Bioactive Lipid Species and Metabolic Pathways in Progression and Resolution of Nonalcoholic Steatohepatitis

Bioactive lipid species and lipid metabolic pathways in NASH progression and resolution

Running title: emerging lipid species in NASH

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Abstract

The liver-related burden of NASH is growing as a consequence of increased disease prevalence and of the lack of an effective treatment.

A decade has passed since initial lipidomic analyses on NAFLD and our knowledge of NASH as a lipotoxic disease has considerbly expanded: identifying novel lipid species and metabolic pathways as potential pathogenic factors and therapeutic targets, including enzymes involved in fatty acid and triglyceride synthesis, polyunsaturated-derived eicosanoids, and bioactive sphingolipids, which are reviewed here. Furthermore, the concept of NASH as a pro-resolving defective disease and the role of specialized proresolving lipid molecules in the resolution of inflammation and regression of fibrosis in NASH are discussed.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world and an emerging risk factor for liver-related complications, including cirrhosis and hepatocellular carcinoma[1]. NAFLD encompasses a histological spectrum ranging from simple steatosis (nonalcoholic fatty liver, NAFL) to non-alcoholic steatohepatitis (NASH)[1], with variable degrees of fibrosis, the latter having the potential to progress to cirrhosis[2].

The liver-related burden of NASH is growing and NASH is projected to be the leading indication for liver transplantation by 2020, as a consequence of increased disease prevalence and of the lack of an effective treatment[3].

Lipotoxicity, defined as abnormal cellular lipid composition, leading to toxic lipid accumulation, organelle dysfunction, cell injury and chronic inflammation, is the hallmark of NASH[2,4], Our understanding of the main lipid species and pathways involved in lipotoxicity in NASH has susbtantially advanced in the last decade due to the progress in lipidomics, a subcategory of metabolomics that uses analytical chemistry techniques like mass spectrometry(MS) and chromatography to identify and quantify the diverse lipid species contained in biological samples[2]. The analysis of data derived from from liver and blood samples of patients across the whole NAFLD spectrum generated specific lipid signatures which are associated with different stages of liver disease[5,6,7,8,9,10,11,] (**Table 1**). The interpretation of these signatures not only disclosed novel metabolic pathways potentially responsible for liver disease progression or resolution, paving the way to new therapeutic approaches, but allowed also the identification of non-invasive diagnostic biomarkers of NASH and of fibrosis, which currently can be most reliably identified by invasive liver biopsy[1].

Lipidomic studies revealed that, although most hepatic lipids in NAFLD accumulate in the form of triglycerides (TGs), TGs are an inert form of lipid storage and protect against cell lipotoxicity, which is determined by the accumulation of toxic lipids, including intermediates in TG synthesis

(saturated fatty acids, SFAs, and free cholesterol, FC), ceramide and/or by a deficiency in lipid species that mantain cellular integrity including phospholipids, ω3-polyunsaturated Fatty Acids(PUFAs) or PUFA-derived Specialized Proresolving Mediators (SPMs)[10,11].

We will review recent advances regarding lipid species involved in NASH progression and resolution and discuss mechanistic insights and potential therapeutic targets[11]. We will focus on different types of free fatty acids (FFAs), phospholipids, sphingolpids, and PUFA-derived eicosanoids and SPMs, The role of cholesterol accumulation in the pathogenesis of NASH has been recently reviewed elsewhere[12].

Role of saturated fatty acids in the pathogenesis and progression of NASH

Studies with stable isotopes demonstrated that in NAFLD, hepatic FFAs derive from lipolysis of adipose tissue TG (60% of hepatic FFAs), from *de novo* lipogenesis (25%), which is inappropriately upregulated, and from dietary TG(15%)[13].

In NAFLD, as in other insulin resistant states, the liver is chronically exposed to high levels of circulating FFAs due to unrestricted lipolysis of adipose tissue TGs; additionally, hepatic uptake of circulating FFAs, which involves a tetrameric plasma membrane protein complex that comprises plasma membrane fatty acid-binding protein (FABP), caveolin-1, fatty acid translocase (FAT/CD36) and calcium independent membrane phospholipase A2 (iPLA2β), is uprregulated and contributes substantially to circulating FFA internalization[14].

The saturated fatty acids (SFAs) palmitate (PA, C16:0) and stearate (C18:0) are major components of the diet, are synthesized via *de novo* lipogenesis and accumulate in the steatotic liver, paralleling liver disease severity[8-10]. These SFAs possess substantial lipotoxicity through a variety of mechanisms: they can bind to plasma membrane Toll-Like Receptor (TLR)-4 and death receptor TNF-related apoptosis-inducing ligand (TRAIL)-2, triggering caspase-dependent hepatocyte apoptosis and inflammasome-mediated pro-inflammatory cytokine secretion, or can enter the cell

and trigger endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and c-Jun N-terminal kinase (JNK) activation.

JNK belongs to the mitogen-activated protein kinase (MAPK) family and is a major mediator of hepatic lipotoxicity: in hepatocytes JNK inactivates Insulin Receptor Substrate (IRS)-1, inducing insulin resistance, interacts with the outer membrane mitochondrial protein SH3BP5 (Sab) to impair respiration, and induces reactive oxygen species (ROS) generation[15]. Furthermore, JNK suppresses Peroxisome Proliferator-Activated Receptor (PPAR)-α-mediated Fibroblast Growth Factor (FGF)-21 expression and mitochondrial and peroxisomal ß-oxidation, and activates the proapoptotic protein p53-upregulated modulator of apoptosis(PUMA)[16,17](**Figure 1A**)

Notably, although hepatocytes are the primary target of SFA lipotoxicity, nonparenchimal cells are also involved: SFAs activate TLR-4 on hepatic stellate cells (HSCs) to secrete the chemokine monocyte chemoattractant protein(MCP)-1 and activate JNK in Kupffer cells and macrophages to induce proinflammatory M1 polarization, chemotaxis and secretion of profibrogenic factors Transforming Growth Factor(TGF)-ß and Tissue Inhibitor of Metalloproteinase(TIMP)-1[15,18](**Figure 1B**).

On this basis, two strategies have been proposed to antagonizehepaticSFA lipotoxicity: the modulation of nuclear transcription factors involved in SFA metabolism, and selectiveinhibition of enzymes involved in key steps of lipid synthesis.

Modulation of nuclear transcription factors involved in lipid metabolism

LXR and SREBP inhibition

Nuclear receptors are ligand-activated transcription factors thatregulate the expression of target genes to affect diverse cellular processes, including lipid metabolism. A number of these nuclear receptors (reviewed in[2]), including liver X receptors (LXRs), Farnesoid X receptor (FXR), and

PPAR-α, PPAR-β (also called PPAR-δ) and PPAR-γ regulate hepatic lipid metabolism and are being targeted by emergingpharmacological therapies.

Liver X Receptor(LXR)-α modulation

There are two LXRs, LXR-α and LXR-β, with considerable sequence homology and the same ligand binding, but with different tissue distribution: LXRα is highly expressed in the liver, adipose tissue and macrophages, whereas LXRβ is expressed in many tissues[19].

LXR-α is a key regulator of whole-body FFA and cholesterol metabolism: upon activation, LXRα upregulates hepatic SREBP-1c-mediated *de novo* lipogenesis and inhibits VLDL catabolism, promoting hepatic steatosis, large VLDL triglyceride production and hyperlipidemia[20](**online Supplementary Table 1**). LXR-α activation promotes also net cholesterol loss from the body by upregulating ABCA1, ABCG5/G8 in macrophages, hepatocytes and enterocytes and downregulating intestinal NPC1L1 expression, thereby enhancing reverse cholesterol transport and intestinal excretion. Furthermore, LXR-α increases the hepatic trascription of cytochrome P450 7A1 (CYP7A1), the rate-limiting enzyme in bile acid synthesis, promoting cholesterol conversion to bile acids, and accelerates LDL-receptor degradation, thereby reducing hepatocyte cholesterol uptake[12].

25-Hydroxycholesterol-3-sulfate (25HC3S) is an endogenous sulfated oxysterol that suppresses LXR/SREBP-1 activation and NF-κB–mediated pro-inflammatory response[21], showing potent anti-steatotic, anti-inflammatory and anti-fibrotic activity in rodent models of NASH[22].

25HC3S (Dur-928) is being developed as an oral agent for the treatment of NASH and proved safein a phase Ib study in patients with NASH (ANZCTR number**:**ACTRN12615001355561).

Farnesoid X Receptor agonists

Originally known for its function of bile acid sensor in enterohepatic tissues, farnesoid X receptor (FXR) has recently emerged as a master regulator of lipid metabolism and of inflammatory and fibrogenic processes. FXR is expressed mainly in the liver, intestine, kidney and adrenal glands, and at lower levels in adipose tissue. In the liver, FXR activation reverses lipotoxocity by increasing FA β-oxidation, suppressing SREBP-1c-mediated *de novo* lipogenesis and promoting cholesterol excretion by ABCG5/G8 transporters and has insulin-sensitizing, anti-inflammatory and anti-fibrotic properties (**online Supplementary Table 1)**[2]**.** The semi-synthetic bile acid FXR agonist obetichoic acid (OCA) was evaluated in NASH, with favourable effects on NASH resolution and fibrosis improvement but unwanted changes in blood cholesterol levels (increased LDL-C and decreased HDL-C)[23]. The impact of OCA on long-term clinical outcomes in noncirrhoticNASH will be clarified by the ongoing 5-year phase III REGENERATE trial (ClinicalTrials.gov ID: NCT02548351). Other non-steroidal FXR agonists (Px-102, Px-104,LMB763, Gs-9674) are being evaluated in phase I-IIa RCTs in NASH (ClinicalTrials.gov Identifier: NCT01998659, NCT0199910, NCT02854605).

PPAR agonists

PPARs belong to the nuclear receptor superfamily and they can be classified into 3 isotypes designated PPAR-α, PPAR-β (also called PPAR-δ) and PPAR-γ. PPARs form heterodimers with RXR and the PPAR:RXR heterodimer extensively regulates genes involved in metabolism inflammation and fibrogenesis[2], promptingdevelopment of PPAR agonistsfor NASH therapeutics[2](**online Supplementary Table 1**).

PPAR-α and PPAR-δ have overlapping metabolic effects and complementary tissue distribution, which led to the development of dual PPARα/δ agonists. These agents reversed hepatic toxic lipid accumulation by upregulating mitochondrial and peroxisomal fatty acid β-oxidation and microsomal ω-oxidation and ketogenesis in hepatic parenchimal and nonparenchymal cells,

improving NASH and fibrosis in preclinical models of NASH[24,25]. Furhtermore, the lipid-oxidizing effects of PPARα/δ agonists in skeletal muscle and adipose tissue improvedsystemic insulin resistance and adipose tissue dysfunction (**online supplementary Table 1**).

Elafibranor, a dual PPARα/δ agonist, reversed NASH without fibrosis worsening and improved blood lipid profileand glucose utilization in a phase IIb RCT[26], while the selective PPAR-δ agonist seladepar (MBX-8025)improved liver enzymes, inflammatory markers, insulin resistance and atherogenic dyslipidemia in dyslipidemic patients[27].

PPAR-γ is expressed byadipocytes, immune cells, including macrophages Kupffer cells, andHSCs, and to a lower extent by hepatocytes and skeletal miocytes.

PPAR-γ activation reverses lipotoxocity by enhancing adipocyte differentiation and insulin sensitivity and promoting adiponectin secretion, thereby reducing lipotoxic SFA overflow to the liver; furthermore, PPAR-γ upregulation shifts SFA-induced macrophage and Kupffer cell polarization from an M1 to an M2 phenotype[18], and reverses HSC transdifferentiation to myofibroblasts[28](**online supplementary Table 1**).

On this basis, dual PPAR-α/γagonistsand panPPARagonists have been developed to combine the lipid-oxidizing properties of PPAR-α/δ agonists with the insulin sensitizing effects of PPAR-γ agonists.

Saroglitazar, a dual PPAR-α/γ agonist, and IVA337(Lanifibranor), a pan-PPAR agonist, increased the expression of β-oxidation-related and fatty acid desaturation-related enzymes and ameliorated diet-induced NASH and fibrosis with an efficacy superior to single class PPAR agonists[29,30] .

Saroglitazar improved liver enzymes in diabetic NAFLD patients[31] and a small, phase IIa clinical trial on biopsy-proven NASH patients has been completed (CTRI registration no.: CTRI/2010/091/000108).

Inhibition of enzymes involved in *de novo* lipogenesis and triglyceride synthesis

Acetyl-CoA carboxylase (ACC) inhibition

ACC catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA, the first, rate-limiting step in *de novo* lipogenesis[19].

ACC exists as two tissue-specific isozymes that are encoded by separate genes and display distinct cellular distributions: ACC1 is a cytosolic enzyme present in lipogenic tissues (liver, adipose); ACC2 is a mitochondrial surface-associated isozyme present in oxidative tissues (liver, heart, skeletal muscle)[19].

In the liver, cytosolic malonyl-CoA formed by ACC1 is used primarily for *de novo* lipogenesis, whereas mitochondrial malonyl-CoA formed by ACC2 acts primarily as an allosteric inhibitor of mitochondrial fatty acid uptake by carnitine palmitoyltransferase (CPT)-1, resulting in a subsequent reduction in mitochondrial fatty acid β-oxidation[19]. Thus, inhibition of the ACCs may reduce *de novo* lipogenesis and increase fatty acid ß-oxidation, two key steps in fatty acid metabolism that are dysregulated in human and experimental NASH[19]: consistently, mice with constitutively activated ACC have enhanced lipogenesis, hepatic insulin resistance and develop NASH and fibrosis, which are prevented by genetic ACC ablation and reversed by pharmacological ACC inhibition[32].

Following these preclinical results, the orally available, liver-targeted, selective small molecule allosteric ACC1/2 inhibitor GS-0976 (formerly NDI-010976) suppressed *denovo* lipogenesis and improved MRI-assessed steatosis and serum fibrosis markers in a small phase 2a RCT enrolling noncirrhotic NASH patients[33].

Diacylglycerol acyltransferase (DGAT)-1 and DGAT-2 inhibition

Acyl-CoA:diacylglycerol acyltransferase (DGAT) is an ER-membrane-bound enzyme that catalyzes the final step in TG synthesis: diglyceride (DAG) esterification by long-chain acyl-CoA esters. DGAT exists in two isoforms encoded by two distinct genes. DGAT-1 is most highly expressed in the small intestine enterocytes, where it participates to the lipolysis-reesterification cycle by reassemblingTG from dietary FFAs in the process of intestinal fat absorption to form

chylomicrons[19]. DGAT-2 is expressed primarily in the liver, adipose tissue and skin, where it synthesizes TG from *de novo* synthesized FFAs and newly formed diglycerides (DAGs)[34].

DGAT-1 knockout (*DGAT1*$^{-/-}$) mice are viable and have modest reductions in tissue TG, whereas DGAT-2 knockout (*DGAT2*$^{-/-}$) mice have severe lipopenia(~90 % reduction in whole-body TG) and impaired skin barrier function (including the inability to retain moisture), and die shortly after birth[35].

Due to the data from DGAT knockout mouse models and to the finding that dietary SFA absorption is upregulated in NASH patients[36], most research focused on selective DGAT-1 inhibitors for the treatmernt of obesity, NASH and hyperlipidemia. Pradigastat, a potent selective DGAT-1 inhibitor, slightly reduced MRI-assessed liver fat after 24 weeks, but diarrhea and steatorrhea due to fat malabsorption affected up to 86% of patients on active treatment[37].

Growing data suggest that pharmacological DGAT-2 inhibition is an effective and safe therapeutic option, due to the central role of this isoenzyme in hepatic TG metabolism: unlike DGAT-1 inhibition, selective hepatic DGAT-2 inhibition with small molecule inhibitors improved high fat diet-induced hepatic steatosis, fibrosis and insulin resistanceby down-regulating SREBP-1c-mediated lipogenesis, protein kinase C(PKC)-mediated insulin resistance, and NF-κB-mediated inflammation and fibrgenesis and upregulated fatty acid oxidative and thermogenic pathways, including CPT-1 and uncoupling protein(UCP)-2[38,39,40].

Role of glycerophospholipid lysophosphatidylcholine (LPC) in the pathogenesis and progression of NASH

LPC is a glycerophospholipid generated from phosphatidylcholine (PC) by partial hydrolysis of one fatty acid group. This reaction can be catalyzed intracellularly by phospholipase A2(PLA2) or extracellularly by plasma lecithincholesterol acyltransferase (LCAT)[19].

The relative contribution of these two pathways to hepatic LPC accumulation *in vivo* remains to be elucidated. PLA2-generated LPC is an important mediator of SFA-induced lipotoxicity: in

cultured hepatocytes, incubation wih LPC triggered cell apoptosis, while PLA2 inhibition reduced intracellular LPC and palmitate-induced apoptosis[14,41]. Consistently, hepatic LPC content is increased in rodent models and in human NASH and parallels liver disease severity[5,8,10].

Beside lipoapoptosis, which is triggered by JNK and ER stress activation, other mechanisms for LPC lipotoxicity have been identified: LPC impairs hepatic mitochondrial oxidative phosphorylation, causing mitochndrial dysfunction[42], induces pro-inflammatory and pro-fibrogenic extracellularvescicle (EV) release from hepatocytes[43,44] and is converted into the potent profibrogenic phospholipid lysophosphatidic acid (LPA) by the enzyme autotaxin[45](**Figure 1**; **online supplementary Table 2).**

Most of these lipotoxic mechanisms overlap with those of SFA, suggesting that LPC generation may be a major downstream effector of SFA citotoxicity. An additional, important mechanism of lipotoxicity is the depletion of membrane PC caused by PLA2 activation[14,46]. PC is the most abundant phospholipid in mammalian cells, is essential for cell membrane integrity and is also an important feedback inhibitor of SREBP1c-mediated *de novo* lipogenesis[47]. Hepatocytes have a high demand for PC, which is used for the production of VLDL and is secreted in bile, making these cells particularly liable to PC depletion.

The NASH liver is characterized by hepatic PC depletion (**Table 1**) [9,10] and disruption of hepatocyte membrane functional integrity, which results in release of lipotoxic lipids into the extracellular space, hepatocyte apoptosis, inflammation, and liver disease progression[46,48]. Remarkably, this chain of events was interrupted by genetic or pharmacological PLA2 inhibition, which normalized intracellular PC pool, prevented diet-indued NAFLD and reversed established NASH and fibrosis[14,49].

Bioactive Sphingolipids: role of the "sphingolipid rheostat" in the pathogenesis and treatment of NASH

Sphingolipids are a lipid species containing a sphingosine (18-carbon amino alcohol) backbone that is N-acylated with various fatty acids. Sphingolipids are ubiquitous components of eukaryotic cell membranes and are both structural lipids and potent signaling molecules regulating key cellular functions, like cell growth and survival, proliferation, differentiation, migration and immune responses in health and disease states, including metabolic disorders and NASH[50]. Furthermore, ceramide, sphingosine, and sphingosine-1-Phosphate (S1P) are readily interconvertible and the delicate balance of their relative levels has been termed the "sphingolipid rheostat", as it can direct cellular fate to opposing directions: as an example, ceramide and sphingosine induce cell apoptosis, senescence, and growth arrest while S1P promotes cell survival, growth, and proliferation[50].

Role of ceramide in NASH

Ceramide consists of an amino group of a sphingoid base, typically sphingosine, bound to a saturated or monounsaturated fatty acyl chain, and constitutes the hydrophobic core of all complex sphingolipids, including sphingomyelin, cerebrosides and gangliosides[50]. Ceramide can be synthesized *de novo* from serine and palmitate by the sequential action of three ER-resident enzymes—serine palmitoyltransferase (SPT), ceramide synthase (CeS) and dihydroceramide desaturase (DES) — or generated from hydrolysis of plasma membrane sphingomyelin into ceramide and phosphocholine by the enzyme sphingomyelinase (SMase)(**Figure 2**).

Lipidomic studies indicate hepatic ceramide content is increased in NAFLD and correlates with liver disease severity[5-10]. Hepatic ceramide overload results from both increased hydrolysis of sphingomyelin by acid sphingomyelinase (ASMase), one isoform of SMase which is upregulated in NASH by proinflammatory stimuli, including reactive oxygen species (ROS), TNF-α, death receptor ligands[51], and from increased availability of SFAs, the limiting substrate for *de novo* ceramide synthesis. Additionally, recent data indicate the intestine is a relevant source of ceramide, whose synthesis in ileum and cecum is stimulated by the activation of a bile acid/intestinal FXR axis(**Figure 2**)[52]. Supportive of a key role of intestine-derived ceramide in liver injury and

metabolic dysregulation in NASH, intestine-specific genetic or pharmacological FXR inhibition reduced circulating ceramide levels by 30-50%, enhanced adipose tissue browning, and improved hepatic insulin resistance and liver injury in HFD obese mice; remarkably, these effects were reversed by ceramide injection, confirming the benefits of intestinal FXR inhibition are mediated by ceramide reduction.

Accumulating evidence suggests ceramide is a key mediator of SFA lipotoxicity, which requires ceramide accumulation and is prevented by inhibiting ceramide formation: for example, studies using lipid infusion revealed that ceramide formation is required for SFA-induced inhibition of Akt-mediated insulin signaling and insulin resistance[53]. The mitochondria are another major cellular target of ceramide, which impairs fatty acid β-oxidation through inactivation of complex II and IV of the electron transport chain and promotes ROS production, TG accumulation and insulin resistance[54,55](**Figure 2**). Further mechanisms of hepatic lipotoxicity of ceramide overload in NASH include disruption of calcium homeostasis in the ER, which leads to ER-stress-mediated apoptosis Nlrp3 inflammasome activation impairment of autophagy, and upregulation of hepatic hepcidin, which leads to hepatic iron overload[56,57,58,59,60]. Additionally, ASMAse activation may promote liver injury independently of ceramide accumulation by disrupting methionine and phosphatidylcholine metabolism, which promotes lysosomal membrane permeabilization[58] and directly activates HSCs[61](**Figure 2**).

On this basis, several strategies aiming at relieving hepatic ceramide accumulation have been investigated, including inhibiting ceramide hydrolysis from sphingomyelin or ceramide *de novo* biosynthesis or enhancing ceramide degradation. ASMase can be inhibited byfunctional inhibitors of acid sphingomyelinases (FIASMAs), a heterogeneous group of weakly basic and highly lipophilic molecules (**Table 2**), which accumulate into the lysosome, detach ASMase from the inner lysosomal membrane and induce its degradation by lysosomial proteases[62].

Reduction of ceramide *de novo* synthesis can be achieved through inhibition of the enzymes involved in ceramide synthesis, i.e., SPT, CeS or DES or through inhibition of intestinal FXR activation **(Table 2)**.

Treatment of genetically induced (ob/ob) and HFD–induced obese rodents with the natural fungal metabolite myriocin, a specific inhibitor of SPT, decreased circulating ceramides and body weight and improved glucose tolerance, hepatic insulin resistance, steatosis and fibrosis[63,64].

However, complete whole-body inhibition of ceramide synthesis may have significant side effects, including neurodegeneration[65], because of the pivotal role of ceramide in the formation of other sphingolipid derivatives that are essential to cell membrane function and intracellular signaling pathways. Hence, selective intestinal FXR inhibition may overcome the limitation of whole-body ceramide synthesis inhibition: the bile acid derivative glycine-β-muricholic acid (Gly-MCA), reduced systemic ceramide levels and improved obesity and NAFLD without affecting CNS ceramide synthesis[52]. The challenge will be to restrict FXR inhibition to the intestine, as hepatic FXR activation actually improved NASH[23].

Another strategy to limit side effects of whole-body ceramide depletion derives from the finding that most lipotoxic effects of ceramide are mediated by C16:0 ceramide, which predominantly accumulates in the liver and adipose tissue of NASH patients[10,55], but not by very-long acyl-chain fatty acid (VLCFA) ceramides[54,55].

In mammals, CeS comproses six isoforms (CerS1–6), which differ in their acyl-chain specificities, tissue distribution, and transcriptional, and post-translational regulation. In the liver, CeS2 uses very long acyl-chain C22–26 fatty acids, while CeS5-CeS6 use long-chain C14–16 acyl-chain fatty acids[66]. Therefore, selective inhibition of CeS5-6 may prevent accumulation of toxic C16:0 ceramide without depriving the organism of VLCFA ceramides.

Compound ST1072 inhibits preferentially CerS4 and CerS6[67], but to date has not been evaluated in NASH or associated metabolic disorders. CerS activity can also be modulated by phosphorylation or deacetylation, which opens up further therapeutic options for selective inhibition of C16:0

ceramide production[68]. Finally, Dihydroceramide desaturase (DES)-1 inhibitor fenretinide has been shown to inhibit CeS5, downregulate the levels of long acyl-chain ceramides in favour of VLCFA ceramides[69] and to improve diet-induced obesity and NAFLD[70]

Another therapeutic strategy is to enhance ceramide degradation by the enzyme acid ceraminidase, which is an important mediator of the biological actions of adiponectin[71](**Figure 2**). Acid ceraminidase overexpression reduced hepatocyte apoptosis, liver inflammation and fibrosis and prevented high fat diet-induced NASH and insulin resistance in transgenic mice[72].

Role of Sphingosine-1-Phosphate (S1P) in NASH

Once generated from ceramide deacylation, sphingosine can be phosphorylated by sphingosine kinases (SphKs) to form Sphingosine-1-Phosphate(S1P)(**Figure 2**). There are two pathways of S1P degradation: reversible dephosphorylation to sphingosine by nonspecific phosphatases, and by two S1P-specific phosphatases, SPP1 and SPP2; and irreversible cleavage by S1P lyase (SPL), which leads to the formation of phosphatidylethanolamine and hexadecenal. The latter is the only exit pathway for degradation of sphingoid bases in mammalian cells(**Figure 2**).

Although the ready interconvertibility of ceramide, sphingosine, and S1P and the opposing effects of these sphingolipidson cell growth and survival led tocoin the term "sphingolipid rheostat"[50], growing data support a more complex picture for S1P, which can exert diverse and even opposing actions, depending on the subcellular compartment of synthesis, on the cellular molecular target, and on the cell type.

In humans two sphingosine kinase isoforms (SphK1 and SphK2) exist, with different subcellular localization, resulting in cellular S1P compartmentalization[73]. Upon activation, SphK1 translocates to the plasma membrane, where it catalyses the formation of S1P from plasma membrane-associated sphingosine: here, generated S1P is preferentially released extracellularly to activate a family of five cell surface G-protein coupled receptors (GPCRs), named S1PR1-5.

Conversely, SphK2 localizes in intracellular compartments, including the nucleus, the ER and the mitochondria, where newly generated S1P acts directly on intracellular targets in a receptor-independent mode: in the nucleus, S1P arrests DNA synthesis by inhibiting Histone Deacetylases (HDAC)-1/2; in the ER, S1P contributes to TNF receptor-associated factor 2 (TRAF2) activation, which is required for NF-kB activation[74], and in the mitochondria, S1P interacts with prohibitin 2 to regulate respiration and triggers BAK-dependent cell apoptosis[73].

The cellular compartmentalization of SphKs and S1P is therefore so critical for the regulation of cellular functions that artificially targeting SK1 to the ER or nucleus can allow the otherwise pro-survival enzyme to promote apoptosis[75].

The expression of the two SphK isoforms also varies across different tissues/organs, with SK1 most highly expressed in lung, spleen and leukocyte and SK2 being the predominant isoform in the kidney and liver[73-75]. However in NASH hepatic SphK1 expression is considerably upregulated[76, 77] as a result of SFA overflow to the liver and of the stimulatory effect of pro-inflammatory cyokine TNF-α and IL-1 elevation, which may play a critical role in promoting liver injury.

The complex interaction of the aforementioned factors explains the results of studies evaluating the role of SpK1/ SphK2 and of S1P receptor (S1PR) modulation in NASH.

The functional manipulation of SphK isoforms in diet-induced NASH models demonstrated that SphK1 activation promotes NASH, enhances secretion of pro-inflammatory cytokines TNF-α and IL-6 and inhibits secretion of the anti-inflammatory adiponectin and IL-10 adipokines[78], while SphK2 activation has opposite effects[79]. However, since SphK activation uses ceramideas a substrate to synthesize S1P, intracellular levels of these two sphingolipids are often inversely related, making it is difficult to dissect the effects of changes in S1P levels from the changes in ceramide concentrationfollowing functional SphK manipulation[78,79].

Studies using whole-body genetic or pharmacological manipulation of SIPRs[80] indicate S1PR1 and S1PR3 are the crucial receptor subtypes mediating not only hepatic necro-inflammation but also fibrosis progression: in human fibrotic liver SphK1 S1P and S1PR(1,3) expression were increased

irrespective of the etiology of fibrosis; S1PR(1,3) were massively upregulated and exerted a powerful migratory action on human myofibroblasts, whereas S1PR2, which inhibited myofibroblast migration, was downregulated[81, 82](**Figure 2**). Furthermore, TGF-β is a potent inducer of SphK1, but not of SphK2 expression, and S1P is a crucial mediator of intracellular TGF-β signaling[83] .

The therapeutic implications of S1PR antagonism in NASH are unexplored.

Administration of FTY720 (fingolimod), a functional S1PR1 antagonist approved for the treatment of multiple sclerosis, showed substantial antisteatotic, anti-inflammatory and anti-fibrotic activity in diet-induced rodent models of NASH, and S1PR(1,3)antagonist VPC23019 inhibited HSC activation in cell cultures[79,81].

However, it should be noted that the functional effects of S1P axis activation may be even opposing, depending on the cell type involved: as an example, activation of S1PR1 in endothelial sinusoidal cells promotes regeneration and suppresses fibrosis in the liver[84], and adipocyte-specific inhibition of S1P synthesis leads to lipodystrophy, NASH and insulin resistance[85]. Ongoing nanotechnology approaches may develop liver-specific FTY720 nanoparticles and limit off-target effects of S1PR antagonists[86]. A deeper knowledge of the down-stream cellular signaling pathways and functional effects of S1P in different organs and tissues would allow more targeted therapeutic interventions.

Monounsaturated Fatty Acid (MUFA) in NASH

The most abundant and well-studied MUFAs in NAFLD are palmitoleic acid, a 16 carbon length monounsaturated fatty acid (C16:1) and oleic acid, a 18 carbon length MUFA (C18:1), which are generated by the enzyme stearoyl CoA desaturase-1 (SCD1) from SFA palmitic and stearic acid, respectively[19].

Despite the fact that they contribute to steatosis, these MUFAs are less lipotoxic than SFAs:

when individually studied, these MUFAs induce apoptosis, but this effect is minimal, compared with SFAs and they greatly attenuated palmitate-induced apoptosis in cultured hepatocytes[87]. The cytoprotective effects of MUFAs may depend on their lower ability to trigger ER stress and PUMA activation and to a more efficient incorporation into TG: consistently, in cultured hepatocytes the combination of a MUFA with palmitate or SCD1 upregulation mitigated palmitate-induced apoptosis but enhanced TG accumulation while, in the setting of impaired TG synthesis, oleate accumulaion induced lipotoxicity[88].

Polyunsaturated Fatty Acid (PUFA) biosynthetic pathways in the pathogenesis and treatment of NASH

Twenty-carbon and more than 20-carbon polyunsaturated fatty acids (PUFAs) include two classes of PUFA, i.e., n-6 (ω-6) and n-3 (ω-3) series, named after the position of the first unsaturation counting from the methyl end of the fatty acid, the so-called omega-C.
ω-6 PUFAs include dihomo-γ-linolenic (DGLA, 20:3n-6) and arachidonic acid (AA, 20:4n-6),while ω-3 PUFAs include eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)(**Figure 3**).
Mammals do not have the necessary fatty acid desaturases to convert oleic acid (18:1*n*-9) into linoleic acid (LA, 18:2n-6) and α-linolenic acid(ALA, 18:3n-3), which must therefore be introduced with the diet and are referred to as dietary essential fatty acid (**Figure 3**). The conversion rates of 18-carbon fatty acids to 20-carbon PUFAs are less than 5-10%[89]: hence, fish and their oils, which contain high levels of these PUFAs, such as EPA and DHA, are the primary source of these FAs.
The relevance of ω-6 and ω-3 PUFAs in numerous cellular biological functions has emerged in the latest years[90]. PUFAs modulate cell membrane fluidity and permeability, membrane microdomain composition and hormone receptor binding, a critical step in receptor signaling: as an example, ω-3 PUFAs increase membrane fluidity, resulting in an enhanced number of membrane insulin

receptors, an increased affinity of insulin to its receptors and an improved insulin sensitivity[91]. Furthermore, PUFAs have been found to modulate the expression of genes involved in lipid metabolism, redox balance, inflammation and fibrogenesis through interacting with nuclear receptors and transcription factors in different experimental models of NASH[92,93,94, 95](**online Supplementary Table 3**).

Finally, PUFAs are released by PLA2 from cell membrane phospholipids to become substrates for the synthesis of eicosanoids and specialized proresolving mediators (SPMs) by the coordinated activities of cyclooxygenases (COX-1 a constitutive enzyme, or COX-2 an inducible enzyme), lipoxygenases (5-, 12-, or 15-LOX), or cytochrome P450 monoxygenases: while ω-6 PUFA arachidonic acid gives rise to the pro-inflammatory 2-series PGs and TXs and to the 4-series LTs, ω-3 PUFAs EPA and DHA give rise to the potent specialized pro-resolving molecules(SPMs) lipoxins, resolvins, maresins and protectins, which drive inflammatory response resolution(see below)[11](**Figure 4).**

The synthesis of highly unsaturated fatty acids such as the ω-3 EPA and DHA (synthesized from α-linolenic acid), and the ω-6 AA (synthesized from linoleic acid) is a multi-stage process requiring successive actions by elongase and desaturase enzymes. In humans, three desaturases occur: Fatty Acid Desaturase 1 (FADS1, or Δ5 desaturase), Fatty Acid Desaturase 2 (FADS2, or Δ6 desaturase), and Stearoyl CoA Desaturase(SCD)-1 (or Δ9 desaturase, where Δ indicates the number of the first carbon atom that forms the double bond relative to the carboxyl group)[89].

FADS1 and FADS2 are membrane-bound enzymes that catalyze the rate-limiting steps in the formation of long-chain PUFAs AA, EPA AND DHA(**Figure 3**). SCD-1 forms the MUFA oleic acid (18:1) from stearic acid (18:0), a saturated fatty acid either synthesized in the body from palmitic acid or ingested directly.

Lipidomic analyses coupled with enzymatic activity and gene expression profiling of human and mouse liver tissue highlighted a major dysregulation in hepatic long chain fatty acid (LCFA) desaturation and an unbalanced ω-6 to ω-3 ratio, with impaired fluxes toward the omega-3 fatty

acids pathway resulting in an increased omega-6 to omega-3 ratio as a key pathogenic factor in NASH[5,9,10,96] .

Growing evidence support a central role for impaired FADS1 activity in dysregulated long chain fatty acid (LCFA) fluxes and liver injury in NASH[9,10]. Impaired FADS1 activity acts as a bottleneck leading upstream to the accumulation of SFA and of preferential ω-6 PUFA-derived proinflammatory eicosanoids and downstream to the deficiency in VLCFA, which are substrates for the synthesis of phospholipids, essential components of cell membranes

Studies using pharmacological FADS1 activity manipulation demonstrated that this enzyme is a key determinant of membrane phospholipid composition and proinflammatory versus proresolving lipid mediator balance[97]. Hepatic FADS1 inhibition with Antisense Oligonucleotides (ASOs) yields two major pathophysiological consequences promoting NASH development: downstream, it alters the composition and quantity of membrane phospholipid leading to cell membrane phospholipid deficiency, hepatocyte membrane integrity disruption, cell necrosis and extracellular leak of lipotoxic lipids, which contribute to liver injury[9,10]. Upstream, the impaired FADS1 activity leads to an imbalance in desaturation fluxes toward the preferential synthesis of ω-6-derived pro-inflammatory eicosanoids PGs, TXs and LTs (see below) at the expense of ω-3-derived specialized pro-resolving lipid mediators(SPMs), further promoting chronic hepatic inflammation (**Figure 3-4**)[97]. Importantly, the proinflammatory effects of FADS1 deficiency are non limited to hepatocytes, but occur also in cultured macrophages, where FADS1 knockout induced classic M1 polarization and suppressed pro-resolving M2 activation programs[97]. In contrast with these proinflammatory effects, FADS1 inhibition has anti-lipogennic effects by inhibiting LXR/SREBP-1c-mediated lipogenesis, which may explain why pharmacological FADS1 inhibition reduced hepatic steatosis body weight and insulin resistance[97,98] .

It should be noted that the impact of FADS1 activity on livdr injury is diet-specific and is afected by the relative ω-3 and ω-6 PUFA intake:

Mechanisms underlying impaired FADS1 activity in NASH are various: dietary FAs regulate hepatic *FADS1* and *FADS2* gene expression and the activity is reduced by PUFAs in human HepG2 cells[99] and in rodents fed PUFA-enriched diets[100]. A genetic component has also been proposed, as functional genetic polymorphisms in *FADS1* gene have been found to modulate human hepatic lipid composition and content[101].

The dominant strategy adopted to restore a normal hepatic hepatic ω-6/ω-3 PUFA ratio has been dietary supplementation with varying doses and formulations of ω-3 PUFAs with different biological activity, which showed consistent benefits on liver injury and metabolic parameters in diet-induced models of NASH[93-95,97]. However, the analysis of RCTs with PUFAs conducted to date in NASH, demonstrated that ω-3 supplementation rescued hepatic ER stress, mitochondrial dysfunction and deregulated *de novo* lipogenesis[102], improved steatosis and cardiometabolic profile, but did not affect histological necro-inflammation, NAFLD disease activity and fibrosis [90,103,]. Potential reasons for the discrepant results of PUFA supplementation on steatosis versus those on necroinflammation and fibrosis include the heterogeneity of the type (with DHA showing more consistent anti-inflammatory and anti-fibrotic activity that EPA in preclinical models[94, 95]), doses and formulation of ω-3 PUFAs used in different RCTs. Furthermore, while the antisteatogenic and metabolic effects of PUFAs are mediated directly by PUFA interaction with transcription factors involved in lipid metabolism, the anti-inflammatory effcts are mediated by PUFA-derived SPMs[93] and it may be more difficult to reach hepatic ω-3 PUFA concentrations high enough to enhance generation of anti-inflammatory/pro-resolving mediators, as the largest amount of dietary FAs undergoes β-oxidation (20–30%) or storage as TGs in adipocytes (15–80%)[19]. Furthermore, in NASH SMPs may be more rapidly inactivated by eicosanoid oxidoreductases and/or by the SPM receptor down-regulation observed in obesity-related condition(discussed below)[104].

Other approaches to replace or complement dietary supplementation have been tested preclinically to re-equilibrate hepatic ω-6/ω-3 ratio: the introduction of ω-3 desaturase (*FAT-1)* activity, which is

capable of generating ω-3 from ω-6 PUFAs and is absent in humans, restored a normal hepatic ω-3 PUFA content and reversed steatosis and necro-inflammatory changes in transgenic mice fed a HFD diet[93].

Another focus of research is the development of inhibitors of SCD-1, which is the critical control point regulating *de novo* lipogenesis, triglyceride synthesis and FA oxidation through the generation of MUFAs[105]. A small molecule SCD-1 inhibitor ameliorated hepatic steatosis and necroinflammation in diverse nutritional models of NASH[106], and aramchol, a conjugate of cholic and arachidic acid that inhibits SCD-1 and *de novo* lipogenesis, given for 3 months significantly decreased hepatic fat content by 12.5% in a phase II RCT enrolling NAFLD patients[107]. Treatment was not associated with improvement in liver enzymes, raising a concern that the reduction in hepatic fat was not accompanied by an improvement in inflammation or cellular injury. This is important as a potential drawback of SCD-1 inhibition is the increase of substrate SFAs, which are lipotoxic and may exacerbate inflammation[108]. A phase IIb study with histological end-points is currently evaluating the effects of higher aramchol doses (400 and 600 mg/day) in non-cirrhotic biopsy-proven NASH (clinicaltrials.gov.ID: NCT02279524)(**Table 2**).

Eicosanoids and Specialized Proresolving Mediators in NASH

A hallmark of obesity-related disorders is a state of chronic low-grade inflammation, which in NASH recognizes the adipose tissue and the liver as major targets. In abdominal adipose tissue, the inflammatory state is fuelled by an expansion of adipose tissue pro-inflammatory macrophage pool[2], resulting in adipose tissue and systemic insulin resistance, unrestrained lipolysis and release of lipotoxic fatty acids, and pro-inflammatory cytokine and chemokine secretion[2,109]. In the liver, chronic unresolved inflammation evokes a wound healing process, which includes fibrogenesis and persistent production of new extracellular matrix to replace damaged tissue, resulting in tissue scarring, fibrosis and eventually cirrhosis. Among the different inflammatory mediators, mounting experimental and human evidence supports a key role for eicosanoids and Specialized Proresolving

mediators (SPMs) in determining the immunometabolic milieu of NASH, with relevant therapeutic implications.

Eicosanoids (from the Greek term *"eicosa-",* "twenty") is the collective term for a family of signaling lipid molecules derived from the oxidation of straight-chain 20C-containing PUFAs, mainly AA, but also DGLA and EPA (**Figure 4**)[110]. Multiple subfamilies of eicosanoids exist, including prostaglandins(PGs), thromboxanes(TXs), leukotrienes(LTs), lipoxins(LXs) and resolvins(Rvs), with functionally heterogeneous, and even opposing activities on inflammation, immune and cardiovascular system, metabolism, cell growth and tisue remodeling. Eicosanoids act as autocrine, paracrine or endocrine signaling agents through binding to surface G-protein coupled receptors(GPCRs) on target cells. Unlike FAs, eicosanoids are not stored within cells but rather synthesized from ω-6 and ω-3 PUFAs contained in membrane phospholipids upon the activation of phospholipases A2's (PLA2s). Following their release from cell membranes, ω-6 and ω-3 PUFAs are further metabolized through various pathways involving the enzymes cyclooxygenases (COXs) and the thre major lipoxygenases 5-, 12- and 15-lipoxygenase(5-, 12-, 15-LOXs).

Parallel to the emerging role of pro-inflammatory eicosanoids, an important role of novel, pro-resolving lipid molecules, collectively named SPMs, in enhancing self-limitation and resolution of chronic inflammation and, more recently, of tissue fibrosis, is being recognized: SPMs act on key molecular pathways to restore a normal cell homeostasis and tissue architecture in different organs, including the steatotic liver[11](**Table 3**).

These SPMs are also synthesized from omega-3 PUFAs EPA and DHA by the coordinate action of 5-, 12- and 15-LOX, and include lipoxins (LXs, derived from lipoxygenase interaction products), resolvins(Rvs, derived from resolution phase interaction products), which were classified as either E-series Rvs(if generated from EPA) or D-series Rvs (if derived from DHA)), protectins(PDs), and maresins(MaRs, from macrophage mediators in resolving inflammation), which derive from DHA[7] **(Figure 4).**

Data from diet-induced models of NASH and cell cultures disclosed an imbalance between pro-inflammatory eicosanoids and anti-inflammatory/pro-resolving SPMs in the liver and adipose tissue as a central pathogenic feature of liver injury in NASH, and suggest the restoration of a normal pro/anti-inflammatory balance as a feasible and effective therapeutic target[11,111,112].Current approaches aim either at antagonizing proinflammatory eicosanoids or at enhancing SPM actions.

Antagonizing proinflammatory eicosanoids

1) Group IVA phospholipase A2 inhibition

Phospholipases A2 (PLA2s) catalyzes the hydrolysis of the *sn*−2 fatty acid subsistent from membrane glycerophospholipids, generating lysophospholipids and arachidonic acid (AA), a substrate for the synthesis of eicosanoids prostaglandins (PGs) and leukotrienes (LTs) by cyclooxygenases and lipoxygenases[113](**Figure 4**).

The over 20 isozymes of mammalian PLA2s identified to date have been classified into intracellular (groups IV and VI PLA2s) and secretory forms(groups I, II, III, V, X, and XII PLA2s). Among them, the groupIV calcium-dependent cytosolic PLA2α(PLA2α), received most attention because of its central role in AA generation, its broad expression, and its active participation in cell metabolism[63].

Although groupIV PLA2α is constitutively expressed in most cells and tissues, several post-translational mechanisms regulate its activity under different pathophysiological conditions: beside Ca(2+) binding to its C2 domain, groupIV PLA2α is functionally linked to cell surface receptors, that modulate its activity through phosphorylation by protein kinases, including ERK1/2 and p38 MAPK, S-nitrosylation through interaction with nitric oxide (NO), and interaction with proteins[vimentin , cPLA2-interacting protein (PLIP)] and phospholipids[63].

Therefore, diverse extracellular signaling molecules, including pro-inflammatory and pro-fibrotic cytokines (IL-1, TNF-α, Angiotensin II)and growth factors(EGF), converge on group IV PLA2α, whchcatalizes the first, rate-limiting step in the AA cascade.

Mounting evidence suggest a critical role for group IVPLA2α in the onset of obesity-related disorders. In the liver and adipose tissue, group IVPLA2α is required to synthesize AA-derived PGs and LTs by COXs and LOXs (discussed below)(**Figure 4**).

A central role for group IV PLA2α in hepatic lipid metabolism emerged from high fat diet(HFD)-induced rodent models, where genetic group IV PLA2α deletion prevented the development of NASH and, more intriguingly, pharmacological inhibition of groupIV PLA2α with orally active compounds, including the ω3-polyunsaturated fatty acid derivatives AVX001 and AVX002 and the indole derivative ASB14780, reversed established steatohepatitis and fibrosis[49,117]. ·

AVX001 was safe and effective in patients with mild-to-moderate psoriasis[114] but has not been evaluated in NASH (**Table 2**).

2) Cyclooxygenase-2 (COX-2) pathway inhibition

COX is a membrane-bound byfunctional enzyme that catalyzes the first two committed steps in the pathwayleading to the formation of PGs and TX, namely cyclooxygenation and peroxidation

There are 2 COX isoforms: COX-1 is ubiquitous and isconstitutively expressed throughout the gastrointestinalsystem, the kidneys, the vascular smooth muscle andplatelets, while COX-2 is undetectable in most tissues, but its expression can be induced by a variety of stimuli related to inflammatory response and is therefore referred to as the inducible COX isoform. However, this distinction is not completely true, as COX-1 can be induced undercertain conditions and COX-2 has been shownto be constitutively expressed in the brainand the kidneys[43].

In methionine-choline deficient (MCD) diet and HFD-induced NASH models, hepatic and adipose COX-2 expression is significantly increased, as a result of NF-κB and IL-1 axis activation, and correlates with the severity of steatohepatitisIn[115,116].

The effects of COX-2 activation on liver disease appear largely mediated by the generation of PGs, including15-deoxy-PGI_2 and PGE_2, which enhance triglyceride storagenecro-inflammation and fibrosis by a variety of mechanisms: autophagy inhibition[117], enhancement of lipid droplet

formationandadipogenesis[118], increased chemokine MCP-1 secretion and TGF-β1-induced HSC activation **(online supplementary Table 3)**.

Intriguingly, liver histology, adipose tissue inflammation and metabolic abnormalities were improved to a similar extent by celecoxib, a selective COX-2 inhibitor, or by PGE receptor antagonists, indicating that the effects of COX-2 activation in NASH are largely PGE2-mediated[115,119] . PGE2 induces hepatocyte TG accumulation and apoptosis, promotes Kupffer cell activation and adipose tissue inflammation and dysfunction[120, 121, 122, 123,124] **(online supplementary Table 3**); furthermore, growing evidence suggests COX-2-driven PGE-2 biosynthesis promotes obesity-associated hepatocellular carcinoma and breast cancer through Prostaglandin E receptor 4(PTGER4)-mediated suppression of antitumor immunity and enhanced transcription of CYP-19, CYP-181 and aromatase-catalyzed estrogen biosynthesis[125]**(online supplementary Table 3**)).

Several issues regarding the safety of COX-2 inhibition remain: PGE2 enhances macrophage polarization switch from a pro-inflammatory M1 to a pro-resolving M2 phenotype in adipose tissue, thereby initiating inflammation resolution[126,127].

Secondly, COX-2 is constitutively expressed in endothelium and kidney, where it contributes to the synthesis of the protective PGI2 and it is still debated if COX-2 inhibition increases CVD risk[128].

3) 5-lipoxygenase (5-LOX)/leukotriene pathway inhibition

Arachidonate 5-lipoxygenase (5-LOX) is a calcium-requiring, ATP-requiring, iron-requiring enzyme that catalyses the two-step lipoxygenation of arachidonic acid to formbioactivepro-inflammatory lipids leukotriene (LT) LTB4 and cysteinyl-leukotrienes (Cys-LTs) LTC4, LTD4 and LTE4[11]. Furthermore, 5-LOX is also involved with 15-LOX in the synthesis of Lipoxins LXA4 and LXB4 which have anti-inflammatory and pro-resolving properties (see below)[7](**Figure 4**).

5-LOX is expressed primarily in inflammatory cells including polymorphonuclear leukocytes, eosinophils, monocytes, mast cells, and B-lymphocytes, where LTs exert potent pro-inflammatory actions bypromoting recruitment and chemotaxis, partly through NF-κB activation[111].

In the liver, Kupffer cells express 5-LOX and synthesize LTB4 and cysteinyl-LTs, the latter being also produced by hepatocytes through transcellular metabolism of LTA4 secreted by Kupffer cells[129]. LTspromoteKupffer cell and HSC activation. A similar role for adipocyte 5-LOX in mediating adipose tissue inflammation has been found in experimental models of obesity[129] . Notably, the adipose tissue and liver from mice with high-fat diet (HFD)-induced NASH showed increased expression of 5-LOX and its products[130] , and in humans the progression from healthy liver to NASH is paralleled by an increased formation of 5-LOX products[6]. Furthermore, functional 5-LOX manipulation studies corroborate a causal role for 5-LOX in liver injury: genetic deletion of 5-LOX protected from HFD-induced obesity, insulin ressitance and NASH[131, 132], and pharmacological 5-LOX inhibition activated AMPK activation, reduced NF-kB activation and lipolysis and improved insulin resistance, steatosis and necroinflammatory changes in NASH[129,131]. On this basis, an orally available small molecule 5-LOX inhibitor, MN-001 (tipelukast), which acts also as LTD4 receptor antagonist and phosphodiesterases (PDE) 3/4 inhibitor, reduced inflammation and fibrosis and proinflammatory and profibrogenic gene expression in an advanced NASH model[133] and was FDA-approved for a Phase IIa RCT in NASH patients with advanced fibrosis[134].

Beside 5-LOX inhibition, another strategy to antagonize the proinflammatory actions of LTs is receptor antagonism, and, intriguingly, inhibition of the LTB4 receptor 1 (Ltb4r1) achieved the same anti-inflammatory and insulin sensitizing effects in the liver and adipose tissue as 5-LOX inhibition[135], indicating that specific LT receptor antagonism could achieve the benefits of 5-LOX inhibition while preserving 5-LOX-mediated synthesis of pro-resolving mediators lipoxins.

Enhancing resolution of inflammation and fibrosis with specialized proresolving mediators (SPMs)

It is being increasingly recognized that the resolution of inflammation is not a passive phenomenon, deterimined by dilution and dissipation of inflammatory mediators, but an active process which is

orchestrated by a temporally regulated, sequential secretion of pro-resolving molecular signals which lead to self-limitation and resolution of the inflammatory response and restore tissue function[7,136].Tightly connected to inflammation, , liver fibrosis is currently considered a wound healing response to chronic, unresolved tissue injury, which is primarily driven by inflammatory and immune mediated mechanisms[2]. Experimental models indicate liver fibrosis is not an unidirectional, irreversible process but can be reversed through four steps, which may coexist independently of each other: cessation of chronic damage, shifting the balance from inflammation to resolution , deactivation of myofibroblasts, matrix degradation (reflected by an altered balance between matrix stabilizing and matrix degrading factors)[2](**Table 3**).

Unlike their precursors ω-3 PUFAs, which act at the micromolar to millimolar range, SPMs exert their biological actions in the picomolar to nanomolar range, with concentrations as low as10nM producing a 50 percent reduction in PMN transmigration in models systems[137].

SPMs not only attenuate the inflammatory response but also expedite its resolution by shifting the key cells of innate and adaptive immunity, i.e., macrophages and T cells, from a pro-inflammatory to a pro-resolving phenotype: SPMs enhance monocyte migration, macrophage polarization into a M2 phenotype[138], promoting macropage autophagy[139] and clearance of apoptotic cells (efferocytosis), and shift $CD4^+$ T cell differentiation from a pro-inflammatory T_h1/T_h17 to a pro-resolving T_{reg} phenotype[140].

In HFD-induced rodent models of NASH, in precision-cut liver slices and in cultured primary hepatocytes Kupffer cells and adipocytes, Maresin 1(MaR1) administered at physiological, nanomolar concentrations prevented palmitate- and hypoxia-induced ER stress and apoptosis and enhanced Kupffer cell phagocytosis[112].

Recent studies indicate that in obesity-associated NASH the formation of SPM in the inflamed liver and adipose tissue is severely deregulated[141], and that the administration of SPMs ameliorates inflammatory metabolic and histological features of obesity-related NASH through mechanisms strictly involved in NASH pathogenesis, inclusing insulin sensitivity, triglyceride accumulation, cell

apoptosis and fibrogenesis.More specifically, LXA4, RvD1 RvE1, Protectin D1 (PD1) and MaR1 improved adipose tissue inflammation and insulin resistance and hepatic fat infiltration and insulin resistance and reduced hepatocyte ER stress-induced apoptosis through Akt and AMPK activation and JNK inhibition; and PD1 and MaR1 increased expression of adiponectin, a key anisteatotic, anti-inflammatory and antifibrogenic adipokine, to a similar extent as thiazolidinediones[142, 143, 144, 145,146]

SPM demostrated also potent anti-fibrotic properties preclinically: Resolvin D1, Resolvin E1, Protectin DX and MaR1 attenuated fibrosis progression and, more intriguingly, reversed established fibrosis in preclinical models of hepatic, renal and pulmonary fibrosis[147, 148], at least in part through suppressing and reversing TGF-β1/Smad2/3-induced epithelial-to-mesenchimal transition (EMT) of epithelial cells, which provide up to 40% of extracellular matrix-deposing myofibroblasts, and restoring a normal tissue levels of Matrix metalloproteinase(MMPs), which are crucial for ECM resorption[149, 150,151,152].

A key issue to the clinical development of SPMs is the achievement of therapeutically effective SPMs concentrations to activate resolution signals in target tissues, including the live and adipose tissue, as SPMs are rapidly inactivated by eicosanoid oxidoreductases and leukocyte receptors for SPMs appear to be down-regulated in obesity-related conditions[104].

Pharmacological strategies to enhance biological activity and selective delivery of SPMs to target organs, are being investigated: thesynthetic oxidoreductase-resistant RvD1 analogue benzo-diacetylenic-17R-RvD1-methyl ester (BDA-RvD1) showed a 3.5-fold higher potency than the natural compound in resolving ischemia-reperfusion-induced lung injury[153], while the incorporation of SPMs into liposomes (e.g., Lipo-RvD1) should enhance selective clearance by macrophages[154].

A RCT evaluating safety and efficacy of RX10045, a synthetic RvE1 analog, on dry eye disease, has been completed (ClinicalTrials.gov ID: NCT00799552).

Concluding remarks and future directions

The health-related burden of NASH is increasing all over the world along with the obesity epidemic. There are no approved treatments for NASH, as lifestye intervention is often hampered by low patient compliance and requires long-term committent. Proposed pharmacological options do not seem to reverse more advanced stages of disease and therefore are unlikely to change the natural history of the disease or are encumbered by unwanted effects[155]. Treatment of NASH is challenging, as progression from steatosis to NASH and fibrosis is likely a multi-factorial process, involving varied molecular pathways that may operate in different patient subsets at different stages of the disease. In the last decade our knowledge of NASH as a lipotoxic disease has considerably expanded the spectrum ofnew lipid species and pathways involvedin NAFLD progression. These advances in basic science are expected to make up to clinical stage of development, posingatwofold challenge for future research: first, the discovery of safe and effective pharmacological tools to antagonize the diverse lipotoxic species. Secondly, once a variegated pharmacological armamentariumis available, will be the selection of the optimal therapeutic strategy for each patient: in this context recent advances inlipidomics, genetics and epigenetics systems biology technologies will likelyenable treatments tailored to individual genomic and lipidomic profiles[156.]. As a example, the I148M variant of patatin-like phospholipase domaincontaining 3 (PNPLA3)protein,an intracellular lipase involved in lipid droplet (LD) remodelling, ia a major genetic determinant of the full spectrum of NAFLD across different ethnicities and of response to PUFA treatment[157], independently of traditional metabolic risk factors[158]. The mutant (I148M) variant protein, with loss of enzymatic activity, accumulates on LD surface by evading proteasomaldegradationandimpairs triglyceride mobilization from the LD by other lipases[159]: in hepatic stellate cells this impaired the release of retinol and lipids from lipid droplets, resulting in a more fibrogenic phenotype[160], while the precisemolecular mechanisms underlying lipotoxicity of this variant in hepatocytes remain to be defined. A potentialtherapeuticimplication of recent advances in PNPLA3 biology could be the development of agents that decrease PNPLA3 protein

levels and improve liver disease by reducing the impairment in LD remodeling in NASH carriers of the PNPLA3 I148M mutant allele, in an individualized pharmacogenomic approach.

FIGURE LEGENDS

Figure 1. Mechanisms of saturated fatty acid (SFA) lipotoxicity.

Panel A. SFAs possess substantial lipotoxicity for hepatocytes through a variety of mechanisms: they can bind to plasma membrane Toll-Like Receptor (TLR)-4 and to death receptor TNF-related apoptosis-inducing ligand (TRAIL)-R2, inducing caspase 8 activation, which cleaves Bid into its truncated form (tBid) and triggers caspase-dependent hepatocyte apoptosis.

SFA can also enter hepatocyte and trigger ceramide-dependent inflammasome-mediated pro-inflammatory cytokine secretion, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and c-Jun N-terminal kinase (JNK) activation.

JNK inactivates Insulin Receptor Substrate (IRS)-1, inducing insulin resistance, interacts with the outer membrane mitochondrial protein SH3BP5 (Sab) to impair respiration, and induces reactive oxygen species (ROS) generation.

Furthermore, JNK suppresses Peroxisome Proliferator-Activated Receptor (PPAR)-α-mediated Fibroblast Growth Factor (FGF)-21 expression and mitochondrial and peroxisomal ß-oxidation, and activates the proapoptotic proteins p53-upregulated modulator of apoptosis (PUMA) and Bim. PUMA and Bim belongs to the Bcl2-2 Homology(BH3)-only protein family: both proteins are major mediators of SFA-induced lipoapoptosis through Bax activation, resulting in mitochondrial outer membrane permeabilization (MOMP) with cytochrome c liberation and in lysosome permealization with cathepsin B release.

SFA and and SFA-derived LPC and ceramide all lead to the release of Extracellular vescicles (EVs) from hepatocytes. Recent studies have defined multiple roles of lipotoxic EVs in lipotoxicity and NASH pathogenesis through cell-to-cell communication via various cargoes. CXCL10 and ceramide-enriched EVs mediate monocyte/macrophage chemotaxis to the liver, TRAIL-enriched EVs contribute to macrophage activation, and miR-128-3p-laden EVs enhance HSC proliferation and activation in vitro.

Panel B. Mechanisms of SFA lipotoxicity on nonparenchimal liver cells.

Nonparenchimal cells are also involved in SFA lipotoxicity: SFAs activate TLR-4 on hepatic stellate cells (HSCs) to secrete the chemokine monocyte chemoattractant protein(MCP)-1 and activate JNK in Kupffer cells and macrophages to induce proinflammatory M1 polarization, chemotaxis and secretion of profibrogenic factors Transforming Growth Factor(TGF)-ß and Tissue Inhibitor of Metalloproteinase(TIMP)-1

Abbreviations: CXCL10: C-X-C motif ligand; MOMP: mitochondrial outer membrane permeabilization; PUMA: p53-upregulated modulator of apoptosis; CHOP: CAAT/enhancer binding homologous protein; JNK: c-Jun N-terminal kinase; LPC: lysophosphatidylcholine; LPA: lisophosphatidic acid; TRAIL: TNF-related apoptosis-inducing ligand; BID, pro-apoptotic BCL-2 interacting domain; CHOP, CCAAT/enhancer-binding homologous protein; Bim, Bcl-2 protein family member; SFA, saturated fatty acid; FoxO3a, forkhead box-containing protein, class O member 3a; TNFa, tumor necrosis factor a; NF-kB: Nuclear Factor-kB; ROS, reactive oxygen species; ER, endoplasmic reticulum; MCP: monocyte chemoattractant protein.

Figure 2. Metabolic circuits of Ceramide (CER), sphingosine (Sph) and Sphingosine-1-Phosphate (S1P) synthesis and catabolism (the “sphingolipid rheostat”) and cellular targets of sphingolipid lipotoxicity.

Panel A. Ceramide can be synthesized *de novo* from serine and palmitate by the sequential action of three ER-resident enzymes—serine palmitoyltransferase (SPT), ceramide synthase (CeS) and dihydroceramide desaturase (DES). — or generated from hydrolysis of plasma membrane sphingomyelin into ceramide and phosphocholineby the enzyme sphingomyelinase.
Once generated from ceramide deacylation, sphingosine can be phosphorylated by sphingosine kinases (SphKs) to form S1P. There are two pathways of S1P degradation: reversible dephosphorylation to sphingosine by nonspecific phosphatases, and by two S1P-specific phosphatases, SPP1 and SPP2; and irreversible cleavage by S1P lyase (SPL), which leads to the formation of phosphatidylethanolamine and hexadecenal. The latter is the only exit pathway for degradation of sphingoid bases in mammalian cells.

Ceramide inhibits Akt phosphorylation and activation, thereby impairing Akt-mediated insulin signaling and promoting insulin resistance, an effect that is antagonized by S1P.
The mitochondria are another major cellular target of ceramide, which impairs fatty acid β-oxidation through inactivation of electron transport chain (ETC) complex II and IV and promotes ROS production and TG accumulation. Furthermore, ceramide triggers BAX-dependent mitochondrial membrane permeabilization and cytochrome c release, leading to apoptosis
Further mechanisms of hepatic lipotoxicity of ceramide overload in NASH include disruption of calcium homeostasis in the ER, which leads to ER-stress-mediated apoptosis
Ceramide synergizes with S1P to activate SREBP-1c and SREBP-2 to enhance *de novo* lipogenesis and cholesterol synthesis, and NF-kB and Nlrp3 inflammasome to induce proinflammatory cytokine and chemokine secretion.
Further mechanisms of lipotoxicity include impairment of autophagy, and upregulation of hepatic hepcidin, which leads to hepatic iron overload. Additionally, ASMAse activation may promote liver injury independently of ceramide accumulation by disrupting methionine and phosphatidylcholine metabolism, which promotes lysosomal membrane permeabilization and directly activates HSCs.

Panel B.

Recent data indicate the intestine is a relevant source of ceramide, whose synthesis in ileum and cecum is stimulated by the activation of a bile acid/intestinal FXR axis: the activity of FXR in the epithelial cells of the ileum is inhibited by different intestinal bile acids, including cholic acid (CA), chenodeoxycholic acid (CDCA) and β-muricholic acid β-MCA), while FXR activation upregulates ceramide synthesis.

In adipocytes, ceramide impairs adipose function through increase ER stress, decreasing the ration of beige to white adipocytes SphK1 activation induces an increase in secretion of TNF-α and IL-6 and inhibits secretion of adiponectin and IL-10, promoting inflammation and insulin ressitance.

Furthermore, in adipocytes and macrophages, ceramide activates Nlrp3 inflammasome to induce proinflammatory cytokine and chemokine secretion.

In HSCs, SIPR(1, 3) activation induces activation, migration and HSC transdifferentiation into myofibroblasts

Finally, SIPR1 activation in endothelial cells enhances integrity and barrier function

Figure 3. Metabolism of. Scheme of short-, long- and n-6 and n- 3 PUFA biosynthesis leading to membrane phospholipids synthesis.

The metabolism of PUFA is a complex process involving several enzymes of desaturation, elongation, and *β*-oxidation. Shown here is the pathway of both n-6 and n-3 PUFA metabolism to more unsaturated, long-chain members of each family.

The long chain saturated fatty acids and unsaturated fatty acids of the n-3, n-6, n-7 and n-9 series can be synthesized from myristic acid (C14:0) and palmitic acid (C16:0). Long-chain fatty acids of the n-6 and n-3 series can also be synthesized from precursors obtained from dietary precursors to elongation (ELOVL) and desaturation steps as indicated in these pathways.

Abbreviations. ACC: acetyl-CoA carboxylase; ELOVL: elongase of very

long chain fatty acid; FASN: fatty acid synthase; FADS: fatty acid desaturase; SCD: stearoyl-CoA desaturase.

Figure 4. Lipids derived from arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

AA is metabolized by cyclooxygenases (COX) 1/2 to prostaglandins(PGs) and thromboxanes(TXs) and by 5-lipoxygenase (5-LOX) to leukotrienes (LTs) which are involved in the initiation of the inflammatory response (red colour).

Hydroxyeicosatetraenoic acids (HETEs) and lipoxins are also synthesized from arachidonic acid and here 5-, 12- and 15-LOX and cytochrome (Cyt) P450 are involved.

Eicosapentaenoic acid is metabolized to 3-series PGs by COX and 3-series TXs by 5-LOX (weak pro-inflammatory properties, pink colour) and to E-series resolvins (anti-inflammatory and pro-resolving actions, green colour) by CYP450 and 5-LOX.

Resolvins of the D-series, protectins and maresins are derived from docosahexaenoic acid.

Lipoxins, resolvins, protectins and maresins have anti-inflammatory and pro-resolving actions.

Table 1. Main lipidomic studies in NAFLD patients

Author	**Population (n)**	**Biological Sample**	**Technique**	**Findings in NASH relative to NAFL**
Puri 2007 [5]	Non-cirrhotic NASH(n=9) NAFL(n=9) Control(n=9)	Liver	**TLC**	↓ PC ↑lysoPC ↓DHA/EPA/AA ↑ω-6/ω-3 PUFA ratio ↑FC

Puri 2009 [6]	Non-cirrhotic NASH(n=50) NAFL(n=25) Control(n=50)	Plasma	TLC	↓DHA//DPA ratio ↓ MUFAs ↓ plasmalogens ↑5-, 8-, 11-, 15-HETE
Gorden 2015 [7]	Cirrhosis (n=20) NASH(n=20) NAFL(n=17) Control(n=31)	Plasma	LC-MS	↓ lysoPE ↑ PE ↑ Cer, DH-Cer and DH-deoxyCer ↓ Sph
Zhou 2016 [8]	NASH(n=69) NAFL(n=117) Non-NASH(n=249) Non-NAFL(n=132)	Plasma	UPLC-MS	↑SFA(14:0/16:0/18:0) ↑MUFA(44:1/54:1) ↓ sphingomyelin ↓lysoPC
Chiappini 2017 [10]	NASH(n=15) NAFL(n=39) Control(n=7)	Liver	LC-MS	↑SFA(14:0/16:0/18:0) ↑MUFA(16:1/18:1) ↑ω-6/ω-3 PUFA ratio ↑Cer(C16/C18) ↓phospholipids (PC/PE/PI/PS) ↑ sphingomyelin

Abbreviations: AA: arachidonic acid; TLC: thin-layer chromatography; FC: free cholesterol; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; Cer: ceramide; DH: dihydro; DHA: docosahexanoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentanoic acid; LysoPC: lysophosphatidylcholine; HETE: hydroxyeicosatetraenoic acid; LC: liquid chromatography; MS: mass spectrometry; Sph: sphingosine; UPLC: ultra-performance liquid chromatography.

Table 2. Molecular targets and pharmacological agents targeting lipotoxicity in NASH

Molecular target	**Agent**	**Biological effect**	**Furthest developmental stage in NASH**	
			Preclinical	**Clinical (RCT ID number)**
LXR	25HC3S	LXR/SREBP-1c inhibition NF-κB inhibition	+	Ib: ACTRN12615000267550
FXR	Obeticholic acid	FXR activation	+	III: Clinicaltrials.gov ID: NCT02548351
	Px-104	FXR activation	+	IIa: Clinicaltrials.gov ID: NCT0199910
	LMB763	FXR activation	+	IIa: Clinicaltrials.gov ID: NCT02913105
	Gs-9674	FXR activation	+	IIa: Clinicaltrials.gov ID: NCT02854605
PPAR-α/δ	Elafibranor	PPAR-α/δ activation	+	IIa: Clinicaltrials.gov ID: NCT01694849
PPAR-δ	MBX-8025(selade	PPAR-δ activation	Preclinical	-

	par)			
PPAR-α/γ	Saroglitazar	PPAR-α/γ activation	+	IIa: CTRI/2010/091/000108
PPAR-α/δ/γ	IVA337 (Lanifibranor)	PPAR-α/δ/γ activation	preclinical	-
ACC1/2	GS-0976	ACC1/2 inhibitor	+	IIa Clinicaltrials.gov ID: NCT02856555
DGAT-1	Pradigastat	DGAT-1 inhibitor	+	IIa Clinicaltrials.gov ID: NCT01811472
PLA2	ASB14780, AVX001, AVX002	PLA2 inhibition	Preclinical	-
Autotaxin	PF8380	Autotaxin inhibitor	Preclinical	-
Ceramide	fenretinide	DES-1 and CeS5 inhibitor	Preclinical	-
	ST1072	CeS(4,6) inhibitor	Preclinical	-
	myriocin, FTY720	SPT inhibitor	Preclinical	-
	Gly-MCA	Intestinal FXR inhibition	Preclinical	-
	FIASMAs: TCAs, SSRIs,	Inhibitors of ceramide hydrolysis from sphingomyelin by SMase	Preclinical	-

	CCBs			
S1P	fingolimod	S1P type 1 receptor (SIPR1) antagonist	Preclinical	-
	VPC23019	S1PR(1,3)) antagonist	Preclinical	-
ω-3 PUFA	ω-3 fish oil	ω-3 PUFA supplementation	+	IIa∗: ClinicalTrials.gov: NCT00681408, NCT01154985, NCT01992809
SCD-1	aramchol	SCD-1 inhibitor	+	IIa: NCT01094158, NCT02279524
5-LOX and LTD4	tipelukast	5-LOX inhibitor, LTD4 receptor antagonist and PDE 3/4 inhibitor	+	IIa: NCT02681055
LTB4	CP105696	LTB4 receptor antagonist	Preclinical	-
Resolvin D1	BDA-RvD1 Lipo-RvD1	RvD1 analogues	Preclinical	-
Resolvin E1	RX10045	RvE1 analogue	Preclinical	-

Abbreviations: ACC: Acetyl-CoA carboxylase; TCA: tricyclic antidepressants; SSRI: selective serotonin-reuptake inhibitors; CCBs: calcium channel blockers; 25HC3S: 25-Hydroxycholesterol-3-sulfate; PLA2: phospholipase A2; CeS: ceramide synthase; DES: Dihydroceramide desaturase; S1P: Sphingosine-1-Phosphate; SPT: serine palmitoyltransferase; SMase: sphingomyelinase; SCD-1: Stearoyl Coenzyme A Desaturase-1; LOX: lipoxygenase; LT: leukotriene; PDE: phosphodiesterase,

∗ only RCT with post-treatment liver histology are reported.

Table 3. Involvement of Specialized Proresolving Mediators(SPMs) in the different steps of fibrosis resolution in NASH

Step 1: Cessation of chronic injury to the liver (allowing hepatocyte recovery and modulating the microenvironment)			
SPM	**Molecular mechanism**	**Cellular pathway**	**Ref**
MaR1 **RvD1**	**Hepatocyte:** ↓ PA- and hypoxia-induced ER stress ↓ SREBP-1c activation ↑ PPAR-α, and PGC-1α activation→ ↑ mitochondrial function ↑AMPK phosphorylation ↑ Akt phosphorylation ↑ autophagy	↓ JNK activation →↓ apoptosis ↓ CHOP activation→↓ apoptosis ↑ antiapoptotic miRNA signature ↑ mitochondrial ß-oxidation→↓ steatosis ↓ *de novo* lipogenesis→↓steatosis	112 146 143
RvD1-D6 RvE1-E2-E3, MaR1, PD1	**Adipocyte**: ↑AMPK phosphorylation ↑ Akt phosphorylation ↑ autophagy ↑ PPAR-γ expression ↑ IRS-1/IRS-2 expression ↑ GLUT-2/-4 expression	↓ secretion of pro-inflammatory adipokines (IL-1, TNF-α, IL-6) ↑ secretion of adiponectin ↑ insulin sensitivity and FFA oxidation ↓ lipolysis→↓ flow of toxic SFA to the liver	112 144 145
Step 2. Intrahepatic balance switch from pro-inflammatory to restorative(phenotypic adjustments of immune cells, especially induction of restorative macrophages),			
SPM	**Molecular mechanism**	**Cellular pathway**	**Ref**
MaR1	**Kupffer cell:** ↓ PA- and hypoxia-induced ER stress	↑efferocytosis of apoptotic cells and cellular debris ↓ LTB4 and TNF-α secretion	112
LXA4 15-epi-LXA4 RvD1 RvE1 PD1	**Macrophage**: Switch from a M1 pro-inflammatory to a M2 pro-resolving phenotype ↑ autophagy	↑ MAPK/HSP27 activation→↑ IL-10 secretion ↑PI3K/Akt signaling→↑ efferocytosis ↓MCP-1, TNF-α, IL-1 secretion	138 139

RvD1-D2 MaR1	**Adaptive immune T cells** Shift from a pro-inflammatory to a pro-resolving phenotype	Switch $CD4^+$ T cell differentiation from a pro-inflammatory T_h1/T_h17 phenotype to a pro-resolving T_{reg} phenotype ↓TNF-α and IL-17 secretion ↑ IL-22 secretion	140
Step 3. Deactivation of myofibroblasts (by senescence, apoptosis and inactivation)			
SPM	**Molecular mechanism**	**Cellular pathway**	**Ref**
LXA4, **RvD1,** **RvE1,** **MaR1**	↓TGF-β1/Smad2/3-axis activation	↓ epithelial-to-mesenchimal transition (EMT) and HSC activation	149 150 151 152
Step 4. Extracellular matrix degradation			
SPM	**Molecular mechanism**	**Cellular pathway**	**Ref**
RvD1, **RvE1,** **PD1**	**Kupffer cells, macrophages, HSCs** restored balance between matrix stabilizing and matrix degrading factors/enzymes	↓TIMP-1 secretion and ↑ MMP-9/12 activity →↑ ECM degradation	148 151 152

Abbreviations: ACC: acetyl-CoA carboxylase; FAS: fatty acid synthase; PA: palmitate; CHOP: CCAAT/enhancer-binding protein homologous protein; SREBP-1, ↑ PPAR-α, and PGC-1α ; CPT-1a: carnitine palmitoyltransferase, ACOX-1: acyl-coenzyme A oxidase

JNK: c-Jun N-terminal kinase;

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Online supplementary table

Table 1. Nuclear transcription factors Liver X receptor(LXR),FXR, SREBF-2/miRNA-33a, PXR, PPAR-α, -PPAR-δ in the pathogenesis and treatment of NAFLD.

Liver X receptor(LXR)-α		
Cells	**Molecular targets**	**Biological effect**
Hepatocyte, enterocyte, macrophage	↑ SREBP-1c and ChREBP activity	↑ hepatic *de novo* lipogenesis, steatosis and large VLDL secretion
	↑ CYP7A1 expression	↑ cholesterol conversion to bile acids
	↓ NPC1L1expression	↓ intestinal cholesterol absorption
	↑ macrophage, intestinal and hepatic ABCG5/G8 expression	↑ cholesterol reverse transport and excretion into bile and gut lumen
	↑ macrophage and hepatic ABCA1 expression	↑ cholesterol efflux to acceptor apoA-I to form HDL-C
	↑ hepatic CEH-->↑ FC availability for ABC transporters	↑ cellular free cholesterol efflux
	↑ hepatocyte CD36 expression	↑ uptake of plasma oxLDLs and FFAs
	↑ LDLR degradation	↓ uptake of plasma LDL
	↑ Angptl3 secretion →LPL inhibition	↓ VLDL catabolism
	↓ apoA-V secretion	VLDL catabolism
Macrophage, KC	↓ secretion of proinflammatory cytokines IL-1/TNF-α	↓ hepatic inflammation
HSC	↓ HSC activation	↓ hepatic fibrosis
Farnesoid X Receptor		

Cell type	Molecular mechanism	Biological action
hepatocyte	↓ SREBP-1c-mediated lipogenesis	Reduced hepatic steatosis
	↑ PPAR-α-mediated FFA β-oxidation	
	↓ gluconeogenesis	
	↑ IRS-1 phosphorylation and coupling with the PI-3K	Enhanced insulin sensitivity
	↑AdipoR2 expression	
	↑ CYP7A1 andABCG5/G810 expression	Enhanced bile acid synthesis and cholesterol excretion into bile
	↓ hepatic lipase activity and ApoC-III/apoA-1 synthesis	Reduced plasma HDL-C
	↓ VLDL secretion and HDL-Csynthesis	Reduced plasma TG
	↑ApoC-II synthesis and VLDLR-mediated uptake of VLDL	
	↓NF-κB activation	Reduced inflammation
Marophage, KC	↓ NF-κB activation→↓ MCP-1 and TGF-ß secretion	Reduced inflammation and fibrogenesis
HSC	↓ TGF-ß-R expression	Reduced fibrogenesis
Adipocyte	↑ PPAR-γ expression	Improved adipose tissue dysfunction
	↑ adiponectin and AdipoR2 expression	
	↓ TNF-α secretion	

Enterocyte	Enhanced gut barrier function and secretion of antibacterial factors angiogenin, iNOS, IL-18 Enhanced FGF-19 secretion	Reduced bacterial endotoxemia Increased bile acid synthesis, EE and fat oxidation
PPAR-α		
Cell type	**Molecular mechanism**	**Biological action**
Hepatocyte, Miocyte, Adipocyte	↑ expression of : acyl-CoA synthetase, CPT1A, VLCAD/LCAD/MCAD, acyl-CoA dehydrogenase, trifunctional protein HADHB, ACOX1, L-bifunctional protein EHHADH ↑ CYP4A and HMGCA activity ↑ FATP, CD36, L-FABP activity ↑ LPL activity and reduced apoC-III ↑ apo-AI/apo-AII synthesis	Increased mitochondrial and peroxisomal ß-oxidation Increased ω-oxidation Increased ketogenesis Enhanced FFA uptake Enhanced lipolysis of TG Increased HDL-C levels
Hepatocyte	↑ p65 binding to NF-κB response element of C3 promoter→ reduced complement C3 secretion ↓ NF-κB activation ↑ FGF21 expression ↓ expression of IL-6 , IL-1,TNF-α, ICAM-1, VCAM-1 Increased catalase activity	Reduced inflammatory response and endothelial dysfunction ↑ metabolic effects of PPAR-α Enhanced H_2O_2 detoxification
PPAR-β(δ)		
Cell type	**Molecular mechanism**	**Biological action**

Hepatocyte	↑ mitochondrial β-oxidation	Improved hepatic steatosis and insulin resistance
	↑ ABCA1 expression	Increased HDL-C levels
Macrophage KC	↓ M1/M2 phenotype ratio ↓ NF-κB activation and TGF-ß1 secretion	Reduced inflammatory and fibrogenesis
Adipocyte, miocyte	↑ PGC-1α-mediated mitochondrial biogenesis and ß-oxidation ↑ mitochondrial UCP-1/3 expression ↑ LPL expression	Enhanced fat oxidation and EE Reduced plasma TG
Enterocyte	↓ NPC1L1 expression and cholesterol reabsorption from bile and intestine	Reduced cholesterol accumulation
PPAR-γ		
Cell type	**Molecular mechanism**	**Biological action**
Adipocyte	↑ subcutaneous preadipocyte differentiation and apoptosis of insulin-resistant visceral adipocytes ↑ insulin signalling and adiponectin secretion ↑ GLUT1/GLUT4 translocation ↑ release of pro-inflammatory FFAs and cytokines TNF-α, resistin and IL-6	Enhanced insulin sensitivity and subcutaneous fat storage Enhanced glucose disposal and FFA oxidation Reduced systemic inflammation
Skeletal miocyte, hepatocyte	↑ GLUT1/GLUT4 expression and translocation to the cell surface ↓ gluconeogenesis (adponectin-mediated)	Enhanced insulin sensitivity, glucose disposal and FFA oxidation
Macrophage KC	M2 phenotype switching ↓ NF-κB activation	Anti-inflammatory and pro-resolving effect

HSC	Inhibition of TGFβ-1/Smad3-signaling pathway Induction of cell apoptosis	Anti-fibrotic effects

Abbreviations: ABC. ATP-binding cassette; CYP7A1: Sterol 7α hydroxylase; 27OHC: 27-hydroxycholesterol; 7OHC: 7-hydroxycholesterol; 22(R)OHC: 22(R)-hydroxycholesterol, 24(S): 24(S)-hydroxycholesterol,

27OHC: 27-hydroxycholesterol 24(S), 25OOC: 24(S), 25-epoxycholesterol 25OHC3S:25-hydroxycholesterol-3-sulfate , 22(S)OHC: 22(S)-hydroxycholesterol;

SAA: serum amyloid A protein; LE: late endosome; LY: lysosome;

PM: plasma membrane; IL: interleukin; HMGCoAR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; NPC1, NPC2: Niemann-Pick C1, C2; NPC1L1: Niemann-Pick C1-like 1; oxLDL: oxidized low density lipoproteins;PPAR: peroxisome proliferator-activated receptor; SHP: small heterodimeric partner; StARD4:Steroidogenic acute regulatory protein D4; LE/LY: late endosomes/lysosomes; PEPCK: phosphoenol-pyruvate carboxykinase; IRS-1: insulin-receptor substrate-1; TGF: transforming growth factor; CEH: cholesteryl ester hydrolase; LDLR: low density lipoprotein receptor; FFA: free fatty acids; HSC:hepatic stellate cells; VLDL: very low density lipoproteins

Online supplementary Table 2. Role of group IVA phospholipase A2 and cyclooxygenase (COX)-2-generated mediators in the pathogenesis of NASH and hepatic fibrosis

group IVA phospholipase A2		
15-deoxy-PGI2 and PGE2-mediated		
Cell type and molecular pathways	**Cellular effect**	**Biological effect**

Hepatocyte, adipocyte: **→↓cAMP synthesis→**↓ HSL activity ↓ VLDL-TG secretion ↑**SREBP-1c activation→**↑ ***de novo*** **lipogenesis** ↑ **DGAT-2 activation→**↑ **TG synthesis** ↓ constitutive autophagy **Kupffer cells, HSCs**: ↑ MCP-1 secretion→ monocyte recruitment to the liver ↑ TGF-ß secretion→HSC activation **NOX activation→**↑ **ROS production**	Lipid storage cell death monocyte recruitment HSC activation	**Steatosis** **Obesity** **Necrosis** **Inflammation** **Fibrosis**
Lysophosphatidylcholine-mediated		
Hepatocyte, adipocyte: ↓ mitochondrial oxidative phosphorylation ↑ **ER stress** ↑ JNK activation **HSC:** ↑ activation by autotaxin-generated LPA	Cell apoptosis HSC activation	necrosis fibrogenesis
Cyclooxygenase(COX)-2		
Cell type and molecular pathways	**Cellular effect**	**Biological effect**

Hepatocyte: ↓ **PGC1α→** ↓CPT1 activity ↓ adipose triglyceride lipase ↓ MTTP synthesis ↓ Akt /↑ p53 signaling ↑ JNK expression	↓ **mitochondrial β-oxidation** ↓ **TG hydrolysis** ↓ **VLDL secretion** ↑ **apoptosis** ↑ **proinflammatory cytokine secretion**	**Steatosis** **Necro-inflammation**
Kupffer cells: ↑ oncostatin M → ↑ SOCS3 secretion by hepatocytes →↓ CPT1 in hepatocytes	↓ Akt activation ↓ mitochondrial FFA β-oxidation	**Insulin ressitance** **Steatosis**
HSCs: ↑ PGE2 secretion→↓ regulatory T cells (Treg) activation	↓ antitumor immunity	**HCC progression**
Adipocyte: ↑ **pro-inflammatory cytokine and chemokine MCP-1 secretion** ↑ **macrophage polarization switch from a M1 to a M2 phenotype**	macrophage recruitment resolution of inflammation	**Enhanced induction and resolution of inflammation**

Abbreviations: ABCA1: ATP-binding cassette transporters A1; ACC: acetyl-CoA carboxylase; AMPK: adenosine-monophosphate kinase; **Ccl3 : chemokine(C-C motif ligand 3 ;** CD36: cluster of differentiation-36; CHOP: C/EBP homologous protein; CPP: calciprotein particles; CPT-1: carnitine palmitoyltransferase-I**;** ; ER: endoplasmic reticulum; FAS: fatty acid synthase; FFA: free fatty acids; FXR: farnesoid X-receptor; GLUT: glucose transporter; HMG-CoAR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IL: interleukin; 11β-HSD1: 11β-hydroxysteroid dehydrogenase type 1; IRE1α : inositol requiring element 1α ; IRS-1: insulin receptor substrate-1; KLF: Kruppel-like factor; LDL: low-density lipoprotein; LDL-R: low-density lipoprotein receptor; LPA: lysophosphatidic acid; MCP-1: monocyte chemotactic protein-1; NO: nitric oxide; NOX:

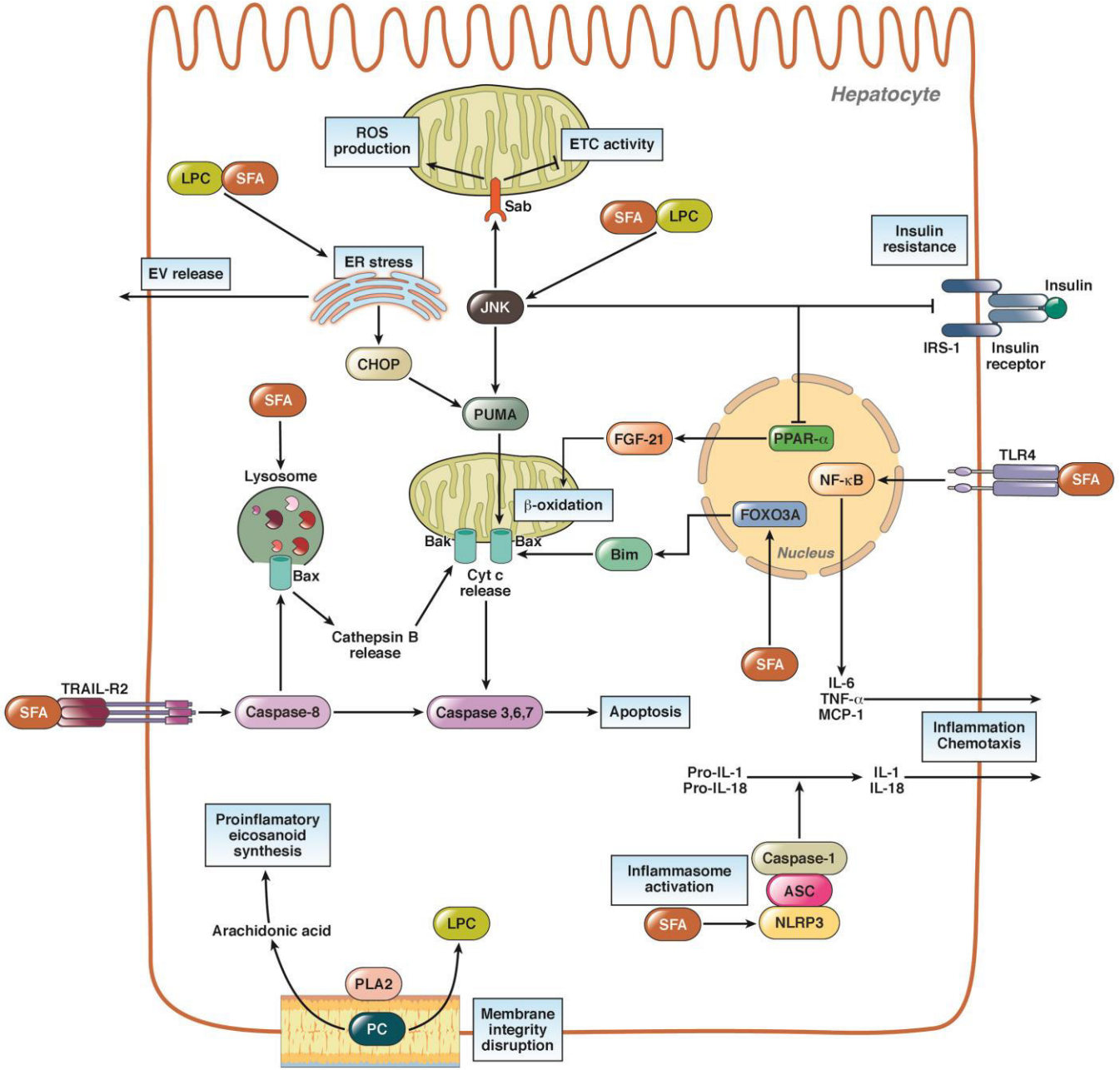
NADPH oxidase; OCA: obeticholic acid; PCSK9 : proprotein convertasesubtilisin kexin 9 ; PGC-1α: peroxisome proliferator-activated receptor-γ coactivator-1 α; ROS: reactive oxygen species; SCD-1: stearoyl-CoA desaturase-1; SOD2: superoxide dimutase-2; SR-A1: scavenger receptor-A1; SOCS3: suppressor of cytokine signaling 3; SR-B1: scavenger receptor-B1; SREBP: sterol-responsive element binding protein; STAT3: signal transducer and activator of transcription; TGF-β: transforming growth factor- β; TLR: toll-like receptor; TNF: tumor necrosis factor; VLDL: very low density lipoprotein; VSCMs: vascular smooth muscle cells;

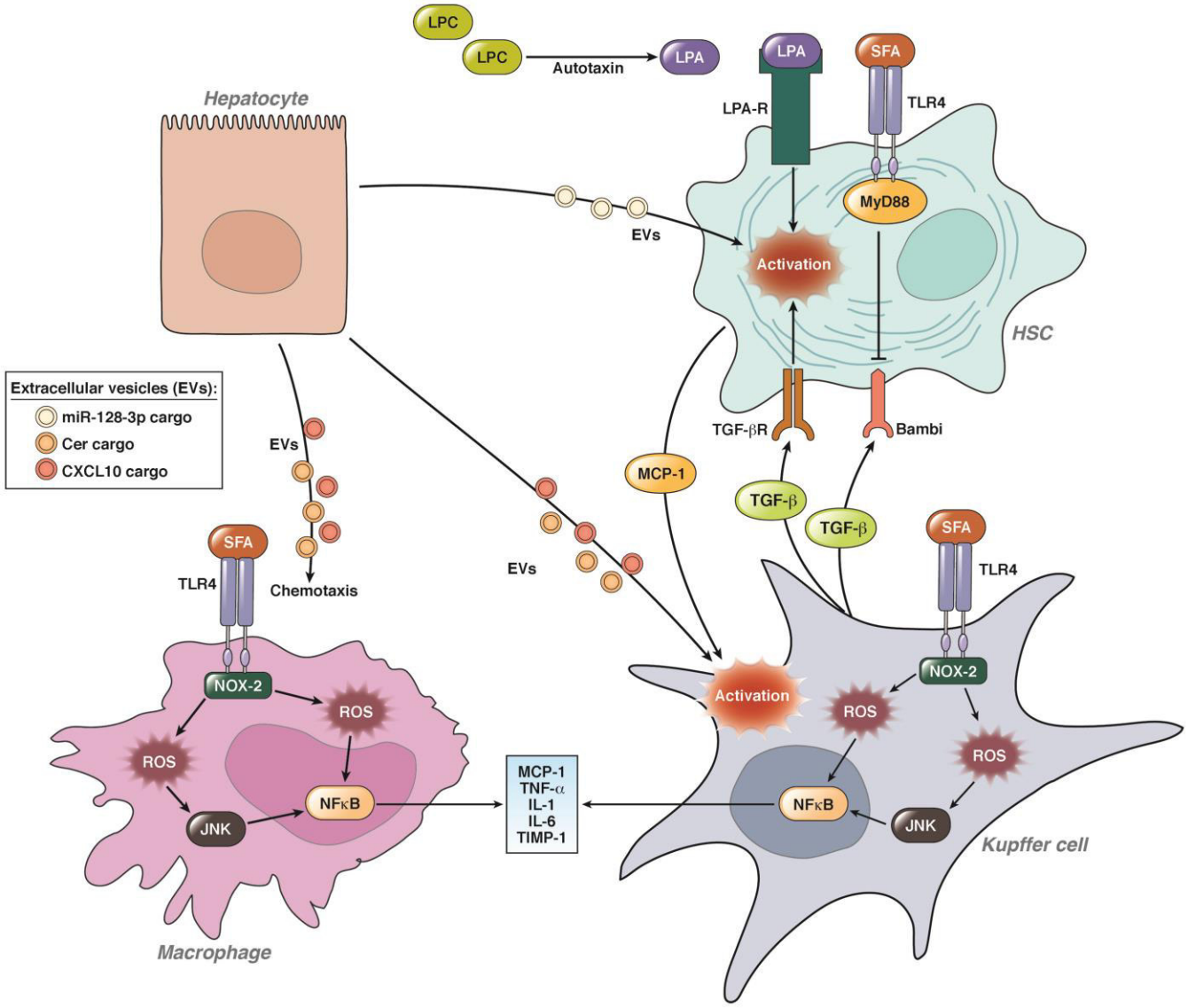
Online Supplementary Table 3. Main pathways and biological effect of Polyunsaturated Fatty Acids (PUFAs)

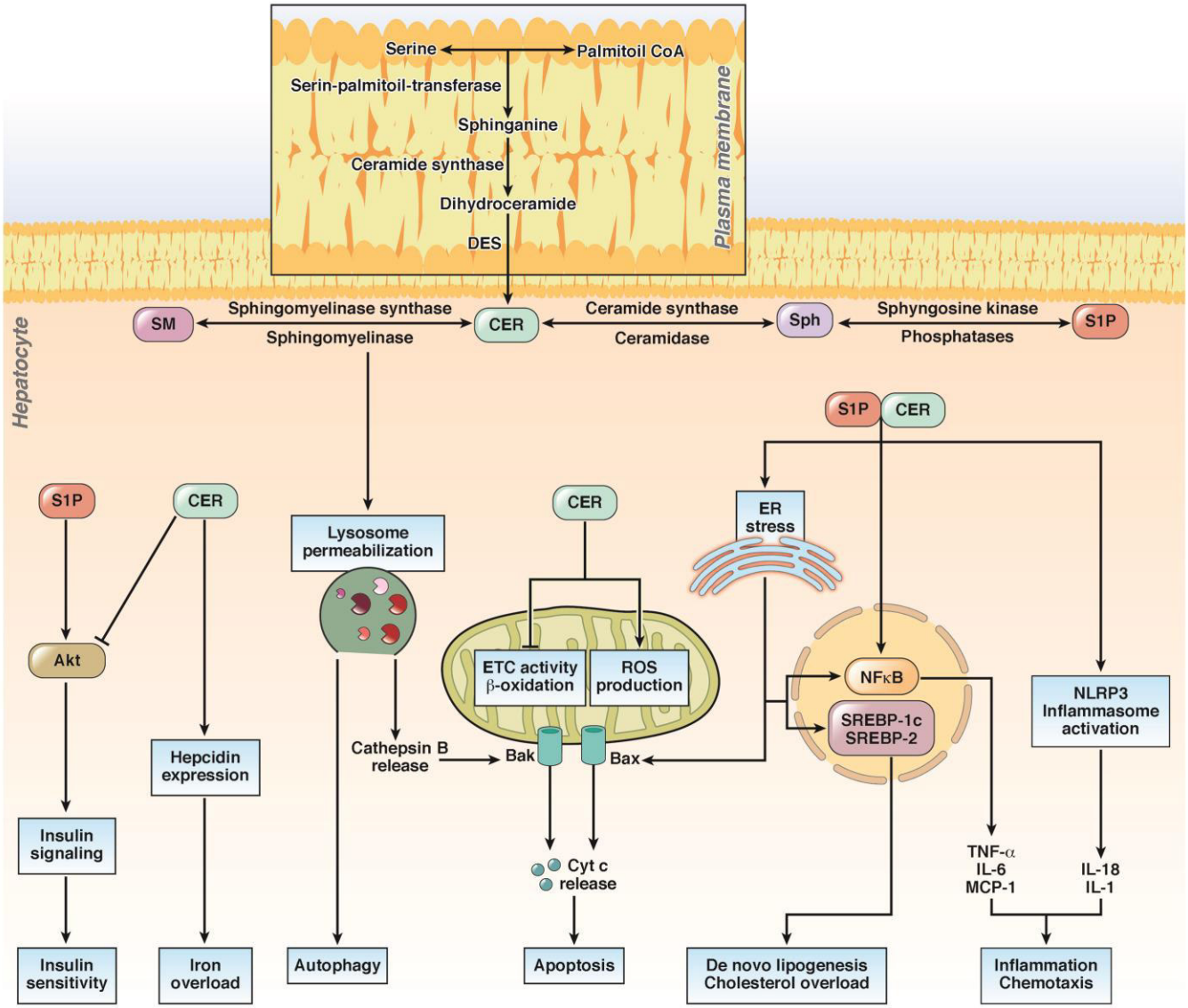
Hepatocyte			
Type of PUFA	**Transcription factor/cellular pathway**	**Cellular molecular mechanism**	**Biological effect**
EPA, DHA	↓ LXR/SREBP-1c activation	↓ *de novo* lipogenesis	↓ hepatic triglyceride
EPA, DHA	↓ ChREBP activation	↓ *de novo* lipogenesis ↓ glycolysis	↓ hepatic triglyceride
EPA, DHA	↓ SREBP- activation	↓ cholesterol synthesis	↓ hepatic cholesterol
EPA	↑PPAR-α activation	↑ fatty acid β-oxidation	↓ hepatic triglyceride
DHA	↓ NF-κB activation [46]	↓ apoptosis ↓ pro-inflammatory IL-1 secretion	↓ hepaticnecro-inflammation
DHA	↓ NLRP3 inflammasome activation[46]	↓ caspase-1 activation ↓ IL-1 secretion	↓ necro-inflammation
DHA, EPA	↑ FXR activation	↓ cholesterol and FA synthesis	↓ hepatic triglyceride and cholesterol

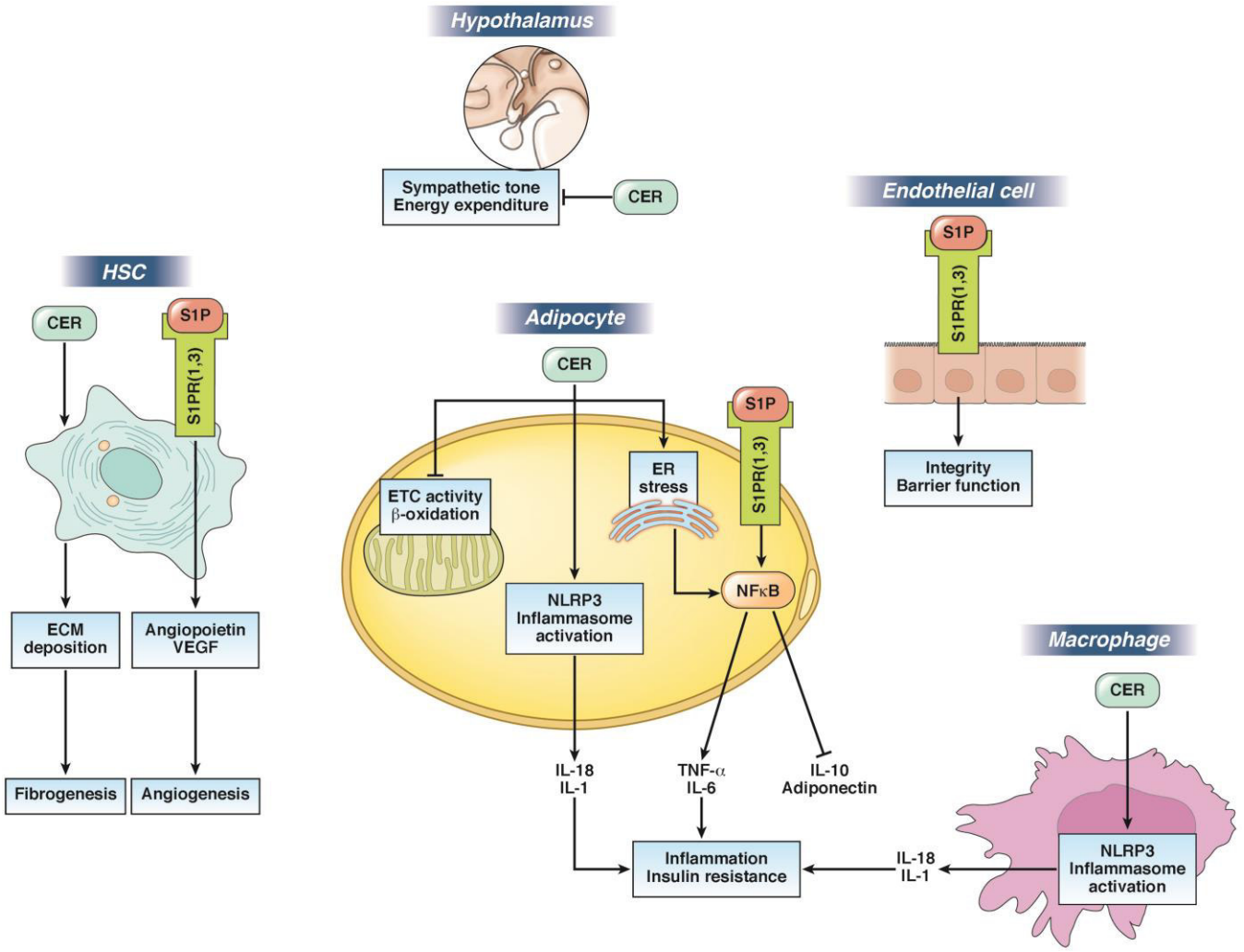
DHA, EPA	↓HNF-4α activation	↓ VLDL assembly and secretion	↓ plasma triglyceride
DHA	↓Tolllike receptor-2/4/9 [48]	↓proinflammatory cytokine secretion	↓necroinflammation
DHA	↓ NOX expression[48]	↓ superoxide/hydrogen peroxide production	↓ oxidative stress
Macrophage/Kupffer cells			
DHA	↑ PPAR-γ activation	Polarization to a M2, proresolving phenotype	Anti-inflammation Fibrosis resolution
Hepatic Stellate Cell (HSC)			
DHA	↓ TGFβ-Smad3-Col1A1 axis activation	↓ collagen deposition	↓ fibrosis

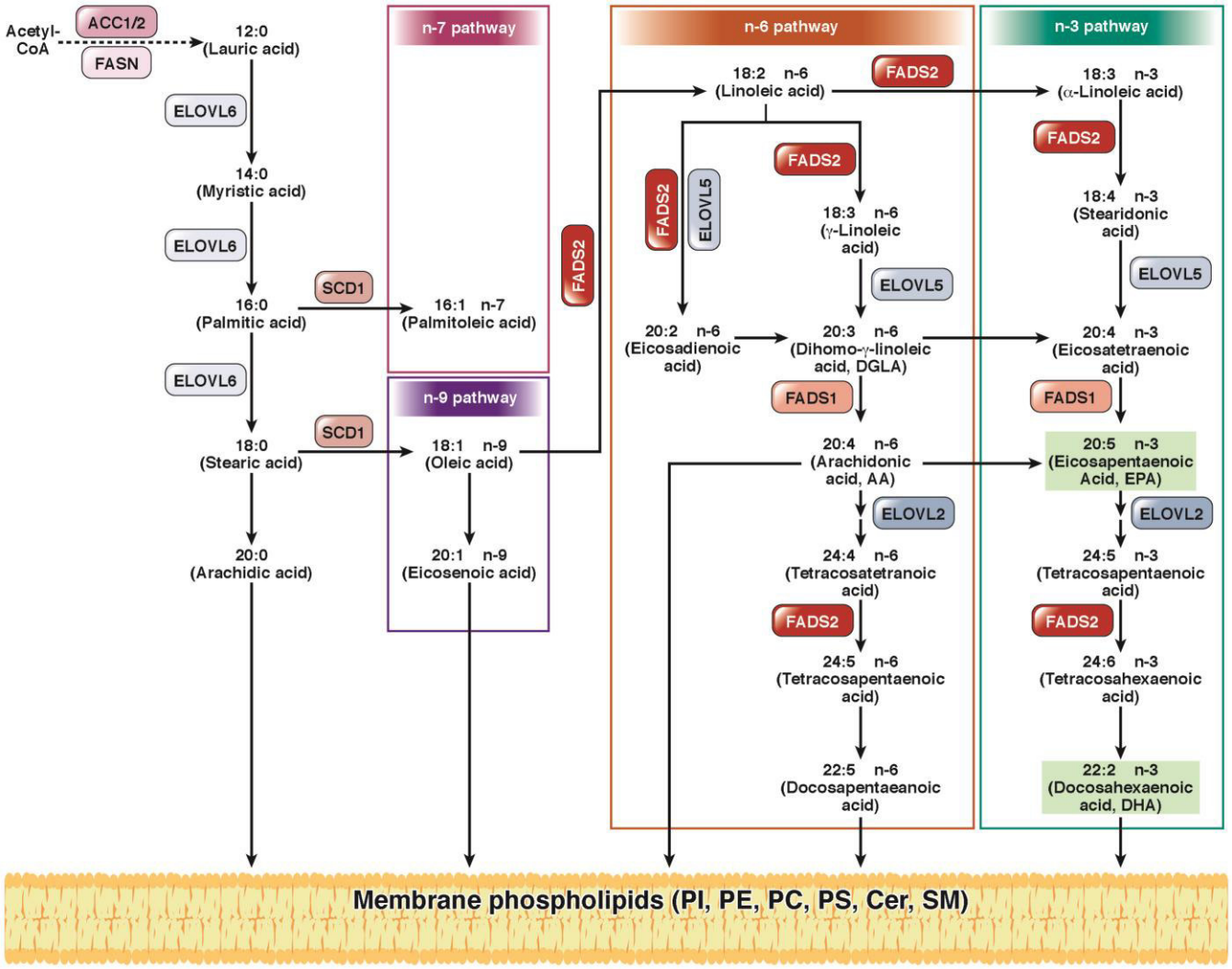
Abbreviations: SREBP-1c: sterol regulatory element-binding protein 1c; ChREBP: carbohydrate response element binding protein; Col1A1: collagen 1A1; TGFβ: transforming growth factor-β; mothers against decapentaplegic homolog (Smad)3; NF-κB: nuclear factor-kappa B; NOD-like receptor protein 3 (NLRP3) inflammasome, LXR: liver X receptor

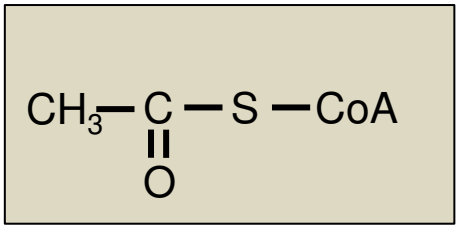

Hepatocyte
ROS production
ETC activity
Sab
LPC
SFA
SFA
LPC
Insulin resistance
EV release
ER stress
JNK
Insulin
IRS-1
Insulin receptor
CHOP
PUMA
SFA
FGF-21
PPAR-α
TLR4
SFA
Lysosome
β-oxidation
NF-κB
FOXO3A
Bak
Bax
Bim
Nucleus
Bax
Cyt c release
Cathepsin B release
SFA
IL-6
TNF-α
MCP-1
TRAIL-R2
SFA
Caspase-8
Caspase 3,6,7
Apoptosis
Inflammation Chemotaxis
Pro-IL-1
Pro-IL-18
IL-1
IL-18
Proinflamatory eicosanoid synthesis
Caspase-1
Inflammasome activation
ASC
SFA
NLRP3
Arachidonic acid
LPC
PLA2
PC
Membrane integrity disruption


LPC
LPC
Autotaxin
LPA
LPA
SFA
LPA-R
TLR4
Hepatocyte
MyD88
EVs
Activation
HSC
Extracellular vesicles (EVs):
miR-128-3p cargo
Cer cargo
CXCL10 cargo
TGF-βR
Bambi
EVs
MCP-1
TGF-β
TGF-β
SFA
TLR4
Chemotaxis
EVs
SFA
TLR4
NOX-2
Activation
NOX-2
ROS
ROS
ROS
ROS
NFκB
NFκB
JNK
JNK
MCP-1
TNF-α
IL-1
IL-6
TIMP-1
Kupffer cell
Macrophage

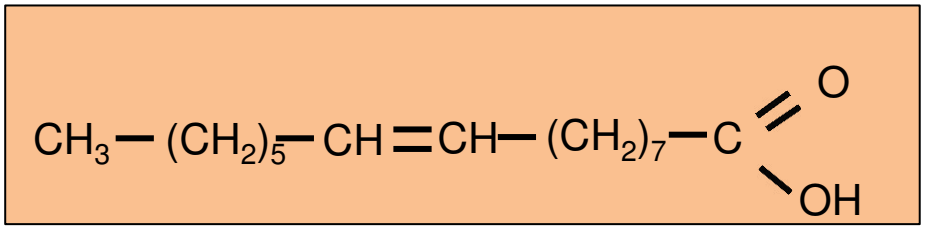

Serine
Palmitoil CoA
Serin-palmitoil-transferase
Sphinganine
Ceramide synthase
Dihydroceramide
DES
Plasma membrane
Hepatocyte
SM
Sphingomyelinase synthase
Sphingomyelinase
CER
Ceramide synthase
Ceramidase
Sph
Sphyngosine kinase
Phosphatases
S1P
S1P
CER
S1P
CER
Akt
Insulin signaling
Insulin sensitivity
Hepcidin expression
Iron overload
Lysosome permeabilization
Autophagy
CER
ETC activity β-oxidation
ROS production
Cathepsin B release
Bak
Bax
Cyt c release
Apoptosis
ER stress
NFκB
SREBP-1c
SREBP-2
De novo lipogenesis
Cholesterol overload
NLRP3 Inflammasome activation
TNF-α
IL-6
MCP-1
IL-18
IL-1
Inflammation
Chemotaxis


Hypothalamus
Sympathetic tone
Energy expenditure
CER
Endothelial cell
S1P
S1PR(1,3)
Integrity
Barrier function
HSC
CER
S1P
S1PR(1,3)
ECM
deposition
Angiopoietin
VEGF
Fibrogenesis
Angiogenesis
Adipocyte
CER
S1P
S1PR(1,3)
ETC activity
β-oxidation
ER
stress
NFκB
NLRP3
Inflammasome
activation
IL-18
IL-1
TNF-α
IL-6
IL-10
Adiponectin
Inflammation
Insulin resistance
Macrophage
CER
IL-18
IL-1
NLRP3
Inflammasome
activation

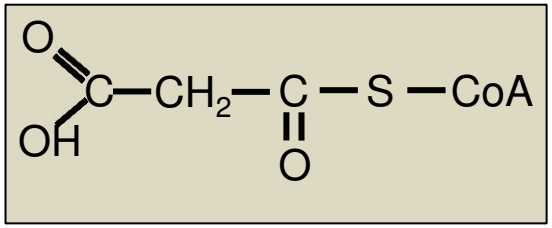

Acetyl-CoA
ACC1/2
FASN
12:0 (Lauric acid)
ELOVL6
14:0 (Myristic acid)
ELOVL6
16:0 (Palmitic acid)
SCD1
16:1 n-7 (Palmitoleic acid)
ELOVL6
18:0 (Stearic acid)
SCD1
18:1 n-9 (Oleic acid)
20:0 (Arachidic acid)
20:1 n-9 (Eicosenoic acid)
n-7 pathway
n-9 pathway
FADS2
n-6 pathway
18:2 n-6 (Linoleic acid)
FADS2
FADS2
ELOVL5
18:3 n-6 (γ-Linoleic acid)
FADS2
ELOVL5
20:2 n-6 (Eicosadienoic acid)
20:3 n-6 (Dihomo-γ-linoleic acid, DGLA)
FADS1
20:4 n-6 (Arachidonic acid, AA)
ELOVL2
24:4 n-6 (Tetracosatetranoic acid)
FADS2
24:5 n-6 (Tetracosapentaenoic acid)
22:5 n-6 (Docosapentaeanoic acid)
n-3 pathway
18:3 n-3 (α-Linoleic acid)
FADS2
18:4 n-3 (Stearidonic acid)
ELOVL5
20:4 n-3 (Eicosatetraenoic acid)
FADS1
20:5 n-3 (Eicosapentaenoic Acid, EPA)
ELOVL2
24:5 n-3 (Tetracosapentaenoic acid)
FADS2
24:6 n-3 (Tetracosahexaenoic acid)
22:2 n-3 (Docosahexaenoic acid, DHA)
Membrane phospholipids (PI, PE, PC, PS, Cer, SM)



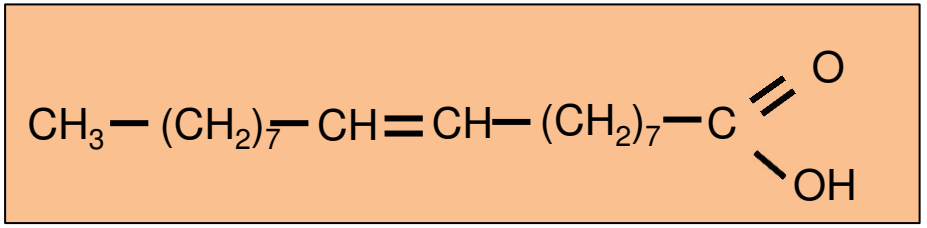
Acetil-CoA



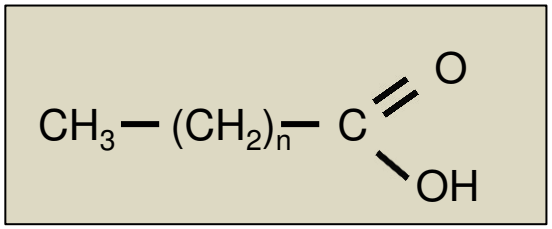
Palmitoleic acid



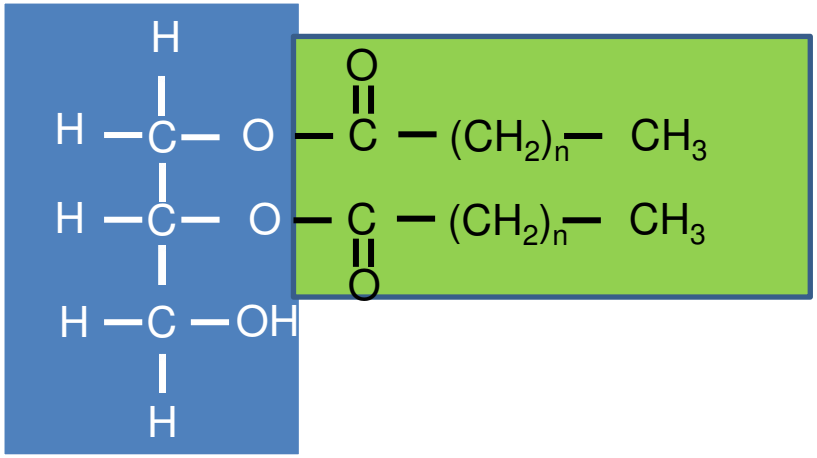
Malonil-CoA



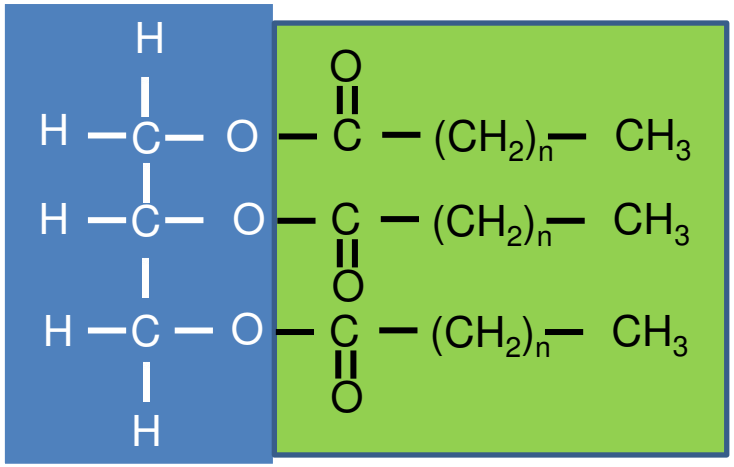
Oleic acid



Miristic Acid (n=12); 14:0
Palmitic Acid (n=14); 16:0
Stearic Acid (n=16); 18:0

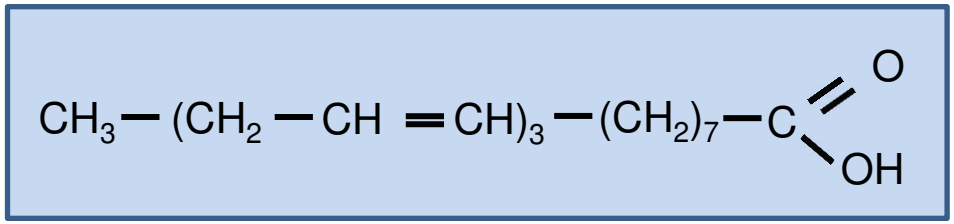


Diacylglycerol

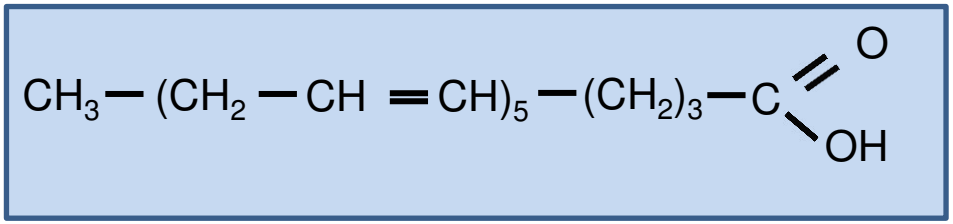


Triacylglycerol

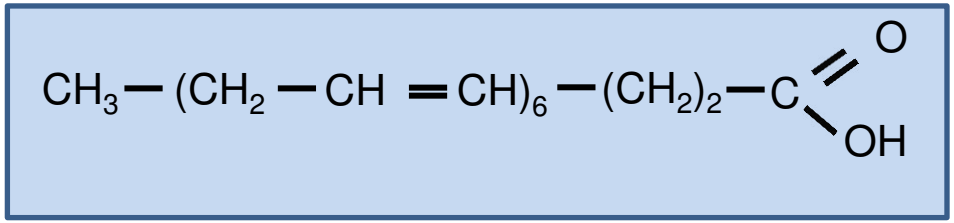
ω-3 fatty acids



α-Linolenic acid

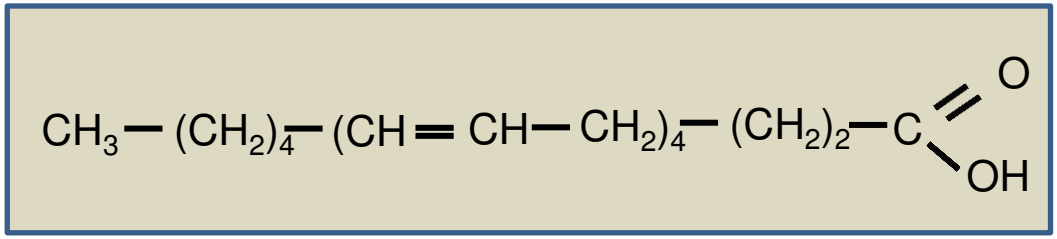


Eicosapentaenoic acid

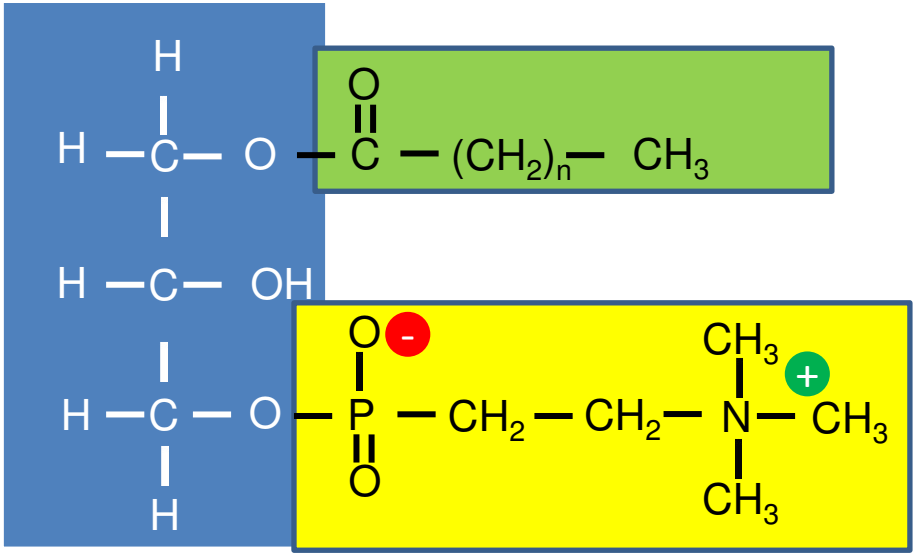


Docosahexaenoic acid

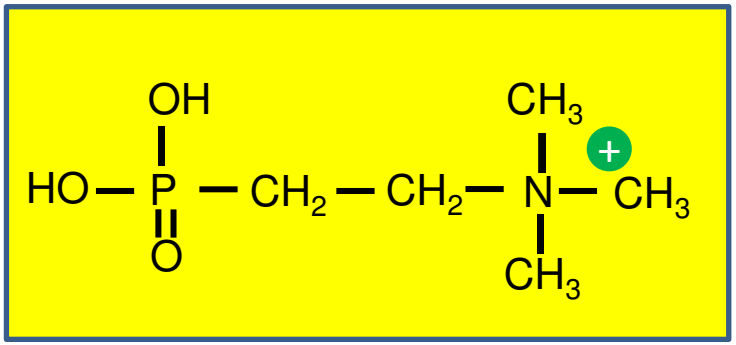
ω-6 fatty acids



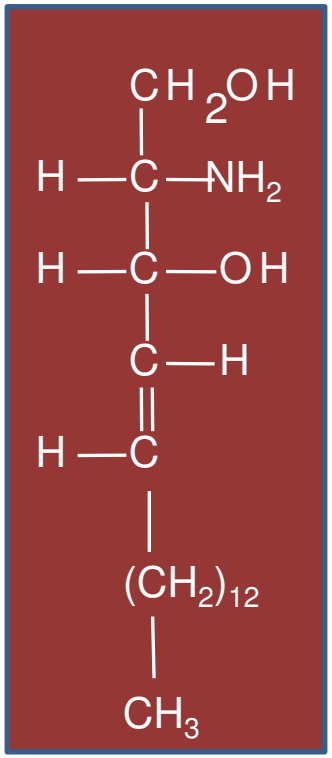
Arachidonic acid



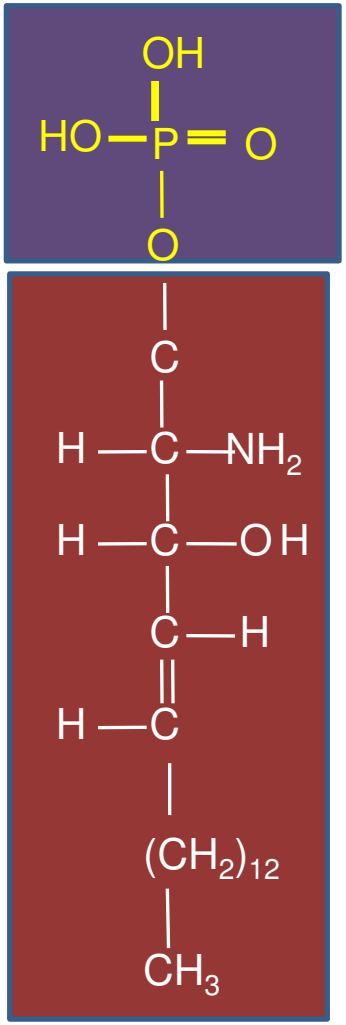
Lysophosphatidylcholine



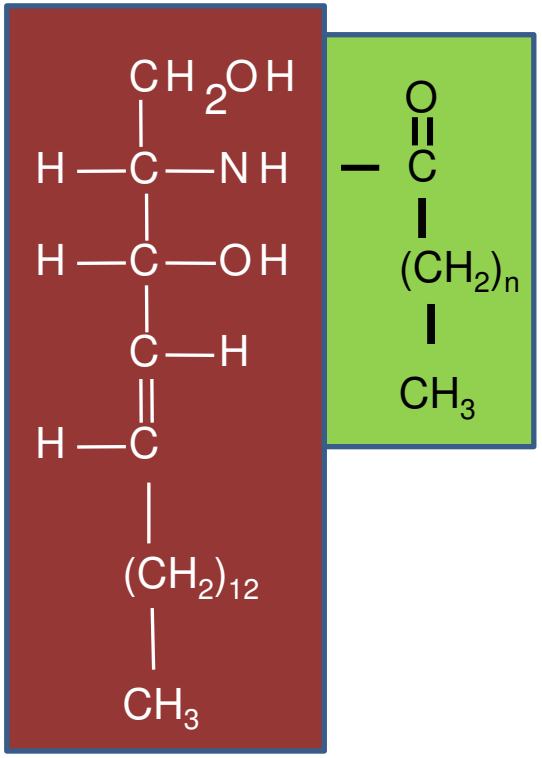
Phosphocholine



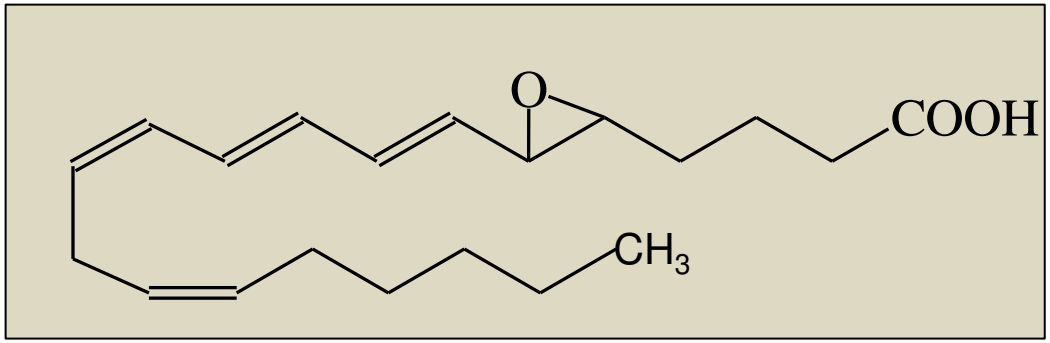
Sphingosine



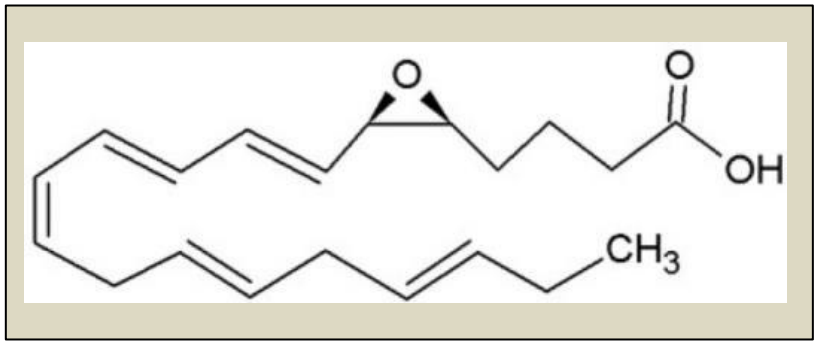
Sphingosine-1-phosphate



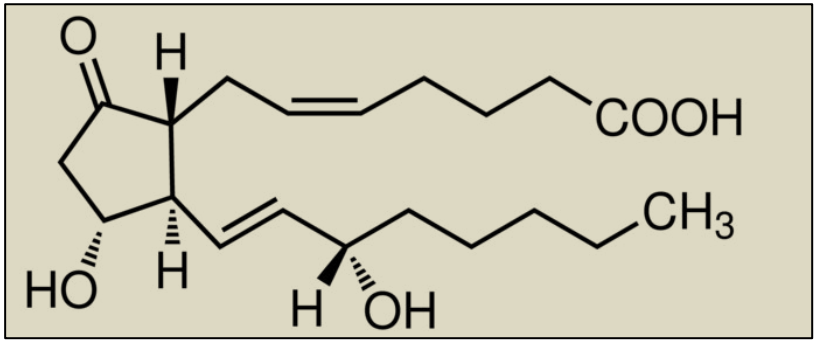
Ceramide



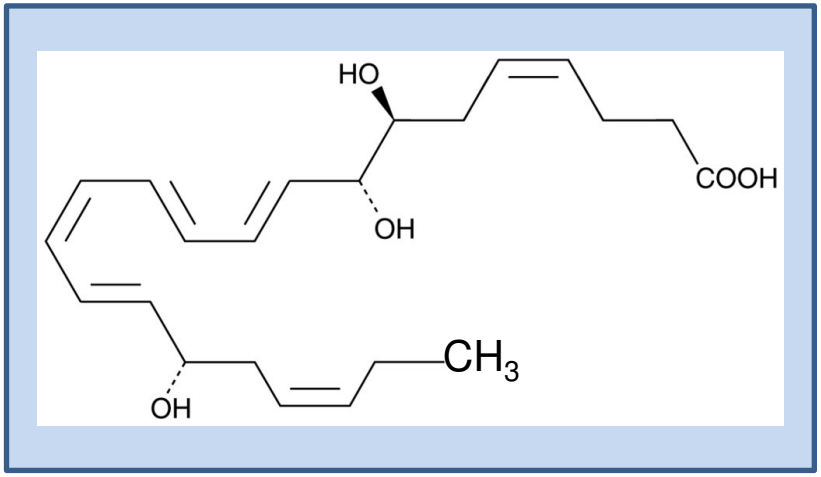
Leukotriene A



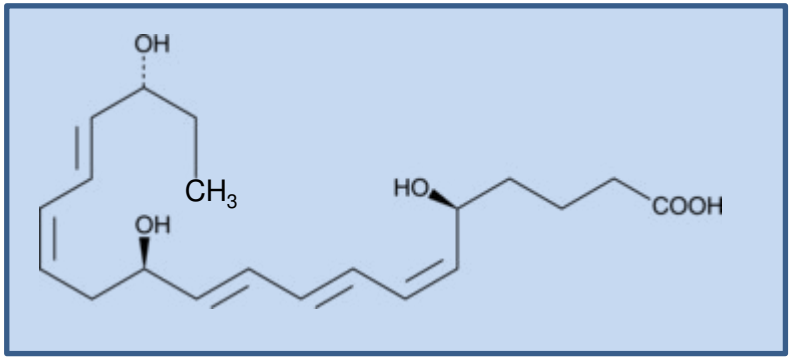
Leukotriene A5



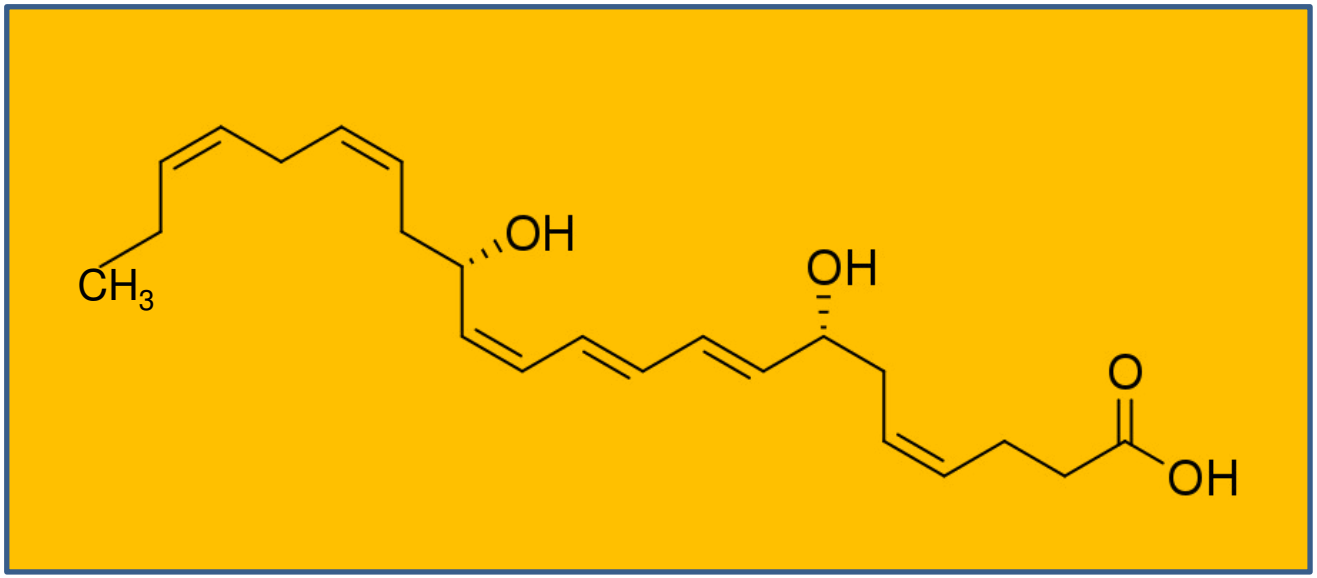
Prostaglandin E2



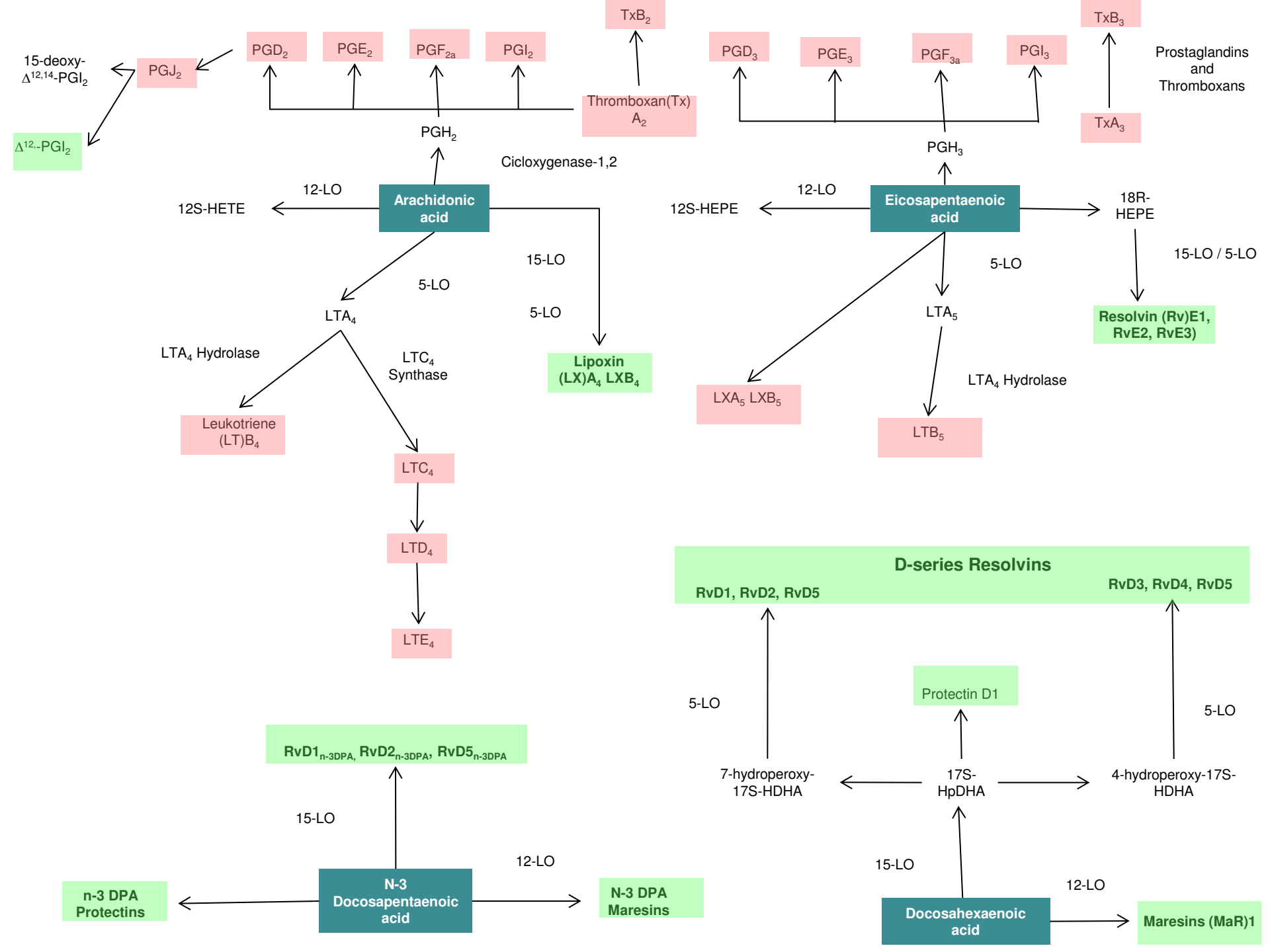
Resolvin D1



Resolvin E1



Maresin 1


15-deoxy-Δ12,14-PGI2
PGJ2
Δ12,-PGI2
PGD2
PGE2
PGF2a
PGI2
TxB2
Thromboxan(Tx) A2
PGH2
Cicloxygenase-1,2
12-LO
12S-HETE
Arachidonic acid
15-LO
5-LO
5-LO
LTA4
LTA4 Hydrolase
LTC4 Synthase
Leukotriene (LT)B4
LTC4
LTD4
LTE4
Lipoxin (LX)A4 LXB4
PGD3
PGE3
PGF3a
PGI3
TxB3
TxA3
Prostaglandins and Thromboxans
PGH3
12-LO
12S-HEPE
Eicosapentaenoic acid
18R-HEPE
15-LO / 5-LO
5-LO
LTA5
Resolvin (Rv)E1, RvE2, RvE3)
LXA5 LXB5
LTA4 Hydrolase
LTB5
D-series Resolvins
RvD1, RvD2, RvD5
RvD3, RvD4, RvD5
5-LO
Protectin D1
5-LO
7-hydroperoxy-17S-HDHA
17S-HpDHA
4-hydroperoxy-17S-HDHA
RvD1n-3DPA, RvD2n-3DPA, RvD5n-3DPA
15-LO
12-LO
n-3 DPA Protectins
N-3 Docosapentaenoic acid
N-3 DPA Maresins
15-LO
12-LO
Docosahexaenoic acid
Maresins (MaR)1