Elucidation of CYP Inducibility and Inflammatory Response via Cytokine Release in Non-Alcoholic Fatty Liver Disease

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citable link</td>
<td><a href="https://nrs.harvard.edu/URN-3:HUL.INSTREPOS:37365074">https://nrs.harvard.edu/URN-3:HUL.INSTREPOS:37365074</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
Elucidation of CYP Inducibility and Inflammatory Response via Cytokine Release in Non-Alcoholic Fatty Liver Disease

Peony D. Banik

A Thesis in the Field of Bioengineering and Nanotechnology for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2020
Abstract

Non-alcoholic fatty liver disease (NAFLD) is an emerging global health issue that has recently reached 20% in global prevalence and is projected to be the main cause of liver morbidity in the next decade. The lack of accurate human-relevant in vitro systems has caused a barrier in drug development and in the study of cytochrome P450 enzyme expression under hepatosteatotic conditions. Enzyme families CYP1, CYP2, and CYP3 are responsible for the metabolism of more than 60% of all clinically relevant drugs and so, with the growing prevalence of NAFLD, it is likely that NAFLD patients exhibit alterations in these vital drug-metabolizing enzymes and may have risk for harmful drug-drug interactions (DDIs). Here, an in vitro model of NAFLD was engineered using primary human hepatocytes to incorporate clear markers of NAFLD in its progression to non-alcoholic steatohepatitis (NASH) such as inflammation and lipid accumulation. Primary human hepatocytes were cultured in a collagen sandwich configuration that present visible and quantifiable lipid accumulation while maintaining viability and hepatic differentiation over 7 days of cultures and expressing biological markers of NAFLD. Pro-inflammatory cytokines MCP-1, IP-10, IL-8, and VEGFa displayed significant upregulation in the Steatotic states as compared to the Healthy control. These changes were accompanied by significant downregulation of CYP3A4 and CYP1A1 expression and upregulation of CYP2B6 expression in Steatotic cultures upon drug exposure. As there are no approved drug treatments available for NAFLD, this study will
give insight into how the diseased state of the liver may affect the metabolism of drugs taken by patients diagnosed with NAFLD.
Dedication

To my father, who taught me the value of sincere work.

To my mother, who taught me that even the largest task can be accomplished one step at a time.

And to my sister, for being my biggest cheerleader.
Acknowledgments

I would first like to thank my Thesis Director, Dr. Berk Usta of the Center for Engineering in Medicine (CEM) at Harvard Medical School/Massachusetts General Hospital. I express my sincere gratitude to Dr. Usta for his continuous support of my Master’s degree research, for his patience, motivation, enthusiasm, and immense knowledge – a true master, as his name already implies. I would like to thank you for your valuable suggestions and for allowing me to grow as a research scientist.

I would also like to extend my deepest gratitude to my colleague and friend, Camilo Rey Bedón. Many of the experiments and results described in this thesis were accomplished with his help and support; I would like to thank him for carrying out endless experiments together to make this project happen. His vision, sincerity, and knack for solving seemingly convoluted problems cannot be understated. Thank you for your friendship, empathy, and sense of humor.

I would also like to thank Dr. Korkut Uygun and Sinan Özer of the Center for Engineering in Medicine (CEM) at Harvard Medical School/Massachusetts General Hospital as my supervisors; they have been supportive since the days I began working at CEM. I very much appreciate their unfailing support and continuous encouragement.

Finally, I would like to thank my family whose love and guidance are with me in whatever I pursue. This accomplishment would not have been possible without them. Thank you.
Table of Contents

Dedication .................................................................................................................................................. v
Acknowledgments ......................................................................................................................................... vi
List of Tables ............................................................................................................................................... ix
List of Figures ............................................................................................................................................... x
Chapter I. Introduction.................................................................................................................................. 11
  Non-Alcoholic Fatty Liver Disease (NAFLD): A Growing Pandemic .................................................. 11
  Progression of NAFLD ............................................................................................................................ 13
  Pathogenesis of NAFLD ........................................................................................................................... 15
  Multiple-Hit Hypothesis of NAFLD .......................................................................................................... 17
  Understanding the Pathogenesis of NAFLD in vitro ............................................................................. 21
  Drug Metabolism and Cytochrome P450 in NAFLD ........................................................................... 23
  NAFLD as an Inflammatory Disease ........................................................................................................ 25
Chapter II. Materials and Methods ........................................................................................................... 27
  Primary Human Hepatocyte Culture ......................................................................................................... 27
  Induction of Steatosis ............................................................................................................................... 29
  Drug Treatment ......................................................................................................................................... 30
  Lipid Accumulation .................................................................................................................................... 31
  Urea and Albumin Production ................................................................................................................. 31
  Cytokine Release ....................................................................................................................................... 32
  qRT-PCR ..................................................................................................................................................... 33
Chapter III. Results ....................................................................................................................................... 35
Primary Human Hepatocytes Cell Culture Media Optimization and Viability .....35
Verification of Steatosis and Quantification of Lipid Accumulation ................39
Cytokine Release Profile.............................................................................43
CYP450 Transcription Regulation.............................................................44
Drug-Mediated CYP450 Transcription Regulation ....................................46
Chapter IV. Discussion ...............................................................................48
References..................................................................................................57
List of Tables

Table 1. Liver donor information including sex, age, donor path, cause of death, BMI and WIT ................................................................. 29
Table 2. Hepatic culture media composition. .................................................. 30
Table 3. Gene targeting primers ..................................................................... 34
List of Figures

Figure 1. Non-alcoholic fatty liver disease prevalence rates. ...........................................13
Figure 2. Multiple-Hit Hypothesis model of Pathogenesis of NAFLD/NASH. ..............20
Figure 3. Media Optimization ..................................................................................36
Figure 4. Urea Secretion and Albumin Synthesis .............................................................38
Figure 5. Bright-field images of culture of 7 days .........................................................40
Figure 6. Lipid accumulation upon palmitic acid (PA) and oleic acid (OA) exposure. ....41
Figure 7. Quantification of Lipid Accumulation ..............................................................42
Figure 8. Cytokine Release Profile ..............................................................................43
Figure 9. Basal Expression of CYPs and TFs .................................................................45
Figure 10. Drug Response .........................................................................................47
Chapter I.
Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD): A Growing Pandemic

Non-alcoholic fatty liver disease (NAFLD) is a clinical syndrome that is characterized by predominant macrovesicular fat accumulation, or steatosis in the liver that can progress into non-alcoholic steatohepatitis (NASH), liver cirrhosis, liver cancer and ultimately death (Tanaka et al, 2019). NAFLD is projected to be the main cause of liver morbidity in the next decade and is now considered to be one of the most common liver diseases in the world (Moore et al, 2010). NAFLD includes a wide spectrum of pathological diseases that range from hepatic steatosis to liver fibrosis and is associated with an increased risk of all-cause mortality which is contributed by liver- and non-liver related deaths including coronary artery disease and diabetes (Than et al, 2015).

As the world proceeds to embrace the spread of sedentary lifestyle and the Western diet, the prevalence of NAFLD has increased alarmingly in both children and the elderly in many countries across the world (Tanaka et al, 2019). Currently, approximately 25% of adults in the United States have fatty liver disease without excessive ethanol consumption (Tanaka et al, 2019). Roughly a third of all individuals in annual health checkups were found to have NAFLD in Japan, translating to an estimated 20 million NAFLD patients (Eguchi et al, 2012). It has been shown that the total prevalent NAFLD cases estimate to 243.67 million people in China in 2016 and is expected to increase 29.1% to 314.58 million people in the year 2030 (Estes et al, 2018). And so, NAFLD is
the most common chronic liver disease in the Western world having been associated with
the discernible rise in obesity and Type II diabetes, however, the disease still manifests in
Asian countries as well in which the general population has lower BMI and no records of
insulin resistance (Hruby and Hu, 2016). Moreover, the prevalence of NAFLD has
doubled in 20 years – while other liver diseases stayed flat, currently standing at 20-30%
in the West and 5-18% in Asia (Benedict and Zhang, 2017; LaBrecque, 2014).
Additionally, the presence of NAFLD in individuals with Type 2 diabetes mellitus is
shown to be much higher than that of the general population at 40% to 70% (Argo et al,
2009). All in all, NAFLD is alarmingly becoming the most common liver disease in the
world with its global prevalence is estimated at 24% in which the highest reported rates
are from South America and the Middle East, followed by Asia, the United States, and
Europe (Younossi et al, 2017). A compounding factor of concern is the significant
underdiagnosis of fatty liver disease and the inability to efficiently track the progression
of the disease (Merrell and Cherrington, 2011). Overall there is a clear clinical need for
additional investigations into the characterization of NAFLD and a better understanding
of the metabolic and inflammatory consequences associated with the disease.
Figure 1. Non-alcoholic fatty liver disease prevalence rates.

NAFLD prevalence rates reported from Asia, Europe, Middle East, North America and South America.

Vernon et al. (2011) Alimentary Pharmacology & Therapeutics

Progression of NAFLD

First illustrated about 30 years ago, NAFLD was described as lipoprotein abnormalities in patients diagnosed with fibrosis and hepatitis (Adler and Schaffner, 1979), however, it is only recently that the scope of the disease has been better understood by the scientific community. NAFLD is a spectrum of progressive liver diseases that encompass a series of stages. The mild and early stage of NAFLD, termed,
NAFL is a condition defined by excessive fat accumulation in the form of triglycerides (steatosis) in the liver with > 5% of hepatocytes (Anstee et al, 2013). Despite nonalcoholic steatohepatitis (NASH) being a more progressive type of NAFLD, NAFL still carries a clinical burden as it is associated with cardiovascular diseases (CVD) and complications. This risk of CVD has attracted intense scientific appeal over the past decade as it has important implications for strategies such as screening and surveillance for the increasing number of NAFLD patients (Bhatia et al, 2012).

Histologically, the potentially progressive form of NAFLD has been referred to as NASH with a significant rate (25-59%) of progression from NAFL to NASH (Anstee et al, 2013). This progression is accompanied by increasing oxidative stress, pro-inflammatory cytokines, and activation of stellate cells along with other indications and is considered the tipping point to further stages of NAFLD (Masarone et al, 2018; Boecksman et al, 2018). NAFLD and its more severe form, NASH, represent the hepatic manifestation of the metabolic syndrome which includes risk factors such as obesity, insulin resistance, Type II diabetes and hypertension (Boecksman et al, 2018). The later stages of NASH can further progress to cirrhosis which is one of the leading causes of hepatocellular carcinoma which, if salvageable, consequently leads to the need for liver transplant putting more pressure on the organ donor shortage crisis (Boecksman et al, 2018).
Pathogenesis of NAFLD

The comprehension of NAFLD/NASH pathogenesis is key to innovating proper therapeutic interventions for the disease. The mechanism underlying the progression of NAFLD/NASH is multifaceted and complex however much research has been dedicated to gaining a deeper understanding of these mechanisms. Steatosis is caused by dysregulations in the synthesis and transport of triglycerides in various areas of the body. The sources of the accumulated lipids include increased \textit{de novo} lipogenesis (DNL), increased fatty acid reflux associated with diet and peripheral tissues and a decrease in the removal of triglycerides (Merrell and Cherrington, 2011). \textit{De novo} lipogenesis (DNL) is the metabolic pathway that synthesizes fatty acids from excess carbohydrates which then are incorporated into triglycerides (TGs) for energy storage. Under normal conditions, DNL mainly takes place in the liver and in adipose tissue (Ameer et al, 2014). The lipogenic pathway modulates the flow of carbons from glucose to fatty acids and consists of a series of enzymatic reactions. The reactions begin with the conversion of citrate to acetyl-CoA which is then carboxylated to malonyl-CoA. The metabolic key rate-limiting enzyme that brings about the conversion of malonyl-CoA to palmitate is fatty acid synthase (FASN); the major product of DNL, palmitate, is later further converted into complex fatty acids (Ameer et al, 2014). These fatty acids are usually absorbed from the circulation into the hepatocytes, and so an increase in DNL would, therefore, subsequently cause an overall increase in circulating fatty acids in the bloodstream and lipid accumulation in the hepatocytes (Tanaka et al, 2019).

The onset of NAFLD is triggered by the accumulation of triacylglycerol (TAG) in hepatocytes. The fatty acid product of the the \textit{de novo} lipogenesis pathway described is
catabolized by β-oxidation in the mitochondria or peroxisomes wherein the excess amounts are then converted into TAG which are stored as lipid droplets in hepatocytes. The TAG present in lipid droplets is hydrolyzed and then secreted into the blood circulation as low-density lipoprotein particles. The disruption of these pathways could lead to the development of hepatosteatosis (Tanaka et al, 2017). The dysregulation of nuclear receptors in hepatocytes is a major characteristic of NASH and it is these transcription factors that regulate an array of mechanisms, including lipogenesis and β-oxidation, which implicate liver pathophysiology. The β-oxidation of fatty acids in hepatocytes is regulated mainly by nuclear receptors such as peroxisome proliferator-activated receptor α, or PPARα. The downregulation of PPARα has been shown to be associated with NAFLD/NASH and may partially account for the correlation between NAFLD/NASH and insulin resistance (Boeckmans et al, 2018).

Another contributing factor to NAFLD/NASH is adipocyte dysfunction since approximately 60% of fatty acids in the liver arise from adipose tissue (Donnelly et al, 2015). Studies have demonstrated that, in steatotic mice, enhanced induced white adipose lipolysis assists fatty acid mobilization from adipose tissue to the liver therefore increasing hepatic oxidative stress which ultimately leads to the development of steatohepatitis (Tanaka et al, 2014).

TAGs that are stored in hepatocytes as lipid droplets are not considered to be toxic as several studies have shown little to no correlation between the accumulation of TAG and NAFLD severity. In addition, hepatosteatosis is known to attenuate as the disease progresses to fibrosis (Tanaka et al, 2019). That being said, precursors and intermediates of TAG, such as palmitate, are likely to be the culprit in being harmful to
hepatocytes. For instance, palmitate increases oxidative and endoplasmic reticulum stress which may lead to lipoapoptosis (Akazawa et al, 2018). Furthermore, lipid-rich cells are more susceptible to lipid peroxidation causing mitochondrial and endoplasmic reticulum dysfunction - this is just one example of lipotoxicity, one the major sources for hepatocyte injury in NASH. These damaged hepatocytes release various pro-inflammatory mediators, such as cytokines, which recruit immune cells and activate Kupffer and stellate cells which amplify cell death and inflammation - a major distinguishing feature of NAFLD’s transition to NASH (Tanaka et al, 2019).

Multiple-Hit Hypothesis of NAFLD

There have been different theories created to understand the multifaceted nature of NAFLD/NASH. Initially, the ‘two hits hypothesis’ was created in which hepatic accumulation was said to be attributed to high fat diet, sedentary lifestyle, obesity and insulin resistance - denoted as the ‘first hit’ in this hypothesis. The aforementioned would sensitize the liver to further damage, denoted as the ‘second hit,’ which pathologically activates the fibrogenesis and inflammatory cascades characteristic to NASH (Buzzetti et al, 2016). Later studies discounted the ‘two hits hypothesis’ deeming it as too simplistic to properly mimic the complexity of NAFLD/NASH and its multiple parallel mechanistic layers which work synergistically in the progression of the disease. As a result, a multiple-hit hypothesis has taken precedence over the amiss ‘two-hit hypothesis.’
The multiple-hit hypothesis recapitulates multiple factors that contribute to NAFLD and NASH that function synergistically (Figure 2). As described previously, the accumulation of TAG in conjunction with the increase if FFA release into circulation are major sequential attributes to the pathogenesis of NAFLD and NASH. However, there are many other layers that are responsible for the onset of NAFLD and NASH. Insulin resistance is one of the key factors in the development of steatosis and is physiologically established via dietary habits, environmental factors and genetic factors along with obesity caused by adipocyte proliferation and changes in the intestinal microbiome (Buzzetti et al, 2016). In particular, excess caloric intake which allude to high glucose, high fructose, high fat and/or high cholesterol are environmental factors associated with steatosis. The excess dietary glucose and fructose enter the liver and contribute to the accumulation of TAG via DNL. In addition, hyperinsulinemia or hyperglycemia increase DNL, therefore further impairing β-oxidation and enhancing fatty acid intake (Kim et al, 2018).

Another ‘hit’ of the multiple-hit hypothesis includes hepatocyte-derived factors that can act on non-parenchymal cells which would contribute to the progression of NASH. In response to various stimuli such as excessive lipids, hepatocytes, along with these non-parenchymal cells such as hepatic stellate cells and Kupffer cells, produce chemokines and pro-inflammatory cytokines. The release of chemokines and cytokines is markedly lower from hepatocytes than from stellate cells and Kupffer cells however both contribute to infiltration of macrophages into the liver and activation of stellate cells - a clear marker of NAFLD transition to NASH (Kim et al, 2018; Tanaka et al, 2019).
Hepatocytes release extracellular vesicles which have been reported to occur at increased levels in NASH mouse models and in patients with NASH (Povero et al, 2015). The extracellular vesicles released from hepatocytes act as messenger signals when treated with lipotoxic fatty acids which in turn mediate intercellular communication between hepatocytes and stellate cells and Kupffer cells, further intensifying inflammation and fibrosis. Due to its key role in the pathogenesis of NASH, there have been efforts to block the release of extracellular vesicles as a therapeutic consideration (Kim et al, 2018).

As part of the multiple-hit hypothesis, contributions of adipose tissue and intestine have been recently studied to determine inter-organ crosstalk and its effect on NASH. Growing evidence suggests that the adipose tissue-liver and intestine-liver axes play crucial roles in the pathogenesis of NASH (Vonghia et al, 2015). Previous studies have shown that white adipose tissue (WAT) systematically affects hepatic inflammation via impaired suppression of insulin-mediated lipolysis (Bijnen et al, 2018). In addition to WAT, brown adipose tissue (BAT) is also associated with the progression of NAFLD as it has been demonstrated that obese mice fed with high fat diet (HFD) display mitigated hepatic steatosis post transplation of BAT (Bijnen et al, 2018). The intestine-liver axis is also vital in understanding the progression of NAFLD as emerging evidence suggests that lipid intermediates from the intestine trigger the onset of NAFLD/NASH and insulin resistance (Jiang et al, 2015). Furthermore, intestine-derived hormones such as Fibroblast Growth Factor 19 (FGF19) and Glucagon-Like Peptide 1 (GLP-1) impact the development of NAFLD/NASH as well which has led to studies regarding hormone-based therapy for treatment of NASH (Wiest et al, 2017).
Figure 2. Multiple-Hit Hypothesis model of Pathogenesis of NAFLD/NASH. Kim et al, 2018. Frontiers in Endocrinology.
Understanding the Pathogenesis of NAFLD in vitro

The most definitive method of diagnosing NAFLD pathogenesis is with a liver biopsy however this is significantly complicated for studying a disease in which there are multiple stages and due to the overall process of identifying patients for obtaining tissue samples (Merrell and Cherrington, 2011). The limitations of this method have pushed the field into investigating other techniques of accurately modeling NAFLD and currently, there are multiple animal models that feature genetically obese rodents such as the use of obese Zucker rats to represent NAFLD (Larter and Yeh, 2008). However, in most patients, the development of NAFLD takes many years and is a result of multiple compounding factors, as described in the multiple-hit hypothesis, that are not able to be replicated in rodent models since their liver pathology rarely progresses beyond steatosis to NASH (Kanuri and Berghain, 2013). Although obese animal models are commonly available, we have yet to create an ideal animal model of NAFLD that would be an apt representation, displaying both steatosis and inflammation as well as hepatocyte injury. Nonetheless, it is difficult to accurately recapitulate NAFLD in humans as these animal models disregard other factors such as physical activity, environmental influences, and genetics (Kanuri and Berghain, 2013).

Several human-based in vitro systems have been established to investigate NAFLD. Hepatoma-derived cell lines are widely used and include HepG2, Huh7, and HepaRG cells however they exhibit an abnormal genotype and lack population diversity due to the cancerous background of these cell lines (Boecksman et al, 2018). Most importantly, these cell lines demonstrate poor liver-specific metabolic competence causing these cell lines to be an insufficient choice in modeling NAFLD (Boecksman et
This is in vast contrast to primary human hepatocytes which are considered the gold standard for optimal hepatic in vitro modeling (Muller and Sturla, 2019). Primary human hepatocytes exhibit high functionality relative to the human organ in vivo and are considered to be highly reliable for predictive results in both toxicological and pharmacological settings (Muller and Sturla, 2019). Fresh primary human hepatocytes are obtained from liver resections or whole liver organs that are deemed non-transplantable or are cryopreserved by commercial vendors. One cause for concern for this approach is donor variability which can be viewed as beneficial in order to relate individual variations in genetic polymorphisms while donor difference can lead to poor reproducibility and discrepancies in experimental results (Muller and Sturla, 2019). Nonetheless, primary human hepatocytes are the most ideal choice for studying and modeling NAFLD and its various key stages due to their ability to most accurately mimic the physiological hepatic state (Muller and Sturla, 2019).

To induce intracellular lipid accumulation, oleic acid and palmitic acid are often used in static culture (Boecksman et al, 2018). Oleic acid and palmitic acid are naturally occurring fatty acids, respectively unsaturated and saturated, found in various animal and vegetable fats and oils (Juarez-Hernandez et al, 2016). It has been found that saturated fatty acids, like palmitic acid, are more toxic than unsaturated fatty acids through the investigation of various ratios of oleic acid to palmitic acid in hepatic cell culture (Gómez-Lechón et al, 2007). Further research into the concentrations derived from the differences in free fatty acids and plasma levels of healthy and NASH patients has led to achieving the optimal oleic acid:palmitic acid ratio to achieve a lipotoxic hepatic cell
culture system as a tool for understanding the complex disease progression of NAFLD (Feaver et al, 2016).

Drug Metabolism and Cytochrome P450 in NAFLD

The most frequently prescribed drugs are cleared by detoxification through metabolism which is the main function of the liver. There are a variety of enzymes that overlap in substrate specificity that is expressed in the liver and are divided into Phase I and Phase II drug-metabolizing enzymes (DMEs) (Merrell and Cherrington, 2011). Phase I reactions are oxidizing reactions that typically convert a parent drug into a polar metabolite by adding or exposing functional groups whereas Phase II reactions are conjugating reactions wherein an endogenous substrate combines with the polar metabolite to form an even more polar conjugate. Since there is usually an excess of the endogenous substrates, which include sugar, glutathione or sulfur groups, Phase II reactions are rarely rate-limiting, except in some cases of acute toxicity (Na et al, 2018). In contrast, Phase I reactions are carried out by cytochrome P450 (CYPs) enzymes are rate-limiting and are clinically significant for drug-drug interactions (DDIs). DDIs occur when multiple drugs are present, the influence of one drug on a cytochrome enzyme can affect the downstream metabolic rate of another drug altering its toxicity and dosage efficacy.

CYPs are the major enzymes involved in drug metabolism; the P450 isoforms CYP3A4, CYP1A1, CYP2B6, CYP2C9, and CYP2E1 contributes to the metabolism of
the vast majority of drugs (Zanger et al, 2008). CYP3A4 is of most importance as the most abundant CYP in the human liver that metabolizes the majority of drugs (Anzenbacher et al, 2001). CYP1A, CYP2B6, CYP2C9 and CYP2E1 account for approximately 9%, 4%, 17%, and 2%, respectively, of the hepatically cleared drugs found to be subject to Phase I reactions (Zanger et al, 2008). Many nuclear receptors, classified as transcription factors (TFs), regulate CYP expression and therefore control the metabolism of their endogenous ligands (Table 1). For instance, TF pregnane X receptor (PXR) regulates the expression of CYP3A via activation of toxic bile acids which are substrates for CYP3A - the most predominant hepatic CYP (Williams et al, 2004). The inadvertent activation of PXR can lead to undesirable DDIs. For example, troglitazone activates PXR at therapeutic doses and is metabolized by CYP3A to a potentially toxic quinone, which caused fatal hepatotoxicity in rare cases. Closely related drugs rosiglitazone and pioglitazone, which do not activate PXR, do not form reactive quinones and are not hepatotoxic (Willson and Kliewer, 2002). Several other drug-metabolizing genes are activated by other receptors, including constitutive androstane receptor (CAR) and the aryl hydrocarbon receptor (AhR). Drug inducers Rifampicin, Omeprazole, and Phenytoin commonly prescribed drugs that are widely used to assess CYP inducibility. Rifampicin is known to activate the PXR transcription factor, which regulates CYP3A4 expression; Omeprazole activates the AHR transcription factor, which regulates CYP1A1 expression; and Phenytoin activates the CAR transcription factor, which is known to regulate both CYP3A4 and CYP2B6. As PXR and CAR are capable of transcriptional induction of an array of drug-metabolizing genes that cover all phases of
hepatic drug disposition, there is considerable interest in defining their relative contributions to CYP-mediated DDIs (Wang et al, 2013).

NAFLD as an Inflammatory Disease

Inflammatory diseases such as NAFLD and NASH can modulate drug pharmacokinetics through several mechanisms, thus drug metabolism is highly affected by inflammation (Morgan, 2009). The inflammation is generally caused by the activation of the immune system in response to tissue damage or infectious agents (Morgan, 2009). As a result, inflammatory mediators such as proinflammatory cytokines regulate acute phase protein synthesis inflammation (Medzhitov, 2008). As the most important site for drug clearance, the liver is susceptible to inflammatory stimuli that downregulate activity and expression of most CYP enzymes which can then cause reduced clearance and increased drug toxicity (Jover et al, 2002). Cancer is a compelling case in which inflammation causes altered drug metabolism. Expression of acute phase proteins is upregulated via transcriptional activation of major as inflammatory cytokines such as TNF-α, interleukins and interferons reach the liver. Subsequently, these pathways induce expression of nuclear factors such as activation of mitogen-activated protein kinases that modulate the expression of hepatocyte nuclear factors such as C/EBβ and NFkB which controls transcription of DNA, cytokine production and cell survival (Elfaki et al, 2018).

While it is known that different cytokines suppress specific P450 genes, transcriptional regulation of CYPs by inflammatory mediators has been demonstrated in
only a few cases and is largely unknown (Elfaki et al, 2018; Morgan et al, 2002). Even in the case of hepatic viral infection, different virus types seem to either regulate CYP activity in no clear pattern, suggesting that detailed studies are needed. Therefore, there is a need to better understand the effects of inflammation on DDI pharmacodynamics, which will improve our ability to control this variability in DDIs.
The following section describes the material, methods and techniques used throughout the experimentation required for this study. In brief, primary human hepatocytes were used to develop an *in vitro* model of NAFLD, which was characterized through multiple assays to validate cell health, lipid accumulation and inflammatory response. Ultimately, this NAFLD model, as well as the healthy culture, were exposed to various drugs and their transcriptomic response to such chemicals was assessed via qRT-PCR.

**Primary Human Hepatocyte Culture**

Cryopreserved primary human hepatocytes were obtained through the Cell Resource Core (CRC) at the Massachusetts General Hospital (MGH). This study used three different donors with varying age, gender and BMI, to better account for person to person variations. The donor information can be found in Table A.

The cryopreserved hepatocytes were thawed by rapidly swirling the cryovials in a 37°C water bath until there were no visible ice crystals in the liquid. Then, each tube is transferred into a 40 mL mixture of 40% Percoll® (Millipore Sigma, USA) in DMEM - Dubelcco’s Modified Eagle Medium (Thermo Fisher Scientific, USA), which is then gently mixed and centrifuged at 100g for 10 minutes. The viable cells pellet at the bottom of the tube. The suspending media is then aspirated, and the pellet is resuspended in 5mL of fresh seeding media, made with William’s Medium E (Millipore Sigma) supplemented
with supplemented with 10% fetal bovine serum (FBS, Sigma, St Louis, MO, USA), 0.5 U/mL insulin, 7 ng/mL glucagon, 20 ng/mL epidermal growth factor, 7.5 ug/mL hydrocortisone, 200 U/mL penicillin, 200 ug/mL streptomycin, and 50 ug/mL gentamicin. The resuspended cells were counted using a Cellometer K2 fluorescent viability cell counter (Nexcelom Bioscience, Lawrence, MA, USA) following the manufacturer instructions. Following the gold standard of in vitro NAFLD modeling, a 2.5 dimension (2.5D) sandwich culture model of NAFLD with the viable cryopreserved human hepatocytes will be utilized (Dunn et al, 1989). Models that are one cell layer thick but support the apical and basal polarization of the primary hepatocytes and the formation of bile canalicular networks are classified as 2.5D. In addition to this polarization, the 2.5D models were chosen for providing long-term stability for hepatic phenotype which is important for developing disease models, like NAFLD, where the pathologies might take time to develop in vitro. Here, the viable cells were then seeded onto a pre-collagen coated 12 well plate (Corning, New York, NY) at a cell density of 600,000 cells per well and incubated at 37°C and 10% CO2. After 24 hours, the media is aspirated from each well and 200µL of a top gel consisting of 1.25 mg/mL rat tail collagen and 10X DMEM is applied and allowed to polymerize at 37°C for one hour before adding the appropriate Healthy or Steatotic media. The cells are put back into the incubator and media is replenished every day for the following 7 days of culture.
Table 1. Liver donor information including sex, age, donor path, cause of death, BMI and WIT.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sex</th>
<th>Age</th>
<th>DCD/DBD</th>
<th>Cause of Death</th>
<th>BMI</th>
<th>WIT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>Female</td>
<td>47 yo</td>
<td>DCD</td>
<td>Anoxia</td>
<td>46.9</td>
<td>17 mins</td>
</tr>
<tr>
<td>Donor 2</td>
<td>Male</td>
<td>52 yo</td>
<td>DCD</td>
<td>Anoxia</td>
<td>27.6</td>
<td>29 mins</td>
</tr>
<tr>
<td>Donor 3</td>
<td>Male</td>
<td>36 yo</td>
<td>DCD</td>
<td>Head Trauma</td>
<td>38.4</td>
<td>19 mins</td>
</tr>
</tbody>
</table>

**Induction of Steatosis**

In order to induce steatosis or maintain the cells in a healthy lean state, the media composition was altered to include fatty acids, and high glucose and insulin. The healthy medium is based on William's Medium E supplemented with 100 µU/mL insulin, 7 ng/mL glucagon, 20 ng/mL epidermal growth factor, 7.5 µg/mL hydrocortisone, 200 U/mL penicillin, 200 µg/mL streptomycin, and 50 µg/mL gentamicin. The steatosis medium includes the addition of 800µM oleic acid (OA), 400µM palmitic acid (PA), 1000µU/mL Insulin and 20mM D-Glucose outlined in Table 2. So, the steatotic media includes a total of 1.2mM fatty acids as a 2:1 ratio of OA:PA, ten times more insulin and twice the glucose than the basal lean media. The cells were exposed to the corresponding media after the polymerization of the collagen top gel and the media was replenished every 24 hours.
Table 2. Hepatic culture media composition.

<table>
<thead>
<tr>
<th></th>
<th>Healthy Culture</th>
<th>Steatotic Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>2g/L D-Glucose</td>
<td>4g/L D-Glucose</td>
<td></td>
</tr>
<tr>
<td>690 pM Insulin</td>
<td>6900 pM Insulin</td>
<td></td>
</tr>
<tr>
<td>7 ng/mL Glucagon</td>
<td>7 ng/mL Glucagon</td>
<td></td>
</tr>
<tr>
<td>7.5 ug/mL Hydrocortisone</td>
<td>7.5 ug/mL Hydrocortisone</td>
<td></td>
</tr>
<tr>
<td>20 ng/mL EGF</td>
<td>20 ng/mL EGF</td>
<td></td>
</tr>
<tr>
<td>5% BSA</td>
<td>5% BSA</td>
<td></td>
</tr>
<tr>
<td>200 U/mL Penicillin</td>
<td>200 U/mL Penicillin</td>
<td></td>
</tr>
<tr>
<td>200 µg/mL Streptomycin [100x]</td>
<td>200 µg/mL Streptomycin [100x]</td>
<td>800µM of Oleic Acid</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>400µM of Palmitic Acid</td>
</tr>
</tbody>
</table>

Drug Treatment

On day 5 of culture, the cells were exposed to either 25µM Rifampicin, 50µM Omeprazole, 50µM Phenytoin, or 0.5% DMSO for control in their corresponding healthy or steatotic media. Rifamycipin, Omeprazole and Phenytoin are prototypical inducers of PXR, AHR and CAR respectively. All samples were normalized to a total of 0.5% DMSO vehicle, which is known to be a weak transcription inducer of multiple CYP450 enzymes at this concentration. The drug treatment was replenished on day 6 and on day 7 the cells were lysed using 400µL per well of TriZol Reagent (Life Technologies) for further gene expression assessment.
Lipid Accumulation

The main characteristic of NAFLD \textit{in vitro} models is the recapitulation of lipid accumulation in hepatocytes in the form of intracellular lipid vesicles. The accumulation is noticeable in the microscopic phenotype of hepatocytes and is quantifiable using hydrophobic dyes that exhibit affinity to lipids such as AdipoRed (Lonza Group AG, Switzerland). AdipoRed Assay Reagent is a lipophilic compound that specifically partitions into the fat droplets in mammalian cells and fluoresces at 572 nm.

On days 0, 1, 3, 5, and 7, Healthy and Steatotic cultures were washed with 500 uL PBS and then fixed with 500 uL 4% paraformaldehyde in PBS for 15 minutes at room temperature after which the cultures were washed again with PBS and stained with 400 uL of AdipoRed for 15 minutes at room temperature. The cultures were then washed with PBS and fluorescent images were obtained using the EVOS fluorescence microscope (Thermo Fisher Scientific, USA). The obtained images were transferred to ImageJ (National Institutes of Health, USA) to quantify lipid accumulation based on total image intensity.

Urea and Albumin Production

To assess the function of the hepatocytes, media samples were collected every other day of the culture period and were tested for urea and albumin secretion. To test for urea, a colorimetric BUN assay was performed with the use of the Stanbio™ BUN Diagnostic Set (Stanbio Laboratories, Cardiff, Wales) with the protocol provided by the
manufacturer. Briefly, the urea assay reagent was prepared by mixing one part of the color reagent with two parts of the acid reagent. Standards were prepared and 10 uL of the standards or media samples were plated on a 96 well flat bottom plates, after which 150 uL of the urea reagent mixture was added. After an incubation at 60°C for 90 minutes, the plate was allowed to cool down (5-10 min) and the absorbance was measured at 520 nm.

To test for albumin, a human ALB solid-phase sandwich ELISA was performed with the use of the Intvitrogen Albumin Human ELISA kit (Thermo Fisher Scientific, USA) with the protocol provided by the manufacturer. Briefly, the assay is designed to measure the amount of target is bound between a matched antibody pair. The provided microplate is pre-coated with a target-specific antibody on which 100 uL of samples, standards and controls are added to each well and bind to the immobilized antibody (capture). The addition of 100uL of biotinylated antibody (detector) creates a sandwich wherein the tetramethylbenzidine (TMB) substrate solution added reacts with this enzyme-antibody-target complex to form a measurable signal at 450 nm that is proportional to the concentration of target present in the original sample.

Cytokine Release

Samples were collected on Days 1 and 7 from both Healthy and Steatotic cultures to obtain a profile of cytokine release during the progression of NAFLD and lipid accumulation. To this end, the Cytokine Human Magnetic 30-Plex Panel for Luminex™
platform (Thermo Fisher Scientific, USA) was used to quantify human cytokines, chemokines and growth factors in the tissue culture supernatant with the protocol provided by the manufacturer. In brief, premixed antibody beads in which internally dyed magnetic microspheres with red and infrared fluorophores of differing intensities are added to each well. Samples are appropriately diluted with provided assay diluent and then are added to each well along with standards and controls. These beads are covalently bound to biotinylated antibodies to create a sandwich immunoassay. The samples are analyzed on a Luminex™ MAGPIX instrument to determine concentration of samples from the standard curve using the curve fitting software.

qRT-PCR

The total RNA was initially isolated from each lysed sample through a phase separation by mixing the 400µL of sample in TriZol reagent with 70µL of 1-Bromo-3-Chloropropane (BCP, Sigma Aldrich) and centrifuging at 15,000g for 10 mins. After centrifugation the aqueous solution was separated and mixed with one volume of 70% EtOH. The sample was further purified using a PureLink RNA Mini Kit (Life Technologies) according to the manufacturer instructions and eluted in 45µL of 18Ω di-water.

The RNA concentration and quality of each sample was measured and assessed using a NanoDrop 2000 (Thermo Fisher Scientific). Approximately 750ng of RNA were converted into cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA)
following the manufacturer instructions. Afterwards, the cDNA samples were diluted 5X with 18Ω di-water.

The qRT-PCR reactions were then performed using PowerUp SYBR green master mix (Life Technologies) as 10µL reactions on 384well plates using a Viia7 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The relative expression was quantified through the comparative CT method, normalizing all the samples to GAPDH and 18S housekeeping genes. Primers for each gene target are displayed in Table 3.

### Table 3. Gene targeting primers.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer Sequence (5’-3’)</th>
<th>Reverse Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>GCATGTCTGAGTACGCACGG</td>
<td>CGAGCGACCAAGGAACCA</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>TCCCAGCTCAGCTCAGTACC</td>
<td>GCCGACATGGAGATTTGGGAA</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>GGACATGAACAAACCCTCAGGAC</td>
<td>TCAACTGCAGTGTTTTCCAAGC</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>AAGCGCTGCTGGACTACAAG</td>
<td>GGAGGTTGGTCAGGGAAAAC</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>AATCACTGTTGGCGTGGGG</td>
<td>AATGGGCAAAGTCACAGTGGGA</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>CTAACACCACATGACCAGCCTC</td>
<td>CCAGGTTACCGTGGAAGACG</td>
</tr>
<tr>
<td>PXR</td>
<td>AGGGGAGAAGTCGGAGCAAA</td>
<td>TGGGTCTCACCTCCAGGTTT</td>
</tr>
<tr>
<td>AHR</td>
<td>TCGTCTAAGGTGTCTGCTGGA</td>
<td>CGCAAACAAAGCCAACGTGAGG</td>
</tr>
<tr>
<td>CAR</td>
<td>CTGGTCACACACTTTCGAGA</td>
<td>GATTCCACAGCTGCTCCCT</td>
</tr>
</tbody>
</table>
Chapter III.

Results

The objective of this study is to understand the drug metabolic responses and inflammatory cytokine profile in non-alcoholic fatty liver disease (NAFLD) in its progression to its more severe form, non-alcoholic steatohepatitis (NASH). To do this, an in vitro model of NAFLD in primary human hepatocytes was constructed and characterized wherein primary human hepatocytes were successfully cultured for 7 days and exhibited excellent morphology and quantifiable macrosteatotic lipid accumulation. These primary human hepatocytes were subjected to drug induction for 48 hours by three different drugs in order to elucidate the difference in drug metabolic response and risk to potential drug-drug interactions (DDIs) between healthy and steatotic livers. Supernatant samples from the same culture constructs were analyzed using a panel of 30 cytokines to assess inflammation markers corresponding to NAFLD. Through evaluation of drug inducibility and inflammatory response, elucidation of the difference in response to drugs and risk for potential DDIs between the Healthy and Steatotic state will be achieved.

Primary Human Hepatocytes Cell Culture Media Optimization and Viability

Primary human hepatocytes were cultured for a period of 7 days such that lipid accumulation could progress to macrosteatotic level which is hypothesized to be a tipping point towards the NAFLD/NASH stage. Subsequently, the media was optimized to
ensure maintenance of culture for a period of 7 days and was initially screened by testing urea production.

As detoxification is a vital function of the liver, ammonia is an extremely toxic base that is produced during the deamination of amino acids. Hepatocytes are the main cell type which metabolizes ammonia into urea, and so urea production is a common marker of specific hepatic function. Here, urea production is measured as an initial screening of which media was best suited for hepatic cultures of at least 7 days. The media supplemented with 7.5 ug/mL hydrocortisone, 20 ng/mL EGF and 7 ng/mL glucagon showed a clear, stable urea production in both Healthy and Steatotic cultures (Figure 3) and allowed for continuation on to multiple primary human hepatocyte cultures with validated supplemented media.

Figure 3. Media Optimization. 
*Media containing supplements hydrocortisone, EGF, and glucagon were optimal for sustaining long-term culture.*
After determining the optimal supplementation of cell culture media, primary human hepatocytes were plated in collagen sandwich cultures from 3 donors (Table 2) in 12 well plates for 7 days. The aspirated media was stored at -80°C for post hoc analysis of urea production and albumin synthesis. Urea production of Donor 2 and Donor 3 was very well maintained (Figure 4b, c); however, Donor 1 displayed a decrease in production particularly in the Healthy culture from day 5 onward (Figure 4a). Albumin synthesis was well maintained and stable for all three donors (Figure 4).
Figure 4. Urea Secretion and Albumin Synthesis. 
Healthy and Steatotic cultures exhibit stable urea secretion and albumin synthesis over 7-day culture period.
Verification of Steatosis and Quantification of Lipid Accumulation

The majority of in vitro NAFLD platforms utilize free fatty acids to supplement media to induce hepatic steatosis. Here, primary human hepatocytes are treated with palmitic acid (PA) and oleic acid (OA) to induce lipid accumulation. As demonstrated in Figure 5, bright-field images indicated a significant increase of lipid droplets in the cell cytoplasm over 7 days in the Steatotic culture while the Healthy culture remained lean.

Representative pictures are shown in Figure 6 of lipid content by AdipoRed™ assay (green). All assays were performed on each donor culture on days 0, 1, 3, 5, and 7. Hoescht stain (blue) was used for its high affinity to DNA and therefore its allocation to the nuclei of the hepatocytes. As a derived version of NileRed, AdipoRed™ presents high affinity for lipids and is fluorescent in the 485-572 nm spectrum. The stained images confirm the increase in lipid accumulation over time for the Steatotic cultures and also suggest an increase in size distribution. The lipid vesicles reach sizes equal or greater than that of the cell nuclei. The total intensity of the images was quantified in ImageJ (Figure 7), displaying significant increase in lipid accumulation from day 1 to 5 for all donors, as well as a non-significant increase in day 7 of steatotic culture.
Figure 5. Bright-field images of culture of 7 days.
*Steatotic cultures show clear progression of lipid of accumulation while Healthy culture remain lean with little to no lipid droplets in cell cytoplasm.*
Figure 6. Lipid accumulation upon palmitic acid (PA) and oleic acid (OA) exposure. 
AdipoRed (green) stain represent lipid content and DAPI (blue) localizes to nuclei over 7-day culture period.
Figure 7. Quantification of Lipid Accumulation.
Lipid droplet grow both in size and number over 7-day culture period in primary human hepatocytes (PHH). Lipid accumulation (a) is measured on days 0, 1, 3, 5, and 7 and is normalized to day 0 (b), validating progression of lipid accumulation in NAFLD in vitro model.
Cytokine Release Profile

The aspirated media of Healthy day 1 and 7 as well as Steatotic day 7 were analyzed for secreted cytokines using the Invitrogen 30-Plex Cytokine panel for Luminex platform. Of the given targets, MCP-1, VEGFa, IP10, IL-8 and IL-1ra showed a definitive signal. For pro-inflammatory cytokines MCP-1 and IP-10, there was a significant increase for the Steatotic culture compared to both the Healthy control and the initial state (Figure 8a, b). VEGFa also showed the highest secretion in the Steatotic culture, however, this increase was significant compared to the lean control but not the initial state (Figure 8c). For the case of IL-8, both Healthy and Steatotic cultures presented significant elevated values compared to the initial state (Figure 8d). For the anti-inflammatory cytokine IL-1ra, there was a significant elevated secretion only for the Healthy culture compared to both the initial state and Steatotic culture, both of which remained low (Figure 8e).

![Graphs showing cytokine release profile](image)

**Figure 8. Cytokine Release Profile.**

*MFIs of Healthy and Steatotic states are obtained from day 7 of culture period and are normalized to day 1 Healthy state. Pro-inflammatory cytokines a) MCP-1, b) IP-10, c) VEGFa, and d) IL-8 are upregulated while e) IL-1ra is downregulated in the Steatotic state.*
CYP450 Transcription Regulation

For the assessment of the drug metabolism in the NAFLD in vitro model, the transcriptional expression of key CYP450 enzymes in all three donors was analyzed through qRT-PCR. Of the CYP450 enzymes of interest, we found a dramatic downregulation of less than 0.2-fold for CYP3A4, 2C9 and 1A1 in the Steatotic cultures compared to their Healthy counterpart, while CYP2B6 and 2E1 were upregulated to approximately 4.9 and 1.9 respectively (Fig. 9a). We also further looked at the transcriptional level of three key transcription factors that regulate CYP450 enzymes, AHR, CAR and PXR, to find that only CAR and PXR were downregulated to less than 0.5-fold in the Steatotic cultures compared to the Healthy control (Fig. 9b).
Figure 9. Basal Expression of CYPs and TFs.
*Fold expression of each a) cytochrome P450 enzyme and b) transcription factors were measured in Healthy and Steatotic states before exposure to drug inducer.*
Drug-Mediated CYP450 Transcription Regulation

For further analysis of the transcriptional regulation of the CYP450 enzymes in the NAFLD model, the cultures were exposed to drug inducers Rifampicin, Omeprazole, Phenytoin or a vehicle control in the last 48 hours of the 7-day culture period. Then, the cells were lysed and analyzed through qRT-PCR to determine the difference in the response to each one of these drugs between Steatotic and Healthy cultures. Of these three drugs, rifampicin is known to activate the PXR transcription factor, which regulates CYP3A4 expression; Omeprazole activates the AHR transcription factor, which regulates CYP1A1 expression; and Phenytoin activates the CAR transcription factor, which is known to regulate both CYP3A4 and CYP2B6. For CYP3A4, we found that despite the downregulation of basal expression of the Steatotic culture compared to the Healthy control, both states were highly inducible to a similar level when rifampicin was introduced into the system (Fig 10a). However, when phenytoin was introduced, only the Healthy cultures presented a clear induction to 10-Fold, while the Steatotic culture remained downregulated (Fig. 10a). For CYP1A1, omeprazole was able to induce the transcription of this enzyme to more than 10-Fold for both states Healthy and Steatotic cultures, despite the downregulation of CYP1A1 in the basal Steatotic control (Fig10b). As for CYP2B6, the basal expression of the Steatotic control already presented an upregulation of 5-Fold compared to the Healthy control, which was further induced to 25-fold when phenytoin was introduced into the system. The healthy cultures also presented a 5-fold induction in the presence of phenytoin (Fig.10c).
Figure 10. Drug Response.

Fold expression in Healthy versus Steatotic states after exposure to drug inducer.
Chapter IV.

Discussion

The major focus of this study was to develop an *in vitro* NAFLD model that incorporates key aspects of NAFLD and assess inflammation and drug response in this diseased state. NAFLD is a clinical syndrome characterized by predominant steatosis that can progress to NASH, liver cirrhosis, hepatic carcinoma and ultimately death (Tanaka et al, 2019). Its prevalence has alarmingly escalated in populations across the world reaching a global prevalence of 25% (Younossi et al, 2017). Despite the high prevalence of NAFLD, there are currently no therapies available to NAFLD patients (Boeckmans et al, 2018). As NAFLD continues to progressively affect individuals, it is imperative that the disease be studied efficiently to properly treat those diagnosed with NAFLD. As a common trigger of NAFLD/NASH, hepatic inflammation is considered to be one of the main causes of hepatic tissue damage (Del Campo et al, 2018). This hepatic inflammation assists the progression of NAFLD to NASH to cirrhosis and, finally, hepatic carcinoma (Boeckmans et al, 2018). As a downstream effect of NAFLD, damaged liver tissue and the accumulated adipose tissue highly express various pro-inflammatory cytokines as the disease transitions to steatohepatitis (Gao and Tsukamoto, 2017). Here, we studied cytokine release profile as feedback to mediate acute phase response (APR), a systemic reaction against tissue injury (Ramadori and Armbrust, 2001).

Although there are currently no drugs on the market that directly treat NAFLD, it is of extreme importance to understand the implications the disease has on drug
metabolism as hepatocytes from steatotic livers often exhibit altered metabolic capacity with varying changes in expression in many cytochrome P450 enzymes and their corresponding transporter proteins (Donato et al, 2006).

In this work, the in vitro NAFLD model created utilized primary human hepatocytes for its advantageous features described in order to accurately recapitulate the human physiological state of the liver. Stable cell culture of primary human hepatocytes was achieved through various rounds of media optimization. The cell culture medium composition required modifications to properly sustain the culture for 7 days such that lipid accumulation could reach levels similar to that of NAFLD/NASH patients. To do so, supplements hydrocortisone, epidermal growth factor (EGF) and glucagon were vital in maintaining primary human hepatocyte culture for the desired culture period. The concentrations of these supplements used in this investigation were chosen as they had the maximum effect in maintaining specific hepatic functions such as urea secretion. Detoxification is a vital function of the liver wherein hepatocytes almost exclusively metabolize the main toxic product of deamination of amino acids, ammonia, into much less toxic urea. Here, it is clear that supplements were vital in the longterm maintenance of primary human hepatocytes as the Healthy medium in which supplements are absent displayed a steep decline in urea production from day 3 while supplemented Healthy and Steatotic media were producing significantly more urea for 7 days (Figure 3).

Establishing the ideal media composition was vital in proceeding to expand the study to using primary human hepatocytes from 3 human donors. The donors (Table 1) were selected based on the ability to attach efficiently to collagen-coated plates and vary in age, sex, and BMI. Primary human hepatocyte cultures from these 3 donors were
maintained with the optimized supplemented medium for 7 days and showed stable urea secretion and albumin synthesis (Figure 4). Albumin is almost exclusively produced by the liver and is the most abundant blood protein. As such, in addition to urea, it is considered to be an important marker for the synthetic metabolism of hepatocytes. All primary hepatocytes in culture from each donor display stable metabolic activity in regard to their sustained urea production and albumin synthesis (Figure 4).

Most epithelial cells have a single apical and a single basal surface, however hepatocytes are unique in that they exhibit a belt of apical surface which are bile canaliculi and therefore have two basal surfaces. These bile canaliculi surround each cell and divide them into two sinusoidal surfaces which are each in contact with the extracellular matrix. Hence, a cellular polarity evocative of the in vivo state needs to be maintained in order for hepatocytes to function properly (Dunn et al, 1991). By using the well-established collagen sandwich configuration in which the hepatocytes are sandwiched between two layers of collagenous matrix, the hepatocytes are in an environment that more closely resembles in vivo cell geometry (Dunn et al, 1989). Images of the plated hepatocytes were obtained every other day of the culture period of both Healthy and Steatotic states (Figure 5). As observed, elements of sinusoidal spatial architecture are present in our model - bile canaliculi formation as bright white outlines of each cell is visible and maintained beginning on day 1, and the distinct polygonal morphology of hepatocytes was maintained throughout the 7-day period. In addition, an in vivo-like phenotype is evidenced by the polarized architecture, a vital indicator for differentiated hepatic function and longevity, throughout the culture period.
A healthy liver and steatotic liver were simulated in our model in vitro, in order to best recapitulate NAFLD/NASH. This model better represents a healthy liver compared to other conventional in vitro systems as glucose and insulin levels are similar to that of physiological levels (LeCluyse et al, 2012). This is crucial in enforcing de novo lipogenesis related to glucose and insulin, making the Healthy state a meaningful control to which to compare the Steatotic state. According to the pathogenesis of NAFLD, lipid droplets accumulate due to both increased de novo lipogenesis due to elevated glucose and insulin levels and free fatty acid circulation (Donnelly et al, 2005). Lipid accumulation was induced in the Steatotic state hepatocytes by incorporation of free fatty acids oleic acid and palmitic acid as well as exposure to elevated glucose and insulin levels similar to levels found in patients diagnosed with NAFLD. The mixture of oleic acid and palmitic acid are meant to mimic concentrations of these factors in patients with NAFLD. Images in Figure 5 showed little to no lipid droplets visible within the cell in the Healthy state. To assess lipid accumulation, hepatocytes in the Steatotic state were stained with AdipoRed (Figure 6). Distinct lipid droplets are visible in increasing size and distribution over the culture period in all 3 donors. These changes were quantified using ImageJ wherein cells from all donors show a 4-fold increase in steatosis normalized to their day 1 states (Figure 7). Moreover, the formation of lipid droplets larger than the nuclei is clearly present and, if able to displace the nucleus, is indicative of macrovesicular steatosis (Gluchowski et al, 2017).

There are 5 major cytokines that provided a clear signal over the culture period in both Healthy and Steatotic states, MCP-1, IP-10, VEGFa, IL-8 and IL-1ra (Figure 8). Monocyte chemoattractant protein-1 (MCP-1) is among the pro-inflammatory
molecules that are upregulated in human adipose tissue which adheres to the results here in which MCP-1 is upregulated in the Steatotic state as compared to the Healthy state on day 7 of the culture period (Figure 8a). As a member of the cysteine-cysteine chemokine family, MCP-1 promotes the migration of inflammatory cells by chemotaxis and integrin activation (Boring et al, 1998). In addition, it has been shown MCP-1 deficiency improves insulin resistance and hepatic steatosis (Nio et al, 2012). We hypothesize that the upregulation of MCP-1 in the Steatotic state signifies the progression of NAFLD into the NASH state as this suggests an increase in inflammation and potentially insulin resistance over the culture period. IP-10, otherwise known as C-X-C motif chemokine 10, is produced in hepatocytes as well as macrophages, hepatic stellate cells, and endothelial cells. IP-10 plays a role in the pathogenesis of steatohepatitis via induction of inflammation, oxidative stress, and lipogenesis (Zhang et al, 2014). A recent study reports that lipid-accumulated hepatocytes release extracellular vesicles (EVs) that contain IP-10 and have a key role in the recruitment of macrophages in NAFLD (Ibrahim et al, 2016). As a potential therapeutic target for the treatment of NASH, progressive liver injury and insulin resistance in multiple studies (Xu et al, 2016; Chang et al, 2015), the increase of IP-10 secretion in the Steatotic state (Figure 8b) further validates the presence of inflammation in the Steatotic state and the onset of liver injury and insulin resistance.

VEGFa, or vascular endothelial growth factor A, again is upregulated in the Steatotic state as compared to the Healthy state (Figure 8c) and is known to mediate inflammation in NASH wherein hepatocytes are the main source of secretion under various liver stress stimuli. It has been reported that NASH patients exhibit elevated
levels of VEGFa (Tarantino et al, 2009; Yoneda et al, 2007) which correlate to our findings. IL-8, or interleukin 8, is a chemoattractant that gives rise to neutrophil infiltration and is a strong predictor of increased fibrotic liver injury. Previous reports have shown that IL-8, along with MCP-1, may reflect upregulated gene expression during liver fibrosis in NAFLD (Glass et al, 2018). Here, we see a slight upregulation in the Steatotic state (Figure 8d). Although not particularly substantial, the difference between Healthy and Steatotic states may have been more distinct if the culture period was extended to further induce lipid accumulation and hepatocyte injury.

The anti-inflammatory cytokine interleukin 1 receptor antagonist (IL-1ra) is highly expressed in healthy livers (Tilg et al, 2016). Our results indicate a significant downregulation of IL-1ra in the Steatotic state (Figure 8e) and abide by the conclusions made in other studies suggesting that IL-1ra could be a potential therapeutic target in mediating and controlling the progression of inflammation in NAFLD patients.

One of the many changes in cellular metabolism found in NAFLD is the dysregulation of CYP450 enzymes observed in patients as well as in other in vitro models (Fisher et al, 2009; Kostrzewski et al, 2017). The dysregulation of CYP450 enzymes is critical to the development of new therapeutic drugs as patients in the NAFLD spectrum could present an altered rate of drug clearance, which could cause increased drug toxicity, inefficient treatment dosage, or rare drug-drug interactions compared to healthy individuals. This system’s drug metabolism profile lines up with previous studies, showing the expected downregulation of CYP3A4, 1A1 and 2C9, as well as the upregulation of CYP2B6 (Jamwal et al, 2018; Fisher et al, 2009). This shows that the created system has the ability to model the intricate mechanisms that affect the regulation
of drug metabolism in NAFLD patients and can, therefore, be used to study the cause and effects of lipid accumulation in the metabolism of xenobiotics.

In order to further understand the cause and effect of the dysregulation of CYP450 enzymes in NAFLD, the model was subjected to three drug treatments with Rifampicin, Omeprazole, or Phenytoin. These drugs are used in the pharmaceutical research industry to induce drug-mediated regulation of specific CYP450 enzymes (Lynch and Price, 2007). Each drug activates a specific transcription factor in the hepatocytes’ cytoplasm, which causes it to be translocated into the nucleus, where it binds to CYP450 promoters to induce transcription. Rifampicin is a well-known and established activator of the PXR transcription factor, which causes the upregulation of CYP3A4 (Luo, 2002). Omeprazole activates the AHR transcription factor, which upregulates CYP1A1 (Quattrochi and Tukey, 1993). Finally, Phenytoin was used as a CAR-specific activator, which is known to upregulate CYP2B6 as well as CYP3A4 (Wang et al, 2004). It is important to note that Phenobarbital is more common and thought to be a stronger activator of CAR, but it also presents promiscuity towards PXR activation. Therefore, Phenytoin was used as it has been demonstrated that it activates CAR specifically, and not PXR (Wang et al, 2004).

Our results suggest that the PXR inducibility mechanism is still intact in NAFLD. Despite the dramatic downregulation of CYP3A4 in the Steatotic state compared to Healthy control, both systems showed a significant induction to similar mRNA levels when Rifampicin is introduced into the system. For this to be the case, PXR needs to be readily available in the hepatocytes. This also corroborated the idea that, despite being one of the main regulators of CYP3A4, PXR is not the cause of the initial downregulation.
of the enzyme during the disease. This idea was initially suggested in a PXR-knockout rodent model, which showed a PXR-independent downregulation of CYP3A4 (Jamwal et al, 2018). Now we further demonstrate that the PXR molecule is also still able to activate CYP3A4 in NAFLD, and possibly maintain the consequent DDIs during the disease.

On the contrary, Phenytoin was only able to induce CYP3A4 transcription in the Healthy state, while the Steatotic state remained impaired in the presence of the inducer. This demonstrates that the CAR induction of this enzyme is impaired in NAFLD and might suggest that the transcription factor is inhibited and could be one of the causes for the initial downregulation of CYP3A4 in a steatotic liver.

For the case of CYP1A1, our results show that the functionality of the AHR induction mechanism is retained in NAFLD. Similar to Rifampicin, Omeprazole was able to induce the transcription of CYP1A1 to comparable levels in both the Steatotic and Healthy cultures, despite the basal downregulation of the enzyme in the Steatotic control. This again suggests that AHR mediated DDIs can still occur in NAFLD patients and may suggest that the functionality of this transcription factor is not responsible for the impaired transcription of CYP1A1 in NAFLD.

Currently, there is no consensus on the effect of NAFLD on CYP2B6 regulation, mainly due to the lack of research on this particular enzyme. Some studies have found an increase in the expression of this protein in the progression of NAFLD to NASH, but other models have shown downregulation of the enzyme (Fisher et al, 2009; Pant et al, 2009). Our results show a clear increase in the mRNA expression of CYP2B6 across all donors, as well as an increase in the induction sensitivity to Phenytoin. Interestingly,
these results suggest that the CAR mediated induction of CYP2B6 is still functional under steatosis, and potentially has become more sensitive. This seems to contradict the functionality of CAR for the induction of CYP3A4 through the same pathway, but this could still be explained through other regulatory mechanisms. It can be postulated that epigenetic factors are impairing the ability of CAR to bind the CYP3A4 promoter specifically, while CYP2B6 could have become more readily available, also explaining its increase during the disease and CAR mediated inducibility.

We have shown how this system is able to model the lipid accumulation, inflammatory response, drug metabolism transcriptome and CYP450 drug-mediated transcription regulation of NAFLD in an *in vitro* culture model. We used cryopreserved human hepatocytes, the gold standard for *in vitro* drug metabolism assays, to study the increase of intracellular lipid vesicles during long-term steatosis, the secretion of cytokines that are key to the transition to NASH, and finally the transcription regulation of CYP450 enzymes and the functionality of key transcription factors during NAFLD. To our knowledge, this is the first study on the induction of CYP450 enzymes on an *in vitro* NAFLD model. We have also demonstrated how drug inducibility can be drastically affected in steatotic hepatocytes compared to their healthy counterpart, which suggests that NAFLD patients could present abnormal or unique DDIs. As the epidemic of NAFLD keeps growing without any approved therapeutics, it becomes increasingly critical to understand the regulation and effects of this disease on the drug metabolism capabilities of the liver, not only for the discovery of NAFLD therapeutics, but also for the potential interactions between the disease and unrelated treatments.
References


Benedict, M. & Zhang, X. Non-alcoholic fatty liver disease: An expanded review. World J Hepatol 9, 715-732 (2017)5468341


Moore JB. Non-alcoholic fatty liver disease: the hepatic consequence of obesity and the metabolic syndrome. Proc Nutr Soc 2010; 69: 211-220 [PMID: 20158939 DOI: 10.1017/ S0029665110000030]


Oseini AM, Sanyal AJ. Therapies in non-alcoholic steatohepatitis (NASH). Liver Int. 2017;37:97-103. DOI: 10.1111/liv.13302


Than NN, Newsome PN. A concise review of non-alcoholic fatty liver disease. Atherosclerosis 2015; 239: 192-202 [PMID: 25617860 DOI: 10.1016/j.atherosclerosis.2015.01.001]


