Lipidomics in pathogenesis, progression and treatment of nonalcoholic steatohepatitis (NASH): Recent advances

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Lipidomics in pathogenesis, progression and treatment of nonalcoholic steatohepatitis (NASH): recent advances

Running title: lipidomics in NASH

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease affecting up to 30% of the general adult population. NAFLD encompasses a histological spectrum ranging from pure steatosis to non-alcoholic steatohepatitis (NASH). NASH can progress to cirrhosis and is becoming the most common indication for liver transplantation, as a result of increasing disease prevalence and of the absence of approved treatments. Lipidomic readouts of liver blood and urine samples from experimental models and from NASH patients disclosed an abnormal lipid composition and metabolism. Collectively, these changes impair organelle function and promote cell damage, necro-inflammation and fibrosis, a condition termed lipotoxicity. We will discuss the lipid species and metabolic pathways leading to NASH development and progression to cirrhosis, as well as and those species that can contribute to inflammation resolution and fibrosis regression. We will also focus on emerging lipid-based therapeutic opportunities, including specialized proresolving lipid molecules and macrovesicles contributing to cell-to-cell communication and NASH pathophysiology.

Abbreviations: ACC: Acetyl-CoA carboxylase; APCI, atmospheric pressure chemical ionization; ACLY: Adenosine triphosphate (ATP) citrate lyase; AMPK: AMP-activated kinase; APPI, atmospheric pressure photoionization; COX: cyclooxygenase; CPT: carnitine palmitoyl-transferase; CXCL10: chemokine (C-X-C motif) ligand 10; CYP: cytochrome P450; DAMP: damage-associated molecular pattern; DGAT: Diacylglycerol acyltransferase;

EDPs: epoxydocosapentaenoic acids; EEQs: epoxyeicosatetraenoic acids; EETs: epoxyeicosatrienoic acids; ESI, electrospray ionization; EV: extracellular vesicle; FABP: fatty acid-binding protein; FAT: fatty acid translocase; GPCRs: G-protein coupled receptors; 25HC3S: 25-Hydroxycholesterol-3-sulfate; HETE: hydroxyeicosatetraenoic acid; HPLC: high pressure liquid chromatography; IRS: Insulin Receptor Substrate; IS, internal standard; JNK: c-Jun N-terminal kinase; LC, liquid chromatography; LOX: lipoxygenase; LPC: lysophosphatidylcholine; LXR: Liver X Receptor; MALDI, matrix-assisted laser desorption/ionization; MLK3: mixed lineage kinase 3; MOMP: mitochondrial outer membrane permeabilization; MRI-PDFF: magnetic resonance imaging-proton density fat fraction; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NEFA: non-esterified fatty acid; PAR: Poly ADP-ribose; PARG: poly-ADP-ribose glyco-hydrolase; PARP: Poly (ADP-ribose) polymerases; PGC1α:peroxisome proliferator activated receptor cofactor-1α ; PKC: protein kinase C; PPAR: Peroxisome Proliferator-Activated Receptor; PUMA: p53-upregulated modulator of apoptosis; QTOF MS: quadrupole time-of-flight mass spectrometry; ROCK1: Rho-associated protein kinase 1; SCD1: stearoyl CoA desaturase-1; she: soluble Eypoxide Hydrolase; SFA: saturated fatty acid; SH3BP5: SH3 domain-binding protein 5; SIMS, secondary ion mass spectrometry; SPM: specialized proresolving mediators; SREBP: sterol regulated element binding protein; STARD11: StAR-related Lipid Transfer Domain 11; STAT: signal transducer and activator of transcription; TIC, total ion chromatogram; TAG: triacylglycerol; TIMP: Tissue Inhibitor of Metalloproteinase; TLR: Toll-Like Receptor; TRAIL: TNF-related apoptosis-inducing ligand;
UPLC: ultrahigh-pressure liquid chromatography; VEGF-A: vascular endothelial growth factor A; XIC: extracted ion chromatogram.

1. Introduction: lipidomics contribution to NASH

The term "lipidome" describes the full lipid profile in a cell, tissue, organ, or biological system and is a subcategory of "metabolome" which encompasses the other three main classes of biological molecules (proteins and/or amino-acids, carbohydrates, nucleic acids) (**Figure 1 panel A**).

Lipidomics is the large-scale study of lipid molecule networks and pathways in biological systems at a cellular, tissue and biological system level; it identifies and quantifies the diverse lipid species and their related metabolic pathways and networks in biological samples using analytical chemistry techniques, including mass spectrometry (MS) and chromatography [1].

The discipline of lipidomics was first defined in 2003 [2] and has largely expanded in recent years, due to the advances in MS and in computational methods to analyze the thousands of raw data, together with the acknowledgement of the importance of lipids in many metabolic disorders, including obesity, diabetes and nonalcoholic fatty liver disease (NAFLD).

A typical workflow of lipidomic analysis of biological samples is reported in **Figure 1 panel B**: the steps include sample preparation, MS-based analysis and data acquisition, and data processing. For a detailed description of analytical MS-based techniques readers are referred to existing reviews [1].

Two main techniques are used in MS-based lipidomic analysis, i.e., targeted and untargeted (ie, systems-level) lipidomics.

Targeted lipidomics requires a pre-separation using ultrahigh-pressure liquid the chromatography/high pressure liquid chromatography (UPLC/HPLC) method for one specific, predefined lipid class. A shortcoming of targeted lipidomics is that it usually focuses on one or a few lipid species, while the lipidome encompasses over 180000 different lipids. As most lipid changes occurring over the course of a disease cannot be predicted, this approach may miss changes in many lipid classes associated with pathophysiological conditions. Hence, a discovery-based/systems level analysis would be preferable to identify lipids that are potentially relevant for a pathophysiological condition but that that are not targeted by a specific lipidomic platform. To this aim, untargeted or systems-level lipidomics has been developed for discovery-based research. Untargeted lipidomics, which often employs quadrupole time-of-flight mass spectrometry (QTOF MS), offers a broader mass

range and could theoretically evaluate the whole lipidome range. Nevertheless, this technique has its own limitations, as well: it possesses lower sensitivity than targeted lipidomics and can therefore detect only lipids which are present at considerably high levels. Furthermore, untargeted lipidomics has a lower accuracy of quantitation than targeted lipidomics.

For these reasons, a combination of untargeted with targeted lipidomics has been employed to cross-validate findings from each technique in recent clinical or translational research. The addition of multiple targeted lipidomic techniques allows to detect additional data on lipids that are present in low abundance and that could not be assessed by untargeted lipidomics.

NAFLD is a chronic liver disease that affects up to 30% of the general adult population, and up to 80% of obese and diabetic patients all over the world. NAFLD comprises a histological spectrum that ranges from pure steatosis to non-alcoholic steatohepatitis (NASH) with variable degrees of fibrosis.

NAFLD is an emerging risk factor for type 2 diabetes and cardiovascular disease, and NASH is a major risk factor for liver-related complications [3], as the latter can progress to cirrhosis and hepatocellular carcinoma. Consistently, NASH is becoming a major indication for liver transplantation, as a result of the increasing prevalence of this condition and of the lack of an effective treatment [4,5].

Lipotoxicity, defined as an abnormal accumulation of toxic lipids in the cell, resulting in organelle dysfunction, cell injury, chronic inflammatory changes and fibrosis, is a distinctive feature of NASH [4,6],

Over the last decade, the progress in lipidomics, most notably chromatography and spectrometry (MS), allowed the identification and quantitation of the diverse lipid signatures characterizing biological samples [2]: the analysis of liver, blood and urine samples from patients with NAFLD patients across the entire spectrum of liver disease severity yielded distinct lipid signatures which can be detected at different liver disease stages, including an overall enrichment in saturated fatty acids (SFAs), diacylglycerols (DAG), ceramide, free cholesterol and lipoxygenase

(LOX) metabolites and a reduction in n3-polyunsaturated fatty acids (PUFAs) and phospholipids [7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19] (**Table 1**).

The distinct lipid signatures observed in simple steatosis, in NASH and in advanced fibrosis stages unveiled novel metabolic pathways contributing to liver disease progression or resolution and paved the way to novel therapeutic strategies that are currently at preclinical or early clinical stage of development; furthermore, the ability of these lipid signatures to differentiate pure steatosis from NASH, with or without advanced fibrosis, could also be exploited to derive non-invasive diagnostic biomarker panels to identify different NAFLD stages and thus limit the need to perform invasive liver biopsy, currently the gold standard technique to diagnose and stage NASH.

A clearcut concept that emerged from lipidomic analyses is that neutral lipids like triacylglycerols (TAG) and cholesteryl esters (CE), which represent the vast majority of hepatic lipids in NAFLD, are not lipotoxic but represent a form of storage into inert lipids of lipotoxic lipids including SFAs and free cholesterol (FC), which are made harmless through esterification [20].

Hence, lipotoxicity in NASH is rather determined by the balance between the accumulation of toxic lipid intermediates in TG synthesis and phospholipids, and the shortage in protective lipid species, including n3-PUFAs and n3-PUFA-derived Specialized Proresolving Mediators (SPMs) [4-11].

We will review recent insights into lipids involved in NASH progression and resolution and discuss potential therapeutic strategies, including non-esterified fatty acids (NEFAs), phospholipids, sphingolipids, PUFA-derived eicosanoids and SPMs [21]. Furthermore, the role of extracellular vesicles (EVs) in cell-to-cell communication within the liver and among distant organs will be reviewed with special reference to NASH pathogenesis, staging and therapeutics.

2. Lipotoxic fatty acids in the pathogenesis of liver injury in NASH

In NASH the liver is exposed to an increased amount of circulating lipotoxic NEFAs [22], due to two factors: first, an unrestrained lipolysis of NEFAs from adipose tissue TAGs due to adipose tissue insulin resistance [21]; second, an increased hepatic uptake of circulating NEFAs, due to an

upregulation of the tetrameric NEFA protein carrier, constituted by plasma membrane fatty acid-binding protein (FABP), caveolin-1, fatty acid translocase (FAT/CD36) and calcium independent membrane phospholipase A2β (iPLA2β) [23]. In NASH liver, over 60% of hepatic NEFAs derive from adipose tissue, whereas the rest comes from *de novo* lipogenesis (25%) and from the diet (15%) [22]. Not only the amount, but also the type of NEFA is altered in NAFLD, with a predominant accumulation of SFAs, including palmitic and stearic acid, relative to MUFAs and PUFAs. Experimental cellular and animal models indicated SFAs are more lipotoxic than MUFAs and PUFAs and, consistently, the extent of SFA accumulation parallels the severity of liver disease in NAFLD patients [8,9,10].

2.1 Mechanisms of lipotoxicity of saturated fatty acids in NAFLD

Saturated fatty acids (SFAs) exert their effects on the cell through two main mechanisms: they are ligands for plasma membrane receptors or enter the cell and activate intracellular pathways of lipotoxicity.

Plasma membrane receptors mediating SFA lipotoxicity belong to two main functional classes, i.e., death receptor TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 (TRAIL-R2) and Toll-Like Receptor (TLR)-4, the latter belonging to damage-associated molecular pattern (DAMP) receptors family [4,24, 25].

TRAIL-2 signaling pathway activates caspase 8, resulting in direct or Bcl-2-mediated mitochondrial outer membrane permeabilization (MOMP)-mediated activation of caspases 3, 6 and 7, all converging to trigger cellular apoptosis [2,23] (**Figure 2A**).

TLR-4 pathway engages the nuclear transcription factor Nuclear Factor (NF)-κB, which activates pro-inflammatory cytokine secretion [2] and stress kinases, including c-Jun N-terminal kinase (JNK), a mitogen-activated protein kinase (MAPK). Remarkably, SFAs can also activate JNK directly after they have entered the cell, through endoplasmic reticulum (ER) stress initiation or through stimulation of ceramide synthesis (**Figure 2A**).

JNK leads to insulin resistance by inactivating Insulin Receptor Substrate (IRS)-1, impairs mitochondrial respiration and enhances mitochondrial reactive oxygen species (ROS) generation through interaction with the outer membrane mitochondrial protein Sab (SH3 domain-binding protein 5, SH3BP5) [26], and activates the proapoptotic protein p53-upregulated modulator of apoptosis (PUMA) [2,23].

Additional important mechanisms whereby JNK mediates SFA lipotoxicity in hepatocytes involve downregulation of mitochondrial and peroxisomal FA ß-oxidation, which is achieved at a nuclear level through suppression of Peroxisome Proliferator-Activated Receptor (PPAR)-α-stimulated Fibroblast Growth Factor (FGF)-21 expression [2,23,27] (**Figure 2A**).

Notably, SFA exert their lipotoxicity through interaction with hepatic non-parenchymal cells, as well: upon binding membrane receptor TLR-4 of hepatic stellate cells (HSC), SFAs enhance their secretion of monocyte chemoattractant protein (MCP)-1, a potent chemokine for circulating monocytes; furthermore, SFAs trigger proinflammatory macrophages and Kupffer cells M1 polarization, chemotaxis, Transforming Growth Factor (TGF)-ß and Tissue Inhibitor of Metalloproteinase (TIMP)-1 secretion by these cells via JNK activation [25,28] (**Figure 2B**).

2.2 Role of monounsaturated fatty acids in liver injury

Palmitoleic acid (C16:1) and oleic acid (C18:1) are the most abundant and most extensively studied monounsaturated fatty acids (MUFAs) in NASH. These two MUFAs are generated through the action of the enzyme Stearoyl-CoA desaturase-1 (SCD1) on palmitic and stearic acid, respectively [21].

Although these MUFAs contribute to liver fat accumulation in NAFLD, several lines of evidence indicate they are less lipotoxic than SFAs [29]: the individual proapoptotic potency of these two MUFAs resulted much lower than that of SFAs, and when coincubated with SFAs and isolated human and mouse primary hepatocytes, they attenuated palmitate-induced apoptosis [30]. MUFAs show also a lower ability to activate ER stress and PUMA-mediated apoptosis and are more efficiently incorporated into TAG: accordingly, the coincubation of MUFA with palmitate or SCD1 upregulation mitigate palmitate-induced apoptosis but enhance TAG accumulation in primary

hepatocytes while, on a background of impaired TAG synthesis, oleate accumulation induced significant lipotoxicity [31]. This phenomenon has relevant therapeutic implications as SCD-1 inhibitors are currently being evaluated in NAFLD. SCD-1 plays a central role in modulating FA oxidation, *de novo* lipogenesis and TG synthesis through MUFA generation [32]. An orally available SCD-1 inhibitor compound alleviated hepatic steatosis and necroinflammation in mice fed non-fat, high-sucrose or. methionine and choline-deficient (MCD) diets [33], and aramchol, a conjugate of arachidic acid and cholic acid that inhibits SCD-1 and *de novo* lipogenesis, reduced liver fat by 12.5% in NAFLD patients [34]. However, transaminases failed to normalize with aramchol and raised the concern that hepatic fat reduction was not accompanied by a reduced liver injury. Furthermore, intestinal-specific SCD-1 deletion decreased hepatic MUFA proportion, increased hepatic TAG accumulation, exacerbated hepatic inflammation and fibrosis and enhanced the development of hepatocellular carcinoma (HCC) in diverse diet-induced rodent models of NASH, disclosing a protective role of gut-derived MUFAs against NASH and HCC [35].
Similar concerns arose after the results of the ARRIVE trial, where aramchol 600 mg daily failed to reduce magnetic resonance imaging (MRI)-proton density-assessed hepatic fat fraction (PDFF), whole body or muscle fat content or to improve magnetic resonance elastography (MRE)-assessed liver stiffness, an estimate of liver fibrosis, in patients with HIV-related NAFLD [36].
These findings corroborate the concerns of an increased accumulation of lipotoxic SFAs with SCD-1 inhibitors [37]. The effects of two different doses of aramchol (400 and 600 mg/day) on histological end-points are currently being evaluated in non-cirrhotic patients with NASH [**supplementary Table 1**].

3. Therapeutic strategies to antagonize SFA lipotoxicity

Beside SCD-1 inhibition, two main therapeutic strategies are being pursued to antagonize hepatic SFA lipotoxicity: the inhibition of enzymes regulating key steps in lipid biosynthesis and the modulation of nuclear transcription factors which are master regulators of SFA metabolism.

3.1 Inhibition of key enzymes involved in *de novo* lipogenesis

3.1.1 ATP-Citrate Lyase inhibition

Adenosine triphosphate (ATP) citrate lyase (ACLY) is a cytoplasmic enzyme catalyzing the conversion of citrate to acetyl-CoA, which is essential for fatty acid and cholesterol biosynthesis. Hence, ACLY is a key check point of cellular metabolism placed at a crosslink between carbohydrate oxidation, which produces energy and yields citrate as an intermediate, and energy storing via *de novo* lipogenesis and cholesterol synthesis [38,39]. ACLY is abundant in lipogenic tissues, but is also expressed in myeloid-derived cells where it plays an important function in immune regulation [40]. Besides providing the building blocks for lipid synthesis, ACLY modulates also the extramitochondrial acetyl-CoA pool utilized as an acetyl-donor for the acetylation of multiple transcription factors, enzymes and histones involved in metabolism and inflammation. Therefore, ACLY exerts also post-translational epigenetic regulation of genes involved in key steps of metabolism and inflammatory response. As an example, in monocytes ACLY activation increased proinflammatory cytokine TNF-α and IL-8 production gene through increased histone acetylation of their gene promoters, and ACLY inhibition prevented monocyte proinflammatory cytokine production following lipopolysaccharide (LPS) stimulation [41,42].
In the NASH liver, the number of acetylated proteins is significantly increased and involves key regulators of cell metabolism and apoptosis, including sterol regulated element binding protein (SREBP), peroxisomal proliferator activated receptor gamma coactivator-1 (PGC-1), fork head box protein O1 (FoxO1), NF-κB and p53, and their functional modification is a key mediator of lipotoxicity [43]. In rodents and in obese patients with NAFLD, hepatic ACLY is upregulated and parallels liver disease severity [44,45,46], providing the rationale for testing ACLY inhibitors in these patients. Liver-specific ACLY inhibition with siRNA or with the long-chain diacid. Bempedoic Acid (ETC-1002, 8-Hydroxy-2,2,14,14-tetramethylpentadecanedioic acid), whose cholesterol-lowering efficacy and safety were recently confirmed in two Phase III clinical trials [47,48], improved steatosis, necroinflammation, dyslipidemia and glucose homeostasis in leptin-receptor-deficient and HFD-induced rodent models of NASH [42,43]. The hepatic histological

improvement was accompanied by a reduction in FA and cholesterol biosynthesis, by an upregulation of AMPK and PGC-1α-mediated mitochondrial biogenesis and by hepatic macrophage polarization toward a pro-resolving M2 phenotype[40,41].

3.1.2 Acetyl-CoA carboxylase inhibition

Acetyl-CoA carboxylase (ACC) catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA, the initial, rate-limiting step in *de novo* lipogenesis [21]. Two ACC isozymes exist, which are encoded by separate genes and show different intracellular distribution: ACC1 is a cytosolic enzyme which is abundant in lipogenic tissues with high lipogenic activity, including the liver and adipose tissue; ACC2 is a mitochondrial enzyme which is abundant in tissues with high substrate oxidative rates (liver, skeletal and cardiac muscle) [21].

Hepatocytes utilize malonyl-CoA formed in the cytosol by ACC1 primarily for *de novo* lipogenesis, while ACC2-derived mitochondrial malonyl-CoA acts predominantly as an allosteric inhibitor of mitochondrial carnitine palmitoyl-transferase (CPT)-1, reducing mitochondrial FA uptake and β-oxidation [21]. Thus, ACC inhibition can correct two crucial steps of lipid metabolism that are dysregulated in NASH, i.e., increased *de novo* lipogenesis and impaired ß-oxidation [21]: in transgenic mouse models with ACC gene gain- and loss-of function, constitutive ACC activation induced hepatic insulin resistance, enhanced lipogenesis, and promoted NASH and fibrosis development, which were prevented or reversed by genetic or pharmacological ACC inactivation [49,50,51]. On the basis of these preclinical data, a liver-specific oral allosteric ACC1/2 inhibitor, GS-0976 (Firsocostat), downregulated *de novo* lipogenesis and ameliorated radiological features of steatosis and serum fibrosis markers in noncirrhotic NASH patients [52].

3.1.3 Fatty Acid Synthase inhibition

Mammalian, Fatty Acid Synthase (FASN) (also referred to as type I FASN) is a dimer of two 270 kDa subunits, arranged head-to-tail, a spatial arrangement that enables fatty acid assembly to occur at the interface [53]. Each subunit of FASN is comprised of an acyl carrier protein (ACP) domain and six different catalytic domains [49]. The structural and functional organization of these domains

facilitates the individual reactions to be carried out in the lengthy iterative process starting from acetyl-CoA and malonyl-CoA to produce palmitate (**Figure 3**).

Tissue microarray technology and immunohistochemistry analyses of human NAFLD livers demonstrated a significant correlation of FASN expression with the degree of hepatic steatosis; furthermore, tissue expression of SREBP1, the main transcriptional regulator of FASN, paralleled FASN expression levels in human and experimental NAFLD, suggesting that FASN inhibition may represent a therapeutic strategy in NAFLD by reducing *de novo* lipogenesis, a key step in hepatic lipotoxicity [52,53].

TVB-2640 (denifanstat) is an oral, first-in-class, small-molecule reversible FASN inhibitor that completed phase 1 for treatment of solid tumors [54]. TVB-2640 had a reported FASN IC50 value of 50 nM and showed predictable exposure in phase 1, with a 16-hour half-life [55]. The dose-limiting toxicities were believed to be on-target activities.

The effect of pharmacological FASN inhibition (FASNi) with TVB-2640 was evaluated in human cell cultures and in three diet induced mouse NASH models: in human primary liver microtissues, FASNi decreased TAG content, consistent with direct anti-steatotic activity. In human hepatic stellate cells, FASNi reduced markers of fibrosis including collagen1α (COL1α1) and α-smooth muscle actin (αSMA). In CD4+ T cells exposed to NASH-related cytokines, FASNi decreased production of Th17 cells, and reduced IL-1β release in LPS-stimulated PBMCs [50,51].

FASNi prevented development of hepatic steatosis and fibrosis diet induced NASH models, and was also able to reduce NAFLD activity score, fibrosis score, ALT and TAG levelsi n mice with established NASH [50,51]. Importantly, FASNi was also able to reduce development of hepatocellular carcinoma (HCC) by 85% [56].

These results demonstrate that FASNi attenuates inflammatory and fibrotic drivers of NASH by direct inhibition of immune cells and HSCs, beyond decreasing fat accumulation in hepatocytes. FASN inhibition therefore provides an opportunity to target three key hallmarks of NASH.

Following phase I confirmation of up to 90% inhibition of fasting *de novo* lipogenesis in humans with metabolic abnormalities [57], the effect of TVB-2640 in NASH is currently being evaluated in the

phase 2 FASCINATE-1 trial [ClinicalTrials.gov: NCT03938246] [58]: after 12 weeks of therapy, TVB-2640 significantly reduced liver fat and dose-dependently improved biochemical, inflammatory, and fibrotic biomarkers of NASH as compared with placebo.

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

Diacylglycerol acyltransferase (DGAT) catalyzes the diacylglycerol (DAG) esterification by long-chain acyl-CoA esters, which represents the last step in TAG biosynthesis.

There are two distinct DGAT isoforms. DGAT-1 is predominantly expressed in enterocytes, where it reassembles the TG from dietary FAs in the lipolysis-reesterification process, which is required for intestinal fat absorption and chylomicron secretion [21]. DGAT-2 is expressed predominantly in hepatocytes, adipocytes and skin keratinocytes, where it assembles TAG from *de novo* synthesized NEFAs and DAGs [59].

Gain-of-function and loss-of-function transgenic rodent models shed light into the biological role of this enzyme. *DGAT-1*knockout (*DGAT1*$^{-/-}$) mice show slight reduction in tissue TAG but are viable, while *DGAT2*$^{-/-}$ mice have severe lipopenia (∼90 % reduction in whole-body TAG), defective skin barrier function and die soon after birth [60]. Based on these early data from transgenic mouse models and on the observation that intestinal dietary SFA absorption is increased in patients with NASH [61], selective DGAT-1inhibitors were developed for the treatment of metabolic disorders, including NASH: pradigastat, a potent, selective, DGAT-1 inhibitor, administered for 24 weeks, improved liver fat in NAFLD patients, but its use was affected by a high frequency of unwanted effects: over 80% of patients treated with this drug showed diarrhea and steatorrhea due to fat malabsorption [62].

More recent experimental evidence, however, suggests that DGAT-2 inhibition may be as safe as effective: in high fat diet (HFD)-induced NASH models, selective hepatic small molecule DGAT-2 inhibitors ameliorated insulin resistance and histological features of progressive NASH through upregulation of FA oxidation and thermogenesis and down-regulation of protein kinase C (PKC), SREBP-1c-mediated *de novo* lipogenesis, and NF-κB pathway activation [63,64,65].

3.2. Modulation of nuclear transcription factors to tackle lipotoxicity

Nuclear transcription factors are proteins that control the rate of transcription of specific genes from DNA to messenger RNA by binding to promoter or enhancer regions of the genes.
Some nuclear transcription factors, including Farnesoid X receptor (FXR), liver X receptors (LXRs), and Peroxisome Proliferator-Activated Receptors (PPARs) are master regulators of key steps in hepatic and extrahepatic lipid metabolism and of inflammation and fibrogenesis and are currently being explored as potential pharmacological targets for NASH treatment.

FXR modulation

FXR was initially identified for its function of bile acid sensor in enterohepatic tissues, but its central role in regulating lipid metabolism, inflammation and fibrogenesis has been subsequently disclosed [4]. FXR is prominently expressed by the liver, intestinal and renal cells and adrenal glands, and to a lower extent by adipocytes. Hepatic FXR plays insulin-sensitizing, anti-lipotoxic, anti-inflammatory and anti-fibrotic properties through several mechanisms: increased mitochondrial β-oxidation, suppression of SREBP-1c-mediated *de novo* lipogenesis and enhancement of cholesterol excretion by ABCG5/G8 transporters (**supplementary Table 2).** Obeticholic acid (OCA,6α-ethyl-chenodeoxycholic acid), a semi-synthetic derivative of chenodeoxycholic acid with potent and selective FXR agonist activity, improved steatohepatitis and fibrosis in NASH patients, but long-term safety and tolerability concerns were raised by increased LDL-C and decreased HDL-C levels and by the onset of pruritus, which affected as many as 50% of patients on OCA [66]; furthermore, NASH resolution occurred at a much lower rate with OCA than with other pharmacological agents, and the % NASH resolution as compared with placebo was significant for diabetic, but not for nondiabetic NASH patients [67,68]. Data on efficacy, tolerability and safety of OCA in noncirrhotic NASH patients were confirmed in the long-term in the REGENERATE trial [69]. In an attempt to eliminate unwanted effects of OCA, other non-steroidal FXR agonists (Gs-9674, LMB763, Px-102, Px-104) have been designed and are currently being tested in NASH in phase I-IIa RCTs (**supplementary Table 1**).

Liver X Receptor (LXR)-α modulation

LXR comprises two isoforms, LXR-α and LXR-β, with high homology in their sequence and similar ligand binding affinities, but distinct tissue distribution: LXR-α can be found in the liver, macrophages and adipose tissue, while LXR-β is ubiquitous [38].

LXR-α regulates whole-body cholesterol and FA metabolism: LXR-α activation promotes cholesterol unloading by upregulating both reverse cholesterol transport and cholesterol excretion in the intestinal lumen. These effects are achieved at the molecular level through up-regulation of ABCA1 and ABCG5/G8 in hepatocytes, macrophages, and enterocytes and down-regulation of intestinal Niemann–Pick C1-like 1 protein (NPC1L1), which mediates cholesterol resorption from gut lumen. Furthermore, LXR-α activation upregulates the key enzyme in hepatic bile acid synthesis, i.e., cytochrome P450 7A1 (CYP7A1), thereby favouring cholesterol conversion to bile acids, and enhances lysosomal degradation of LDL-receptor, thus limiting cholesterol uptake by hepatocytes.

Nevertheless, LXR-α activation conveys also unwanted effects, as LXR-α augments hepatic *de novo* lipogenesis by upregulating SREBP-1c expression and downregulates VLDL catabolism, promoting liver TAG accumulation and circulating TAG elevation [70] **(supplementary Table 2**). Recently, 25-Hydroxycholesterol-3-sulfate (25HC3S), an endogenous cholesterol sulfate metabolite and LXR activator, that is able to suppress both LXR/SREBP-1c and the pro- inflammatory transcription factor NF-κB activation [71], has attracted considerable attention for the treatment of NASH: 25HC3S (Dur-928) significantly improved steatosis necroinflammation and fibrosis in experimental models of diet-induced NASH [72] and did not raise major safety concerns in NASH in a phase Ib RCT (**supplementary Table 1**).

PPAR modulation

PPARs comprise three isotypes (PPAR-α, -δ and -γ), which upon forming heterodimers with Retinoid X Receptor (RXR), modulate the transcription of key genes involved in metabolism, inflammation

and fibrogenesis [7]. On this basis, PPAR modulation is being considered a major avenue of research in the field of NASH therapeutics [8] (**Supplementary Table 1-2)**.

The similar metabolic activity and complementary tissue distribution of PPAR-α and PPAR-δ prompted the development of dual PPARα/δ agonists. These agents reversed hepatic lipotoxicity and improved NASH, hepatic fibrosis, insulin resistance and adipose tissue dysfunction in preclinical models through a varied of mechanisms, including upregulation of all cellular FA oxidative pathways (mitochondrial and peroxisomal β-oxidation, microsomal ω-oxidation) and of ketogenesis [73,74] **(supplementary Table 2**).

Early clinical experience with dual PPARα/δ agonists is encouraging: in a phase IIb RCT, elafibranor, a dual PPARα/δ agonist, induced NASH resolution, improved glycaemic control and dyslipidaemia [75], while seladepar (MBX-8025), a selective PPAR-δ agonist, improved plasma lipids, transaminases, insulin resistance, and markers of inflammation in patients with atherogenic dyslipidemia [76].

PPAR-γ is prominently expressed by adipocytes, macrophages and Kupffer cells, by HSCs, and to a lesser extent by hepatocytes and skeletal myocytes.

PPAR-γ activation unloads the liver from lipotoxic SFAs by enhancing adipocyte differentiation and sensitivity to insulin and by enhancing secretion of the adipokine adiponectin [4]. Furthermore, PPAR-γ activation directly ameliorates inflammation and fibrosis by shifting SFA-induced macrophage polarization from a pro-inflammatory M1 to a pro-resolving M2 phenotype [77], and by reversing HSC trans-differentiation into myofibroblasts [78] **(supplementary Table 2**).

On this basis, dual PPAR-α/γ agonists and pan-PPAR agonists, which combine the PPAR-α/δ-mediated lipid pro-oxidizing effects with the PPAR-γ-mediated insulin sensitization, have been developed and tested in NASH.

The dual PPAR-α/γ agonist saroglitazar, and the pan-PPAR agonist Lanifibranor (IVA337) enhanced FA β-oxidation and desaturation and ameliorated diet-induced steatohepatitis and fibrosis with a potency superior to individual class PPAR agonists [79,80] . Both drugs confirmed their

histological and metabolic benefits in two recently completed phase 2 RCTs [81 82] (**supplementary Table 2**).

Chiglitazar, a novel orally administered, non-thiazolidinedione small-molecule pan-PPAR agonist, is currently in phase 2 clinical development for the treatment of NASH [83].

4. Role of Poly (ADP-ribose) polymerases (PARPs) in NASH

Poly (ADP-ribose) polymerases (PARPs) are a family of 17 nuclear enzymes catalyzing poly-ADP-ribosylation, i.e., covalent binding of negatively charged poly-ADP-ribose chains, of diverse target molecules.

PARPs are involved in a number of cellular processes including DNA repair, genomic stability, and programmed cell death. PARPs initiate an immediate cellular response to metabolic, chemical, or radiation-induced single-strand DNA breaks (SSB) by signaling the enzymatic machinery involved in the SSB repair: once PARP detects a SSB, it binds to the DNA, undergoes a structural change, and covalently attaches ADP-ribose moieties to amino acids (predominantly glutamic and aspartic acid) of target proteins. Subsequently, poly-ADP-ribose (PAR) chains of various length and branching complexity are synthesized, forming O-glycosidic bonds between the ADP-ribose molecules. The newly synthesized PAR chain acts as a signal for the other DNA-repairing enzymes and then is degraded via poly-ADP-ribose glyco-hydrolase (PARG).

Importantly, NAD+ serves as substrate for ADP-ribose monomer generation, which, together with direct hexokinase activity inhibition by PARPs and glycolysis impairment, may promote NAD+ and ATP depletion and contribute to necrotic cell during PARPs overactivation [84]. PARPs are composed of four domains: a DNA-binding domain, a caspase-cleaved domain, an auto-modification domain, and a catalytic domain. The DNA-binding domain is composed of two zinc finger motifs. In the presence of damaged DNA, the DNA-binding domain binds the DNA and induce a conformational shift. It has been shown that this binding occurs independently of the other domains. This is integral in a programmed cell death model based on caspase cleavage inhibition of PARP. The

auto-modification domain is responsible for releasing the protein from the DNA after catalysis. Also, it plays an integral role in cleavage-induced inactivation.

Pharmacological PARP inhibitors (Olaparib, Rucaparib, Niraparib, Talazoparib, and Veliparib) are currently used for the treatment of several solid malignancies, which are more dependent on PARP than regular cells, with a favorable side effect profile [85].

PARP activation promotes also lipotoxicity, inflammation and NASH development and progression through multiple mechanisms, summarized in **Table 2**.

In hepatocytes, lipid reactive oxygen species and ox-LDLs trigger DNA damage and PARP1/2 activation [86,87], which depletes NAD+ stores, required by sirtuin SIRT1 and other NAD+dependent enzymes, and directly PARylates target proteins liver X receptor (LXR)α, PPARα, insulin receptor and AMP-activated kinase (AMPK) activation, thus impairing FA oxidation and insulin sensitivity [88,89,90]. Furthermore, PARP1, PARP2 and PARP7 PARylate and inhibit the transcriptional activity of PGC1α, a nuclear receptor cofactor driving mitochondrial biogenesis and gluconeogenesis suppression, thereby aggravating mitochondrial dysfunction and insulin resistance [91].

Beside suppressing FA oxidation, PARP1 activation promotes *de novo* lipogenesis by upregulating the transcription factor SREBP1 and the enzymes DGAT1 and DGAT2 [92], while

PARP7, upon activation by short chain fatty acids or other polycyclic aromatic compounds, represses aryl hydrocarbon receptor (AHR), thereby impairing the detoxification of toxic lipid species [93].

PARP activation exerts also a direct hepatic proinflammatory action through Kupffer cells and circulating macrophage activation, as documented in animal disease models and in a human study [94,95]: PARP1 interacts and promotes activation of a large set of proinflammatory transcription factors in mononuclear immune cells, most prominently NFκB, a key mediator of metabolic inflammation in obesity-related disorders [96,97,98]. PARP1 triggers also the differentiation of T cells into effector T cells such as T helper 1 (Th1), T helper 2 (Th2), and inhibits T cell differentiation into regulatory T cells (Tregs) [99,100], with both these immune cell types playing an emerging role in hepatic inflammation in NASH [4].

Adipose tissue differentiation and metabolism is another key metabolic step modulated by PARP activation. Consistently, PARP1 overactivation has been involved in adipose tissue dysfunction [101], by acting in two ways: first, PARP1 orchestrates the expression of a large set of transcription factors and enzymes involved in adipocyte differentiation, C/EBP-δ-β and PPAR) γ1 and PPARγ2, which upregulate a set of genes involved in adipose tissue expansion, including lipoprotein lipase (LPL), fatty acid transporters (CD36 and aP2), TAG storage protein (perilipin), and adipokines (e.g., leptin, adiponectin) [102] (**Table 2**); second, PARPs activation impair PGC1α-mediated mitochondrial biogenesis and insulin sensitivity and promote NFkB-mediated secretion of proinflammatory cytokines, eventually leading to inflammatory infiltration low-grade adipose tissue inflammation and eventually fibrosis; all these alterations were prevented by PAPR1 deletion in mice [103,104].

Another emerging PARP target that has been implicated in the pathogenesis of NASH is gut microbiota, as hepatic inflammation is triggered by bacterial products translocated to circulation through a leaky gut [105]. PARP1 deletion modulated the diversity of the gut microbiome in rodents, with an increased relative abundance of *Firmicutes*, especially butyrate-producing *Clostridium spp*, known for their beneficial effects on epithelial integrity and the Treg cell compartment [106]. The modulatory effect of PARP1 on cells of the adaptive immune system, mainly Tregs, has been implicated in gut microbiota composition modulation [107].

Based on the above-mentioned actions of PARP activation, PARP inhibition has been evaluated as a therapeutic strategy in *in vivo* NASH models: pharmacological inhibition of PARP with natural (puerarin) or synthetic (olaparib, AIQ, PJ34) molecules or genetic deletion of PARP1 was protective in diverse models of NASH induced by the high-fat high-sucrose [108,109], hypercaloric high-fat diet [110,111], methionine-choline deficient (MCD) diet [106,107] or high fructose diet (HFD) [112]. Intriguingly, PARP inhibition with olaparib not only prevented NASH development, but also reversed the already established pathology [104].

Collectively, these data highlight the high translational potential of PARP inhibitors for NASH therapeutics, as PARPs pervasively mediate hepatic and extrahepatic lipotoxicity and PARP inhibitors are already clinically available for treatment of cancer.

5. Role of lysosomal acid lipase deficiency in NASH

Lysosomal acid lipase (LAL) is a lysosomal enzyme that hydrolyzes CEs and triglycerides that have been previously internalized via low-density lipoprotein receptor (LDL-R) -mediated endocytosis, to produce free cholesterol and free fatty acids (FFAs).

Lysosomal Acid Lipase Deficiency (LAL-D) is an autosomal recessive disorder, with an estimated prevalence between 1:40000–1:300000 [113], characterized by tissue accumulation of cholesteryl esters (CEs) and triglycerides, caused by mutations of the gene encoding LAL, namely LIPA gene. LAL deficiency prevents breakdown of lipid esters and trafficking of lipids from the lysosome into the cytoplasm, disrupting the negative feed-back exerted by intracellular cholesterol concentration on its synthesis and on LDL-R-mediated cholesterol uptake [21]. The consequence is cellular cholesterol overload, which is most evident in those tissues involved in receptor-mediated lipoprotein endocytosis and lysosomal degradation, including the liver, spleen, adrenal glands, lymph nodes, intestinal mucosa, vascular endothelium and skeletal muscle (**Figure 4**). The phenotypic spectrum of LAL-D is highly variable, depending on the residual LAL activity and on environmental factors: the absolute enzyme deficiency causes the more severe form known as Wolman disease (WD), which manifests itself during the first six months of life, and it is rapidly fatal within one year of age. The production of an enzyme with a reduced residual activity causes the cholesteryl ester storage disease (CESD), which has a later age of clinical presentation, ranging from 5 to 44 years or over, a milder clinical course, and remains often unrecognized, since clinical features overlap with other common conditions, most commonly NAFLD and familial hypercholesterolemia [114,115] (**Table 3**).

Recent independent cross-sectional and retrospective data indicates that LAL deficiency may play an unrecognized role in the pathogenesis of lean (i.e., with a Body Mass Index ≤ 25 kg/m^2) NAFLD patients, which represent 20% of the whole NAFLD population and retain a similar liver-related risk as obese NAFLD patients [116,117,118,119,120,121,122]. A distinctive feature of LAL-D as compared with non-LAL-D-related NAFLD is the progressive course of liver disease, which progresses to fibrosis, cirrhosis or liver transplantation over a median of 3.1 years from first clinical manifestation [123, 124]. Premature atherosclerosis and cardiovascular disease have also been reported in LAL-D, but firm evidence for an increased cardiovascular mortality in LAL-D is lacking [125,126].

The key clinical, radiological, pathological features that may help differentiate LAL-D from common NAFLD are reported in **Table 3** [127,128,129,130]: notably, usual therapeutic measures for NAFLD and statin therapy often fail to improve liver histology or halt liver fibrosis progression in patients with LAL-D [114].

The diagnosis relies on measuring LAL activity with Dried blood spot (DBS) assay, which measures LAL activity in peripheral leucocytes [131] and may be an accurate screening tool, and confirmation with LIPA gene testing. Currently, however, over 40 loss-of-function mutations, producing lower LAL activities, have been identified [132]: the most common mutation is the E8SJM variant, which has a carrier frequency of 1:200 in Western countries and has been identified in 50-70% of patients with LAL-D [133].

The introduction of enzyme replacement therapy with Sebelipase α, a recombinant human LAL enzyme, for up to 76 weeks has been shown to be safe and effective and reversed cirrhotic stages of fibrosis in the Acid Lipase Replacement Investigating Safety and Efficacy (ARISE) trial [134].

Based on these considerations, the American Gastroenterological Association (AGA) has recently recommended screening for LAL-D in lean NAFLD patients [135].

6. Extracellular vesicles in NASH: mediators of liver injury, biomarkers of disease severity and therapeutic tools

Extracellular vesicles (EVs) are small membrane vesicles released in a highly regulated manner from damaged or activated cells. Based on their biogenesis and release into extracellular space, EVs can be classified into three broad categories [136,137]: (a) exosomes, (b) microvesicles or microparticles, and (c) apoptotic bodies (**Figure 5**). Furthermore, a fourth, larger size (1–10 μm in diameter), EV population has been recently identified from highly migratory cancer cells and named oncosomes [138].

EVs are membranous vesicles excreted by a cell that can contain almost any cellular molecule with different biological activities, including non-coding RNAs (microRNA, mitochondrial associated tRNA, longRNA, small nuclear RNA, Ro associated Y-RNA), messenger RNAs (mRNAs), DNAs, proteins, and lipids. EVs possess surface molecules that target them to recipient cells, where they can vehicle signaling by interacting with specific receptors, can be internalized by endocytosis and/or phagocytosis or fuse with the plasma membrane to deliver their cargo to the recipient cell, thereby mediating cell-to-cell communication. Growing cellular, animal and human data suggest that EVs derived from hepatocytes, from hepatic non-parenchymal cells [Kupffer cells, HSCs, endothelial cells, T lymphocytes, natural killer (NK) cells, natural killer T (NKT)cells], and from adipocytes, are key mediators of lipotoxicity and of liver disease progression in NAFLD [4].

Early observations demonstrated that circulating EV number is significantly increased in mouse models and NAFLD patients and parallels the severity of liver histological inflammation, representing a potential noninvasive marker to differentiate NAFLD from NASH [139,140]. Later mechanistic studies disclosed the accumulation of lipotoxic lipids, including SFA, free cholesterol, phospholipids and sphingolipids induces the release of great quantities of EVs from hepatocytes, which contribute to key inflammatory steps involved in fatty liver progression to NASH and cirrhosis, namely, necro-inflammation, angiogenesis, and fibrosis (**Table 4; Figure 2B**).

When characterizing the cell sources and temporal sequence of extracellular EV release in murine models of high fat-induced NASH, Li et al. found that hepatocyte-derived EVs increase at earlier

stages, when the predominant histologic lesion is steatosis, remain elevated with NASH progression and are followed by macrophage- and neutrophil-derived EVs' increase [141]. The correlation between circulating hepatocyte-derived EVs levels and inflammation, NAS, and fibrosis strengthened the rationale for quantifying hepatocyte-derived EVs to predict severity of NASH and hepatocellular injury.

Molecular pathways regulating EV release from hepatocytes are also being unraveled.

Fukushima et al. demonstrated that the release of palmitate-stimulated EVs from hepatocytes is critically dependent on *de novo* synthesis of ceramide, which is trafficked by the ceramide transport protein, StAR-related Lipid Transfer Domain 11 (STARD11): using quantitative proteomic analysis of palmitate-stimulated EVs in control and STARD11 knockout hepatocyte cell lines, they showed that STARD11-deficient cells do not release palmitate-stimulated EVs [142]. The same group showed that ceramide trafficking modulates also the protein cargoes composition of EVs in hepatocyte cell lines and NASH patients: compared with palmitate-stimulated EVs from STARD11-/- cells, those secreted from wild-type cells were significantly enriched in histone H3.3 (H3F3A), intercellular adhesion molecule 1 (ICAM1), embigin (Emb), haptoglobin (Hp), immunoglobulin superfamily member 3 (IGSF3), Vanin-1 and other damage-associated pattern (DAMP) molecules which are able to modulate immune cell responses and able to promote liver inflammation in NASH [143].

Povero et al. reported that SFA-laden hepatocytes release microparticles (MPs) in a caspase-3-dependent-manner, which are internalized via a Vanin-1(VNN1)-mediated uptake by endothelial cells and activate them to promote a pro-angiogenic milieu [144]. Remarkably, genetic ablation of caspase 3 or RNA interference directed against VNN1 prevented the development of steatohepatitis-induced pathological angiogenesis in the liver and resulted in a loss of the proangiogenic effects of microparticles, indicating a potential therapeutic target for lipid-induced NASH.

The same group reported that MPs released from fat-laden hepatocytes are avidly internalized by HSCs, where they down-regulate PPAR-γ and induce HSC activation at least in part through delivery of encapsulated miR-128-3p [145] (**Figure 2B, Table 4**). Loss- and gain-of-function studies identified miR-128-3p as a central modulator of the effects of EVs on HSC activation.

Another group of researchers reported that the axis mixed lineage kinase 3 (MLK3)/signal transducer and activator of transcription (STAT) 1 is a critical mediator of the release of Hep-EVs following exposure of hepatocytes to lipotoxic lipids: these EVs carry chemokine (C-X-C motif) ligand 10 (CXCL10) [146,147], a potent macrophage chemoattractant which is able to activate hepatic Kupffer cells and recruit monocyte-derived macrophages during NASH progression [4] (**Figure 2B, Table 4**). Genetic deletion or pharmacological inhibition of MLK3/STAT1 or pretreatment with anti-CXCL10 antibodies prevented CXCL10 enrichment in EVs and proinflammatory macrophage activation, respectively.

Hirsova et al. showed that EVs released from hepatocytes, following activation by death receptor 5 (DR5) after coincubation with lysophosphatidylcholine (LPC), contained tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and were able to activate mouse bone marrow-derived macrophages via TRAIL-mediated activation [148]. Interestingly, the inactivation of the DR5 signaling pathway or of Rho-associated protein kinase 1 (ROCK1) inhibitors reduced circulating EV levels and improved necroinflammatory and fibrotic features in rodent models of diet-induced NASH. Beside SFA and their metabolite LPC, other lipid species have been documented to induce EVs release from hepatocytes.

Zhao et al found that hepatocyte cholesterol loading, a condition frequently encountered in animal and human NASH, impairs hepatocyte lysosomal function and promotes exosome release that induce macrophage pro-inflammatory M1 polarization via their exosomal miR-122-5p cargo [149] **(Figure 2B, Table 4)**. Importantly, hepatocyte miR-122-5p downregulation inhibited exosome-induced activation of macrophages and improved inflammation.

EVs also contribute to the proinflammatory effect of hepatocyte ER stress: following lipotoxic stimuli, damaged hepatocytes release C16:0 ceramide-enriched Hep-EVs in an inositol requiring enzyme1α (IRE1α)-dependent manner (**Figure 2B, Table 4**). These EVs possessed the whole enzymatic apparatus to metabolize ceramide (discussed below) and were able to activate macrophages and promote chemotaxis through formation of sphingosine-1-phosphate (S1P) from C16:0 ceramide [150]. Intriguingly, a critical proinflammatory role for EV S1P enrichment was

demonstrated by Liao et al., who showed that macrophage chemotaxis was significantly reduced by pharmacological inhibition of SphK1 and SphK2 in EV S1P or by $S1P_1$ receptor 1(S1PR1) antagonists, without affecting the number of EVs released [151].

Hepatocyte-derived DNA-containing EVs have been involved in steatohepatitis development, as well: Garcia-Martinez et al. showed that plasma from NASH mice and patients is enriched with high concentrations of hepatocyte—derived MPs containing mitochondrial DNA (mtDNA), which activate Kupffer cells in a TLR9-dependent manner, as pharmacological TLR9 antagonism prevented high fat diet-induced steatohepatitis development and reversed established NASH in diet-induced mouse models of NASH [152] **(Table 4).**

While hepatocyte-derived EVs play a crucial role in modulating non-parenchymal cells and promoting liver injury since early stages of NASH, EVs derived from non-parenchymal liver cells and extrahepatic cells also contribute to liver disease progression and extrahepatic complications of NASH.

Kornek et al. showed that EVs from infiltrating activated inflammatory CD4+ and CD8+ T cells transfer membrane molecules, including CD147, to HSCs, promoting fibrolytic activation and downregulation of profibrogenic activity of these cells [153].

Myofibroblasts are also a prominent source of EVs that vehicle potent proangiogenic factors, including Hedgehog (Hh) and vascular endothelial growth factor A (VEGF-A), which are transferred into ECs resulting in a pro-angiogenic switch in sinusoidal ECs followed by capillarization [154,155] **(Table 4).**

Activated HSCs, the key players in hepatis fibrosis deposition, can secrete profibrogenic connective tissue growth factor (CCN2)-containing EVs to activate quiescent HSCs in a paracrine manner during various forms of liver injury [156] **(Table 4).**

Finally, extrahepatic organs and tissues like adipose tissue can also communicate to the liver via EV release. Adipose tissue expansion and inflammation is a component of obesity-associated liver disease in NASH; Eguchi et al showed that hypertrophied adipocytes release large amounts of EVs, whose plasma levels are elevated in diet-induced mouse models and patients with NASH [157,158],

where they modulate systemic and hepatic insulin resistance and inflammatory response [159,160]: consistently, the administration of EVs from obese mice into lean littermates induced acute hepatic inflammation.

In conclusion, EV-mediated cell-to-cell and organ-to-organ communications may represent central events in a complex and multifactorial diseases like NASH. While a potential therapeutic strategy is to antagonize the proinflammatory, pro-fibrogenic and proangiogenic cargoes of these EVs, another promising therapeutic approach to NASH is the synthesis and delivery to the liver of EVs with antiinflammatory and antifibrotic properties. The use of EVs, especially the exosome subpopulation, as vehicles for RNA interference (RNAi) and drug delivery has generated remarkable interest. EVs are bioavailable, biocompatible and resistant to RNases and proteases [161]. These characteristics make EVs ideal vehicles for the delivery of drugs, proteins, miRNAs, silencing RNA (siRNA) and other molecules that would otherwise be rapidly degraded. Furthermore, the liver is a primary site of EV uptake after intravenous delivery [162], and growing experimental evidence showed the potential use of EVs (predominantly exosomes) in delivering targeted RNA-based therapies in the context of liver diseases.

EVs could efficiently deliver a mimic or inhibitor of miR-155 (an important mediator of liver inflammation in alcoholic hepatitis) to macrophages and hepatocytes both *in vitro* and *in* vivo and delivery of the miR-155 inhibitor to macrophages reduced TNF protein production by >50% [94, 163]. *In vivo*, EVs successfully delivered an exogenous miR-155 mimic to the liver and isolated hepatocytes in *mir-155*-knockout mice.

Another example of the potential of EVs as therapeutic tool to vehicle drugs is represented by curcumin-encapsulated exosomes [164]. Curcumin, the bioactive pigment of turmeric which has polyphenolic-hydrophobic components, is an attractive molecule for the treatment of a variety of liver diseases due to its potent antioxidant, anti-inflammatory and anti-fibrotic properties [4]. However, due to its significant intestinal and liver metabolism, low stability, quick systemic elimination and its hydrophobic property with low solubility, curcumin has limited bioavailability. Curcumin-encapsulated exosomes showed higher bioavailability, solubility and safety, and reached high

concentrations in the blood without toxic effects and immune stimulation, proving more effective than other pharmacological curcumin formulations in delivery this drug to target organs.

Human liver stem cells (HLSCs) represent another therapeutically promising source of EVs. HLSCs are an easily obtainable and expandable stem cell population derived from human adult liver cells. HLSCs play a central role in wound-healing responses to restore liver homeostasis following injury and exhibit *in vitro* and *in vivo* anti-inflammatory and anti-fibrotic effects in different models of diet-induced NASH by regulating specific genes without the need for differentiation into mature hepatocytes[165,166]. HLSC-EV cargoes may modulate their anti-inflammatory and anti-fibrotic effects. Most of the molecules identified were growth factors, cytokines, enzymes like Matrix Metallo-Peptidases and glutathione reductase, transmembrane tyrosine kinase receptors, and miRNAs involved in fibrogenesis regulation like miRNA-21, miRNA-24, miRNA-30a, miR-92a-3p, miRNA-29a, and the let-7 family [165,166].

7. Role of lysophosphatidylcholine in NASH

Lysophosphatidylcholine (LPC, 2-acyl-sn-glycero-3-phosphocholine) is a phospholipid that results from the hydrolysis of an acyl group from the *sn-1* position of phosphatidylcholine (PC). The hydrolysis can occur intracellularly by action of phospholipase A2 (PLA2) or in the extracellular space by action of lecithin-cholesterol acyltransferase (LCAT) [21]. It is still unclear which one of these two pathways contributes more substantially to LPC accumulation observed in human NASH liver. Nevertheless, recent animal and human data link the extent of hepatic LPC accumulation to the severity of liver disease in NASH and place LPC as a central mediator of SFA-induced lipotoxicity through several mechanisms [2-10]: in primary hepatocytes, LPC triggered cell apoptosis, while downregulation of PLA2 activity reduced intracellular LPC and SFA-induced apoptosis [14,167]; beside JNK- and ER stress-mediated lipoapoptosis, LPC impaired hepatic mitochondrial oxidative phosphorylation, resulting in mitochondrial dysfunction [168], and enhanced extracellular with pro-inflammatory and pro-fibrogenic EV release from hepatocytes [169,170]; furthermore, LPC is converted by the extracellular enzyme autotaxin into lyso-

phosphatidic acid (LPA), a potent profibrogenic phospholipid molecule [171] (**Figure 2B**; **online supplementary Table 3).** An additional, relevant lipotoxic mechanism is plasma membrane PC depletion mediated by the activation of PLA2 [14,172]: PC is the main phospholipid in mammal cells, where it is required for the integrity of all cellular membranes and is a feedback inhibitor of SREBP1c-mediated *de novo* lipogenesis [173]. Hepatocytes physiologically require high amounts of PC for VLDL secretion and also excrete PC into bile, which makes these cells prone to PC depletion.

The above mentioned physiological factors and the inappropriate PLA2 activation observed in NASH explain why hepatic PC depletion is a prominent feature of NASH (**Table 1**) [9,10]. Plasma membrane PC depletion disrupts the functional integrity of hepatocyte plasma membrane, causing extracellular release of lipotoxic lipids, hepatocyte apoptosis, inflammation, and progression of liver injury [48,174]. Experimental PLA2 inhibition halted this chain of events, restored cellular PC pool, preventing the development of NAFLD and even reversing inflammatory and fibrotic changes in established NASH experimentally [14,175].

8. Role of Sphingolipids in liver injury in NASH

Sphingolipids are complex lipids sharing a common structural feature, a sphingoid base backbone that is comprised of an alkyl chain of 18 carbon atoms with one to three hydroxyl groups and one amino group at position 2 or 1 of the alkyl chain. The sphingoid base is usually linked to a FA via an amide bond.

This lipid species can be found ubiquitously in cell membranes, where they function as both structural components and signaling molecules to regulate essential cellular activities, including growth, proliferation, migration, differentiation, and survival. Furthermore, the 3 main sphingolipids ceramide, sphingosine, and S1P can be rapidly interconverted, and the delicate balance of their relative concentrations can trigger even opposite biological processes, deserving the term of "sphingolipid rheostat" (**Figure 6A**): consistently, ceramide and sphingosine trigger cell growth arrest, apoptosis and senescence, while S1P enhances cell survival, growth, and proliferation

[172]. Recently, the immunomodulatory role of sphingolipids and of the sphingolipid rheostat has been the subject of intense research in different fields of health and disease, including metabolic disorders like NASH[176].

Role of ceramide in NASH

Ceramide can be synthesized from palmitate and serine by the sequential action of three enzymes located in the ER—serine palmitoyl transferase (SPT), ceramide synthase (CeS) and dihydro-ceramide desaturase (DES) — or formed by the enzyme sphingomyelinase (SMase) through hydrolysis of plasma membrane sphingomyelin (**Figure 6A**).

In patients with NAFLD, hepatic ceramide levels are increased, closely correlating with histological severity [5-10]. Hepatic ceramide accumulation derives from both increased sphingomyelin hydrolysis by acid sphingomyelinase (ASMase), one isoform of Smase whose activity is stimulatedby inflammatory stimuli (TNF-α, reactive oxygen species, death receptor ligands) operatingin NASH [177], and from increased SFA availability, which are required for *de novo* ceramide synthesis. Additionally, recent data suggest the intestine is a key contributor to whole-body ceramide pool and to liver injury in NASH, and that intestinal FXR is a master regulator of ileocecal ceramide synthesis (**Figure 6B**) [178]: gut-specific genetic or pharmacological downregulation of FXR reduced ceramide levels in the blood by 30-50%, ameliorated hepatic insulin sensitivity and liver disease and enhanced adipose tissue oxidative capacity in obese rodents fed a HFD, effects that were all abolished by ceramide administration. Similarly, to LPC, mounting data support ceramide as a key mediator of SFA lipotoxicity, which is abrogated or greatly reduced through inhibition of ceramide formation. SFA-induced inhibition of insulin signaling through the fosfoinositide 3-chinasi (PI3K)/Akt pathway and consequent insulin resistance are prevented by blocking ceramide synthesis [179]. Furthermore, ceramide impairs mitochondrial complex II and IV function of the electron transport chain and mitochondria fatty acid β-oxidation, resulting in reactive oxygen species production, triglyceride accumulation and insulin resistance [180,181] (**Figure 6A**). In the liver, ceramide exerts its lipotoxicity also by disrupting ER calcium homeostasis and triggering cell apoptosis, impairing autophagy, and upregulating Nlrp3 inflammasome and hepcidin expression, the latter resulting in

hepatic iron overload [182,183,184,185,186]. Finally, ASMAse activation *per se* induces liver injury through disruption of methionine-phosphatidylcholine cycle, resulting in lysosomal membrane permeabilization [60] and activation of HSC-mediated fibrogenesis [187] (**Figure 6A-B**).

In the latest years, several strategies to unload the liver of ceramide accumulation have been tested, including inhibition of ceramide hydrolysis from sphingomyelin by ASMases, inhibition of ceramide *de novo* biosynthesis by ER enzymes SPT, CeS and DES, and enhancement of ceramide degradation by the enzyme acid ceramidase.

ASMases can be inhibited by several functional inhibitors of acid sphingomyelinases (FIASMAs). FIASMAs are a heterogeneous class of weakly basic, highly lipophilic compounds which share the ability to accumulate into the lysosome where inhibit ASMase via an indirect, functional mechanism: they insert into the inner leaf of the lysosomal membrane and subsequently cause membrane-associated enzymes, such as ASMases, to detach. Upon detachment from the membrane, these enzymes are cleaved and degraded within lysosomes [188].

D*e novo* ceramide biosynthesis can be reduced by inhibiting the three enzymes responsible for ceramide synthesis or through intestinal FXR downregulation **(supplementary Table 1)**. In genetic or dietary-induced rodent models of obesity, myriocin, a fungal metabolite which inhibits SPT: in diet-induced obesity and NAFLD, myriocin reduced circulating ceramide levels and whole-body and liver fat accumulation and improved hepatic insulin resistance, necroinflammation and fibrosis [189,190]. However, recent data from rodent models suggest that whole-body inhibition of ceramide synthesis may have detrimental effects, including neurodegeneration [191], because ceramide is essential for the formation of other sphingolipid derivatives that are physiologically required for correct cell membrane function and signaling. For this reasons, strategies targeting intestine-specific FXR inhibition are being evaluated with encouraging, safer results than whole-body ceramide synthesis inhibition: accordingly, glycine-β-muricholic acid (β-MCA), a bile acid derivative selective intestinal FXR inhibitor, ameliorated systemic ceramide overload and improved obesity and NAFLD without impairing ceramide synthesis in CNS [54]. Tissue selectivity of FXR modulation appears therefore a crucial requisite to obtain therapeutic effects with FXR modulation: while intestinal FXR

inhibition improved liver disease, in the liver FXR activation, rather than inhibition, has been documented to improve liver disease in NASH.

A different strategy to avoid the consequences of whole-body ceramide depletion relies on the observation that ceramides with different N-acyl side chains (C(14:0)-Cer - C(26:0)-Cer) possess distinct roles in cell signaling, implying different pathophysiological roles: lipotoxic effects of ceramide are mediated by a specific type of ceramide, i.e., C(16:0)-Cer, which accounts for most of the ceramide amount accumulated in the liver and adipose tissue in NASH [10,57] but not for the whole-body pool of very-long acyl-chain fatty acid (VLCFA) ceramides [56,57].

Humans possess six isoforms of CeS (CerS1–6), with different acyl-chain specificity, tissue distribution, and regulation at transcriptional and post-translational level. CeS2 utilizes very long acyl-chain (C22–26) fatty acids, while CeS5-CeS6 utilize long-chain (C14–16) acyl-chain fatty acids [192]. Therefore, specific CeS5-6 inhibition could theoretically prevent toxic C16:0 ceramide accumulation without depleting VLCFA ceramide pool: on this basis, ST1072, a relatively selective inhibitor of CerS4 and CerS6, was developed and tested in cellular lines with encouraging results, but the potential biological impact of these compounds has to be evaluated [193].

Another therapeutic strategy to reduce ceramide synthesis relies on the finding that CerS activity can be modulated at post-translational level through phosphorylation or deacetylation [194]: Novgorodov et al reported that SIRT3 regulates ceramide biosynthesis in mitochondria via deacetylation of ceramide synthase (CerS) 1, 2, and 6 and that SIRT3 inhibition unloaded CNS cells of ceramide, rescued mitochondrial dysfunction and ROS generation and attenuated cell dysfuntion in experimental ischemia-induced brain injury [125].

The synthetic retinoid derivative fenretinide, a dihydroceramide desaturase (DES)-1 inhibitor, which specifically inhibits CeS5, reduced the synthesis of long acyl-chain ceramides and shifted intracellular ceramide composition in favour of VLCFA ceramides [195], thereby improving experimental NASH. Fenretinide will be soon evaluated in patients with biopsy-proven NASH [196].

A final therapeutic approach to deplete ceramide stores is to promote ceramide degradation by acid ceraminidase, which is the focus of recent research as it seems to play a central role in the intracellular actions of adiponectin [197] (**Figure 6**A). In transgenic mouse models of diet-induced obesity, acid ceraminidase overexpression reduced hepatocyte apoptosis, preserved insulin sensitivity and prevented the development of the whole histological spectrum of NASH [198].

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

Sphingosine (2-amino-4-trans-octadecene-1,3-diol) is another component of the "sphingolipid rheostat" and is formed from ceramide diacylation (**Figure 6A**). Cellular sphingosine can then undergo either reacylation by ceramide synthase or phosphorylation by sphingosine kinases (SphKs) to form Sphingosine-1-Phosphate (S1P). S1P can then be degraded through either reversible dephosphorylation to sphingosine by phosphatases or irreversibly cleaved to ethanolamine phosphate and *trans*-2-hexadecenal by a pyridoxal 5′-phosphate-dependent S1P lyase (SPL). The latter represents the only degradative pathway for sphingoid bases in mammals. Emerging evidence suggests a complex pathophysiological role for S1P, which can exert even opposite actions, depending on the cell type, on the cellular compartment where it is synthesized, and on the molecular target in the cell.

The conversion of sphingosine to S1P occurs by the action of two sphingosine kinases (SphK1 and SphK2). These two isoforms possess distinct subcellular localization, which result in cellular compartmentalization of S1P [199]. In response to stimuli like growth factors, cytokines and hormones, SphK1 translocates from the cytosol to the plasma membrane, where it forms S1P. S1P is then subsequently transported to the extracellular space via several transporters, including ATP-binding cassette (ABC) transporters ABCA1, ABCC1, and ABCG2. In the extracellular space, S1P binds and signals through a class of five G protein-coupled receptors (S1PR1–5). S1PR1–3 is ubiquitously expressed, while S1PR4 is mainly expressed by immune cells, and S1PR5 can be found in the spleen and in the central nervous system.

In plasma, S1P is vehicled by HDL-associated apoM, which carries 66% of circulating S1P, whereas albumin carries about 30% of S1P [130]. The liver secretes most of circulating apoM and

thus it plays a central role in regulating blood S1P levels. Consistently, apoM-deficient mice display a 50% lower plasma S1P level, while hepatic apoM overexpression through an adenoviral vector conveys an increased plasma S1P level [130].

In contrast to SphK1, SphK2 is mainly localized in the nucleus, ER and inner mitochondrial membrane: SphK2-mediated increase in intracellular S1P concentration within these organelles, has distinct biological effects: S1P can act in a receptor-independent manner. S1P arrests DNA synthesis through inhibition of Histone Deacetylases (HDAC)-1/2 in the nucleus, thus affecting epigenetic modulation of specific target genes; in the ER, S1P enhances TNF receptor-associated factor 2 (TRAF2)-mediated NF-kB activation [200], while in the mitochondria, S1P binds the inner mitochondrial membrane mitophagy receptor prohibitin 2 (PHB2) to impair mitochondrial respiration and trigger BAX-dependent cellular apoptosis [76].

Hence, subcellular compartmentalization of SphKs and S1P is a critical determinant of S1P biological actions, and accordingly, targeting SK1 either to the ER or to the nucleus can have opposing functions, with either prosurvival or proapoptotic consequences [201].

The expression of the two SphKs in different tissues and organs also varies broadly, with SK1 most highly expressed in leukocytes, lung and spleen and SK2 being predominant in the liver and kidney [195-197].

Substantial human and experimental data indicate hepatic SphK1 expression is upregulated in NAFLD [202,203] where his activity is stimulated by increased hepatic SFA availability and by inflammatory cytokines, most prominently TNF-α and IL-1. However, when trying to assess the role of SpK1/SphK2 and of S1P receptors (S1PRs) in the pathogenesis of liver disease in NASH, these studies gave often discrepant results, probably due to the complex interactions among the cellular compartment where S1P is synthesized, the target cell type, and even the molecular target within the cell.

Growing evidence indicates both SphK1 and SphK2 have been involved in hepatic insulin resistance and glucose homeostasis: SphK1-knockout mice fed a high fat diet develop diabetes, whereas wild type animals show only mild glucose intolerance and develop a compensatory

hyperinsulinemia [204]. This phenotype is explained in part by the protective role of S1P on pancreatic β-cell survival, which limits β-cell degeneration and preserves insulin secretion [135], and in part by hepatic consequences of SphK1 activation: consistently, in the KK/Ay diabetes-prone mouse model, liver-specific adenoviral SphK1 gene delivery enhanced the phosphorylation of insulin signaling kinases like Akt and glycogen synthase kinase (GSK)-3β and improved blood glucose levels and dyslipidemia [205].

SphK2 as well has been shown to be abundantly expressed in the liver, where it plays a key role in modulating insulin sensitivity [206]: in KK/Ay mice fed a HFD, adenoviral-mediated SphK2 overexpression elevated intracellular S1P levels and stimulated Akt phosphorylation, alleviating glucose intolerance and insulin resistance [137].

Extracellular S1P has also been involved in modulation of insulin action: elevated circulating S1P levels were found in diabetic animal models and in patients with type 2 diabetes [207], and recent data indicate that S1P counteracts insulin signaling, promoting hepatic insulin resistance [208]: Fayyaz et al., demonstrated that palmitate is metabolized to S1P in hepatocytes and that S1P levels are elevated in the liver of HFD-fed obese rodents [139]. Molecular mechanisms connecting S1P to insulin resistance have been unraveled: S1P inhibits insulin signaling in hepatocytes through a receptor-dependent process, which seems mediated by the S1PR2 receptor subtype, as pharmacological antagonism of this receptor almost completely abolishes the ability of S1P to promote insulin resistance [139]. Consistently, the small molecule compound FTY720, which acts on all S1P receptors except for S1PR2, does not affect insulin signaling [137]. S1P has been also implicated in the protective actions of adiponectin [209].

Adiponectin promotes ceramide degradation by enhancing the activity of acid ceraminidase, thereby diminishing the accumulation of ceramide, but it also augments S1P formation. This change of the ceramide/S1P balance has been proposed to modulate the action of insulin [140] in ASMase-deficient mice, which show an improved glucose tolerance, an increased formation of glycogen and an enhanced lipid accumulation in the liver [139].

SphK and subsequent S1P formation have recently been found to modulate hepatic lipid accumulation [210]: a lipidomic study evaluating progressive NAFLD stages in mice fed a high fat diet indicated that sphingosine, S1P, dihydrosphingosine, and dihydrophingosine 1-phosphate increased early during high-fat high-cholesterol diet [141]: increased FA availability activated hepatic SphK1 and increased liver fat deposition [211]. Similarly, SphK1 overexpression or S1P administration increased liver steatosis, which was ameliorated by siRNA-mediated downregulation of S1PR2 and S1PR3 receptor subtypes [212].

Beside modulating hepatic lipid accumulation, S1P seems also involved in the progression of simple steatosis to NASH under high fat diets [213]: in mouse models of NASH, SphK1 activation promoted NF-κB activation, cytokine secretion, immune cell infiltration and NASH development, which were prevented by SphK1 gene deletion [144,214].

While SphK1 seems to contribute to NASH development, the role of SphK2 in NASH appears more controversial: hepatic SphK2 overexpression in mice fed a HFD increases hepatic FA oxidation and ameliorates hepatic steatosis, whereas SphK2 knockout mice rapidly develop fatty liver [137,215], indicating that also SphK2 modulates hepatic lipid metabolism. It has been suggested that SphK2 acts by increasing nuclear S1P levels, which in turn inhibits specific HDACs, increasing histone acetylation and thereby upregulating core genes involved in lipid metabolism [145].

To further complicate the interpretation of the biological role of the two SphK isoenzymes in hepatic metabolism, it should be noted that intracellular S1P levels are inversely related to those of ceramide, which is the substrate used by SphKs to synthesize S1P[140,141]. Therefore, it is difficult to separate the effects of changes in S1P levels from those in ceramide concentration following functional SphK manipulation.

While the biological function of SphK1 and SphK2 are debated, data regarding S1PR subtypes are more consistent, with S1PR1 and S1PR3 being responsible for most proinflammatory effects of S1P, which are abrogated by selective knockdown of this receptor subtypes [144].

S1P is also an emerging contributor to liver fibrosis: S1P activates HSCs to proliferate, migrate, increase ECM deposition and secrete proangiogenic factors, including angiopoietin 1 and VEGF [216,217]. Both receptor-independent and receptor-mediated actions contribute to the profibrotic action of S1P. Intracellular S1P seems a critical mediator of TGF-β-induced expression of collagen in HSCs, which is required for human fibrosis development [218].

However, most data indicate a receptor-mediated profibrogenic action of S1P: in the experimental bile duct ligation model, knockout of the S1PR2 protected mice from fibrosis development and pharmacological S1PR2 antagonist with the small-molecule inhibitor JTE-013 ameliorated established liver fibrosis [219].

S1PR3 is another receptor subtype that is upregulated during liver fibrosis [220] and seems critical for liver fibrosis development: S1P-induced migration and activation of HSCs were observed in response to S1PR3 activation [221]. Further, S1PR3 mediated bone marrow-derived cell (BMDC) recruitment to the liver, whereas S1PR3 silencing reduced both the homing of BMDC to the liver and their trans-differentiation into myofibroblasts [222].

While S1PR1 and S1PR3 exert profibrotic effects, S1PR2 appears to protect from liver fibrosis by inhibiting myofibroblast migration: consistently, in human liver samples with advanced fibrosis, S1PR (1,3) expression was massively upregulated while S1PR2 expression was downregulated [223,224] (**Figure 6B**).

The therapeutic implications of S1PR antagonism remain still unexplored in NASH: FTY720

FTY720 (fingolimod) is a small molecule compound that can be phosphorylated to FTY720-phosphate, which is a functional antagonist of S1PR1 receptor subtype and has been developed for the treatment of multiple sclerosis [225]: in diet-induced NASH models, FTY720 demonstrated decreased hepatic lipid accumulation, immune cell infiltration, inflammation and fibrosis, and S1PR (1,3) antagonist VPC23019 inhibited HSC activation *in vitro* [151].

Notably, biological consequences of S1P axis activation can be even opposing depending on the cell type: S1PR1 activation in hepatic endothelial sinusoidal cells has a pro- regenerative and anti-fibrotic effects [226], while adipocyte-specific inhibition of S1P synthesis promotes to

lipodystrophy, NASH and insulin resistance [227]. Therefore, tissue selectivity is a central issue for pharmacological S1P axis modulation. On this basis, ongoing nanotechnology is developing liver-specific FTY720 nanoparticles to enhance selectivity and potency and reduce off-target effects of S1PR antagonists [228].

9. Role of Polyunsaturated Fatty Acids (PUFAs) in NASH

Long chain polyunsaturated fatty acids (LCPUFAs) comprise ≥20 carbon PUFAs, which can be divided into two biochemically and biologically distinct classes, i.e., n6- and n3-PUFAs. n6-PUFAs include dihomo-γ-linolenic (DGLA, 20:3n-6) and arachidonic acid (AA, 20:4n-6), while n3-PUFAs include eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (**Figure 7**).

Humans do not possess the fatty acid desaturases required 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 essential fatty acids (**Figure 7**). Less than 5-10% of 18-C FAs is converted to 20-C PUFAs [229]: therefore, fish oils, which contain high levels of EPA and DHA, are the primary source of n3-PUFAs.

The importance of n6 and n3 PUFAs for numerous biological functions in the cell has been recently highlighted [230] (summarized in **Supplementary Table 4**). PUFAs are pleiotropic molecules, as they possess a variety of biological functions by different molecular mechanisms and targets. PUFAs regulate receptor expression on membrane surfaces and hormone binding by modulating cell membrane fluidity and permeability and membrane microdomain composition: n3 PUFAs enhance membrane fluidity and facilitate insulin receptors to reach plasma membrane, resulting in improved insulin sensitivity [231]. Furthermore, PUFAs directly interact as ligands with nuclear receptors and transcription factors, including PPAR-α, and membrane G protein-coupled receptors (GPCRs), free-fatty acid receptor (FFAR)1 and (FFAR)4 (also known as

GPR40 and GPR120, respectively) to modulate key genes involved in glucose and lipid metabolism, redox pathways, inflammation and fibrogenesis [232,233,234,235,236,237].

Beside these receptor-mediated actions, PUFAs are components of cell membrane phospholipids and are hydrolyzed by PLA2 to become substrates for eicosanoid and SPM synthesis by cyclooxygenases (COX-1 and COX-2), lipoxygenases (5-, 12-, or 15-LOX), or cytochrome P450 monoxygenases: while n6-PUFA AA originates the pro-inflammatory 2-series PGs and TXs and the 4-series LTs, n3-PUFAs EPA and DHA are precursors of SPMs, potent inducers of inflammation resolution(discussed below)[11, 238] (**Figure 8).**

The synthesis of highly unsaturated fatty acids, such as the n3-PUFA EPA and DHA, and the n6-PUFA AA requires the sequential action of elongases and desaturases (**Figure 7**). Humans possess three desaturases: Fatty Acid Desaturase 1 (FADS1, or Δ5 desaturase), Fatty Acid Desaturase 2 (FADS2, or Δ6 desaturase), and SCD)-1 (or Δ9-desaturase).

FADS1 and FADS2 are membrane-bound and are involved in the synthesis of long-chain PUFAs AA, EPA and DHA (**Figure 7**). As previously discussed, SCD-1 forms the MUFAs oleic acid and palmitoleic acid from SFA either synthesized in the body or introduced with the diet (**Figure 7**).

In vivo lipidomic analyses coupled with enzymatic activity and gene expression profiling highlighted a major dysregulation in hepatic LCPUFA desaturation fluxes and an increased n6-to-n3 ratio [5,9]. Functional manipulation of these key enzymes points to preferential fluxes toward the n6-PUFA pathway as a major pathogenic factor in liver disease progression in NASH [239]. More specifically, an insufficient FADS1 activity seems to play a pivotal role in the dysregulated LCFA desaturation fluxes and in liver injury [9]. FADS1 represents a bottleneck in PUFA metabolic fluxes (**Figure 7)** and functional hepatic FADS1 manipulation showed that a defective FADS1 activity conveys two important pathophysiological consequences promoting steatohepatitis [240]: downstream, it alters the quantity and composition of phospholipids in hepatocyte cell membranes, resulting in membrane phospholipid deficiency, membrane disruption, and extracellular leak of lipotoxic lipids, thus perpetuating liver injury [9]. Upstream, defective FADS1 activity results in SFA accumulation and shifts desaturation fluxes toward the preferential synthesis of n6-derived

pro-inflammatory eicosanoids PGs, LTs and TXs at the expense of n3-derived SPMs, with pro-inflammatory consequences in the liver (**Figure 7-8**). The proinflammatory consequences of FADS1 deficiency are not restricted to hepatocytes, but affect also hepatic macrophages and Kupffer cells, where FADS1 deficiency induces pro-inflammatory M1 polarization at the expense of pro-resolving M2 polarization programs [168]. It is important to note that, beside these pro-inflammatory consequences, FADS1 inhibition has also anti-lipogenic effects through inhibition of LXR/SREBP-1c-mediated *de novo* lipogenesis, which may explain why FADS1 inhibitors reduce whole-body and hepatic fat accumulation and improve insulin resistance [168,241].

Several potential mechanisms have been proposed to underlie the impaired FADS1 activity in NASH: a genetic component has been involved, as functional *FADS1* gene polymorphisms modulate human hepatic lipid composition and content [242]. Furthermore, hepatic *FADS1* and *FADS2* gene expression are modulated by dietary FA intake and their activity in cultured hepatocytes and in rodent models is down-regulated by PUFA supplementation [243,244].

Therefore, the impact of FADS1 activity on metabolic fluxes of LCPUFAs depends on the availability of its substrate lipids and on the n3-to-n6 ratio in dietary PUFAs: for this reason, the term FADS1 insufficiency more properly describes the functional state of this enzyme in conditions like Western diets, characterized by unbalanced, higher dietary n6/n3 PUFA ratio, which shifts desaturation fluxes toward the synthesis of n6-derived proinflammatory mediators (**Figure 7**). These fluxes underlie the observed discrepancies between functional studies, which measured FADS1 activity as product-to-precursor ratio and found a decreased FADS1 activity, and epigenetic studies assessing FADS1 mRNA or protein, which found an enhanced gene expression, which was however insufficient to metabolize the increased FA substrate load [164].

On this basis, the most widely adopted approach to restore a normal n6-to-n3 PUFA ratio in the liver has been dietary n3-PUFAsupplementation: however, while n3 PUFA supplementation consistently rescued hepatic ER stress, mitochondrial dysfunction and improved liver injury and metabolic parameters in diet-induced models of NASH [96-98,100], in NASH trials n3-PUFA supplementation ameliorated steatosis and cardio-metabolic profile, but did not affect disease

activity and did not improve the most relevant histological features of NASH, i.e., necro-inflammation and fibrosis, which characterize progressive liver disease [245].

There are multiple potential reasons explaining the discrepancy between steatosis improvement and the lack of efficacy on necroinflammation and fibrosis, including a considerable heterogeneity of n3-PUFA type (DHA showed more consistent anti-inflammatory and anti-fibrotic activity that EPA [97,98]), doses and formulations used in different RCTs. More remarkably, while the anti-steatogenic and metabolic effects of PUFAs are a result of direct interaction of PUFAs with nuclear transcription factors (**Supplementary Table 4**), anti-inflammatory and anti-fibrotic effects may predominantly result from the formation of PUFA-derived metabolites like SPMs [164]. The generation of these SPMs requires higher local, hepatic n3-PUFA concentrations, which may not be achieved with usual doses, as most dietary FAs undergo β-oxidation (20–30%) or are stored in adipocytes as TAG (15–80%) [57]. Furthermore, data from patients with NASH indicate that SPMs may be more extensively inactivated by eicosanoid oxidoreductases and there may be a SPM receptor down-regulation in NASH and obesity, resulting in relative SPM resistance [246].

For these reasons, it has been estimated that the efficacious n3-PUFA dose used to improve necroinflammation and fibrosis in mice (150 mg per mouse/day, human equivalent dose 32.5 g/day) is not feasible for human consumption [247].

For these reasons, additional approaches involving nanomedicine techniques are currently being evaluated preclinically to re-equilibrate hepatic n6/n3 ratio, including the introduction of n3 desaturase (*FAT-1)* activity, which can generate n3- from n6-PUFAs and is absent in humans. Such approach, currently at preclinical stage, normalized hepatic n3-PUFA stores and reversed steatosis and necro-inflammatory changes in transgenic mice fed a HFD diet or ethanol [248,249].

Another approach, which achieved clinical stage of development, is the synthesis of structurally engineered fatty acids (SEFAs).

10. Role of structurally engineered fatty acids for the treatment of NASH

Structurally engineered fatty acids (SEFAs) were developed to enhance the pharmacodynamic effects of naturally occurring fatty acids via positional modifications affecting and redirecting their metabolic fate. Three SEFAs are currently under clinical development. The most advanced SEFA candidate, icosabutate [2-(5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaen-1- yloxy) butanoic acid], is a semi-synthetic eicosapentaenoic acid (EPA) derivative currently evaluated for NASH in the Phase 2b randomized ICONA trial (NCT04052516). Icosabutate is structurally designed to avoid incorporation into complex lipids via an ethyl-group in the α- position and to resist β-oxidation via incorporation of an oxygen atom into the β-position (**Table 5**). These modifications confer several benefits over natural LCFAs for the treatment of both hyperlipidemia and NASH (**Table 5**). First, unlike unmodified LCFAs that are esterified into TAG in enterocytes and transported to the periphery in chylomicrons, icosabutate resists esterification, escapes systemic distribution into adipose tissue, skeletal muscle, and other organs and targets directly the liver via the portal vein [250]. Second, high doses of n3-LCPUFAs enhance their own utilization as a cellular energy source, a metabolic fate that is avoided with icosabutate [251]. Finally, icosabutate's resistance to incorporation into hepatocyte cellular membranes and complex lipids may also be advantageous via a reduction in hepatic lipid peroxidation that is observed in response to treatment with unmodified n3-LCPUFAs [252]. Collectively, these structural modifications results in a high hepatic concentration of extracellular, non-esterified, icosabutate and high n3-LCPUFA receptor/pathway targeting [202,203]. *In vitro* studies confirmed that icosabutate is a potent agonist of n3-LCPUFA receptors, including PPAR-α, FFAR1 and FFAR4, is minimally incorporated into membrane phospholipids [204], and has a prominent inhibitory effect on the hepatic pro-inflammatory arachidonic acid cascade [253], much greater extent than unmodified EPA [204]. The latter finding has several potential explanations: high hepatic concentrations of non-esterified icosabutate can compete for enzymes of the AA cascade without prior release from membrane phospholipids. Alternatively, this finding may be secondary to a more potent down-regulation of the inflammatory response with a resultant decrease in PLA2 activity in response to icosabutate supplementation. This possibility is supported by the finding that icosabutate reduces not only

hepatic AA stores but also the conversion of linoleic acid and AA to their oxygenated metabolites, such as hydroxyeicosatetraenoic acids (HETEs), which have been linked to hepatic lipotoxicity, NASH development and progression [203,254]. In addition to decreases in AA oxygenated metabolites, icosabutate has been shown to reduce the hepatic concentrations of multiple other NASH-associated lipotoxic lipid [38] species, including diacylglycerols, bile acids, and ceramides [202,203,204,205]. Consistent with these *in vitro* actions on cellular molecular pathways for lipotoxicity, *in vivo* studies in diverse diet-induced rodent models of NASH demonstrated a reduction in hepatocyte apoptosis, Kupffer cell activation and hepatic myo-fibroblast proliferation, differentiation and pro-fibrotic gene expression (**Table 5**). In an interim analysis of the ICONA trial carried out after 16 weeks of treatment, icosabutate treatment was associated with a significant reduction in markers of liver injury, inflammation, and fibrogenesis along with improvements in glycemic control and atherogenic lipid profile [255].

11. Role of Eicosanoids and Specialized Proresolving Mediators in progression and resolution of liver disease in NASH

Chronic low-grade inflammation is the hallmark of obesity-related disorders and in NASH has adipose tissue and the liver as its major sources and targets at the same time. In visceral adipose tissue, inflammation is supported by an expansion of the pro-inflammatory macrophage pool, which results in insulin resistance, unrestrained lipolysis and release of lipotoxic FAs, pro-inflammatory cytokine and chemokines [256,257,258]. In the liver, prolonged inflammation determines a wound healing process, which involves continued deposition of production of extracellular matrix and fibrogenesis and eventually results in tissue scarring and cirrhosis.

Among different mediators involved in fibrosis induction and resolution in NASH, growing evidence supports a key role for eicosanoids and SPMs.

Eicosanoids (from the Greek term *"eicosa-",* "twenty") are lipid signaling molecules made by the enzymatic or non-enzymatic oxidation of arachidonic acid or other 20C-containing PUFAs,

including AA, DGLA and EPA (**Figure 7**) [259]. Various eicosanoid subfamilies exist, including PGs, TXs, LTs, lipoxins (LXs) and resolvins (Rvs), with heterogeneous and even opposite activities on metabolism, inflammation, and tissue remodeling. Eicosanoids exert their actions through binding to surface G-protein coupled receptors (GPCRs) in an autocrine, paracrine or endocrine manner. Differently from parent FA compounds, eicosanoids do not undergo storage within cells but are readily synthesized from membrane phospholipids following PLA2s activation. Upon their release from cell membranes, n6- and n3-PUFAs are metabolized by the two enzymes COXs, by three lipoxygenases and by cytochrome P450 oxidases [260] (**Figure 8-9).**

SPMs restore normal cell homeostasis and tissue physiology enhances self-limitation and resolution of tissue inflammation and fibrosis in different organs, including the liver (**supplementary Table 5**).

Recent *in vitro* and *in vivo* data showed an imbalanced action of pro-inflammatory eicosanoids and pro-resolving SPMs in NASH, and support restoration of a normal pro-inflammatory eicosanoid/SPM balance as an effective and feasible therapeutic target in this condition [11,261,262]. This objective could be achieved by two strategies, aiming either at proinflammatory eicosanoid antagonization or at restoring pro-resolving SPM content and action.

11.1 Proinflammatory eicosanoid antagonization

11.1.1 Group IVA phospholipase A2 (PLA2) inhibition

PLA2 catalyzes the hydrolysis of membrane glycerophospholipids to release AA which is subsequently metabolized into eicosanoids in the AA cascade, and lysophospholipids [263] (**Figure 2A**). Over 20 PLA2s isozymes exist in mammals and they have been grouped into intracellular (groups IV and VI PLA2s) and secretory isoenzymes (groups I, II, III, V, X, and XII PLA2s). Among them, group IV calcium-dependent cytosolic PLA2α (PLA2α), received much attention because of its wide tissue distribution and its central role in cell metabolism and AA generation [65].

Group IV PLA2α is constitutively expressed but its activity undergoes substantial post-translational regulation and is modulated by diverse extracellular signaling pathways, including cytokines (IL-1, TNF-α, Angiotensin II) and growth factors (i.e., Epidermal Growth Factor, EGF) with pro-inflammatory and pro-fibrotic actions. Human and animal data support a crucial role for liver and adipose tissue group IV PLA2α as a mediator of obesity-related disorders. In HFD mice, PLA2α knockout prevented NAFLD development and pharmacological inhibition of group IV PLA2α with small molecule compounds, including AVX001, AVX002 and ASB14780, reversed steatohepatitis and fibrosis [107,193]. Among these compounds, AVX001 has been tested in patients with psoriasis [264] but not in patients with NASH (**supplementary Table 1**).

11.1.2 5-lipoxygenase(5-LOX)/leukotriene pathway inhibition

5-LOX catalyzes the two-step lipoxygenation of AA to form leukotriene (LT) LTB4 and cysteinyl-leukotrienes (Cys-LTs) LTC4, LTD4 and LTE4, which possess strong proinflammatory activity. In addition, 5-LOX participates together with 15-LOX in the synthesis of the anti-inflammatory and pro-resolving lipoxins LXA4 and LXB4 (discussed below) (**Figure 8**).

5-LOX is expressed mainly in immune cells (neutrophils, eosinophils, monocytes, B-lymphocytes and mast-cells), where LTs are potent pro-inflammatory and chemotactic mediators for circulating monocytes [180].

Liver resident macrophages Kupffer cells express 5-LOX and can secrete LTB4 and cysteinyl-LTs; the latter can also be synthesized by hepatocytes through transcellular metabolism of LTA4 molecules secreted by Kupffer cells [265]. LTs are potent activators of Kupffer cell and HSCs and are emerging inflammatory mediators in NASH: in diet-induced NAFLD mouse models, 5-LOX and its downstream molecules are markedly upregulated [266] and in NAFLD patients the progression from healthy liver to steatohepatitis parallels the increased levels of 5-LOX pathway metabolites. Functional 5-LOX studies support a central role for 5-LOX in liver injury: 5-LOX gene knockout abolished the systemic and hepatic consequences of HFD, including obesity, insulin resistance and NASH [267,268], while treatment with pharmacological 5-LOX inhibitors enhanced AMPK activation, reduced NF-kB activation and ameliorated liver histology in experimental

models of diet-induced NASH [131,133]. Following these encouraging preclinical data, MN-001 (tipelukast), a small molecule 5-LOX and phosphodiesterase (PDE) 3-4 inhibitor and LTD4 receptor antagonist, which provided hepatoprotection in an advanced NASH rodent model [269], has entered clinical development and is currently being evaluated in NASH patients with advanced fibrosis [270]. LT receptor antagonism is a different approach to antagonize proinflammatory LTs and could offer the incremental advantage over 5-LOX inhibition of sparing 5-LOX-mediated synthesis of the SPM lipoxins: consistently, LTB4 receptor 1 (Ltb4r1) antagonism showed similar benefits as 5-LOX inhibition in the liver and adipose tissue [271].

11.1.3 Cyclooxygenase-2 (COX-2) inhibition

COX is a membrane-bound enzyme catalyzing the initial two biosynthetic steps in PG and TX pathways, namely cyclooxygenation and peroxidation.

Of the 2 major COX isoforms, COX-1 is ubiquitously and constitutively expressed throughout the gastrointestinal system, the kidneys, the platelets and vascular smooth muscle, while COX-2 is referred to as the inducible COX isoform, because it is constitutively undetectable, but can be induced by numerous proinflammatory stimuli. However, this rigid distinction has been overcome, as COX-1 can be induced and COX-2 is constitutively expressed in the CNS and the kidneys [45]. Several lines of evidence involve COX-2 in the pathogenesis of liver injury through several biological mechanisms (**supplementary Table 3**). In diet-induced models of steatohepatitis, liver and adipose COX-2 expression is significantly increased, as a result of NF-κB and IL-1 axis activation, and correlates with histological liver disease severity [272,273]; consistently, COX-2 inhibitors attenuated diet-induced NASH and improved hepatocyte autophagy and senescence [274,275]. Recent *in vivo* mouse models demonstrated that the hepatic effects of COX-2 activation are largely mediated by proinflammatory PG generation, including15-deoxy-PGI2 and PGE2 [276,277]: consistently, treatment with PGE receptor antagonists achieved the same degree of metabolic and histological improvement as treatment with the selective COX-2 inhibitor celecoxib [191,278,279,280,281,282,283]. Another interesting implication of COX-2 inhibition is the potential role of COX-2 in carcinogenesis, as PGE-2 biosynthesis by COX-2 appears a crucial

promoter of obesity-associated hepatocellular carcinoma and breast cancer, an effect involving Prostaglandin E receptor 4(PTGER4)-mediated suppression of antitumor immunity and enhanced transcription of CYP-19, CYP-181 and aromatase-catalyzed estrogen biosynthesis [284] (**supplementary Table 3**). Furthermore, other major concerns regarding COX-2 inhibition safety exist: PGE2 synthesis is a central requisite for the initiation of inflammation resolution, as its synthesis switches macrophage polarization from a pro-inflammatory M1 to a pro-resolving M2 phenotype [285,286]. Furthermore, endothelial and renal constitutive expression of COX-2 contributes to the synthesis of the protective PGI2 and it has been suggested that COX-2 inhibition may increase cardiovascular disease risk [287].

11.1.4 Cytochrome P450 (CYP), epoxyeicosatrienoic acids (EETs) and soluble Epoxide Hydrolase (sEH) pathway

Beside cyclooxygenase (COX) and lipoxygenase (LOX) pathways, eicosanoids can be synthesized through the cytochrome P450 (CYP): CYP pathway products can be further modified by epoxygenase or hydroxylase enzymes to synthesize epoxyeicosatrienoic acids (EETs) from arachidonic acid, epoxyeicosatetraenoic acids (EEQs) from EPA or epoxydocosapentaenoic acids (EDPs) from DHA, [288] (**Figure 9**). These epoxy PUFA derivatives act at cellular level through three molecular pathways: two involve their binding to cell-surface receptors, i.e., a specific G-protein coupled receptor that leads to increased cAMP levels and a non-specific receptor which binds also other lipid-soluble agonists; the third putative pathway involves direct intracellular epoxy incorporation into cell phospholipids and interaction with cytoplasmic transport proteins, including FABPs, and nuclear transcription factors, most notably PPAR-γ and NF-κB [289]. By acting through these pathways, epoxy derivatives exert a potent anti-inflammatory, vasodilatory and insulin sensitizing action, reduce ER stress and restore autophagy in different tissues and organs, including the liver, kidney, adipose tissue and heart [290,291]. It has been shown that EEQs and EDPs exert more potent antiinflamatory actions than EETs [291]. PUFA-derived epoxydes are metabolized by the ubiquitous ctosolic enzyme soluble Eypoxide Hydrolase (sEH). sEH forms a homodimer composed of two 60-kDa monomers,

and each monomer has two different enzymatic activity domains: the C-terminal domain, comprising an α/β hydrolase fold with epoxide hydrolase activity which binds to epoxides specifically and converts them to corresponding diols, and the N-terminal with phosphatase activity. The hydrolysis of epoxy compounds to diols attenuates much of their physiological actions.

Perturbations in the cytochrome P450-sEH pathway have been found in NASH and implicated in the pathogenesis of liver injury in this condition.

Hepatic sEH expression is increased and epoxides consensually decreased, in diverse models of diet-induced NASH mice in palmitate-treated hepatocytes as well as in the liver of patients with NASH, paralleling the severity of liver histology and of glucose and lipid metabolism dysregulation [292,293,294]; these abnormalities were prevented or reversed by genetic or pharmacological sEH inhibition or EETs administration [295]. These changes were paralleled by the downregulation of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and chemokines (CXCL1, MCP-1) of SREBP1c and NF-κB expression in hepatocytes and macrophages and upregulation of PPAR-α [295].

On this basis, the safety and efficacy of several small molecule sEH inhibitors, including 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), trans-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid(t-TUCB) and trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB), is being evaluated in different metabolic and related cardiovascular diseases in preclinical and early phase clinical trials [296,297].

11.2 Specialized proresolving mediators (SPMs): key mediators of resolution of inflammation and fibrosis

The inflammatory response must be actively terminated when no longer needed to prevent unnecessary "bystander" damage to tissues. Failure to do so results in chronic inflammation, and tissue destruction. In the recent years it has become clear that resolution of inflammation is not a passive phenomenon, determined by dissipation of inflammatory signals and mediators but rather, it is an active process which is finely triggered and directed by a temporally regulated, sequential secretion of pro-resolving signals. These signals self-limit and resolve the inflammatory process

eventually restoring tissue integrity and function [11,298]. Liver fibrosis is a wound healing response to prolonged, unresolved tissue injury, which is primarily driven by inflammatory and immune mediators [2]. Similar to inflammation, recent experimental evidence showed liver fibrosis can be reversed even at its most advanced stage, i.e., cirrhosis, and that fibrosis reversal occurs through different steps, which may coexist independently of each other [2]. A pivotal role of SPMs in fibrosis reversal, as well as in inflammation resolution, has been recently proposed (**supplementary Table 5**). SPMs are a heterogeneous family of PUFA-derived lipid species that comprise lipoxins (LXs, whose name derives from lipoxygenase interaction products), resolvins (Rvs, from "resolution phase interaction products"), which belong to either E-series Rvs (if derived 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 **(Figure 8).** Unlike their precursors n3-PUFAs, which exert their actions at the micromolar-to-millimolar range, SPMs are active in the picomolar-to-nanomolar range [299].

SPMs expedite the inflammatory response resolution by shifting innate and adaptive immunity cells, i.e., macrophages and T cells, from a pro-inflammatory M1 to a pro-resolving M2 phenotype [300]: specifically, SPMs promote macrophage autophagy [301], enable monocyte migration and clearance of apoptotic cells (efferocytosis), and shift $CD4^+$ T cell phenotype from a pro-inflammatory T_h1/T_h17 to a pro-resolving T_{reg} one [302].

Recent evidence from experimental models of diet-induced fatty liver and from patients with NASH suggests that SPM formation is severely deregulated in the liver and adipose tissue [303,304], and that the administration of different SPMs improved insulin sensitivity, lipid deposition, cell apoptosis and fibrogenesis, resulting in histological improvement of NASH in diverse experimental models of NASH: Lipoxin LXA4, Resolvins RvD1 and RvE1, MaR1 and Protectin D1 (PD1) improved adipose tissue inflammation and insulin resistance, reduced hepatocyte ER stress-induced apoptosis through Akt and AMPK activation and JNK inhibition and through enhances adiponectin expression[305,306,307,308,309,310]. Beside the pro-resolving effects on inflammation, SPM demonstrated also direct anti-fibrotic and metabolic effects in various

preclinical models of organ fibrosis : Resolvin D1, Resolvin E1, Protectin DX and MaR1 attenuated fibrosis progression and reversed established fibrosis [311, 312] through mechanisms only partly identified, which include: downregulation of TGF-β1/Smad2/3-induced epithelial-to-mesenchymal transition (EMT) of epithelial cells, and restoration of physiological Matrix metalloproteinase (MMP) levels, which are necessary for extracellular matrix resorption [313, 314,315,316].
RvD1 and MaR1 also enhance mitochondrial oxidative phosphorylation, fatty acid β-oxidation and bioenergetic metabolic flux, providing hepatic metabolic protection from steatotic, pro-inflammatory and fibrogenic insults [317].
A critical obstacle to be overcome for the pharmacological development of SPMs is the attainment of therapeutically effective local SPM concentrations at the sites of injury, as SPMs are rapidly inactivated by eicosanoid oxidoreductases and SPM receptors seem down-regulated in obese patients [174]. Several pharmacological strategies to enhance biological activity and selective delivery of SPMs via nanomedicine techniques to target organs are being investigated. These include the synthesis of oxidoreductase-resistant SPM analog mimetics and the incorporation of SPMs into liposomes, with promising results in preclinical models: accordingly, the synthetic oxidoreductase-resistant RvD1 analogue benzo-diacetylenic-17R-RvD1-methyl ester (BDA-RvD1) showed a 3.5-fold higher potency than the natural compound [318], while liposomal Lipo-RvD1showed enhanced selective clearance by hepatic macrophages [319]. The safety and efficacy of the synthetic RvE1 analog RX10045 is currently being investigated in patients with dry eye disease [320].

12. Future perspectives

The health-related burden of NASH is increasing worldwide along with the obesity epidemic and NASH is becoming the leading indication for liver transplantation. There are no approved treatments for NASH, as lifestyle intervention have limited effectiveness. Proposed pharmacological options do not seem to reverse more advanced fibrosis stages of disease and therefore are unlikely to change the natural history of the disease or are encumbered by unwanted effects [321]. Treatment of NASH is challenging, as progression from steatosis to NASH and

fibrosis is likely a multi-factorial process, involving varied molecular pathways operating in different patient subsets and at different stages of liver disease. Therefore, interventions that may be effective at reversing simple steatosis or mild NASH may not be able to reverse more advanced fibrotic stages. Within this context, new lipid species, SPMs, have been implicated in steatohepatitis and fibrosis resolution in experimental models and observational human studies: however, several issues regarding a definite caudal role of SPMs in inflammation and fibrosis resolution in NASH remain, due to current challenges in analytical methods to detect and quantify these metabolites and to knowledge gaps regarding the signaling of the proposed G-protein-coupled SPM receptors [322].

A decade has passed since the first lipidomic analyses in NAFLD [86] and our knowledge of NASH as a lipotoxic disease has considerably expanded the spectrum of new lipid species and pathways involved in NAFLD progression.

Furthermore, the discovery of the potential of EVs as noninvasive markers for NASH staging and as therapeutic targets has allowed substantial advances in our understanding of NASH pathophysiology, diagnosis and treatment. These advances in basic science are expected to make up to clinical stage of development, as to date the only lipidomic approach to NASH, i.e., ω-3 PUFA supplementation, still has to prove its effectiveness at arresting or reversing NASH progression. Hence these advances pose a twofold challenge for future research: first, the discovery of safe and effective pharmacological tools to antagonize the diverse lipotoxic species. The second issue, once several drugs receive approval for NASH, will be the selection of the optimal therapeutic strategy for each patient: in this context recent advances in lipidomics, genetics and epigenetics systems biology technologies will likely enable more individualized treatments tailored to metabolic profiles.

Table 1. Lipidomic studies in NAFLD patients

Author	**Population (n)**	**Tissue**	**Technique**	**Findings NASH versus simple steatosis**
Puri 2007 [7]	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 [8]	Non-cirrhotic NASH(n=50) NAFL(n=25) Control(n=50)	Plasma	TLC	↓DHA//DPA ratio ↓ MUFAs ↓ plasmalogens ↑5-, 8-, 11-, 15-HETE
Barr 2012 [11]	NAFLD(n=246) NASH(n=131)	Plasma	UPLC−MS	↑15-,12-HETE, 5-, 9-,11-HETE
Gorden 2015 [12]	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[14]	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 [15]	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
Hall 2017 [17]	NAFL(n=11) NASH(n=9)	Liver	LC-MS/MS	↑MUFA(18:1) ↑LOX-derived 9-/13-HODE
Velenosi 2022 [18]	NAFLD(n=37) Controls(n=10)	Plasma	UPLC-MS	↑ postprandial hepatic-derived DAG
Zhu 2022 [19]	NAFL(n=43) NASH(n=40)	Urine	LC-MS/MS	↑urinary EV content in: SFA (18:0), LPC (22:6/0:0), and MUFA (18:1) ↓ PI (16:0/18:1)

Abbreviations: AA: arachidonic acid; TLC: thin-layer chromatography; EV: extracellular vesicles; 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; PI: Sph: sphingosine; UPLC: ultra-performance liquid chromatography.

Table 2: Poly (ADP-ribose) polymerases (PARPs) and liver injury in NASH.

PARP activators	PARP enzyme	Molecular target	Cellular effect	Biological effect	Therapeutic tool
Hepatocyte					
ROS-mediated DNA damage; Oleic acid; oxLDLs	PARP1 PARP2	LXRα, PPARα, AMPK, insulin receptor PARylation NAD+ depletion by: ●↑NAD+ consumption by polyADPrybolylation ●↓NAD+ synthesis through anaerobic glycolysis (Pang 2013; Fang 2014; Pang 2015)	SIRT1, LXRα, PPARα and AMPK inactivation insulin receptor downregulation energy store (ATP) depletion	↓ FFA oxidation ↓ insulin sensitivity cell necrosis	panPARPi natural (puerarin) or synthetic (Olaparib, PJ34, AIQ)
	PARP1 PARP2 PARP7	PGC1α inactivation by PARylation (Wang 2020)	Impaired mitochondrial biogenesis Increased gluconeogenesis		
	PARP1	SREBP-1c upregulation by PARylation (Huang 2018)	↑ DGAT1 and DGAT2 expression	↑*de novo* lipogenesis	
SCFA	PARP7	AHR inactivation by PARylation (Jin 2017)	Impaired toxic lipid species detoxification	↑lipotoxicity	
ROS, endotoxin, DAMPs	PARP1	NF-κB PARylation and activation	Increased pro-inflammatory cytokine and chemokine expression	↑inflammation	
Kupffer cells and macrophages					
DAMPs ROS	PARP1	PARylation of NF-κB subunit p65/RelA (Bohio 2019)	↑transcription of NF-κB-regulated genes Proinflammatory activation, chemotaxis and secretion of	Inflammation	

		HMGB1 PARylation→ nuclear HMGB1 release→TLR-2/4 binding (Yang 2014)	proinflammatory cytokines		
T-lymphocyte					
	PARP1/2	Chromatin PARylation Foxp3 inactivation Gu 2020; Nasta 2010)	↑T cell differentiation into Th1 and Th2 ↓T cell differentiation into Treg	Inflammation	
Adipocyte					
	PARP1	C/EBPβ and C/EBPδ PPARγ1-2	Adipocyte differentiation LPL, CD36, aP2TG, perilipin, adipokines (e.g., leptin, adiponectin)	adipocyte hyperplasia and hypertrophy	
		PGC1α inactivation by PARylation (Wang 2020)	Impaired mitochondrial biogenesis Increased gluconeogenesis	Insulin resistance	
		NF-κB-mediated pro-inflammatory cytokine(CRP, IL6) and chemokine (MCP-1) expression	Macrophage, Th1, Th2 infiltration	Low-grade adipose tissue inflammation	INO1001
Gut microbiota					
		↓ Treg in mucosal lamina propria (Larmonier 2016)	unhealthy gut microbiota composition and leaky gut	Low-grade endotoxemia	

Abbreviations: AHR: aryl hydrocarbon receptor; AMPK: AMP-activated kinase; ROS: Reactive oxygen species; SIRT1: sirtuin; KC: Kupffer cell; LPL: lipoprotein lipase; LXR: liver X receptor;

PARylation: polyADPrybolylation; HMGB1: high-mobility group box 1; PGC1α: Peroxisome proliferator activated receptor cofactor-1α ; PPAR: peroxisome proliferator activated receptor.

Table 3. Main features of Lysosomal Acid Lipase Deficiency (LAL-D) and diagnostic hints in patients with suspected NAFLD.

Clinical features (prevalence)		**Comments**
Liver abnormalities (86%)	Progressive liver damage from steatosis to cirrhosis	LAL-D should be considered in suspected NAFLD if: -non-obese individuals or if -no improvement in liver injury despite appropriate weight loss or statin therapy(Himes, Bernstein)
Dyslipidaemia (87%)	Increased LDL-C and/ reduced HDL-C, with or without hypertriglyceridaemia	Features differentiating LAL-D from Familial Hypercholesterolemia: -no family history of dyslipidemia and/or premature CAD in first-degree relatives -no confirmed mutation -liver disease -sustained cholesterol elevation despite lipid-lowering therapy(Quinn)
Cardiovascular (43%)	Accelerated atherosclerosis	No conclusive evidence for an increased cardiovascular mortality in LAL-D
Spleen (36%)	Splenomegaly, hypersplenism-related pancytopenia	DBS-determined LAL activity is influenced by peripheral leukocyte and platelet count (Tovoli)
Intestinal (22%)	Malabsorption, growth retardation	

Adrenal gland (50%)	Calcifications	Highly suggestive for LAL-D
Liver imaging		**Comments**
^{1}H-MRS	Identifies and quantifies hepatic CE accumulation associated with LAL-D.	Non-invasive method to suggest the presence of LAL-D and monitor treatment response in patients with LAL—D
Liver histopathology		**Comments**
H&E staining	Diffuse, intense microvescicular steatosis	Non-specific (Hulkova)
UV light autofluorescence	intracellular ceroid accumulation in macrophages/Kupffer cells and the absence of lipopigment in hepatocytes	Highly specific
immunohistochemistry	luminal (cathepsin D) and membrane (LAMP-1, LAMP-2, LIMP-2) lysosomal markers around lipid vacuoles	Highly specific

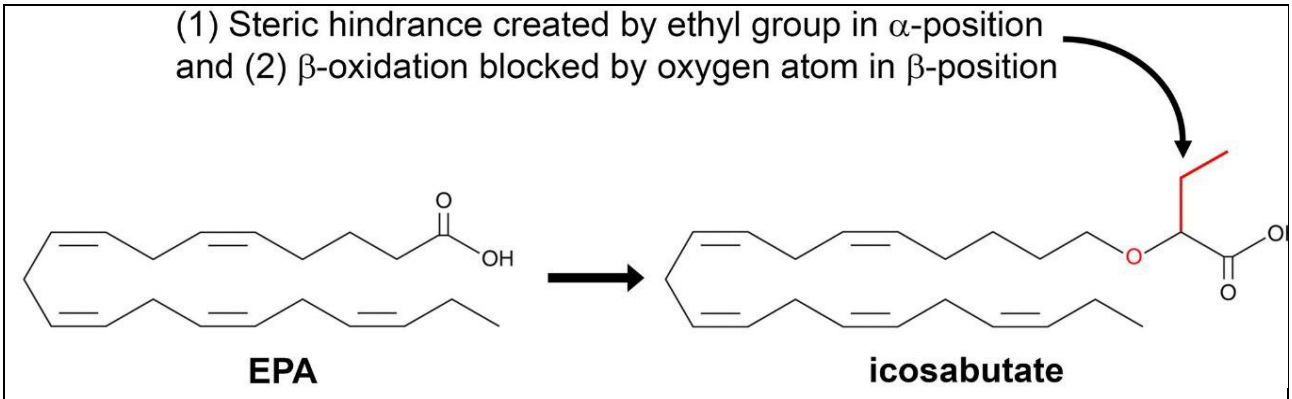
Table 4. Extracellular vescicles (EVs) associated with liver injury in NASH.

Hepatocyte-derived EVs						
Trigger of EV release	**EV type**	**Cargo**	**Cellular effect**	**Biological effect**	**Therapeutic target**	**Study**
SFA→caspase-3 activation	MP	Vaninin-1	Vaninin-1-dependent uptake and activation of Ecs	angiogenesis	Caspase-3 inhibitors, anti-Vaninin-1 antibodies	Povero 2013
Palmitate	MP	miR-128-3p	PPAR-γ down-regulation and HSC activation	Fibrogenesis	antagomiR-128-3p	Povero 2015
Palmitate, LPC→ MLK3/STAT1 activation	Ex, MP	CXCL10	KC activation, macrophage recruitment	Inflammation	MLK3 inhibitors, STAT1 inhibitors,anti-CXCL10 Abs	Ibrahim
Palmitate, LPC→ROCK1 activation	Ex, MP	TRAIL	Bone marrow-derived macrophage activation	Inflammation	ROCK1 inhibitors	Hirsova
Cholesterol	Ex	miR-122-5p	Macrophage M1 polarization	Inflammation	anti-miR-122-5p	Zhao
Palmitate	Ex MP	Ceramide	Macrophage chemotaxis via formation of S1P	Inflammation	sphingosine kinase inhibitors, S1P receptor antagonists	Kakazu
Palmitate→ sphingosine kinase 1/2 activation	Ex, MP	S1P	Macrophage chemotaxis	Inflammation	sphingosine kinase inhibitors, $S1P_1$ receptor antagonists	Liao
Palmitate	MP	mtDNA	TLR-9-mediated KC activation	Inflammation	TLR9 antagonists	Garcia-Martinez I
Activated CD4+ and CD8+ T cell- derived EVs						

Proinflammatory stimuli	MP	CD147	NF-κB activation in HSCs	Fibrolysis upregulation Fibrogenesis downregulation	Activated T cell-derived EVs	Kornek
Myofibroblast- derived EVs						
Proangiogenic stimuli	MP	Hedgehog	EC activation	angiogenesis	Hedgehog antagonists	Witek
Proangiogenic stimuli -	MP	VEGF-A	EC activation	angiogenesis	VEGFR-R2 antagonists	Lemoinne
Activated HSC- derived EVs						
Profibrogenic stimuli	ExC	CCN2	Quiescent HSC activation	Fibrogenesis	CCN2 inhibitor	Charrier
Adipoocyte-derived EVs						
Palmitate, cholesterol	Ex	miR-34a	suppression of Krüppel-like factor 4 (Klf4) expression and of macrophage M2 polarization	Systemic and hepatic inflammation and insulin resistance	antagomiR-34a	Pan
Palmitate	MP	miR-155	SOCS1 suppression→STAT 1 activation→M1 macrophage polarization	Adipose and systemic inflammation	antagomiR-155	Zhang

Abbreviations: Ex: exosome; MLK3: mixed lineage kinase 3; MP: microparticles; CCN2: connective tissue growth factor (CCN2) CXCL10: chemokine (C-X-C motif) ligand 10; EC: endothelial cell; KC: Kupffer cell; HSC: hepatic stellate cell; LPC: lysophosphatidylcholine; ROCK1: Rho-associated protein kinase 1; S1P. sphingosine-1-phosphate;STAT: signal transducer and activator of transcription; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand;

Table 5. Main molecular, kinetic and biological differences between natural EPA and icosabutate



Limited potential as liver targeted drugs due to systemic distribution, incorporation into complex lipids, and use as energy in peripheral tissue	Designed to facilitate direct hepatic uptake and minimize systemic distribution & accumulation in complex lipids
Utilisation as energy source in liver	Unavailable for hepatic β-oxidation
Required dose for activation of FFAR not feasible for human consumption	Liver-targeted: achieves concentrations required to activate FFARs
Increases liver's susceptibility to oxidative stress	Reduces hepatic oxidative stress
No effect on markers of liver damage, inflammation and fibrosis	↓ hepatocyte apoptosis ↓ hepatocyte macropages and Kupffer cell activation ↓hepatic myofibroblast proliferation, activation to HCSs and pro-fibrotic cell expression

Supplementary Table 1. Pharmacological agents targeting lipotoxicity in NASH

Class	Agent	Mechanisms of lipotoxicity targeted by the drug	Development stage in NASH	Trial name/ clinicaltrials.gov ID
ACLY inhibitors	Bempedoic Acid	ATP-Citrate Lyase inhibition	preclinical	-
ACC1/2 Inhibitors	GS-0976 Fircosostat PF-05221304		II	NCT02856555 NCT03987074 NCT03776175 NCT03436420
FASN inhibitors	TVB-2640 FT-4101	FASN	II	NCT04004325 NCT03938246 NCT02948569 NCT03938246
SCD-1 inhibitors	Aramchol	SCD-1 inhibition	III	NCT01094158, NCT02279524 NCT02684591
DGAT-1/2 inhibitors	Pradigastat PF-06865571, IONIS DGAT2Rx(siRNA) SNP-610(CYP2E1 inhibitor)	IONIS DGAT2Rx(siRNA) SNP-610 (CYP2E1 inhibitor)	II	NCT01811472 NCT03776175 NCT03334214 NCT03468556
n3 PUFA	**ω3 fish oil**		II	NCT00681408, NCT01154985, NCT01992809
FXR	Obeticholic acid	FXR activation	III	REGENERATE trial (NCT02548351)
	Px-104	FXR activation	IIa	NCT0199910

	LMB763	FXR activation	IIa	NCT02913105
	Gs-9674	FXR activation	IIa	NCT02854605
LXR	25HC3S	LXR/SREBP-1c inhibition NF-κB inhibition	Ib	ACTRN12615000267550
PPAR-α/δ	Elafibranor	PPAR-α/δ activation	IIa	NCT01694849
PPAR-δ	MBX-8025 (seladepar)	PPAR-δ activation	Preclinical	
PPAR-α/γ	Saroglitazar	PPAR-α/γ activation	IIa	CTRI/2010/091/000108
PPAR-α/δ/γ	IVA337 (Lanifibranor) Chirglitazar	PPAR-α/δ/γ activation	IIa IIa	NATIVE(NCT03008070) NCT05193916
PARPs	Puerarin, olaparib, AIQ, PJ34	PARP inhibition	Preclinical	-
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, CCBs	Inhibitors of ceramide hydrolysis from sphingomyelin by SMase	Preclinical	-
S1P	fingolimod	S1P type 1 receptor (SIPR1) antagonist	Preclinical	-
	VPC23019	S1PR(1,3)) antagonist	Preclinical	-
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; FASN: fatty acid synthase; 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; PARPs: Poly (ADP-ribose) polymerases

∗ only RCT with post-treatment liver histology are reported

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, CCBs	Inhibitors of ceramide hydrolysis from sphingomyelin by SMase	Preclinical	-
S1P	fingolimod	S1P type 1 receptor (SIPR1) antagonist	Preclinical	-
	VPC23019	S1PR(1,3)) antagonist	Preclinical	-
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	-

Supplementary Table 2. Nuclear transcription factors involved in lipotoxicity in NASH.

Farnesoid X Receptor (FXR)		
Cell type	**Molecular mechanism**	**Biological action**
Hepatocyte	↓ SREBP-1c-mediated *de novo* lipogenesis ↑PPAR-α-mediated FFA β-oxidation ↓gluconeogenesis	↓ hepatic steatosis
	↑ IRS-1 phosphorylation and coupling with the PI-3K ↑ AdipoR2 expression	Enhanced insulin sensitivity
	↑CYP7A1 andABCG5/G810 expression	Enhanced bile acid synthesis and cholesterol excretion into bile
	↓ hepatic lipase activity and ApoC-III/apoA-1 synthesis ↓ VLDL secretion and HDL-C synthesis	Reduced plasma HDL-C
	↑ ApoC-II synthesis and uptake of VLDL	Reduced plasma TG
	↓ 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 ↑ adiponectin and AdipoR2 expression ↓ TNF-α secretion	Improved adipose tissue dysfunction
Enterocyte	Enhanced gut barrier function and secretion of antibacterial factors angiogenin, iNOS, IL-18	Reduced bacterial endotoxemia Increased bile acid synthesis and fat oxidation

	Enhanced FGF-19 secretion	
Liver X Receptor (LXR)-α		
Cells	**Molecular targets**	**Biological effect**
Hepatocyte, enterocyte, macrophage	↑ SREBP-1c- and ChREBP-mediated *de novo* lipogenesis	↑ hepatic steatosis and VLDL secretion
	↑ CYP7A1 expression	↑ cholesterol conversion to bile acids
	↓ NPC1L1 expression	↓ intestinal cholesterol absorption
	↑ macrophage, intestinal and hepatic ABCG5/G8 expression	↑ cholesterol reverse transport and excretion into bile and intestine
	↑ 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 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 and TNF-α	↓ hepatic inflammation
HSC	↓ HSC activation	↓ hepatic fibrogenesis
Peroxisome Proliferator-Activated (PPAR)-α		
Cell type	**Molecular mechanism**	**Biological action**
Hepatocyte, Miocyte, Adipocyte	↑ expression of CPT1A and enzymes involved in mitochondrial and peroxisomal ß-oxidation, ω-oxidation and ketogenesis ↑ FATP, CD36, L-FABP activity	Improved hepatic steatosis and insulin resistance Enhanced FFA uptake

	↑ LPL activity and reduced apoC-III ↑ apo-AI/apo-AII synthesis	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	Improved hepatic steatosis 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	Enhanced insulin sensitivity and subcutaneous fat storage

	↑ insulin signalling and adiponectin secretion ↑ GLUT1/GLUT4 translocation ↑ release of pro-inflammatory FFAs and cytokines TNF-α, resistin and IL-6	Enhanced glucose disposal and FFA oxidation Reduced 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	M1-to-M2 phenotype switching ↓ NF-κB activation	Anti-inflammatory and pro-resolving actions
HSC	Inhibition of TGFβ-1/Smad3-signaling pathway Induction of cell apoptosis	Anti-fibrotic actions

Abbreviations: ABC. ATP-binding cassette; CYP7A1: Sterol 7α hydroxylase; NPC1, NPC2: Niemann-Pick C1, C2; NPC1L1: Niemann-Pick C1-like 1; PPAR: peroxisome proliferator-activated receptor; 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

Supplementary Table 3. Mediators of lipotoxicity generated by group IVA phospholipase A2 and cyclooxygenase (COX)-2 in NASH

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-1cactivation→↑ *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 cell, macrophage:	↓ Akt activation	Insulin ressitance

↑ oncostatin M → ↑ SOCS3 secretion by hepatocytes →↓ CPT1 in hepatocytes ↑ M1-to-M2 polarization switch	↓ mitochondrial FFA β-oxidation Pro-resolving phenotype of macrophage	Steatosis resolution of inflammation
HSCs: ↑ PGE2 secretion→↓ regulatory T cells (Treg) activation	↓ antitumor immunity	HCC progression
Adipocyte: ↑ pro-inflammatory cytokine and chemokine MCP-1 secretion	macrophage recruitment	Enhanced induction of inflammation

Abbreviations:ABCA1: **A**TP-binding cassette transporters A1; ACC: acetyl-CoA carboxylase; AMPK: adenosine-monophosphate kinase; Ccl3 : chemokine(C-C motif ligand 3 ; CD36: cluster of differentiation-36; CPT-1: carnitinepalmitoyltransferase-I**;** ; ER: endoplasmicreticulum; FAS: fattyacid synthase; FFA: free fattyacids; FXR: farnesoid X-receptor; GLUT: glucose transporter; HMG-CoAR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase; IL: interleukin; IRS-1: insulinreceptor substrate-1; LDL: low-density lipoprotein; LDL-R: low-densitylipoproteinreceptor; LPA: lysophosphatidic acid; MCP-1: monocyte chemotactic protein-1; NO: nitric oxide; NOX: NADPH oxidase; ; PGC-1α: peroxisomeproliferator-activatedreceptor-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;

Supplementary Table 4. Molecular targets of Polyunsaturated Fatty Acids (PUFAs)

Hepatocyte			
Type of PUFA	**Transcription factor/cellular pathway**	**Cellular molecular mechanism**	**Biological effect**
EPA, DHA	↑ FFAR1(GPR40) activation ↑FFAR4(GPR120) activation	↑ERK-mediated mitophagy	↓ oxidative stress
EPA, DHA	↓ LXR/SREBP-1c activation	↓ *de novo* lipogenesis	↓ hepatic TAG
EPA, DHA	↓ ChREBP activation	↓ *de novo* lipogenesis ↓ glycolysis	↓ hepatic TAG
EPA, DHA	↓ SREBP- activation	↓ cholesterol synthesis	↓ hepatic cholesterol
EPA, DHA	↑PPAR-α activation	↑ fatty acid β-oxidation	↓ hepatic TAG
DHA	↓ NF-κB activation [46]	↓ apoptosis ↓ pro-inflammatory IL-1 secretion	↓ hepatic necro-inflammation
DHA	↓ NLRP3 inflammasome activation[46]	↓ caspase-1 activation ↓ IL-1 secretion	↓ necro-inflammation
DHA, EPA	↑ FXR activation	↓ cholesterol and FA synthesis	↓ hepatic TAG and cholesterol
DHA, EPA	↓HNF-4α activation	↓ VLDL assembly and secretion	↓ plasma TAG
DHA	↓Toll-like receptor-2/4/9 [48]	↓proinflammatory cytokine secretion	↓necroinflammation
DHA	↓ NOX expression[48]	↓ superoxide/hydrogen peroxide production	↓ oxidative stress
Macrophage/Kupffer cells			
DHA EPA	↑ PPAR-γ activation ↑ GPR120 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; TAG: triacylglycerol

Supplementary Table 5. Specialized Proresolving Mediators (SPMs) and hepatic fibrosis resolution in NASH

Step 1: Cessation of hepatic chronic inflammation **(allowing hepatocyte recovery and modulating the microenvironment)**		
SPM	**Molecular mechanism**	**Cellular pathway**
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
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
Step 2. Phenotype switch of hepatic immune cells from pro-inflammatory to restorative		
SPM	**Molecular mechanism**	**Cellular pathway**
MaR1	**Kupffer cell:** ↓ PA- and hypoxia-induced ER stress	↑efferocytosis of apoptotic cells and cellular debris ↓ LTB4 and TNF-α secretion
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
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
Step 3. Inactivation of fibrosis deposition by myofibroblasts		
SPM	**Molecular mechanism**	**Cellular pathway**
LXA4, RvD1, RvE1, MaR1	↓TGF-β1/Smad2/3-axis activation	↓ epithelial-to-mesenchimal transition (EMT) and HSC activation
Step 4. Extracellular matrix degradation		
SPM	**Molecular mechanism**	**Cellular pathway**
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

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;

FIGURE LEGENDS

Figure 1. Lipidomics workflow and methodology

Panel A. General scheme showing the relationships of the lipidome to the genome, transcriptome, proteome and metabolome. Lipids also regulate protein function and gene transcription as part of a dynamic "interactome" within the cell.

Panel B. Workflow of Lipidomic Analysis. Lipidomic analysis of biological samples includes sample preparation, mass spectrometry-based analysis (i.e., MS data acquisition), and data processing.

Lipidomic analysis by mass spectrometric techniques includes targeted and untargeted (ie, systems-level) lipidomics. In targeted lipidomics, a pre-separation using UPLC/HPLC for one particular lipid class is developed. The shortcoming of targeted lipidomics is that it focuses on one or a few specific lipid classes, while the lipidome comprises over 180000 lipids and most lipid changes occurring over the course of a disease cannot be anticipated a priori. Hence, a discovery-based/systems level analysis is required to identify lipids that are not included within a targeted lipidomic platform, and systems-level or untargeted lipidomics is more efficient than targeted lipidomics in discovery-based research.

In untargeted lipidomics, quadrupole time-of-flight (QTOF) mass spectrometry is often employed. Although this technique offers a broader mass range and, it has significantly lower sensitivity than a targeted lipidomic approach, and can thus assess only high abundance lipids. Furthermore, the accuracy of quantitation is limited in untargeted analyses. Therefore, a combination of untargeted lipidomics with targeted methods has been employed to cross-validate findings from each technique.

Abbreviations: APCI: atmospheric pressure chemical ionization; ESI: electrospray ionization; HPLC: high pressure liquid chromatography; MALDI: Matrix-Assisted Laser Desorption/Ionization; MS: mass spectrometry

Figure 2. Mechanisms underlying saturated fatty acid (SFA) and lysophosphatidylcholine (LPC) lipotoxicity.

Panel A. Pathways of saturated fatty acid (SFA) and lysophosphatidylcholine (LPC) lipotoxicity in hepatocyte.

SFAs activate TLR-4 and death receptor TRAIL-R2.

TLR-4 upregulates Nuclear Factor (NF)-κB-mediated synthesis of pro-inflammatory cytokines pro-IL-1 and pro-IL-18, TNF-α, IL-6, and stress kinases like JNK.

TRAIL-2 pathway triggers caspase 8-mediated activation of caspases 3, 6 and 7, which execute apoptosis, either directly or through MOMP and subsequent cytochrome c release.

MOMP is executed by engagement of proapoptotic Bcl-2 family proteins Bim, Bid, Bakand Bax. Bak and Bax oligomerize to form pores (Mitochondrial Apoptosis-induced Channel, MACs) on the mitochondrial outer membrane. Caspase 8 cleaves Bid, a member of the BCL-2 family, into its truncated form (tBid), which translocates to mitochondria outer membrane, and induces intramembranous BAK oligomerization into a pore and consequent cytochrome c efflux.

SFAs can also induce MOMP by enhancing expression of the proapoptotic Bcl-2 family protein Bim through upregulation ofFoxO3a, and activation of PUMA by activation of JNK and ER stress.

Additionally, SFAs also initiate the lysosomal pathway of apoptosis by inducing Bax-mediated lysosomal permeabilization and subsequent cathepsin B release.

Beside these membrane receptor-mediated mechanisms of lipotoxicity, SFAs can also enter the cell and trigger ceramide-mediated endoplasmic reticulum (ER) stress and NLRP3 inflammasome, and JNK activation.

ER stress upregulates CHOP-mediated PUMA expression and induces the release of Extracellular vesicles (EVs), which exert their lipotoxicity through their different cargoes: CXCL10 and ceramide-enriched EVs are chemotactic for circulating monocytes, TRAIL-enriched EVs contribute can activate macrophages, and miR-128-3p-laden EVs activate HSCs to fibrosis deposition.

JNK is a mitogen-activated protein kinase(MAPK) that induces insulin resistance by inactivating Insulin Receptor Substrate(IRS)-1, impairs mitochondrial respiration and promotes ROS generation

by interacting with the outer membrane mitochondrial protein Sab (SH3BP5), downregulates PPAR-α-mediated FGF)-21 expression and fatty acid ß-oxidation, and activates the proapoptotic proteins PUMA and Bim.

Lysophosphatidylcholine (LPC) is generated intracellularly by PLA2. Lipotoxic mechanisms of LPC largely overlap with those of SFAs. An additional mechanism of lipotoxicity is membrane PC depletion, which disrupts plasma membrane integrity and causes hepatocyte apoptosis and the release of proinflammatory lipid molecules.

Panel B. Mechanisms of SFA and LPC lipotoxicity in hepatic non-parenchymal cells.

SFAs activate TLR-4 on Kupffer cells and macrophages and on HSCs, promoting activation of these cells through different pathways: in macrophages and Kupffer cells, TLR-4 pathway activation triggers NOX-2-mediated ROS generation, and upregulates NF-κB pathway.

NF-κB stimulates pro-inflammatory and profibrotic cytokine and chemokine secretion.

In HSCs, TLR-4 removes the membrane TGF-β1 pseudoreceptor Bambi, thereby sensitizing HSC to TGF-β1-mediated activation.

EVs carrying different cargoes are released by hepatocytes and activate Kupffer cells, macrophages and HSC, while LPC is converted by the extracellular enzyme autotaxin to LPA, a potent activator of HSCs.

Abbreviations: ASC: Apoptosis-associated speck-like protein containing a CARD; BID: pro-apoptotic BCL-2 interacting domain; Bim: Bcl-2 protein family member; CXCL10: C-X-C motif ligand; Cer: Ceramide; CHOP: CAAT/enhancer binding homologous protein; ER, endoplasmic reticulum; FoxO3a: forkhead box-containing protein, class O member 3a; JNK: c-Jun N-terminal kinase;LPA: lisophosphatidic acid; LPC: lysophosphatidylcholine; MCP: monocyte chemoattractant protein; MOMP: mitochondrial outer membrane permeabilization; NLRP3: nucleotide-binding and oligomerization domain-like receptor pyrin domain-containing protein; 3PUMA: p53-upregulated modulator of apoptosis; TRAIL: TNF-related apoptosis-inducing ligand; SFA, saturated fatty acid;

TNFα: tumor necrosis factor α; NF-kB: Nuclear Factor-kB; PC: phosphatidylcholine; ROS: reactive oxygen species;

Figure 3. ACP, acyl carrier protein; Fatty acid synthase (FASN). Fatty acid synthesis is initiated by malonyl-CoA. The different iteration of the cycle following the action of the four enzymes of the complex (β-ketoacyl synthase, KS, β-keto reductase, KR, dehydratase, DH, enoyl reductase, βER) add two atoms of carbon to produce fatty acyl-CoA.

Figure 4. Role of lysosomal acid lipase (LAL) in hepatocyte lipid metabolism (Panel A) and of LAL-deficiency in NASH pathogenesis (panel B).

Panel A: LAL hydrolyzes cholesteryl ester (CE) and triglyceride (TG) in the lysosome to release fatty acids (FAs) and free cholesterol (FCHOL). Modified low-density lipoprotein (mLDL) internalized through scavenger receptor-mediated endocytosis is an important source of CE and TG for lysosomal hydrolysis. The hydrolyzed FAs and FCHOL can be re-esterified and form lipid droplets in the endoplasmic reticulum for storage. Lipid droplets can be delivered to the lysosome for LAL-mediated hydrolysis via autophagy to provide energy supply and maintain cellular homeostasis. The engulfed apoptotic cells by macrophages through a process called efferocytosis also deliver neutral lipids to the lysosome, and LAL is essential for maintaining the efferocytosis capacity of macrophages. The lipolytic products of LAL have active biological roles. Hydrolyzed FAs are substrates for fatty acid oxidation (FAO) and synthesis of very low-density lipoprotein (VLDL). CE-derived FAs also provide precursors for the synthesis of lipid mediators that have a broad spectrum of functional impact on inflammatory response and resolution. Lysosomal flux of FCHOL is essential for the production of oxysterol that triggers the activation of liver-X-receptor (LXR), resulting in increased ABCA1 expression and cholesterol efflux to remove cholesterol from the cells. Under normal circumstances, oxysterol (oxidized derivatives of cholesterol)-dependent activation of the nuclear liver X receptor (LXR), acting on the promoter of the ABCA1 gene, leads to an expression of the ABCA1 gene.

Panel B

The abnormal lipid profile in patients with LAL-Deficiency (LAL-D) (elevated serum total cholesterol, high LDL-C, low HDL-C, elevated triglycerides) can be explained by a reduction in the formation of mature HDL (α-HDL particles). Lipidation of apolipoprotein A-I (apoA-I) by the membrane lipid transporter ATP-binding cassette transporter A1 (ABCA1) is the rate-limiting step in HDL particle formation and the rate of release of cholesterol from late endosomes or lysosomes is a major regulator of ABCA1 expression and activity. In LAL-D, the trap of cholesteryl esters in the lysosomes leads to reduction of free intracellular cholesterol and thus decreased oxysterol formation and the resultant reduced activation of ABCA1 expression as well as decrease of free cholesterol in the subcellular compartments and in the plasma membrane that is available for high density lipoprotein (HDL) particle formation. In the presence of the LAL defect, esterified cholesterol and triglycerides accumulate in the lysosome, and the reduced availability of free cholesterol and fatty acids activate SREBPs. This results in increased intracellular cholesterol synthesis by the action of HMG-CoA-Reductase, esterification of cholesterol by the action of ACAT on VLDL, increased uptake of circulating LDL.

Kupffer cells accumulate FCHOL through uptake of oxidized LDLs by scavenger receptors CD 36 and SR-A and become activated, secreting proinflammatory adipokines (IL-1, TNF-α) and the profibrogenic cytokine Transforming Growth Factor (TGF)-β1, which activates hepatic stellate cells (HCS), promoting hepatic fibrosis. The ability of intracellular free cholesterol overload to directly activate HSCs, without the mediation of KCs through the toll-like receptor (TLR)-4-dependent pathway and trigger hepatic fibrogenesis has been well-documented in mice on high-cholesterol diets and in Niemann–Pick type C1-deficient mice, the latter spontaneously accumulating intracellular free cholesterol.

HSC accumulate cholesterol through oxLDLs uptake by LOX-1 receptors and possibly by other yet poorly characterized receptor and non-receptor pathways. High intracellular cholesterol levels up-regulates toll-like receptor (TLR)-4, which in turn down-regulates, through the adaptor molecule MyD88, the membrane receptor Bambi, a pseudoreceptor for TGF-β1 with negative regulatory

function. The removal of this inhibitor sensitizes HSC to activation by TGF-β1 and secretion of chemotactic factors monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein 1β (MIP1β), that recruit circulating macrophages to the liver to become Kupffer cells. Kupffer cells, in turn, secrete TGF-β and further activate HSC in a paracrine manner.

In **adipocytes** a reduced activation of PPARγ impaired GLUT4 translocation to plasma membrane and fatty acid synthesis leading to atrophy of adipocyte.

Macrophages uptake ox-LDL through scavenger receptors and become lipid-overloaded cells.

Figure 5. Types and origins of extracellular vesicles (EVs).

Exosomes are complex vesicles (30–150 nm in diameter) generated by the interior budding of endosomal membranes to form large multivesicular bodies (MVBs). MVBs are late endosomes containing multiple intraluminal vesicles (ILVs) that were formed by the invagination of the endosomal membrane. As the transfer of MVBs progresses, on the one hand, MVBs can fuse with the plasma membrane and release ILVs as exosomes; on the other hand, MVBs can also be degraded by lysosomes with which they fuse. Multiple types of stimuli affect MVB degradation and can alter the amount and content of exosomes. Such changes in exosome composition play essential roles in the signal transduction in different cell types and different organs. Exosomes play an important role not only in cellular homeostasis, but also in the pathogenesis of major human diseases. Growing evidence suggested that exosomes carry material from one cell to other cell for initiation of disease. Further, exosomes have been implicated for a promising source of disease-associated biomarkers, and may eventually be used as delivery vehicle for targeted biological therapies.

Microvesicles are extracellular vesicles (100 to 1,000 nm in diameter) produced by budding from the plasma membrane.

Apoptotic vesicles are larger (100–5,000 nm in diameter) particles formed by large-scale plasma membrane blebbing, and are released during apoptotic cell death.

Figure 6. Metabolism and cellular targets of Ceramide (CER), sphingosine (Sph) and Sphingosine-1-Phosphate(S1P) (the "sphingolipid rheostat").

Panel A. Biosynthetic and catabolic pathways of ceramide are represented.

Sphingosine (Sph)is generated through ceramide deacylation and can be phosphorylated by sphingosine kinases (SphKs) to form sphingosine-1 phoshate (S1P). S1P can be degraded to sphingosine through reversible dephosphorylation by nonspecific phosphatases and by two S1P-specific phosphatases, sphingosine1-phosphate phosphatase 1(SPP1) and sphingosine-1-phosphate phosphatase 2 (SPP2); or can be irreversibly cleaved by S1P lyase (SPL), leading to the formation of phosphatidylethanolamine and hexadecenal.

Ceramide (CER) impairs Akt-mediated insulin signaling, an effect that is antagonized by S1P.

Ceramide impairs mitochondrial fatty acid β-oxidation by electron transport chain (ETC) complex II and IV inactivation, enhances reactive oxygen species (ROS) production and lipid accumulation.

Ceramide exerts its lipotoxicity also through include BAX-dependent mitochondrial outer membrane permeabilization (MOMP) and endoplasmic reticulum (ER) stress activation, both triggering apoptosis, by enhancing hepcidin-mediated overload, through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and NLR family pyrin domain containing 3 (Nlrp3) inflammasome activation, through sterol regulatory element-binding protein 1c (SREBP-1c) and sterol regulatory element-binding protein 2 (SREBP-2) upregulation, which increase *de novo* lipogenesis and cholesterol synthesis, and through autophagy impairment.

Additionally, acid sphingomyelinase (ASMAse) activation promotes liver injury independently of ceramide accumulation by disrupting phosphatidylcholine (PC) metabolism.

Panel B.

The intestine provides nearly 50% of circulating ceramide, whose synthesis in enterocyte is modulated by a bile acid/intestinal farnesoid X receptor (FXR) axis: FXR activity and ceramide synthesis in the intestinal epithelium are inhibited by intestinal bile acids cholic acid (CA),

chenodeoxycholic acid (CDCA) and β-muricholic acid (β-MCA), while FXR activation stimulates ceramide synthesis.

In adipocytes, ceramide induces adipose dysfunction by increasing ER stress and impairing oxidative capacity. SIPR (1,3) activation stimulates TNF-α and IL-6 secretion and inhibits adiponectin and IL-10 secretion. In adipocytes and adipose tissue macrophages, ceramide activates Nlrp3 inflammasome-mediated proinflammatory cytokine secretion.

In HSCs, SIPR (1,3) activates HSCs while in endothelial cells SIPR1 activation increases integrity and barrier function

Figure 7. Scheme of short-, long- and n-6 and n-3 PUFA biosynthesis leading to membrane phospholipids synthesis.

Shown here is the pathway of both n-6 and n-3 PUFA metabolism to more unsaturated, long-chain members of each family.

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 8. 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).

Hydroxy eicosatetraenoic 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 [260].

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.

Figure 9. Cytochrome P450 (CYP) pathway, epoxyeicosatrienoic acids (EETs), soluble Epoxide Hydrolase (sEH) and hydroxysaeicosatrienoic acids (HETEs) pathway.

Abbreviations: ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; CYP450: cytochrome P450; EET: epoxyeicosatrienoic acid; EEQ: epoxyeicosatetraenoic acid; EDP: epoxydocosapentaenoic acid; sEH: soluble epoxide hydrolase; DHET: dihydroxyeicosatrienoic acid; DiHETE: dihydroxyeicosatetraenoic acid; DiHDPE: dihydroxyeicosatetraenoic acids**.**

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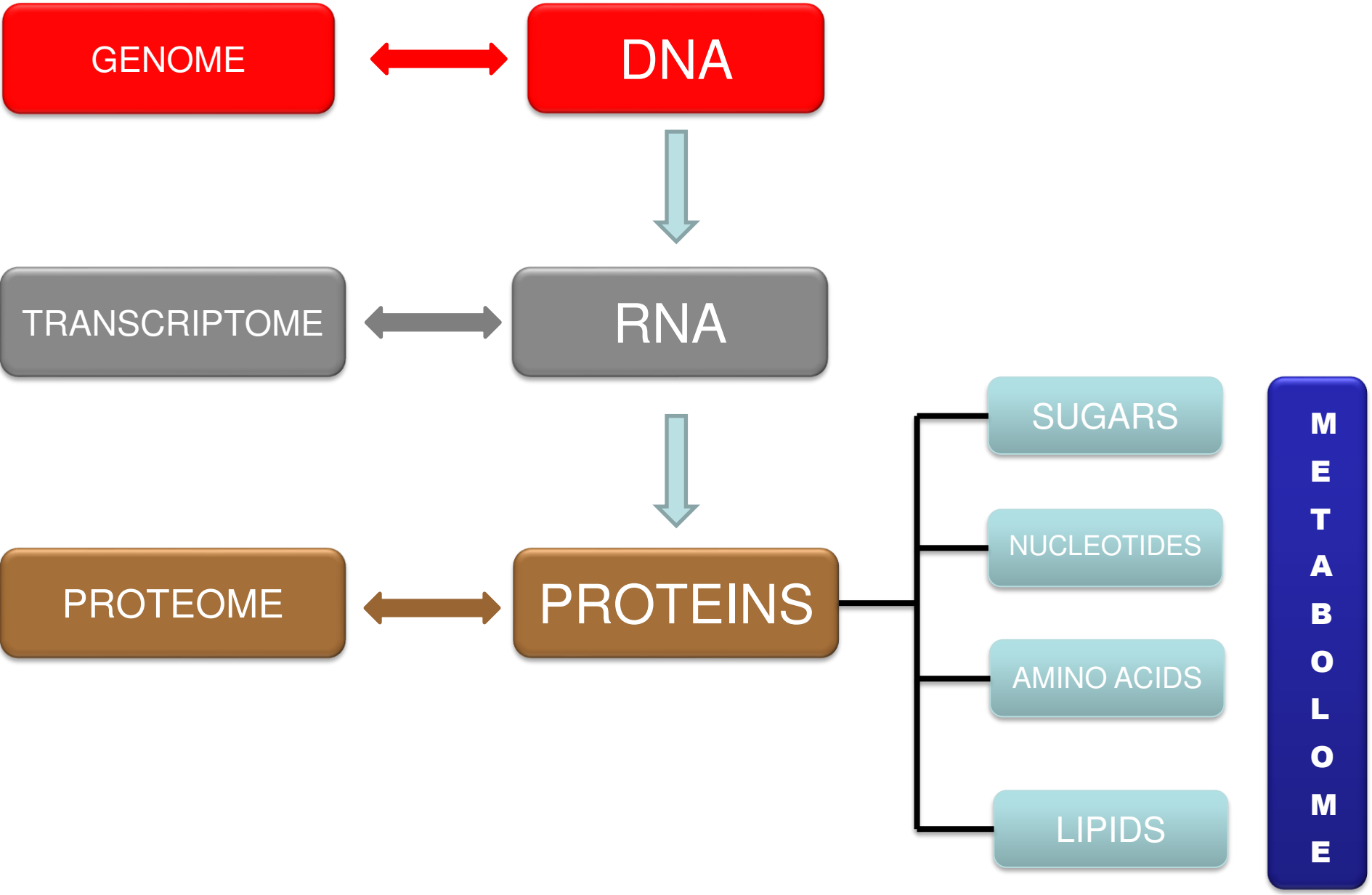
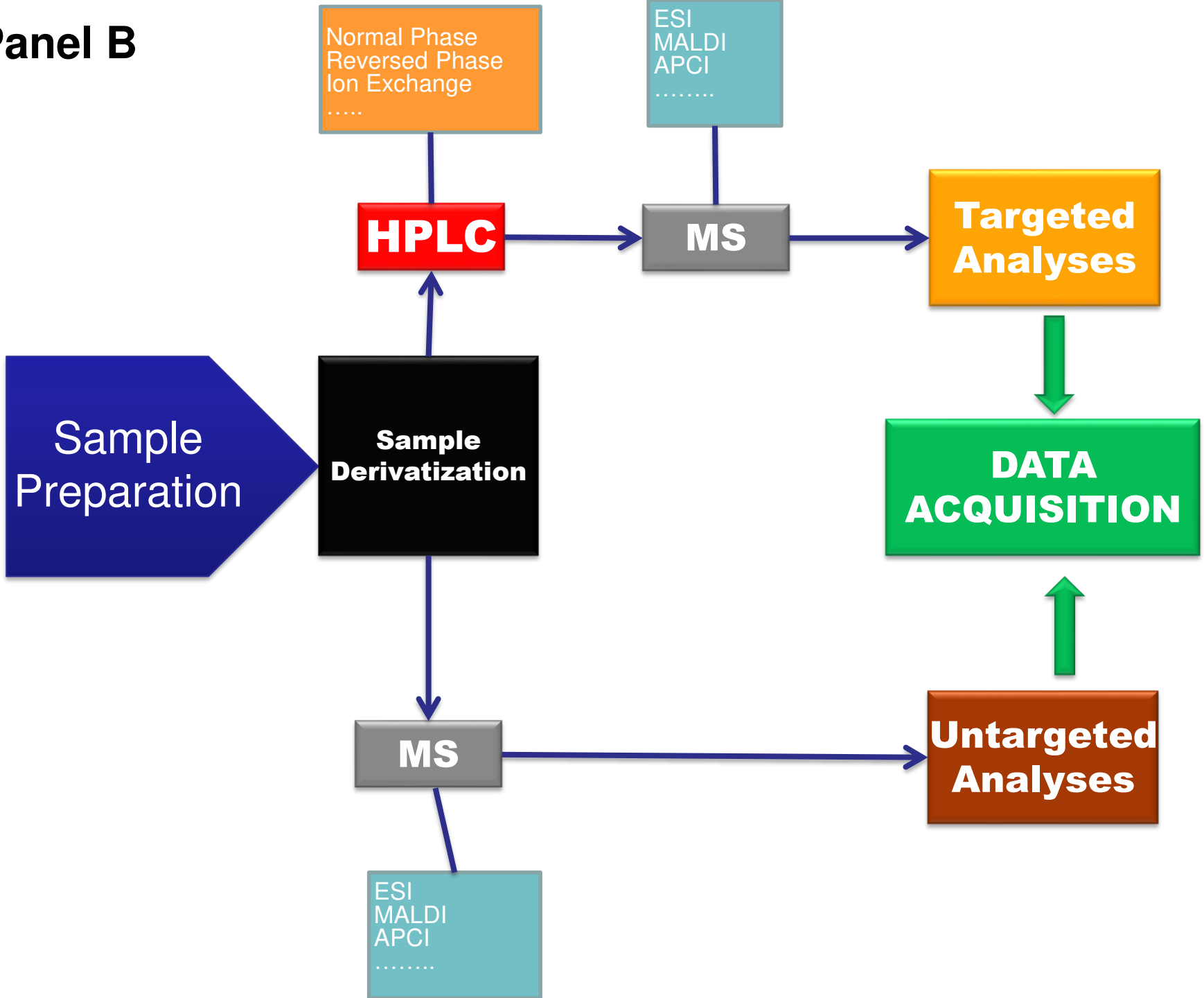
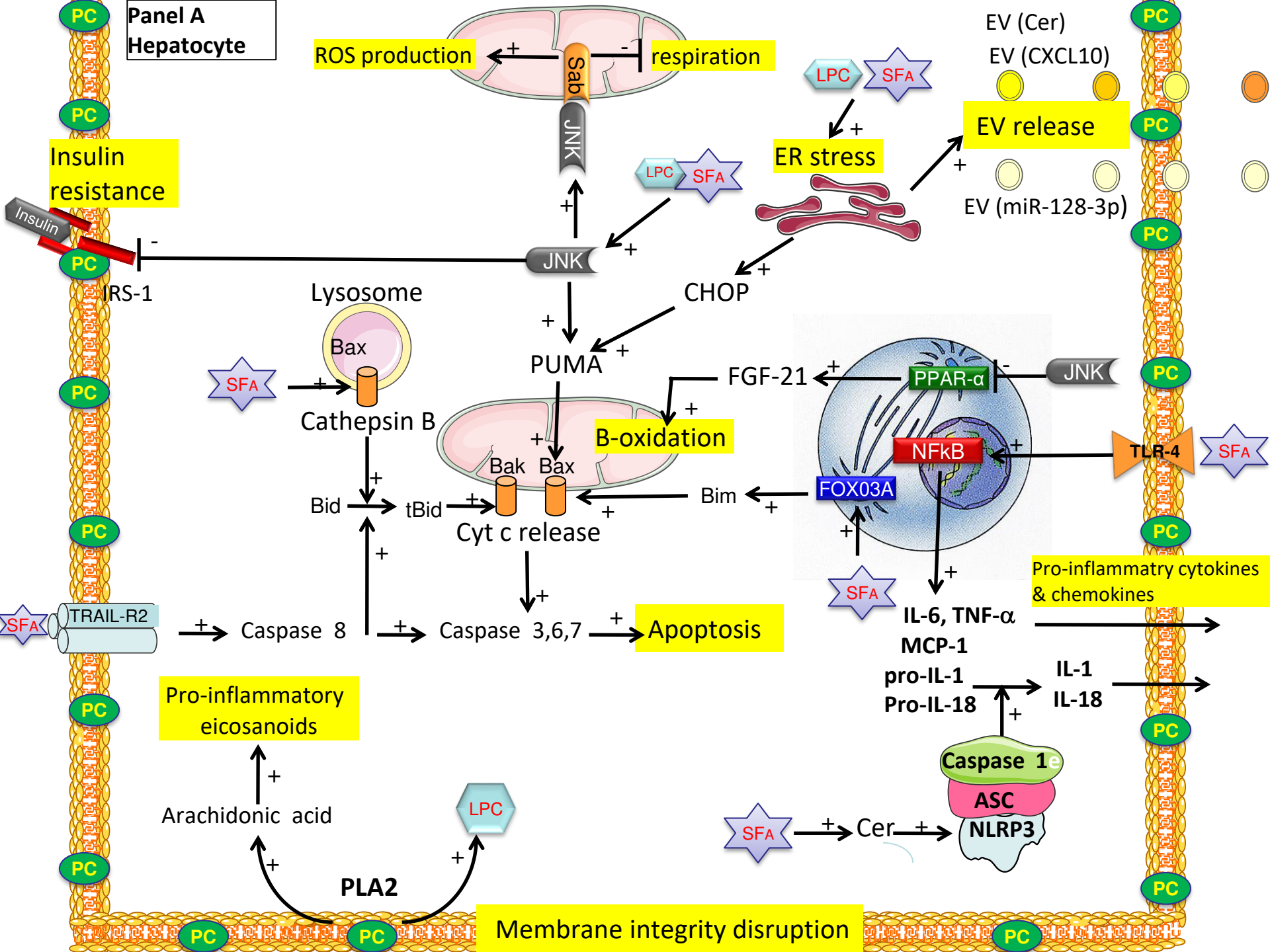
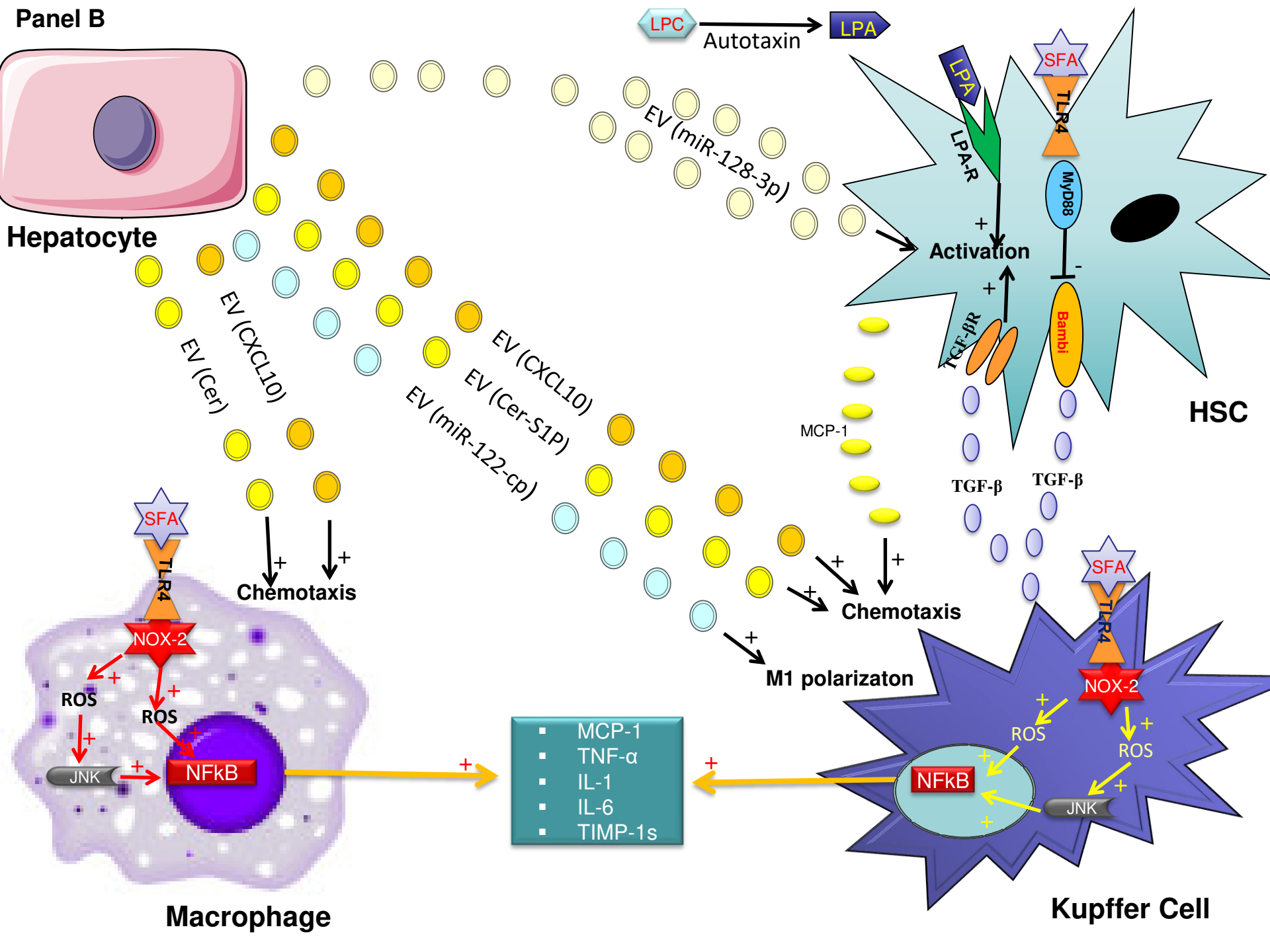
Panel A


GENOME
DNA
TRANSCRIPTOME
RNA
PROTEOME
PROTEINS
SUGARS
NUCLEOTIDES
AMINO ACIDS
LIPIDS
METABOLOME

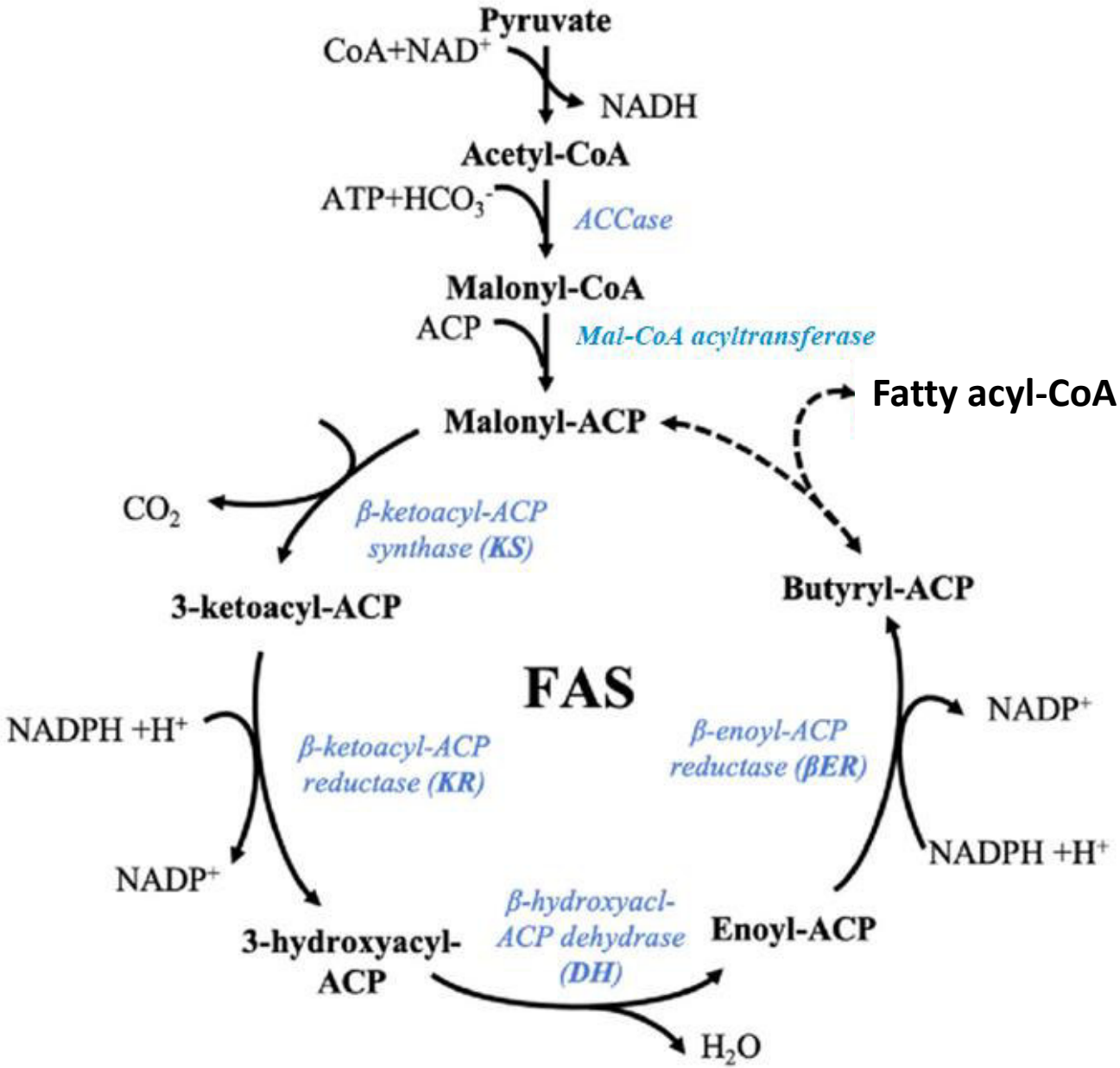
Panel B

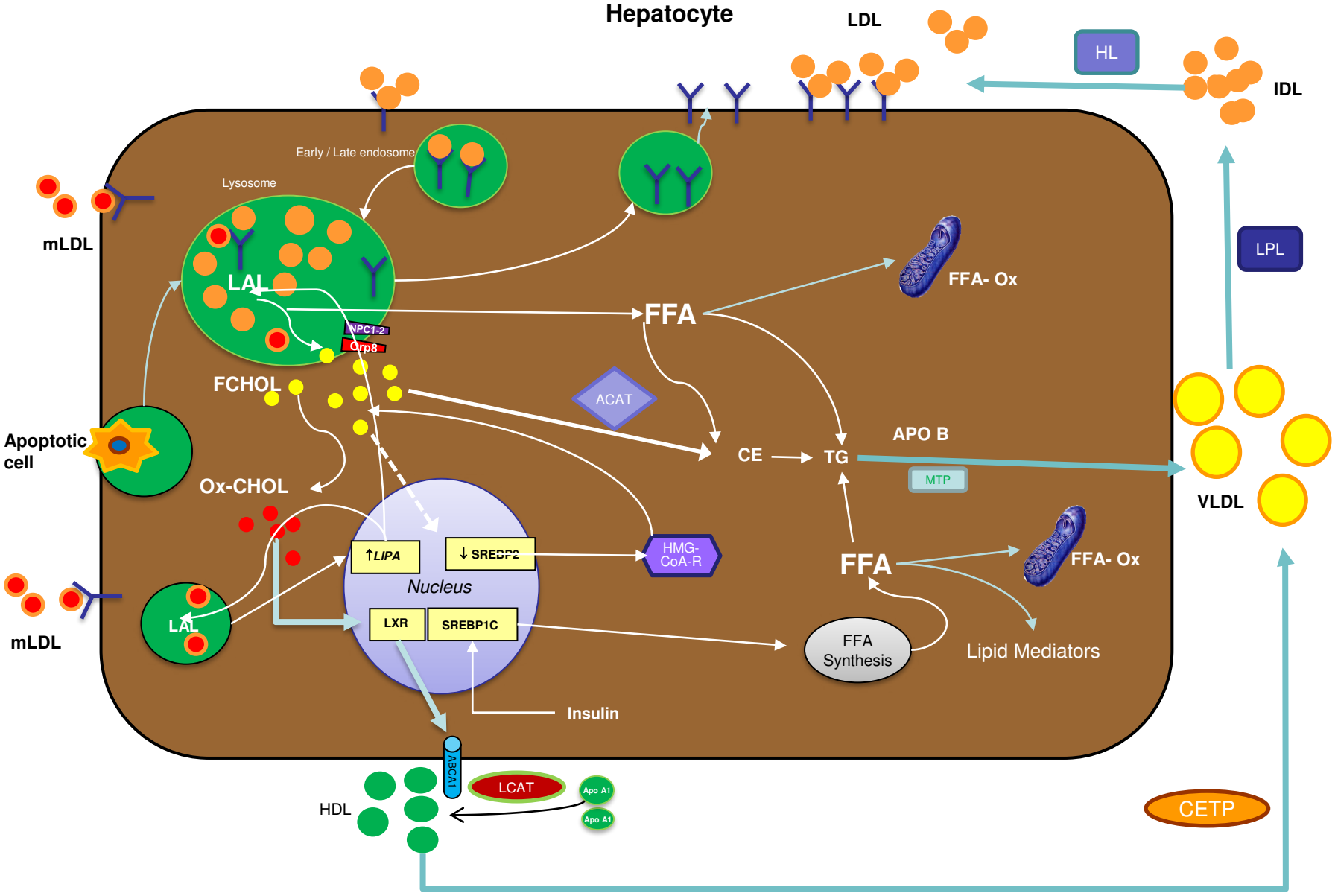

Normal Phase
Reversed Phase
Ion Exchange
.....
ESI
MALDI
APCI
.....
HPLC
MS
Targeted Analyses
Sample Preparation
Sample Derivatization
DATA ACQUISITION
MS
Untargeted Analyses
ESI
MALDI
APCI
.....

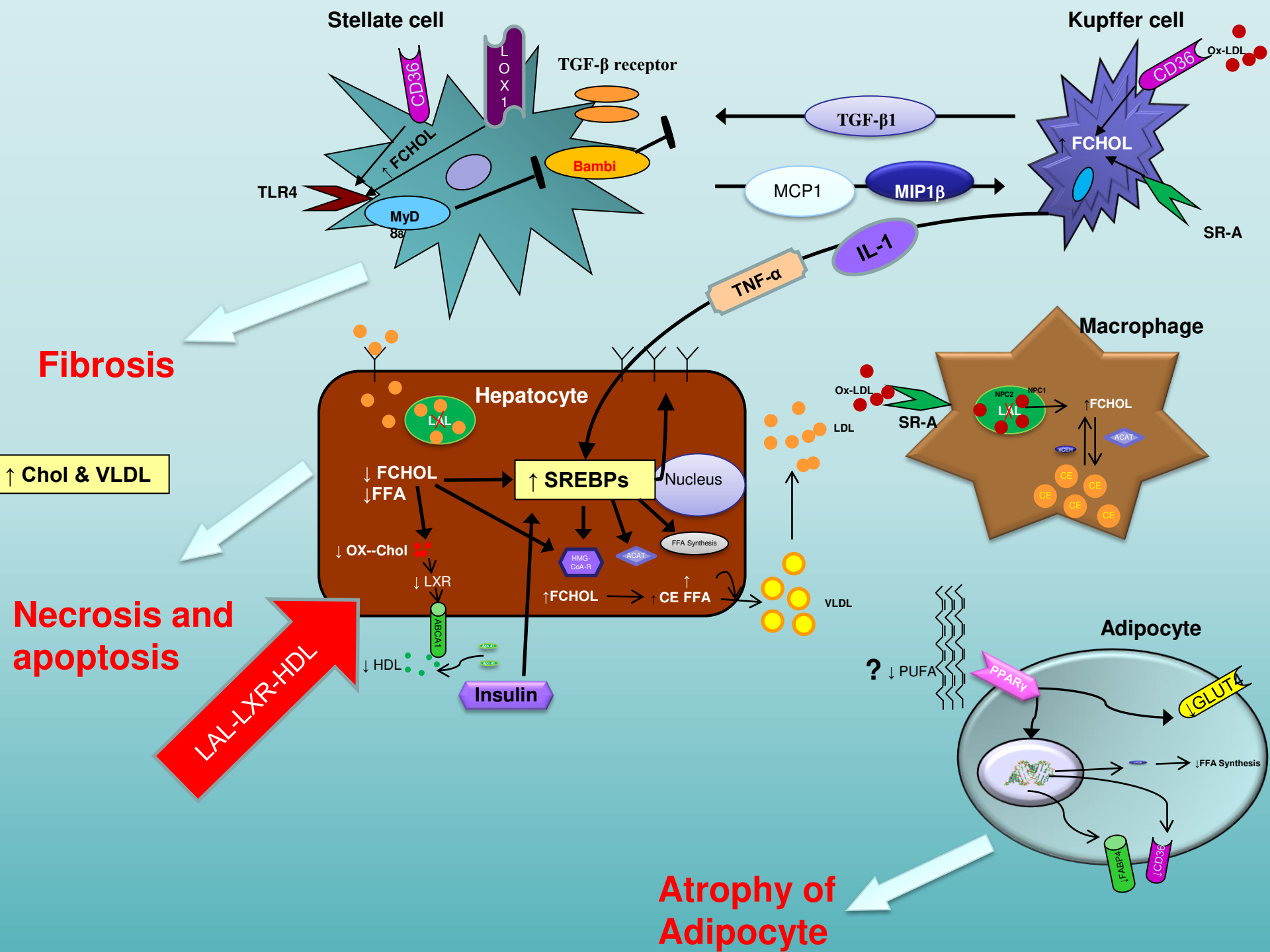

Panel A
Hepatocyte
ROS production
respiration
Sab
JNK
Insulin resistance
Insulin
IRS-1
JNK
LPC
SFA
ER stress
EV release
EV (Cer)
EV (CXCL10)
EV (miR-128-3p)
CHOP
Lysosome
Bax
SFA
Cathepsin B
PUMA
FGF-21
B-oxidation
PPAR-α
JNK
NFkB
TLR-4
SFA
FOX03A
Bak
Bax
Bid
tBid
Bim
Cyt c release
SFA
Pro-inflammatry cytokines & chemokines
IL-6, TNF-α
MCP-1
pro-IL-1
Pro-IL-18
IL-1
IL-18
SFA
TRAIL-R2
Caspase 8
Caspase 3,6,7
Apoptosis
Caspase 1
ASC
NLRP3
SFA
Cer
Pro-inflammatory eicosanoids
Arachidonic acid
LPC
PLA2
Membrane integrity disruption
PC

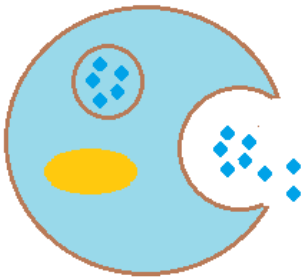

Panel B
Hepatocyte
LPC
Autotaxin
LPA
EV (miR-128-3p)
EV (CXCL10)
EV (Cer)
EV (CXCL10)
EV (Cer-S1P)
EV (miR-122-cp)
MCP-1
LPA
LPA-R
SFA
TLR4
MyD88
Activation
TGF-βR
Bambi
HSC
TGF-β
TGF-β
Chemotaxis
Chemotaxis
M1 polarizaton
SFA
TLR4
NOX-2
ROS
ROS
JNK
NFkB
Macrophage
MCP-1
TNF-α
IL-1
IL-6
TIMP-1s
SFA
TLR4
NOX-2
ROS
ROS
NFkB
JNK
Kupffer Cell

Fatty Acid Synthesis



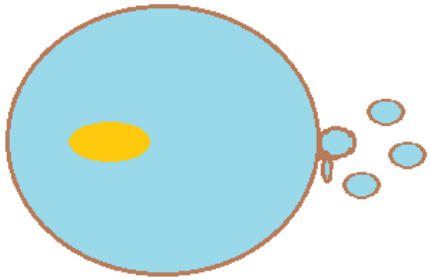

Hepatocyte
LDL
HL
IDL
Early / Late endosome
Lysosome
mLDL
LAL
FFA
FFA- Ox
NPC1-2
Crp8
FCHOL
ACAT
LPL
Apoptotic cell
Ox-CHOL
CE
TG
APO B
MTP
VLDL
↑LIPA
↓ SREBP2
HMG-CoA-R
Nucleus
LXR
SREBP1C
FFA
FFA- Ox
mLDL
LAL
FFA Synthesis
Lipid Mediators
Insulin
ABCA1
LCAT
Apo A1
Apo A1
HDL
CETP


Stellate cell
CD36
LOX1
TGF-β receptor
↑FCHOL
TLR4
MyD 88
Bambi
TGF-β1
MCP1
MIP1β
Kupffer cell
Ox-LDL
CD36
↑FCHOL
SR-A
IL-1
TNF-α
Fibrosis
Hepatocyte
LAL
↓FCHOL
↓FFA
↑SREBPs
Nucleus
↓OX--Chol
↓LXR
ABCA1
↓HDL
HMG-CoA-R
ACAT
FFA Synthesis
↑FCHOL
↑CE FFA
Insulin
LDL
VLDL
Macrophage
Ox-LDL
SR-A
NPC2
NPC1
LAL
↑FCHOL
ACAT
nCEH
CE
↑Chol & VLDL
Necrosis and apoptosis
LAL-LXR-HDL
? ↓PUFA
PPARγ
Adipocyte
↓GLUT4
↓FFA Synthesis
↓FABP4
↓CD36
Atrophy of Adipocyte



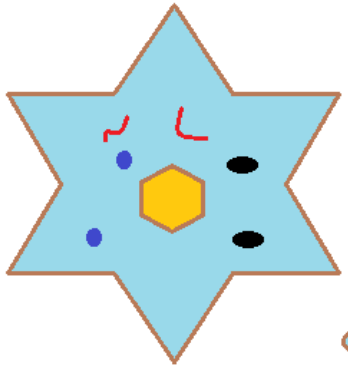
Intracellular Vesicular Traffic

Exosomes (30 – 150 nm)



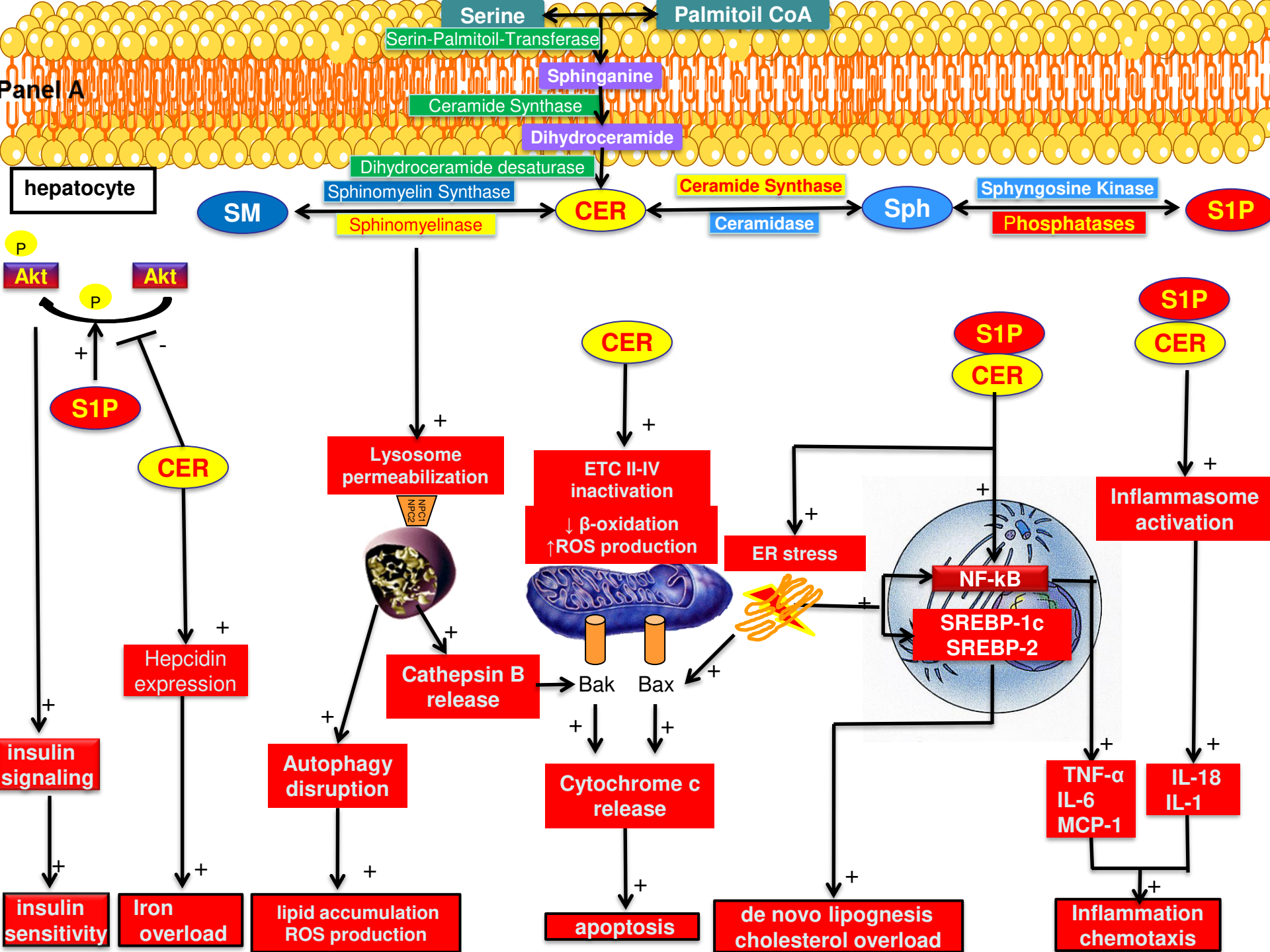
Plasma Membrane Shedding

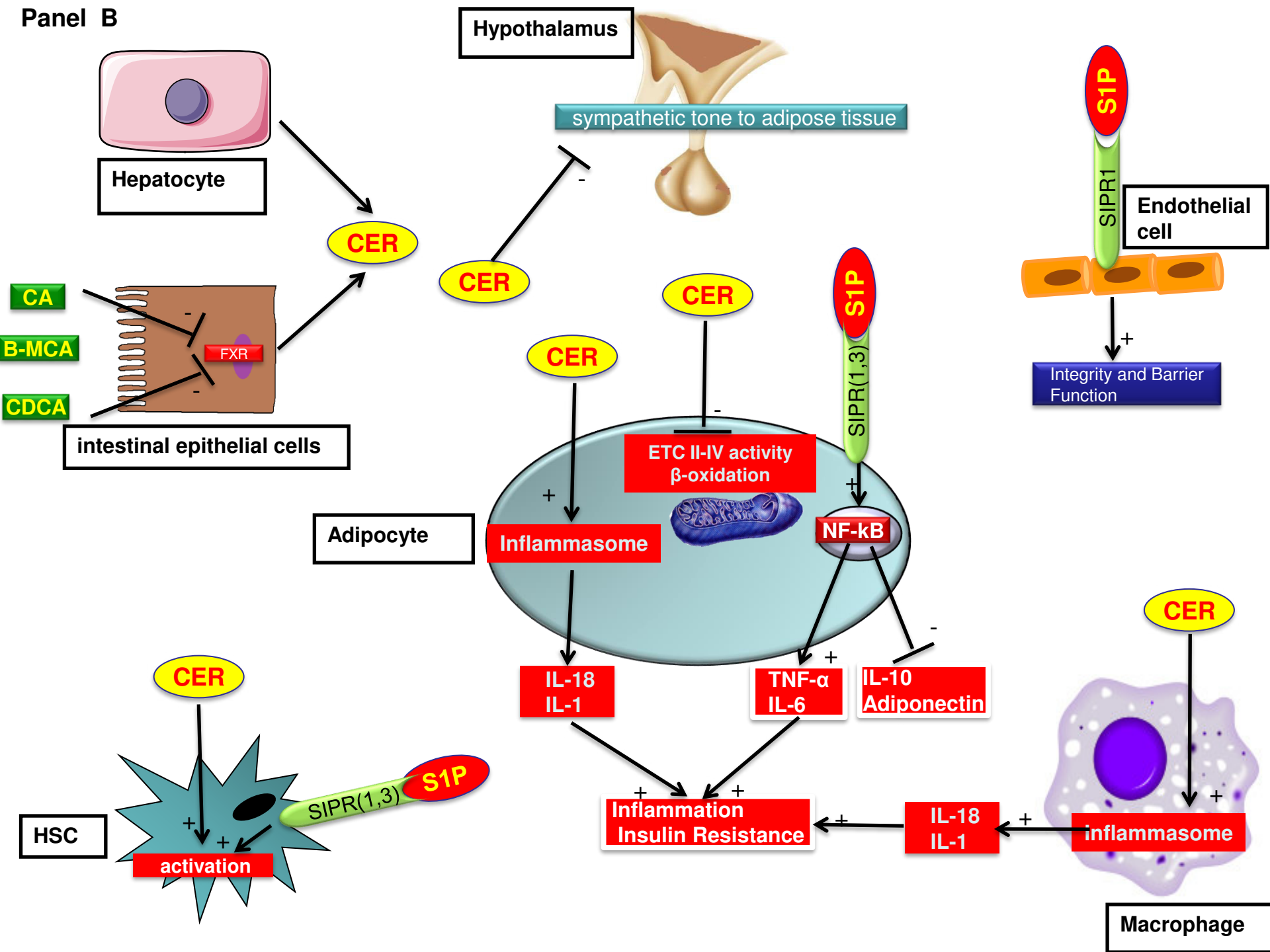
Microvescicles (100–1000 nm)

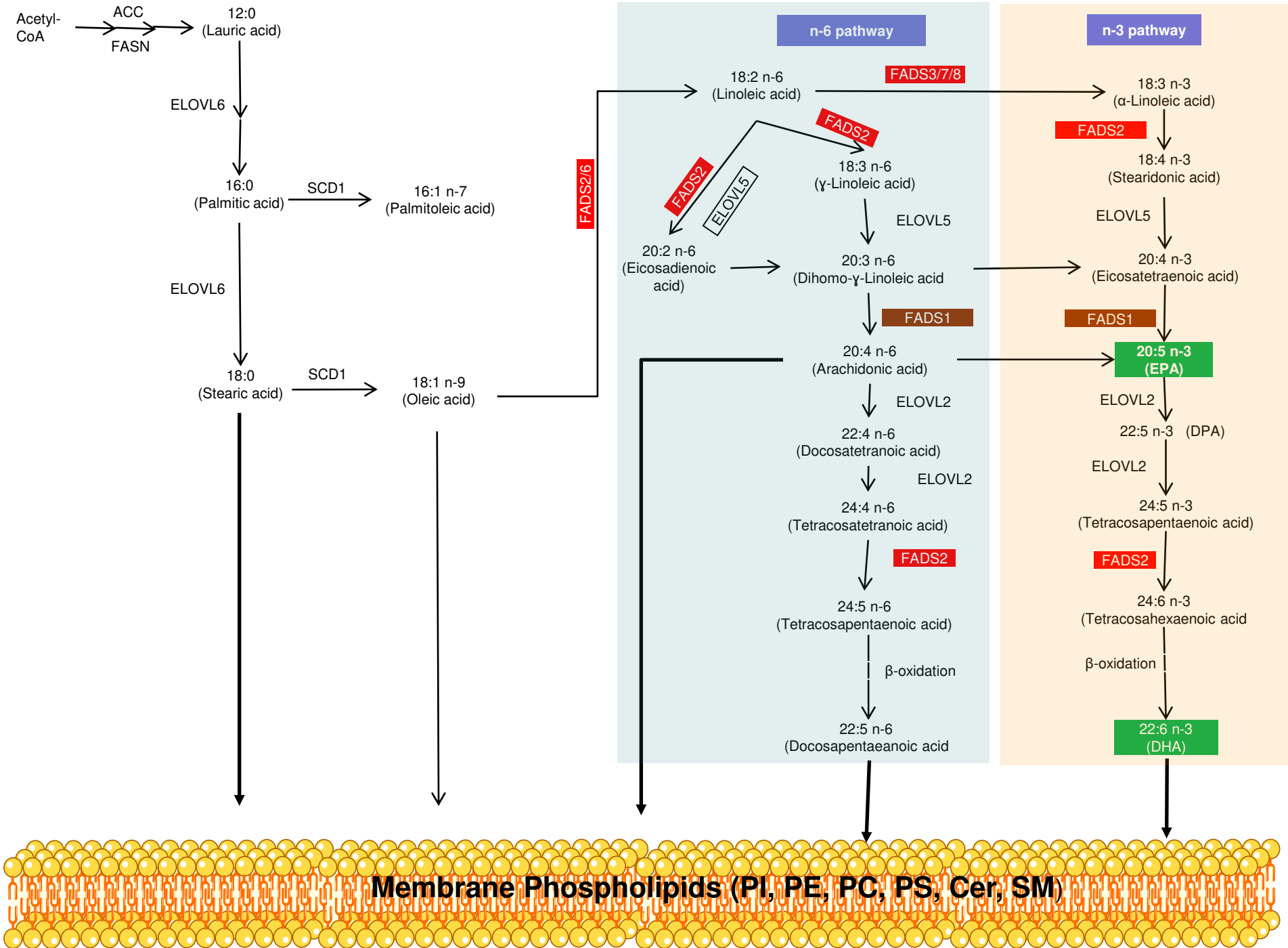


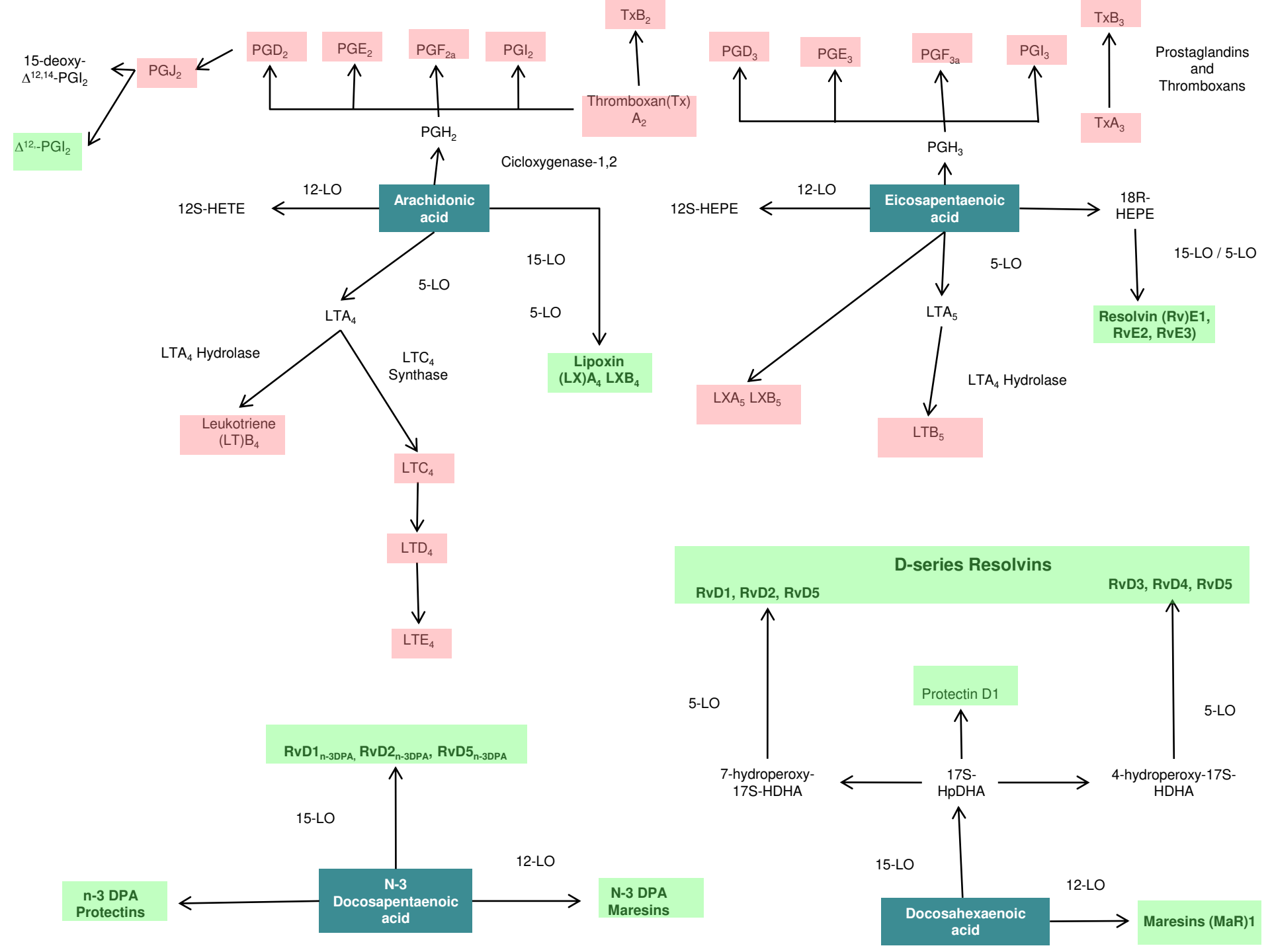
End Death Products

Apoptotic Vescicles (100-5000 nm)


Panel A
hepatocyte
Serine
Palmitoil CoA
Serin-Palmitoil-Transferase
Sphinganine
Ceramide Synthase
Dihydroceramide
Dihydroceramide desaturase
Sphinomyelin Synthase
SM
Sphinomyelinase
CER
Ceramide Synthase
Ceramidase
Sph
Sphyngosine Kinase
Phosphatases
S1P
P
Akt
Akt
P
+
-
S1P
CER
+
insulin signaling
+
insulin sensitivity
+
Hepcidin expression
+
Iron overload
+
Lysosome permeabilization
NPC1
NPC2
+
Cathepsin B release
+
Autophagy disruption
+
lipid accumulation ROS production
CER
+
ETC II-IV inactivation
↓ β-oxidation
↑ROS production
Bak
Bax
+
+
Cytochrome c release
+
apoptosis
S1P
CER
+
ER stress
+
NF-kB
SREBP-1c
SREBP-2
+
de novo lipognesis
cholesterol overload
S1P
CER
+
Inflammasome activation
+
TNF-α
IL-6
MCP-1
+
IL-18
IL-1
+
Inflammation
chemotaxis


Panel B
Hypothalamus
sympathetic tone to adipose tissue
Hepatocyte
CER
CER
CA
B-MCA
CDCA
FXR
intestinal epithelial cells
S1P
SIPR1
Endothelial cell
Integrity and Barrier Function
CER
CER
S1P
SIPR(1,3)
ETC II-IV activity
β-oxidation
Adipocyte
Inflammasome
NF-kB
IL-18
IL-1
TNF-α
IL-6
IL-10
Adiponectin
CER
CER
HSC
activation
SIPR(1,3)
S1P
Inflammation
Insulin Resistance
IL-18
IL-1
inflammasome
Macrophage


Acetyl-CoA
ACC
FASN
12:0 (Lauric 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)
FADS2/6
n-6 pathway
18:2 n-6 (Linoleic acid)
FADS3/7/8
FADS2
18:3 n-6 (γ-Linoleic acid)
FADS2
ELOVL5
ELOVL5
20:2 n-6 (Eicosadienoic acid)
20:3 n-6 (Dihomo-γ-Linoleic acid
FADS1
20:4 n-6 (Arachidonic acid)
ELOVL2
22:4 n-6 (Docosatetranoic acid)
ELOVL2
24:4 n-6 (Tetracosatetranoic acid)
FADS2
24:5 n-6 (Tetracosapentaenoic acid)
β-oxidation
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 (EPA)
ELOVL2
22:5 n-3 (DPA)
ELOVL2
24:5 n-3 (Tetracosapentaenoic acid)
FADS2
24:6 n-3 (Tetracosahexaenoic acid
β-oxidation
22:6 n-3 (DHA)
Membrane Phospholipids (PI, PE, PC, PS, Cer, SM)


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

Cytochrome P450 (CYP), epoxyeicosatrienoic acids (EETs) and soluble Epoxide Hydrolase (sEH) pathway