3rd GLOBAL LIVER HEALTH FORUM

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Importance of hepatic membrane protection against NAFLD/NASH

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Disclosures

- Prof. Wei An has nothing to disclose

Overview

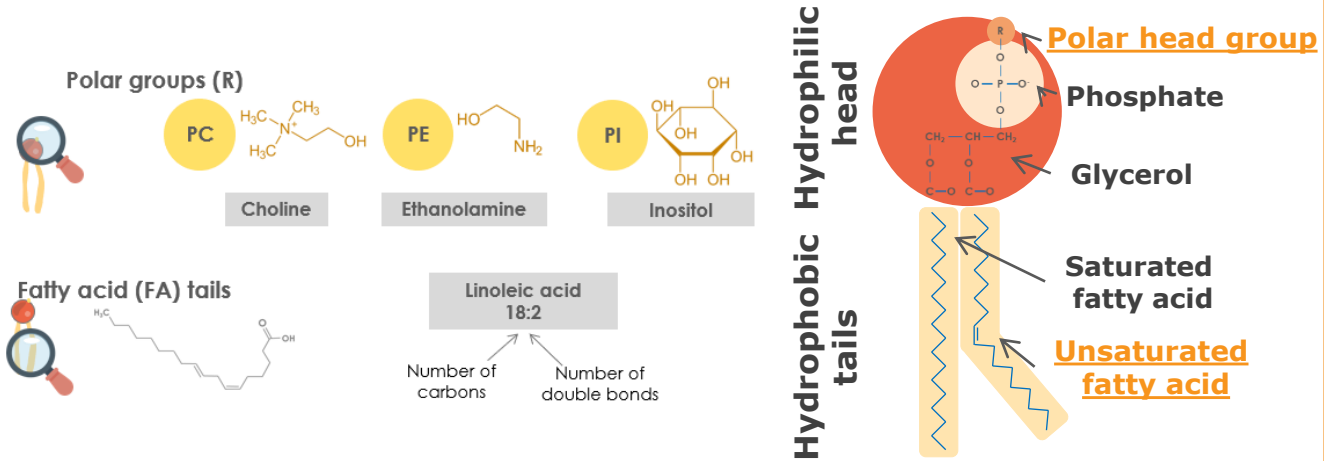
1. Cellular and subcellular membranes
2. Preclinical and clinical evidence of EPL
3. Mitochondria-associated membranes and hepatotoxicity
4. Summary

Cellular and subcellular membranes

Phospholipids are essential components of cellular and subcellular membranes

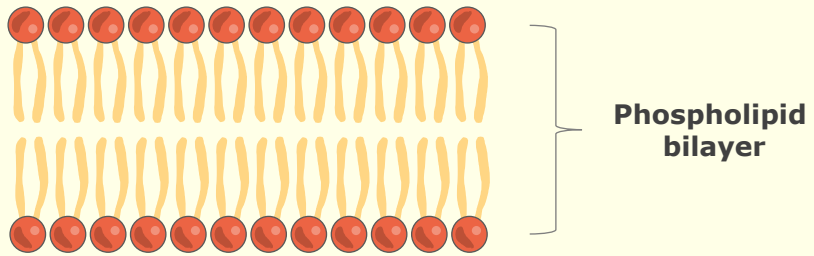
The most abundant phospholipids in mammalian tissues are:[1]

- Phosphatidylcholine (**PC**)
- Phosphatidylethanolamine (**PE**)



Phospholipid functions

- Provide cell **integrity**[2]
- Support membrane **fluidity** and **permeability**[2]
- Help transport molecules
- The **PC/PE ratio** is a key regulator of membrane integrity[1,2]

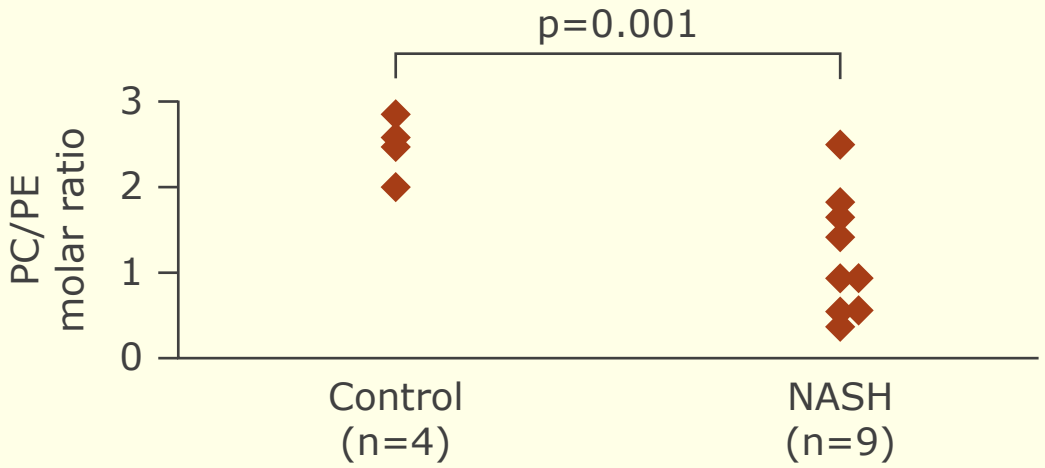


PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol

1. Li Z, et al. Cell Metab 2006;3(5):321–31; 2. Escribá PV, et al. J Cell Mol Med 2008;12(3):829–75

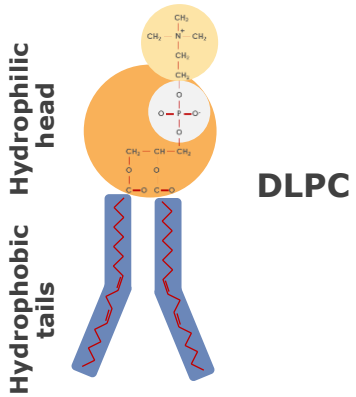
EPLs are associated with improved membrane stability

- Damage to liver cell membranes and organelles originate from reduced phospholipid levels or altered phospholipid composition, and lead to decreased membrane fluidity[1]
- Hepatic PC/PE ratios in patients with NASH have been shown to be significantly lower than those in healthy controls[2]



EPL

- DLPC is the lead compound in the active ingredient whose phosphatidylcholine molecules have specified amounts of 72–96% of phospholipids in EPL[1]



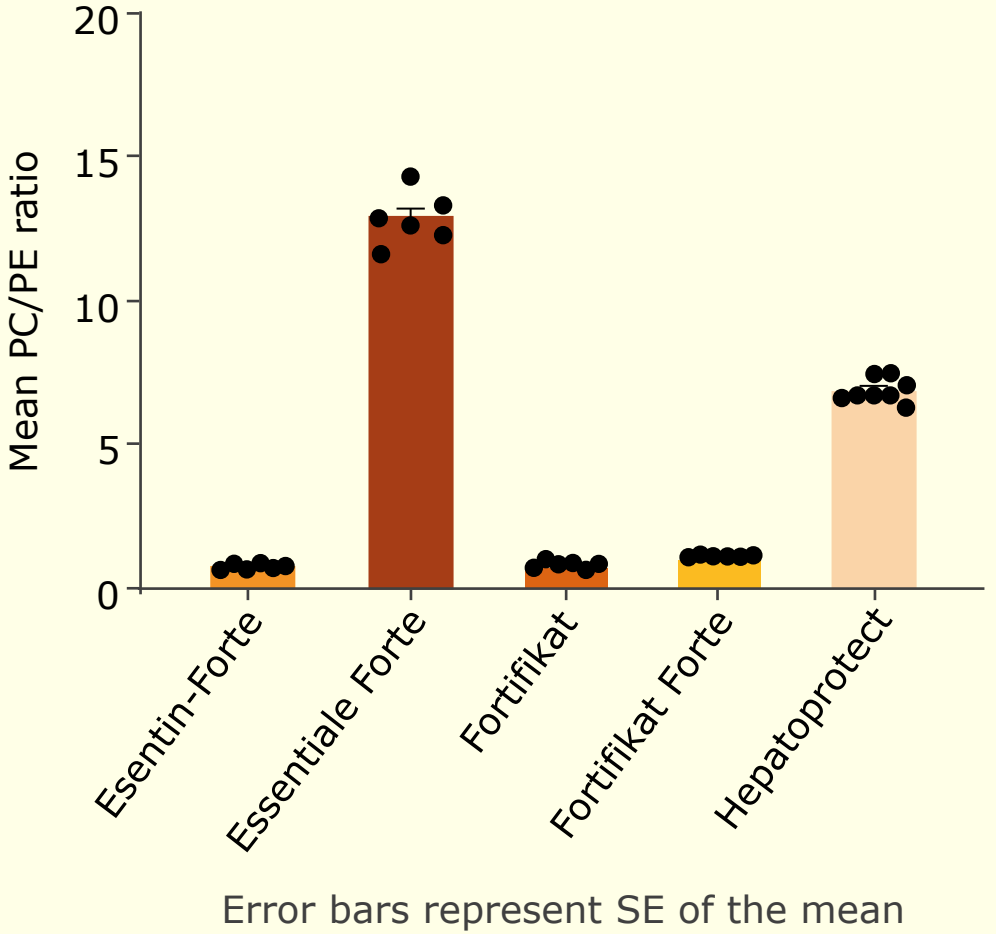
EPL administration significantly increases its percentage in the **membranes** of **hepatocytes**, **blood corpuscles** and **pancreatic tissue**, **among other tissues**[1]

Liver biopsy samples were collected from four healthy persons (control) and nine NASH patients, and PC and PE were quantified
DLPC, 1,2-di-linoleoyl phosphatidylcholine; EPL, essential phospholipids; NASH, non-alcoholic steatohepatitis; PC, phosphatidylcholine; PE, phosphatidylethanolamine

1. Gundermann KJ, et al. Clin Exp Gastroenterol 2016;9:105–17; 2. Li Z, et al. Cell Metab 2006;3(5):321–31

Essentiale Forte has shown the highest PC:PE ratio among other EPL preparations

- Essentiale Forte had a **significantly higher PC:PE (12.9 ± 2.2) ratio** compared with each of the other generic PPCs investigated
- The higher PC:PE ratio observed in Essentiale Forte suggests that it may have a **substantial clinical benefit** in the early treatment of hepatic disorders
- **PC 36:4, 36:3 and 36:5** with high linolenic and linolenic acid content was the most common species detected in all preparations
- A **significantly higher** distribution of PC species 36:4 was seen in Essentiale Forte when compared directly with each of the other PPC preparations, except Esentin



Error bars represent SE of the mean

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PPC, polyenylphosphatidylcholine; SE, standard error

Lüchtenborg C, et al. Lipids 2020;55(3):271–8

Preclinical and clinical evidence of EPLs

How familiar are you with the preclinical evidence supporting the use of EPL in patients with NAFLD?

1. I am fully up to date with the latest data on EPL
2. I am aware of some data on the use of EPL
3. I am aware of data for other hepatoprotective agents, but not for EPL
4. I am not aware of data supporting EPL use in patients with NAFLD

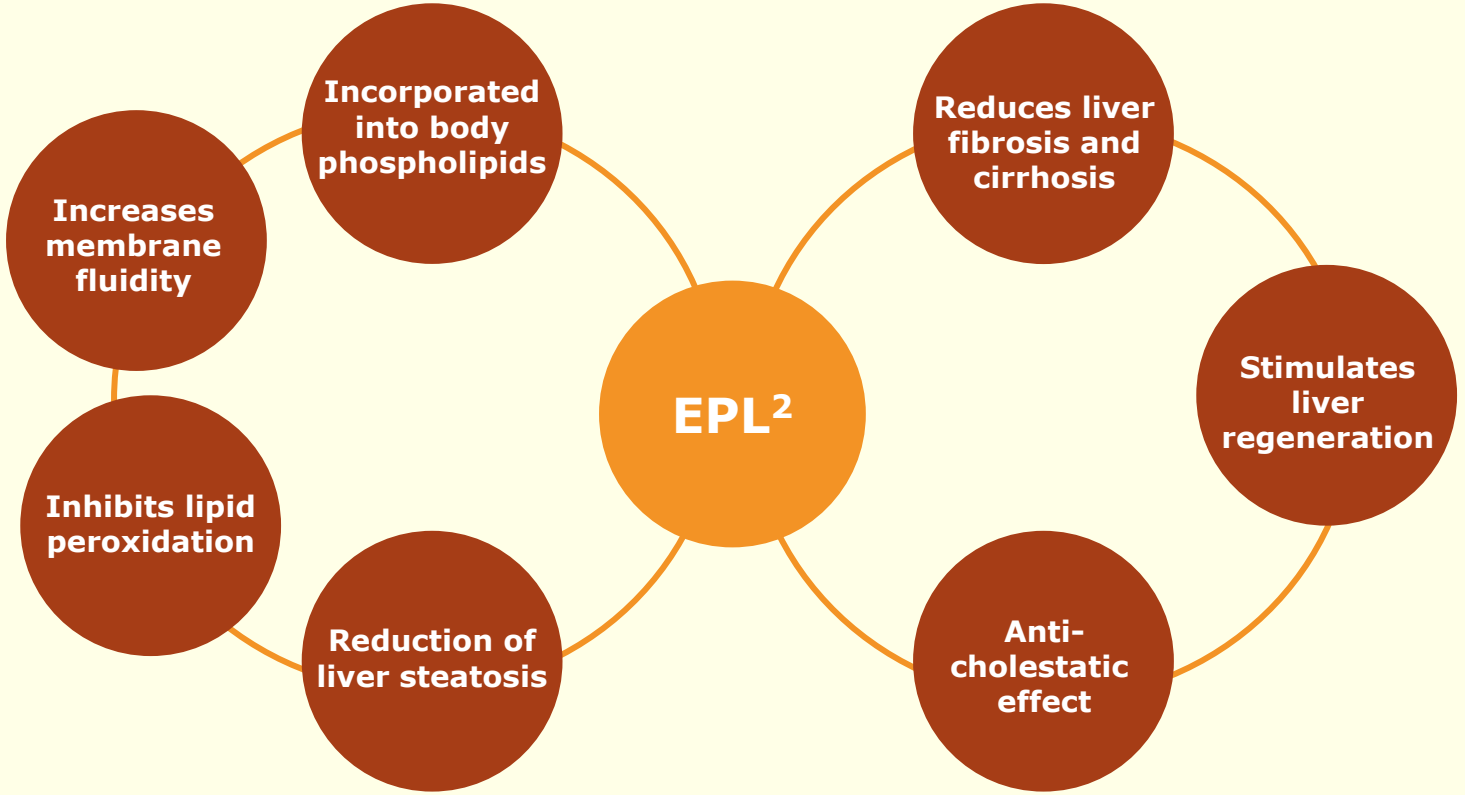
EPL, essential phospholipid; NAFLD, non-alcoholic fatty liver disease

Preclinical evidence has revealed various functions of EPL

Though EPL preparations have been used in humans since 1957, very little is known about their MoA at the cellular level[1]

Preclinical trials have provided insights into multiple MoA of EPL that are potentially involved in their hepatoprotective effects[2]

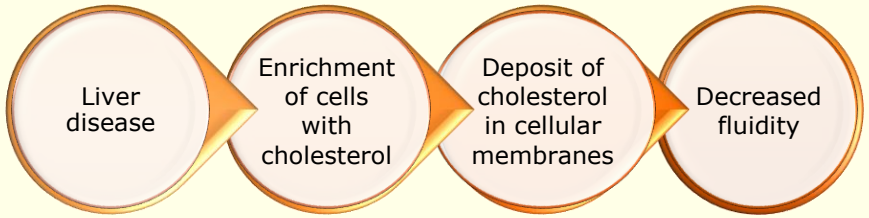
These potential MoA of EPL are amenable to further study *in vitro*



EPL, essential phospholipids; MoA, mechanism of action

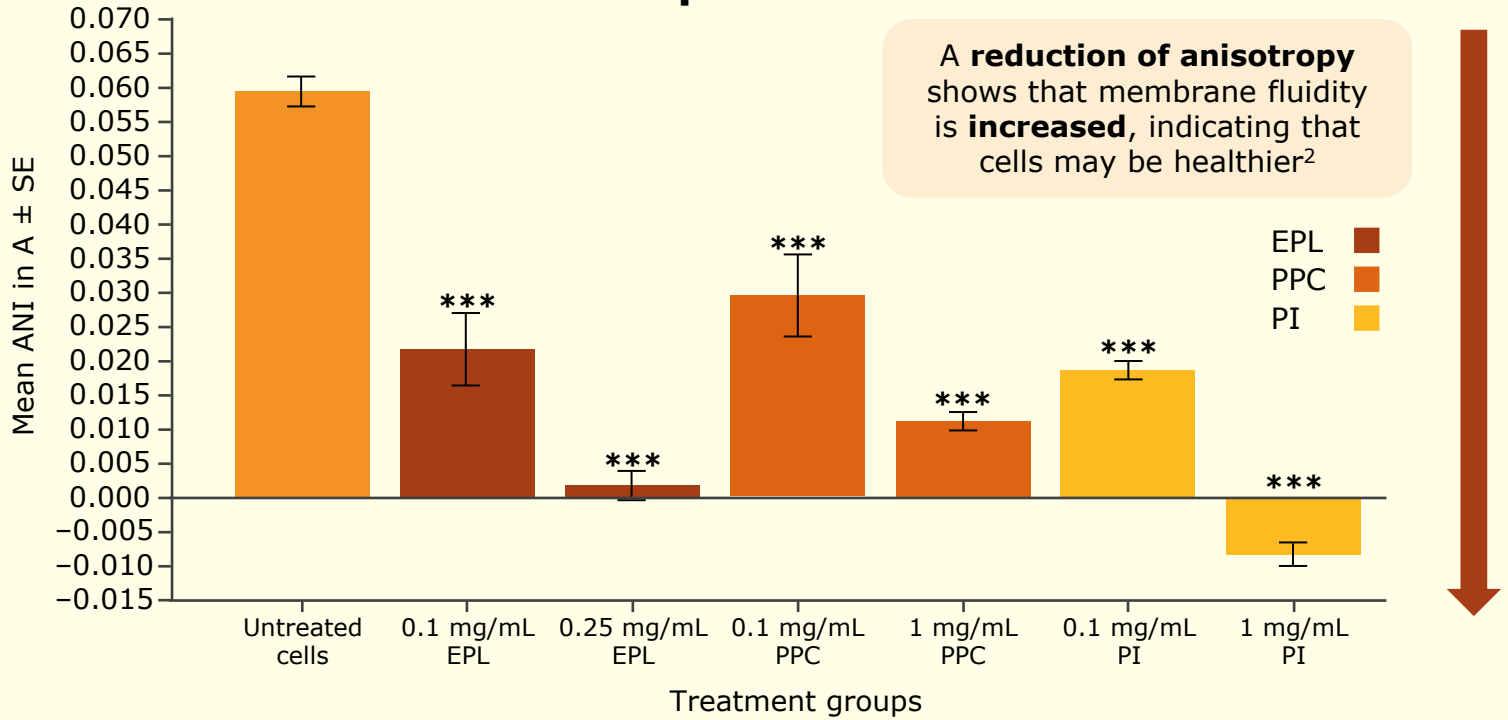
1. Küllenberg D, et al. Lipids Health Dis 2012;11:3; 2. Gundermann KJ, et al. Pharmacol Rep 2011;63:643–59

EPL have been shown to increase membrane fluidity



- **PPC contributes to increasing membrane fluidity by sequestering excess cholesterol** in erythrocyte membranes[1]

Effect of EPL, PPC and PI on anisotropy in the HepG2 cell line†2



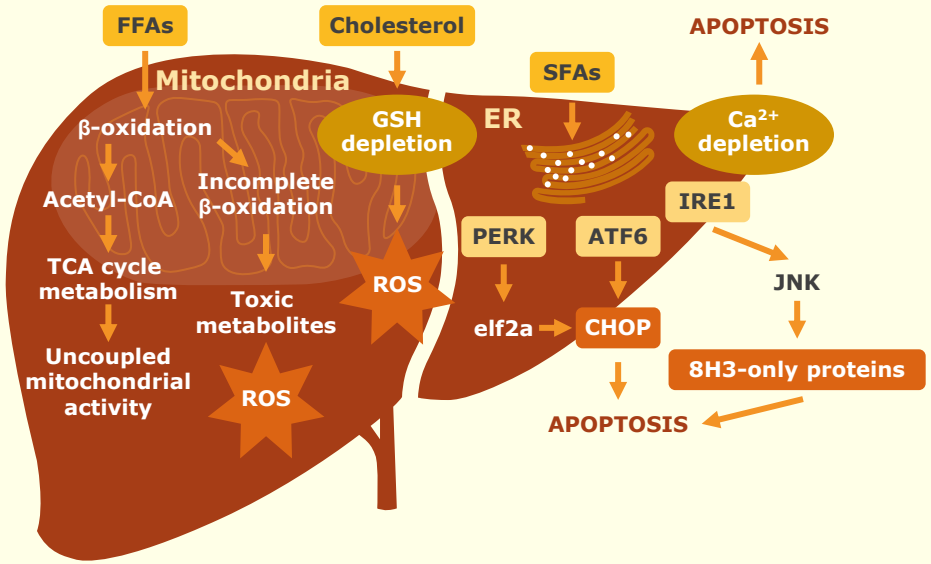
Anisotropy values significantly decreased with all three phospholipids in HepG2 cell cultures compared with untreated cells[2]

† Values shown are mean ± SE for four separate experiments; n=1 well for each concentration of each compound per experiment; ***p<0.001 versus untreated cells. HepG2 refers to a human liver cancer cell line.
ANI, anisotropy, EPL, essential phospholipids; PI, phosphatidylinositol; PPC, polyenylphosphatidylcholine; SE, standard error

1. Gheorghe, L. Ro Med J 2020;67(Suppl); 2. Wupperfeld D, et al. Euro Fed Lipid Congress 2021

Lipid peroxidation can lead to inflammation and liver damage

- Several circulating biomarkers of lipid peroxidation have been identified in NAFLD and linked with disease severity[1]



Malondialdehyde (MDA) and 4-Hydroxinonenal (4-HNE)

- **PPC** (600 mg/kg/day orally) **reduced in-vitro MDA level**
- **DLPC significantly reduced 4-HNE production in rats by 36%** ($p<0.001$)[3]
- EPL (100–300 mg/kg) **reduced lipid peroxidation**[4]

Hydrogen peroxide (H_2O_2) and glutathione

- EPL (100–300 mg/kg) **reduced H_2O_2 production** by liver microsomes[4]
- EPL (100–300 mg/kg) **increased the concentration of glutathione** that scavenges H_2O_2[4,5]
- EPL (100–300 mg/kg) **reduced lipid peroxidation**[4]

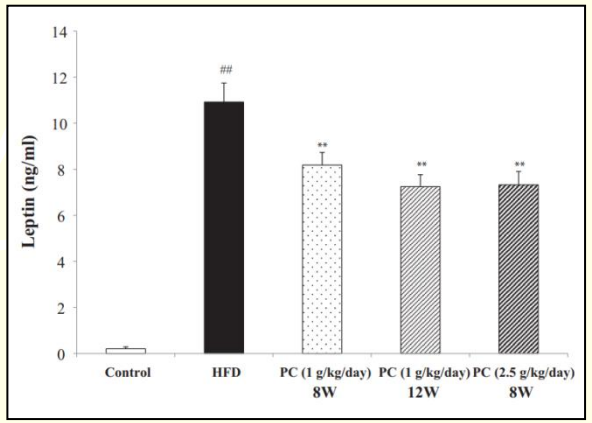
DLPC, dilinoleoylphosphatidylcholine; EPL, essential phospholipid; ER, endoplasmic reticulum; FFA, free fatty acid; GSH, glutathione; PPC, polyenylphosphatidylcholine; MDA, plasma malondialdehyde; NAFLD, non-alcoholic fatty liver disease; ROS, reactive oxygen species; SFA, saturated fatty acid
Image adapted from Svegliati-Baroni et al. Free Radic Biol Med 2019;144:293–309

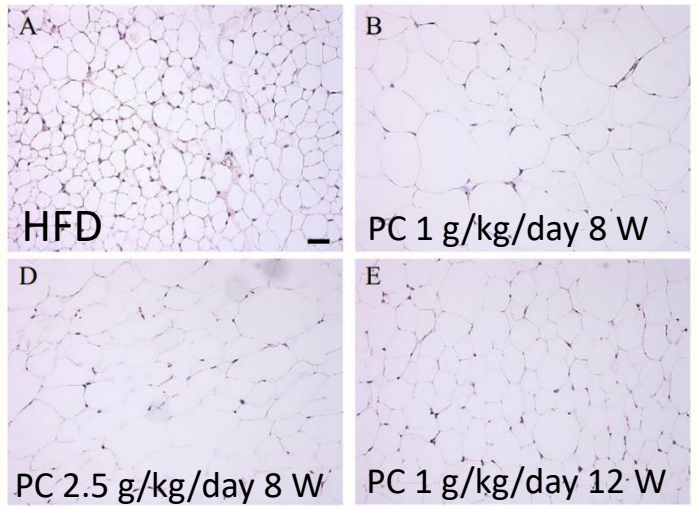
1. Svegliati-Baroni G, et al. Free Radic Biol Med 2019;144:293–309; 2. Martelli A, et al. Med Sci Res 1989;17:995-6; 3. Gheorghe, L. Ro Med J 2020;67(Suppl);
4. Klinger W, et al. Z Gastroenterol 1991;29(Suppl. 2):14–17; 5. Gusdon A, et al. Oxid Med Cell Longev 2014; Article ID 637027

Leptin is also implicated in the pathogenesis of NAFLD

- Leptin has been demonstrated to have antisteatotic effects[1]
- Key functions of leptin include limiting triglyceride storage in hepatocytes and preventing increases in lipotoxicity[1]
- Serum leptin levels have previously been associated with NAFLD through insulin secretory dysfunction and insulin resistance[2]

EPL decreases NAFLD-induced **leptin secretion and reduces lipid accumulation in the liver[3]**





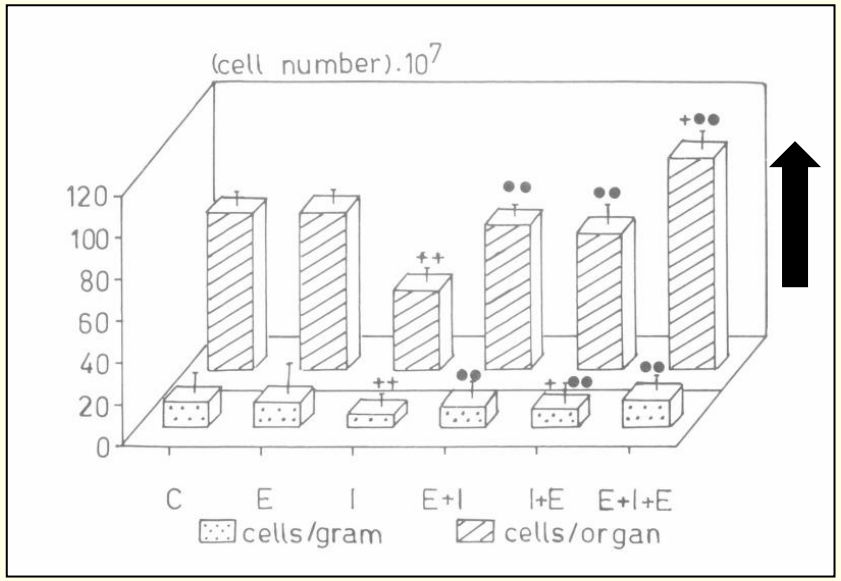
- Recent studies in mice fed a high-fat diet indicated that treatment with Essentiale® (550 mg/kg/day orally) for several weeks **reduced lipid content of liver tissue** in patients with NAFLD

EPL, essential phospholipids; NAFLD, non-alcoholic fatty liver disease; mRNA, messenger ribonucleic acid

1. Jiménez-Cortegana C, et al. Biomedicines 2021;9:762; 2. Wong V, et al. Gut 2010;59:969–74; 3. Lee HS, et al. Life Sci 2014;118:7–14

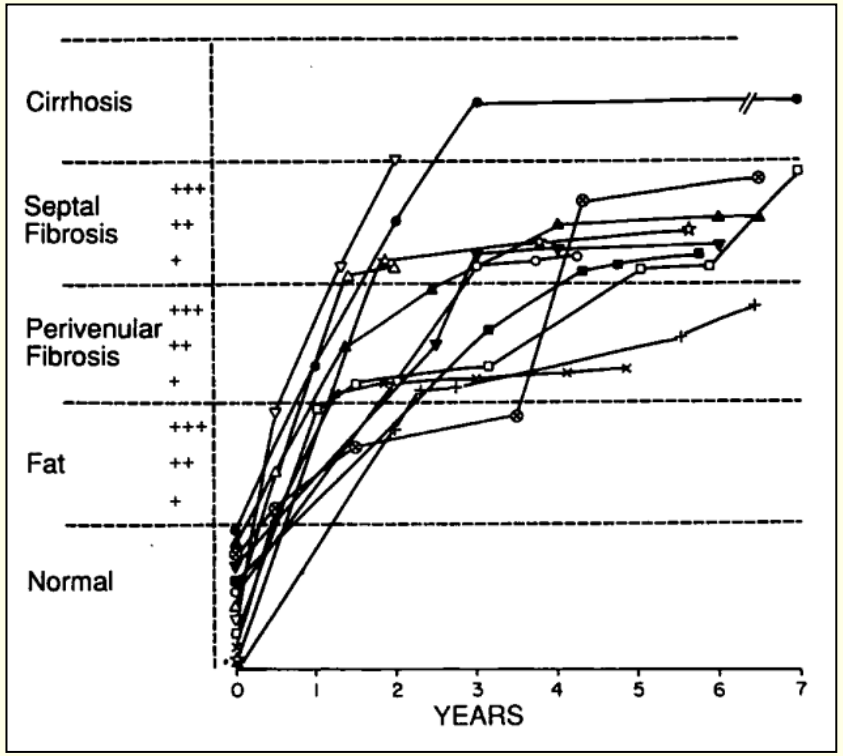
Targeting liver damage with EPL may have therapeutic benefits

Effect of EPL on liver regeneration



EPLs treatment could enhance hepatocyte cellularity and liver regeneration after partial hepatectomy or body irradiation[1]

DLPC treatment demonstrated several anti-fibrotic mechanisms[2]



Studies suggest EPL increases liver regeneration and reduces liver fibrosis

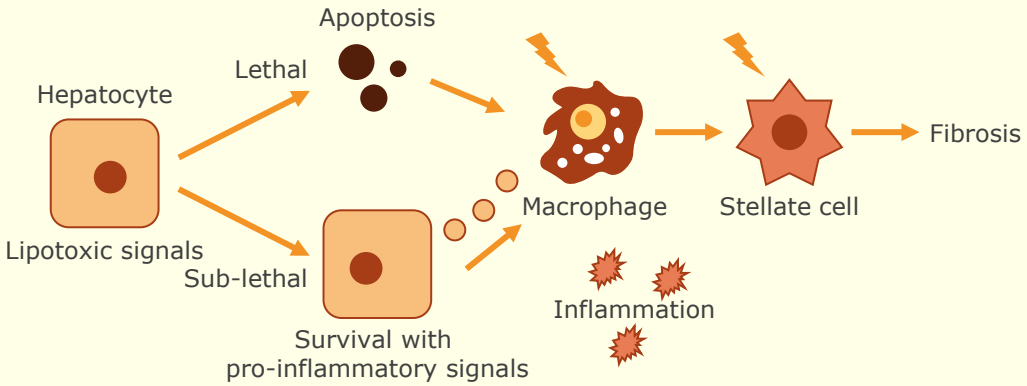
EPL, essential phospholipids; SE, standard error

1.Kropáčová K, Mišúrová E. Physiol Res 1995;44:241–7; 2. Lieber CS, et al. Hepatology 1990;12:1390–8

Preventing hepatocyte apoptosis may help to maintain healthy hepatic function

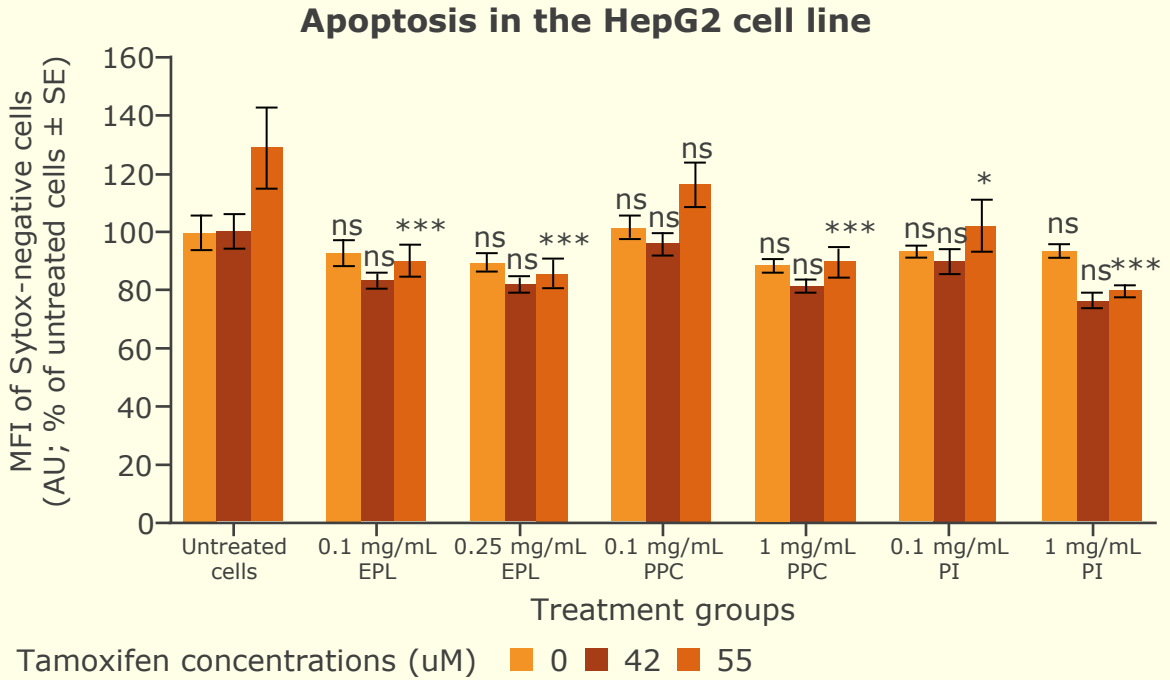
Apoptosis in liver cells[1]

- Programmed cell death (50–70 x 10^9 cells/day)
- Highly regulated
- Extrinsic and intrinsic pathways



- Lethal lipotoxic signals triggered by free fatty acids lead to hepatocyte apoptosis, which are engulfed by macrophage, initiating inflammatory and fibrotic reactions[2]

Effect of EPL, PPC and PI on apoptosis in the HepG2 cell line[3]



Tamoxifen-induced apoptosis was significantly decreased by both EPL and PI in HepG2 cells

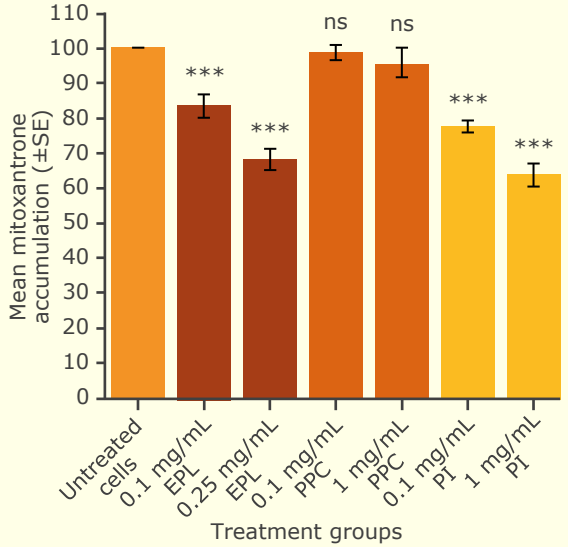
AU, arbitrary units; EPL, essential phospholipids; ns, not significant; MFI, mean fluorescent intensity; PI, phosphatidylinositol; PPC, polyenylphosphatidylcholine; SE, standard error
Figure adapted from Akazawa Y, et al. J Gastroenterol 2018:53;893–906

1. Maros T, et al. Arzneimittelforschung 1973;23:1538–42; 2. Akazawa Y, et al. J Gastroenterol 2018;53:893–906; 3. Wupperfeld D, et al. Euro Fed Lipid Congress 2021

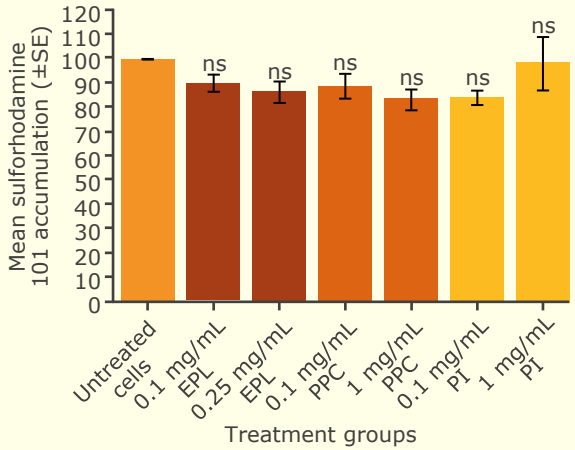
Liver cells also express various transport proteins which reply on membrane integrity to function

Effect of EPL, PPC and PI on hepatocellular transport protein activity in the HepG2 cell line

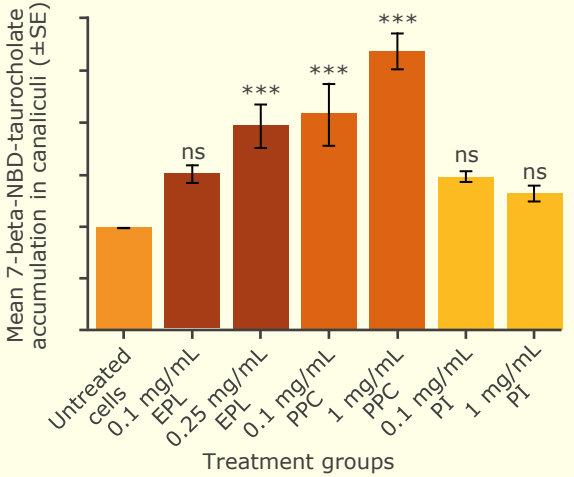
BCRP-mediated function significantly increased with EPL and PI†



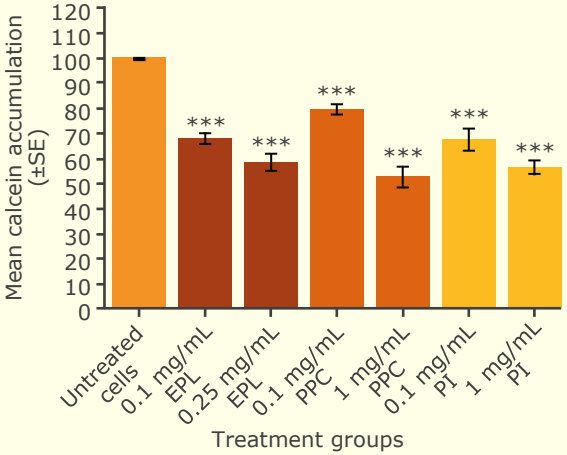
MRP-2-mediated function was not affected†



BSEP-mediated function was significantly increased with EPL and PPC†



P-GP-mediated function was significantly increased with EPL, PPC and PI†

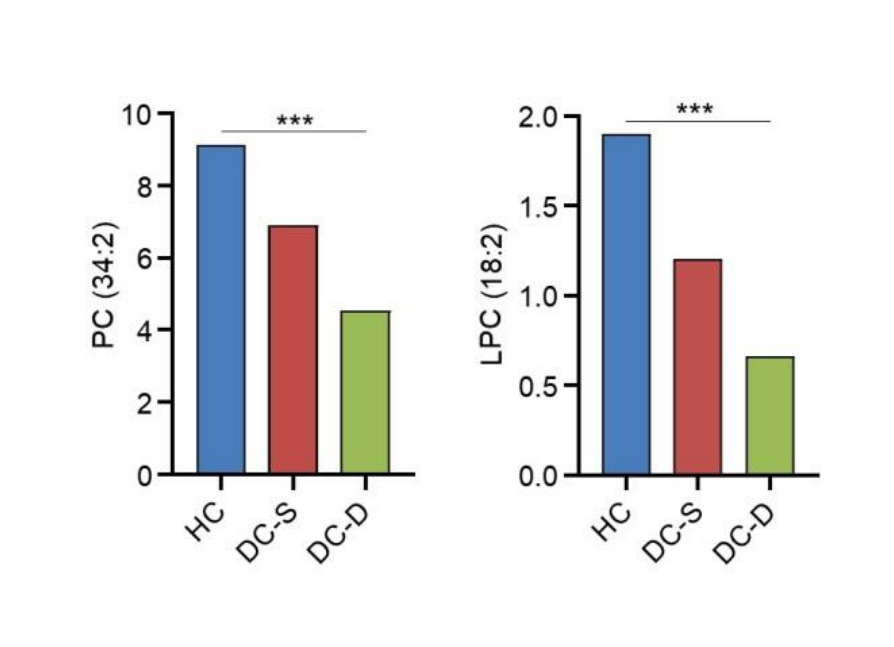


EPL increased hepatocellular extracellular transport involving the BSEP, BCRP and P-GP transport proteins. More protein transport could indicate healthier cells

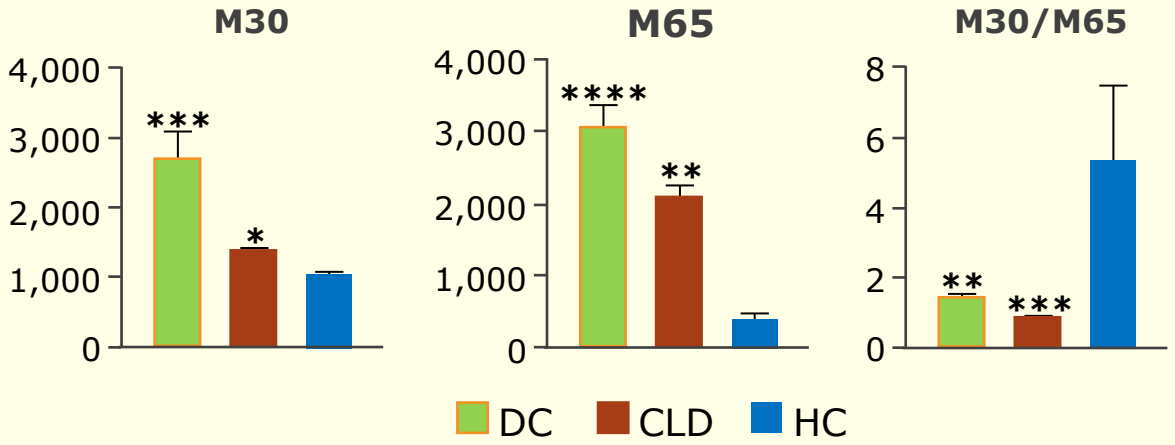
Values shown are mean ± SE (cellular substrate accumulation as percentage of untreated cells)
***p<0.001 versus untreated cells; †Compared with untreated cells, a significant change in substrate accumulation from untreated cells indicates increased hepatocyte transport function and could indicate healthier cells
BCRP, breast cancer resistance protein; BSEP, bile salt export protein; EPL, essential phospholipids; MRP-2, multi-drug resistance-associated protein 2; ns, not significant; P-GP, P-glycoprotein; PI, phosphatidylinositol; PPC, polyenylphosphatidylcholine; SE, standard error
Wupperfeld D, et al. Euro Fed Lipid Congress 2021

Clinical studies using metabolic profiling have also linked hepatic damage to cell membrane composition

Plasma metabotyping in patients with decompensated cirrhosis[1] (N=248)†



Comparison of markers of cell death in peripheral blood (Day 1)



Treatment with EPL increases the DLPC content of hepatocyte membranes,[2] and molecular profiling of patients with liver disease may influence future treatments

†External validation of NMR results for reduced LPC and PC was confirmed using UPLC-TOF-MS, *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001
CLD, chronic stable liver disease; DC, decompensated cirrhosis; DLPC, 1,2-di-linoleoyl phosphatidylcholine; EPL, essential phospholipids; HC, healthy control; LPC, lysophosphatidylcholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; UPLC-TOF-MS, reversed-phase ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry

1. McPhail M, et al. J Hepatol 2016;64:1058–67; 2. Gundermann KJ, et al. Clin Exp Gastroenterol 2016;9:105–17

EPLs exert a triple action in liver diseases

Active regeneration of liver cells[1–3]

- **Increases mitotic activity**
- **Increases DNA content**

Detoxification effects[4–6]

- **Inhibits lipid peroxidation**
- **Prevents liver steatosis, fibrosis, and cirrhosis**

Protective effects on cell level[1,3,6]

- **Increases membrane fluidity and transport**
- **Protects and increases genetic material**
- **Reduces apoptosis**

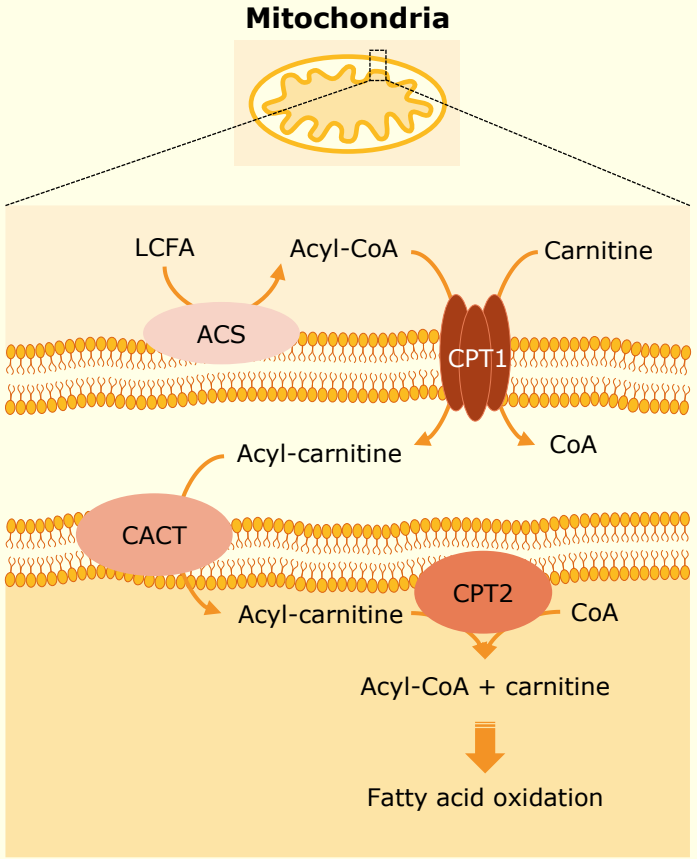
EPL, essential phospholipids

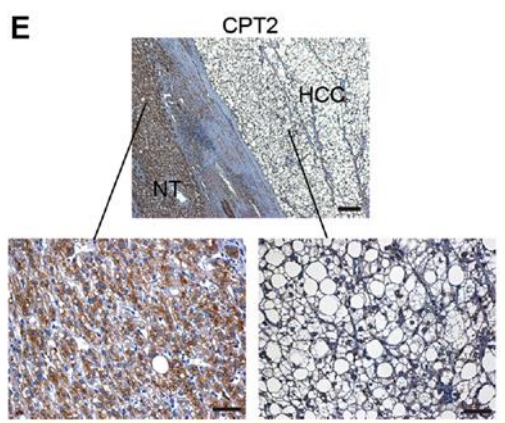
1. Demirbilek S, et al. Hepatol Res 2006;34:84-91; 2. Martelli A, et al. Med Sci Res 1989;17: 995–96; 3. Kropáčová K, Mišúrová E. Physiol Res 1995;44:241–7; 4. Klinger W, et al. Z Gastroenterol 1991;29(suppl 2):14-17; 5. Holeček M, et al. Arzneimittelforschung 1992;42:337-339; 6. Wupperfeld D, et al. Euro Fed Lipid Congress 2021

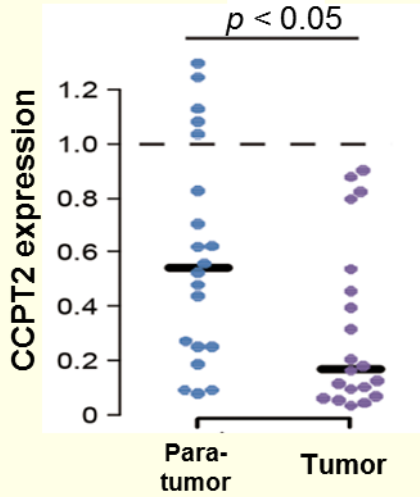
Mitochondria-associated membranes and hepatotoxicity

Mitochondria play a crucial role in regulating cell death and other processes

- Mitochondria are associated with NASH/NAFLD due to their effect on **hepatic lipid homeostasis, ROS production** and **lipid peroxidation, cytokine release** and **cell death**[1]
- Mitochondrial membranes are also composed of phospholipids and house a **variety of proteins for transport and signalling**[2]





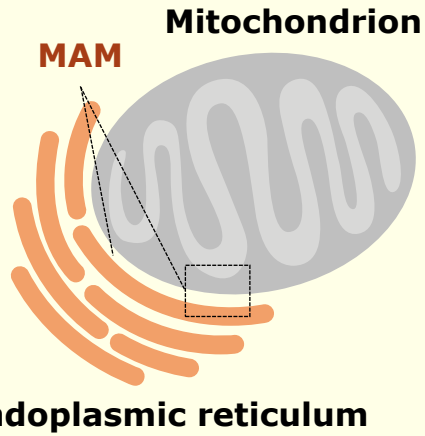


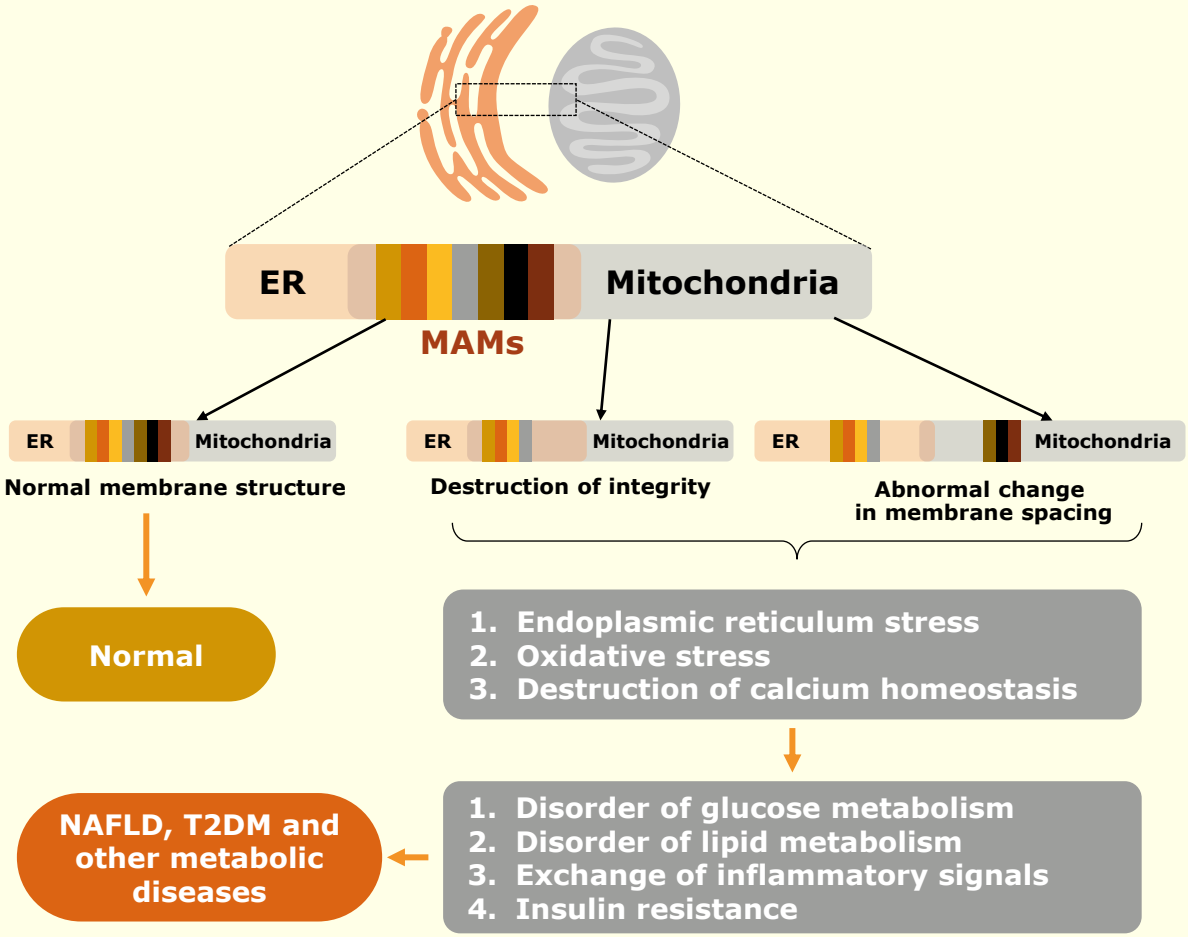
ACS, acylcarnitines; CoA, coenzyme; CPT1, carnitine palmitoyltransferase 1; CACT, carnitine-acylcarnitine translocase; CPT2, carnitine palmitoyltransferase 2; LCFA, long-chain fatty acid; MAM, mitochondrial associated membrane; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species
Image provided and approved by speaker

1. Begriche K, et al. Mitochondrion 2006;6:1–28; 2.Wang J, et al. Lipids Health Dis 2020;19:72 3. Fujiwara N,et al. Gut. 2018 Aug;67(8):1493-1504.

Mitochondria-associated membranes participate in the control of hepatic metabolism

- Disruption of the **structural** and **functional** integrity of MAMs can lead to a variety of **hepatic metabolic diseases**
- The **dysregulation** between **mitochondria and the ER** continues to be implicated in metabolic diseases



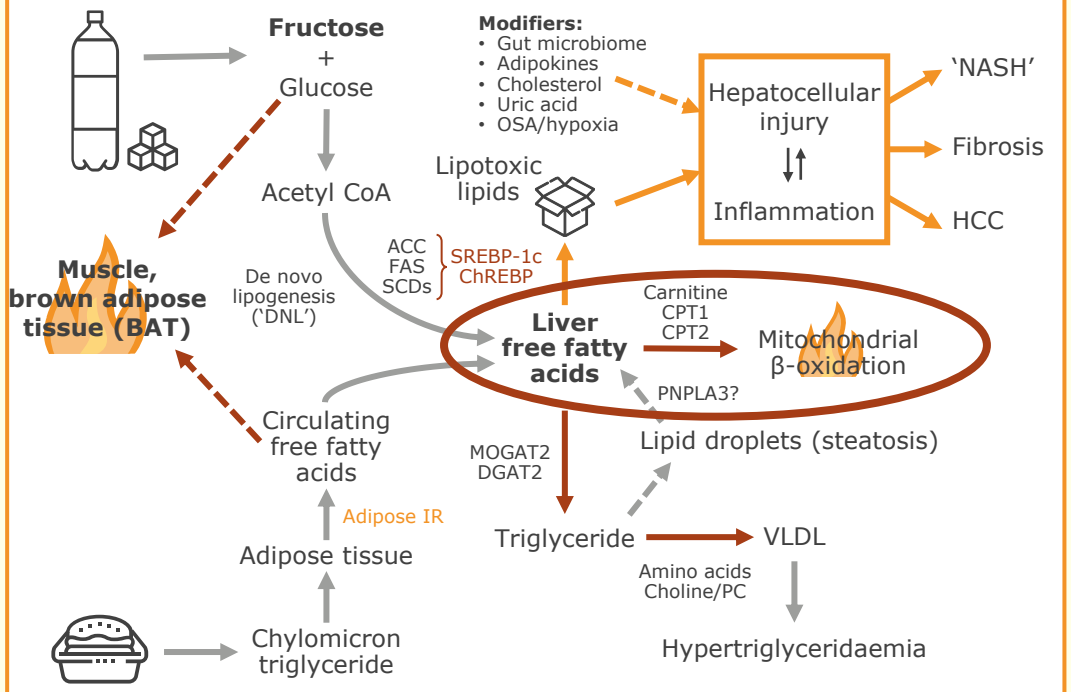


ER, endoplasmic reticulum; MAM, mitochondrial associated membrane; NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus

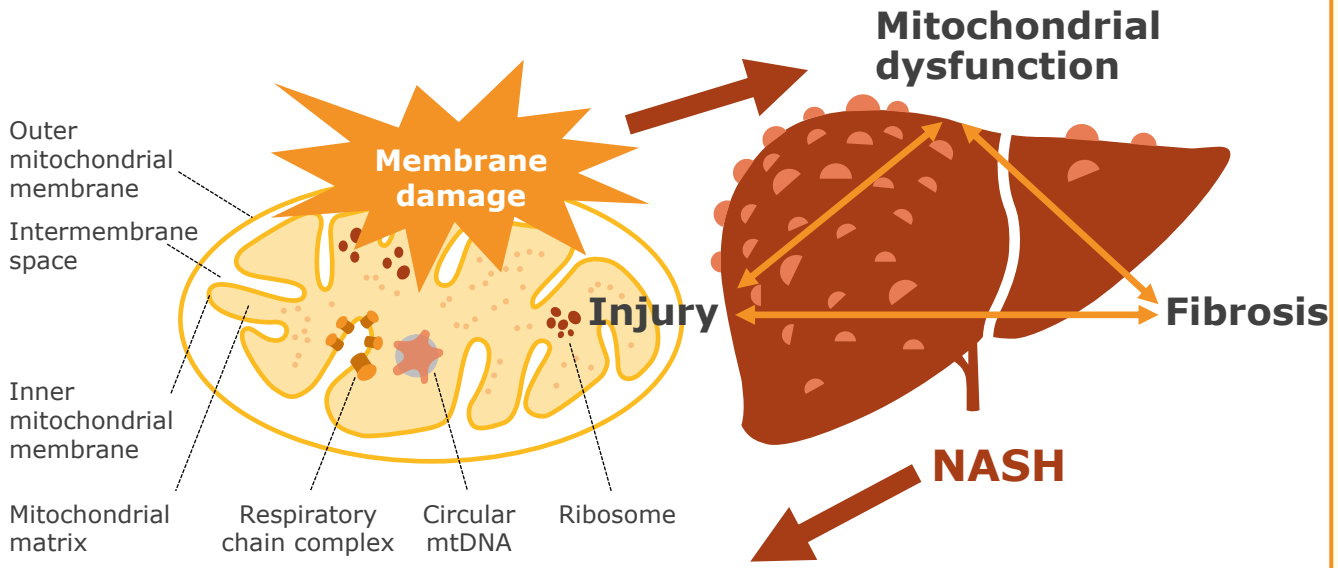
Wang J, et al. Lipids Health Dis 2020;19:72

Mitochondria play a pivotal role during the transition from simple steatosis to NAFLD

Mitochondrial fatty acid β-oxidation is a second line of defense against lipotoxicity[1]



Loss of mitochondrial membrane integrity and permeability can lead to NAFLD[1,2]



- **Loss of mitochondrial membrane potential**
- **mPTP openings (induced ROS release)**

ACC, acetyl-CoA carboxylase; ChREBP, carbohydrate-response element-binding protein; CoA, coenzyme A; CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine Palmitoyltransferase 2; DGAT, Diacylglycerol acyltransferases; FAS, fatty acid synthase; HCC, hepatocellular carcinoma; IR, insulin resistance; MOGAT, Monoacylglycerol O-Acyltransferase; mPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial deoxyribonucleic acid; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PC, phosphatidylcholine; PNPLA3, patain-like phospholipase domain-containing protein 3; ROS, reactive oxygen species; SREBP-1c, Sterol regulatory element-binding protein-1c; SCD, Stearoyl-CoA desaturase; SS, simple steatosis; VLDL, very low-density lipoprotein

Figure adapted from Neushwander-Tetri B, et al. BMC Med 2017;15:45

1. Li X, et al. Cell Death Discov 2020;6:80; 2. Lee J, et al. Arch Pharm Res 2019;42:935–46

Summary

1. Disruption to cellular and subcellular membranes, including MAM, have been linked to NAFLD/MAFLD in humans
2. Targeting cellular and molecular mechanisms related to membrane protection against cell damage may benefit treatment outcomes
3. EPL are used as treatment due to their hepatoprotective properties, including against lipotoxicity and protection of genetic material
4. Further insights into the MoA of NAFLD/MAFLD can help to determine future therapies

EPL, essential phospholipids; MAFLD, metabolic-associated fatty liver disease; MAM, mitochondrial associated membranes; MoA, mechanism of action; NAFLD, non-alcoholic fatty liver disease