Universidade de Lisboa
Faculdade de Farmácia

Role of mitofusin-2 and microRNA-222-3p in modulating lipid metabolism during non-alcoholic fatty liver disease

Diogo Alexandre Espada Fernandes

Trabalho de Campo orientado pelo Professor Doutor Rui Eduardo Mota Castro, Professor Auxiliar e coorientado pelo Investigador André Daniel Lopes Simão.

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has a 25% prevalence worldwide and comprises a range of liver lesions, from simple steatosis to non-alcoholic steatohepatitis (NASH), which can then further progress to cirrhosis and, eventually, hepatocellular carcinoma (HCC). Lipotoxic metabolic injury of the liver typically arises from an inadequate lifestyle, including sedentarism, high fat diets and excessive caloric intake. Excessive fat accumulation, along with generation of reactive oxygen species (ROS), leads to mitochondrial and endoplasmic reticulum (ER) stress, as well as inflammation and apoptosis. Mitochondria are responsible for performing oxidative phosphorylation, lipidic β-oxidation, but are also very dynamic organelles, which respond to external stimuli with cycles of fusion and fission events. These processes of are mediated by a several different proteins including mitofusin-2 (MFN2). Noteworthy, MFN2 is found downregulated in NAFLD. In parallel, microRNAs (miRNAs or miRs) have been shown to also play a role in NAFLD pathogenesis. In particular, miR-222-3p was described to be up-regulated in NAFLD and is a putative post-transcriptional inhibitor of MFN2.

In this work, we aimed to evaluate the role of miR-222-3p and MFN2 in modulating lipid metabolism and ER stress in an animal model of diet-induced NAFLD. Our results showed that the steatosis and NAS scores were clearly elevated in MCD+HFD-fed mice, with both tending to decrease upon treatment with antagomir-222-3p. Results further showed a clear impairment of lipid metabolism enzymes, as well as ER stress mediators, in animals fed the MCD+HFD diet. The overall protective effects of antagomir-222-3p in our model of experimental NAFLD could be due to the observed rescue of FATP5, CPT1 and FASN mRNA expression in NASH animals, as well as the inhibition of ER stress mediators PERK and ATF6. Most of these protective effects were independent of hepatocyte MFN2. Overall, our findings highlight the critical role of antagomiR-222-3p in ameliorating NAFLD/NASH pathogenesis, in particular deregulated lipid metabolism and ER stress, for which it could constitute a promising therapeutic strategy for treating NAFLD.

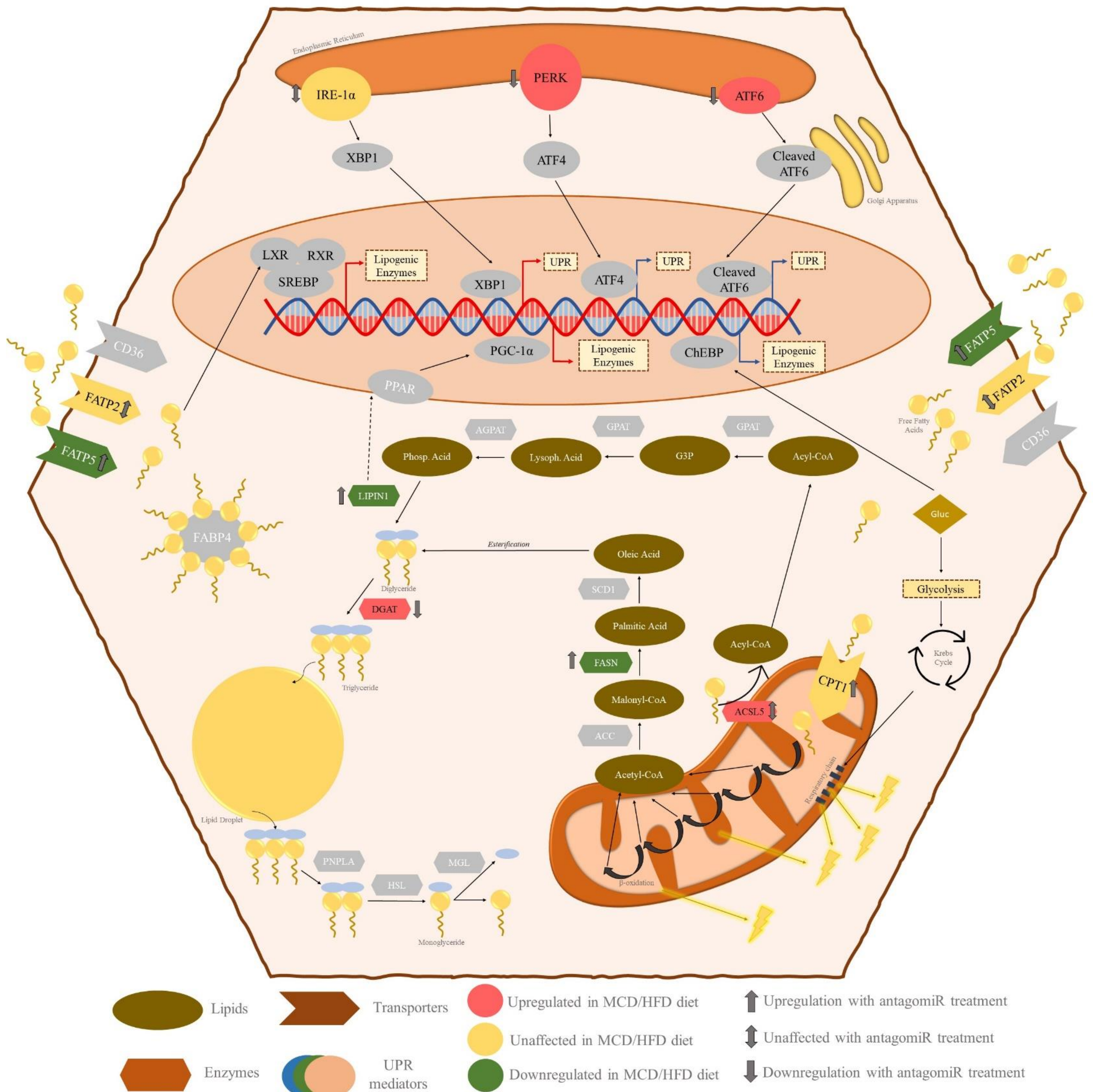
Keywords: Endoplasmic Reticulum Stress; Lipid metabolism; microRNAs Mitochondria; Non-Alcoholic Fatty Liver Disease.

Resumo

O fígado gordo não alcoólico (FGNA) tem uma prevalência mundial de 25% e compreende uma gama de lesões hepáticas, desde a esteatose simples até a esteato-hepatite não alcoólica (EHNA), podendo evoluir para cirrose e, eventualmente, carcinoma hepatocelular (CHC). A lesão metabólica do fígado causada por lipotoxicidade geralmente surge de um estilo de vida inadequado, incluindo sedentarismo, dietas ricas em gordura e ingestão calórica excessiva. A acumulação excessiva de gordura, em conjunto com a geração de espécies reativas de oxigénio (ROS), leva ao stress mitocondrial e do retículo endoplasmático (RE), bem como inflamação e apoptose. As mitocôndrias são responsáveis por realizar a fosforilação oxidativa, β-oxidação lipídica, mas também são organelos muito dinâmicos, que respondem a estímulos externos com ciclos de eventos de fusão e fissão. Estes processos são mediados por várias proteínas, incluindo a mitofusina-2 (MFN2). Notavelmente, MFN2 é encontrado regulado negativamente no FGNA. Em paralelo, foi demonstrado que os microRNAs (miRNAs ou miRs) também desempenham um papel na patogénese do FGNA. Em particular, o miR-222-3p foi descrito como regulado positivamente no FGNA e é um inibidor pós-transcricional da MFN2. Neste trabalho, objetivamos avaliar o papel do miR-222-3p e MFN2 na modulação do metabolismo lipídico e do stress do RE num modelo animal de FGNA induzida por dieta. Os resultados mostraram que os scores de esteatose e NAS foram elevados em ratinhos alimentados com MCD+HFD, com ambos tendendo a diminuir após o tratamento com antagomir-222-3p. Os resultados mostraram ainda um claro comprometimento das enzimas do metabolismo lipídico, bem como dos mediadores de stress do RE, nos modelos animais. Os efeitos protetores do antagomir-222-3p no modelo experimental podem ser devidos ao resgate observado da expressão de mRNA de FATP5, CPT1 e FASN em animais NASH, bem como a inibição dos mediadores de stress de ER PERK e ATF6. A maioria desses efeitos protetores foi independente da MFN2 do hepatócito. No geral, nossos resultados destacam o papel crítico do antagomiR-222-3p na melhoria do FGNA, em particular no metabolismo lipídico e stress RE, para o qual pode constituir uma estratégia terapêutica promissora para o tratamento do FGNA.

Palavras-chave: Fígado Gordo Não Alcoólico; Metabolismo Lipídico; microRNA; Mitocôndria; Stress do Retículo Endoplasmático.

Graphical Abstract



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5 anos do Mestrado Integrado em Ciências Farmacêuticas a terminar com este trabalho, do qual estou tão orgulhoso.

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Abbreviations

ACC	Acetyl-Coenzyme A Carboxylase
ACSL	Acyl-Coenzyme A Synthetase Long Chain
ALT	Alanine Transaminase
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ANOVA	Analysis of Variance
AST	Aspartate Transaminase
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
BiP	Binding Immunoglobulin Protein
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
ChREBP	Carbohydrate-Responsive Element Binding Protein
CoA	Coenzyme A
CPT	Carnitine Palmitoyltransferase
DG	Diacylglycerides
DGAT	Diglyceride Acyltransferase
DNA	Deoxyribonucleic Acid
DNL	De novo Lipogenesis
dNTPs	Deoxyribonucleotide Triphosphate
ER	Endoplasmic Reticulum
ERN	Endoplasmic Reticulum to Nucleus Signalling
ERR	Estrogen-Related Receptor
FA	Fatty Acid
FABP	Fatty Acid Binding Protein

FASN	Fatty Acid Synthase
FATP	Fatty Acid Transporter Protein
FFA	Free Fatty Acid
GPAT	Glycerol-3-Phophate Acyltransferase
HCC	Hepatocellular Carcinoma
HFD	High-Fat Diet
HPRT	Hypoxanthine-Guanine Phosphoribosyltranferase
HTN	Hypertension
IL	Interleukin
IR	Insulin Resistance
IRE	Inositol-Requiring Enzyme
KO	Knock-Out
LD	Lipid Droplet
LT	Liver Transplantation
LXR	Liver X Receptor
MCD	Methionine-Choline Deficient
MFN	Mitofusin
MG	Monoglyceride
MGL	Monoacylglycerol Lipase
miRNA/miR	Micro Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
MS	Metabolic Syndrome
NAFLD	Non-Alcoholic Fatty Liver Disease
NAS	Non-Alcoholic Fatty Liver Disease Score
NASH	Non-Alcoholic Steatohepatitis

NF-kB	Nuclear Factor kappa-light-chain-enhancer of Activated B Cells
nt	Nucleotide
OA	Oleic Acid
OMM	Outer Mitochondrial Membrane
OXPHOS	Oxidative Phosphorilation
PA	Palmitic Acid
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PERK	PRKR-like endoplasmic reticulum kinase
PGC	Proliferator-Activated Receptor Gamma Coativator
PNPLA	Patatin-Like Phospoholipase
PPAR	Proliferator-Activated Receptor Gamma
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Reverse Transcription
SCD	Stearoyl-CoA Denaturase
SIRT	Sirtuin
SREBP	Sterol- Regulatory Element Binding Protein
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TNF	Tumour Necrosis Factor
UPR	Unfolded Protein Response
VLDL	Very-Low Density Lipoprotein
WT	Wild-type
XBP	X-Box Binding Protein

μg Micrograms

μL Microliters

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1 Introduction

1.1 Non-alcoholic fatty liver disease

Over the 21st century, non-alcoholic fatty liver disease (NAFLD) evolved from a relatively unknow liver disease with an abnormal histology to the most prevalent chronic liver disease, with the fastest progression to cirrhosis and hepatocellular carcinoma (HCC) (1). It has an estimated worldwide prevalence of 25% among the adult population (1), increasing every year, further constituting the major indication for liver transplantation (LT) (1–4).

1.1.1 Pathogenesis and Progression

NAFLD can be defined by the accumulation of lipids within hepatocytes that can progress to more severe forms of the disease, namely non-alcoholic steatohepatitis (NASH), that can further progress to liver fibrosis, cirrhosis and, eventually, HCC (1,3–5). As NAFLD evolves to subsequent stages, the prognostic is more unlikely to be favourable (2,4–6). Some authors consider NAFLD as the liver manifestation of metabolic syndrome (MS), since this disease is intimately related with obesity, hypertension (HTN), insulin resistance (IR), type 2 diabetes mellitus (T2DM) and high levels of serum triglycerides (TG) (1,4,7)

Even though it can be one of the causes of liver cancer and eventually death, in the early stages of the disease it can be reversible with the diminution of fat consumption on diet and a healthier lifestyle. The early forms of NAFLD and NASH are reversible, but once liver fibrosis develops, the disease becomes irreversible (Figure 1.1).

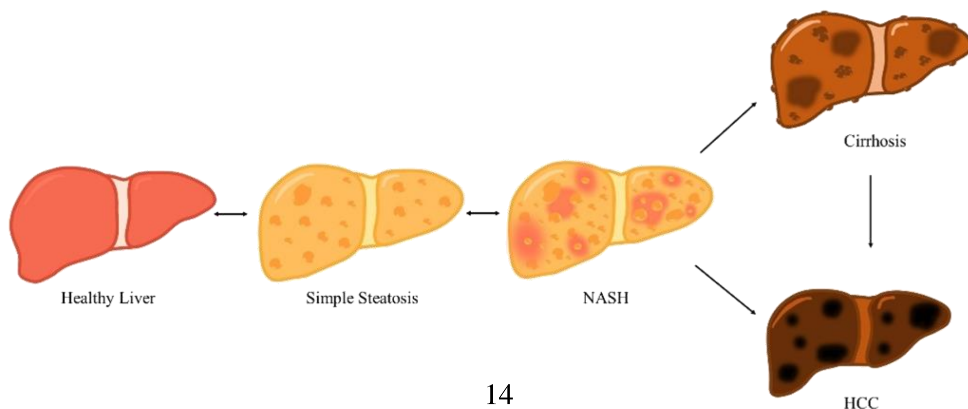


Figure 1.1 | Stages of NAFLD. Liver disease may start with lipid accumulation within hepatocytes, leading to a state called simple steatosis. Once the inflammatory process begins, the disease becomes more severe with different characteristics, giving rise to NASH. If not early diagnosed and/or treated, fibrosis ensues, and the disease becomes irreversible. With a continuous stimulus, the liver damage becomes even worse, hepatocytes start to die by apoptotic processes and liver failure becomes more likely. The last state of liver damage is hepatocellular carcinoma, highly harmful and lethal.

Obese individuals tend to accumulate more visceral fat than healthy individuals, that have mostly subcutaneous fat. This type of adiposity content is more dangerous and propitious to give rise to NAFLD, since it is associates with an elevation of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) and a diminution of adipokines, like adiponectin (7). In addition, visceral fat is more propense to suffer lipolysis, releasing free fatty acids (FFAs) into blood circulation that will reach the liver, and is more insulin resistant than other types of adipose tissue, due to reduced levels of endogenous adiponectin (8–10). This hormone has also a direct effect in the liver mitochondria, increasing its enzymatic activity, controlling glucose homeostasis, leading to fatty acid oxidation, and reducing the activity of enzymes involved in fatty acid production (9). Thus, decreased circulating adiponectin levels will increase liver fat accumulation, enhancing NAFLD, by promoting chronic systemic inflammation, IR, and hepatic damage (11).

1.1.1.1 Lipid metabolism

Liver is the essential organ where lipid metabolism occurs, regulating the homeostasis through different biochemical processes. It is responsible for capturing and metabolizing fatty acids (FA) to synthesize new molecules, when needed, to redistribute through the body (12). Normally, these processes are balanced according to the organism needs, but metabolic changes will disrupt those pathways and perturb the normal lipid homeostasis, creating or enhancing diseases, such as NAFLD (12). NAFLD is much more prevalent among obese individuals, who tend to consume a lot more lipids and carbohydrates than the daily reference intake, namely glucose and fructose, leading to a phenomenon called lipo- and glucotoxicity. Glucose toxicity relates to an overstimulation of the pancreas, increasing insulin release and, therefore,

promoting IR (13). On the other hand, fructose is typically metabolized in the small intestine. Once inside the organism, fructose reaches the liver through the portal system, and is immediately transformed into fructose-1-phosphate, a major substrate for fatty acid synthesis, promoting de novo lipogenesis (DNL). When both glucose and fructose levels are above the healthy threshold, carbohydrate-responsive element-binding protein (ChREBP) and sterol-regulatory element-binding protein 1c (SREBP1c) (5) are activated, resulting in the transcription of genes that encode lipogenic enzymes (7,13), enhancing lipid accumulation, as stated below.

The major source of hepatic lipids is the uptake of FA from circulation (14,15). The uptake is mediated by two major transport proteins – the cluster of differentiation 36 (CD36), whose expression was shown to correlate with intrahepatic lipid accumulation (12,14), and by fatty acid transporter proteins (FATP) 2 and 5, the two isoforms expressed in the liver. CD36 expression is regulated by the liver X receptor (LXR) (12), a transcription factor of several lipogenic enzymes. Once in the cell, FA cannot diffuse around the cytosol since they have its hydrophobic portion, so they bind to the fatty acid binding protein 4 (FABP4), that facilitates the mobility and use of these by the cell. Additionally, this FA pool binding to FABP4 is thought to be a storage/protective mechanism for toxic lipids (16).

In metabolic diseases, NAFLD included, lipid metabolism is deregulated, promoting liver lipid accumulation.

1.1.1.1.1 Mitochondrial β-oxidation

Mitochondrial β-oxidation is the process of metabolizing lipids to generate adenosine tri-phosphate (ATP). Under physiological circumstances, this process occurs when glucose concentrations are low, providing the necessary energy for the organism. However, in cases of lipid overabundance as in NAFLD, this process also takes place regarding the elimination of those. The FAs enter the mitochondria trough carnitine palmitoyltransferase 1 (CPT1) (12), and the cycle of successive reactions to metabolize the long chain begins. This process will produce one acetyl-coenzyme A (acetyl-CoA) for each cycle, that goes back to the cytosol.

However, the excess of acetyl-CoA will enhance the production of more TG (Figure 1.2) since it is the main substrate for de novo lipogenesis (12,14). Alongside,

this process will generate reactive oxygen species (ROS) as a by-product, just like the respiratory chain from glycose metabolism does. The overstimulation of these processes will increase the ROS levels within hepatocytes and enhance the metabolic dysfunction with mitochondrial disarrangements, allowing the NAFLD vicious cycle to continue.

1.1.1.1.2 De novo lipogenesis

DNL is the process in which the liver produces new fatty acids, such as TGs, from acetyl-CoA, produced by glycolysis and mitochondrial β-oxidation of FA. In healthy individuals, this process normally occurs while fasting, but it was shown that DNL is elevated among NAFLD/NASH patients (12,17).

SREBP and ChREBP (18) are the main mediators of DNL. Both transcription factors were shown to be upregulated in NAFLD. Since they also are stimulated by insulin, it would be expectable that in a state of IR, DNL would not be that enhanced. However, it seems that there is a state of selective IR, where the stimuli of DNL does not get compromised (12,19). The activation of genes by these regulation factors will result in enhanced expression of lipogenic enzymes.

As Figure 1.2 shows, DNL begins with the transformation of acetyl-CoA in malonyl-CoA through the action of acetyl-CoA carboxylase (ACC), which is then metabolized into palmitic acid (PA) in a reaction catalysed by fatty acid synthase (FASN). After that, the result product can be elongated and denatured by stearoyl-CoA denaturase (SCD) giving rise to oleic acid (OA), that after esterification forms diacylglycerides (DG), that are finally stored as TG, after diglyceride acyltransferase (DGAT) catalyses the reaction (12,14).

Besides, a parallel and much more prevalent (14) process may occur beginning with acyl-CoA, who is produced through the activity of acyl-CoA synthetase long chain family member 5 (ACSL5). This molecule is not an intermediary of any process, and like acetyl-CoA it originates as a by-product of another metabolic reactions. It is produced in order to activate FA directly into its own metabolic reactions. After binding itself to FAs, the resulting molecule might be esterificated into glycerol-3-phosphate, in a reaction catalysed by the glycerol-3-phosphate acyltransferase (GPAT). After that, GPAT catalyses its change into lysophosphatidic acid, which gives rise to phospatidic

acid, a direct precursor of DG, trough lipin. These are later turned into TG and stored into lipid droplets (LD) promoting ballooning and steatosis (14).

These processes may be seen as protective (12,14,20), but when in excess may also cause cell inflammation and ultimately death, like in NAFLD, where hepatocytes suffer a metabolic reprogramming.

1.1.1.1.3 Lipolysis

Lipolysis is the process where, from the LD storage within the cell, FFAs are released. Mechanistically, as seen in Figure 1.2, it starts with the transformation of TG into DG again, through patatin-like phospholipase domain-containing 3 (PNPLA3), and after that are hydrolysed again into monoglyceride (MG) by the hormone sensitive lipase (HSL). Finally, in order to release the FFA, the monoacylglycerol lipase (MGL) separates the glycerol group.

In NAFLD, this process is slowed, accompanied with a downregulation of the enzymes needed for the process. Interestingly, PNPLA3 mutation in a single nucleotide is highly correlated with a propensity to develop the disease, which corroborates the importance of the gene in the pathogenesis and progression of the disease.

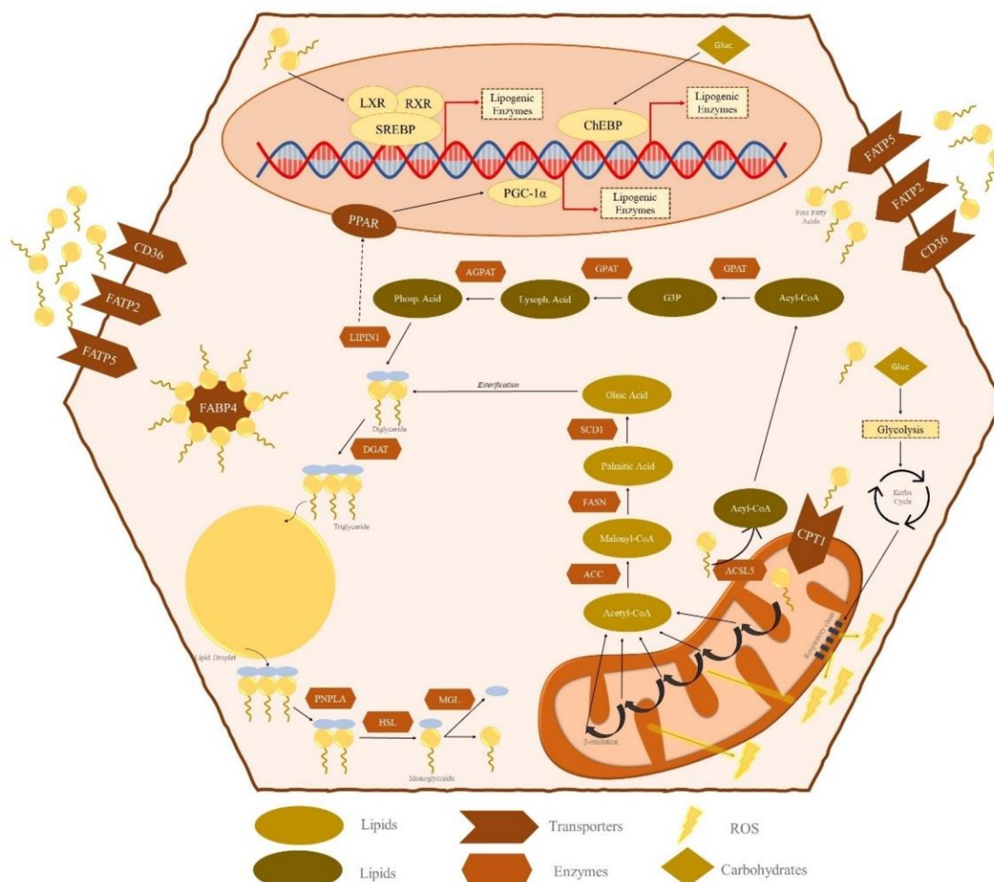


Figure 1.2 | Hepatocyte lipid metabolism. Elevated levels of FFA are a hallmark of the MS and NAFLD itself. Lipids enter the hepatocyte through specific transporters and are bound to a protein that allows their motion within the aqueous medium. When in excess, FFA are metabolised into TGs in order to be stored as lipid droplets, that will originate the ballooning and steatosis state of the cell. Mitochondria is an essential organelle in this metabolism, since it can activate the acyl-CoA pathway that will lead to TG synthesis. On the other hand, through β-oxidation of long-chain FAs, acetyl-CoA is produced and its itself metabolised through a different pathway into TGs. The result is a lipid droplet full of TGs in the cytosol. When needed, the reverse process can occur, liberating FA from the droplet. Along all these metabolic processes, ROS are produced, enhancing cell damage when in excess, and the transcription of lipogenic enzymes is also overstimulated.

Altogether, the deregulation of these three metabolic processes will deregulate lipid metabolism within hepatocytes, enhancing lipid accumulation and leading the cell to metabolic reprograming, ballooning, steatosis, and inflammation.

1.1.1.2 Mitochondrial dysfunction and endoplasmic reticulum stress

Mitochondria play crucial roles in apoptosis, intracellular calcium and lipid homeostasis and stress response (21,22), being also structurally dynamic organelles (22). In response to many different stimuli, these two-membrane organelles can undergo fusion, in which two mitochondria give rise to one bigger mitochondrion, and fission, in which a single mitochondrion can divide itself into two mitochondria. Therefore, mitochondria modulate their morphology according to the cell needs by coordinating these two processes to maintain their size, shape, and distribution within the cell (22). Pathological states, such as NAFLD, may interfere with this balance, favouring one process over the other, leading to mitochondrial dysfunction.

Under physiological and healthy conditions, mitochondria are responsible for generating ATP, being referred many times as "the powerhouse of the cell". In this process, ROS are generated as a secondary product (11,23). On the other hand, under stressful conditions, the cell can suffer many injuries, including metabolic reprogramming, which can lead to a bigger propensity to fat synthesis, enhancing NAFLD (11). In this case, there is a lot more need for the cell to perform oxidative phosphorylation (OXPHOS) which will also increase the electron supply from

mitochondria and therefore generate more ROS as a by-product. ROS and mitochondria dysfunction can engage in a vicious cycle where mitochondrial dysfunction produces ROS and ROS itself enhances mitochondrial dysfunction and liver impairment (11,23,24). With that in mind, mitochondria are a potential therapeutic target for the treatment, diagnosis and/or prevention of NAFLD.

The endoplasmic reticulum (ER) is an organelle that is intimately connected with mitochondria and is responsible for many vital cellular processes, namely synthesis, maturation and protein folding, some lipid synthesis and calcium homeostasis, key for ER-mitochondrial connection (13,25,26). ER stress is normally mediated by a specific response of this organelle, the unfolded protein response (UPR), responding to deficiently folded proteins to re-establish organelle homeostasis (13). This can be seen as a survival mechanism performed by the ER. Therefore, a constant activation of the UPR will result in activation of apoptosis (13,25). Under normal circumstances, several regulatory ER proteins are bound to an intraluminal chaperon binding immunoglobulin protein (Bip/GRP78), in its inactive state. However, under stressful conditions, these proteins will be released from this complex and activate intracellular signalling pathways that lead to the UPR (13,27).

The UPR can follow three pathways, as shown in Figure 1.3. These are the inositol-requiring enzyme 1 (IRE1), codified by the endoplasmic reticulum to nucleus signalling (ERN) gene, in which X-box binding protein 1 (XBP1) is activated and then enhances transcription of UPR mediators; the PRKR-like endoplasmic reticulum kinase (PERK), that activates the activating transcription factor (ATF) 4; and the activating ATF6 (13,27), that translocates to the Golgi apparatus to be activated and then promotes UPR.

The main goal of these pathways is to restore ER homeostasis under stress situations such as those created by misfolded proteins, high concentrations of FFAs or oxidative stress (13). More studies in humans need to be performed, but it was already proven that, in yeasts, ER stress and the UPR is also a source of ROS (25,28), associated with oxidative stress and cell damage. Interestingly, mitofusin-2 (MFN2) is the protein responsible to tether the mitochondria to the ER. Compromising this connection when MFN2 levels are downregulated, such as in NAFLD, might also be a trigger for ER stress and the UPR, enhancing disease pathogenesis through yet another pathway (29,30).

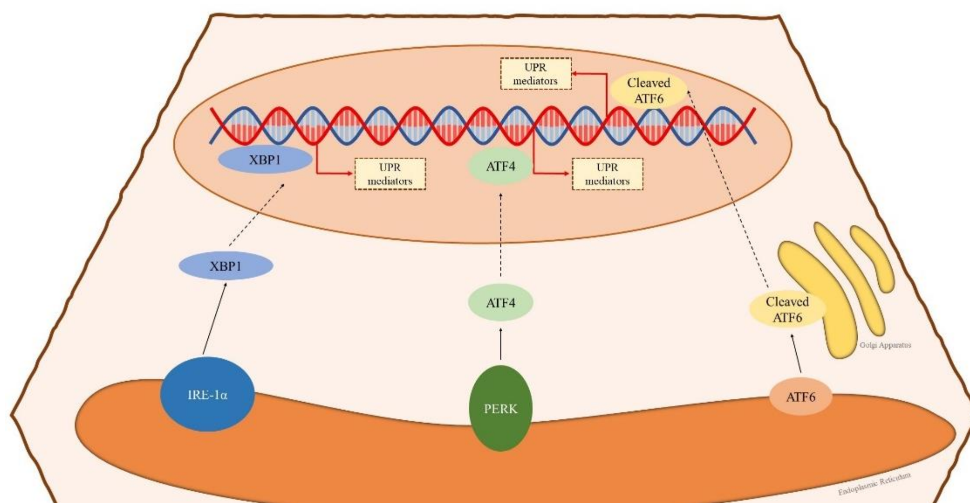


Figure 1.3 | The Unfolded Protein Response. The UPR is a mechanism of survival promoted by the ER in order to maintain cell viability. The UPR is triggered by certain stimuli, such as FFA excess and oxidative stress, hallmarks of NAFLD. The three main pathways that trigger the UPR are the IRE-1α, PERK and ATF6, that will lead to the activation of transcription factors in order to transcript genes that encode the response.

1.2 Mitofusin-2 and NAFLD

It is known that mitochondria are highly dynamic organelles, playing crucial roles in apoptosis, intracellular calcium homeostasis and stress response (21,22). Reacting to many different stimuli, these two-membrane organelles can undergo fusion and fission events. Therefore, mitochondria modulate their morphology according to the cell needs by coordinating these two processes to maintain their size, shape, and distribution

within the cell (22). Pathological states may interfere with this balance, favouring one process over the other, leading to mitochondrial dysfunction. Fusion is the process by which two mitochondria merge, giving rise to a single mitochondrion. Exacerbation of this process will create a hyperfused mitochondria network (22). Mitochondria fusion appears to be stimulated by cell survival-related mechanisms, since it can enhance OXPHOS and ATP production, as well as protect cells against engulfment by immune cells during starvation (22,31). In NAFLD, a few reports have indicated increased mitochondria fission and decreased fusion alongside decreased MFN2 expression, a GTPase located in the outer mitochondrial membrane (OMM) and whose main functions are assuring mitochondrial fusion and ER tethering, in the adipose tissue and the liver (22). Further, MFN2- knock out (KO) animal models develop NASH in a most rapid way than wild-type (WT) animals (22). Altogether, MFN2 might represent a potential therapeutic target for NAFLD. Additionally, MFN2 gene promoters were found to be downregulated in response to certain stimuli, such as oxidative stress, obesity, and T2DM (30).

The first step of protein synthesis is the biogenesis of messenger ribonucleic acid (mRNA) from the cellular deoxyribonucleic acid (DNA). Thus, the transcription of mRNA needs to be promoted, and in the case of MFN2, this is done by peroxisome proliferator-activated receptor gamma coactivator one alpha (PGC-1α), a transcriptional activator, after peroxisome proliferator-activated receptor gamma (PPAR-γ) activation. PGC-1α enhances the activity of the MFN2 gene promoter, which binds the nuclear hormone estrogen-related receptor alpha (ERRα), a nuclear receptor leading to mRNA transcription. PGC-1β is another isoform of the PGC that can also modulate MFN2 expression, but into basal and not stressful conditions. Interestingly, the opposite also happens. In a metabolic healthy situation, like physical exercise (49), PGC levels are increased, enhancing MFN2 synthesis, to promote mitochondrial fusion, to perform OXPHOS more efficiently.

Besides that, sirtuin-1 (SIRT1) is another protein that has metabolic roles within hepatocytes by being directly involved in cholesterol and fat metabolism, as well as in glucose homeostasis. It is, along with adenosine monophosphate-activated protein kinase (AMPK), an upstream regulator of PGC-1α, which is itself a MFN2 regulator. AMPK is an enzyme sensible to ATP levels, promoting mitochondrial biogenesis (32). Thus, an imbalance in the homeostasis of this axis will ultimately lead to mitochondrial

dysfunction. AMPK and SIRT1 work in a synergistic way, enhancing each other activities in a positive feedback loop. Also, fructose was shown to have an active role in inflammatory and IR processes, as well as to deregulate SIRT1 directly (33). Mechanistically, SIRT1 suppresses SREBP-1c and ChREBP activity, which will ultimately lead to decreased levels of TG in the liver. By being repressed, these factors will remain activated, both due to SIRT1 deregulation; and carbohydrates direct action. The homeostatic balance of these regulatory processes is crucial for maintenance of mitochondrial dynamics.

In NAFLD, PGC-1α activity is downregulated, which will consequently lower the *Mfn2* gene transcription. At the same time, SREBP activity is enhanced, increasing lipid synthesis and accumulation (Figure 1.4).

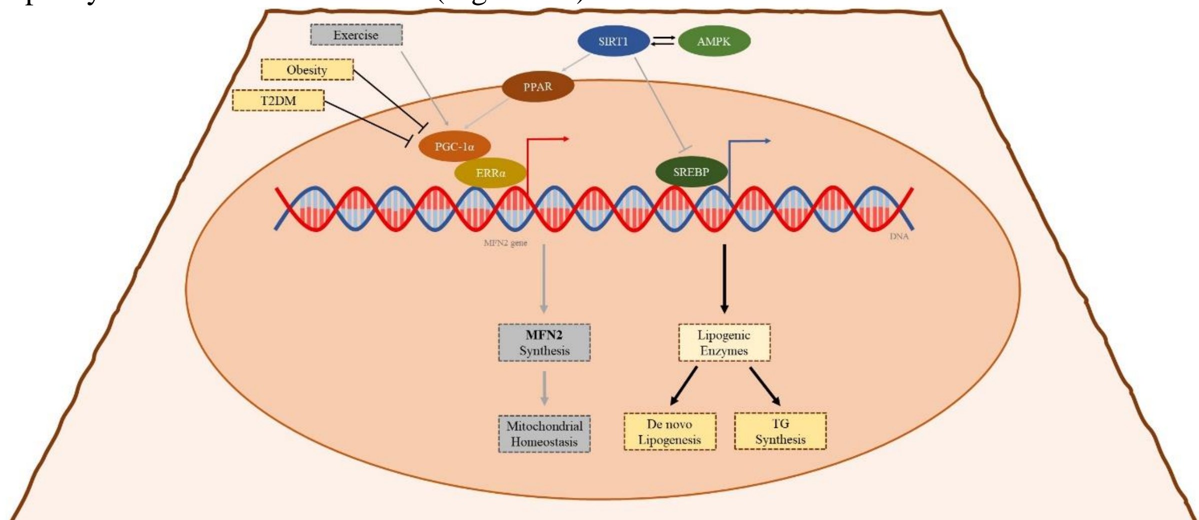


Figure 1.4 | *Mfn2* gene regulation in NAFLD. *Mfn2* regulators are inhibited due to the deregulated metabolic state of the organism, like obesity and T2DM, hallmarks of NAFLD. This will result in decreased MFN2 mRNA synthesis, which will ultimately enhance NAFLD pathogenesis and progression. At the same time, SREBP inhibition is decreased, which leads to de novo lipogenesis and TG synthesis, enhancing lipid accumulation within the hepatocytes.

MFN2 is regulated at both pre- and post-transcriptional levels. Ultimately, any deregulation in the process will lead to mitochondrial and ER dysfunction, ROS production, lipid metabolism impairment and NAFLD pathogenesis.

1.3 microRNAs and NAFLD

microRNAs (miRNAs/miRs) are small non-coding RNA molecules that regulate gene expression at the post-translational level. With their imperfect complementarity, these 22 nucleotides (nt) long RNAs, they bind to mRNA after transcription and prevent its connection to the ribosome where translation happens, as shown in Figure 1.5.

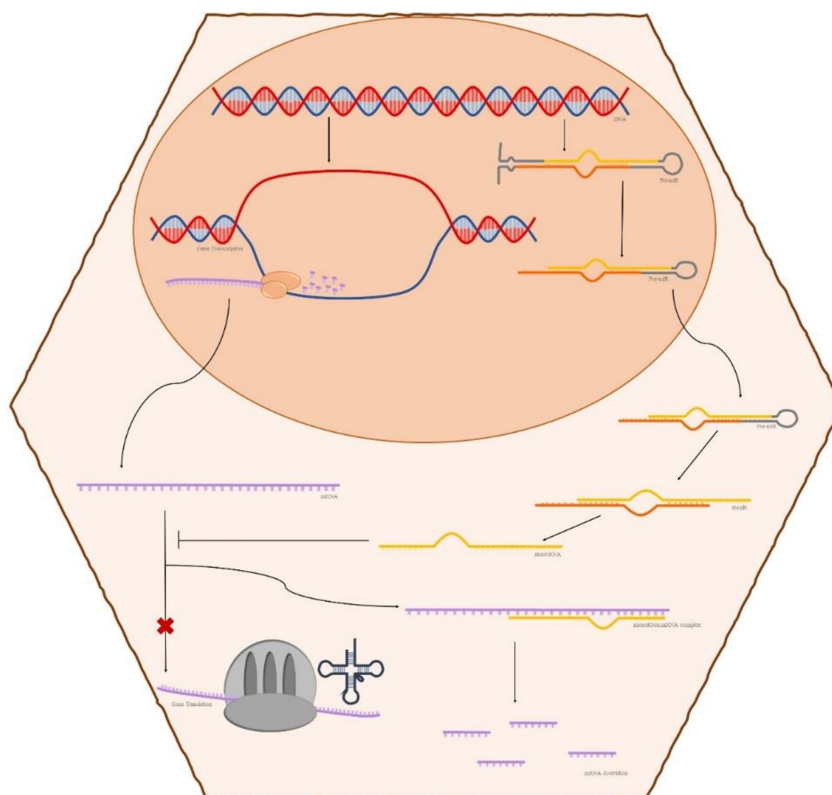


Figure 1.5 | Synthesis and mechanism of action of microRNAs. After being transcribed, microRNAs undergo a maturation process, going from pri-miR to pre-miR and finally to mature miRNA. This 22 nt molecule, binds to a mRNA and prevents the translation step to occur, therefore stopping protein synthesis and leading to mRNA inhibition or destruction.

1.3.1 MicroRNA-222-3p

A recent report has suggested that miR-222-3p is a direct inhibitor of MFN2 and that its liver ablation ameliorates NAFLD and NASH phenotypes (34). In fact, miR-222-3p is one of the most up-regulated miRNAs in the liver of experimental NAFLD mice. Ablation of this post-transcriptional regulator in these models led to reduced hepatic steatosis, inflammation, and fibrosis (34). On the other hand, overexpression of

miR-222-3p aggravated NASH and led to a faster progression to inflammation and fibrosis (34). Interestingly, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) can promote miR-222-3p transcription, creating a positive feedback loop enhancing NAFLD, since inflammation is another hallmark of the disease. This miRNA was found to be upregulated in every NAFLD stage, from simple steatosis up until HCC, being one of the miRNAs that is most upregulated in this lethal cancer, as well in adipocytes of obese animal models and human patients. With that in mind, miR-222-3p might be a special candidate for a novel therapeutic approach since it is directly connected with MFN2 and contributes to oxidative stress, inflammation, fibrosis, steatosis, and triglycerides accumulation, which are the main hallmarks of NAFLD. Liver specific ablation of this regulatory RNA counteracted the NAFLD phenotype, resulting in decreased fibrosis, inflammation, and steatosis. Not surprisingly, liver enzymes such as alanine transaminase (ALT) and aspartate transaminase (AST) were also found decreased, depicting improved liver disease.

Thus, this molecule seems to be a promising candidate for an effective therapy for NAFLD. Also, it is expected that miR-222-3p may also have some influence on other disease features and pathological mechanisms, which requires more studies. Also, PGC-1α was also described as a target of miR-222-3p, which therefore might further reduce MFN2 levels, indirectly (35).

2 Objectives

Previous results from our group showed that mice fed with a methionine and choline deficient diet combined with a high fat diet (MCD/HFD) - a NAFLD animal model - have decreased MFN2 protein levels and increased miRNA-222-3p expression in the liver (Figure 2.1). In addition, treating these animals with an inhibitor of miRNA-222-3p – antagomir-222-3p – slightly increased MFN2 expression.

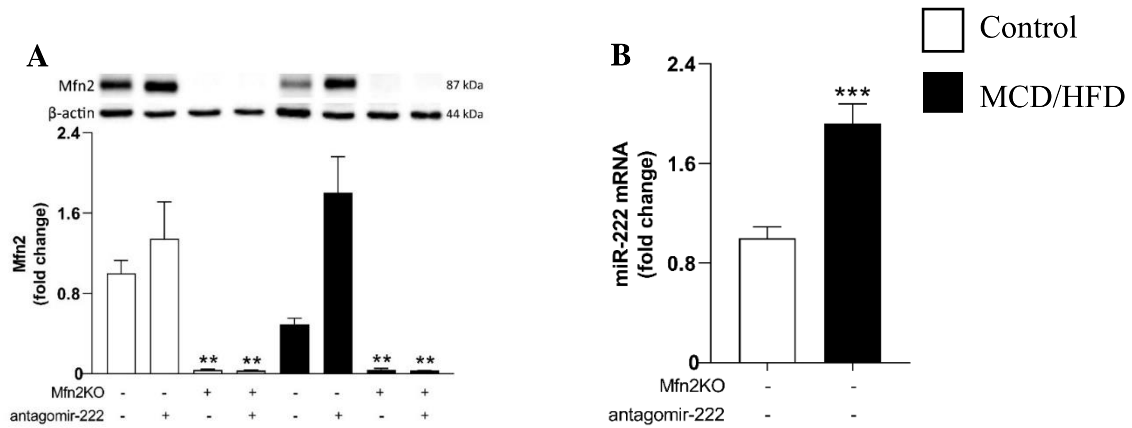


Figure 2.1 | Preliminary results. A – MFN2 protein levels are decreased in the livers of MCD/HFD-fed mice, comparing with controls. When treated with antagomiR-222-3p, MFN2 protein expression increased. B – In MCD/HFD-fed mice, miR-222-3p expression is increased.

Alongside these findings, MFN2-KO animal models showed an accumulation of TG within the liver, suggesting a propension to develop steatosis and therefore NAFLD, as shown in Figure 2.2.

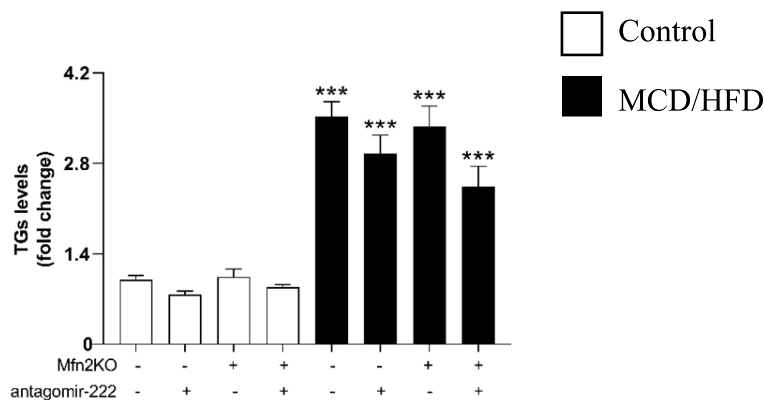


Figure 2.2 | Preliminary results II. Triglycerides levels are increased in MCD+HFD diet. Also, the treatment with antagomiR-222-3p suggests a possible protective role in the lipid accumulation.

With that in mind, the goals of this project were to:

1. Evaluate the capacity of the MCD+HFD diet to induce a NAFLD-like phenotype in mice.
2. Understand how the diet and treatment with antagomiR-222-3p affect lipid metabolism pathways, using both MFN2-KO animals and WT mice.
3. Unravel the effects of the putative NAFLD-inducing diet, MFN2 and antagomiR-222-3p in the liver ER stress response.

3 Materials and Methods

3.1 Animal Models

10-weeks-old C57BL6 male mice (Charles River Laboratories International, Inc., Wilmington, MA, USA; n=76) were randomly assigned into 8 groups, according to Table 3.1.

Table 3.1 │ Animal groups division, according to the study variables. MCD: Methionine-Choline Deficient; HFD: High Fat Diet; *Mfn2*: Mitofusin-2; KO: Knock-Out.

Group	n	MCD+HFD Diet	*Mfn2*-KO	AntagomiR-222
1	10	-	-	-
2	9	-	-	+
3	8	-	+	-
4	8	-	+	+
5	11	+	-	-
6	10	+	-	+
7	10	+	+	-
8	10	+	+	+

Mice (Charles River Laboratories International, Inc.; n=41) were fed with a MCD diet (TestDiet®, St. Louis, MO, USA) combined with a high fat diet HFD (Research Diets Inc., New Brunswick, NJ, USA) supplemented with 0.1% L-methionine on drinking water for 3 weeks, compared with a standard diet (SD; n=35). The water was supplemented with methionine on a low dose, even though the diet is free of it, because a diet with a total ablation of this component would cause mice weight loss, leading to a non-similar feature of NAFLD.

As we aimed to study MFN2 activity and if any potential therapeutic effects of antagomiR-222-3p were dependent on MFN2 status, both MFN2-WT (n=40) and MFN2 hepatocyte-specific KO (n=36) mice were used. Genetically modified animal models were generated by a *Cre-Lox* hepatocyte-specific KO (36).

To analyse the possible therapeutic effect of silencing miR-222-3p, mice were treated randomly with either phosphate-buffered saline (PBS), that works as a placebo (n=39), or with a specific antagonist of miR-222-3p - antagomiR-222-3p (Qiagen©, Hilden, Germany; n=37). Mice received 3 subcutaneous injections once a day for 3 consecutive days of either PBS or antagomiR-222 at 16 mg/kg body weight (Qiagen©), as shown in Figure 3.1.

After 3 weeks on the diet, animals were fasted for 4 hours, anesthetized using isoflurane, and then sacrificed by cervical dislocation. After that, the liver samples were extracted, cut, flash-frozen in liquid nitrogen, and further stored at -80ºC.

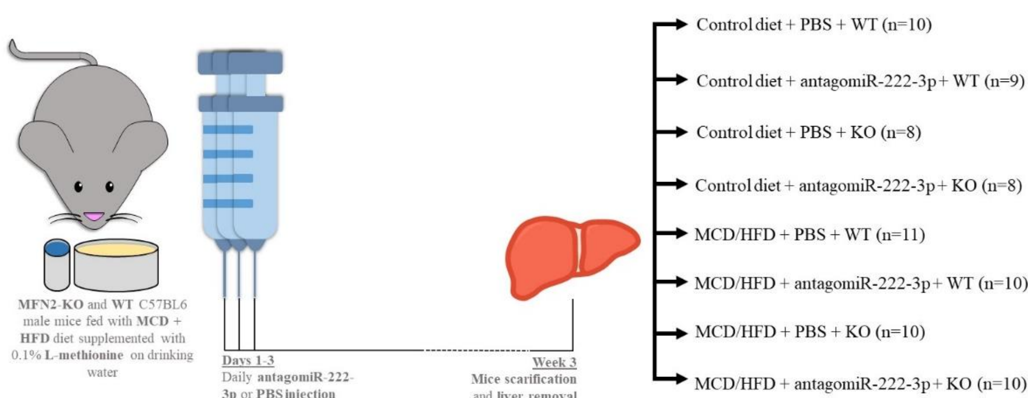


Figure 3.1 | Animal models. C57BL6 male mice were fed either with a standard diet (n=35) or with MCD+HFD diet (n=41) and treated with three injections in consecutive days of PBS as placebo (n=39) or antagomiR-222-3p (n=37). MFN2-KO animal models were used (n=36) facing MFN2-WT (n=40). After three weeks mice were sacrificed, and livers was removed.

All animal experiments were carried out with the permission of the local animal ethical committee in accordance with the EU Directive (2010/63/EU), Portuguese law (DL 113/2013) and all relevant legislations. The experimental protocol was approved by Direção Geral de Alimentação e Veterinária, Portugal. Animals received humane care in a temperature-controlled environment with a 12-hour light-dark cycle, complying with the Institute's guidelines, and as outlined in the "Guide for the Care

and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). All experiments were performed by an investigator accredited for directing animal experiments (FELASA level C).

3.2 Histopathological analysis

Liver sections were coloured with the haematoxylin-eosin method and blinded evaluated and scored by an experienced pathologist, in an microscopical analysis. Steatosis was graded from 0 to 3 based on the percentage of steatotic hepatocytes (0: <5%; 1: 5-33%; 2: 34-66%; 3: >66%). Inflammation was graded from 0 to 3 by the presence or absence of inflammatory cells under microscopic examination at x200 magnification (0: absent; 1: <2 foci per field; 2: 2-4 foci per field; 3: >4 foci per field). NAFLD Score (NAS) was also calculated concerning ballooning, steatosis and inflammation grades and divided into steatosis (NAS ≤ 2), “borderline” NASH (NAS 3-4) and NASH (NAS ≥ 5) (37,38).

3.3 RNA Extraction

RNA extraction begins with tissue lysis to release cell content. This process was performed in the TissueLyser II (QIAGEN®, Hilden, Germany) in an eppendorff containing 1 milliliter (mL) of TRIzol™ (Thermo Fisher Scientific, Waltham, MA, USA) to denature proteins, split nucleoprotein complexes, and inhibit RNAses. After that, chloroform was added and a high-speed centrifugation at 12000 *g* performed. RNA was then isolated by removing the aqueous phase. A precipitation step was carried out, with isopropanol, and the RNA was then washed with ethanol at 75% and dissolved in Milli-Q® (Merck KGaA, Darmstadt, Germany) sterile water and stored at -80ºC.

3.4 RNA Purification

RNA purification was done to ameliorate the ratios of contamination with both proteins and alcohols. For that, RNA was precipitated again, with sodium acetate 3M, with a pH of 5.2, and pure ethanol. After this step, RNA was washed between two to

three times, with ethanol at 75%, as in the extraction step. By the end of the washes, the RNA pellet was dried and redissolved in Milli-Q ® (Merck KGaA) water.

3.5 RNA Quantification

Qubit™ 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentration of RNA in solution. This fluorescence method is important so that we can use the same amount of RNA in the next steps, ensuring that the differences seen are from the gene expression in the cell and not the amount of sample used.

3.6 Gene Expression Assays

3.6.1 Reverse Transcription – cDNA Synthesis

To be able to study the expression of the target gene, complementary DNA (cDNA) synthesis is required, since the polymerase chain reaction (PCR) is meant to amplify only DNA. A reverse transcription (RT) was performed, synthetizing a complementary DNA.

The reaction was performed based on the SYBR™ Green (Meridian LifeScience, BioLine®, Tennessee, USA) method. 1 microgram (μg) of RNA from each sample was reverse-transcribed into cDNA in a non-specific way by using 2 microliters (μL) of random hexamers per duplicate (NZYTech, Lda, Lumiar, Portugal) – random sequences of six nucleotides that will work as primers. 1 μL of deoxyribonucleotides triphosphate (dNTPs) and 2 μL of the reverse transcriptase enzyme (NZYTech, Lda, Lumiar, Portugal) was used. Along with that, a RNAse inhibitor (NZYTech, Lda, Lumiar, Portugal) was used to suppress enzymes that could degrade the samples, as well as a reaction buffer (NZYTech, Lda, Lumiar, Portugal), the medium with all the cofactors required. The temperature cycles shown in table 3.2 were performed in the VWR Thermal Cylcer XTender[96] (VWR International, LLC, USA).

Table 3.2 │ SYBR™ Green RT protocol

Step	Temperature (ºC)	Time (min)
1	25	10:00
2	50	50:00
3	85	05:00

3.6.2 Quantitative Polymerase Chain Reaction

The PCR is a technique to amplify and quantify DNA. Along with DNA polymerase, nucleotides, and the correct medium and cofactors, contained in the SYBR™ Green solution (Meridian LifeScience, BioLine®), with the right temperature cycles as shown in table 3.3, the DNA is amplified. Also, the SYBR™ Green I Dye (Meridian LifeScience, BioLine®) was used, increasing its signal proportionally to the amount of new synthetized DNA, binging itself to the double stranded DNA.

As SYBR™ Green (Meridian LifeScience, BioLine®) protocol generates cDNA of all genes in sample, the PCR must be specific to amplify only the cDNAs of interest, and for that specific primers are used, which sequences are in table 3.4. The reaction took place in the Quantstudio™ 7 Flex real-time PCR instrument (Applied Biosystems, Thermo Fisher Scientific).

Table 3.3 │ SYBR™ Green quantitative PCR protocol

Step	Temperature (ºC)	Time (min)
1	95	02:00
2 (× 40 cycles)	95	00:05
	60	00:30
3	95	00:15
4	60	01:00
5	95	00:15

Table 3.4 │ Primers used in SYBR™ Green qPCR protocol

Gene	Primer	Sequence
FASN	Forward	5’- AAG TTG CCC GAG TCA GAG AAC C -3’
	Reverse	5’- ACT CAT AGA GCC CAG CCT TCC ATC -3’
FATP-2	Forward	5’- GAT ACT TTC CGG TGG AAA GG -3’
	Reverse	5’- TTC GAC CCT CAT GAC CTG -3’
FATP-5	Forward	5’- TAC AAG TTG GAG CCA CCT G -3’
	Reverse	5’- TCA CCC ACA TAC AAG ATC ACT G -3’
DGAT-2	Forward	5’- AGT TTC CTG GCA TAA GGC CC -3’
	Reverse	5’- TGG GA ACCA GAT CAG CTC CAT -3’
LXR	Forward	5’- TGT GCG CTC AGC TCT TGT C -3’
	Reverse	5’- CTC CGT TGC AGA ATC AGG AGA A -3’
LIPIN-1	Forward	5’- CAT ACA AAG GCA GCC ACA CG -3’
	Reverse	5’- GAG CTC CTT CAC CGT CAC AA -3’
INSIG-1	Forward	5’- ACC ATC GCC TCC CTA GCT AC -3’
	Reverse	5’- CCC ATA GCT AAC TGT CGT CCT -3’
CPT-1	Forward	5’- TGG ACC CAA ATT GCA GTG GT -3’
	Reverse	5’- GCA TCT CCA TGG CGT AGT AGT -3’
ACSL-5	Forward	5’- TTA AAC TTG GCG GGG TGA GA -3’
	Reverse	5’- CTG TGT AGC TCC TTT CGC CA -3’
PERK	Forward	5'- GGA CTC CTG TCT TGG TTG GG -3'
	Reverse	5'- TGG GGC TGA GGA TGG AAA AG -3'
ATF-6	Forward	5'- ATT TGG GAG GTG GAA GTG GG -3'
	Reverse	5'- AAG CCA CAG GTC CTC TTT AGG -3'
ERN-1	Forward	5’- ACC TGC CCA AAC ATC GAG A -3’
	Reverse	5’- CTG AGA TAC GGT GGT CGG TG -3’

HPRT	Forward	5'- GGT GAA AAG GAC CTC TCG AAG TG -3'
	Reverse	5'- ATA GTC AAG GGC ATA TCC AAC AAC A -3'

The relative amount of genes was determined by the threshold cycle ($2^{-\Delta\Delta CT}$) method, where $\Delta\Delta CT = (CT_{Target} - CT_{HPRT})_{sample} - (CT_{Target} - CT_{HPRT})_{calibrator}$. It is important to notice that in this protocol, using SYBR™ Green primers, a correction on the CT value must be done with a calibration curve previously done for the primers. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene is used as the control gene, since its expression is regular across the groups.

With this correction and with the normalization with the control gene and the control group, the results become expressed in fold change from the control.

3.7 Statistical Analysis

Statistical analysis was performed with GraphPad Prism 9.3.0 software (GraphPad Software, Inc., San Diego, CA, USA). The collected data was statistically tested for normal distribution through the Shapiro-Wilk normality test. Data was analyzed one-way analysis of variance (ANOVA) with Tukey post hoc. In case of passing the normality test, this was the used method. In case of not corresponding to a gaussian distribution, a non-parametric ANOVA was performed with a Dunn's test post hoc. Values of $p < 0.05$ were considered statistically significant.

4 Results

4.1 The MCD+HFD diet induces a NAFLD/NASH phenotype in mice

Histopathologic analysis (Figure 4.1A) showed that animals fed the MCD+HFD diet displayed liver histology characteristic of a NAFLD/NASH phenotype. Vacuolization, indicative of lipid accumulation within hepatocytes, is visible in the liver sections of animals fed the MCD+HFD diet, comparing with the control, standard diet (SD). MCD+HFD-fed MFN2-KO animals appeared to have slightly worsen steatosis, while treatment with antagomiR-222-3p had a protective effect in both WT and MFN2-KO mice, diminishing the number of lipid vacuoles. The steatosis score (Figure 4.1B) was in agreement with these observations, although it should be noted that no statistical differences were found. As for the NAS score, it was found to be significantly elevated in MCD+HFD-fed mice ($p<0.05$ *vs* control), further suggesting that this diet efficiently induced NAFLD in these animals (Figure 4.2C). Unlike for the steatosis score, MCD+HFD-fed MFN2-KO animals displayed a slightly lower NAS score comparing with MCD+HFD-fed WT animals. Still, the antagomiR-222-3p treatment led to the lowest values of the NAS score.

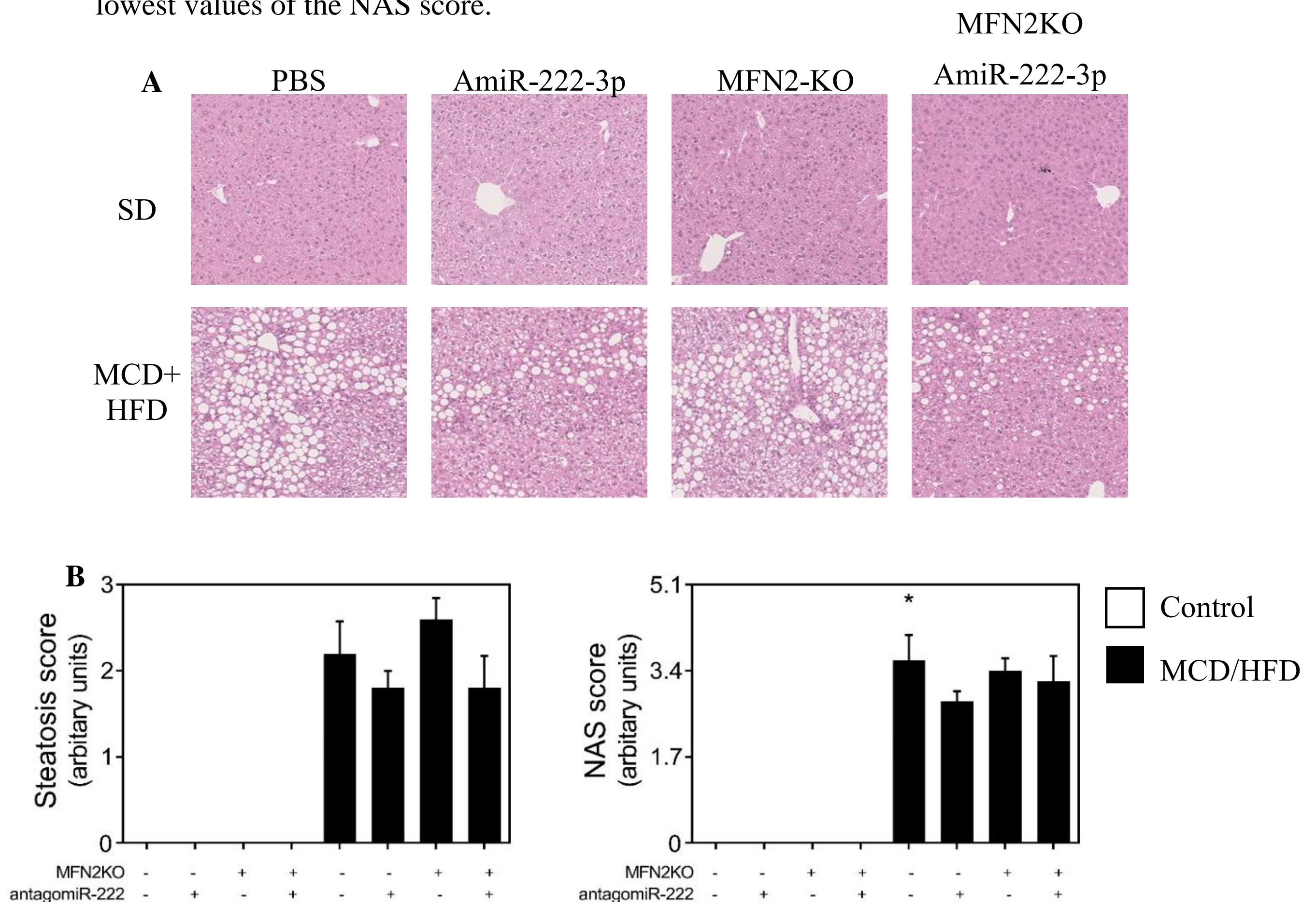
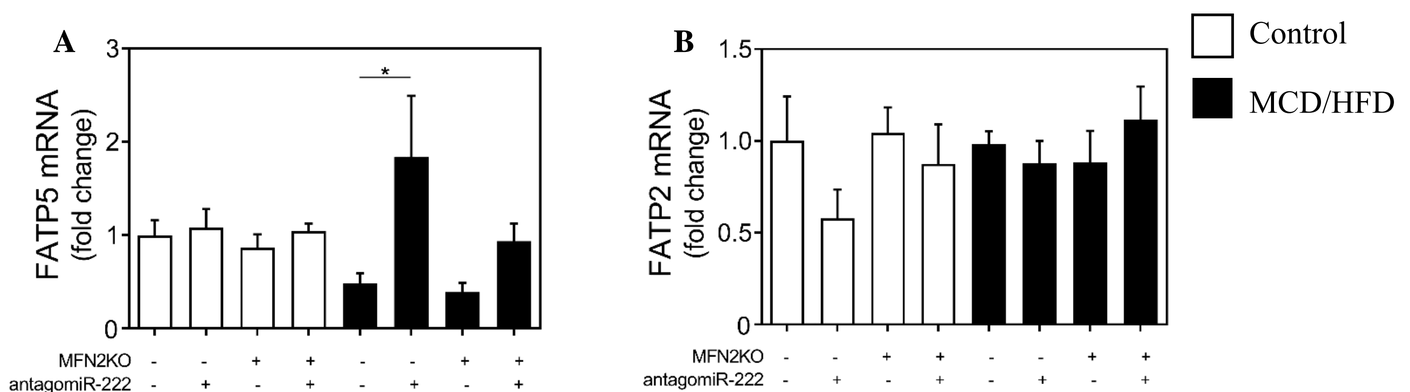


Figure 4.1 | Histopathological NAFLD analysis. Liver sections were coloured with the haematoxylin-eosin method and blinded evaluated by an experienced pathologist, in an microscopical analysis and scored concerning steatosis and NAS. A – Histological images show lipid accumulation in MCD+HFD-fed animals, with a putative protective effect of antagomiR-222-3p. B – Steatosis score was calculated taking into account the percentage of steatotic hepatocytes, C – NAS was calculated as described in Materials and Methods. *p<0.05

4.2 AntagomiR-222-3p modulates liver expression of fatty acid transporters in MCD+HFD-fed mice

Hepatocyte fatty acid transporters, namely FATP5 and FATP2, as well as CPT1, a mitochondria fatty acid transporter were studied. Results showed that, even though both isoforms of FATP are expressed in the liver, FATP5 is more altered in experimental NAFLD (Figure 4.2A and B). Despite no statistical differences being found, this receptor was consistently down-regulated in MCD+HFD-fed mice, irrespective of MFN2 expression. Noteworthy, upon antagomiR-222-3p treatment, FATP5 levels were significantly increased in MFN2-WT mice ($p<0.05$ *vs* PBS) but only slightly increased in MFN2-KO mice, suggesting 1) a protective role of FATP5 in NAFLD and 2) an at least partially MFN2-dependent modulation by antagomiR-222-3p. On the other hand, no significant changes in FATP2 expression were found (Figure 4.2B). Despite MCD+HFD-fed mice exhibiting similar liver CPT1 expression levels as control animals, antagomiR-222-3p significantly increased its expression in MFN2-WT mice ($p<0.05$ *vs* PBS) but not in MFN2-KO mice, reinforcing the notion that increased expression of fatty acid transporters might be protective in NAFLD and that antagomir-222-3p mediates this effect - at least partially - via MFN2.



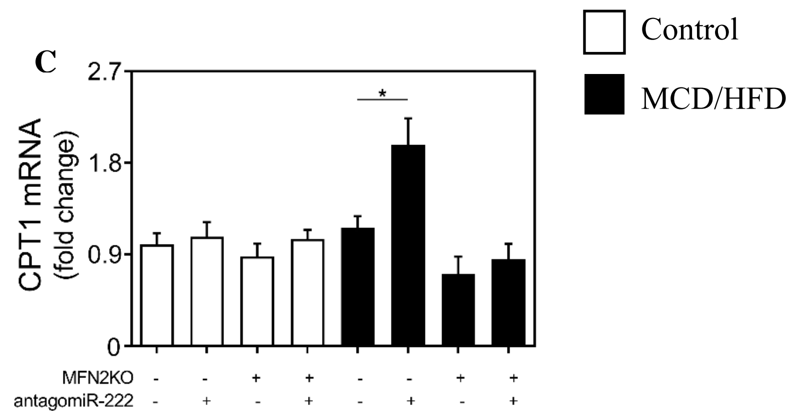


Figure 4.2 | AntagomiR-222-3p treatment increases liver expression of fatty acid transporters. C57BL6 male mice were fed either with a standard diet (n=35) or with MCD+HFD diet (n=41) and treated with PBS as placebo (n=39) or antagomiR-222-3p (n=37). MFN2-KO animals were used (n=36) facing MFN2-WT (n=40). RNA extraction and gene expression analysis trough RT-PCR was performed for (A) FATP5, (B) FATP2 and (C) CPT1. *p<0.05

4.3 Lipid metabolizing enzymes are altered in hepatocytes with steatosis and are slightly modulated by antagomiR-222-3p

Lipid metabolism enzymes are typically deregulated in NAFLD. Our results showed that, in MCD+HFD-fed mice, DGAT2 and ACSL5 were slightly upregulated, whereas LIPIN1 and FASN were downregulated (*p<0.05 vs. controls for FASN) (Figure 4.3). For up-regulated genes, antagomiR-222-3p had marginally protective effects, mostly for DGAT2. In turn, protective effects appeared to be superior for downregulated genes, in both MFN2 WT and KO mice, for which a MFN2-dependent role of antagomiR-222-3p appears not to exist in these settings. Noteworthy, MCD+HFD-induced FASN down-regulation was less statistically significant in animals concomitantly treated with antagomiR-222-3p (0.01 vs. 0.001 in MFN2 WT animals and 0.05 vs. 0.001 in MFN2 KO animals).

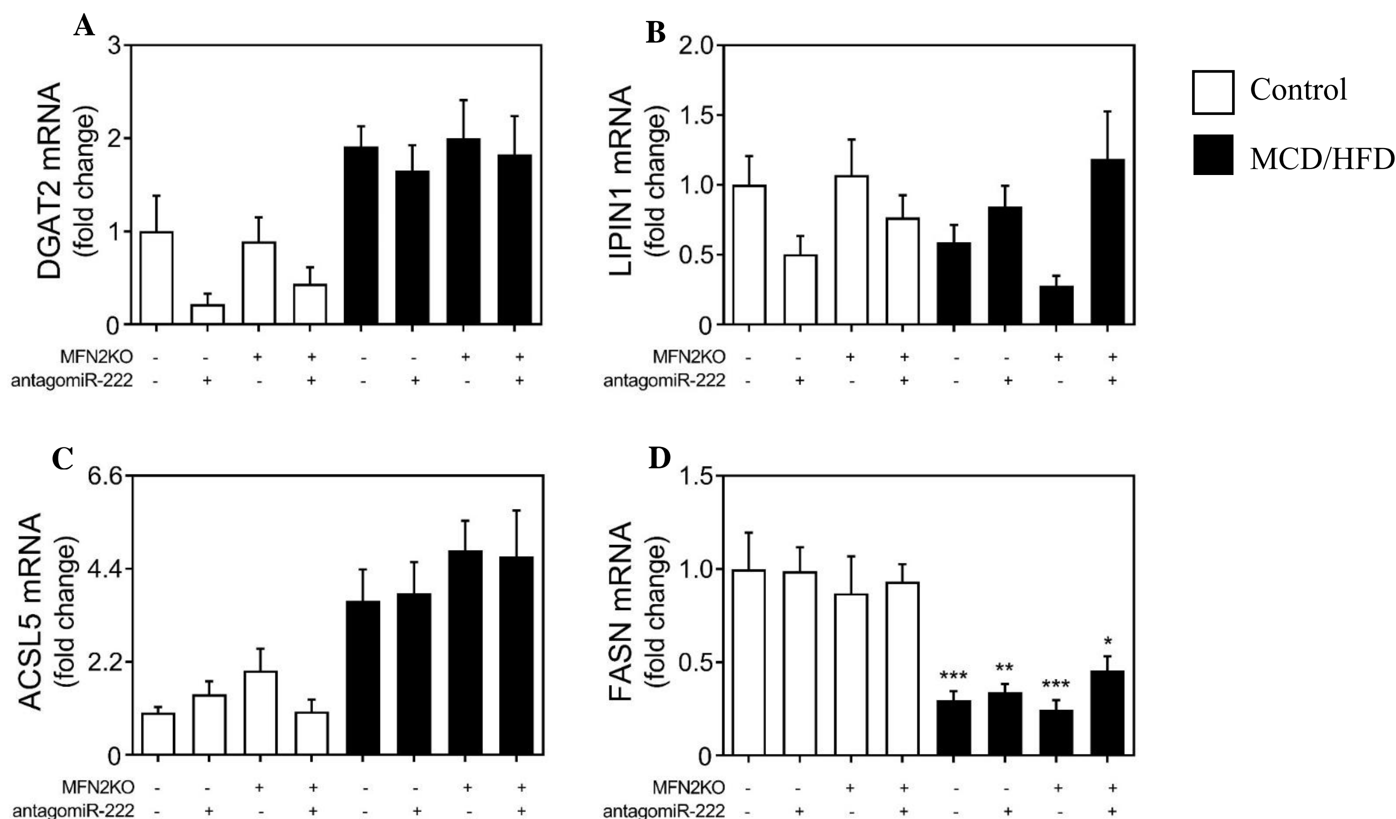


Figure 4.3 | Lipid metabolism enzymes are deregulated in NAFLD and partially modulated by antagomir-222-3p. C57BL6 male mice were fed either with a standard diet (n=35) or with MCD+HFD diet (n=41) and treated with PBS as placebo (n=39) or antagomiR-222-3p (n=37). MFN2-KO animals were used (n=36) facing MFN2-WT (n=40). RNA extraction and gene expression analysis trough RT-PCR was performed for (A) DGAT2, (B) LIPIN1, (C) ACSL5 and (D) FASN. *p<0.05, **p<0.01, ***p<0.001 vs respective control.

4.4 Endoplasmic reticulum stress mediators are deregulated in MCD+HFD-fed mice and are slightly modulated by antagomiR-222-3p

Our results showed that the main mediators of the UPR were deregulated in MCD+HFD-fed mice, comparing with SD-fed mice (Figure 4.4). In particular, PERK and ATF6 liver mRNA expression was increased ($p<0.05$ for ATF6), while no changes were observed for IRE1. MFN2 ablation led to increased PERK expression and decreased ATF6 and IRE1 expression in MCD-HFD-fed mice, comparing with MFN2 WT mice, although these results were not statistically significant. The antagomir-222-3p treatment very slightly prevented PERK mRNA increase in both MFN2 WT and KO

mice. This inhibitory effect was more evident for ATF6 ($p<0.05$ in MFN2 WT mice). No major effects were observed for IRE1.

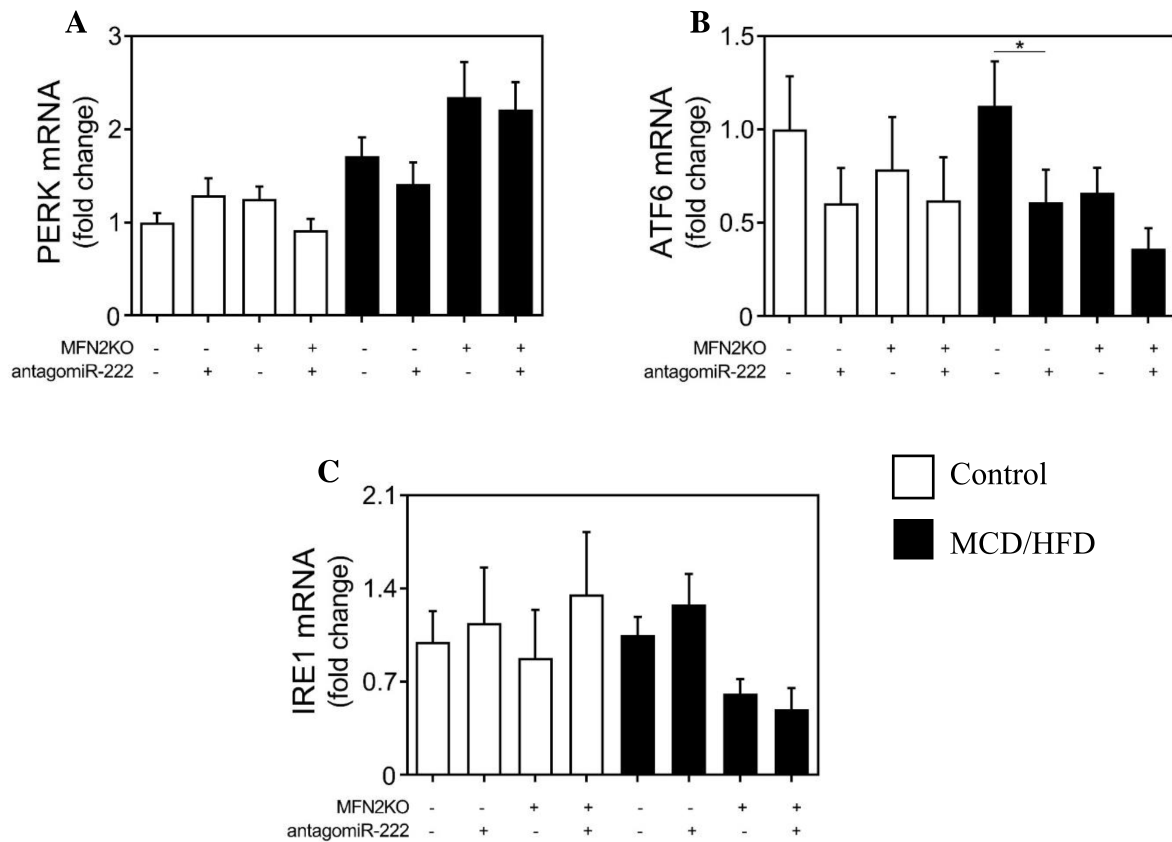


Figure 4.4 | Hepatic UPR gene expression is increased in MCD-HFD-fed mice and partially modulated by antagomir-222-3p. C57BL6 male mice were fed either with a standard diet (n=35) or with MCD+HFD diet (n=41) and treated with PBS as placebo (n=39) or antagomiR-222-3p (n=37). MFN2-KO animals were used (n=36) facing MFN2-WT (n=40). RNA extraction and gene expression analysis trough RT-PCR was performed for (A) PERK, (B) ATF6 and (C) IRE1. *$p<0.05$

5 Discussion

In this work, we established that our MCD diet model combined with HFD induced NASH-like liver features in a short period of time, including steatosis and dysregulated lipid metabolism. The MCD diet, besides having high sugar and fat (39), has not methionine nor choline, which are needed substrates for mitochondrial β-oxidation of fatty acids and very low density lipoproteins (VLDL) (40). That said, mice will not be able to metabolize fat, leading to hepatic accumulation and therefore ballooning and steatosis, which will evolve to NAFLD and NASH, paired with the characteristic fibrosis and cell death (41). Apart from that, other studies have shown that male C57BL6 mice also develop inflammation with the MCD diet, in a short period of time (42), making this diet useful for studying NAFLD/NASH. However, the systemic response of mice in this model alone does not fully reflect the human disease, since it can cause weight loss and hypoglycaemia (43). To overcome that and to mimic the human disease, a HFD was used along with the MCD diet. As the name suggests, it is a diet enriched in fat (39) that not only induces NAFLD, NASH and HCC, but also creates a metabolic state of IR, obesity, hyperglycaemia, hypercholesterolemia, and hypertriglyceridemia, along with the inflammation, fibrosis, mitochondrial dysfunction, and oxidative stress (43), i.e., the hallmarks of the human NAFLD. As a result, our results showed that the steatosis and NAS scores were clearly elevated in MCD+HFD-fed mice, confirming the capacity of this diet to induce NAFLD. Interestingly, upon antagomiR-222-3p treatment, both scores tended to decrease, suggesting that silencing miR-222-3p might afford therapeutic effects in ameliorating NAFLD.

Given that steatosis was deeply altered in our experimental model, we next evaluated expression of lipid metabolism enzymes in the liver. Only one of the FATP isoforms showed to be altered by the diet, namely FATP5. This may be due to some specificity of the isoform in FFA uptake since FATP5 is more correlated with NAFLD lipid transport and accumulation (44). In parallel, CPT1 mitochondrial transporter mRNA expression was not affected by the MCD-HFD diet. Nonetheless, upon antagomiR-222-3p treatment, both FATP5 and CPT1 expression levels were increased. The ultimate effect of this modulation could be the increased influx of lipids into hepatocytes and, subsequently, into the mitochondria, to be metabolized and then stored

in lipid droplets, which could be seen as a protective mechanism for the cell, avoiding toxic lipids to be free, at least in some stage of the disease. Of note, antagomir-222-3p-induced FATP5 and CPT1 expression appears to be at least partially dependent on MFN2, as this effect was less pronounced in MFN2 KO animals. Still, the overall meaning of the miR-222-3p: MFN2 pathway in NAFLD deserves further exploitation, as the effect of antagomir-222-3p in the histological analysis – and other lipid metabolism genes - seemed to be independent of MFN2.

Before being stored, lipids need to be metabolised into TGs. Our results showed that the acyl-CoA pathway appears to be particularly modulated in our NAFLD animal model. ACSL5, that converts FA into acyl-CoA was clearly upregulated, as well as DGAT, an enzyme that turns DG into TG. Corroborating this data, FASN, that participates in the acetyl-CoA pathway, was significantly downregulated. Upon treatment with antagomiR-222-3p, FASN expression was slightly increased. This could translate into an accelerated lipid metabolism to synthetize TG, which would again constitute part of the overall protective effects of antagomir-222-3p in NAFLD. Surprisingly, antagomir-222-3p failed to induce major changes in DGAT and ACSL5 mRNA expression. In turn, LIPIN1 expression was positively – though not statistically significant – modulated by antagomir-222-3p. This might have a protective effect since it is one of the enzymes in the pathway of TG synthesis, to store it in lipid droplets.

The ER stress response was shown to be increased in mice fed the MCD+HFD diet, namely mRNA expression of PERK and ATF6, though not IRE1α. The UPR consists of a chain of reactions triggered by many cell-injury factors (27), like ROS and lipid excess. Thus, it is expectable for this process to be exacerbated in MCD+HFD animals. In these animals, antagomiR-222-3p decreased the expression of both PERK and ATF6 and this effect could be, once again, partially dependent on MFN2. As such, inhibition of ER stress could represent an additional mechanism by which antagomir-222-3p exerts its protective effects. However, more studies should be performed, as these results were not statistically significant.

6 Conclusions and Future Perspectives

In this work, it was firstly established that the MCD and HFD diets, together, can induce NAFLD efficiently in a short period of time, with some of the same features as human NAFLD. One of these features is the deregulated hepatic lipid metabolism. The liver is one of the main organs responsible for metabolizing fat. As such, the study of metabolic impairment in this organ is very important to better understand not only NAFLD pathogenesis but also the details of the antagonizing effects of antagomiR-222-3p treatment, as well as the role of MFN2 in both scenarios. We can conclude that lipid metabolism is clearly impaired in our experimental model of NAFLD, from lipid entrance within hepatocytes to its metabolization and storage. From the pathways studied, the acyl-CoA showed to be the more affected. This pathway not only metabolizes fat, but it is also connected to mitochondrial proteins, which may suggest a relationship between mitochondrial impairment and fat metabolism. Along with it, antagomiR-222-3p showed its protective effect along the pathway, eliciting a putative therapeutic role for treating NAFLD. Hepatocyte-specific MFN2 ablation appeared to affect the protective mechanisms of antagomir-222-3p only partially, suggesting that additional targets play a more substancial role in mediating its ameliorating effects. As such, more studies need to be performed to better understand the role of miRNA-222-3p and its relationship with MFN2 in NAFLD lipid metabolism.

Along with these findings, we can also conclude that ER stress is augmented in our experimental NAFLD model, possibly playing a functional role in disease triggering and progression. Similarly to lipid metabolism, antagomir-222-3p treatment attenuated ER stress, as seen by the inhibition of PERK and ATF6 mRNA expression, which may thus constitute another mechanism by which the silencing of miR-222-3p affords protective effects against NAFLD. Even though these results are promising, more studies should be done in order to realise the connection between the UPR and mitochondrial deregulation, and how miR-222-3p modulates it.

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