The role of MBOAT7 on fatty liver disease

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"Science, my boy, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth."

Jules Gabriel Verne
Journey to the center of the Earth

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the main health disorder in internal medicine, affecting one third of the population worldwide. Environmental and genetic factors contribute to NAFLD susceptibility and progression. Amongst these, a genetic variant in the *membrane bound O-acyltransferase domain-containing 7* (*MBOAT7*), also known as *lysophosphatidylinositol acyltransferase 1* (*LPIAT1*), robustly contributes to the entire spectrum of NAFLD.

MBOAT7 encodes for an O-acyltransferase that catalyses the transfer of free fatty acids to lysophospholipids, allowing the remodelling of phospholipids. The *MBOAT7 rs641738 C>T* genetic variant is associated with the development and progression of NAFLD and related end stage liver disease, namely cirrhosis and hepatocellular carcinoma (HCC). Despite the interest in MBOAT7, very little is known about the mechanisms by which this protein contributes to the pathogenesis and progression of fatty liver disease.

In this thesis, we unveiled the topological organization, we assessed the enzymatic activity, we identified the catalytic site, and we unravelled how MBOAT7 depletion causes hepatic fat accumulation via a novel metabolic pathway.

In paper I, by using a combination of *in silico* and *in vitro* approaches, we showed that MBOAT7 is a multispanning membrane protein strongly attached to endomembranes by six transmembrane domains (TMDs) and two putative re-entrant loops.

In paper II, by producing in large scale the human MBOAT7 in the yeast species *Pichia pastoris*, we described MBOAT7 as an O-acyltransferase that esterifies free polyunsaturated fatty acids (PUFAs) to the *sn-2* position of lysophosphatidylinositol (LPI), releasing newly acyl-chain remodelled phosphatidylinositol (PI). Moreover, missense

mutations at the position 321 and 356 of the protein almost abolished the enzymatic activity, indicating that this is the catalytic dyad for the O-acyl transferase activity of MBOAT7.

In paper III, we showed that MBOAT7 depletion led to triglycerides and collagen accumulation in 2D and 3D hepatic models. Similarly, hepatic specific *MBOAT7* knock-out mice developed steatosis and fibrosis. We demonstrated that MBOAT7 depletion on the one hand reduced the PI acyl chain remodelling rate, and on the other hand it boosted the PI synthesis and degradation. PI breakdown can be catalysed by a phospholipase C-like protein that releases newly synthesized diacylglycerols (DAGs). DAGs can undergo a final esterification step resulting in triacylglycerol (TAG) synthesis, the main lipid component of liver fat.

In conclusion, our studies demonstrate that MBOAT7: 1) is an integral membrane protein anchored to endomembranes by six TMDs; 2) has an O-acyltransferase activity that preferentially esterifies PUFAs to LPI with a catalytic site composed of the Asparagine and Histidine in position 321 and 356 of the protein, respectively; and 3) MBOAT7 depletion causes higher liver fat content by increasing triglyceride synthesis mediated by a novel non-canonical bio-metabolic pathway.

Keywords

MBOAT7, O-acyltransferase, phosphatidylinositol, arachidonic acid, NAFLD, steatosis.

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SAMMANFATTNING PÅ SVENSKA

Non-alcoholic fatty liver disease (NAFLD) är den främsta sjukdomen inom internmedicinen och en tredjedel av världens befolkning är drabbad. Miljö och genetiska faktorer bidrar till benägenheten att utveckla sjukdomen samt till dess progression. En av dessa faktorer är en genetisk variant i *membrane bound O-acyltransferase domain-containing 7* (*MBOAT7*), även känd som *lysophosphatidylinositol acyltransferase 1* (*LPIAT1*), vilken starkt bidrar till alla spektra av NAFLD.

MBOAT7 kodar för ett O-acetyltransferas som katalyserar omvandlingen av fria fettsyror till lysofosfolipider, och som därmed medger modifiering av fosfolipider. Den genetiska varianten *MBOAT7 rs641738 C>T* är associerad med utvecklingen och progressionen av NAFLD och slutstadiet av leversjukdom; cirros och hepatocellulär cancer (HCC). Trots intresset för MBOAT7 är mycket lite känt kring de mekanismer som gör att detta protein bidrar till patogenesen och progressionen av fettleversjukdom.

I denna avhandling förklarar vi proteinets topologiska uppbyggnad, vi utvärderar dess enzymatiska aktivitet samt identifierar dess katalytiska domän, och vi visar hur en nedreglering av MBOAT7 leder till ansamling av leverfett via en ny metabol signalväg.

I artikel I visar vi genom en kombination av in silico- och in vitro-studier att MBOAT7 är ett polytopiskt membranprotein som är starkt bundet till endomembranet genom sex transmembrana domäner (TMD) och två. förmodade inåtgående öglor.

I artikel II visar vi, genom att i stor skala producera humant $MBOAT_7$ i jästarten *Pichia pastoris,* att MBOAT7 är ett O-acetyltransferas som förestrar fria fleromättade fettsyror (PUFA)

till *sn-2*-positionen i lysofosfatidylinositol (LPI) vilket leder till bildning av fosfatidylinositol (PI) med modifierade acylkedjor. Vidare visar vi att missensmutationer i position 321 och 356 i proteinet nästan helt upphäver den enzymatiska effekten, vilket indikerar att detta är den katalytiska domänen för O-acetyltransferas-aktiviteten hos MBOAT7.

I artikel III visar vi genom 2D- och 3D-modeller av levern att nedreglering av MBOAT7 leder till ansamling av triglycerider och kollagen. På motsvarande sätt utvecklade möss med leverspecifik knock-out av MBOAT7 steatos och fibros. Vi visar att nedreglering av MBOAT7 reducerar hastigheten med vilken acylkejorna av PI modifieras, medan den samtidigt ökar syntesen och degraderingen av PI. Nedbrytningen av PI kan katalyseras genom ett fosfolipas C-liknande protein som utsöndrar nysyntetiserat diacylglycerol (DAG). DAG kan genomgå ett slutligt förestringssteg vilket resulterar i syntes av triacylglycerol (TAG), den huvudsakliga lipid-komponenten i leverfett.

Samanfattningsvis demonstrerar våra studier att MBOAT7: 1) är ett integralt membranprotein som är bundet till endomembranet via sex TMDs; 2) har en O-acetyltransferas-aktivitet som företrädesvis förestrar PUFAs till LPI med en katalytisk domän bestående av aspargin och histidin i position 321 och 356 i proteinet; 3) nedreglering av MBOAT7 leder till ökad mängd leverfett genom ökad syntes av triglycerider, vilket medieras av en ny, icke kanonisk biometabol signalväg.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I.
Caddeo A*, Jamialahmadi O*, Solinas G, Pujia A, Mancina RM, Pingitore P, Romeo S.
MBOAT7 is anchored to endomembranes by six transmembrane domains.
J Struct Biol. 2019 Jun 1;206(3):349-360

II.
Caddeo A*, Hedfalk K, Romeo S, Pingitore P.
LPIAT1/MBOAT7 contains a catalytic dyad transferring polyunsaturated fatty acids to lysophosphatidylinositol.
Biochim. Biophys. Acta, Mol. Cell. Biol. Lipids 2021 Jan 26;158891

III.
Tanaka Y*, Shimanaka Y*, Caddeo A*, Kubo T, Mao Y, Kubota T, Kubota N, Yamauchi T, Mancina RM, Baselli G, Luukkonen P, Pihlajamäki J, Yki-Järvinen H, Valenti L, Arai H, Romeo S, Kono N.
LPIAT1/MBOAT7 depletion increases triglyceride synthesis fueled by high phosphatidylinositol turnover.
Gut. 2020 Apr 6. pii: gutjnl-2020-320646

CONTENT

ABBREVIATION

αSMA • Smooth muscle α-actin

AA • Arachidonic acid

ACC • Acetyl-CoA carboxylase

ACS • Acetyl-CoA synthetase

ACTA2 • Actin alpha 2

ALT • Alanine transaminase

ANOVA • Analysis of variance

APOE • Apolipoprotein E

ASD • Autistic spectrum disorders

AST • Aspartate transaminase

Asn • Asparagine

BMI • Body mass index

CAD • Coronary artery disease

CDP-DAG • Cytidine diphosphate diacylglycerol

CDS • Cytidine diphosphate diacylglycerol synthase

CL • Cardiolipin

CNX • Calnexin

CoA • Coenzyme A

ChREBP • Carbohydrate response element binding protein

DAG • Diacylglyceride

DGAT • Diacylglycerol O-acyltransferase

DNL • *De novo* lipogenesis

EPA • Eicosapentaenoic acid

ER • Endoplasmic reticulum

FA • Fatty acid

FAS • Fatty acid synthase

FBS • Fetal bovine serum

FPP • Fluorescence protease protection

GAPDH • Glyceraldehyde 3-phosphate dehydrogenase

GCKR • Glucokinase regulatory protein

GPAM • Glycerol-3-phosphate acyltransferase, mitochondrial

HBV • Hepatitis B virus

HCC • Hepatocellular carcinoma

HCV • Hepatitis C virus

HEK293T/17 • Human embryonic kidney cells 293

HEPG2 • Liver hepatocellular carcinoma cell line

HFD • High fat diet

His • Histidine

HSD17B13 • Hydroxysteroid 17-beta dehydrogenase 13

Huh-7 • Hepatocyte-derived carcinoma cell line

ID • Intellectual disability

IP1 • Inositol monophosphate

$\mathbf{K_{cat}}$ • turnover number

KO • Knock out

LD • Lipid droplet

LPA • Lysophosphatidic acid

LPE • Lysophosphatidylethanolamine

LPI • Lysophosphatidylinositol

LPIAT1 • Lysophosphatidylinositol acyltransferase-1

LPS • Lysophosphatidylserine

LX2 • Human hepatic stellate cell line

MAM • Mitochondria-associated membranes

MBOAT7 • Membrane bound O-acyltransferase domain containing 7

MMP1 • Matrix metalloproteinase-1

MMP2 • Matrix metalloproteinase-2

MRI • Magnetic resonance imaging

MUFA • Monounsaturated fatty acid

NAFLD • Non-alcoholic fatty liver disease

NASH • Non-alcoholic steatohepatitis

Ni • Nickel

OA • Oleic acid

PA • Phosphatidic acid

PA • Palmitic acid

PC • Phosphatidylcholine

PDGF • Platelet-derived growth factor

PE • Phosphatidylethanolamine

PG • Phosphatidylglycerol

PI • Phosphatidylinositol

PI3P • Phosphatidylinositol 3-phosphate

PIP2 • Phosphatidylinositol 4,5-bisphosphate

PIP3 • Phosphatidylinositol 3,4,5-trisphosphate

PIS • Phosphatidylinositol synthase

PLA2 • Phospholipase A2

PLC • Phospholipase C

PNPLA3 • Patatin like phospholipase domain containing 3

PS • Phosphatidylserine

PUFA • Polyunsaturated fatty acid

SA • Stearic acid

SD • Standard deviation

SDS • Sodium dodecyl sulfate

SEM • Standard error of the mean

SM • Sphingomyelin

SNP • Missense nucleotide polymorphism

SREBP1c • Sterol regulatory element binding protein 1c

SREBP2 • Sterol regulatory element binding protein 2

T2D • Type 2 diabetes

TAG • Triacylglyceride

TGF-β • Transforming growth factor beta

TIMP1 • Tissue inhibitor of matrix metalloproteinases 1

TIMP2 • Tissue inhibitor of matrix metalloproteinases 2

TLC • Thin layer chromatography

TM6SF2 • Transmembrane 6 superfamily member 2

TMD • Transmembrane domain

ULA • Ultra-low attachment

VLDL • Very low density lipoprotein

V_{max} • Maximal velocity

WT • Wild type

1. INTRODUCTION

This thesis examines the role of the membrane bound O-acyltransferase domain-containing 7 (MBOAT7) protein in the pathogenesis of non-alcoholic fatty liver disease (NAFLD).

The thesis is composed of three studies in which, respectively, I solved the topology of the MBOAT7 protein in hepatic cells [1], described the enzymatic activity and identified the catalytic site of the protein [2], and showed how MBOAT7 depletion causes a higher phosphatidylinositol (PI) turnover resulting in hepatic fat accumulation [3].

1.1. Lipids in human cells

Lipids are a group of biomolecules soluble in organic solvents. Lipids show an extremely heterogeneous structure due to the varying length of their C-chain and high number of biochemical transformations to which they can be subjected.

Based on their structures and roles in cells, lipids are divided into several groups, such as phospholipids, ceramides, sterols and fatty acids. Lipids are used as structural components of membranes, signalling molecules, energy source and storage [4]. On the one hand, they are stored into lipid droplets (LDs) in form of triacylglycerides (TAGs), which are neutral lipids composed of three fatty acids esterified to a glycerol backbone [5]. On the other hand, lipases can hydrolyse the bonds between the glycerol backbone and fatty acids composing TAGs, releasing free fatty acids which are then used as an energy source.

Cells show varied lipid composition at different stages of cell cycle. Moreover, the lipid distribution amongst the organelles of a

cell is high diverse [6]: this suggests the importance of lipids in carrying out various functions to which they are delegated.

1.1.1. Fatty acids

Fatty acids are carboxylic acids that differ in C-chain length and degree of unsaturation. Saturated fatty acids have no double bonds in their long hydrocarbon chains, whilst unsaturated fatty acids show one or more double bonds. Unsaturated fatty acids with single double bond are classified as monounsaturated fatty acids (MUFAs), and those with more than one double bond as polyunsaturated fatty acids (PUFAs) (**Figure 1**). Furthermore, fatty acids can undergo elongation, desaturation, oxidation and hydroxylation processes, generating a higher lipid diversity [7].

Fatty acids are usually esterified to more complex lipids, such as TAGs, phospholipids or esters. They are introduced into the body by diet, broken down by bile salts in the small intestine, absorbed via intestine capillaries and then re-esterified to TAGs in the lumen. TAGs are incorporated into chylomicrons and carried to liver, skeletal muscles, and adipose tissue, where they can be stored or used as energy source.

PUFAs are essential dietary molecules that, once introduced by diet, are rapidly incorporated into lysophospholipids by acyltransferases, where they are physiologically stored. High levels of free PUFAs can lead to the synthesis of newly-remodelled TAGs [8]. PUFAs are involved in the communication amongst cells, in particular in the inflammatory signalling through the biosynthesis of lipid mediators. Amongst PUFAs, ω-3 eicosapentaenoic acid (20:5, EPA) is the precursor of resolvins and protectins [9], that are pro-resolving lipid mediators. ω-6 arachidonic acid (20:4, AA) is the precursor of leukotrienes, thromboxanes, prostaglandins (proinflammatory lipid mediators) and prostacyclins (anti-inflammatory lipid mediators), metabolites synthesised via the lipoxygenase and cyclooxygenase pathways [10, 11], respectively.

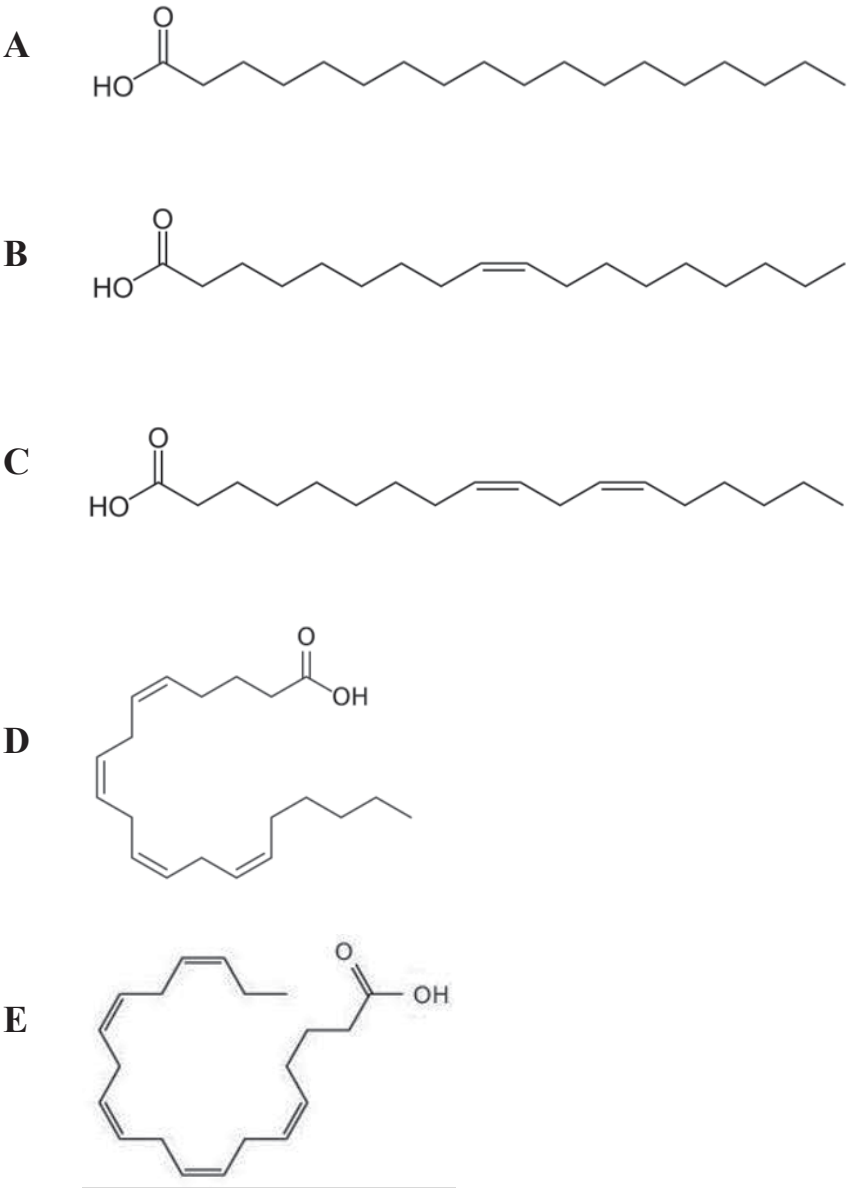

Figure 1 Structural representation of five fatty acids with different degree of saturation. A) stearic acid (18:0); B) oleic acid (18:1); C) linoleic acid (18:2); D) arachidonic acid (20:4); E) eicosapentaenoic acid (20:5).

1.1.2. Phospholipids

Phospholipids are amphiphilic molecules composed of a hydrophilic and negatively charged phosphate group “head”, a glycerol backbone, and two hydrophobic fatty acyl "tails" (**Figure 2**). The hydrophilic head can be modified by the addition of organic molecules, resulting in the subsequent synthesis of different phospholipids, such as phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), or phosphatidylserine (PS).

Phospholipids are the main components of biological membranes. Their acyl-chain composition affects membrane fluidity, curvature and dynamics, regulating all the pathways occurring within the lipid bilayer [12], such as molecular trafficking and cell signalling [13]. Phospholipids are also precursors of lipid mediators involved in signal transduction, such as eicosanoids [10].

The fatty acid tail composition of phospholipids shows high diversity: saturated and MUFAs are preferentially esterified at the *sn-1* position, whilst PUFAs at the *sn-2* position [14]. The acyl-chain incorporation occurs either during *de novo* synthesis, called the Kennedy pathway [15], or during the acyl-chain remodelling pathway, called the Lands´ cycle [16, 17], by the activity of acyltransferases and phospholipases.

The incorporation of fatty acids into lysophospholipids requires the addition of a molecule of coenzyme A (CoA) to each free fatty acid moiety by acetyl-CoA synthetases (ACSs), which regulate their turnover and availability.

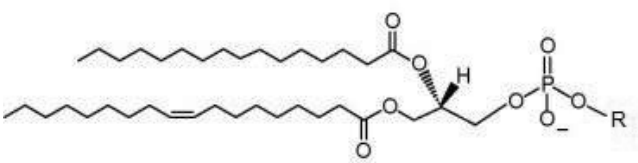

Figure 2. Structural representation of a phospholipid.

1.1.3. Lipids in cell membranes

Cellular and subcellular organelle membranes are composed of a lipid bilayer consisting primarily of lipids, proteins and carbohydrates, in different amounts and proportions. Membrane lipid composition changes dynamically [18], affecting the lipid membrane homeostasis, fluidity, curvature and biological function.

Glycerophospholipids are the most abundant lipids in cell membranes, of which PC is the major structural phospholipid [19]. Membrane regions with a high content of unsaturated fatty acids are thinner and more fluid compared to regions containing a lower percentage of unsaturated lipids [19]. Indeed, higher PUFAs content causes a decrease in membrane rigidity [20]. Different cell types have their own specific capacities to be enriched in phospholipids based on the expression level of enzymes involved in the lipid synthesis and remodelling.

Membrane proteins are directly anchored to the lipid bilayer and, based on their interactions with the membrane, they can be classified in a) peripheral proteins, weakly bound to the surface of membranes by electrostatic interactions or hydrogen bonds, and easily detachable by pH changes; and b) integral proteins, embedded in the lipid bilayer and removable only by harsh detergents. Integral membrane proteins spanning the lipid bilayer of the membrane from side to side, once or multiple times, are called transmembrane proteins. To span the membrane thickness, each transmembrane domain (TMDs) must be composed of at least 20 amino acids organized in a structural motif called alpha-helix.

Lipid composition affects the thickness of the membrane and, consequently, the conformation, localization, substrate recruitment, and activity of transmembrane proteins [21]. To maintain lipid homeostasis, cells can sense lipid levels, or the level of their lipid precursors, and regulate the expression of transcriptional factors or enzymes involved in the lipid metabolism, such as the sterol regulatory element-binding protein 2 (SREBP2) in response to sterol deficiency [22].

The importance of the membrane lipid composition is underlined by the severity of diseases caused by mutations in

enzymes involved in the lipid metabolism, such as the Sjögren-Larsson syndrome [23] and loss of function mutations in *MBOAT7 locus* [24-26], which lead to aberrant lipid composition resulting in biological dysfunctions. The mechanisms by which these changes in membrane properties cause such pathogenic phenotypes still remain unknown.

1.1.4. Phosphatidylinositol

Phosphatidylinositol (PI) is one of the major eukaryotic glycerophospholipids [27]. In the liver, 5% of total phospholipids are PI species. PI, particularly enriched in the endoplasmic reticulum (ER) [28], is synthesized from its lipid precursor phosphatidic acid (PA), which undergoes two consecutive enzymatic reactions catalysed by cytidine diphosphate diacylglycerol synthase (CDS) and PI synthase [29], respectively.

Newly-synthesised PIs are composed by an inositol head group, a phosphate group, a glycerol backbone and two non-polar fatty acyl tails (**Figure 3**). Newly-synthesized PIs are usually highly enriched in saturated or monounsaturated fatty acids, such as palmitic acid (16:0, PA), stearic acid (18:0, SA) or oleic acid (18:1, OA) in their *sn-1* and *sn-2* positions. The acyl-chain composition of PI is then modified by deacylation and reacylation processes occurring in the membranes via the Lands´ cycle during which PUFAs, such as arachidonic acid (20:4, AA) or eicosapentaenoic acid (20:5, EPA), are esterified at the *sn-2* position of lysophospholipids [30, 31].

Recently, it has been shown that AA-containing PI inhibits the production of newly-synthesised PI by downregulating CDS activity [3]. On the contrary, depletion of AA-containing PI, caused by dysregulation in the remodelling pathway, leads to the upregulation of CDS and PI synthase. Thus, newly-synthesised PIs are used as substrate by a phospholipase C-like protein for the synthesis of DAGs which are eventually converted to TAGs [3].

The inositol polar head ring of PI can be reversibly phosphorylated at one, two or three positions by lipid kinases to synthesize seven phosphorylated derivatives called phosphoinositides, such as phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 3,4,5-trisphosphate (PIP3), all of which are molecules involved in cell signalling, protein recruitment, membrane traffic regulation and heterogeneity.

The intracellular synthesis and degradation of phosphoinositides are regulated by the activity of specific kinases, phosphatases and phospholipases [32] [33].

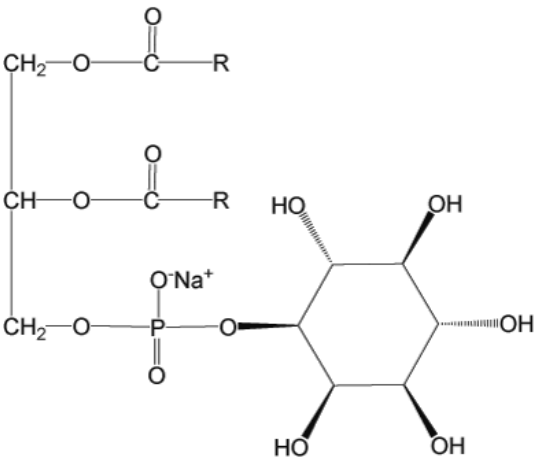

Figure 3. Structural representation of phosphatidylinositol (PI).

1.1.5. Phospholipid remodelling via the Lands´ cycle

In 1956, Kennedy and Weiss showed for the first time that glycerophospholipids are synthesized from diacylglycerols (DAG) via a *de novo* pathway called the Kennedy pathway [15]. The acyl-chain composition of phospholipids is modulated by phospholipases and acyl-transferases, which catalyse the hydrolysis and esterification of free fatty acids to lysophospholipids, respectively. Saturated and MUFAs are usually transferred to the *sn-1* position, whilst PUFAs are linked at the *sn-2* position. This acyl-chain remodelling pathway, called the Lands´ cycle, occurs in the ER [34], and it is composed by a series of deacylation and reacylation reactions [16, 17]. Free fatty acids and lysophospholipids availability depends on the balance between phospholipid acylation and hydrolysis [35], that changes depending on the physiological state of the cells.

Specifically, MBOAT7 protein transfers free arachidonoyl-COA to the *sn-2* position of lysophosphatidylinositol (LPI), releasing newly-remodelled PIs (**Figure 4**) [2]. PI can be used as substrate by phospholipase A2 (PLA2) that catalyses the hydrolysis of the bond between the fatty acid in *sn-2* position and the glycerol backbone, releasing free AA and LPI [36]. The incorporation of PUFAs, previously bio-activated to acyl-CoAs by ACSs, results in the remodelling of phospholipids and membranes in which they are enclosed [14].

The expression of enzymes involved in the remodelling of phospholipids is critical for the control of the fluidity and composition of membranes, for the availability of free fatty acids, signalling molecules and lipid mediators [37].

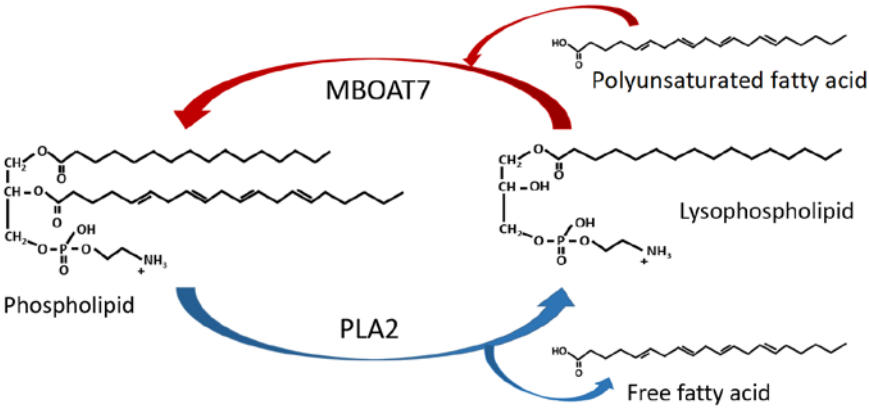

Figure 4. Acyl chain remodelling of phospholipids via the Lands´ cycle. Abbreviations: MBOAT7, Membrane bound O-acyltransferase domain-containing 7; PLA2, phospholipase A2.

1.2. Hepatocyte lipid metabolism

Liver and adipose tissue are the responsible organs for lipid and carbohydrate metabolisms. Hepatic lipid metabolism occurs in hepatocytes that receive, use, store or secrete lipids [38]. The metabolic balance between TAG uptake and secretion, and TAG synthesis and hydrolysis, is crucial for the hepatic lipid homeostasis. Abnormalities in the hepatic function can promote metabolic disorders, including fatty liver diseases [39].

Hepatic *de novo* lipogenesis (DNL) is a bio-metabolic pathway characterized by the synthesis of fatty acid chains from acetyl-CoA subunits produced during glycolysis, that are then esterified to a glycerol backbone to synthesize TAGs. It is driven by high glucose or fructose availability, and by transcriptional regulation of enzymes involved in the fatty acid metabolism [40].

These pathways are mediated by the activity of regulator elements, such as the sterol regulatory element binding protein 1c (SREBP1c) and the carbohydrate response element binding protein (ChREBP). Specifically, SREBP1c is triggered by increased insulin signaling under feeding conditions, and transcriptionally activates genes involved in fatty acids synthesis, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) [41]. ChREBP, instead, is activated by increased glucose levels and it upregulates pyruvate kinase, ACC and FAS [42].

Glucose, through glycolysis, provides acetyl-CoA subunits for the synthesis of fatty acids, that are then esterified to the glycerol backbone required for the final synthesis of TAGs [43]. Recently, it has been shown that fructose, by skipping the regulatory steps of glycolysis, can promote both DNL and repress hepatic fatty acid catabolism and insulin signaling [44], contributing to fat infiltration in the liver. Thus, higher DNL rates contribute to NAFLD [45] and development of type 2 diabetes mellitus (T2D).

In addition, mitochondrial dysfunctions can affect beta-oxidation rate causing TAG accumulation in cells [46]. Hence, TAGs are stored as lipid droplets and secreted into the blood stream as very low density lipoproteins (VLDL) via exocytosis. During insulin

resistance, patients show liver steatosis, caused by increased TAG synthesis and reduced lipolysis, and hypertriglyceridemia, caused by the upregulation of genes involved in VLDL secretion [47].

1.2.1. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is considered the most common cause of liver disease in Western countries [48, 49], and it is projected to further increase all over the world [50].

NAFLD is a pathological condition described as intrahepatic lipid accumulation of more than 5% of liver weight. It is described as a complex disease [51] characterized by increased liver fat content in absence of alcohol abuse, steatogenic medical prescriptions, viral infections, or metabolic disorders [52].

The spectrum of NAFLD comprehends a variety of conditions: simple steatosis is the main causal risk factor for the development of non-alcoholic steatohepatitis (NASH), characterized by hepatocellular ballooning and lobular necroinflammation [53, 54]. Approximately 40% of these cases can further progress to fibrosis and cirrhosis (**Figure 5**) and, amongst these, 2% of individuals are eventually diagnosed with hepatocellular carcinoma (HCC) [55]. Fat accumulation in liver is a leading risk factor for long-term hepatic injury and metabolic disorders, such as insulin resistance, hypertension and T2D [54].

To date, despite the improved knowledge on genetic determinants and prognostic biomarkers, there are no approved pharmacological drugs or therapies for the treatment of NAFLD, other than a healthy lifestyle, physical exercise and low-fat diet. The heterogeneity of NAFLD could be the reason why the drugs tested till now did not result in an improved liver clinical picture without worsening insulin resistance, hyperglycemia or risk of cardiovascular disease. This underlies the importance of individualized therapeutic approaches for the treatment of NAFLD.

Moreover, individuals with end-stage NAFLD or HCC can undergo bariatric surgery or liver transplantation to reduce mortality and metabolic co-morbidities linked to obesity and liver disease [56].

Despite the lack of an effective pharmacological therapy, approximately 5% of individuals diagnosed with NAFLD die from hepatic or cardiovascular complications [51], amongst which coronary artery disease (CAD) is the leading cause of death [57].

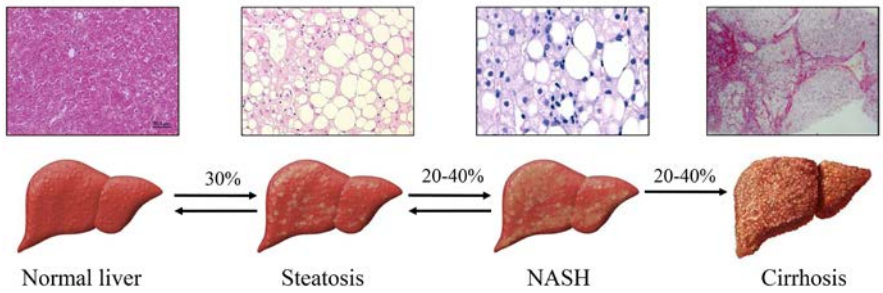

Figure 5. Natural progression of fatty liver disease. Images are courtesy of Professor Luca Valenti, University of Milan, Italy. Abbreviations: NASH, non-alcoholic steatohepatitis.

1.2.2. NAFLD diagnosis

Liver steatosis is associated with elevated levels of circulating aspartate transaminase (AST) and alanine transaminase (ALT), hepatic injury, insulin resistance, T2D and hypertension [54].

The diagnosis of NAFLD usually starts by detecting abnormal levels of circulating liver transaminases and lipids on overweight individuals. Diagnosis is confirmed by a non-invasive assessment of liver fat content by abdominal ultrasound, x-ray based techniques, magnetic resonance imaging (MRI) or elastography [58].

Despite being more invasive and uncomfortable for the patient, liver biopsy followed by microscopic examination remains the gold-standard technique for characterizing hepatic alterations [59].

The detection of liver fat accumulation, lobular inflammation, hepatocellular ballooning and fibrotic scarring results in a diagnosis confirmation.

1.2.3. Environmental factors leading to NAFLD

Environmental determinants, such as dietary factors, obesity, T2D, metabolic syndrome, poor physical activity, hepatitis B virus (HBV) and C virus (HCV) infections, and the use of some drugs (e.g. Tamoxifen), are triggering factors for liver fat accumulation and NAFLD development [60]. These environmental factors, along with genetic risk determinants, are the main components to understand the individual variability found in NAFLD patients.

In Western societies the number of overweight and obese individuals is drastically increasing due to an unhealthy lifestyle. Obese people with metabolic disease, in addition to a higher risk of developing cardiovascular diseases, show a higher prevalence of NAFLD than healthy individuals [61].

The degree of liver steatosis is associated with higher incidence rate of insulin resistance and T2D [54, 62], specifically 85% of NAFLD carriers have pre-diabetes or T2D. In addition, NAFLD is associated with excess body weight and insulin resistance, even though lean patients with NAFLD show less insulin resistance and a better clinical picture.

Recently, some studies showed that the gut microbiome composition can induce fat accumulation and liver inflammation, predisposing individuals to NAFLD [63, 64].

1.2.4. Genetic factors leading to NAFLD

NAFLD genesis, progression and pathological phenotype are associated with common genetic variations in genes involved in lipid metabolism [48] [65], such as *PNPLA3* [66] (the *rs738409 C>G* variant is the strongest genetic determinant of NAFLD), *MBOAT7* [67], *TM6SF2* [68], *GCKR* [54], *HSD17B13, APOE* and *GPAM* [69, 70] genes. The genetic association power of these risk variants with NAFLD is directly proportional to their impact on liver fat accumulation [54]. Other pathological variants in genes involved in hepatocyte mitochondrial dysfunction, insulin resistance, inflammatory response and fibrogenesis are associated with the development of hepatic disorders.

Epidemiological studies show that relatives of individuals with NAFLD have a higher risk to develop liver disease, and that variability in hepatic fat content is due to genetic factors [71]. Accordingly, studies in twins [72, 73] showed that fatty liver disease is strongly influenced by inherited factors [51], and that monozygotic twins have a more similar phenotype than dizygotic twins.

Interestingly, the prevalence of NAFLD is not homogeneous amongst ethnicities: Hispanics have higher prevalence than Caucasians and African-Americans [74].

Human molecular genetics studies shed light on new biological pathways involved in the pathogenesis of NAFLD, and confirmed that heritability strongly influences the heterogeneous aetiology of NAFLD [75]. The pathogenic role of genetic risk variants predisposing to NAFLD is magnified by the presence of long-term enviromental risk factors.

A novel potential approach for NAFLD diagnosis could be the identification of individuals having a family medical history of liver disorders, followed by detecting genetic risk variants predisposing to fatty liver disease [76]. This non-invasive proceeding can allow the tracking of individuals genetically predisposed to NAFLD, resulting in the introduction of opportune lifestyle changes timely avoiding future development of liver-related disorders. Moreover, improvements in diagnostic accuracy and patient safety are needed

to increment the cost-effectiveness ratio of analysis and individual healthcare.

In conclusion, NAFLD is a highly heritable multifactorial disorder [71] driven by genetic susceptibility and environmental factors [51, 77, 78].

1.2.5. Acyl-chain remodelling in NAFLD

The acyl-chain remodelling of phospholipids is an orchestrated process, namely the Lands´ cycle, composed of deacylation and reacylation reactions. Phospholipase A2 (PLA2), the main phospholipase involved in the phospholipid remodelling, catalyses the deacylation of unsaturated phospholipids releasing free PUFAs, that eventually can be used as substrate for the synthesis of prostanoids (prostaglandins, prostacyclins and thromboxanes) or eicosanoids (leukotrinenes) by cyclooxygenases or lipoxygenases, respectively. MBOAT7, one of the main acyl-transferases involved in the hepatic phospholipid remodelling, catalyses the re-esterification of free PUFAs to lysophospholipids, incrementing the degree of unsaturation of phospholipids.

Changes in the gene and protein levels of these enzymes, due to tissue insults or inflammatory processes, cause a rearrangement of cellular membranes and hepatic structures resulting in organ recovery or failure [79]. Thus, *MBOAT7* expression levels decrease along with the degree of liver inflammation and injury [80]. On the contrary, the correlation between NAFLD and PLA2 requires further investigation as findings are still inconsistent [81, 82].

1.3. MBOAT superfamily

Membrane bound O-acyltransferase (MBOAT) superfamily is composed of enzymes involved in lipid biosynthesis [83-85], membrane lipid remodelling [86-88], cell development [89], embryogenesis [90], and in the transferring of free acyls to the hydroxyl groups of lipid substrates [91] in order to synthesize newly-remodelled lipids. Each member of the MBOAT superfamily has a different affinity for specific acyl donors and acceptors [88]. Amongst them, MBOAT1 is involved in the acyl-chain remodelling of phosphatidylserine (PS) and phosphatidylethanolamine (PE) [92], and MBOAT5 in phosphatidylcholine (PC) [93, 94].

Structurally, members of the MBOAT superfamily are membrane proteins attached to endomembranes by a variable number of transmembrane domains (TMDs) [95]. These proteins share a preserved homology domain containing a conserved Histidine (His) and Asparagine (Asn) residues [30, 91] (both hydrophilic amino acids) that are likely part of the putative catalytic site of these proteins.

1.3.1. MBOAT7

Membrane bound O-acyltransferase domain-containing 7 (*MBOAT7*) gene, also known as *lysophosphatidylinositol acyltransferase 1 (LPIAT1),* is located in the chromosome 19 and encodes for a 472 amino acids-long integral membrane O-acyltransferase widely expressed in human tissues [67]. MBOAT7 is a six TMDs protein anchored to endomembranes [1], such as ER, LD and mitochondria-associated membranes (MAM) [67].

MBOAT7 contributes to the acyl-chain remodelling of phospholipids, preferentially transferring PUFAs, such as arachidonoyl-CoA (used as an acyl donor) to the *sn-2* position of lysophospholipids, especially LPI (used as an acyl acceptor) [12, 88].

Carrying out its O-acyltransferase activity, MBOAT7 contributes to increase the desaturation degree of phospholipids.

Hence, MBOAT7 is involved in the arachidonate metabolism, a minor component of phospholipids used for the synthesis of eicodanoids [88] and its oxygenated derivatives involved in the inflammatory response and cell signalling [96].

In rodents, *Mboat7*-deficient mice (*Mboat7*$^{-/-}$) show reduced AA-containing PI, PI3P, and PIP2 [12], confirming the role of MBOAT7 in the arachidonate metabolism and in the remodelling of PI.

1.3.2. MBOAT7 and liver disease

The *rs641738 C>T* genetic variant in the *locus* containing the *MBOAT7* gene has been identified as a novel susceptibility risk factor for chronic liver disease related to alcohol abuse [97] and, soon after, for NAFLD [67].

The *MBOAT7 rs641738 C>T* genetic variant, having a minor allele frequency of 0.42 in Europeans and 0.33-0.34 in Africans and Hispanics, is a missense nucleotide polymorphism (SNP) associated with a higher risk of alcohol-related cirrhosis [97], the entire spectrum of NAFLD (steatosis, hepatic inflammation, fibrosis) [67, 98 99, 100], and an increased risk of liver inflammation and fibrosis development in patients with chronic hepatitis B and C infections [101, 102]. The minor *MBOAT7 rs641738 C>T* variant is associated with reduced *MBOAT7* gene expression and protein levels [67].

The importance of MBOAT7 in the hepatic arachidonate metabolism is underlined by the fact that carriers of the *rs641738 C>T* genetic variant showed lower levels of 20:4-PI/total PI and 20:5-PI/total PI ratios, and higher concentration of 18:1-PI/total PI and 18:2-PI/total PI in plasma [67], while all the other lipid classes, such as ceramides, free fatty acids and triglycerides, remained unchanged in plasma [67] and liver [98].

In severely obese patients, hepatic *MBOAT7 mRNA* levels decreased with the severity of hepatic injury, regardless of liver

inflammation, T2D or genetic background [80]. Moreover, MBOAT7 hepatic expression is reduced compared to normal weight people, irrespective to the presence of the *rs641738* gene variant [103]. This has been further confirmed by comparing lean mice to obese mice in which the hepatic expression of *Mboat7* was drastically reduced [103]. In mice, *Mboat7* knock-out causes hepatic steatosis, liver injury, elevated levels of AST and ALT in the blood [103], and a decrease in the PUFAs-containing PI concentration in liver [104].

Interestingly, hepatic *Mboat7* was down-regulated in NAFLD and hyperinsulinemia mouse models, resulting in an increased fat accumulation in liver compared to lean mice [80]. Mice with insulin resistance and hyperinsulinemia showed reduced *Mboat7 mRNA* levels, regardless of the diet. Accordingly, increased insulin levels after refeeding or insulin injections caused a decrease of *Mboat7 mRNA* and protein levels in mouse hepatocytes [80]. Indeed, *Mboat7* ASO-mediated knockdown mice showed reduced hepatic insulin resistance and hyperinsulinemia [103].

Moreover, Mboat7 liver-specific deletion increases SREBP-1c activity which leads to a higher *de novo* fat synthesis rate in mice [105]. Recently, we showed that depletion of MBOAT7 causes an increased PI turnover that promotes TAG accumulation and fibrosis in both *in vivo* and *in vitro* models [3].

All these data confirmed the role of MBOAT7 in long-term hepatic fat accumulation and progression to chronic liver disease.

1.3.3. MBOAT7 and brain disease

The *MBOAT7* is a susceptibility risk gene for mental disorders as it plays an important role in neurodevelopment and brain homeostasis. In mammalian brain, MBOAT7 is highly expressed and AA-PI is one of the most abundant phospholipids [106].

Individuals homozygous for pathogenic variants in the *MBOAT7* gene showed intellectual disability (ID) [25], epilepsy, cerebellar atrophy [107] and autistic spectrum disorders (ASD) [26], characterized by delayed motor milestones, poor coordination [24] and seizure. The frequencies of these pathogenic variants are higher in individuals born from consanguineous parents belonging to populations in which consanguineous marriages are more common [108].

In mice, Mboat7 is required for cortical lamination [96]. Specifically, *Mboat7*$^{-/-}$ mice, in which Mboat7 activity has strongly been reduced, are smaller and show severe developmental brain defects [104], atrophy of the cerebral cortex and hippocampus, abnormal cortical lamination, a higher number of apoptotic cells in the cortex [30], and they die within few days after birth.

In *Mboat7*$^{-/-}$ mice, brain tissues contained less 20:4-PI and increased levels of 18:0-PI [104], confirming the role of Mboat7 in the acylation of LPI via the Lands´ cycle.

1.3.4. MBOAT7 topology

The topological organization of a protein gives important information about its interactions, substrate recognition, activity and role in the cell. The correct topology of a membrane protein is crucial to carry out its physiological function in the cell and to interact with the cellular environment, substrates or cofactors [109].

Several methods can be used to assess the topology of a membrane protein, from computer-based predictive algorithms

based on different approaches, such as hydropathy analysis, artificial neural networks or statistical analysis, to experimental approaches.

MBOAT superfamily is composed of transmembrane proteins with a number of TMDs ranging from 2 to 12 [86, 110]. Despite showing that MBOAT7 localizes in ER, MAM and LD [67], the number, localization and orientation of the TMDs of MBOAT7 were still unknown. By combining *in silico* and *in vitro* analyses, we showed that MBOAT7 is a multispanning transmembrane protein anchored to endomembranes by six TMDs and two putative re-entrant loops [1]. Moreover, we determined that the N- and C-terminal of MBOAT7 face the cytosolic side of endomembranes, and that the putative catalytic site of MBOAT7, composed of the asparagine in position 321 (Asn-321) and the histidine in position 356 (His-356), faces the lumen of cellular organelles.

1.3.5. MBOAT7 enzymatic activity

MBOAT7 is an O-acyltransferase that transfers free acyl donors to lipid acceptors via the Lands´ cycle. It has been showed that mboa-7, the homologous protein of MBOAT7 in *Caenorhabditis elegans,* is the responsible enzyme for the incorporation of PUFAs into PI [30]. In mice, *Mboat7* knockdown results in changes in LPI and PI hepatic levels, suggesting a role of the enzyme in the PI metabolism [103]. Moreover, MBOAT7 showed acyltransferase activity using LPI as lipid acceptor in microsomes of human neutrophils [88].

Homology modelling analyses suggested the presence of a putative catalytic dyad composed of a conserved asparagine and a preserved histidine [30]. In human, the Asn-321 and His-356 form the putative luminal enzymatic site of MBOAT7 [1]. Despite the growing interest in MBOAT7, the exact enzymatic activity of the protein and the identification of its catalytic site were still uncertain.

To gain insight into the enzymatic activity of MBOAT7, we produced the human MBOAT7 protein in the yeast species *Pichia pastoris*. MBOAT7 was purified by nickel-chromatography and incubated with a combination of radiolabelled and unlabelled lipid

donors (free fatty acids) and unlabelled lipid acceptors (lysophospholipids). The released radiolabelled phospholipids were separated by thin-layer chromatography (TLC) and measured by liquid scintillation counting. MBOAT7 showed the highest catalytic efficiency by transferring PUFAs, such as AA and EPA, to LPI [2].

Moreover, missense mutations at the presumptive enzymatic site of MBOAT7 strongly inhibited the O-acyltransferase activity of the protein, supporting the belief that Asn-321 and His-356 compose the conserved catalytic dyad of MBOAT7.

1.3.6 MBOAT7 depletion causes hepatic steatosis and fibrosis

MBOAT7 rs641738 C>T pathological variant causes a reduction in the *MBOAT7* gene expression and protein synthesis levels [67]. This genetic variant has been associated with increased liver fat content, NASH and fibrosis [67], but the mechanism behind this pathological phenotype was still unknown.

In paper III, we showed that *MBOAT7* depletion in mice liver, in hepatic 2D cell cultures (Huh-7 and HepG2 cells) and 3D spheroids, composed of hepatocytes and hepatic stellate cells (HepG2/LX-2), caused steatosis, hepatitis and fibrosis [3]. In addition, depletion of MBOAT7 in human hepatocytes resulted in a higher *de novo* TAG synthesis rate without affecting TAG degradation, secretion or fatty acid catabolism. *Mboat7* knock-out mice showed a decrease in AA-PI content and an increase in other PI species in liver, whereas the content of other phospholipids remained unchanged. These data confirmed the role of MBOAT7 in the remodelling of AA-containing PI.

MBOAT7 depletion in hepatocytes led to a reduced acyl chain remodelling of PI and, consequently, to an accelerated synthesis of PI mediated by increased activation of cytidine diphosphate diacylglycerol synthase 2 (CSD2), an enzyme usually inhibited by AA-containing PI. Moreover, MBOAT7 depletion enhances PI

degradation rate, resulting in a higher PI turnover. The larger PI availability can be used as substrate by a phospholipase C-like protein to synthesize new DAGs, precursors of TAGs, resulting in an increased TAG synthesis and accumulation in liver [3].

2. METHODOLOGICAL CONSIDERATIONS

In the following paragraphs, considerations about selected methods are discussed. Specific details can be found in the "Material and methods" sections of the three enclosed publications.

2.1. *In vitro* model systems

2.1.1. Pichia pastoris

Pichia pastoris is a methylotrophic yeast that belongs to the class Ascomycetes. *P. pastoris* is widely used as an expression system for the production of heterologous mammalian proteins [113-114] as it is easy to manipulate genetically, has a high growth-rate and achieves very high cell density in the culture [115]. In particular, it is used for the production of membrane proteins that undergo correct post-translational modifications, folding and insertion into membrane bi-layer [116].

The genome of *P. pastoris* contains two alcohol oxidase genes, *Aox1* and *Aox2*, which include strongly inducible promoters allowing yeasts to use methanol as a carbon source for growth and energy [117]. Since methanol is used as energy source and inducer of the recombinant proteins expression, and since it inhibits the yeast growth at high concentrations, the addition of sorbitol to the medium can be beneficial to improve cell yields [118]. Nevertheless, cell growth is strongly repressed in the presence of glucose [116].

In paper II, we used *P. pastoris* as a transformation host for the production of human MBOAT7 in large-scale. We introduced the human *MBOAT7* gene (previously gene optimized for *P. pastoris*

and subcloned into pPICZB vector) under the control of the *Aox1* promoter, and we induced the protein production by the addition of methanol-sorbitol to the medium. The insertion of a His6-tag at the C-terminal of *MBOAT7* allowed the subsequent purification of the protein by nickel-affinity chromatography.

2.1.2. Cell 2D models

In paper I, human embryonic kidney cells 293 stably expressing the SV40 large T antigen (HEK293T/17) were used to perform all the *in vitro* experiments.The stable expressed SV40 large T antigen [119] binds the SV40 enhancers of expression vectors to increase the expression of recombinant proteins. HEK293T/17 are epithelial adherent cells widely used in molecular biology owing to their high growth rate, resistance and efficiency in producing high amounts of recombinant proteins from plasmid vectors carrying the SV40 origin of replication.

In paper II, HepaRG cells were used to perform all the *in vitro* studies. HepaRG are highly stable, immortalised human hepatic cells used in various fields of research [120] (**Figure 6A**). In our lab, HepaRG cells have been extensively used in the study of genes involved in the pathogenesis of NAFLD.

In paper III, three different hepatic cell lines were used. Huh-7 cells are well-differentiated immortal hepatocyte-derived carcinoma cell line (**Figure 6B**). *In vitro* experiments were repeated in a second hepatic cell line, namely HepG2, to confirm data collected in Huh-7 cell line. HepG2 cells are a well-differentiated hepatocellular carcinoma cell line widely used for the analysis of lipid metabolism in hepatocytes (**Figure 6C**). Moreover, a third cell line, consisting of human hepatic stellate cells (LX-2), were used with HepG2 cells as a co-culture for the generation of 3D spheroids. LX-2 cells have been used by our group in previous studies due to their relatively high transfection efficiency, capacity to accumulate retinol and subsequent conversion to retinyl esters [121] (**Figure 6D**).

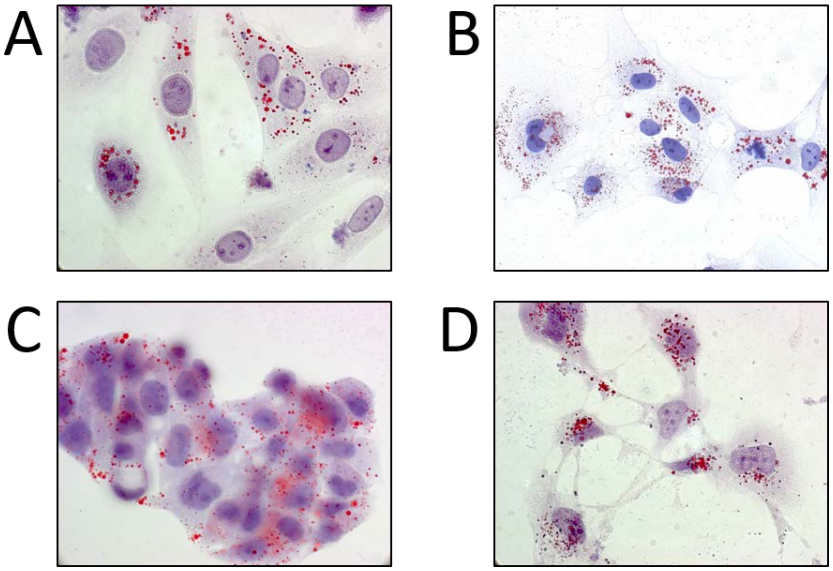

Figure 6. Representative pictures of four hepatic cell types used as experimental 2D models. A) HepaRG cells; B) Huh-7 cells; C) HepG2 cells; D) LX-2 cells.

2.1.3. Cell 3D models

3D spheroids are an innovative *in vitro* tool used in medical research to accurately mimic biological processes, physiological responses, and disease features. Spheroids are miniaturized organs that can recapitulate human organ phenotype. To generate human 3D spheroids we used ultra-low attachment (ULA) plates that force cells to aggregate into a suspended state enabling spheroids formation. This cell culture tool allows cells to interact with each other in a three-dimensional organ-like structure mimicking a more physiologically meaningful *in vitro* microenvironment [122] [123]. Furthermore, more than one cell type can be used to design spheroids (**Figure 7**).

In paper III, a combination of LX-2 and HepG2 cells, in a physiological 1:24 ratio, was used to make liver spheroids to

recapitulate the main features of NAFLD [123]. HepG2 are hepatocytes, the main parenchymal cells of the liver which represent the 80% of the organ volume and cell population. They regulate the synthesis, storage and secretion of TAGs which are stored into LDs, and secreted into the bloodstream in form of VLDL particles.

LX-2 are human hepatic stellate cells and the principal storage site for retinoids [124]. LX-2 cells are involved in the progression of liver fibrosis contributing to collagen infiltration during chronic liver disease. This cell type is extensively used as tools to study the mechanisms behind the hepatic fibrogenesis process as they synthesize a number of proteins involved in the progression and regression of fibrosis, such as matrix metalloproteinases (MMP-1, MMP-2), tissue inhibitor of matrix metalloproteinases (TIMP-1, TIMP-2), platelet derived growth factor receptor β (PDGF-Rβ), smooth muscle α-actin (αSMA), transforming growth factor beta (TGF-β), a major fibrogenic cytokine in liver disease [125-127].

During hepatic stellate cell activation, due to liver damage, there is an increase in mRNA expression of genes involved in the development of steatosis, such as *TGF-β1, TGF-β2, COL1A1,* and *ACTA2* [128, 129].

It is important to point out that both HepG2 and LX-2 cell types used to generate spheroids are homozygous carriers of the PNPLA3 I48M variant, known to increase susceptibility to liver inflammation and injury [130].

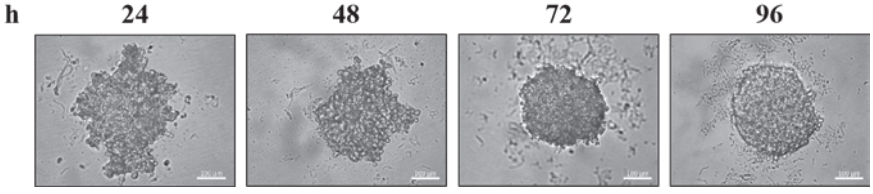

Figure 7. 3D spheroids generation. Pictures were taken after 24, 48, 72 and 96 hours by using Axio Vert.A1 inverted microscope (Carl Zeiss AG). Abbreviations: h, hours.

2.1.4. Fluorescence protease protection assay

Fluorescence protease protection (FPP) assay is a novel technique used to determine the subcellular localization and topology of membrane proteins in living cells [109]. The FPP assay determines whether a fluorescent tag-protein is free in the cytosol, a membrane-associated protein, or a transmembrane protein spanning the thickness of the phospholipid bilayer entirely [131]. Moreover, it defines the topology of the protein, the structural organization of the protein with respect to the membrane in which it is embedded, and the orientation of its TMDs within the membrane.

The FPP assay requires the addition of a fluorescence-tag sequence to the gene of interest. The assay is based on selective permeabilization of the plasma membrane by a cholesterol-binding saponin called digitonin, that allows the access to a non-specific proteinase (proteinase K) able to cleave the fluorescent-tag bound to the protein of interest.

Three endpoints are possible (**Figure 8**): 1) the incubation with digitonin causes the decay of the fluorescence-tag, meaning that the protein of interest is free in the cytosol (e.g. maxFP-Green); 2) the incubation with digitonin followed by proteinase K causes the decay of the fluorescence, meaning that the domain of the protein to which the fluorescence-tag is bound has a cytosolic localization (e.g. Caveolin-1-GFP); or 3) the incubation with digitonin followed by proteinase K does not lead to the disappearance of the fluorescence signal, meaning that the domain of the protein to which the fluorescence-tag is bound has a luminal localization and it is protected from the proteinase K activity by the structure of the membrane (e.g. DsRed2-Mito).

In paper I, by adding a GFP-tag to the N- or C- terminal of thirteen full length or truncated forms of MBOAT7, and subjecting transiently transfected HEK293T/17 cells to the FPP assay, we solved the topological organization of MBOAT7.

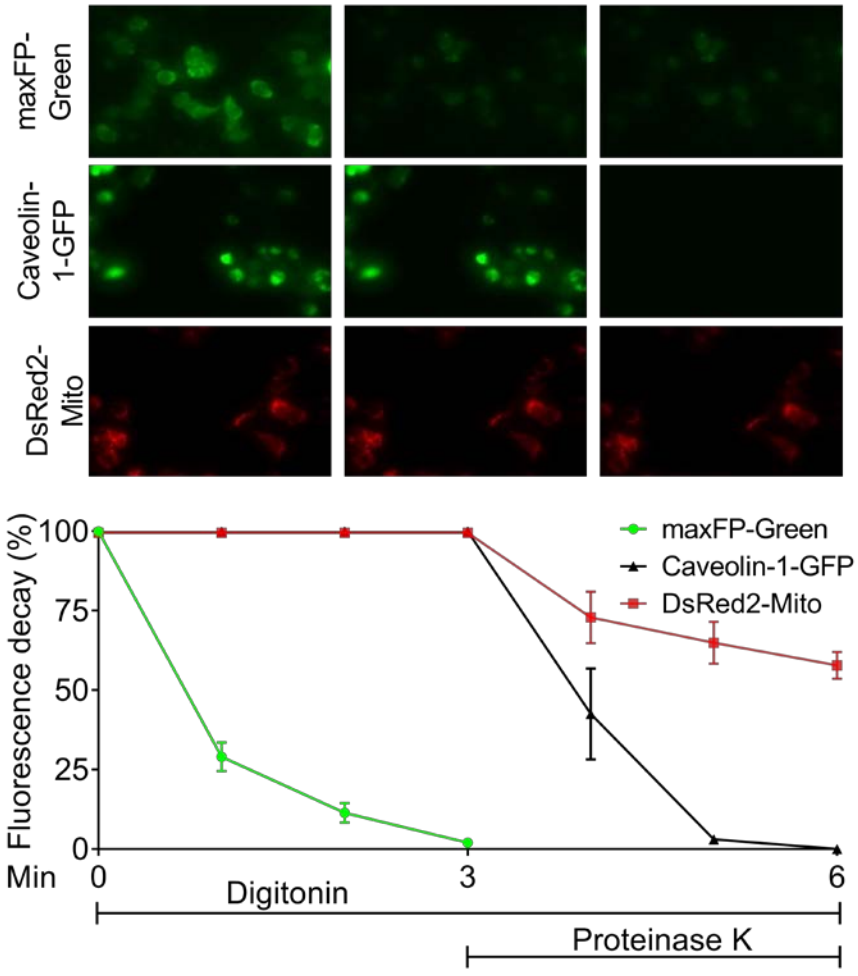

Figure 8. Fluorescence protease protection assay set up by using HEK293T/17 cells transiently transfected with a fluorescent tag-protein free in cytosol (maxFP-Green), a membrane protein whose fluorescent-tag faced the cytosol (Caveolin-1-GFP), or a membrane protein whose fluorescent-tag was luminal and protected by the lipid-bilayer (DsRed2-Mito). Pictures were taken after 0, 3 and 6 minutes by using Axio Vert.A1 inverted microscope (Carl Zeiss AG). The decay of the fluorescence was quantified by using MATLAB (Mathworks). Abbreviations: Min: minutes.

2.2. *In vivo* model systems

2.2.1. Mouse models

Human and mouse share anatomical and metabolic similarities. Mice are widely used as animal models for molecular and genetic studies to better understand the mechanism behind pathologies, including liver disease. One big advantage of the mouse model is the opportunity to generate knock out and knock in models to simulate human genetic diseases. In previous studies, *Mboat7* knock out mice showed high neonatal lethality caused by severe developmental brain defects [96, 104].

In paper III, to avoid mice lethality, we generated tamoxifen-inducible *Mboat7* knock out mice (*Mboat7*$^{-/-}$), in which the *Mboat7* alleles were deleted, by pairing *Mboat7*-floxed mice with transgenic mice carrying tamoxifen-inducible *Cre* transgene. Mice, fed on chow diet, showed normal growth, physiological brain morphology and increased liver TAG content. Moreover, we generated hepatocyte-specific *Mboat7* knockout mice by mating *Mboat7*-floxed mice with *albumin-Cre* mice. These mice showed higher liver weight and increased hepatic neutral fat content[3].

2.2.2. Human cohorts

In paper III, to study the differences in liver phospholipids composition between *MBOAT7 wild type* subjects and individuals carrying the heterozygous or homozygous *MBOAT7 rs641738 C>T* variant, a cohort of 125 obese Finnish subjects [132] was used and lipidomic data, obtained from liver biopsies, were analyzed.

In addition, to study the differences in liver *mRNA* expression levels between individuals carrying the *MBOAT7* wild type gene or the heterozygous or homozygous *MBOAT7 rs641738* variant, a cohort of 125 obese Italian subjects was used. The *mRNA* expression of genes involved in hepatic inflammation and fibrosis, obtained

from percutaneous liver biopsies during bariatric surgery, was analyzed.

Written informed consent was obtained for each individual participating in the studies.

2.3. Statistical analysis

Statistical analysis is applied to a set of data to read and understand the outputs of experimental results. The use of statistical tests aims to test the null hypothesis suggesting no statistical differences between groups. To show how much evidence there is to reject the null hypothesis (e.g. the means of two groups are significantly different), we used inferential statistics and hypothesis testing.

In our studies, a p-value is statistically significant if < 0.05 (level of significance), meaning that there is a 95% chance that the null hypothesis is false. The smaller is the p-value and the stronger is the evidence the null hypothesis can be rejected.

In paper I, values are shown as mean ± standard error of the mean (SEM) and compared using unpaired two-sample two-tailed Student´s test, that examines the null hypothesis of two population means being equal. Standard deviation (SD) measures the variability and the dispersion of data values in a sample. It measures how accurately the mean represents the sample data [133]. SEM is calculated by dividing it by the square root of N (the sample size), resulting always smaller than the SD ($SD/\sqrt{N}$).

In paper II, values are shown as mean ± SD. Groups were compared by using non-parametric Mann-Whitney U test. The kinetic curves were defined by the Michaelis-Menten model. The maximum enzyme velocity at saturated substrate concentration (V_{max}), the affinity of the enzyme for the given substrate (K_m), the turnover number (K_{cat}) and the catalytic efficiency of the enzyme (K_{cat}/K_m) were determined by fitting the experimental data to the kinetic model.

In paper III, values are shown as mean ± SEM and compared using unpaired two samples two-tailed Student´s test, or non-

parametric Mann-Whitney U test, or one-way analysis of variance (ANOVA) with Tukey´s *post hoc* test. One-way ANOVA is used to compare three or more independent groups. To further examine the pairwise difference between groups, a *post hoc* test was performed. Moreover, human data from lipidomic analysis and gene expression are shown as mean ± SD, and p-values were calculated by linear regression analysis adjusted for age, gender and body mass index (BMI).

3. AIMS

The overall goal of this thesis is to better understand the role of MBOAT7 on human genetics of fatty liver disease. The specific aims of the three studies included in this thesis are:

Paper I: to solve the topological organization of MBOAT7 within the phospholipid bilayer of cell endomembranes.

Paper II: to study the enzymatic activity of MBOAT7 and to identify the catalytic site of the protein.

Paper III: to investigate the mechanism by which MBOAT7 depletion causes hepatic fat accumulation.

4. RESULTS

4.1. Paper I: MBOAT7 is anchored to endomembranes by six transmembrane domains

In this study, we solved the topological organization of MBOAT7 in living cells. Although previous works showed that MBOAT7 is highly expressed in the liver and localizes in ER, MAM and lipid droplets [67], the topology of MBOAT7 was unknown.

We performed *in silico* analysis by employing 22 computational methods based on different approaches, from machine learning to amino acid hydropathy analysis, that predicted TMDs ranging from 5 to 12.

In vitro analyses showed that MBOAT7 is a transmembrane protein strongly attached to endomembranes, which can be detached from membranes by using the harsh ionic detergent sodium dodecyl sulfate (SDS).

Moreover, to establish the number, localization and orientation of the TMDs of MBOAT7, we performed the FPP assay on living cells transiently transfected with full-length and truncated GFP-MBOAT7 forms. To confirm FPP assay data, we performed differential membrane permeabilization coupled with immunofluorescence microscopy on cells transiently transfected with full-length FLAG-MBOAT7 forms.

Both *in vitro* approaches showed that MBOAT7 is a transmembrane protein anchored to endomembranes by six TMDs and two presumptive re-entrant loops (**Figure 9**).

Interestingly, the putative catalytic site of MBOAT7, composed by the conserved asparagine in position 321 (Asn-321) and the histidine in position 356 (His-356), faces the luminal side of cellular organelles (**Figure 9**).

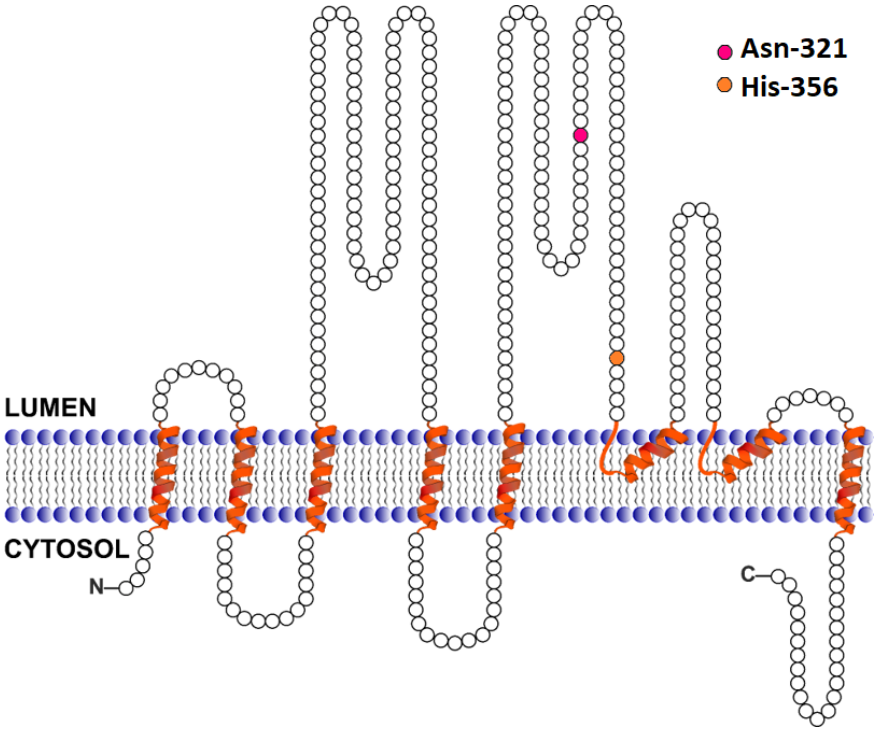

Figure 9. Schematic representation of the topological organization of MBOAT7. Abbreviations: Asn, asparagine; His, histidine.

4.2. Paper II: LPIAT1/MBOAT7 contains a catalytic dyad transferring polyunsaturated fatty acids to lysophosphatidylinositol

Members of the MBOAT superfamily are membrane proteins that catalyze the transfer of free fatty acids to the hydroxyl groups of lipid substrates. These proteins share a conserved putative catalytic dyad composed of a Histidine and an Asparagine.

In this study, we used the methylotrophic yeast *P. pastoris* to produce in large-scale the human MBOAT7 wild type and three mutants that lack one or both amino acids present at the putative catalytic site.

By using a combination of radiolabelled and unlabelled lipid substrates, we demonstrated that purified MBOAT7 wild type preferentially esterifies PUFAs, such as AA and EPA, to the *sn-2* position of lysophospholipids, whilst it showed weak activity in transferring saturated and MUFAs to phospholipids (**Figure 10**). Specifically, MBOAT7 showed the highest catalytic efficiency in transferring free PUFAs to LPI:

Moreover, missense mutations in the catalytic dyad of the protein resulted in the loss of the O-acyltransferase activity, confirming the role of the Asn-321 and His-356 on the enzymatic activity of MBOAT7.

The specificity of the enzymatic reaction was confirmed by the strong reduction of the O-acyltransferase activity of the protein in presence of thimerosal, a strong inhibitor of MBOAT7 [88].

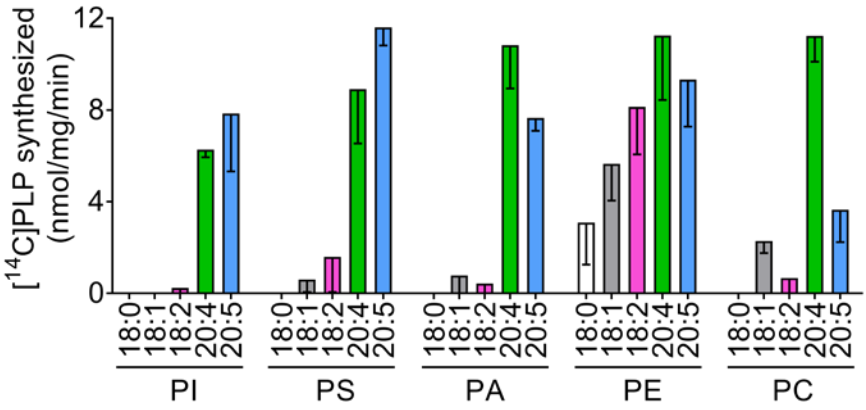

Figure 10. Purified MBOAT7 wild type showed the highest O-acyltransferase activity by using PUFAs as acyl donors. Abbreviations: 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid; 20:5, eicosapentaenoic acid; PI, phosphatidylinositol; PS, phosphatydylserine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PLP, phospholipid.

4.3. Paper III: LPIAT1/MBOAT7 depletion increases triglyceride synthesis fueled by high phosphatidylinositol turnover

The *MBOAT7 rs641738 C>T* genetic variant is associated w ith the full spectrum of NAFLD. Carriers of this pathogenic polymorphism show lower *MBOAT7* gene expression and protein levels.

In this study, we investigated the mechanism by which MBOAT7 depletion leads to hepatic steatosis and fibrosis. We used *in vivo* and *in vitro* approaches, by generating hepatic-specific *Mboat7* knockout (*Mboat7*$^{-/-}$) mice, and by using 2D and 3D human hepatocytes cultures. Specifically, 3D spheroids were composed of hepatocytes and stellate cells in a 24:1 physiological ratio.

Firstly, we showed that *Mboat7*$^{-/-}$ mice developed steatosis and fibrosis when subjected to a high-fat diet. Fat and collagen accumulation, due to MBOAT7 depletion, was confirmed in 2D and 3D cultures. Secondly, by using radiolabelled substrates, we showed an increased *de novo* TAG synthesis rate in cell models.

We determined that MBOAT7 depletion resulted in a decrease in acyl chain remodelling of PI via the Lands´ cycle, and in an increased PI turnover. PI-AA depletion upregulates the enzymatic activity of CDS, which enhances the PI availability for a phospholipase C-like protein that, releasing free inositol monophosphate (IP1), causes a higher synthesis of DAG. Eventually, DAG is easily acylated to form TAGs in the liver (**Figure 11**).

Moreover, lipidomic analysis of human liver tissues of Finnish obese individuals carrying the *MBOAT7 rs641738 C>T* variant [132] showed a reduction in PUFAs-containing PI, whereas other phospholipids remained unaltered.

All together, we described a novel metabolic pathway that connect the higher PI turnover, caused by MBOAT7 depletion, to an increased hepatic TAG synthesis.

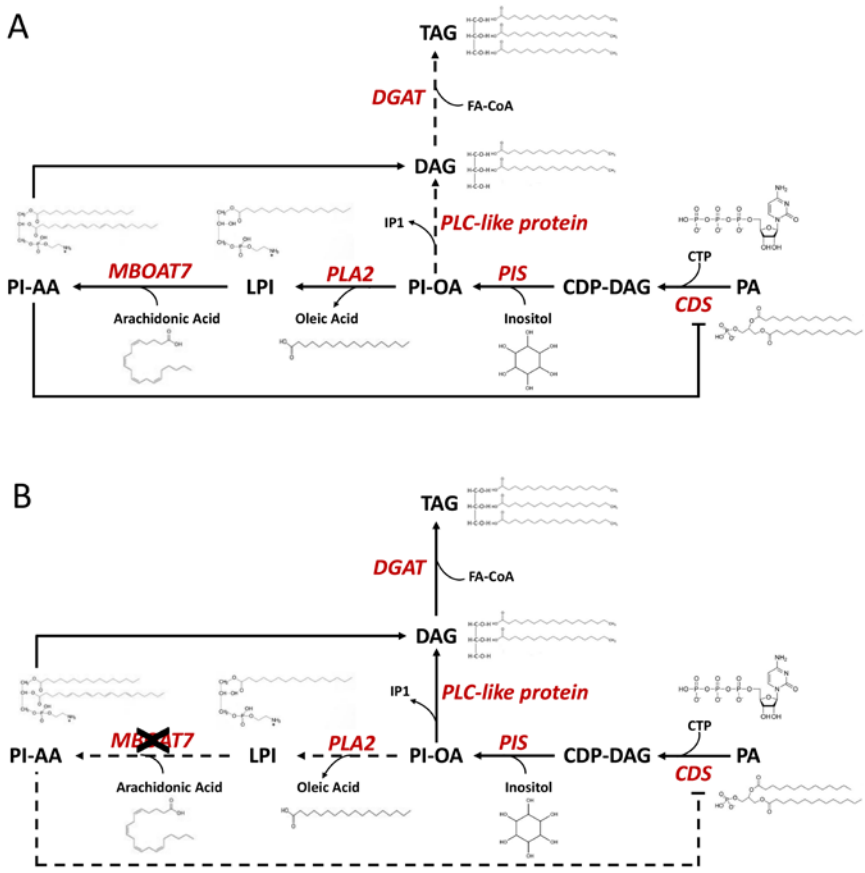

Figure 11. Schematic representation of the mechanism by which A) MBOAT7 catalyses the synthesis of newly remodelled PI; B) MBOAT7 depletion causes increased TAG synthesis. Abbreviations: CDP-DAG, cytidine diphosphate diacylglycerol; CDS, cytidine diphosphate diacylglycerol synthase; DGAT, diacylglycerol O-acyltransferase; DAG, diacylglycerol; IP1, inositol monophosphate; LPI, lysophosphatidylinositol; MBOAT7, membrane bound O-acyltransferase domain-containing 7; PA, phosphatidic acid; PI-OA, phosphatidylinositol with oleic acid; PI-AA, phosphatidylinositol with arachidonic acid; PIS, phosphatidylinositol synthase; PLA2, phospholipase A2; PLC, phospholipase C; TAG, triglyceride.

5. DISCUSSION

MBOAT7 is an O-acyltransferase highly expressed in liver and brain, and involved in the O-acyl chain remodelling of phospholipids. In the last years it has been shown that the single nucleotide polymorphism *rs641738 C>T* in the *MBOAT7 locus* confers increased susceptibility to the full spectrum of NAFLD by reducing the *MBOAT7* gene expression and protein levels.

Missense mutations in the *MBOAT7* gene have been associated with epilepsy, intellectual disability, autistic traits and defects in the brain development. Despite the remarked importance of MBOAT7 in the hepatic lipid metabolism and brain homeostasis, very little is still known about the role of MBOAT7 on fatty liver disease.

In these studies, I focused my work on solving for the first time the topological organization of MBOAT7, on investigating the enzymatic activity and the localization of the catalytic site of MBOAT7, and finally on understanding the metabolic process by which MBOAT7 depletion causes neutral fat accumulation in liver.

In the first paper, by using a combination of *in silico* and *in vitro* analysis, we concluded that MBOAT7 is a multispanning membrane protein anchored to endomembranes by six TMDs and two presumptive re-entrant loops. Based on this structural organization, the putative catalytic dyad of MBOAT7 faces the lumen of organelles. The knowledge of the topology of MBOAT7 and the correct orientation of the catalytic site are key information to better understand the role of the protein in the lipid metabolism, and to develop a plan for the treatment of people carrying *MBOAT7* pathogenic genetic variants.

In the second paper, we established that MBOAT7 is an O-acyltransferase that esterifies free fatty acids to the *sn-2* position of lysophospholipids to release newly-remodelled phospholipids. By using a mixture of radiolabelled and unlabelled acyl donors and acyl acceptors, we demonstrated that MBOAT7 has the highest catalytic

efficiency using PUFAs as acyl-donors and LPI as acyl-acceptor. Furthermore, for the first time we demonstrated that the Asparagine in the amino acid position 321 and the Histidine in position 356 are key amino acids for the enzymatic activity of the protein. Missense mutations in these two amino acids cause a loss of function of the enzyme.

In the third paper, to investigate the role of MBOAT7 in fatty liver disease, we silenced the *MBOAT7* gene in 2D and 3D hepatic cell cultures resulting in the accumulation of neutral fat and collagen. Similarly, Mboat7 depletion in mice led to hepatic steatosis and fibrosis, recapitulating the human phenotype.

To explain how MBOAT7 depletion drives hepatic fat accumulation, we proposed for the first time a pathway connecting the reduction in hepatic PI-AA content to TAG synthesis. Briefly, MBOAT7 depletion causes reduced PI acyl-chain remodelling and increased PI turnover (higher synthesis and degradation rate). Reduced PI-AA content upregulates CDS activity causing an increased PI availability which can be used as substrate by a phospholipase C-like protein for the synthesis of DAGs. Finally, DAGs are converted into TAGs by DAG acyltransferases.

Taken all together, in this thesis we elucidated important aspects of the role of MBOAT7 on the acyl-chain remodelling of phospholipids. Further studies are required to unravel the importance of MBOAT7 in the pathogenesis and progression of non-alcoholic fatty liver disease.

6. CONCLUSION

The main findings of this thesis are:

- MBOAT7 is a multispanning membrane protein attached to endomembranes by six transmembrane domains and two presumptive re-entrant loops. The catalytic dyad, composed of the Asparagine in position 321 and Histidine in position 356, faces the luminal side of endomembranes.

- MBOAT7 is an O-acyltransferase that preferentially transfers free polyunsaturated fatty acids to the *sn-2* position of lysophosphatidylinositol. Missense mutations in the putative catalytic site cause the loss of function of the protein.

- MBOAT7 depletion causes hepatic steatosis via a novel metabolic pathway. Increased hepatic lipogenesis is due to a higher turnover of phosphatidylinositol used as substrate by a phospholipase C-like protein for the synthesis of diacylglycerols, precursor of triacylglycerols (TAG), causing an increased TAG synthesis and accumulation in liver.

7. FUTURE PERSPECTIVE

Recently, MBOAT7 has been associated with the progression of alcoholic and non-alcoholic fatty liver disease (NAFLD) [67, 97], to date the most common problem in internal medicine [49]. Despite this, very little was known about MBOAT7 and its role on fatty liver disease.

In these studies, I investigated some of the unknown aspects of MBOAT7. Specifically, in paper I, I solved the topological organization of MBOAT7, describing the enzyme as an integral membrane protein anchored to endomembranes by six TMDs [I] with its catalytic dyad facing the lumen of organelles. In paper II, I characterized MBOAT7 as an O-acyltransferase that preferentially transfers PUFAs to LPI, releasing newly-remodeled PI. In addition, missense mutations in the putative catalytic dyad caused the loss of function of MBOAT7, underlying the importance of these two residues (Asn-321 and His-356) in the enzymatic activity of the protein. In paper III, by combining *in vitro* analysis, by using 2D and 3D cell cultures, with *in vivo* analysis, by using mice models and human cohorts, we established a non-canonical pathway through which MBOAT7 depletion causes triglyceride accumulation in liver.

Together, the knowledge of the topology of MBOAT7, the amino acid composition and localization of the catalytic dyad, the characterization of the enzymatic activity of the protein, and the description of the mechanism by which MBOAT7 depletion causes hepatic fat accumulation, contribute to better understand the role of MBOAT7 in hepatic diseases.

These new findings may help to develop new strategies for the treatment of NAFLD, in particular of hepatic injuries in patients carrying *MBOAT7* pathological variants.

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