Firouzian H, Assali R, Hekmatdoost A, Ferns G. Effect of a Probiotic and Metformin on Liver Aminotransferases in Non-alcoholic Steatohepatitis: A Double Blind Randomized Clinical Trial. Int J Prev Med. 2013;4(5):531-7. Epub 2013/08/10. PubMed PMID: 23930163; PMCID: PMC3733183.

299. Rahimlou M, Ahmadnia H, Hekmatdoost A. Dietary supplements and pediatric non-alcoholic fatty liver disease: Present and the future. World J Hepatol. 2015;7(25):2597-602. Epub 2015/11/12. doi: 10.4254/wjh.v7.i25.2597. PubMed PMID: 26557952; PMCID: PMC4635145.

300. Yari Z, Rahimlou M, Eslamparast T, Ebrahimi-Daryani N, Poustchi H, Hekmatdoost A. Flaxseed supplementation in non-alcoholic fatty liver disease: a pilot randomized, open labeled, controlled study. Int J Food Sci Nutr. 2016;67(4):461-9. Epub 2016/03/18. doi: 10.3109/09637486.2016.1161011. PubMed PMID: 26983396.

301. Guo Y, Xun Z, Coffman SR, Chen F. The Shift of the Intestinal Microbiome in the Innate Immunity-Deficient Mutant rde-1 Strain of C. elegans upon Orsay Virus Infection. Frontiers in microbiology. 2017;8:933. Epub 2017/06/15. doi: 10.3389/fmicb.2017.00933. PubMed PMID: 28611740; PMCID: PMC5446984.

302. Ley RE. Gut microbiota in 2015: Prevotella in the gut: choose carefully. Nature reviews Gastroenterology & hepatology. 2016;13(2):69-70. doi: 10.1038/nrgastro.2016.4. PubMed PMID: 26828918.

303. Zhao Y, Wu J, Li JV, Zhou NY, Tang H, Wang Y. Gut microbiota composition modifies fecal metabolic profiles in mice. J Proteome Res. 2013;12(6):2987-99. Epub 2013/05/02. doi: 10.1021/pr400263n. PubMed PMID: 23631562.

304. Ferreira DM, Afonso MB, Rodrigues PM, Simao AL, Pereira DM, Borralho PM, Rodrigues CM, Castro RE. c-Jun N-terminal kinase 1/c-Jun activation of the p53/microRNA 34a/sirtuin 1 pathway contributes to apoptosis induced by deoxycholic acid in rat liver. Molecular and cellular biology. 2014;34(6):1100-20. Epub 2014/01/15. doi: 10.1128/MCB.00420-13. PubMed PMID: 24421392; PMCID: PMC3958031.

305. Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, Masciana R, Forgione A, Gabrieli ML, Perotti G, Vecchio FM, Rapaccini G, Gasbarrini G, Day CP, Grieco A. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. Hepatology. 2009;49(6):1877-87. doi: 10.1002/hep.22848. PubMed PMID: 19291785.

306. Brun P, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palu G, Martines D. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. American journal of physiology Gastrointestinal and liver physiology. 2007;292(2):G518-25. Epub 2006/10/07. doi: 10.1152/ajpgi.00024.2006. PubMed PMID: 17023554.

307. Soares JB, Pimentel-Nunes P, Roncon-Albuquerque R, Leite-Moreira A. The role of lipopolysaccharide/toll-like receptor 4 signaling in chronic liver diseases. Hepatol Int. 2010;4(4):659-72. Epub 2011/02/03. doi: 10.1007/s12072-010-9219-x. PubMed PMID: 21286336; PMCID: PMC2994611.

308. Elshaghabee FM, Bockelmann W, Meske D, de Vrese M, Walte HG, Schrezenmeir J, Heller KJ. Ethanol Production by Selected Intestinal Microorganisms and Lactic Acid Bacteria Growing under Different Nutritional Conditions. Frontiers in microbiology. 2016;7:47. Epub 2016/02/10. doi: 10.3389/fmicb.2016.00047. PubMed PMID: 26858714; PMCID: PMC4732544.

309. Purohit V, Bode JC, Bode C, Brenner DA, Choudhry MA, Hamilton F, Kang YJ, Keshavarzian A, Rao R, Sartor RB, Swanson C, Turner JR. Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and medical consequences: summary of a symposium. Alcohol. 2008;42(5):349-61. Epub 2008/05/28. doi: 10.1016/j.alcohol.2008.03.131. PubMed PMID: 18504085; PMCID: PMC2614138.

310. Chawla A, Nguyen KD, Goh YP. Macrophage-mediated inflammation in metabolic disease. Nature reviews Immunology. 2011;11(11):738-49. Epub 2011/10/11. doi: 10.1038/nri3071. PubMed PMID: 21984069; PMCID: PMC3383854.

311. Jiang W, Wu N, Wang X, Chi Y, Zhang Y, Qiu X, Hu Y, Li J, Liu Y. Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of

humans with non-alcoholic fatty liver disease. Scientific reports. 2015;5:8096. doi: 10.1038/srep08096. PubMed PMID: 25644696; PMCID: 4314632.

312. Briskey D, Heritage M, Jaskowski LA, Peake J, Gobe G, Subramaniam VN, Crawford D, Campbell C, Vitetta L. Probiotics modify tight-junction proteins in an animal model of nonalcoholic fatty liver disease. Therap Adv Gastroenterol. 2016;9(4):463-72. Epub 2016/07/02. doi: 10.1177/1756283X16645055. PubMed PMID: 27366215; PMCID: PMC4913342.

313. Alisi A, Carpino G, Oliveira FL, Panera N, Nobili V, Gaudio E. The Role of Tissue Macrophage-Mediated Inflammation on NAFLD Pathogenesis and Its Clinical Implications. Mediators of inflammation. 2017;2017:8162421. Epub 2017/01/25. doi: 10.1155/2017/8162421. PubMed PMID: 28115795; PMCID: PMC5237469.

314. Luther J, Garber JJ, Khalili H, Dave M, Bale SS, Jindal R, Motola DL, Luther S, Bohr S, Jeoung SW, Deshpande V, Singh G, Turner JR, Yarmush ML, Chung RT, Patel SJ. Hepatic Injury in Nonalcoholic Steatohepatitis Contributes to Altered Intestinal Permeability. Cell Mol Gastroenterol Hepatol. 2015;1(2):222-32. Epub 2015/09/26. doi: 10.1016/j.jcmgh.2015.01.001. PubMed PMID: 26405687; PMCID: PMC4578658.

315. Chen P, Starkel P, Turner JR, Ho SB, Schnabl B. Dysbiosis-induced intestinal inflammation activates tumor necrosis factor receptor I and mediates alcoholic liver disease in mice. Hepatology. 2015;61(3):883-94. Epub 2014/09/25. doi: 10.1002/hep.27489. PubMed PMID: 25251280; PMCID: PMC4340725.

316. Luck H, Tsai S, Chung J, Clemente-Casares X, Ghazarian M, Revelo XS, Lei H, Luk CT, Shi SY, Surendra A, Copeland JK, Ahn J, Prescott D, Rasmussen BA, Chng MH, Engleman EG, Girardin SE, Lam TK, Croitoru K, Dunn S, Philpott DJ, Guttman DS, Woo M, Winer S, Winer DA. Regulation of obesity-related insulin resistance with gut anti-inflammatory agents. Cell

metabolism. 2015;21(4):527-42. Epub 2015/04/12. doi: 10.1016/j.cmet.2015.03.001. PubMed PMID: 25863246.

317. Postler TS, Ghosh S. Understanding the Holobiont: How Microbial Metabolites Affect Human Health and Shape the Immune System. Cell metabolism. 2017;26(1):110-30. Epub 2017/06/20. doi: 10.1016/j.cmet.2017.05.008. PubMed PMID: 28625867; PMCID: PMC5535818.

318. Baker SS, Baker RD, Liu W, Nowak NJ, Zhu L. Role of alcohol metabolism in non-alcoholic steatohepatitis. PloS one. 2010;5(3):e9570. Epub 2010/03/12. doi: 10.1371/journal.pone.0009570. PubMed PMID: 20221393; PMCID: PMC2833196.

319. Cope K, Risby T, Diehl AM. Increased gastrointestinal ethanol production in obese mice: implications for fatty liver disease pathogenesis. Gastroenterology. 2000;119(5):1340-7. Epub 2000/10/31. PubMed PMID: 11054393.

320. Small CL, Reid-Yu SA, McPhee JB, Coombes BK. Persistent infection with Crohn's disease-associated adherent-invasive Escherichia coli leads to chronic inflammation and intestinal fibrosis. Nature communications. 2013;4:1957. Epub 2013/06/12. doi: 10.1038/ncomms2957. PubMed PMID: 23748852; PMCID: PMC3938456.

321. Aron-Wisnewsky J, Gaborit B, Dutour A, Clement K. Gut microbiota and non-alcoholic fatty liver disease: new insights. Clin Microbiol Infect. 2013;19(4):338-48. Epub 2013/03/05. doi: 10.1111/1469-0691.12140. PubMed PMID: 23452163.

322. Cummings JH, Macfarlane GT. Role of intestinal bacteria in nutrient metabolism. JPEN Journal of parenteral and enteral nutrition. 1997;21(6):357-65. Epub 1997/12/24. doi: 10.1177/0148607197021006357. PubMed PMID: 9406136.

323. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut microbes. 2016;7(3):189-200. doi: 10.1080/19490976.2015.1134082. PubMed PMID: 26963409; PMCID: 4939913.

324. Clausen MR, Mortensen PB. Kinetic studies on colonocyte metabolism of short chain fatty acids and glucose in ulcerative colitis. Gut. 1995;37(5):684-9. Epub 1995/11/01. PubMed PMID: 8549946; PMCID: PMC1382875.

325. Donohoe DR, Collins LB, Wali A, Bigler R, Sun W, Bultman SJ. The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. Mol Cell. 2012;48(4):612-26. Epub 2012/10/16. doi: 10.1016/j.molcel.2012.08.033. PubMed PMID: 23063526; PMCID: PMC3513569.

326. Bloemen JG, Venema K, van de Poll MC, Olde Damink SW, Buurman WA, Dejong CH. Short chain fatty acids exchange across the gut and liver in humans measured at surgery. Clin Nutr. 2009;28(6):657-61. Epub 2009/06/16. doi: 10.1016/j.clnu.2009.05.011. PubMed PMID: 19523724.

327. van der Beek CM, Bloemen JG, van den Broek MA, Lenaerts K, Venema K, Buurman WA, Dejong CH. Hepatic Uptake of Rectally Administered Butyrate Prevents an Increase in Systemic Butyrate Concentrations in Humans. J Nutr. 2015;145(9):2019-24. Epub 2015/07/15. doi: 10.3945/jn.115.211193. PubMed PMID: 26156796.

328. Ferreira CM, Vieira AT, Vinolo MA, Oliveira FA, Curi R, Martins Fdos S. The central role of the gut microbiota in chronic inflammatory diseases. Journal of immunology research. 2014;2014:689492. Epub 2014/10/14. doi: 10.1155/2014/689492. PubMed PMID: 25309932; PMCID: PMC4189530.

329. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446-50. Epub 2013/11/15. doi: 10.1038/nature12721. PubMed PMID: 24226770.

330. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature. 2009;461(7268):1282-6. Epub 2009/10/30. doi: 10.1038/nature08530. PubMed PMID: 19865172; PMCID: PMC3256734.

331. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013;341(6145):569-73. Epub 2013/07/06. doi: 10.1126/science.1241165. PubMed PMID: 23828891; PMCID: PMC3807819.

332. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(34):13780-5. Epub 2007/08/19. doi: 10.1073/pnas.0706625104. PubMed PMID: 17699621; PMCID: PMC1959459.

333. Kanauchi O, Suga T, Tochihara M, Hibi T, Naganuma M, Homma T, Asakura H, Nakano H, Takahama K, Fujiyama Y, Andoh A, Shimoyama T, Hida N, Haruma K, Koga H, Mitsuyama K, Sata M, Fukuda M, Kojima A, Bamba T. Treatment of ulcerative colitis by feeding with

germinated barley foodstuff: first report of a multicenter open control trial. Journal of gastroenterology. 2002;37 Suppl 14:67-72. Epub 2003/02/08. PubMed PMID: 12572869.

334. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, Ballet V, Claes K, Van Immerseel F, Verbeke K, Ferrante M, Verhaegen J, Rutgeerts P, Vermeire S. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut. 2014;63(8):1275-83. Epub 2013/09/12. doi: 10.1136/gutjnl-2013-304833. PubMed PMID: 24021287.

335. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, Cosnes J, Corthier G, Marteau P, Dore J. Low counts of Faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis. 2009;15(8):1183-9. Epub 2009/02/25. doi: 10.1002/ibd.20903. PubMed PMID: 19235886.

336. Kalina U, Koyama N, Hosoda T, Nuernberger H, Sato K, Hoelzer D, Herweck F, Manigold T, Singer MV, Rossol S, Bocker U. Enhanced production of IL-18 in butyrate-treated intestinal epithelium by stimulation of the proximal promoter region. Eur J Immunol. 2002;32(9):2635-43. Epub 2002/09/11. doi: 10.1002/1521-4141(200209)32:9<2635::AID-IMMU2635>3.0.CO;2-N. PubMed PMID: 12207348.

337. Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, Maruya M, Ian McKenzie C, Hijikata A, Wong C, Binge L, Thorburn AN, Chevalier N, Ang C, Marino E, Robert R, Offermanns S, Teixeira MM, Moore RJ, Flavell RA, Fagarasan S, Mackay CR. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nature communications. 2015;6:6734. Epub 2015/04/02. doi: 10.1038/ncomms7734. PubMed PMID: 25828455.

338. Singh N, Thangaraju M, Prasad PD, Martin PM, Lambert NA, Boettger T, Offermanns S, Ganapathy V. Blockade of dendritic cell development by bacterial fermentation products butyrate and propionate through a transporter (Slc5a8)-dependent inhibition of histone deacetylases. The Journal of biological chemistry. 2010;285(36):27601-8. Epub 2010/07/06. doi: 10.1074/jbc.M110.102947. PubMed PMID: 20601425; PMCID: PMC2934627.

339. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL, Suzuki T, Taylor TD, Itoh K, Kikuchi J, Morita H, Hattori M, Ohno H. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 2011;469(7331):543-7. Epub 2011/01/29. doi: 10.1038/nature09646. PubMed PMID: 21270894.

340. Wrzosek L, Miquel S, Noordine ML, Bouet S, Joncquel Chevalier-Curt M, Robert V, Philippe C, Bridonneau C, Cherbuy C, Robbe-Masselot C, Langella P, Thomas M. Bacteroides thetaiotaomicron and Faecalibacterium prausnitzii influence the production of mucus glycans and the development of goblet cells in the colonic epithelium of a gnotobiotic model rodent. BMC Biol. 2013;11:61. Epub 2013/05/23. doi: 10.1186/1741-7007-11-61. PubMed PMID: 23692866; PMCID: PMC3673873.

341. Vinolo MA, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. J Nutr Biochem. 2011;22(9):849-55. Epub 2010/12/21. doi: 10.1016/j.jnutbio.2010.07.009. PubMed PMID: 21167700.

342. Chang PV, Hao L, Offermanns S, Medzhitov R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proceedings of the

National Academy of Sciences of the United States of America. 2014;111(6):2247-52. Epub 2014/01/07. doi: 10.1073/pnas.1322269111. PubMed PMID: 24390544; PMCID: PMC3926023.

343. Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR, Black JC, Hoffmann A, Carey M, Smale ST. A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell. 2009;138(1):114-28. Epub 2009/07/15. doi: 10.1016/j.cell.2009.04.020. PubMed PMID: 19596239; PMCID: PMC2712736.

344. Kim M, Qie Y, Park J, Kim CH. Gut Microbial Metabolites Fuel Host Antibody Responses. Cell host & microbe. 2016;20(2):202-14. Epub 2016/08/02. doi: 10.1016/j.chom.2016.07.001. PubMed PMID: 27476413; PMCID: PMC4982788.

345. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 2013;504(7480):451-5. Epub 2013/11/15. doi: 10.1038/nature12726. PubMed PMID: 24226773; PMCID: PMC3869884.

346. Singh N, Gurav A, Sivaprakasam S, Brady E, Padia R, Shi H, Thangaraju M, Prasad PD, Manicassamy S, Munn DH, Lee JR, Offermanns S, Ganapathy V. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity. 2014;40(1):128-39. Epub 2014/01/15. doi: 10.1016/j.immuni.2013.12.007. PubMed PMID: 24412617; PMCID: PMC4305274.

347. Opitz CA, Litzenburger UM, Sahm F, Ott M, Tritschler I, Trump S, Schumacher T, Jestaedt L, Schrenk D, Weller M, Jugold M, Guillemin GJ, Miller CL, Lutz C, Radlwimmer B, Lehmann I, von Deimling A, Wick W, Platten M. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature. 2011;478(7368):197-203. Epub 2011/10/07. doi: 10.1038/nature10491. PubMed PMID: 21976023.

348. Alexeev EE, Lanis JM, Kao DJ, Campbell EL, Kelly CJ, Battista KD, Gerich ME, Jenkins BR, Walk ST, Kominsky DJ, Colgan SP. Microbiota-Derived Indole Metabolites Promote Human and Murine Intestinal Homeostasis through Regulation of Interleukin-10 Receptor. The American journal of pathology. 2018;188(5):1183-94. Epub 2018/02/20. doi: 10.1016/j.ajpath.2018.01.011. PubMed PMID: 29454749; PMCID: PMC5906738.

349. Lamas B, Richard ML, Leducq V, Pham HP, Michel ML, Da Costa G, Bridonneau C, Jegou S, Hoffmann TW, Natividad JM, Brot L, Taleb S, Couturier-Maillard A, Nion-Larmurier I, Merabtene F, Seksik P, Bourrier A, Cosnes J, Ryffel B, Beaugerie L, Launay JM, Langella P, Xavier RJ, Sokol H. CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. Nature medicine. 2016;22(6):598-605. Epub 2016/05/10. doi: 10.1038/nm.4102. PubMed PMID: 27158904; PMCID: PMC5087285.

350. Martinez-Augustin O, Sanchez de Medina F. Intestinal bile acid physiology and pathophysiology. World J Gastroenterol. 2008;14(37):5630-40. Epub 2008/10/07. PubMed PMID: 18837078; PMCID: PMC2748196.

351. Ridlon JM, Kang DJ, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. Journal of lipid research. 2006;47(2):241-59. Epub 2005/11/22. doi: 10.1194/jlr.R500013-JLR200. PubMed PMID: 16299351.

352. Levy M, Thaiss CA, Zeevi D, Dohnalova L, Zilberman-Schapira G, Mahdi JA, David E, Savidor A, Korem T, Herzig Y, Pevsner-Fischer M, Shapiro H, Christ A, Harmelin A, Halpern Z, Latz E, Flavell RA, Amit I, Segal E, Elinav E. Microbiota-Modulated Metabolites Shape the Intestinal Microenvironment by Regulating NLRP6 Inflammasome Signaling. Cell. 2015;163(6):1428-43. Epub 2015/12/08. doi: 10.1016/j.cell.2015.10.048. PubMed PMID: 26638072; PMCID: PMC5665753.

353. Duboc H, Rajca S, Rainteau D, Benarous D, Maubert MA, Quervain E, Thomas G, Barbu V, Humbert L, Despras G, Bridonneau C, Dumetz F, Grill JP, Masliah J, Beaugerie L, Cosnes J, Chazouilleres O, Poupon R, Wolf C, Mallet JM, Langella P, Trugnan G, Sokol H, Seksik P. Connecting dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel diseases. Gut. 2013;62(4):531-9. doi: 10.1136/gutjnl-2012-302578. PubMed PMID: 22993202.

354. Sayin SI, Wahlstrom A, Felin J, Jantti S, Marschall HU, Bamberg K, Angelin B, Hyotylainen T, Oresic M, Backhed F. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. Cell metabolism. 2013;17(2):225-35. Epub 2013/02/12. doi: 10.1016/j.cmet.2013.01.003. PubMed PMID: 23395169.

355. Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, Nicholson JK, Holmes E. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. Proceedings of the National Academy of Sciences of the United States of America. 2011;108 Suppl 1:4523-30. Epub 2010/09/15. doi: 10.1073/pnas.1006734107. PubMed PMID: 20837534; PMCID: PMC3063584.

356. Brestoff JR, Artis D. Commensal bacteria at the interface of host metabolism and the immune system. Nature immunology. 2013;14(7):676-84. Epub 2013/06/20. doi: 10.1038/ni.2640. PubMed PMID: 23778795; PMCID: PMC4013146.

357. Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M. A G protein-coupled receptor responsive to bile acids. The Journal of biological chemistry. 2003;278(11):9435-40. Epub 2003/01/14. doi: 10.1074/jbc.M209706200. PubMed PMID: 12524422.

358. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. Science. 1999;284(5418):1362-5. Epub 1999/05/21. PubMed PMID: 10334992.

359. Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Nakamura T, Itadani H, Tanaka K. Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun. 2002;298(5):714-9. Epub 2002/11/07. PubMed PMID: 12419312.

360. Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG, Glass CK. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. Nature. 2005;437(7059):759-63. Epub 2005/08/30. doi: 10.1038/nature03988. PubMed PMID: 16127449; PMCID: PMC1464798.

361. Vavassori P, Mencarelli A, Renga B, Distrutti E, Fiorucci S. The bile acid receptor FXR is a modulator of intestinal innate immunity. Journal of immunology. 2009;183(10):6251-61. Epub 2009/10/30. doi: 10.4049/jimmunol.0803978. PubMed PMID: 19864602.

362. Gadaleta RM, van Erpecum KJ, Oldenburg B, Willemsen EC, Renooij W, Murzilli S, Klomp LW, Siersema PD, Schipper ME, Danese S, Penna G, Laverny G, Adorini L, Moschetta A, van Mil SW. Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. Gut. 2011;60(4):463-72. Epub 2011/01/19. doi: 10.1136/gut.2010.212159. PubMed PMID: 21242261.

363. Kim I, Morimura K, Shah Y, Yang Q, Ward JM, Gonzalez FJ. Spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice. Carcinogenesis. 2007;28(5):940-6. Epub 2006/12/22. doi: 10.1093/carcin/bgl249. PubMed PMID: 17183066; PMCID: PMC1858639.

364. Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. Cancer Res.

2007;67(3):863-7. Epub 2007/02/07. doi: 10.1158/0008-5472.CAN-06-1078. PubMed PMID: 17283114.

365. Wang YD, Chen WD, Wang M, Yu D, Forman BM, Huang W. Farnesoid X receptor antagonizes nuclear factor kappaB in hepatic inflammatory response. Hepatology. 2008;48(5):1632-43. Epub 2008/10/31. doi: 10.1002/hep.22519. PubMed PMID: 18972444; PMCID: PMC3056574.

366. Mencarelli A, Renga B, Migliorati M, Cipriani S, Distrutti E, Santucci L, Fiorucci S. The bile acid sensor farnesoid X receptor is a modulator of liver immunity in a rodent model of acute hepatitis. Journal of immunology. 2009;183(10):6657-66. Epub 2009/11/03. doi: 10.4049/jimmunol.0901347. PubMed PMID: 19880446.

367. Guo C, Qi H, Yu Y, Zhang Q, Su J, Yu D, Huang W, Chen WD, Wang YD. The G-Protein-Coupled Bile Acid Receptor Gpbar1 (TGR5) Inhibits Gastric Inflammation Through Antagonizing NF-kappaB Signaling Pathway. Frontiers in pharmacology. 2015;6:287. Epub 2015/12/24. doi: 10.3389/fphar.2015.00287. PubMed PMID: 26696888; PMCID: PMC4675858.

368. Pols TW, Nomura M, Harach T, Lo Sasso G, Oosterveer MH, Thomas C, Rizzo G, Gioiello A, Adorini L, Pellicciari R, Auwerx J, Schoonjans K. TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading. Cell metabolism. 2011;14(6):747-57. Epub 2011/12/14. doi: 10.1016/j.cmet.2011.11.006. PubMed PMID: 22152303; PMCID: PMC3627293.

369. Wang YD, Chen WD, Yu D, Forman BM, Huang W. The G-protein-coupled bile acid receptor, Gpbar1 (TGR5), negatively regulates hepatic inflammatory response through antagonizing nuclear factor kappa light-chain enhancer of activated B cells (NF-kappaB) in

mice. Hepatology. 2011;54(4):1421-32. Epub 2011/07/08. doi: 10.1002/hep.24525. PubMed PMID: 21735468; PMCID: PMC3184183.

370. Crozet P, Margalha L, Confraria A, Rodrigues A, Martinho C, Adamo M, Elias CA, Baena-Gonzalez E. Mechanisms of regulation of SNF1/AMPK/SnRK1 protein kinases. Front Plant Sci. 2014;5:190. Epub 2014/06/07. doi: 10.3389/fpls.2014.00190. PubMed PMID: 24904600; PMCID: PMC4033248.

371. Hardie DG, Salt IP, Hawley SA, Davies SP. AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. The Biochemical journal. 1999;338 (Pt 3):717-22. PubMed PMID: 10051444; PMCID: 1220108.

372. Herzig S, Shaw RJ. AMPK: guardian of metabolism and mitochondrial homeostasis. Nature reviews Molecular cell biology. 2018;19(2):121-35. Epub 2017/10/05. doi: 10.1038/nrm.2017.95. PubMed PMID: 28974774; PMCID: PMC5780224.

373. Jager S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(29):12017-22. Epub 2007/07/05. doi: 10.1073/pnas.0705070104. PubMed PMID: 17609368; PMCID: PMC1924552.

374. Yang W, Hong YH, Shen XQ, Frankowski C, Camp HS, Leff T. Regulation of transcription by AMP-activated protein kinase: phosphorylation of p300 blocks its interaction with nuclear receptors. The Journal of biological chemistry. 2001;276(42):38341-4. Epub 2001/08/24. doi: 10.1074/jbc.C100316200. PubMed PMID: 11518699.

375. Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, House CM, Fernandez CS, Cox T, Witters LA, Kemp BE. Mammalian AMP-activated protein kinase subfamily. The Journal of biological chemistry. 1996;271(2):611-4. Epub 1996/01/12. PubMed PMID: 8557660.

376. Thornton C, Snowden MA, Carling D. Identification of a novel AMP-activated protein kinase beta subunit isoform that is highly expressed in skeletal muscle. The Journal of biological chemistry. 1998;273(20):12443-50. Epub 1998/06/20. PubMed PMID: 9575201.

377. Cheung PC, Salt IP, Davies SP, Hardie DG, Carling D. Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. The Biochemical journal. 2000;346 Pt 3:659-69. Epub 2000/03/04. PubMed PMID: 10698692; PMCID: PMC1220898.

378. Ross FA, MacKintosh C, Hardie DG. AMP-activated protein kinase: a cellular energy sensor that comes in 12 flavours. FEBS J. 2016;283(16):2987-3001. Epub 2016/03/05. doi: 10.1111/febs.13698. PubMed PMID: 26934201; PMCID: PMC4995730.

379. Birk JB, Wojtaszewski JF. Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. J Physiol. 2006;577(Pt 3):1021-32. Epub 2006/10/14. doi: 10.1113/jphysiol.2006.120972. PubMed PMID: 17038425; PMCID: PMC1890393.

380. Crute BE, Seefeld K, Gamble J, Kemp BE, Witters LA. Functional domains of the alpha1 catalytic subunit of the AMP-activated protein kinase. The Journal of biological chemistry. 1998;273(52):35347-54. PubMed PMID: 9857077.

381. Hawley SA, Fullerton MD, Ross FA, Schertzer JD, Chevtzoff C, Walker KJ, Peggie MW, Zibrova D, Green KA, Mustard KJ, Kemp BE, Sakamoto K, Steinberg GR, Hardie DG. The ancient drug salicylate directly activates AMP-activated protein kinase. Science. 2012;336(6083):918-22. doi: 10.1126/science.1215327. PubMed PMID: 22517326; PMCID: 3399766.

382. Sanders MJ, Ali ZS, Hegarty BD, Heath R, Snowden MA, Carling D. Defining the mechanism of activation of AMP-activated protein kinase by the small molecule A-769662, a

member of the thienopyridone family. The Journal of biological chemistry. 2007;282(45):32539-48. doi: 10.1074/jbc.M706543200. PubMed PMID: 17728241.

383. Scott JW, van Denderen BJ, Jorgensen SB, Honeyman JE, Steinberg GR, Oakhill JS, Iseli TJ, Koay A, Gooley PR, Stapleton D, Kemp BE. Thienopyridone drugs are selective activators of AMP-activated protein kinase beta1-containing complexes. Chemistry & biology. 2008;15(11):1220-30. doi: 10.1016/j.chembiol.2008.10.005. PubMed PMID: 19022182.

384. Gowans GJ, Hawley SA, Ross FA, Hardie DG. AMP is a true physiological regulator of AMP-activated protein kinase by both allosteric activation and enhancing net phosphorylation. Cell metabolism. 2013;18(4):556-66. doi: 10.1016/j.cmet.2013.08.019. PubMed PMID: 24093679; PMCID: 3791399.

385. Steinberg GR, Kemp BE. AMPK in Health and Disease. Physiological reviews. 2009;89(3):1025-78. Epub 2009/07/09. doi: 10.1152/physrev.00011.2008. PubMed PMID: 19584320.

386. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, Alessi DR, Hardie DG. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. Journal of biology. 2003;2(4):28. doi: 10.1186/1475-4924-2-28. PubMed PMID: 14511394; PMCID: 333410.

387. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Current biology : CB. 2003;13(22):2004-8. PubMed PMID: 14614828.

388. Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D. Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-

activated protein kinase in mammalian cells. Cell metabolism. 2005;2(1):21-33. doi: 10.1016/j.cmet.2005.06.005. PubMed PMID: 16054096.

389. Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell metabolism. 2005;2(1):9-19. doi: 10.1016/j.cmet.2005.05.009. PubMed PMID: 16054095.

390. Xie M, Zhang D, Dyck JR, Li Y, Zhang H, Morishima M, Mann DL, Taffet GE, Baldini A, Khoury DS, Schneider MD. A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(46):17378-83. doi: 10.1073/pnas.0604708103. PubMed PMID: 17085580; PMCID: 1859937.

391. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(10):3329-35. Epub 2004/02/27. doi: 10.1073/pnas.0308061100. PubMed PMID: 14985505; PMCID: PMC373461.

392. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, Montminy M, Cantley LC. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science. 2005;310(5754):1642-6. doi: 10.1126/science.1120781. PubMed PMID: 16308421; PMCID: 3074427.

393. Hurley RL, Anderson KA, Franzone JM, Kemp BE, Means AR, Witters LA. The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases.

The Journal of biological chemistry. 2005;280(32):29060-6. Epub 2005/06/28. doi: 10.1074/jbc.M503824200. PubMed PMID: 15980064.

394. Momcilovic M, Hong SP, Carlson M. Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. The Journal of biological chemistry. 2006;281(35):25336-43. Epub 2006/07/13. doi: 10.1074/jbc.M604399200. PubMed PMID: 16835226.

395. O'Neill LA, Hardie DG. Metabolism of inflammation limited by AMPK and pseudo-starvation. Nature. 2013;493(7432):346-55. Epub 2013/01/18. doi: 10.1038/nature11862. PubMed PMID: 23325217.

396. Carling D, Zammit VA, Hardie DG. A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS letters. 1987;223(2):217-22. Epub 1987/11/02. PubMed PMID: 2889619.

397. Munday MR, Campbell DG, Carling D, Hardie DG. Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase. Eur J Biochem. 1988;175(2):331-8. Epub 1988/08/01. PubMed PMID: 2900138.

398. Woods A, Williams JR, Muckett PJ, Mayer FV, Liljevald M, Bohlooly YM, Carling D. Liver-Specific Activation of AMPK Prevents Steatosis on a High-Fructose Diet. Cell reports. 2017;18(13):3043-51. Epub 2017/03/30. doi: 10.1016/j.celrep.2017.03.011. PubMed PMID: 28355557; PMCID: PMC5382239.

399. Li Y, Xu S, Mihaylova MM, Zheng B, Hou X, Jiang B, Park O, Luo Z, Lefai E, Shyy JY, Gao B, Wierzbicki M, Verbeuren TJ, Shaw RJ, Cohen RA, Zang M. AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-

resistant mice. Cell metabolism. 2011;13(4):376-88. Epub 2011/04/05. doi: 10.1016/j.cmet.2011.03.009. PubMed PMID: 21459323; PMCID: PMC3086578.

400. Kawaguchi T, Osatomi K, Yamashita H, Kabashima T, Uyeda K. Mechanism for fatty acid "sparing" effect on glucose-induced transcription: regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. The Journal of biological chemistry. 2002;277(6):3829-35. Epub 2001/11/29. doi: 10.1074/jbc.M107895200. PubMed PMID: 11724780.

401. Hong YH, Varanasi US, Yang W, Leff T. AMP-activated protein kinase regulates HNF4alpha transcriptional activity by inhibiting dimer formation and decreasing protein stability. The Journal of biological chemistry. 2003;278(30):27495-501. Epub 2003/05/13. doi: 10.1074/jbc.M304112200. PubMed PMID: 12740371.

402. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell. 2008;30(2):214-26. Epub 2008/04/29. doi: 10.1016/j.molcel.2008.03.003. PubMed PMID: 18439900; PMCID: PMC2674027.

403. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003;115(5):577-90. Epub 2003/12/04. PubMed PMID: 14651849.

404. Chavez JA, Roach WG, Keller SR, Lane WS, Lienhard GE. Inhibition of GLUT4 translocation by Tbc1d1, a Rab GTPase-activating protein abundant in skeletal muscle, is partially relieved by AMP-activated protein kinase activation. The Journal of biological chemistry. 2008;283(14):9187-95. Epub 2008/02/09. doi: 10.1074/jbc.M708934200. PubMed PMID: 18258599; PMCID: PMC2431020.

405. Kim JH, Park JM, Yea K, Kim HW, Suh PG, Ryu SH. Phospholipase D1 mediates AMP-activated protein kinase signaling for glucose uptake. PloS one. 2010;5(3):e9600. Epub 2010/03/17. doi: 10.1371/journal.pone.0009600. PubMed PMID: 20231899; PMCID: PMC2834755.

406. Ahmadian M, Abbott MJ, Tang T, Hudak CS, Kim Y, Bruss M, Hellerstein MK, Lee HY, Samuel VT, Shulman GI, Wang Y, Duncan RE, Kang C, Sul HS. Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. Cell metabolism. 2011;13(6):739-48. Epub 2011/06/07. doi: 10.1016/j.cmet.2011.05.002. PubMed PMID: 21641555; PMCID: PMC3148136.

407. McGarry JD, Leatherman GF, Foster DW. Carnitine palmitoyltransferase I. The site of inhibition of hepatic fatty acid oxidation by malonyl-CoA. The Journal of biological chemistry. 1978;253(12):4128-36. Epub 1978/06/25. PubMed PMID: 659409.

408. Saggerson D. Malonyl-CoA, a key signaling molecule in mammalian cells. Annu Rev Nutr. 2008;28:253-72. Epub 2008/07/05. doi: 10.1146/annurev.nutr.28.061807.155434. PubMed PMID: 18598135.

409. Lindholm CR, Ertel RL, Bauwens JD, Schmuck EG, Mulligan JD, Saupe KW. A high-fat diet decreases AMPK activity in multiple tissues in the absence of hyperglycemia or systemic inflammation in rats. J Physiol Biochem. 2013;69(2):165-75. Epub 2012/09/04. doi: 10.1007/s13105-012-0199-2. PubMed PMID: 22941749; PMCID: PMC3644018.

410. Yang Z, Kahn BB, Shi H, Xue BZ. Macrophage alpha1 AMP-activated protein kinase (alpha1AMPK) antagonizes fatty acid-induced inflammation through SIRT1. The Journal of biological chemistry. 2010;285(25):19051-9. Epub 2010/04/28. doi: 10.1074/jbc.M110.123620. PubMed PMID: 20421294; PMCID: PMC2885183.

411. Mottillo EP, Desjardins EM, Crane JD, Smith BK, Green AE, Ducommun S, Henriksen TI, Rebalka IA, Razi A, Sakamoto K, Scheele C, Kemp BE, Hawke TJ, Ortega J, Granneman JG, Steinberg GR. Lack of Adipocyte AMPK Exacerbates Insulin Resistance and Hepatic Steatosis through Brown and Beige Adipose Tissue Function. Cell metabolism. 2016;24(1):118-29. Epub 2016/07/15. doi: 10.1016/j.cmet.2016.06.006. PubMed PMID: 27411013; PMCID: PMC5239668.

412. Steinberg GR, Michell BJ, van Denderen BJ, Watt MJ, Carey AL, Fam BC, Andrikopoulos S, Proietto J, Gorgun CZ, Carling D, Hotamisligil GS, Febbraio MA, Kay TW, Kemp BE. Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. Cell metabolism. 2006;4(6):465-74. Epub 2006/12/05. doi: 10.1016/j.cmet.2006.11.005. PubMed PMID: 17141630.

413. Woo SL, Xu H, Li H, Zhao Y, Hu X, Zhao J, Guo X, Guo T, Botchlett R, Qi T, Pei Y, Zheng J, Xu Y, An X, Chen L, Chen L, Li Q, Xiao X, Huo Y, Wu C. Metformin ameliorates hepatic steatosis and inflammation without altering adipose phenotype in diet-induced obesity. PloS one. 2014;9(3):e91111. Epub 2014/03/19. doi: 10.1371/journal.pone.0091111. PubMed PMID: 24638078; PMCID: PMC3956460.

414. Gauthier MS, O'Brien EL, Bigornia S, Mott M, Cacicedo JM, Xu XJ, Gokce N, Apovian C, Ruderman N. Decreased AMP-activated protein kinase activity is associated with increased inflammation in visceral adipose tissue and with whole-body insulin resistance in morbidly obese humans. Biochem Biophys Res Commun. 2011;404(1):382-7. Epub 2010/12/07. doi: 10.1016/j.bbrc.2010.11.127. PubMed PMID: 21130749; PMCID: PMC3061625.

415. Fritzen AM, Lundsgaard AM, Jordy AB, Poulsen SK, Stender S, Pilegaard H, Astrup A, Larsen TM, Wojtaszewski JF, Richter EA, Kiens B. New Nordic Diet-Induced Weight Loss Is

Accompanied by Changes in Metabolism and AMPK Signaling in Adipose Tissue. J Clin Endocrinol Metab. 2015;100(9):3509-19. Epub 2015/07/01. doi: 10.1210/jc.2015-2079. PubMed PMID: 26126206.

416. Ko HJ, Zhang Z, Jung DY, Jun JY, Ma Z, Jones KE, Chan SY, Kim JK. Nutrient stress activates inflammation and reduces glucose metabolism by suppressing AMP-activated protein kinase in the heart. Diabetes. 2009;58(11):2536-46. Epub 2009/08/20. doi: 10.2337/db08-1361. PubMed PMID: 19690060; PMCID: PMC2768176.

417. Hurley RL, Barre LK, Wood SD, Anderson KA, Kemp BE, Means AR, Witters LA. Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular cAMP. The Journal of biological chemistry. 2006;281(48):36662-72. Epub 2006/10/07. doi: 10.1074/jbc.M606676200. PubMed PMID: 17023420.

418. Kim B, Figueroa-Romero C, Pacut C, Backus C, Feldman EL. Insulin Resistance Prevents AMPK-induced Tau Dephosphorylation through Akt-mediated Increase in AMPKSer-485 Phosphorylation. The Journal of biological chemistry. 2015;290(31):19146-57. Epub 2015/06/24. doi: 10.1074/jbc.M115.636852. PubMed PMID: 26100639; PMCID: PMC4521037.

419. Hawley SA, Ross FA, Gowans GJ, Tibarewal P, Leslie NR, Hardie DG. Phosphorylation by Akt within the ST loop of AMPK-alpha1 down-regulates its activation in tumour cells. The Biochemical journal. 2014;459(2):275-87. Epub 2014/01/29. doi: 10.1042/BJ20131344. PubMed PMID: 24467442; PMCID: PMC4052680.

420. Ning J, Xi G, Clemmons DR. Suppression of AMPK activation via S485 phosphorylation by IGF-I during hyperglycemia is mediated by AKT activation in vascular smooth muscle cells. Endocrinology. 2011;152(8):3143-54. Epub 2011/06/16. doi: 10.1210/en.2011-0155. PubMed PMID: 21673100; PMCID: PMC3138225.

421. Horman S, Vertommen D, Heath R, Neumann D, Mouton V, Woods A, Schlattner U, Wallimann T, Carling D, Hue L, Rider MH. Insulin antagonizes ischemia-induced Thr172 phosphorylation of AMP-activated protein kinase alpha-subunits in heart via hierarchical phosphorylation of Ser485/491. The Journal of biological chemistry. 2006;281(9):5335-40. Epub 2005/12/13. doi: 10.1074/jbc.M506850200. PubMed PMID: 16340011.

422. Dagon Y, Hur E, Zheng B, Wellenstein K, Cantley LC, Kahn BB. p70S6 kinase phosphorylates AMPK on serine 491 to mediate leptin's effect on food intake. Cell metabolism. 2012;16(1):104-12. Epub 2012/06/26. doi: 10.1016/j.cmet.2012.05.010. PubMed PMID: 22727014; PMCID: PMC3407689.

423. Cao Q, Cui X, Wu R, Zha L, Wang X, Parks JS, Yu L, Shi H, Xue B. Myeloid Deletion of alpha1AMPK Exacerbates Atherosclerosis in LDL Receptor Knockout (LDLRKO) Mice. Diabetes. 2016;65(6):1565-76. Epub 2016/01/30. doi: 10.2337/db15-0917. PubMed PMID: 26822081; PMCID: PMC4878417.

424. Galic S, Fullerton MD, Schertzer JD, Sikkema S, Marcinko K, Walkley CR, Izon D, Honeyman J, Chen ZP, van Denderen BJ, Kemp BE, Steinberg GR. Hematopoietic AMPK beta1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. The Journal of clinical investigation. 2011;121(12):4903-15. Epub 2011/11/15. doi: 10.1172/JCI58577. PubMed PMID: 22080866; PMCID: PMC3226000.

425. Berod L, Friedrich C, Nandan A, Freitag J, Hagemann S, Harmrolfs K, Sandouk A, Hesse C, Castro CN, Bahre H, Tschirner SK, Gorinski N, Gohmert M, Mayer CT, Huehn J, Ponimaskin E, Abraham WR, Muller R, Lochner M, Sparwasser T. De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. Nature medicine. 2014;20(11):1327-33. Epub 2014/10/06. doi: 10.1038/nm.3704. PubMed PMID: 25282359.

426. Wang Q, Zhang M, Torres G, Wu S, Ouyang C, Xie Z, Zou MH. Metformin Suppresses Diabetes-Accelerated Atherosclerosis via the Inhibition of Drp1-Mediated Mitochondrial Fission. Diabetes. 2017;66(1):193-205. Epub 2016/10/16. doi: 10.2337/db16-0915. PubMed PMID: 27737949; PMCID: PMC5204316.

427. Fullerton MD, Ford RJ, McGregor CP, LeBlond ND, Snider SA, Stypa SA, Day EA, Lhotak S, Schertzer JD, Austin RC, Kemp BE, Steinberg GR. Salicylate improves macrophage cholesterol homeostasis via activation of Ampk. Journal of lipid research. 2015;56(5):1025-33. Epub 2015/03/17. doi: 10.1194/jlr.M058875. PubMed PMID: 25773887; PMCID: PMC4409279.

428. Ouimet M, Ediriweera HN, Gundra UM, Sheedy FJ, Ramkhelawon B, Hutchison SB, Rinehold K, van Solingen C, Fullerton MD, Cecchini K, Rayner KJ, Steinberg GR, Zamore PD, Fisher EA, Loke P, Moore KJ. MicroRNA-33-dependent regulation of macrophage metabolism directs immune cell polarization in atherosclerosis. The Journal of clinical investigation. 2015;125(12):4334-48. Epub 2015/11/01. doi: 10.1172/JCI81676. PubMed PMID: 26517695; PMCID: PMC4665799.

429. Rutherford C, Speirs C, Williams JJ, Ewart MA, Mancini SJ, Hawley SA, Delles C, Viollet B, Costa-Pereira AP, Baillie GS, Salt IP, Palmer TM. Phosphorylation of Janus kinase 1 (JAK1) by AMP-activated protein kinase (AMPK) links energy sensing to anti-inflammatory signaling. Sci Signal. 2016;9(453):ra109. Epub 2016/12/06. doi: 10.1126/scisignal.aaf8566. PubMed PMID: 27919027.

430. Greer EL, Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP, Brunet A. The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription

factor. The Journal of biological chemistry. 2007;282(41):30107-19. Epub 2007/08/23. doi: 10.1074/jbc.M705325200. PubMed PMID: 17711846.

431. Alvarez-Guardia D, Palomer X, Coll T, Davidson MM, Chan TO, Feldman AM, Laguna JC, Vazquez-Carrera M. The p65 subunit of NF-kappaB binds to PGC-1alpha, linking inflammation and metabolic disturbances in cardiac cells. Cardiovasc Res. 2010;87(3):449-58. Epub 2010/03/10. doi: 10.1093/cvr/cvq080. PubMed PMID: 20211864.

432. Jung TW, Lee SY, Hong HC, Choi HY, Yoo HJ, Baik SH, Choi KM. AMPK activator-mediated inhibition of endoplasmic reticulum stress ameliorates carrageenan-induced insulin resistance through the suppression of selenoprotein P in HepG2 hepatocytes. Mol Cell Endocrinol. 2014;382(1):66-73. Epub 2013/09/24. doi: 10.1016/j.mce.2013.09.013. PubMed PMID: 24055274.

433. Dai X, Ding Y, Liu Z, Zhang W, Zou MH. Phosphorylation of CHOP (C/EBP Homologous Protein) by the AMP-Activated Protein Kinase Alpha 1 in Macrophages Promotes CHOP Degradation and Reduces Injury-Induced Neointimal Disruption In Vivo. Circulation research. 2016;119(10):1089-100. Epub 2016/10/30. doi: 10.1161/CIRCRESAHA.116.309463. PubMed PMID: 27650555; PMCID: PMC5085850.

434. Wu Y, Song P, Zhang W, Liu J, Dai X, Liu Z, Lu Q, Ouyang C, Xie Z, Zhao Z, Zhuo X, Viollet B, Foretz M, Wu J, Yuan Z, Zou MH. Activation of AMPKalpha2 in adipocytes is essential for nicotine-induced insulin resistance in vivo. Nature medicine. 2015;21(4):373-82. Epub 2015/03/24. doi: 10.1038/nm.3826. PubMed PMID: 25799226; PMCID: PMC4390501.

435. Cai Z, Ding Y, Zhang M, Lu Q, Wu S, Zhu H, Song P, Zou MH. Ablation of Adenosine Monophosphate-Activated Protein Kinase alpha1 in Vascular Smooth Muscle Cells Promotes

Diet-Induced Atherosclerotic Calcification In Vivo. Circulation research. 2016;119(3):422-33. Epub 2016/06/04. doi: 10.1161/CIRCRESAHA.116.308301. PubMed PMID: 27256105.

436. Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. 2004;23(12):2369-80. Epub 2004/05/21. doi: 10.1038/sj.emboj.7600244. PubMed PMID: 15152190; PMCID: PMC423286.

437. Yang XD, Tajkhorshid E, Chen LF. Functional interplay between acetylation and methylation of the RelA subunit of NF-kappaB. Molecular and cellular biology. 2010;30(9):2170-80. Epub 2010/02/18. doi: 10.1128/MCB.01343-09. PubMed PMID: 20160011; PMCID: PMC2863596.

438. Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature. 2009;458(7241):1056-60. Epub 2009/03/06. doi: 10.1038/nature07813. PubMed PMID: 19262508; PMCID: PMC3616311.

439. Lan F, Cacicedo JM, Ruderman N, Ido Y. SIRT1 modulation of the acetylation status, cytosolic localization, and activity of LKB1. Possible role in AMP-activated protein kinase activation. The Journal of biological chemistry. 2008;283(41):27628-35. Epub 2008/08/09. doi: 10.1074/jbc.M805711200. PubMed PMID: 18687677; PMCID: PMC2562073.

440. Morari J, Torsoni AS, Anhe GF, Roman EA, Cintra DE, Ward LS, Bordin S, Velloso LA. The role of proliferator-activated receptor gamma coactivator-1alpha in the fatty-acid-dependent transcriptional control of interleukin-10 in hepatic cells of rodents. Metabolism: clinical and experimental. 2010;59(2):215-23. Epub 2009/09/22. doi: 10.1016/j.metabol.2009.07.020. PubMed PMID: 19766270.

441. Kim HJ, Park KG, Yoo EK, Kim YH, Kim YN, Kim HS, Kim HT, Park JY, Lee KU, Jang WG, Kim JG, Kim BW, Lee IK. Effects of PGC-1alpha on TNF-alpha-induced MCP-1 and VCAM-1 expression and NF-kappaB activation in human aortic smooth muscle and endothelial cells. Antioxidants & redox signaling. 2007;9(3):301-7. Epub 2006/12/23. doi: 10.1089/ars.2006.1456. PubMed PMID: 17184171.

442. Palomer X, Alvarez-Guardia D, Rodriguez-Calvo R, Coll T, Laguna JC, Davidson MM, Chan TO, Feldman AM, Vazquez-Carrera M. TNF-alpha reduces PGC-1alpha expression through NF-kappaB and p38 MAPK leading to increased glucose oxidation in a human cardiac cell model. Cardiovasc Res. 2009;81(4):703-12. Epub 2008/11/29. doi: 10.1093/cvr/cvn327. PubMed PMID: 19038972.

443. Wang SX, Zhang M, Liang B, Xu J, Xie ZL, Liu C, Viollet B, Yan DG, Zou MH. AMPK alpha 2 Deletion Causes Aberrant Expression and Activation of NAD(P)H Oxidase and Consequent Endothelial Dysfunction In Vivo Role of 26S Proteasomes. Circulation research. 2010;106(6):1117-28. doi: 10.1161/Circresaha.109.212530. PubMed PMID: WOS:000276214000015.

444. Li XN, Song J, Zhang L, LeMaire SA, Hou XY, Zhang C, Coselli JS, Chen L, Wang XL, Zhang Y, Shen YH. Activation of the AMPK-FOXO3 Pathway Reduces Fatty Acid-Induced Increase in Intracellular Reactive Oxygen Species by Upregulating Thioredoxin. Diabetes. 2009;58(10):2246-57. doi: 10.2337/db08-1512. PubMed PMID: WOS:000270776200012.

445. Xie Z, Zhang J, Wu J, Viollet B, Zou MH. Upregulation of mitochondrial uncoupling protein-2 by the AMP-activated protein kinase in endothelial cells attenuates oxidative stress in diabetes. Diabetes. 2008;57(12):3222-30. Epub 2008/10/07. doi: 10.2337/db08-0610. PubMed PMID: 18835932; PMCID: PMC2584127.

446. Schulze PC, Yoshioka J, Takahashi T, He ZH, King GL, Lee RT. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. Journal of Biological Chemistry. 2004;279(29):30369-74. doi: 10.1074/jbc.M400549200. PubMed PMID: WOS:000222531900062.

447. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nature immunology. 2010;11(2):136-40. Epub 2009/12/22. doi: 10.1038/ni.1831. PubMed PMID: 20023662.

448. Dong YZ, Zhang MA, Wang SX, Liang B, Zhao ZX, Liu C, Wu MY, Choi HC, Lyons TJ, Zou MH. Activation of AMP-Activated Protein Kinase Inhibits Oxidized LDL-Triggered Endoplasmic Reticulum Stress In Vivo. Diabetes. 2010;59(6):1386-96. doi: 10.2337/db09-1637. PubMed PMID: WOS:000278844700012.

449. Terai K, Hiramoto Y, Masaki M, Sugiyama S, Kuroda T, Hori M, Kawase I, Hirota H. AMP-activated protein kinase protects cardiomyocytes against hypoxic injury through attenuation of endoplasmic reticulum stress. Molecular and cellular biology. 2005;25(21):9554-75. doi: 10.1128/Mcb.25.21.9554-9575.2005. PubMed PMID: WOS:000232754600036.

450. Dong YZ, Zhang M, Liang B, Xie ZL, Zhao ZX, Asfa S, Choi HC, Zou MH. Reduction of AMP-Activated Protein Kinase alpha 2 Increases Endoplasmic Reticulum Stress and Atherosclerosis In Vivo. Circulation. 2010;121(6):792-803. doi: 10.1161/Circulationaha.109.900928. PubMed PMID: WOS:000274536500010.

451. Qiang XY, Xu LL, Zhang M, Zhang PC, Wang YH, Wang YC, Zhao Z, Chen H, Liu X, Zhang YB. Demethyleneberberine attenuates non-alcoholic fatty liver disease with activation of AMPK and inhibition of oxidative stress. Biochem Bioph Res Co. 2016;472(4):603-9. doi: 10.1016/j.bbrc.2016.03.019. PubMed PMID: WOS:000373753600006.

452. Schmid AI, Szendroedi J, Chmelik M, Krssak M, Moser E, Roden M. Liver ATP Synthesis Is Lower and Relates to Insulin Sensitivity in Patients With Type 2 Diabetes. Diabetes care. 2011;34(2):448-53. doi: 10.2337/dc10-1076. PubMed PMID: WOS:000287433900037.

453. Dzamko N, van Denderen BJW, Hevener AL, Jorgensen SB, Honeyman J, Galic S, Chen ZP, Watt MJ, Campbell DJ, Steinberg GR, Kemp BE. AMPK beta 1 Deletion Reduces Appetite, Preventing Obesity and Hepatic Insulin Resistance. Journal of Biological Chemistry. 2010;285(1):115-22. doi: 10.1074/jbc.M109.056762. PubMed PMID: WOS:000273070100012.

454. Zhang HA, Yang XY, Xiao YF. AMPK alpha 1 overexpression alleviates the hepatocyte model of nonalcoholic fatty liver disease via inactivating p38MAPK pathway. Biochem Bioph Res Co. 2016;474(2):364-70. doi: 10.1016/j.bbrc.2016.04.111. PubMed PMID: WOS:000376221700022.

455. Seo E, Park EJ, Joe Y, Kang S, Kim MS, Hong SH, Park MK, Kim DK, Koh H, Lee HJ. Overexpression of AMPK alpha 1 Ameliorates Fatty Liver in Hyperlipidemic Diabetic Rats. Korean J Physiol Pha. 2009;13(6):449-54. doi: 10.4196/kjpp.2009.13.6.449. PubMed PMID: WOS:000273724900007.

456. Fullerton MD, Galic S, Marcinko K, Sikkema S, Pulinilkunnil T, Chen ZP, O'Neill HM, Ford RJ, Palanivel R, O'Brien M, Hardie DG, Macaulay SL, Schertzer JD, Dyck JR, van Denderen BJ, Kemp BE, Steinberg GR. Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. Nature medicine. 2013;19(12):1649-54. Epub 2013/11/05. doi: 10.1038/nm.3372. PubMed PMID: 24185692; PMCID: PMC4965268.

457. Harriman G, Greenwood J, Bhat S, Huang X, Wang R, Paul D, Tong L, Saha AK, Westlin WF, Kapeller R, Harwood HJ, Jr. Acetyl-CoA carboxylase inhibition by ND-630

reduces hepatic steatosis, improves insulin sensitivity, and modulates dyslipidemia in rats. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(13):E1796-805. Epub 2016/03/16. doi: 10.1073/pnas.1520686113. PubMed PMID: 26976583; PMCID: PMC4822632.

458. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair W, Vasquez DS, Joshi A, Gwinn DM, Taylor R, Asara JM, Fitzpatrick J, Dillin A, Viollet B, Kundu M, Hansen M, Shaw RJ. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science. 2011;331(6016):456-61. Epub 2011/01/06. doi: 10.1126/science.1196371. PubMed PMID: 21205641; PMCID: PMC3030664.

459. Ducommun S, Deak M, Sumpton D, Ford RJ, Nunez Galindo A, Kussmann M, Viollet B, Steinberg GR, Foretz M, Dayon L, Morrice NA, Sakamoto K. Motif affinity and mass spectrometry proteomic approach for the discovery of cellular AMPK targets: identification of mitochondrial fission factor as a new AMPK substrate. Cell Signal. 2015;27(5):978-88. Epub 2015/02/17. doi: 10.1016/j.cellsig.2015.02.008. PubMed PMID: 25683918.

460. Toyama EQ, Herzig S, Courchet J, Lewis TL, Jr., Loson OC, Hellberg K, Young NP, Chen H, Polleux F, Chan DC, Shaw RJ. Metabolism. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. Science. 2016;351(6270):275-81. Epub 2016/01/28. doi: 10.1126/science.aab4138. PubMed PMID: 26816379; PMCID: PMC4852862.

461. Gariani K, Philippe J, Jornayvaz FR. Non-alcoholic fatty liver disease and insulin resistance: from bench to bedside. Diabetes Metab. 2013;39(1):16-26. Epub 2012/12/26. doi: 10.1016/j.diabet.2012.11.002. PubMed PMID: 23266468.

462. Sinha RA, Yen PM. Thyroid hormone-mediated autophagy and mitochondrial turnover in NAFLD. Cell Biosci. 2016;6:46. Epub 2016/07/21. doi: 10.1186/s13578-016-0113-7. PubMed PMID: 27437098; PMCID: PMC4950712.

463. Galloway CA, Lee H, Brookes PS, Yoon Y. Decreasing mitochondrial fission alleviates hepatic steatosis in a murine model of nonalcoholic fatty liver disease. American journal of physiology Gastrointestinal and liver physiology. 2014;307(6):G632-41. Epub 2014/08/02. doi: 10.1152/ajpgi.00182.2014. PubMed PMID: 25080922; PMCID: PMC4166723.

464. Hasenour CM, Ridley DE, Hughey CC, James FD, Donahue EP, Shearer J, Viollet B, Foretz M, Wasserman DH. 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) effect on glucose production, but not energy metabolism, is independent of hepatic AMPK in vivo. The Journal of biological chemistry. 2014;289(9):5950-9. Epub 2014/01/10. doi: 10.1074/jbc.M113.528232. PubMed PMID: 24403081; PMCID: PMC3937663.

465. Kaushik S, Cuervo AM. AMPK-dependent phosphorylation of lipid droplet protein PLIN2 triggers its degradation by CMA. Autophagy. 2016;12(2):432-8. Epub 2016/02/24. doi: 10.1080/15548627.2015.1124226. PubMed PMID: 26902588; PMCID: PMC4835968.

466. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism: clinical and experimental. 2016;65(8):1038-48. doi: 10.1016/j.metabol.2015.12.012. PubMed PMID: 26823198.

467. Dowman JK, Tomlinson JW, Newsome PN. Pathogenesis of non-alcoholic fatty liver disease. QJM : monthly journal of the Association of Physicians. 2010;103(2):71-83. doi: 10.1093/qjmed/hcp158. PubMed PMID: 19914930; PMCID: 2810391.

468. Schippa S, Conte MP. Dysbiotic events in gut microbiota: impact on human health. Nutrients. 2014;6(12):5786-805. doi: 10.3390/nu6125786. PubMed PMID: 25514560; PMCID: 4276999.

469. Sridharan GV, Choi K, Klemashevich C, Wu C, Prabakaran D, Pan LB, Steinmeyer S, Mueller C, Yousofshahi M, Alaniz RC, Lee K, Jayaraman A. Prediction and quantification of bioactive microbiota metabolites in the mouse gut. Nature communications. 2014;5:5492. doi: 10.1038/ncomms6492. PubMed PMID: 25411059.

470. Ghazalpour A, Cespedes I, Bennett BJ, Allayee H. Expanding role of gut microbiota in lipid metabolism. Current opinion in lipidology. 2016;27(2):141-7. doi: 10.1097/MOL.0000000000000278. PubMed PMID: 26855231; PMCID: 5125441.

471. Vernocchi P, Del Chierico F, Putignani L. Gut Microbiota Profiling: Metabolomics Based Approach to Unravel Compounds Affecting Human Health. Frontiers in microbiology. 2016;7:1144. doi: 10.3389/fmicb.2016.01144. PubMed PMID: 27507964; PMCID: 4960240.

472. Lee JH, Wada T, Febbraio M, He J, Matsubara T, Lee MJ, Gonzalez FJ, Xie W. A novel role for the dioxin receptor in fatty acid metabolism and hepatic steatosis. Gastroenterology. 2010;139(2):653-63. doi: 10.1053/j.gastro.2010.03.033. PubMed PMID: 20303349; PMCID: 2910786.

473. Tanos R, Murray IA, Smith PB, Patterson A, Perdew GH. Role of the Ah receptor in homeostatic control of fatty acid synthesis in the liver. Toxicological sciences : an official journal of the Society of Toxicology. 2012;129(2):372-9. doi: 10.1093/toxsci/kfs204. PubMed PMID: 22696238; PMCID: 3491957.

474. Pelclova D, Fenclova Z, Preiss J, Prochazka B, Spacil J, Dubska Z, Okrouhlik B, Lukas E, Urban P. Lipid metabolism and neuropsychological follow-up study of workers exposed to

2,3,7,8- tetrachlordibenzo- p-dioxin. International archives of occupational and environmental health. 2002;75 Suppl:S60-6. doi: 10.1007/s00420-002-0350-4. PubMed PMID: 12397412.

475. Ying W, Cheruku PS, Bazer FW, Safe SH, Zhou BY. Investigation of Macrophage Polarization Using Bone Marrow Derived Macrophages. Jove-J Vis Exp. 2013(76). doi: UNSP e50323

10.3791/50323. PubMed PMID: WOS:000209227800024.

476. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G. METLIN: a metabolite mass spectral database. Therapeutic drug monitoring. 2005;27(6):747-51. PubMed PMID: 16404815.

477. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, Cheng D, Jewell K, Arndt D, Sawhney S, Fung C, Nikolai L, Lewis M, Coutouly MA, Forsythe I, Tang P, Shrivastava S, Jeroncic K, Stothard P, Amegbey G, Block D, Hau DD, Wagner J, Miniaci J, Clements M, Gebremedhin M, Guo N, Zhang Y, Duggan GE, Macinnis GD, Weljie AM, Dowlatabadi R, Bamforth F, Clive D, Greiner R, Li L, Marrie T, Sykes BD, Vogel HJ, Querengesser L. HMDB: the Human Metabolome Database. Nucleic acids research. 2007;35(Database issue):D521-6. doi: 10.1093/nar/gkl923. PubMed PMID: 17202168; PMCID: 1899095.

478. Wolf S, Schmidt S, Muller-Hannemann M, Neumann S. In silico fragmentation for computer assisted identification of metabolite mass spectra. BMC bioinformatics. 2010;11:148. Epub 2010/03/24. doi: 10.1186/1471-2105-11-148. PubMed PMID: 20307295; PMCID: Pmc2853470.

479. Allen F, Pon A, Wilson M, Greiner R, Wishart D. CFM-ID: a web server for annotation, spectrum prediction and metabolite identification from tandem mass spectra. Nucleic acids

research. 2014;42(Web Server issue):W94-9. doi: 10.1093/nar/gku436. PubMed PMID: 24895432; PMCID: PMC4086103.

480. Manteiga S, Lee K. Monoethylhexyl Phthalate Elicits an Inflammatory Response in Adipocytes Characterized by Alterations in Lipid and Cytokine Pathways. Environ Health Perspect. 2017;125(4):615-22. doi: 10.1289/EHP464. PubMed PMID: 27384973; PMCID: PMC5381996.

481. Nagy SR, Sanborn JR, Hammock BD, Denison MS. Development of a green fluorescent protein-based cell bioassay for the rapid and inexpensive detection and characterization of ah receptor agonists. Toxicological sciences : an official journal of the Society of Toxicology. 2002;65(2):200-10. PubMed PMID: 11812924.

482. Reid DT, Eksteen B. Murine models provide insight to the development of non-alcoholic fatty liver disease. Nutrition research reviews. 2015;28(2):133-42. doi: 10.1017/S0954422415000128. PubMed PMID: 26494024.

483. Haukeland JW, Damas JK, Konopski Z, Loberg EM, Haaland T, Goverud I, Torjesen PA, Birkeland K, Bjoro K, Aukrust P. Systemic inflammation in nonalcoholic fatty liver disease is characterized by elevated levels of CCL2. Journal of hepatology. 2006;44(6):1167-74. doi: 10.1016/j.jhep.2006.02.011. PubMed PMID: 16618517.

484. Malhi H, Bronk SF, Werneburg NW, Gores GJ. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. The Journal of biological chemistry. 2006;281(17):12093-101. doi: 10.1074/jbc.M510660200. PubMed PMID: 16505490.

485. Zhao B, Degroot DE, Hayashi A, He G, Denison MS. CH223191 is a ligand-selective antagonist of the Ah (Dioxin) receptor. Toxicological sciences : an official journal of the Society

of Toxicology. 2010;117(2):393-403. doi: 10.1093/toxsci/kfq217. PubMed PMID: 20634293; PMCID: PMC2940411.

486. Kim KA, Gu W, Lee IA, Joh EH, Kim DH. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. PloS one. 2012;7(10):e47713. doi: 10.1371/journal.pone.0047713. PubMed PMID: 23091640; PMCID: 3473013.

487. Pang J, Xi C, Huang X, Cui J, Gong H, Zhang T. Effects of Excess Energy Intake on Glucose and Lipid Metabolism in C57BL/6 Mice. PloS one. 2016;11(1):e0146675. doi: 10.1371/journal.pone.0146675. PubMed PMID: 26745179; PMCID: 4706434.

488. Williams BB, Van Benschoten AH, Cimermancic P, Donia MS, Zimmermann M, Taketani M, Ishihara A, Kashyap PC, Fraser JS, Fischbach MA. Discovery and characterization of gut microbiota decarboxylases that can produce the neurotransmitter tryptamine. Cell host & microbe. 2014;16(4):495-503. doi: 10.1016/j.chom.2014.09.001. PubMed PMID: 25263219; PMCID: 4260654.

489. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest. 2006;116(11):3015-25. doi: 10.1172/JCI28898. PubMed PMID: WOS:000241810900025.

490. Schwartz EA, Zhang WY, Karnik SK, Borwege S, Anand VR, Laine PS, Su Y, Reaven PD. Nutrient modification of the innate immune response: a novel mechanism by which saturated fatty acids greatly amplify monocyte inflammation. Arteriosclerosis, thrombosis, and vascular biology. 2010;30(4):802-8. doi: 10.1161/ATVBAHA.109.201681. PubMed PMID: 20110572.

491. Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MT, Brickey WJ, Ting JP. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. Nature immunology. 2011;12(5):408-15. doi: 10.1038/ni.2022. PubMed PMID: 21478880; PMCID: 4090391.

492. Pagadala M, Kasumov T, McCullough AJ, Zein NN, Kirwan JP. Role of ceramides in nonalcoholic fatty liver disease. Trends in endocrinology and metabolism: TEM. 2012;23(8):365-71. doi: 10.1016/j.tem.2012.04.005. PubMed PMID: 22609053; PMCID: 3408814.

493. Garcia-Ruiz I, Solis-Munoz P, Fernandez-Moreira D, Munoz-Yague T, Solis-Herruzo JA. In vitro treatment of HepG2 cells with saturated fatty acids reproduces mitochondrial dysfunction found in nonalcoholic steatohepatitis. Disease models & mechanisms. 2015;8(2):183-91. doi: 10.1242/dmm.018234. PubMed PMID: 25540128; PMCID: 4314783.

494. Zhang J, Zhao Y, Xu C, Hong Y, Lu H, Wu J, Chen Y. Association between serum free fatty acid levels and nonalcoholic fatty liver disease: a cross-sectional study. Scientific reports. 2014;4:5832. doi: 10.1038/srep05832. PubMed PMID: 25060337; PMCID: 5376058.

495. Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, Fantoni LI, Marra F, Bertolotti M, Banni S, Lonardo A, Carulli N, Loria P. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. Journal of gastroenterology and hepatology. 2009;24(5):830-40. doi: 10.1111/j.1440-1746.2008.05733.x. PubMed PMID: 19207680.

496. Stojsavljevic S, Gomercic Palcic M, Virovic Jukic L, Smircic Duvnjak L, Duvnjak M. Adipokines and proinflammatory cytokines, the key mediators in the pathogenesis of

nonalcoholic fatty liver disease. World J Gastroenterol. 2014;20(48):18070-91. doi: 10.3748/wjg.v20.i48.18070. PubMed PMID: 25561778; PMCID: 4277948.

497. Endo M, Masaki T, Seike M, Yoshimatsu H. TNF-alpha induces hepatic steatosis in mice by enhancing gene expression of sterol regulatory element binding protein-1c (SREBP-1c). Experimental biology and medicine. 2007;232(5):614-21. PubMed PMID: 17463157.

498. Hubbard TD, Murray IA, Bisson WH, Lahoti TS, Gowda K, Amin SG, Patterson AD, Perdew GH. Adaptation of the human aryl hydrocarbon receptor to sense microbiota-derived indoles. Scientific reports. 2015;5:12689. doi: 10.1038/srep12689. PubMed PMID: 26235394; PMCID: 4522678.

499. Jin UH, Lee SO, Sridharan G, Lee K, Davidson LA, Jayaraman A, Chapkin RS, Alaniz R, Safe S. Microbiome-derived tryptophan metabolites and their aryl hydrocarbon receptor-dependent agonist and antagonist activities. Molecular pharmacology. 2014;85(5):777-88. doi: 10.1124/mol.113.091165. PubMed PMID: 24563545; PMCID: 3990014.

500. Lakshman MR, Campbell BS, Chirtel SJ, Ekarohita N. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on de novo fatty acid and cholesterol synthesis in the rat. Lipids. 1988;23(9):904-6. PubMed PMID: 3185127.

501. Wada T, Sunaga H, Miyata K, Shirasaki H, Uchiyama Y, Shimba S. Aryl Hydrocarbon Receptor Plays Protective Roles against High Fat Diet (HFD)-induced Hepatic Steatosis and the Subsequent Lipotoxicity via Direct Transcriptional Regulation of Socs3 Gene Expression. The Journal of biological chemistry. 2016;291(13):7004-16. doi: 10.1074/jbc.M115.693655. PubMed PMID: 26865635; PMCID: 4807284.

502. Gharavi N, El-Kadi AO. Down-regulation of aryl hydrocarbon receptor-regulated genes by tumor necrosis factor-alpha and lipopolysaccharide in murine hepatoma Hepa 1c1c7 cells.

Journal of pharmaceutical sciences. 2005;94(3):493-506. doi: 10.1002/jps.20267. PubMed PMID: 15627257.

503. Drozdzik A, Dziedziejko V, Kurzawski M. IL-1 and TNF-alpha regulation of aryl hydrocarbon receptor (AhR) expression in HSY human salivary cells. Archives of oral biology. 2014;59(4):434-9. doi: 10.1016/j.archoralbio.2014.02.003. PubMed PMID: 24565903.

504. Gorski JR, Weber LW, Rozman K. Tissue-specific alterations of de novo fatty acid synthesis in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-treated rats. Archives of toxicology. 1988;62(2-3):146-51. PubMed PMID: 3196149.

505. Muse ED, Obici S, Bhanot S, Monia BP, McKay RA, Rajala MW, Scherer PE, Rossetti L. Role of resistin in diet-induced hepatic insulin resistance. The Journal of clinical investigation. 2004;114(2):232-9. doi: 10.1172/JCI21270. PubMed PMID: 15254590; PMCID: 449748.

506. Lamas B, Natividad JM, Sokol H. Aryl hydrocarbon receptor and intestinal immunity. Mucosal immunology. 2018;11(4):1024-38. Epub 2018/04/08. doi: 10.1038/s41385-018-0019-2. PubMed PMID: 29626198.

507. Agus A, Planchais J, Sokol H. Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. Cell host & microbe. 2018;23(6):716-24. Epub 2018/06/15. doi: 10.1016/j.chom.2018.05.003. PubMed PMID: 29902437.

508. Frericks M, Meissner M, Esser C. Microarray analysis of the AHR system: tissue-specific flexibility in signal and target genes. Toxicology and applied pharmacology. 2007;220(3):320-32. Epub 2007/03/14. doi: 10.1016/j.taap.2007.01.014. PubMed PMID: 17350064.

509. Senn JJ. Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. The Journal of biological chemistry. 2006;281(37):26865-75. Epub 2006/06/27. doi: 10.1074/jbc.M513304200. PubMed PMID: 16798732.

510. Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore N, Hwang DH. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. The Journal of biological chemistry. 2003;278(39):37041-51. doi: 10.1074/jbc.M305213200. PubMed PMID: 12865424.

511. Huang S, Rutkowsky JM, Snodgrass RG, Ono-Moore KD, Schneider DA, Newman JW, Adams SH, Hwang DH. Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. Journal of lipid research. 2012;53(9):2002-13. Epub 2012/07/07. doi: 10.1194/jlr.D029546. PubMed PMID: 22766885; PMCID: PMC3413240.

512. Nguyen MT, Favelyukis S, Nguyen AK, Reichart D, Scott PA, Jenn A, Liu-Bryan R, Glass CK, Neels JG, Olefsky JM. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. The Journal of biological chemistry. 2007;282(48):35279-92. Epub 2007/10/06. doi: 10.1074/jbc.M706762200. PubMed PMID: 17916553.

513. Osborn O, Olefsky JM. The cellular and signaling networks linking the immune system and metabolism in disease. Nature medicine. 2012;18(3):363-74. Epub 2012/03/08. doi: 10.1038/nm.2627. PubMed PMID: 22395709.

514. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. The Journal of clinical investigation. 2006;116(11):3015-25. Epub 2006/10/21. doi: 10.1172/JCI28898. PubMed PMID: 17053832; PMCID: PMC1616196.

515. Liao F, Andalibi A, deBeer FC, Fogelman AM, Lusis AJ. Genetic control of inflammatory gene induction and NF-kappa B-like transcription factor activation in response to

an atherogenic diet in mice. The Journal of clinical investigation. 1993;91(6):2572-9. Epub 1993/06/01. doi: 10.1172/JCI116495. PubMed PMID: 8514869; PMCID: PMC443320.

516. Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, Hoshino K, Takeuchi O, Kobayashi M, Fujita T, Takeda K, Akira S. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature. 2002;420(6913):324-9. Epub 2002/11/26. doi: 10.1038/nature01182. PubMed PMID: 12447441.

517. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science. 2003;301(5633):640-3. Epub 2003/07/12. doi: 10.1126/science.1087262. PubMed PMID: 12855817.

518. Horng T, Barton GM, Flavell RA, Medzhitov R. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature. 2002;420(6913):329-33. Epub 2002/11/26. doi: 10.1038/nature01180. PubMed PMID: 12447442.

519. Lancaster GI, Langley KG, Berglund NA, Kammoun HL, Reibe S, Estevez E, Weir J, Mellett NA, Pernes G, Conway JRW, Lee MKS, Timpson P, Murphy AJ, Masters SL, Gerondakis S, Bartonicek N, Kaczorowski DC, Dinger ME, Meikle PJ, Bond PJ, Febbraio MA. Evidence that TLR4 Is Not a Receptor for Saturated Fatty Acids but Mediates Lipid-Induced Inflammation by Reprogramming Macrophage Metabolism. Cell metabolism. 2018;27(5):1096-110 e5. Epub 2018/04/24. doi: 10.1016/j.cmet.2018.03.014. PubMed PMID: 29681442.

520. Braunersreuther V, Viviani GL, Mach F, Montecucco F. Role of cytokines and chemokines in non-alcoholic fatty liver disease. World J Gastroenterol. 2012;18(8):727-35. doi: 10.3748/wjg.v18.i8.727. PubMed PMID: 22371632; PMCID: 3286135.

521. Innocentin YLS, Withers DR, Roberts NA, Gallagher AR, Grigorieva EF, Wilhelm C, Veldhoen M. Exogenous Stimuli Maintain Intraepithelial Lymphocytes via Aryl Hydrocarbon Receptor Activation. Cell. 2011;147(3):629-40. doi: 10.1016/j.cell.2011.09.025. PubMed PMID: WOS:000296573700017.

522. Stockinger B, Omenetti S. The dichotomous nature of T helper 17 cells. Nature Reviews Immunology. 2017;17(9):535-44. doi: 10.1038/nri.2017.50. PubMed PMID: WOS:000408686300008.

523. Qiu J, Guo XH, Chen ZME, He L, Sonnenberg GF, Artis D, Fu YX, Zhou L. Group 3 Innate Lymphoid Cells Inhibit T-Cell-Mediated Intestinal Inflammation through Aryl Hydrocarbon Receptor Signaling and Regulation of Microflora. Immunity. 2013;39(2):386-99. doi: 10.1016/j.immuni.2013.08.002. PubMed PMID: WOS:000330948800019.

524. Nguyen NT, Kimura A, Nakahama T, Chinen I, Masuda K, Nohara K, Fujii-Kuriyama Y, Kishimoto T. Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(46):19961-6. doi: 10.1073/pnas.1014465107. PubMed PMID: WOS:000284261800066.

525. Zindl CL, Lai JF, Lee YK, Maynard CL, Harbour SN, Ouyang WJ, Chaplin DD, Weaver CT. IL-22-producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(31):12768-73. doi: 10.1073/pnas.1300318110. PubMed PMID: WOS:000322441500063.

526. Qiu J, Heller JJ, Guo XH, Chen ZME, Fish K, Fu YX, Zhou L. The Aryl Hydrocarbon Receptor Regulates Gut Immunity through Modulation of Innate Lymphoid Cells. Immunity.

2012;36(1):92-104. doi: 10.1016/j.immuni.2011.11.011. PubMed PMID: WOS:000299766000013.

527. Wang XT, Ota N, Manzanillo P, Kates L, Zavala-Solorio J, Eidenschenk C, Zhang J, Lesch J, Lee WP, Ross J, Diehl L, van Bruggen N, Kolumam G, Ouyang WJ. Interleukin-22 alleviates metabolic disorders and restores mucosal immunity in diabetes. Nature. 2014;514(7521):237-+. doi: 10.1038/nature13564. PubMed PMID: WOS:000342663100044.

528. Kimura A, Naka T, Nakahama T, Chinen I, Masuda K, Nohara K, Fujii-Kuriyama Y, Kishimoto T. Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses. The Journal of experimental medicine. 2009;206(9):2027-35. doi: 10.1084/jem.20090560. PubMed PMID: 19703987; PMCID: 2737163.

529. Krishnan S, Ding Y, Saedi N, Choi M, Sridharan GV, Sherr DH, Yarmush ML, Alaniz RC, Jayaraman A, Lee K. Gut Microbiota-Derived Tryptophan Metabolites Modulate Inflammatory Response in Hepatocytes and Macrophages. Cell reports. 2018;23(4):1099-111. Epub 2018/04/26. doi: 10.1016/j.celrep.2018.03.109. PubMed PMID: 29694888.

530. Wu J, Kumar-Kanojia A, Hombach-Klonisch S, Klonisch T, Lin F. A radial microfluidic platform for higher throughput chemotaxis studies with individual gradient control. Lab Chip. 2018;18(24):3855-64. doi: 10.1039/c8lc00981c. PubMed PMID: 30427358.

531. Sule N, Penarete-Acosta D, Englert DL, Jayaraman A. A Static Microfluidic Device for Investigating the Chemotaxis Response to Stable, Non-linear Gradients. Methods Mol Biol. 2018;1729:47-59. doi: 10.1007/978-1-4939-7577-8_5. PubMed PMID: 29429081.