# Essential phospholipids – new perspectives on the MoA

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# Disclosures

- Prof. Fricker has nothing to disclose

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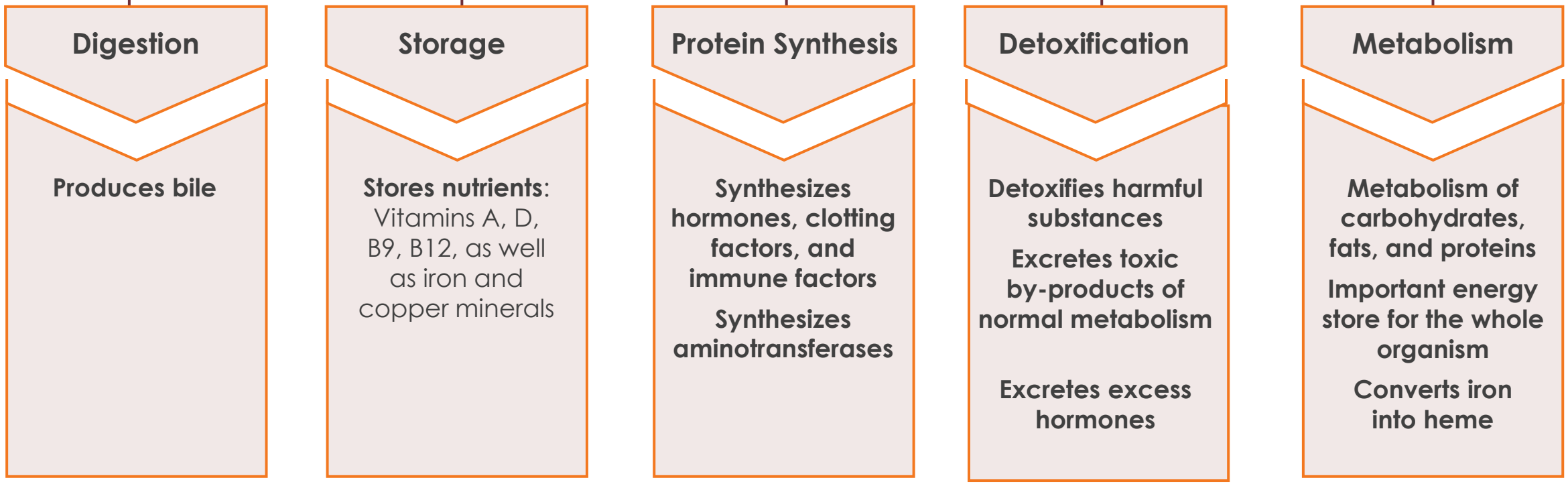
# Liver functions

**The liver is responsible for around 500 functions carried out by hepatocytes**




Franciscus A. HCSP. Version 1. April 2015

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# Liver disease and NAFLD/MAFLD

An estimated 844 million people have chronic liver disease[1]

NAFLD/MAFLD is one of the most common chronic liver diseases worldwide[2]

However, there are currently no FDA-approved drugs for treating NAFLD/MAFLD

FDA, Food and Drug Administration; MAFLD, metabolic-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease

1. Marcellin P and Kutala BK, Liver Int 2018;38 Suppl 1:2–6; 2. Ge X, et al. BMJ Open 2020;10:e036663. doi: 10.1136/bmjopen-2019-036663

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# NAFLD/MAFLD management strategies

**NAFLD/MAFLD management** consists of **treating the liver disease** as well as the associated metabolic **comorbidities** such as obesity, hyperlipidaemia, insulin resistance, and type 2 diabetes mellitus[1]

## Non-pharmacological approaches

A **personalised lifestyle intervention**, focusing on diet and exercise, **to lose weight**[1, 2]

**Weight loss** recommendations are:[1, 2]
3–5% reduction in body weight in patients with steatosis
7–10% reduction in body weight in patients with non-alcoholic steatohepatitis (NASH)

## Treatment of comorbidities

Aimed to **improve liver disease,** should be limited to biopsy-proven **NASH** and **fibrosis**[1]

**Management of comorbidities**, for instance, with:[1,2]
Anti-diabetics
Lipid-lowering agents
Anti-hypertensive agents

## Essential phospholipids

Adjunctive treatment with **Essential phospholipids (EPL)** has been shown to **alleviate symptoms**, inducing **histological changes** and **slowing** the **progression of liver disease**[3]

**EPL treatment has been associated with:**[3]
- **Clinical improvements of steatosis**
- **Anti-fibrotic effects**
- **Anti-oxidants effects**

MAFLD, metabolic-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease

1. Chalasani N, et al. Hepatology 2018;67(1):328–57; 2. Machado MV, et al. World journal of gastroenterology: WJG. 2014;20(36):12956; 3. Gundermann KJ, et al. Clin Exp Gastroenterol. 2016;9:105–117

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# How familiar are you with the mechanism of action of phospholipids in protecting liver health?

1. Very familiar
2. Somewhat familiar
3. Not familiar at all

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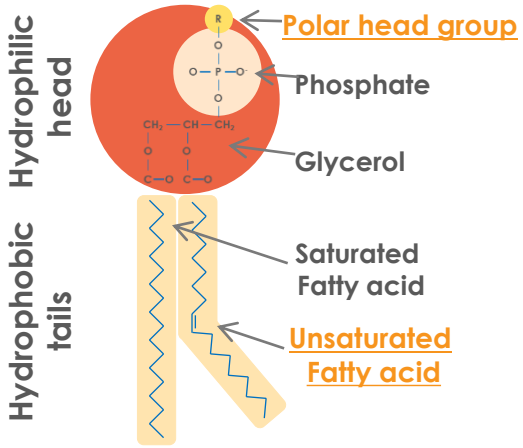
# Importance of phospholipids in cell membranes (1)

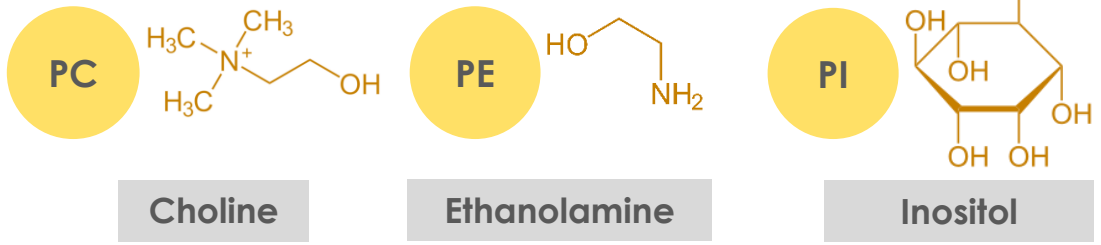
**Phospholipids (PL)** are essential **components of cellular and sub-cellular membranes**.

The most abundant PLs in mammalian tissues are:

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




## Polar groups (R)




## Fatty acid (FA) tails

Linoleic acid 18:2

Number of carbons

Number of double bonds

**Changes in the hepatic PC/PE molar ratio have been linked to the development of NAFLD/MAFLD in humans**

MAFLD, metabolic-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease

Li Z, et al. Cell metabolism 2006;3(5):321–31

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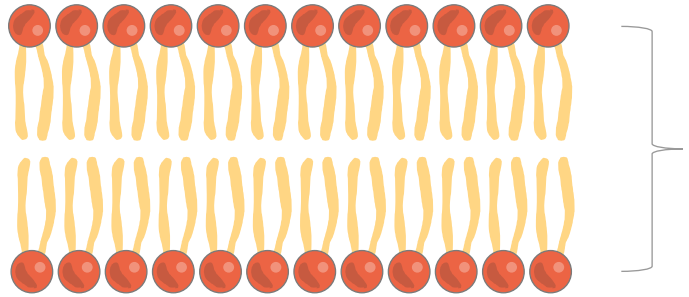
# Importance of phospholipids in cell membranes (2)

**PL functions**

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




**Membranes** constitute a meeting point for lipids and proteins and define the entity of cells and cytosolic organelles[1]

**Damage to liver cell membranes and the organelles originate from reduced phospholipid levels or altered phospholipid composition, and lead to decreased membrane fluidity[3]**

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids

1. Escribá PV, et al. J Cell Mol Med. 2008;12(3):829–75; 2. Li Z, et al. Cell Metab. 2006;3(5):321–31; 3. Gundermann KJ, et al. Clin Exp Gastroenterol. 2016;9:105–117

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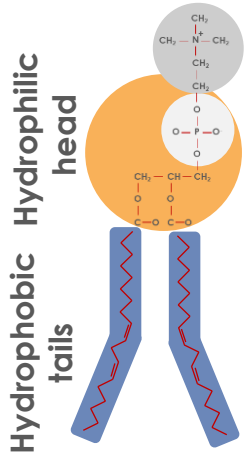
# Essential phospholipids

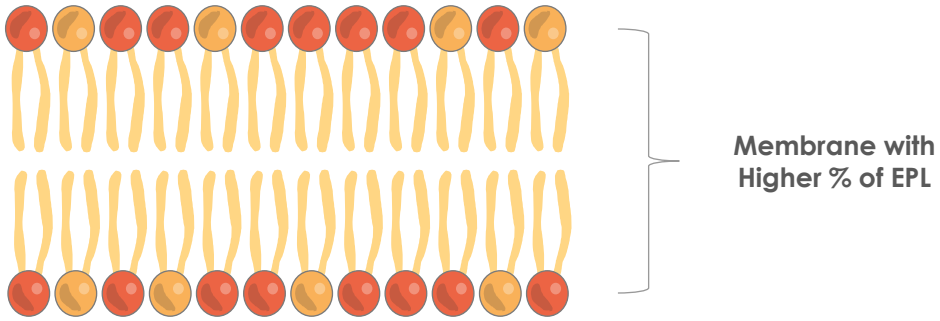
EPL

- **EPL** is a highly purified extract of **polyenylphosphatidylcholine (PPC) molecules** from soybeans[1]
- **1,2-di-linoleoyl phosphatidylcholine (DLPC)** is the lead compound in the active ingredient whose phosphatidylcholine molecules have specified amounts of 72–96% of PL in EPL[1]




DLPC

**EPL administration** significantly increases its percentage in the **membranes** of **hepatocytes**, **blood corpuscles** and **pancreatic tissue**, **among other tissues**[2]




**EPL have shown to exert membrane stabilising, membrane repairing and antioxidant actions that can be used to treat NAFLD/MAFLD[2]**

EPL, essential phospholipids; MAFLD, metabolic-associated fatty liver disease; NAFLD, non-alcoholic fatty liver disease; PL, phospholipids

1. Gundermann KJ, et al. Clin Exp Gastroenterol. 2016;9:105–117; 2. Gundermann KJ, et al. Pharm Rep 2011;63:643–59

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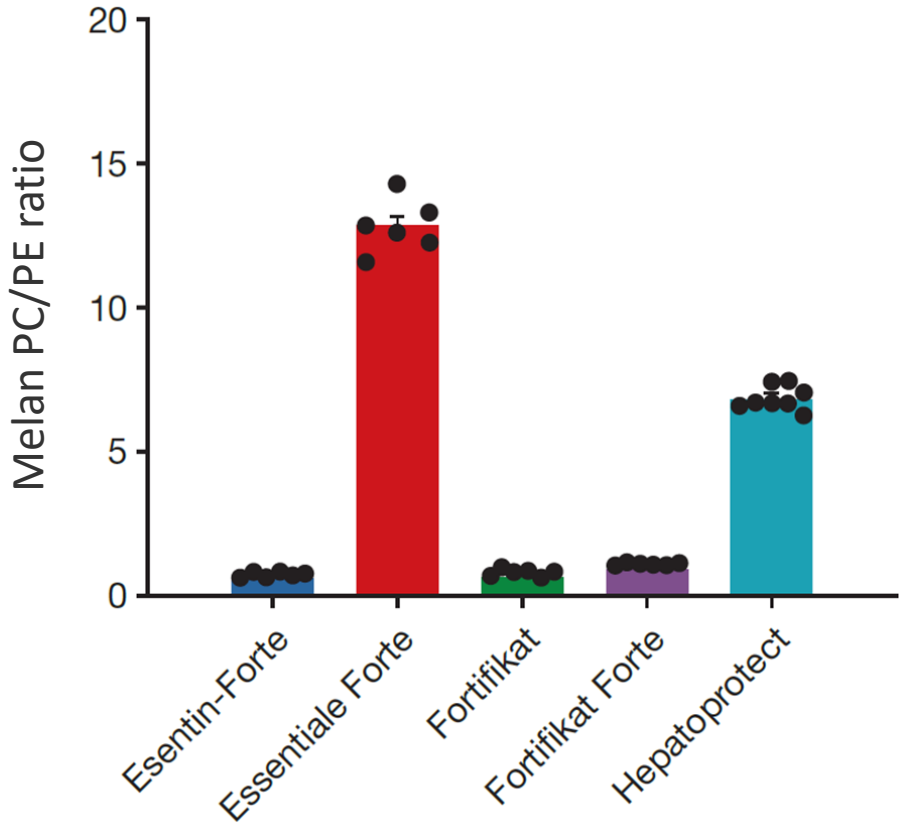
# Essentiale Forte has the highest PC:PE ratio

- 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[1]
- **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 Forte




Error bars represent standard error of the mean

PC, phosphatidylcholine; PE, phosphatidylethanolamine

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

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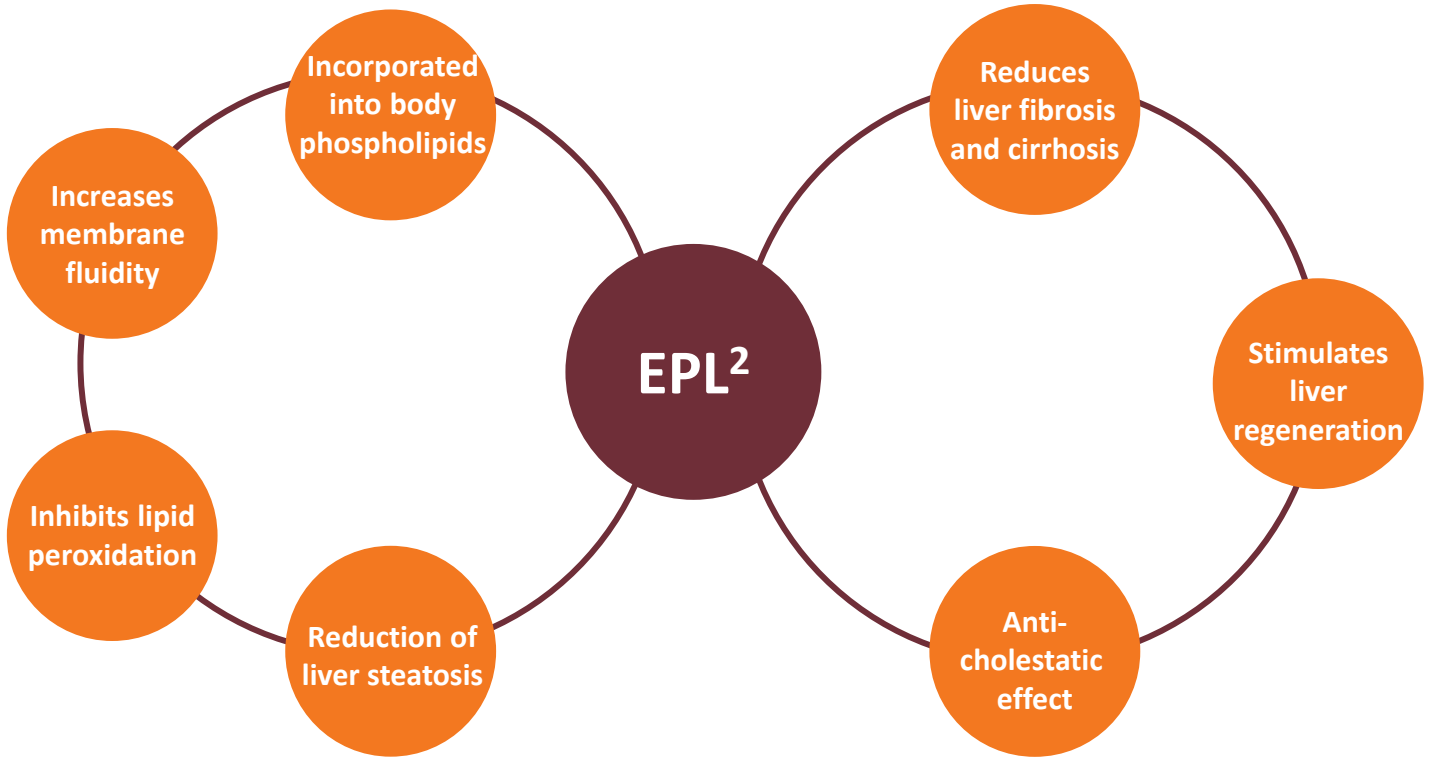
# Pre-clinical evidence for MoA of EPL

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

Pre-clinical 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) doi:10.1186/1476-511X-11-3; 2. Gundermann KJ, et al. Pharmacol Rep. 2011;63:643–659

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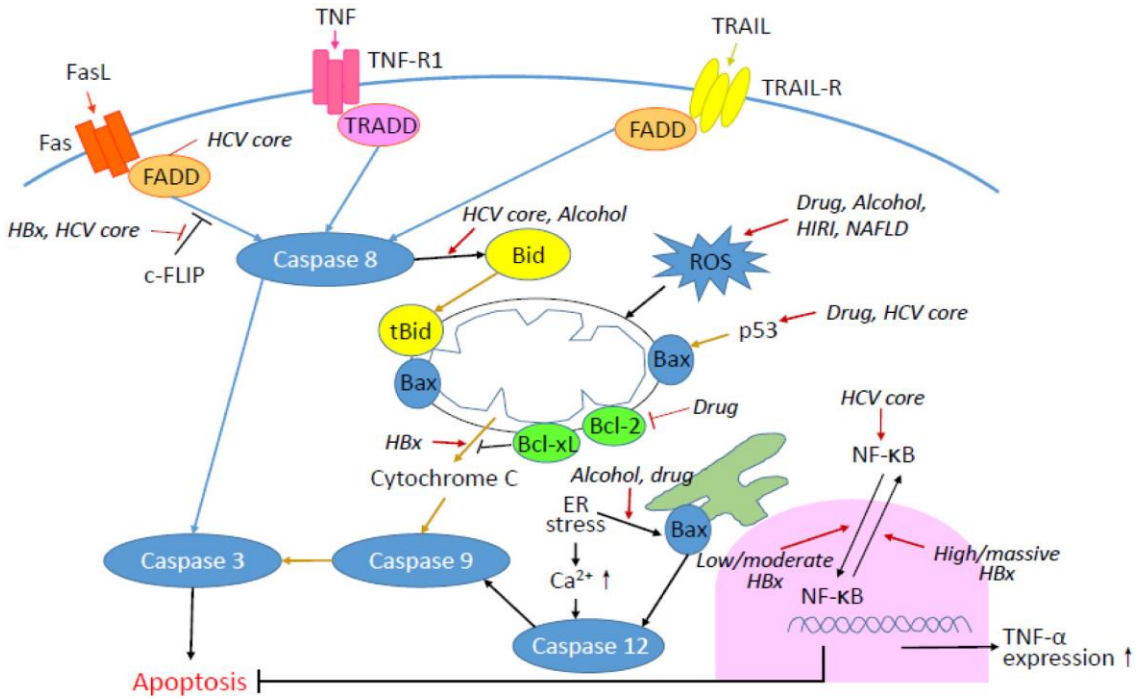
# Importance of apoptosis in liver cells

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

- Blue lines indicate extrinsic pathways, whereas light brown lines indicate intrinsic pathways
- The influence of virus infection, alcohol, fat, ischemia reperfusion and drug on hepatocyte apoptosis is also indicated by italics and red arrows

## Apoptotic pathways in hepatocytes




Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2 protein; Bcl-xL, B-cell lymphoma-extra large protein; Bid, BH3 interacting-domain death agonist; Ca2+, calcium ions; C-FLIPP, cellular FADD-like interleukin-1β converting enzyme inhibitory protein; ER, endoplasmic reticulum; FADD, Fas-associated protein with death domain; FasL, Fas ligand; HBx, hepatitis B virus protein x; HCV, hepatitis C virus; HIRI, hepatic ischemia reperfusion injury; NAFLD, non-alcoholic fatty liver disease; NF-κB, Nuclear factor kappa B; ROS, reactive oxygen species; TNF, tumour necrosis factor; TRADD, TNF-R1-associated death domain protein; TRAIL, TNF-related apoptosis inducing ligand

Cao L et al. J Cell Death 2016;9:19–29

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# Investigation into the impact of EPL, PPC & PI on hepatocyte function – an *in vitro* MoA study

The effects of **EPL** (0.1 and 0.25 mg/mL), and its constituents, **PPC** and **phosphatidylinositol (PI)** (both at 0.1 and 1 mg/mL) in human hepatocyte cell lines (HepG2, HepaRG, steatotic HepaRG) versus untreated cells were assessed in terms of the following parameters:

### Membrane Fluidity

- Assessed by anisotropy using a fluorescent probe

### Cell Apoptosis

- Apoptosis was induced by tamoxifen
- Apoptosis markers caspase-3 and caspase-7 were identified using caspase-3/7 green detection reagent

### Hepatocyte Transport Function

- Transporter activity was detected using fluororescent substrates specific to each of the transporters evaluated (BCRP, MRP-2, BSEP and P-GP)

BCRP, breast cancer resistance protein; BSEP, bile salt export pump; EPL, essential phospholipids; MoA, mechanism of action; MRP-2, multi-drug resistance-associated protein 2; P-GP, permeability glycoprotein; PI, phosphatidylinositol; PPC, polyenylphosphatidylcholine;
Wupperfeld D et al. Euro Fed Lipid Congress 2021 PO; manuscript in development

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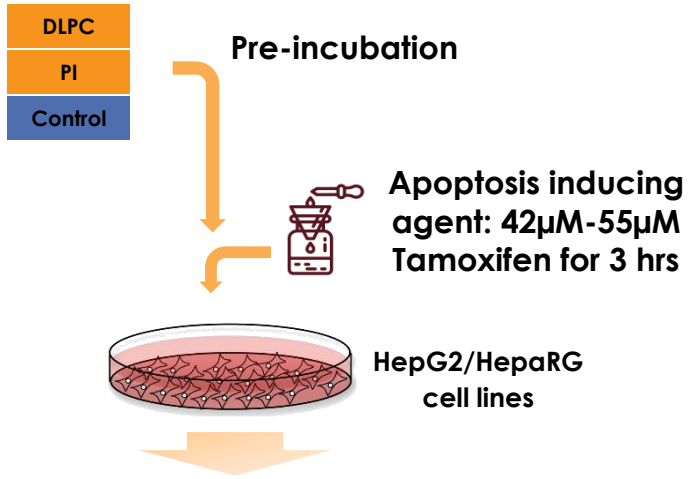
# Investigation into the effect of EPL, PPC & PI on cell apoptosis

- Caspases are enzymes which are **required for completion** of various **apoptotic pathways and stimulation of various cytokines**[1]

- The **determination of hepatocyte caspase activation** in the blood is a **strong** and **independent** predictor of **NAFLD/NASH**[3]

**Aim:**
**Evaluate if Caspase-3/7 become de-activated after EPL, PPC or PI treatment**

**Anti-apoptotic effects of EPL, PPC or PI treatment**




DLPC, 1,2-di-linoleoyl phosphatidylcholine; EPL, essential phospholipids; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PPC, polyenylphosphatidylcholine; PI, phosphatidylinositol
1. Singh S, et al. World J Gastroenterol. 2017;23(36):6549; 2. McArthur K, et al. Trends Cell Biol. 2018;28(6):475-93; 3. Wieckowska A, et al. Hepatology 2006;44(1):27–33

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# Investigation into the effect of EPL, PPC & PI on hepatocyte transport function

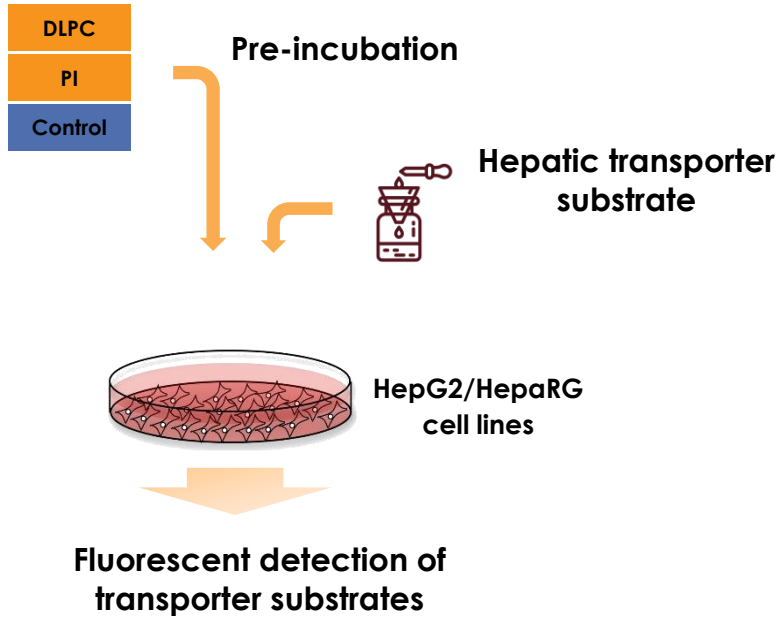
- Liver cells express various **transport proteins** whose function strongly depends on **membrane integrity**

- The main hepatocellular export proteins are:[1]
  - **PgP** → Export of xenobiotics and endogenous metabolites
  - **MRP2** → Export of organic anions and drug conjgates
  - **BCRP** → Export of xenobiotics and endogenous metabolites

    **BSEP** → Bile salt export

- Many of these transport proteins are altered in liver diseases[2]

**Aim:**
**Evaluate if some transporters can be activated by EPL, PPC or PI treatment**




BCRP: Breast cancer resistance protein, BSEP: Bile salt export protein; EPL, essential phospholipids; MRP2: Multidrug resistance-associated protein 2; PgP: p-glycoprotein; PI, phosphatidylinositol; PPC, polyenylphosphatidylcholine

1. Hewitt NJ, et al. Drug metabolism reviews. 2007;39(1):159–234; 2. Thakkar N, et al. J Pharm Sci 2017;106:2282–2294

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# Effects of EPL on hepatocyte membrane fluidity *in vitro*

- Addition of **EPL (0.1 and 0.25 mg/mL)** resulted in a significant increase in membrane fluidity of HepG2 cells compared with untreated cells
- **PPC and PI (0.1 and 1 mg/mL)** also significantly increased membrane fluidity of HepG2 cells compared with untreated cells
- In HepaRG cells, only addition of **PI 1 mg/mL** conferred a significant increase in membrane fluidity compared with untreated cells
- In steatotic HepaRG cells, **PI 1 mg/mL** significantly increased membrane fluidity compared with untreated cells
- EPL and PPC did not have a significant impact on the membrane fluidity of HepaRG and steatotic HepaRG cells at the concentrations tested

EPL, essential phospholipids; PI, phosphatidylinositol; PPC, polyenylphosphatidylcholine;
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# Anti-apoptotic effects of EPL in hepatocytes *in vitro*

## HepG2 cells

- **EPL** (0.1 and 0.25 mg/mL) **significantly reduced** tamoxifen-induced apoptosis (P<0.001)
- **PPC** (1.0 mg/mL) **significantly reduced** tamoxifen-induced apoptosis (P<0.001)
- **PI** (0.1 and 1 mg/mL) **significantly reduced** tamoxifen-induced apoptosis (P<0.05)

## HepaRG cells

- The addition of EPL (0.1 and 0.25 mg/mL), PPC (0.1 and 1.0 mg/mL) or PI (0.1 mg/mL) to tamoxifen-treated HepaRG cells resulted in a **trend for reduced rates of apoptosis**, although this was not significant

## Steatotic HepaRG cells

- The addition of EPL (0.1 mg/mL), PPC (0.1 and 1.0 mg/mL) and PI (0.01 mg/mL) to tamoxifen-treated cells resulted in a **trend for reduced rates of apoptosis**, although this was not significant

EPL, essential phospholipids; PI, phosphatidylinositol; PPC, polyenylphosphatidylcholine;
Wupperfeld D et al. Euro Fed Lipid Congress 2021 PO; manuscript in development

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# Impact of EPL on hepatocellular transport *in vitro* (1)

**BCRP**

- In **HepG2 cells**, EPL (0.1 and 0.25 mg/mL) and PI (0.1 and 1 mg/mL) **significantly increased** BCRP activity compared with baseline
- EPL, PPC and PI had no significant effect on the BRCP activity *in vitro* in either **HepaRG** or **steatotic HepaRG cells**

**MRP-2**

- MRP-2 activity was not affected by any PL in **HepG2 cells**
- **MRP-2 activity was significantly increased** by EPL, PI (1 mg/mL,) and PPC 1 mg/mL in **HepaRG cells** and by PI (1 mg/mL) in **steatotic HepaRG cells** compared with pre-treatment levels

**P-GP**

- **P-GP activity was significantly increased from baseline** by all compounds in **HepG2 cells**
- PI 1 mg/mL significantly increased P-GP activity in **HepaRG** and **steatotic HepaRG cells** from baseline

BCRP, breast cancer resistance protein; EPL, essential phospholipids; MRP-2, multi-drug resistance-associated protein 2; P-GP, permeability glycoprotein; PI, phosphatidylinositol; PL, phospholipids; PPC, polyenylphosphatidylcholine

Wupperfeld D et al. Euro Fed Lipid Congress 2021 PO; manuscript in development

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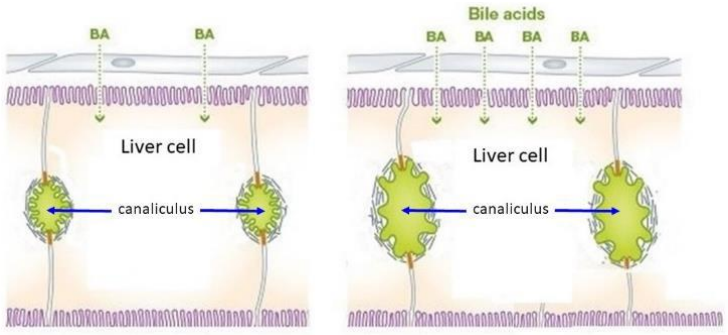
# Impact of EPL on hepatocellular transport *in vitro* (2)

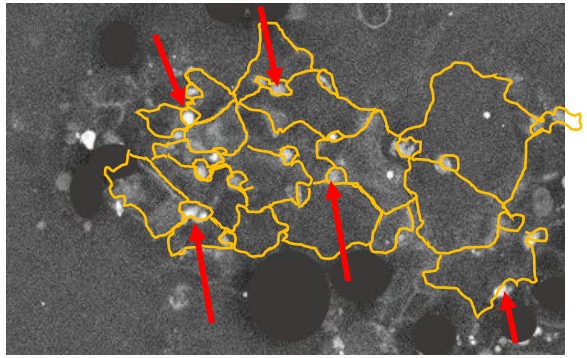
**BSEP**

- BSEP activity in **HepG2 cells** was **significantly increased** after treatment with EPL (0.25 mg/mL), and PPC (0.1 and 1 mg/mL) compared with before treatment ($p<0.001$)
- BSEP activity in **steatotic HepaRG cells** was **significantly increased** by EPL (0.25 mg/mL, $p<0.05$) and PPC (0.1 and 1 mg/mL, $p<0.05$ and $p<0.0001$, respectively) compared with before treatment
- EPL, PI and PPC had no effect on **HepaRG BSEP** activity

**Visualisation of BSEP in untreated and PPC-treated cells**




**Visualisation of BSEP in EPL-treated cells**



Yellow lines on figure represent individual cells; red arrows highlight examples of bile canaliculi structures with accumulated florescent substrate that presents BSEP activity. BSEP, bile salt export pump; EPL, essential phospholipids; PI, phosphatidylinositol; PPC, polyenylphosphatidylcholine

Wupperfeld D et al. Euro Fed Lipid Congress 2021 PO; manuscript in development

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# Conclusions

1. EPL are used to treat liver diseases, including NAFLD/MAFLD, due to their hepatoprotective properties
2. Several potential mechanisms of action of EPL have been elucidated by pre-clinical studies
3. In an *in vitro* study, EPL, PPC, and PI increased hepatocyte membrane fluidity, decreased apoptosis and increased hepatocellular transport
4. EPL may improve liver function and confer hepatoprotective effects via these MoA

EPL, essential phospholipids; MAFLD, metabolic-associated fatty liver disease; MoA, mechanism of action; NAFLD, non-alcoholic fatty liver disease; PI, phosphatidylinositol; PPC, polyenylphosphatidylcholine

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