NON-ALCOHOLIC STEATOHEPATITIS (NASH) MODELS FOR PRECLINICAL TESTING OF CELL DEATH INHIBITORS IN VITRO

by

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DEDICATION

In dedication to my family, especially my father, for their support; to Kamyar for always being there for me, ready to help, never giving up; to Dr. Richard L. Cho for his unwavering dedication and continual support; and to my cat Maia, for making me laugh with her perfectly timed antics that kept my spirits up.

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Secondly, I would like to thank Peter Masschelin for his help with the topic; without his dedicated research, writing on my chosen topic would not have been possible. Also, thank you to Sasha Stoddard for her assistance in the lab and for answering my many questions.

Finally, special thanks to the School of Systems Biology at George Mason University, and the INOVA Health Systems for their resources.

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LIST OF ABBREVIATIONS AND SYMBOLS

Absorption, Distribution, Metabolism, and Excretion ……..ADME
Alanine Aminotransferase…….ALT
Alpha-Smooth Muscle Actin ……....αSMA
Aspartate Aminotransferase……..AST
Cytokeratin 18.....CK-18
Endoplasmic Reticulum …….ER
Fluoromethyl Ketone ……FMK
High-Fat Diet…….HFD
Mallory-Denk Bodies……..…….....……..MBDs
Methionine-Choline Deficient ..... MCD
Monocyte Chemoattractant Protein…… ..MCP-1
Monounsaturated Fatty Acid.....MUFA
Non-Alcoholic Fatty Liver Disease …….NAFLD
Non-Alcoholic Steatohepatitis …… ..…NASH
Polyunsaturated Fatty Acid..... PUFA
Tumor Necrosis Factor Alpha ..... TNF-α

ABSTRACT

NON-ALCOHOLIC STEATOHEPATITIS (NASH) MODELS FOR PRECLINICAL TESTING OF CELL DEATH INHIBITORS IN VITRO

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George Mason University, 2019

Thesis Director: Dr. Ancha Baranova

Given the worldwide prevalence of Non-Alcoholic Fatty Liver Disease (NAFLD), and a substantial proportion of simple steatosis patients progressing to Non-Alcoholic Steatohepatitis (NASH), a condition associated with liver inflammation, and, further, to cirrhosis and end-stage liver failure, the development of *in vitro* models for NAFLD is warranted. Currently used models of this kind are overly simplistic, and incompatible with high-throughput experimentation mode which is necessary for the screening of potential therapeutics capable of the reversal of NASH phenotype. The best way to proceed forward would be with utility of liver-on-a-chip devices harboring established immortal cell lines, for example, HepG2 – a "workhorse" of liver toxicology – or its less malignant counterpart HepaRG. With an aid of these devices, many potential therapeutic compounds may be profiled either as monotherapy, or as synergistic enhancers for other

potential treatments. The targeting of the necroptosis, rather than “classical” apoptosis, is a promising therapeutic option aimed at the delay of progression or even a reversal of NAFLD and other inflammation-associated liver diseases.

CHAPTER ONE: INTRODUCTION

1.1 Epidemiology of Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease worldwide [Vernon G et al., 2011]. According to recent meta-analysis, global prevalence of NAFLD is at approximately 25%, with highest prevalence in the populations of Middle Eastern and South American origin, and lowest in the African nations [Younossi et al., 2016]. Obesity is the most common comorbid condition associated with NAFLD, with approximately 51% of NAFLD patients also being obese, followed by metabolic syndrome (42%) and hypertension (39%) [Younossi et al., 2016].

NAFLD is commonly divided into the following categories: 1) nonalcoholic fatty liver, including so-called isolated hepatic steatosis, a marked intrahepatic lipid accumulation, and 2) nonalcoholic steatohepatitis (NASH), which is a progressive form of NAFLD eventually leading to accumulation of the fibrotic changes and cirrhosis [Rinella, 2015]. Moreover, liver parenchyma affected by NASH is a permissive environment for the development of hepatocellular carcinoma, which comprises 90% of all liver cancers [European Association for the Study of the Liver, 2012]. After the onset of decompensated cirrhosis or hepatocellular carcinoma, liver transplant is the only

curative option available for patients with NASH. The pathway for the development of NASH and its advanced forms is depicted at **Figure 1.**

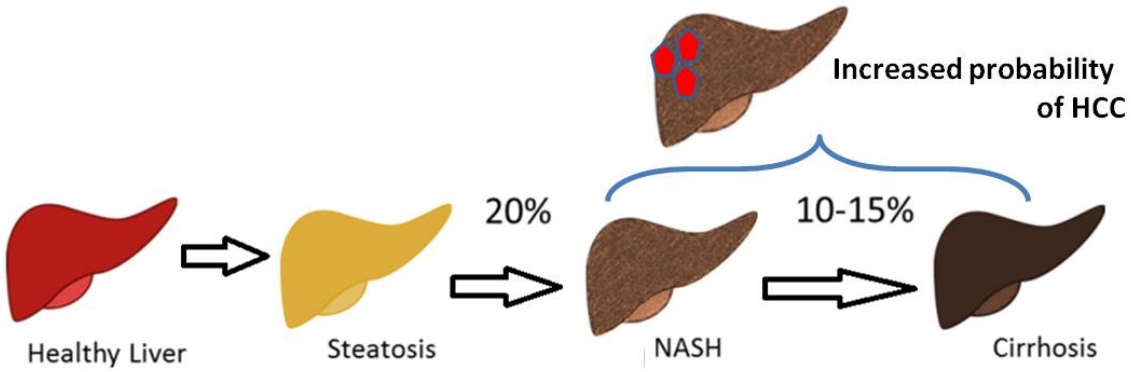

Figure 1 Progression of NAFLD to NASH and to Cirrhosis

1.2 Diagnostics of Nonalcoholic Fatty Liver Disease

The lack of patient awareness and proper screening explains common finding of the progressive form of NAFLD or NASH, which is already at the cirrhotic stage at the time of initial diagnosis. Liver biopsies are a proven diagnostic modality for NAFLD and NASH. Progression of NAFLD to NASH has been associated with marked alterations in hepatocyte histology. On a biopsy, histological hallmarks of NASH are clearly visible and include the presence of ballooned hepatocytes with Mallory-Denk bodies (MBDs) - aggregates of intermediate filament proteins, inflammation, large lipid droplets indicative of steatosis and more or less evident fibrotic changes [Lakhani HV et al, 2018]. Unfortunately, liver biopsy is a highly invasive procedure, commonly associated with

substantial discomfort, and some morbidity, spurring the need for the development of less invasive techniques.

Examples of slide sections representing various histologically defined forms of NAFLD can be seen in Figure 2.

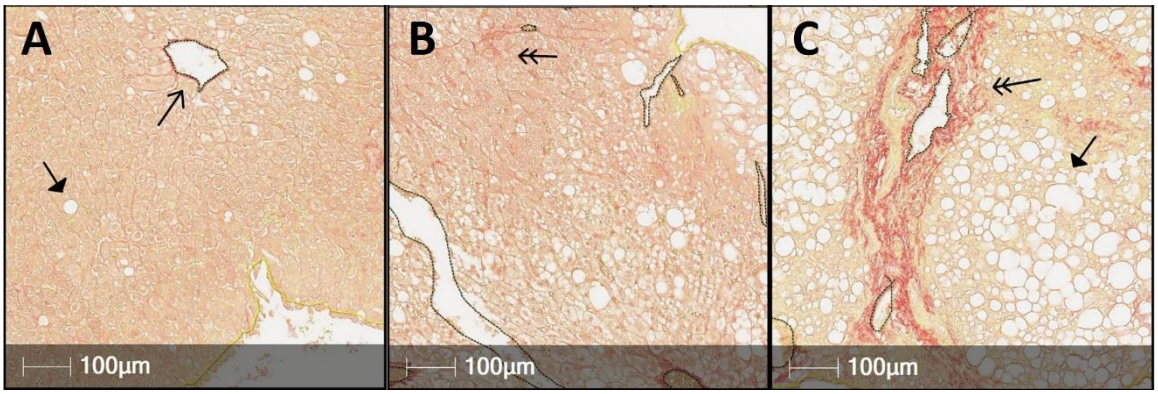

Figure 2 Stained sections of liver biopsy material collected from patients with various forms of NAFLD.
A: Mildly abnormal liver parenchyma with less than 5% of slide displaying steatosis, signs of fibrosis are minimal, a majority of hepatocytes are normal. B: In NAFLD, there is moderate presence of steatosis and fibrosis, amounts of normal hepatocytes are reduced. C: In NASH, both steatosis and fibrosis are severe, with a few functional hepatocytes remaining. Arrows pointing at contoured white areas point at sinusoids, which are excluded from histological assessment. Double arrow points at collagen deposits stained with Sirius Red, which are indicative of a degree of fibrosis. Closed arrows point at relatively large lipid droplet. (From Thesis work of Sasha Stoddard, MS, with permission and some changes).

So far, a number of minimally invasive diagnostic techniques are proposed for screening at-risk cohorts, including patients with obesity, metabolic syndrome, type 2 diabetes, or hyperlipidemia. These techniques include ultrasonic abdominal imaging, elastography or biomarker panels [Castera L et al., 2019]. It is of note, that commonly used measurements of aminotransferase enzymes activities, so called AST and ALT, are

known for their low sensitivity [Uslusoy HT et al, 2009]. In-office ultrasound was suggested as a first line screening tool for defining steatosis, but this modality is not specific enough to distinguish NASH from NAFLD. Magnetic Resonance imaging is capable of accurate quantification of steatosis or defining the stage of fibrosis, but is too expensive for establishing as routine practice. Much promoted use of transient elastography (FibroScan), which measures the stiffness of the liver parenchyma is much more sensitive to the stage of liver fibrosis, than to the degree of liver fatness. In addition, this technique is less precise when used in obese patients in the need of assessment for NAFLD and NASH [Arora A and Sharma P, 2012].

In the last decade, a variety of serum-based biomarkers and their combinations were proposed as liver biopsy alternatives for identification of patients at high risk of the development of NASH and advanced fibrosis [Vilar-Gomez E and Chalasani N, 2018; Page S et al., 2013; Younossi ZM et al., 2010]. For some of these biomarkers, their alterations are secondary to pathophysiological processes affecting liver parenchyma; these biomarkers are called “indirect”. Other markers of liver dysfunction reflect the extent of pathophysiological process, for example, deposition of the collagen, directly. Both direct and indirect biomarkers may quantify the fibrosis (NAFLD fibrosis score, FibroTest®, ELF™, FIB-4 index and BARD score) or inflammation (e.g. circulating fragments of cytokeratin-18, and some cytokines), or both (combinatorial panels) **(Figure 3)**. While these non-invasive tests showed a promise in several studies, their NASH cut-off values for the use in clinical practice have not yet been validated, and may be population-specific. In the future, patients might be diagnosed and treated according to

their molecular signatures, built upon the genomic profile of the entire patient's body, transcriptomics of liver parenchyma, or proteomic and metabolomic biomarkers present in the patient's blood.

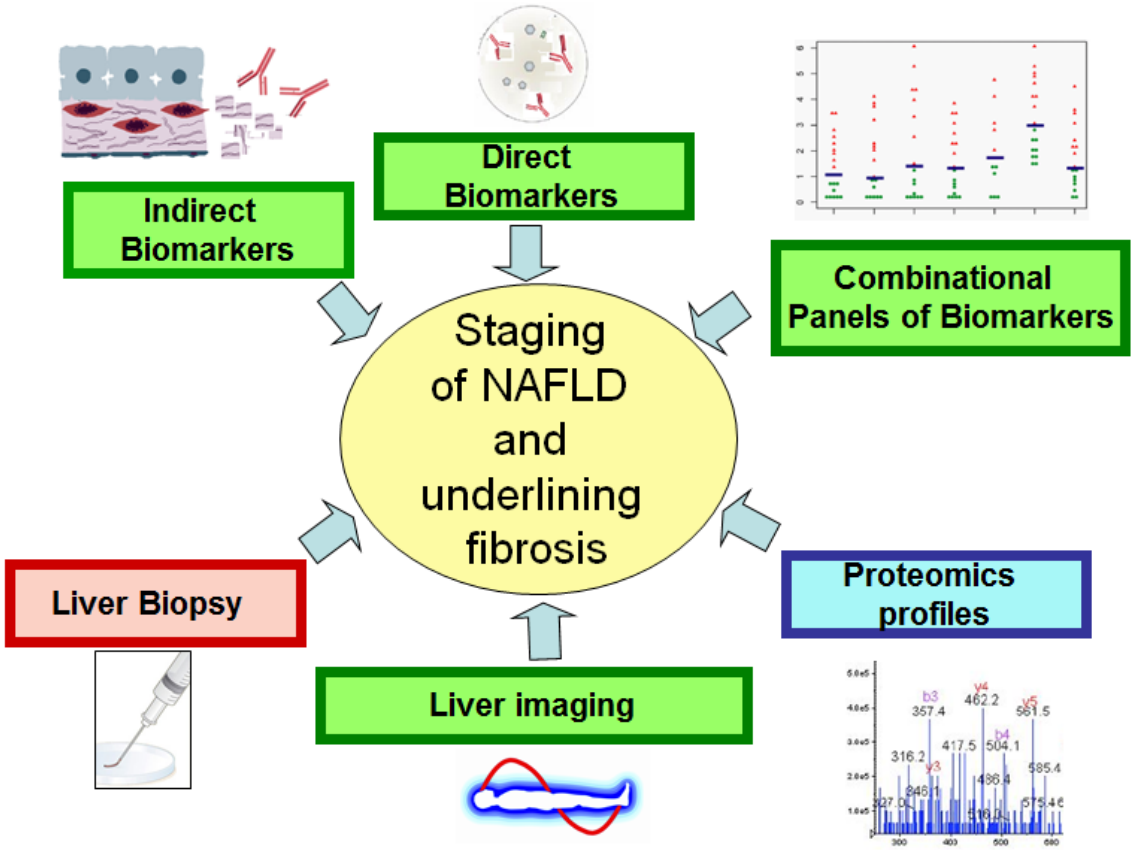

Figure 3 Various diagnostic modalities for the staging of NAFLD and underlining fibrosis.

1.3 Pathophysiology of Nonalcoholic Fatty Liver Disease

Pathophysiology of NAFLD is multifaceted and understood incompletely. Currently, a multiple-hit theory explaining the progression of NAFLD to NASH prevails. This theory has replaced previously proposed two-hit model, which was well-supported by evidence obtained in animal models, with a first hit being the development of insulin resistance which sensitizes the liver to further damage by second pro-inflammatory hit [Day CP, James OF, 1998]. In humans, pathogenesis of NAFLD is more complex than in genetically simplified animal models. “Two-hit” model is now regarded as overly simplistic to accurately depict the driving forces behind NAFLD, and, because of that, obsolete. Current understanding of NAFLD describes it as necroinflammatory liver condition propagated by multiple pathogenetic mechanisms which compound each other. These mechanisms involve various cell populations comprising liver parenchyma and rely on long distance communications with other organs, most importantly, with visceral adipose tissue. Deterioration of the liver develops slowly, under constant insult of oxidative stress, which is enhanced in insulin- resistant and lipid-laden cells, and sets stage for the propagation of inflammatory cascades, collagen deposition, and hepatocyte death [Manne et al., 2018].

The overwhelming prevalence of non-alcoholic liver disease in the general population significantly influences outcomes of drug therapies for many other chronic conditions. In particular, the processing of xenobiotics in fatty livers is altered, thus, influencing absorption, distribution, metabolism, and excretion (ADME) processes. The presence or absence of hepatic disease status determines expression of ADME-

related gene to a greater degree that age or sex of patients. In particular, in livers of patients with NASH, the genes encoding uptake transporter genes are coordinately down-regulated; because of that NASH patients may require larger dosage of the same drug [Lake AD et al., 2011]. It is hypothesized that the change in gene expression program which limits uptake is a result of a hepatoprotective mechanism that prevents accumulation of toxic intermediates in hepatocytes affected by disease [Lake AD et al., 2011]. In frame of personalized medicine, ADME processes would, evidently, have to be ascertained for NAFLD and non-NAFLD populations differently [Clarke JD, Cherrington NJ, 2015].

Currently, no FDA approved therapies are available for patients with NASH. Recommended treatments for NAFLD emphasize lifestyle changes, including weight loss through exercise and maintaining low sugar diet. So far, representatives of four different drug classes were found to be active against NASH in Phase 2 clinical trials, and have moved into Phase 3. The development of these drug classes aids the treatment of NASH by targeting the reduction of hepatic fat accumulation and metabolic stress, reducing inflammation and injury to the liver, treating NAFLD and its progression at the gut-liver axis, and aiding in the suppression of liver fibrosis. These classes include Farnesoid X nuclear receptor (FXR) agonists, CCR2/CCR5 antagonists, ASK1 inhibitors, and PPARα/δ agonists [Ogawa Y et al, 2018]. There is an understanding that successful reversal of NASH histopathology may require a synergistic treatment with more than one compound.

Considerable heterogeneity of NAFLD with regard to presence of various comorbidities, patients' age and gender, as well as the degree of fibrosis present a substantial obstacle on a path to the development of safe and efficient pharmacological treatments for this condition. Another obstacle is a lack of suitable *in vitro* models which would allow the high-throughput screening of novel therapeutic compounds for this condition.

1.4 Study Aims

1. Provide a rationale for use of apoptosis inhibitors as potential NAFLD therapeutics.
2. Analyze various approaches to establish *in vitro* NAFLD model suitable for preclinical studies of cell death inhibitors.

CHAPTER TWO: A RATIONALE FOR USE OF CELL DEATH INHIBITORS AS POTENTIAL NAFLD THERAPEUTICS

2.1 Apoptosis is Central to Pathophysiology of NAFLD

The process of programmed cell death, or apoptosis, occurs during normal development of human organs and tissue, and in course of aging. Apoptotic cells acquire distinct morphological characteristics, such as shrinkage of the cell, which is typically followed by its fragmentation into membrane-bound apoptotic bodies undergoing rapid consumption by adjacent cells [Saraste A, Pulkki K, 2000]. As opposed to necrosis, apoptosis is an active form of cell death that involves activation of specialized cellular machineries that perform progressive self-destruction of the cell. This homeostatic mechanism maintains correct balance of cell populations in tissues and eliminates the cells with damaged membranes, DNA and/or too many unfolded proteins. The mechanisms of apoptosis are highly complex and sophisticated, and may proceed along one or another energy-dependent cascade of molecular events. The two main routes being either death receptor (DR) pathway (extrinsic mechanism) or mitochondrial pathway (intrinsic mechanism), which is initiated by intracellular stimuli. Both pathways result in initiation of caspase cascade, which culminates in the cleavage of a number of proteins in the cell, followed by ordered cell disassembly. Schematic of extrinsic and intrinsic

apoptosis pathways with key molecular players is depicted at **Figure 4.** When apoptotic mechanisms are disturbed, resulting in too much or too little apoptosis, the tissue homeostasis shifts. The ability to modulate apoptosis has immense therapeutic potential for neurodegenerative and autoimmune diseases, repair of the damage caused by ischemia and many types of cancer.

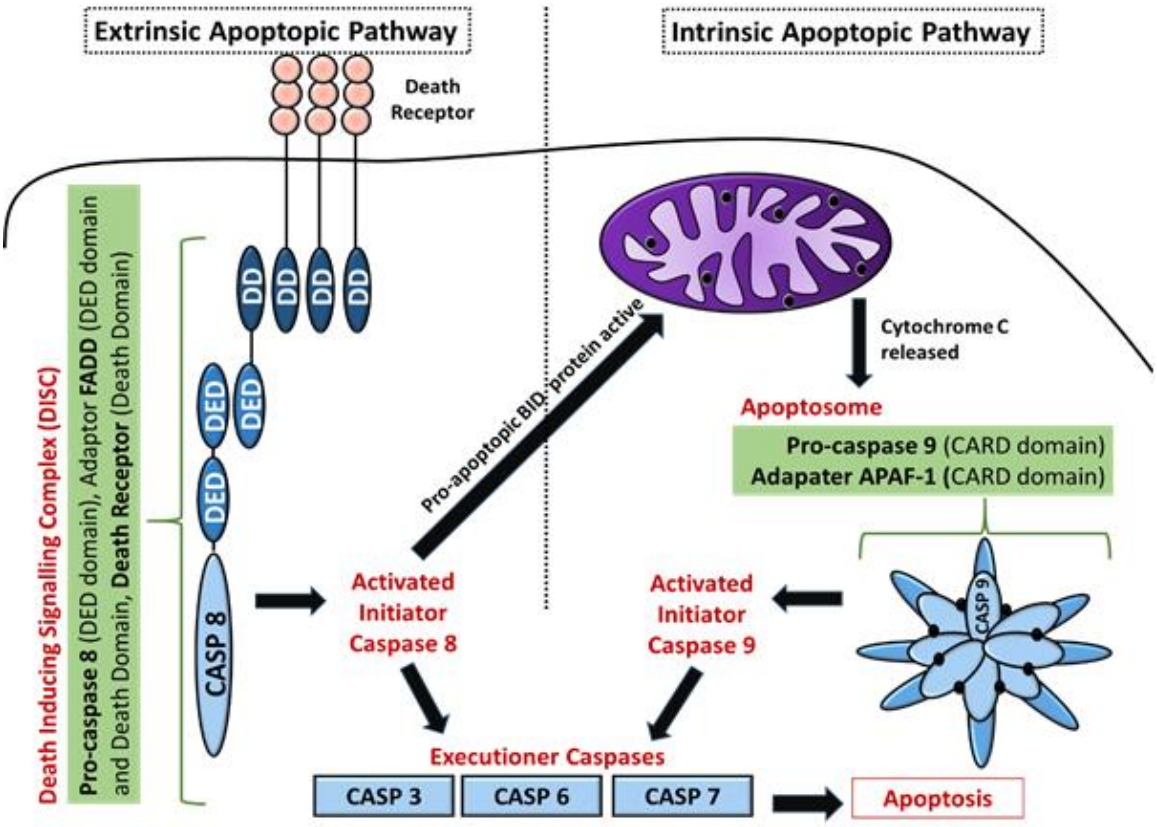

Figure 4 A scheme of extrinsic and intrinsic apoptosis pathways with key molecular players. Abbreviation CASP denotes effector caspase enzymes. This figure is reproduced under Creative Commons license.

Hepatic parenchyma is predominantly filled by hepatocytes. These cells have an extraordinary capacity to regenerate when they face damaging factors, such as bursts of reactive oxygen species (ROS), lack of oxygen, and environmental toxins including alcohol. In acute liver poisoning with chemical toxins such as acetaminophen and carbon tetrachloride (CCl4), liver sinusoids are damaged by necrosis resulting in their disorganization. Damaged hepatocytes induce inflammation of injured tissue by producing damage-associated molecular patterns (DAMPs), which recruit and activate non-parenchymal Kupffer cells. In turn, these resident hepatic macrophages secrete interleukin-6 (IL-6), a paracrine regulator of gene expression for adjacent hepatocytes, which now are pushed to proliferate, typically repairing the damage within 72 hours [Michalopoulos GK, DeFrances MC, 1997].

Chronic liver diseases, such as NAFLD and NASH, are in so-called chronic inflammatory states, and associated with persistent damage to hepatocytes. In this type of inflammation, the remodeling of the tissue and its repair occur simultaneously. Regardless of its etiology, chronic inflammation induces deposition of the collagen in liver parenchyma (a fibrosis) that eventually leads to the loss of its function. A dominant role in fibrosis is played by hepatic stellate cells (HSCs), also known as perisinusoidal cells or pericytes, which turn into myofibroblasts capable of producing a large amount of collagen. Both chronic inflammation and collagen accumulation are propagated by constant inflow of growth factors and cytokines, many of which also play an anti-apoptotic role. As apoptosis is a vital component of wound healing necessary for the

removal of inflammatory cells and the formation of scar tissue [Greenhalgh, 1998], its dysregulation contributes to excessive scarring and fibrosis.

The apoptosis of hepatocytes is critically dependent on both intrinsic and extrinsic apoptotic pathways being intact. In normal hepatocytes, extrinsic signals alone are typically not powerful enough, so the mitochondria-mediated pathway is required for signal amplification [Yin XM et al., 2003]. On the other hand, lipid-laden hepatocytes of fatty livers exhibit remarkable hepatocyte apoptosis [Ribeiro PS et al., 2004]. Key roles in hepatocyte apoptosis of NAFLD are played by mitochondrial dysfunction and endoplasmic reticulum (ER) stress. These two types of pro-apoptotic signals augment each other, which is to be expected due to close physical and functional interactions between ER and mitochondria [Vannuvel K et al., 2016].

Cell death is a necessary function of hepatic homeostasis, with equilibrium between the loss and replacement of hepatocytes. Amounts of apoptosis taking place in healthy or injured liver may be evaluated by monitoring the activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes which are released into the serum by dying cells. In a healthy liver, approximately 0.05% of hepatocytes are being removed by apoptosis at any given time, which is considered a low turnover, and results in low ALT activity in the serum. In settings of NAFLD, hepatocyte turnover increases, resulting in higher rates of cell death and higher amounts of AST and ALT being released [Luedde et al, 2014]. The transition from normal serum activity of AST and ALT enzymes in the blood to elevated levels of these enzymes typically coincides with an onset of active inflammatory process in liver parenchyma and

hallmarks the transition of simple steatosis to NAFLD. These increases are, however, transient as progressive hepatocyte death along with replacement of liver parenchyma with collagen deposits leads to lowered amounts of living hepatocytes and lowered levels of hepatic enzyme released despite an apparent increase in their turnover. An improvement in the control of apoptosis, for example, by pharmacological means, may delay the rate of liver deterioration in patients with NAFLD by preventing the progression of the disease towards advanced stages of fibrosis.

Expression levels of many molecules involved in apoptosis, including that of activated caspases, are commonly elevated in the livers of NASH patients, and may be related to the progression of simple hepatic steatosis to NASH. In particular, there is an increase in activity of caspase-3, which cleaves cytokeratin-18 (CK-18), which is the major intermediate fragment protein in the liver. Cleavage of CK-18 by caspase-3 generates its soluble fragment that is an independent predictor of NASH in patients with hepatic steatosis [Wieckowska A et al., 2006]. Another caspase with levels increased in NASH is caspase 2, an initiator caspase in lipid-induced cytotoxicity, a process which is especially important in context of ectopic lipid accumulation in hepatocytes seen in NAFLD [Johnson ES et al., 2013].

2.2 Pan-caspase inhibitors, their potential as a novel class of NASH drugs and associated concerns

Suppression of the caspases is a well-known way to inhibit apoptosis in research laboratories. Caspase inhibitors can either be peptides, proteins, or small molecule inhibitors. For example, pan-caspase inhibitor Z-VAD-FMK, a cell permeable fluoromethyl ketone (FMK)-derivatized peptide, found its utility as a tool for studying Caspase activity. This inhibitor does not display cytotoxic effects.

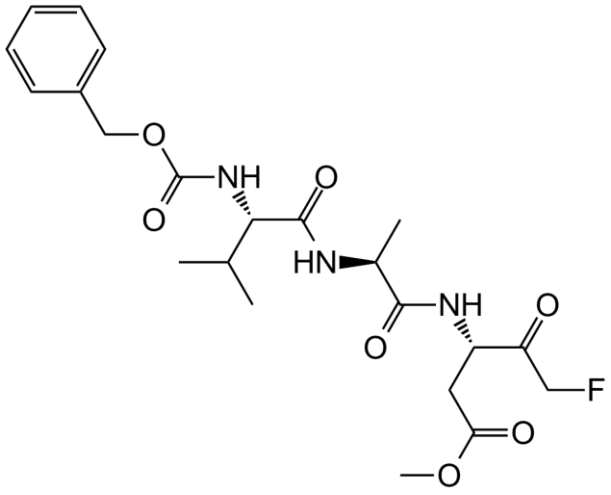

Figure 5 A scheme of extrinsic and intrinsic apoptosis pathways with key molecular players.

Pan-caspase inhibitors are pursued for their ability to treat diseases such as autoimmune disorders and cancer. The small molecule pan-caspase inhibitor VX-166 has garnered interest for its ability to treat fibrosis and sepsis. In murine high fat diet (HFD) model of NASH, administration of Emricasan, a pan-caspase inhibitor, was shown to

ameliorate liver injury as defined by end-point biomarkers of serum aspartate aminotransferase and alanine aminotransferase activities, NAS histological scored as well as the levels of mRNAs encoding for Interleukin-1 beta (IL 1-β), tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein (MCP-1) and C-X-C chemokine ligand-2 (CXCL2). In parallel with the degree of liver inflammation, supplementation with Emricasan also led to diminishing signs of hepatic fibrosis, including expression of α-Smooth Muscle Actin (αSMA), which serves as a marker for hepatic stellate cell activation, fibrosis score, percentage of liver affected by collagen deposition as measured to parenchyma staining with Sirius red, and the levels of expression for genes encoding profibrogenic cytokines [Barrayro FJ et al., 2015]. Similar data were acquired when mice with NAFLD were treated with pan-caspase inhibitor VX-166 [Witek RP et al., 2009; Anstee QM et al., 2010].

Despite great interest generated by initial studies of pan-caspase inhibitors as potential therapeutics, there is a concern about their mechanism of action. Some studies showed that under condition of stress and suppression of apoptosis, cell may go into necrosis or necroptosis instead. In case of apoptosis, there is essentially no inflammatory reaction ensued because: (1) apoptotic cells do not release the content of their cytoplasm into the surrounding tissue; (2) apoptotic bodies are quickly phagocytosed by adjacent cells. As opposed to apoptosis, necrosis begins with a loss of cell membrane integrity and an uncontrolled release of its contents into the extracellular space. In cells adjacent to the dying one, necrosis initiates an inflammatory response, attracting leukocytes which react to this inflammation non-specifically, as to suspect microbial invasion, and release

various damaging substances causing collateral damage to surrounding tissues and inhibiting the healing process. A programmed variant of necrosis is called necroptosis **(Figure 6).** The process of necroptosis is caspase-independent. Because of that, necroptosis serves as an alternative back-up mechanism for the cell death, which activates in cases when apoptosis is inhibited, for example, when apoptosis signaling is blocked by the virus. An exposure to pan-caspase inhibitors is a known factor which activates the program of necroptosis.

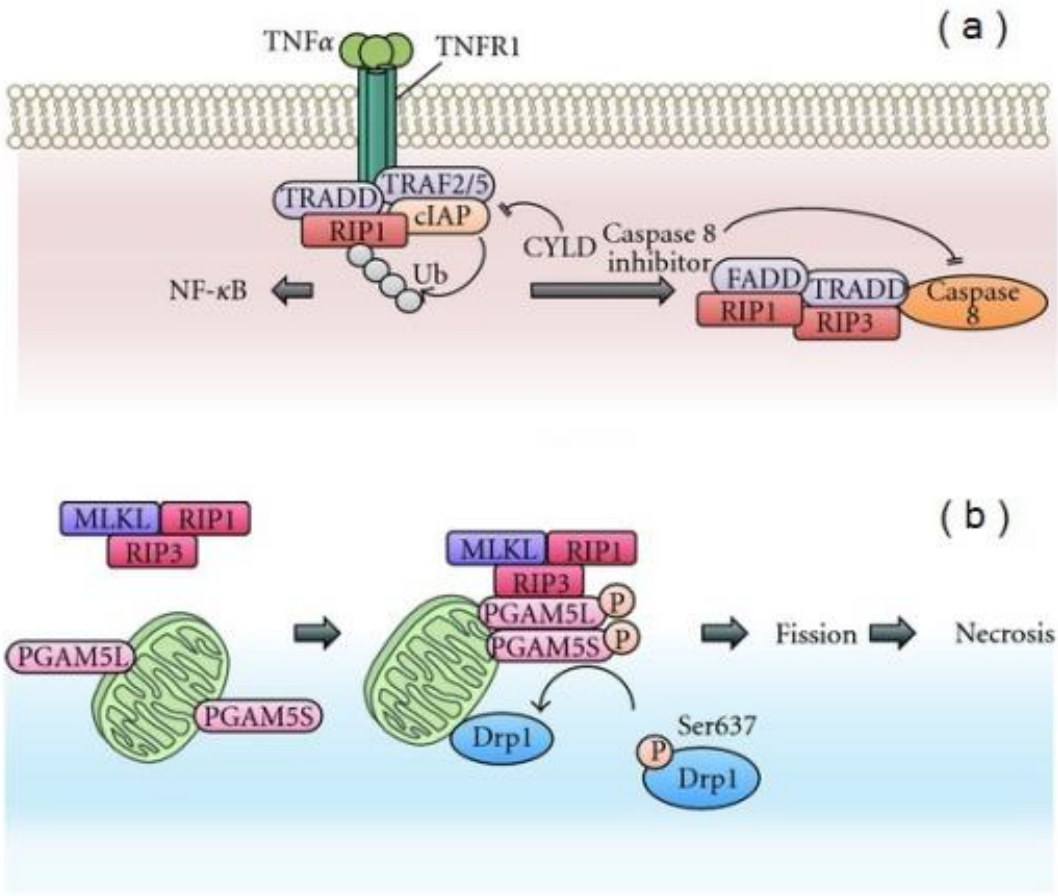

Figure 6 Necroptosis signaling pathways.
(a) Necroptosis may be induced by recruitment of RIP1 to TNF receptors 1 (TNFR1) and TNFR2 upon their binding to TNFα. In presence of pan-caspase inhibitor, for example, Z-VAD-fmk, RIP1 interacts with RIP3 and forms a necrosis-inducing complex. (b) In mitochondria, RIP3 phosphorylates Ser/Thr protein phosphatase PGAM5, thus, enhancing its protein phosphatase activity. Once activated, PGAM5 promotes mitochondrial fission and subsequent necrosis. This figure is reproduced under Creative Commons license [Kanamaru Y et al., 2012].

Notably, both in human and in experimental NAFLD, the levels of circulating biomarkers of necrosis and its inducer TNF-α, as well as the degree of the phosphorylation of RIP3 and mixed lineage kinase domain like pseudokinase MLKL are higher than in control individuals with healthy livers [Afonso MB et al., 2015]. Functional studies in primary hepatocytes collected from mice with fatty livers and

normal livers established the association between TNF-α-induced RIP3 expression and activation of necroptosis [Afonso MB et al., 2015]. Because of that, utilization of specific inhibitors of necroptosis, rather than that of apoptosis, may be plausible avenue for the development of the NAFLD-targeting medications. In addition to end point inhibitors, action upon intracellular targets or cell death effector machinery, one may also consider the molecules interfering with intracellular signals which may activate one or another pathway of the cell death, depending on particular tissue context. All this justifies the development of *in vitro* models of NASH suitable for the screening of cell-death disrupting compounds.

CHAPTER THREE: APPROACHES TO THE DEVELOPMENT OF *IN VITRO* NAFLD MODEL SUITABLE FOR PRECLINICAL STUDIES OF CELL DEATH INHIBITORS

3.1 Why to model Nonalcoholic Fatty Liver Disease *in vitro*

As the prevalence of NAFLD in world populations continues to grow, more emphasis is placed on understanding of the molecular networks driving its progression to NASH, and on a search for safe medications capable of reversing the NASH and the NAFLD phenotypes.

To date, a majority of the basic studies of NAFLD mechanisms as well as preclinical tests for NASH therapy candidate compounds were performed *in vivo*, in rodent knockout or exposure models. Limitations of these models include substantial costs, and incompatibility with high-throughput mode of candidate compound assessments [Van de Bovenkamp et al., 2007]. Moreover, interspecies differences in NASH pathogenesis are substantial. Even when different rodent NAFLD models are compared to each other, for example, high-fat diet fed rats and mice, these models vary dramatically in their ability to reflect various clinical and histopathological features of human NAFLD. In a recent direct metabolomics comparison of liver samples collected from humans and animals affected with NAFLD, many significant trends in progression-related changes were not reflected by the rodent models, with a major contributor to this

discrepancy being the interspecies difference in bile acid composition. Moreover, the levels for the branched chain amino acids, which are fairly commonly disturbed in human NASH patients, were not changed in either high-fat diet (HFD) or methionine-choline deficient (MCD) diet fed animals models of NASH [Han J et al., 2017]. In addition, the widespread use of animal models raised ethical concerns, leading to strong push for its minimization.

3.2 Human *in vitro* models of Nonalcoholic Fatty Liver Disease

To date, several human *in vitro* models of NAFLD have been developed. Some of these models are based on primary cultures of hepatocytes collected from patients with fatty livers [Feldstein AE et al., 2004; Vinciguerra M et al., 2009], and others – on the immortalized hepatocyte cells [Gomez-Lechon MJ et al., 2007] treated with a combination of monounsaturated fatty acids (MUFAs) and saturated fatty acids (SFAs). Primary hepatocytes do not proliferate *in vitro*; the cells harvested from each individual patient could be used only for a week, and for a limited number of tests. Moreover, primary cells tend to lose hepatocyte differentiation over culturing time. Because of that, primary hepatocytes are less amenable to standardization, which makes them less preferable material for building a model of NAFLD than immortalized human cells.

To establish the NAFLD-resembling phenotype, human cell cultures may be treated with long chain fatty acids most abundant in Western diet. Either palmitate (C16:0) or oleate (C18:1), or a mixture of the two may be used. Typically, making

hepatocellular steatosis in cell cultures is achieved within only 48 hours of treatment with the mixture of fatty acids.

It is well recognized that the culture of one cell type (so-called monoculture) may not correctly reflect a pathophysiology of any complex disorder, including NAFLD. Architecture of human liver is far from being simple; it is built by a variety of cooperating cells that perform dozens of functions including large scale production of major protein constituents of the blood components, conversion of glucose to glycogen, its storage and mobilization, biosynthesis of fatty acids, cholesterol, and bile acids as well as and detoxification/ biotransformation of various endogenous and exogenous substances. Because of that, the monoculture model cannot possibly mimic the real multicellular interaction either in the normal or in diseased organ. Understanding of a limited nature of monocultures lead to attempts of establishing a co-culture of hepatocytes with some other types of liver cells, either in cell inserts (also called Transwells), where two cell types may grow on two different surfaces sharing only the culture media [Barbero-Becerra VJ et al., 2015], or in organ-on-a chip devices [Poloznikov A et al., 2018]. These two approaches reproduce intercellular communication more accurately. It is expected the biochemical response to fat accumulation in the co-culture models would be more physiological as well.

In our lab, we previously have undertaken the study aimed at elucidation of the effects of exposure to various lipid species on both the cellular and mitochondrial functions of hepatocytes, in a simple *in vitro* model of NAFLD. I have taken part in this effort. In particular, I have maintained the culture of HepG2 cells and exposed it to oleic

acid. A bulk of experimental results of this study was presented in Master's Thesis work of Peter Masschelin (Masschelin P, Thesis embargoed). While aiding us with major insights into a differential response of HepG2 cells to fatty acid species differing in their length and saturation, this study also uncovered substantial limitations to this model, possibly caused by our inability to measure the proportion of fatty acids remaining in the media at the end of the treatment period, which precluded us from quantifying the amounts absorbed. Further studies would require a development of better *in vitro* models, possibly including those with multiple cell types in co-culture to be subsequently exposed to the media containing high levels of NASH-associated-metabolites, including glucose, insulin, and free fatty acids, or a use of microfluidic platform.

We suggest that HepG2 cells might be replaced by HepaRG cell line, and that such a move to a HepaRG model should become an immortalized hepatocyte standard in research laboratories. HepaRG cells can express both membrane transporters and Phases I and II drug metabolizing enzymes which are normally found in the liver. Furthermore, HepaRG cell line is characterized by gene expression program more closely resembling that of primary human hepatocytes. They also express Cytochromes P450 (CYP) mRNAs at levels comparable to ones observed in primary human hepatocytes. HepG2 cells, by contrast, display little CYP activity; if needed, expression of CYPs in this cell line may be achieved after its transfection with plasmid constructs, which are difficult to regulate at their physiological levels. Notably, functional activities of HepaRG cells remain stable for 1-2 weeks after the 3-4 weeks at confluency [Guillouzo A et al., 2007].

CHAPTER FOUR: CONCLUSION

Given the worldwide prevalence of Non-Alcoholic Fatty Liver Disease, and a substantial proportion of simple steatosis patients progressing to Non-Alcoholic steatohepatitis (NASH), a condition associated with liver inflammation, and, further, to cirrhosis and end-stage liver failure, the development of *in vitro* models for NAFLD is warranted. Currently used models of this kind are overly simplistic, and incompatible with high-throughput experimentation mode which is necessary for the screening of potential therapeutics capable of the reversal of NASH phenotype. It seems that the best way to proceed forward would be with utility of liver-on-a-chip devices harboring established immortal cell lines, for example, HepG2 – a "workhorse" of liver toxicology – or its less malignant counterpart HepaRG. With an aid of these devices, many potential therapeutic compounds may be profiled either as monotherapy, or as synergistic enhancers for other potential treatments. The targeting of the necroptosis, rather than "classical" apoptosis, is a promising therapeutic option aimed at the delay of progression or even a reversal of NAFLD and other inflammation-associated liver diseases.

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