Diploma thesis

EFFECTS OF PHOSPHATIDYLCHOLINE SYNTHESIS ON LRH-1

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Abstract

Liver receptor homologue-1 (LRH-1/NR5A2) is a nuclear receptor and transcription factor that can be activated by ligands and is critically involved in the regulation of liver metabolism. Phospholipids have been discovered as potential LRH-1 ligands and activation of LRH-1 by certain phosphatidylcholines (PCs) has been shown to have physiological effects able to ameliorate non-alcoholic fatty liver disease and improve insulin sensitivity. However, a natural ligand or a metabolic pathway producing a LRH-1 ligand has not yet been defined. Since PCs have been described to activate LRH-1 we hypothesized that metabolic pathways producing PCs may generate an endogenous natural LRH-1 ligand. To test our hypothesize we silenced the two metabolic pathways of endogenous PC production, the Kennedy and the PEMT pathway, and studied effects on mRNA expression of LRH-1 target genes *invitro* in the hepatic cell line HepG2. In addition, we studied LRH-1 dependent gene expression *invivo* in Pemt knockout mice. Pemt knockout mice showed significantly decreased mRNA expression levels of the classical LRH-1 target genes Cyp8b1, Gck and Gnmt. However, also Lrh-1 mRNA itself was significantly decreased. In human *invitro* cell line experiments we robustly knocked down the key rate limiting enzyme of the Kennedy pathway, CTα, and PEMT pathway, PEMT, using siRNA oligonucleotides. In line with the rodent *invivo* experiments siPEMT in HepG2 cells also showed significant reduction of LRH-1 target genes along with significant decrease in mRNA levels of the nuclear receptor LRH-1 itself. CTα knockdown did not have any robust effects on LRH-1 or LRH-1 target genes. Taken together, our experimental evidence suggests that the PEMT pathway, which generates a distinct set of PCs, significantly affects LRH-1 signalling while the Kennedy pathway has only little effect. However, from the experiments performed so far we are unable to differentiate if effects on LRH-1 target genes are caused by reduction of an endogenous ligand - as hypothesized - or rather caused by reduction of LRH-1 itself, or a combination of both. We have also yet not shown if, complementary to PEMT silencing, induction of the PEMT pathway activates LRH-1 signalling. Thus, further studies to answer these questions are warranted. These studies should include overexpression of stable LRH-1, which is unresponsive to gene regulation and gain of function experiments including PEMT overexpression and treatment with PEMT specific PC compounds.

Zusammenfassung

Liver receptor homologue-1 (LRH-1/NR5A2) ist ein Kernrezeptor und Transkriptionsfaktor, der durch Liganden aktiviert werden kann und an der Regulation des Lebermetabolismus maßgeblich beteiligt ist. Phospholipide wurden als potenzielle LRH-1 Liganden identifiziert und es konnte gezeigt werden, dass es durch die Aktivierung von LRH-1 durch spezielle Phosphatidylcholine (PCs) möglich ist die Nicht-alkoholische Fettlebererkrankung günstig zu beeinflussen und die Insulin Senstivität zu erhöhen. Dennoch wurden bisher weder ein natürlicher Ligand noch ein Stoffwechselweg, welcher einen solchen endogenen Liganden produziert, gefunden. Aufgrund der Tatsache, dass PCs als Aktivatoren von LRH-1 beschrieben wurden, formulierten wir die Hypothese, dass Stoffwechselwege, die PCs produzieren, auch einen natürlichen Liganden für LRH-1 hervorbringen könnten. Um unsere Hypothese zu testen wurden die zwei Stoffwechselwege, die für die Produktion von endogenen PCs verantwortlich sind, der Kennedy-Pathway und der PEMT-Pathway, genetisch ausgeschalten und die nachfolgenden Effekte auf die mRNA-Expression von LRH-1 Zielgenen in der hepatischen Zelllinie HepG2 untersucht. Zusätzlich wurde die Expression von LRH-1 abhängigen Zielgenen in Pemt-Knockout Mäusen studiert. Pemt-Knockout Mäuse zeigten eine signifikant niedrigere mRNA Expression der klassichen LRH-1 Zielgene Cyp8b1, Gck und Gnmt. Auch die Lrh-1 mRNA selbts war signifikant reduziert. In den *invitro* Zellkultur Experimenten mit humanen HepG2 Zellen wurden die Schrittmacher Enzyme des Kennedy-Pathways, CTα und des PEMT-Pathways, PEMT, mit Hilfe von siRNA Oligonukleotiden ausgeschalten. In Übereinstimmung mit den *invivo* Maus- Experimenten zeigte der PEMT knockdown in den HepG2 Zellen eine signifikante Redukton der LRH-1 Zielgene und wiederum eine signifikante Reduktion der mRNA Spiegel des Kernrezeptors LRH-1. Die Ausschaltung von CTα wirkte sich weder auf LRH-1 noch dessen Zielgene in signifikanter Weise aus. Die Ergebnisse unserer Experimente legen nahe, dass der PEMT-Pathway, welcher sehr spezifische PCs generiert, einen signifikanten Einfluss auf die Aktivität von LRH-1 hat, während der Kennedy -Pathway keinen solchen Effekt hat. Es ist jedoch aus den bisherigen Experimenten nicht möglich zu sagen, ob die Auswirkungen auf die LRH-1 Zielgene durch eine Reduktion der endogenen Liganden – wie von uns vermutet – zustande kommt, oder ob es durch die Reduktion von LRH-1 selbst bedingt ist oder eine Kombination beider Faktoren vorliegt. Ebenso wurde noch nicht gezeigt ob die Induktion von PEMT, im

Gegensatz zu dessen Ausschaltung, LRH-1 aktiviert. Zur Beantwortung dieser Fragen sind weitere Experimente vorgesehen. Diese Studien sollten die Überexpression von stabilem LRH-1, welches selbst nicht reguliert wird und daher stabil expremiert wird, und sog. „Gain-of-funtion“ Experimente, wie PEMT Überexpression und die Behandlung mit PEMT spezifischen PC Verbindungen, beinhalten.

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List of abbreviations

ABCA1	ATP-binding cassette transporter 1
ABCG5/8	ATP-binding cassette transporter G5/8
ApoA1	Apolipoprotein A1
AR	Androgen receptor
ASBT	Apical sodium-dependent bile acid transporter
BRCA1	Breast cancer-1
BSEP	Bile salt export pump
CA	Cholic acid
CAR	Constitutive androstane receptor
CDCA	Chenodeoxycholic acid
cDNA	Complementary deoxyribonucleic acid
Cel	Carboxyl ester lipase
CETP	Cholesteryl ester transfer protein
CK	Cholin-kinase
COUP -TF	Chicken ovalbumin upstream promoter transcription factor
Cpa1	Carboxypeptidase A1
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CT	CTP-phosphocholine-cytidylyltransferase
Ctrl	Chymotrypsin-like
CYP	Cytochrome P450
DAX-1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region
DBD	DNA binding domain
DLPC	Dilauroyl phosphatidylcholine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiothreitol
DUPC	Diundecanoyl phosphatidylcholine
Ela1	Elastase 1
ER	Estrogen receptor
FXR	Farnesoid-X-receptor
Gck	Glucokinase
Gnmt	Glycine-n-methyltransferase
GR	Glucocorticoid receptor
HDL	High density lipoprotein
HNF-4	Hepatocyte nuclear factor-4
HRE	Hormone responsive elements
HSD3B2	3β-hydroxysteroid dehydrogenase
LBD	Ligand binding domain
LDL	Low density lipoprotein

LRH-1	Liver receptor homologue-1
mRNA	Messenger ribonucleic acid
NAFLD	Non-alcoholic fatty liver disease
NR	Nuclear receptor
Oct4	Octamer-binding transcription factor 4
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamin-N-methyltransferase
PG	Phosphatidylglycerol
PGC-1 α	Peroxisome-proliferator-activated-receptor-γ-co-activator-1α
PIP	Phosphatidylinositol
PPARα	Peroxisome proliferator-activated receptor α
PPARγ	Peroxisome proliferator-activated receptor γ
PR	Progesterone receptor
PTF1-L	Pancreas transcription factor 1-L complex
PTM	Posttranslational modification
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SF-1	Steroidogenic-factor 1
SHP-1	Short heterodimer partner-1
shRNA	Small hairpin ribonucleic acid
siRNA	Silencing ribonucleic acid
SR-B1	Scavenger-receptor-B1
SREBP1c	Sterol regulatory binding protein 1c
StAR	Steroidogenic acute regulatory protein
TR	Thyroid receptor
VDR	Vitamin D receptor
VLDL	Very low density lipoprotein
WT	Wildtype
ZMF	Center for medical research

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Effects of phosphatidylcholine synthesis on LRH1 activity

1. Introduction

1.1 Nuclear receptors

Nuclear receptors (NRs) are ligand-sensitive transcription factors, which can directly link metabolic and environmental stimuli to changes in gene transcription (1). NRs play a key role for cell homeostasis and metabolic adaptation and are therefore also - directly or indirectly - involved in pathogenesis but also treatment of various diseases and metabolic imbalances. Some of the best known examples of NRs as disease modifiers are the androgen receptor (AR) and its role in prostate cancer, estrogen (ER) and progesteron receptor (PR) and breast cancer or the constitutive androstane receptor (CAR) for hepatocellular cancer or the farnesoid X receptor (FXR) and short heterodimer partner-1 (SHP) for cholestatic liver disease and gallstone disease, respectively (2-5). NRs are already widely pharmacologically targeted such as the peroxisome proliferator-activated receptor α (PPARα) by fibrates, the peroxisome proliferator-activated receptor γ (PPARγ) by glitazones, the glucocorticoid receptor (GR) by glucocorticoids, or the thyroid receptor (TR) by thyroid hormones (6-8) . However, not all NRs have identified ligands yet and not all physiological as well as pathophysiological consequences of NR action are established. Thus, identifying new ligands may open up new insights into NRs function and pharmacological strategies.

The NR superfamily in humans consists of 48 members including receptors for steroid hormones, thyroid hormones, various lipids, bile acids or xenobiotics. However, only approximately half of those receptors have well defined ligands whereas some NR remain without any identified ligands up to now and therefore are called “orphan receptors”(1) (Table 1). Recently, new ligands have been identified for some of the former orphan NRs, which are therefore called “adopted NRs”. These newly discovered ligands include, among others, phospholipids as ligands for the liver receptor homologue-1 (LRH-1) (9) Table 1 shows a classification of NRs and their typical ligand, if known (adapted from (1)).

Table 1 Nuclear receptors and their ligands

Endocrine Receptors	Adopted Orphan Receptors	Orphan Receptors
Ligands: High-affinity, hormonal lipids	Low-affinity, dietary lipids	Unknown
ER α,β (*estrogens*) PR (*progesterone*) AR (*testosterone*) GR (*cortisol*) MR (*aldosterone*) RAR α,β,γ (*vitamin A)* TR α,β (*thyroid hormone*) VDR (*vitamin D*)	PXR α,β,γ (*xenobiotics*) PPAR α,β,γ (*fatty acids, prostaglandins, eicosanoids*) LXR α,β (*oxysterols*) FXR (*bile acids*) PXR/SXR (*xenobiotics, steroids*) CAR (*androstane*) SF-1 (*phosphatidylinositol*) LRH-1 (*phosphatidylinositol*)	DAX-1 (*unknown*) SHP (*unknown*) TLX (*unknown*) PNR (*unknown*) NGFI-B α,β,γ (*unknown*) ROR α,β,γ (*cholesterol, ATRA*)? ERR α,β,γ (*unknown*) RVR α,β,γ (*unknown*) GCNF (*unknown*) TR 2,4 (*unknown*) HNF-4 (*fatty acids*)? COUP-TF α,β,γ (*unknown*)

NRs share an overall similar design. Basically, NRs consist of a DNA binding domain (DBD) and a ligand binding domain (LBD). The DBD also called C-region is the most conserved part of a NR with only little or no variation among different species. The DBD consists of two zinc finger motifs, which allow binding to specific locations on the DNA, called hormone responsive elements (HRE). The LBD, or the E-region, is the second most conserved part and it further encompasses a ligand-dependent activation function (AF-2) and dimerization interface. The LBD forms the ligand pocket which can bind the specific ligand. The LBD is connected to the DBD by the hinge-region (D-region). The hinge-region is important for protein flexibility and receptor dimerization. The A/B-region, which is thought to contain ligand-independent activation activity (AF-1), is highly variable and can further modulate NR activity. Some receptors also have an F-region close to the carboxy-terminal end of the NR (1, 10). Among mammals the NR sequences are highly conserved, yet there are clearly distinguishable species-specific variations (11). Figure 1 shows a schematic structure of a NR (adapted from (10)). A three dimensional structure of a typical LBD and DNA binding domain can be found at: https://en.wikipedia.org/wiki/Nuclear_receptor.

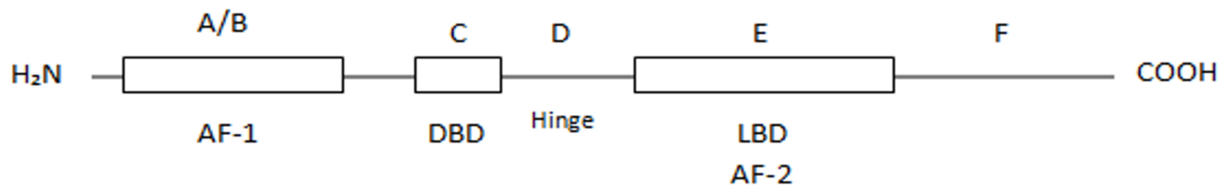

Figure 1. Schematic structure of a nuclear receptor

Nuclear receptors consist of: the ligand binding domain (LBD or C-region) which binds the specific ligand and further encompasses ligand-dependent activation function (AF-2); the DNA binding domain (DBD or E-region), which binds to the response element on the DNA; the D-region (Hinge) connects the LBD and DBD; the A/B-region (AF-1) assumedly contains ligand-independent activation activity; and some NRs also comprise an F-region.

NRs can be divided into four groups regarding their binding characteristics to DNA and dimerization with other NRs. Type I NRs are located in the cytosol when inactive. Upon ligand binding, type I NRs homodimerize with themselves and enter the nucleus to bind to their distinct HREs on the DNA (e.g. AR, ER, GR). Two NR-specific half-sites on the DNA make up the HRE, and the second site is inverted from the first (inverted repeat/IR) (12). Type II NRs remain in the nucleus regardless of ligand-binding. When activated, they function as heterodimers with the common partner retinoid X receptor (RXR). NRs which belong to this class include the TR, retinoic acid receptor (RAR), vitamin D receptor (VDR) or FXR. Type III NRs are similar to Type I, but they bind to direct repeats (e.g. chicken ovalbumin upstream promoter transcription factor (COUP-TF) or hepatocyte nuclear factor-4 (HNF-4)). Type IV receptors, such as steroidogenic-factor 1 (SF-1) and LRH-1, bind to DNA as monomers. Most orphan receptors are members of type III and IV NRs (12, 13). Figure 2 shows the four classes of NRs and examples for each one (adapted from (13)).

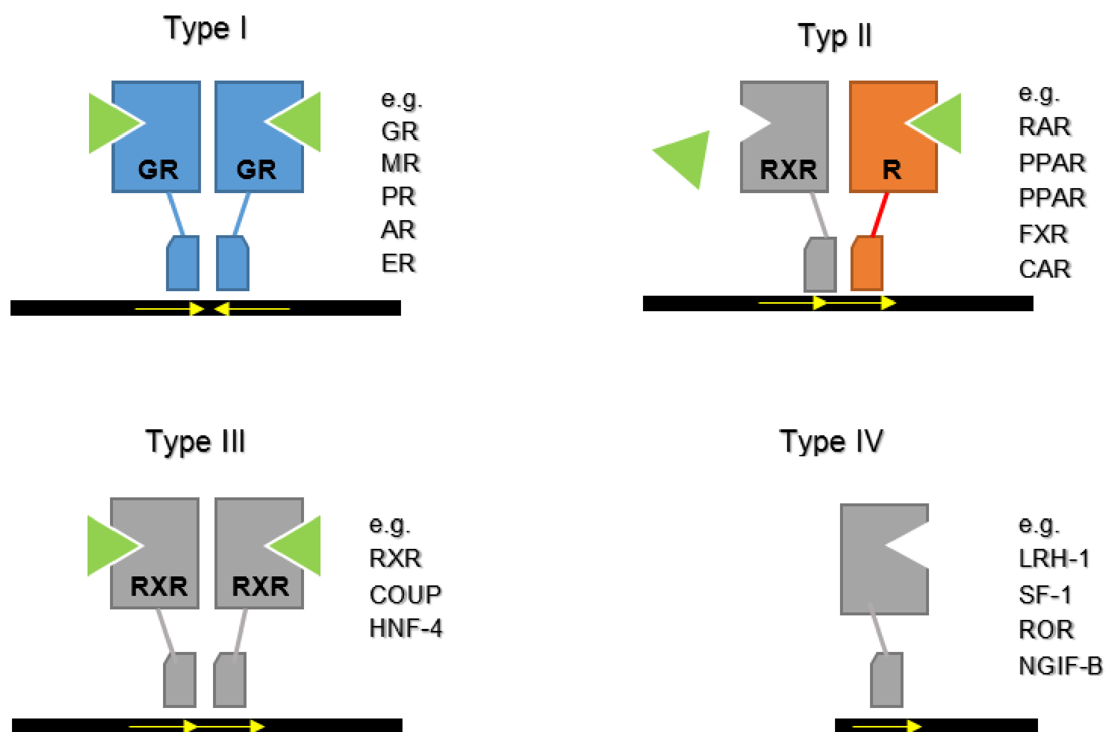

Figure 2. The four classes of nuclear receptors

Nuclear receptors can be classified into four groups based on their DNA binding and dimerization characteristics (adapted from (13)). The yellow arrow represents a NR-specific half site on the DNA. Opposing half sites are called inverted repeats (Type I NRs) and longitudinal half sites are called direct repeats (Type III NRs). See text above for a more detailed explanation.

Following the binding of an agonist ligand, NRs undergo a conformational change, which dissociates co-repressors and enables binding of co-activators. These conformational changes finally result in the assembly of the transcription machinery including the RNA polymerase which starts gene transcription at the transcription start site and upregulates gene expression levels. The opposite occurs when an antagonist binds and gene transcription is shut down. Co-regulators (i.e. co-activators or co-repressors) are critical to NRs function as they either modulate chromatin structure and therefore change the accessibility to promoters or enhancers of target genes or help to stabilize the binding of further co-regulators and the transcription

starting complex (1, 14). Figure 3 gives a schematic overview of NR activation and initiation of target gene transcription (adapted from (15)).

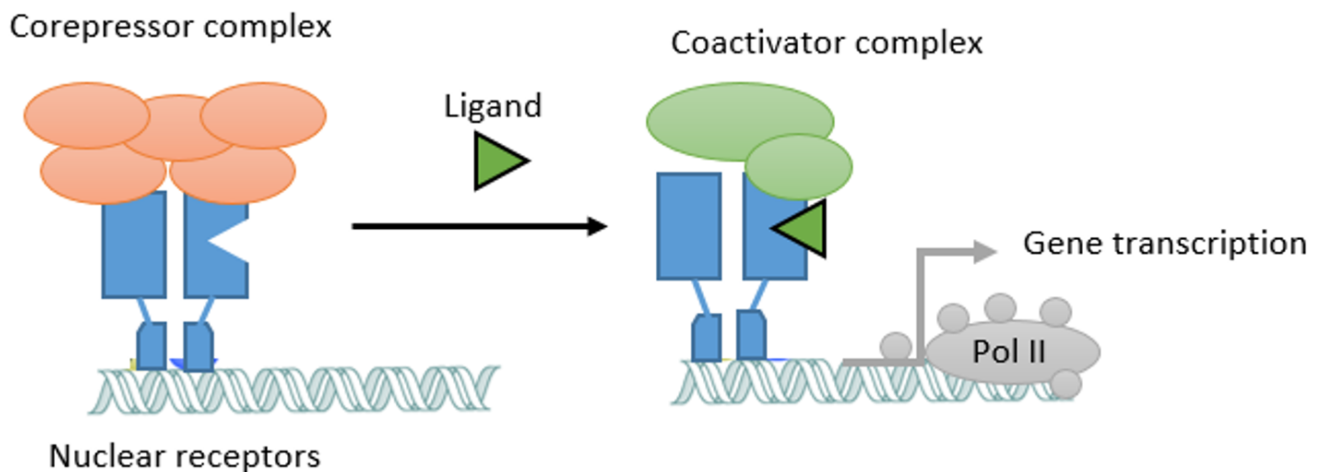

Figure 3. Nuclear receptor activation and transcription of target genes

Upon NR activation by ligands dissociation of corepressors and binding of coactivator occurs. This is followed by the assembly of the transcription machinery including the RNA-polymerase, which leads to increased gene transcription.

Besides activation of NRs by ligands, NRs can also be constitutively active without any ligands or can be regulated by posttranslational modifications (PTMs), such as acetylation, phosphorylation or sumoylation, or “simply” by binding of co-repressors and co-activators.

1.2 Liver receptor homologue-1

LRH-1 (NR5A2) belongs to the group of recently adopted orphan NRs. It is a class IV NR and therefore binds to its specific HRE on the DNA as a monomer. LRH-1 is regulated by ligand-

dependent mechanisms which means that binding of certain molecules alters its activity, as well as ligand-independent mechanisms (10).

Ligand-independent regulation of activity includes post-translational modification (phosphorylation and sumoylation) and interaction with coregulators. Phosphorylation of specific regions of LRH-1 enhances its activity, whereas sumoylation of certain areas has an opposing, repressing effect (16, 17). The most important coregulatory for LRH-1 is small-heterodimer-partner (SHP), which negatively regulates LRH-1 activity. Interestingly SHP is also a direct transcriptional target of LRH-1, thereby creating a negative feedback loop for LRH-1 activity (18).

1.2.1. Distribution and major extrahepatic functions of LRH-1

LRH-1 is expressed in embryonic stem cells, adult liver, intestine, pancreas and ovary cells. It plays a major role in early development, in regulating cholesterol homeostasis and bile acid homeostasis, in steroidogenesis, but also in the pathogenesis of diseases, including breast and pancreatic cancer (19). Figure 4 provides an overview of LRH-1's many functions (adapted from (20)).

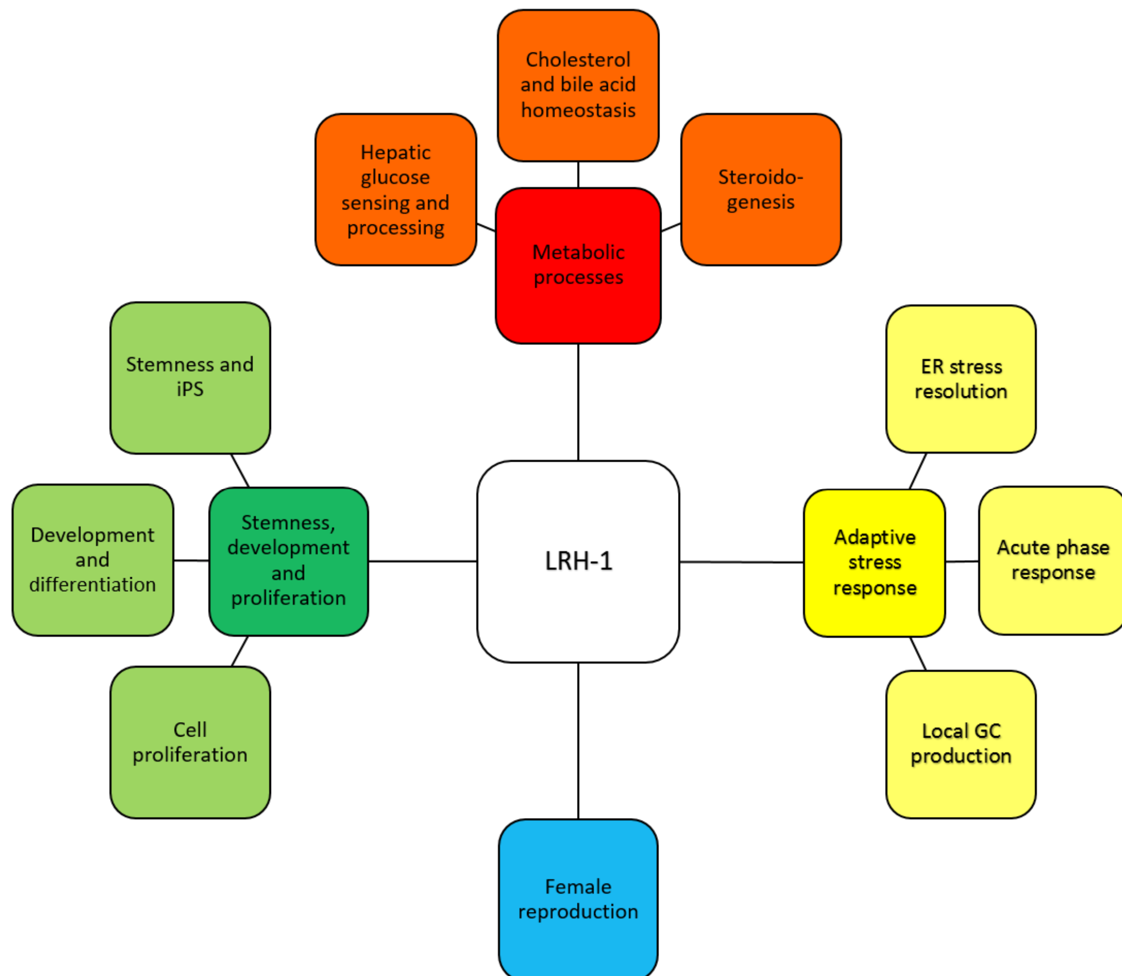

Figure 4. Overview of LRH-1's functions

LRH-1 has various functions: Regulation of metabolic processes (red); adaptive stress response (yellow); female reproduction (blue); stemness, development and proliferation (green) (adapted from (20)).

The fundamental role of LRH-1 in development is underlined by the finding that homozygous LRH-1 gene knockout mice die at a very early embryonic stage since LRH-1 is essential for embryonic development and differentiation by regulating the expression of Oct4, a key molecule for maintaining pluripotency (18).

Tissue specific LRH-1 knockout mice however survive and this has helped to identify the LRH-1 organ specific role. LRH-1 is found normally in relatively low concentrations in breast adipose tissue and there solely in the stromal part, but is highly concentrated in primary invasive breast cancer. Here, LRH-1 is significantly elevated in both tumor epithelial and intra-tumoral stroma cells. The role of LRH-1 in breast cancer is comprehensible, when considering that LRH-1 promotes the transcription of aromatase, the key gene for converting androgens to estrogens. The aromatase enzymatic step becomes the main source of estrogens for postmenopausal women. Estrogens are mitogenic for estrogen-dependent breast cancer mainly in postmenopausal women and thereby LRH-1 promotes tumour growth (19).

Both LRH-1 and its close homologue SF-1 can be found in ovarian cells and they play a major role in steroidogenesis and luteinisation by promoting important target genes like aromatase, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (HSD3B2) and inhibin α subunit (19).

The role of LRH-1 in pancreas is a dual one. Pancreas specific LRH-1 knockout in adult mice has revealed its role in pancreas physiology as key regulator of pancreatic exocrine function. In concert with pancreas transcription factor 1-L complex (PTF1-L) exocrine pancreas specific genes such as carboxyl ester lipase (Cel); elastase 1 (Ela1); chymotrypsin-like (Ctrl); or carboxypeptidase A1 (Cpa1) are regulated LRH-1 dependently (21). On the other hand pancreatic cancer has been linked to LRH-1, too. LRH-1 is highly expressed in ductal pancreatic adenocarcinomas and mediates cancer cell proliferation and differentiation via induction of cyclin D1, cyclin E1 and c-myc (19).

In the intestine LRH-1 has been implicated in intestinal glucocorticoid synthesis and thus regulation of intestinal immunity in response to immunological stress has been discovered. LRH-1 has been shown to activate CYP11A1 and CYP11B1, key enzymes of steroidogenesis. LRH-1 haploinsufficient mice were more likely to develop intestinal inflammation after chemical irritation and they displayed lower glucocorticoid production in the gut. Interestingly, patients with chronic inflammatory bowel disease (IBD, e.g. Crohn's disease or ulcerative colitis) show a decreased expression of LRH-1 in the intestinal mucosa which implies that LRH-1 might also be an interesting pharmacological target for IBD in humans (22). This regulation appears to be particularly important in the gut, but a link between the

LRH-1 homologue SF-1 and CYP11B1 has also been discovered in adrenal glands (23). Thus, it is likely that a similar role of LRH-1 for steroidogenesis in the adrenal glands can be envisioned.

In addition to the role of LRH-1 in the small intestine, more recent findings report involvement of LRH-1 in human colon cancer development and progression. Specifically, in surface cells of cancer tissue, where LRH-1 cannot be detected physiologically, alterations in expression and dislocations of LRH-1 have been found (19, 24).

1.2.2. LRH-1 and liver metabolism

LRH-1 plays an important role in liver metabolism. LRH-1 is involved in four major hepatic metabolic pathways: (I) bile acid metabolism, (II) cholesterol metabolism, (III) hepatic lipogenesis and (IV) glucose homeostasis. In general, LRH-1 activation leads to a lipid-lowering environment and has therefore attracted attention as a potential pharmacological target for hepatic lipid disorders. In the following the major target genes of LRH-1 and its integration in liver lipid metabolism are briefly depicted:

(I) *Bile acid metabolism:* Bile acids are not only simply breakdown products of cholesterol but also function as important signaling molecules in lipid, glucose, and energy metabolism as well as in immune function. LRH-1 critically regulates several enzymatic steps in the generation of and in bile acid metabolism (25). Recent findings in hepatospecific LRH-1 knockout animals suggest a major role for regulation of CYP8B1 transcription and a minor role in CYP7A1 regulation (26, 27). CYP7A1 is the rate limiting enzyme for the conversion of cholesterols to bile acids and loss of CYP7A1 results in elevated cholesterol levels in both liver and serum (28). CYP8B1 is another key enzyme of bile acid synthesis as CYB8B1 determines the amount of cholic acid (CA) over chenodeoxycholic acid (CDCA) in the bile acid pool. CAs increase energy expenditure in rodent brown adipose tissue and prevents from insulin resistance and obesity (29). Increased CYP8B1 levels were also critically associated with the resistance of SHP knockout mice to cholesterol induced fatty liver disease (30). Liver-specific LRH-1 knockout mice showed decreased Cyp8b1 levels along with decreased CA levels and slightly decreased bile acid pool size (26, 27).

LRH-1 also regulates the main canalicular bile acid pump, BSEP, which enhances bile flow and biliary bile acid export. Overexpression of BSEP results in increased lipid export and thus decreased hepatic steatosis (31, 32). In the ileum, apical sodium-dependent bile acid transporter (ASBT) is another positively regulated LRH-1 target gene (33). ASBT reduction leads to loss of intestinal bile acids with consequently increased hepatic cholesterol breakdown for maintaining bile acid supply (34). Taken together, LRH-1 critically determines bile acid composition and pool size.

(II) *Cholesterol metabolism:* LRH-1 regulates all necessary steps and transporters to eliminate excess cholesterol (18, 35). In peripheral tissue excess cholesterol is shuttled through the ABCA1 transporter to ApoA1 (poorly lipidated apolipoproteins) and forms HDL particles. HDL recirculates to the liver and its cholesterol is taken-up by hepatocytes via scavenger-receptor-B1 (SR-B1). Cholesterol can then either be converted to bile acids (see above), or be effluxed actively and passively into the biliary system. This efflux is mediated by cholesterol transporters, ABCG5/8, which are again positively regulated by LRH-1.
In enterocytes the same transporters can be used to return excess cholesterol into the gut lumen. Cholesteryl ester transfer protein (CETP), an enzyme only found in the human organism, transfers cholesterol esters from HDL to ApoB-containing lipoproteins (e.g. LDL, VLDL) in exchange for triglycerides. This may result in additional hepatic cholesterol uptake via LDL-receptors. Summarizing all LRH-1 meditated effects on cholesterol metabolism it can be assumed that LRH-1 has an anti-atherosclerotic effect and may also contribute to anti-steatotic LRH-1 effects on fatty liver by reducing hepatic cholesterol content.

(III) *Hepatic lipogenesis:* Activation of LRH-1 results in suppression of the main transcriptional driver for lipogenesis, sterol regulatory binding protein 1c (SREBP1c) (36, 37). Therefore, activation of LRH-1 and suppression of SREBP1c consequently results in the reversal of a vicious metabolic cycle where increased SREBP1c and *de-novo* lipogenesis lead to increased insulin levels, decreased insulin sensitivity and fatty liver (38).

(IV) *Glucose homeostasis*: LRH-1 also directly regulates hepatic glucokinase, an enzyme responsible for hepatic glucose disposal and lipid homeostasis. The product of glucokinase, glucose-6-phosphate is a substrate for either glycogen synthesis as well as glycolysis and

denovo lipogenesis (39). This turns LRH-1 into a key regulator of proper integration of glucose and lipid metabolism (37, 39). It has been shown in diabetic mouse models that activation of LRH-1 by DLPC, a phospholipid LRH-1 agonist, had a strong impact on glucose metabolism resulting in improved glucose tolerance. In contrast, genetic haploinsufficiency of LRH-1 did not affect glucose metabolism, suggesting that receptor activation has a stronger impact on metabolic effects than inactivation (20).

1.2.3. Ligands for LRH-1

LRH-1 is constitutively active and can drive the transcription of its target genes constitutively (40). The reason for this ligand-independent activity may lie in the N-terminal region of the LBD, where special structural elements that provide an additional layer over the canonical LBD fold were found (41). However, LRH-1's activity is largely regulated by co-activators and co-repressors, some of which are tissue specific, adding further to the complexity of LRH-1 activation and tissue specificity. For example, in the ovary peroxisome-proliferator-activated-receptor-γ-co-activator-1α (PGC-1 α) acts as a co-activator for LRH-1 and mediates the differentiation of granulosa to luteal cells, while DAX-1 blocks this interaction and represses its function. In the liver LRH-1 activity is blocked by SHP-1 (19). Yet, beside constitutive gene regulation, activity of LRH-1 can also be modulated "classically" by ligand binding to the LBD. In 2005 in a series of structural crystallisation studies phospholipids have been identified in the ligand binding pocket of LRH-1 and suggested to be potential ligands of LRH-1 (42-44). In 2011 it was demonstrated that certain phosphatidylcholine (PCs) species indeed ligand-activate LRH-1-driven gene transcription and these PCs could have been recovered in crystallization studies (36, 45). However, it has not been established if endogenously generated phospholipids are able to activate LRH-1 and consequently if phospholipid synthesis is physiologically linked to LRH-1.

Endogenous phospholipids are a heterogeneous group of molecules. They consist of a hydrophilic head, which comprises a negatively charged phosphate group (e.g. phospatidylcholine (PC) or phosphatidylserine (PS)) and two hydrophobic fatty tails that can vary in length and saturation. Crystallographic analysis have shown that specific phospholipids, namely PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and

perhaps even the second messenger phosphatidylinositol (PIPs) interact with the binding pockets of LRH-1 and SF-1, when expressed in bacteria and it is assumed that they may act as functional ligands (9). Furthermore, it appears that changes in the fatty acyl pool of phospholipids may generate signals that can either activate or inhibit LRH-1 (9). While phospholipids with C12-16 saturated fatty acyl groups recruit co-activators, phospholipid molecules containing C18:2 chains rather displace co-activators (46). However, the origin and the role of length or number of double-bonds in the fatty acid chains of the phospholipids are not yet clear. Thus, the definite natural and physiological active ligand (if there is one at all) has not yet been identified and it is not known whether it can be generated endogenously (19).

1.3. Phospholipid synthesis

PC is an essential phospholipid and major component of bilayer membranes. It consists of a hydrophilic choline head and a hydrophobic fatty acid backbone. It is made in every nucleated cell from choline. This choline dependent pathway is responsible for 70% of the total PC pool and is called Kennedy or choline pathway. In addition to that, the PEMT-pathway exists and makes up for the remaining 30%. The PEMT-pathway is only relevant in the liver, but may also be partly active in adipocytes (47).

Kennedy Pathway: Choline is obtained from diet and absorbed in the gut by distinct choline transporters. After uptake the enzyme cholin-kinases (CKα/β) catalyzes phosphorylation of choline to phosphocholine (48). The CTP-phosphocholine-cytidylyltransferase (CT) is the rate-limiting enzyme in the Kennedy pathway. There are two genes, PCYT1A encodes for CTα2 and 3, and PCYT1B encoding for CTβ2 and 3. Whereas CTα is found in every tissue, CTβ is highly enriched in the brain and nervous tissue. CT is activated by binding to the membrane of the endoplasmatic reticulum or the nucleus and is inactive in the soluble form. Interestingly, it's activity is not linked to the cholesterol metabolism but to cell cycle, growth and differentiation (48). The last step is facilitated by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT). This enzyme has never been purified but is speculated to be in excess amounts in every cell (48). Figure 5 shows the PC generation by the Kennedy pathway (right side) (adapted from (48)).

Pemt Pathway: Phosphatidylethanolamin-n-methyltransferase (PEMT) catalyzes the methylation of PE to PC using three methyl-groups coming from S-adenosylmethionine (SAM). PEMT is probably not vital for normal phospholipid metabolism as long as there is sufficient choline uptake from food, but becomes very important for maintaining PC levels and normal liver function when the supply is low. This is suggested by experiments in Pemt knockout mice, which displayed no obvious phenotype when on a chow diet. However, when Pemt knockout mice were put on choline-deficient diet they develop hepatic steatosis, liver failure and die within 3 weeks (48). Figure 5 shows the PC generation by the PEMT pathway (left side) (adapted from (48)).

Are phospholipids produced by either the Kennedy or the PEMT pathways distinct from each other? Analysis of the hepatic phospholipid content in liver and serum of Pemt knockout mice showed significantly lower concentrations of docosahexaenoic acid [22:6(n-3)] and arachidonic acid [20:4(n-6)] as compared to wildtype mice (49). This has been independently confirmed by cell culture experiments using radiolabeled precursors (50). Moreover, several experiments show that the VLDL composition critically depends on PCs derived from the PEMT pathway (51, 52). These findings suggest that PCs produced by the PEMT pathway are partly distinct from PCs formed by the Kennedy pathway and imply that PCs from the PEMT pathway may also have distinct physiological functions.

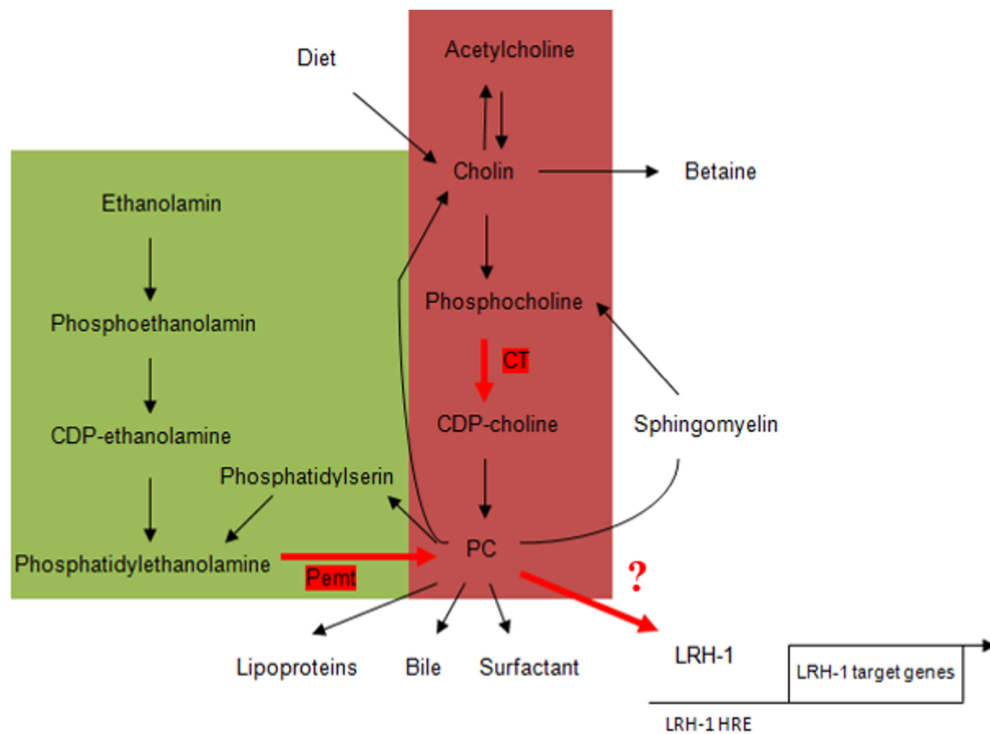

Figure 5. PC generation by the Kennedy and PEMT pathway

PCs can be created by two different metabolic pathways. 70% of endogenous PCs are generated by the Kennedy pathway and the key enzyme CT (red). The PEMT pathway is responsible for the remaining 30% (green). PCs fulfill several important tasks and may even be – as hypothesized - a functional ligand for LRH-1.

2. Hypothesis and Aims:

LRH-1 is a metabolic active NR highly expressed in the liver. Even though phospholipids appear to bind to LRH-1, a natural ligand or endogenous pathways producing the natural ligand have not yet been identified. **Therefore, the overall goal of this study is to test the hypothesis that endogenous phospholipid production may change the activity of LRH-1 in the liver.** More specifically, we want to determine if a distinct PC-generation pathway, i.e. either the Kennedy or the PEMT pathway, may account for potential effects on LRH-1 activity.

Aim: To test if inhibition of either the Kennedy or the PEMT pathway alters LRH-1 activity. In this loss of function assay the rate limiting enzymes of the Kennedy (i.e. PCYT1A) and PEMT (i.e. PEMT) pathway will be genetically silenced and the effects on LRH-1 target gene expression will be determined. This *invitro* assay will be complemented by an analysis of LRH-1 target genes in Pemt knockout mice *invivo*.

3. Significance and novelty

The metabolic syndrome and in particular non-alcoholic fatty liver disease (NAFLD), as the hepatic component of the syndrome is an increasingly recognized health and economic problem in the Western World. Despite intensive research pharmacological treatment options are still limited, which is largely based on incomplete understanding of disease mechanisms and failure of novel treatment concepts in humans. Activation of the NR LRH-1 ameliorates NAFLD and certain phospholipids are functional ligands for LRH-1. Interestingly, it is not known if PCs endogenously produced by the Kennedy or PEMT pathways can affect LRH-1 and thereby impact on liver metabolism.

Identification of an endogenous metabolic pathway that results in changed LRH-1 activity would on one hand deepen our understanding of metabolic liver homeostasis and pathogenesis of metabolic liver diseases such as NAFLD and on the other hand it would reveal a potential target for pharmacological interventions. The activity of both PC generating pathways depends, among other factors, on dietary inputs, i.e. on choline for the Kennedy pathway and methylation donors such as S-adenosylmethionine for the PEMT pathway. Thus dietary supplementations or modifications could be rationally used as a simple therapeutic additive in the treatment of NAFLD.

The hypothesis and the proposed outcome are relevant for both genders.

4. Methods

Mouse tissue:

Frozen liver tissue from male mice with a genetic deletion of Pemt (Pemt$^{-/-}$ mice) and respective wildtype (WT) mice were kindly provided by Prof. Dennis Vance, Edmonton, Alberta (n=4 mice per genotype) (53). Approximately 20mg of liver tissue per mouse were used for RNA isolation to test LRH-1 dependent gene expression. Unfortunately, mice with a defective Kennedy pathway (i.e. liver specific CTα knockout mice) were not available for our studies.

Cell culture and treatment:

For *invitro* studies the human immortal liver cancer cell line HepG2 was used. HepG2 cells were obtained from the cell culture core facility of the ZMF. HepG2 cells were maintained in 6-well dishes containing DMEM media (GIBCO) supplemented with 10% fetal bovine serum and incubated with 5% CO2. Treatment was started when HepG2 cells reached a confluency of about 70-80%. Viability was checked by trypan blue. HepG2 cells were cleaned of medium using PBS. After siRNA treatment (see methods below) cells were incubated for 24h to 48h and harvested by adding 400µl of TRIzol reagent for subsequent RNA isolation. All experiments were performed in triplicates and repeated at least in two independent experiments. Normal human liver tissue from a previous published study with an existing ethical vote has been used to compare baseline expression levels in cell lines to human tissue expression levels (54). All cell culture experiments were performed under the guidance of my supervisors.

RNA-Isolation:

RNA was isolated from 20mg mouse liver tissue and HepG2 cells using 400µl (HepG2 cells) or 800µl (mouse liver tissue) TRIzol Reagent (Ambion 15596026). Tissue was homogenized beforehand using Magnalyzer beads (Roche) and centrifuged two times for 20 min. at 6500rpm. Then 160ul of chloroform was added. The mix was cooled for 15min at 4 °C and then centrifuged again at 13000rpm for another 15 min. at 4 °C. Afterwards we obtained three distinguishable phases: Phenol and chloroform on the bottom, DNA and proteins as the in-

between phase and RNA as a top layer (aequous phase), which was then taken off and mixed with an equal amount of isopropanolol to precipitate the RNA. Following 5 min. of repeated tilting, the tubes were centrifuged once more at 13000rpm for another 15 min. at 4 °C. Subsequently the supernatant was removed and the remaining pellet dissolved in up to 500ul of 70% Ethanol. Next, the mix was centrifuged at 13000rpm for another 15 min. at 4 °C. The supernatant was removed again and the pellet dried before it was dissolved in aqua dest. After that it was incubated at 65°C for 10min and put on ice for at least 30min. Finally RNA concentration was spectrometrically quantified and quality was checked by the 260/280 ratio using Thermo Scientific's NanoDrop2000c. A ratio of 1.8 – 2.0 was regarded as good quality material.

cDNA-Synthesis:

1µg RNA was reversed transcribed into cDNA using the Superscript (Invitrogen) kit. 0,4µl Random Hexamers were used as primers. Transcription was performed using the following protocol: 5 minutes of 65°; 10 minutes of 25°C. Then the mastermix was added to each tube, containing 4µl 5x Buffer; 2µl 0,1M DTT; 0,3µl dNTP; 0,5l dNTP; 0,5µl Superscript; 0,5µl RNAse inhibitor and 2,7µl Aqua dest. Following this procedure, the protocol was continued at 42°C for 90 minutes and 15 minutes of 70°C. Afterwards it was cooled down to 4°C and cDNA stored at 4°C until further qPCR.

Real time qPCR:

2,5µl of the diluted cDNA were used as template. Reaction mix consisted of 1.25µl forward and reverse primer each and 5µl Sybr Green master mix (Invitrogen) containing the polymerase and the nucleotides. PCR was run on a Roche Lightcycler using the following conditions: 95° for 10 minutes; 40 cycles of 95° for 10 seconds, 60° for 15 seconds and 72° for 15 seconds. We used duplicates or triplicates on 96-well plates and duplicates with a Cp deviation >0.5 were excluded. Analysis was performed using the ΔΔCT-method. h18sRNA, h36B4, and mβ-Actin have been used as housekeeping genes for normalization.

Primer design:

Primers were designed using Primer 3 software to avoid primer dimers and hair pins (http://bioinfo.ut.ee/primer3-0.4.0/). Primer specifications were set to annealing temperature of around 60°C, primer size between 18 and 25 nucleotides and primer GC% optimum 50. For specificity of primer binding, primers were blasted against the UCSC database (https://genome.ucsc.edu/).

Table 2. Human primers used for real time qPCR

Name	Forward	Reverse
h18sRNA	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg
h36B4	gcttcattgtgggagcagaca	catggtgttcttgcccatcag
hCTα	atgaggtggtgaggaatgcg	aaacatgcctgcctccttga
hCTβ	cgtattcctctgctggctct	tccctgcttcctttatgtgc
hCYP7A1	cgcaagcaaacaccattcca	ctgtccggatgttgagggag
hCYP8B1	tgctacaggcaggagagttattca	gggagtagacaaaccttgggaaa
hGAPDH	tctcctctgacttcaacagcgac	ccctgttgctgtagccaaattc
hGCK	ctttacacctacctcattgcagg	gcaaggcactatcttctgtccc
hGNMT	tcgctcacttgccagactg	gcaccatgctcgcaatgtttt
hLRH1	cttcctggttactgggcaac	cggcattgacttgttcctg
hPEMT	ccgctctactggaatgtggt	agcaggatggtgacgcttag
hSHP	gcttcaatgctgtctggagtc	cttggaggcctggcacatc

Table 3. Mouse primers used for real time qPCR

Name	Forward	Reverse
mCyp8B1	ttaaggctggcttcctgagc	tcgacggaacttcctgaacag
mGck (39)	acattgtgcgccgtgcctgtgaa	agcctgcgcacactggcgtgaaa
mGnmt	gtgctggacgtagcctgtg	atcacgctgaagccctctt
mLrh-1	tgctggagtgagctcttgattc	gatggtggagtagtccacgtgt
mShp	aagggcacgatcctcttcaa	gtaccagggctccaagact
mβ-Actin	agccatgtacgtagccatcca	tctccggagtccatcacaatg

siRNA treatment:

Small interfering or silencing RNA (siRNA) is a special form of RNA as it does not encode for any proteins. siRNAs are double stranded short molecules (20 – 25 base pairs) which bind to complementary single stranded RNA molecules and interfere with their normal function as the bound mRNA is degraded. Therefore, siRNA treatment is an established method for knockdown experiments.

Silencer® pre-designed siRNA nucleotides specific for PEMT and PCYT1A or scrambled random nucleotides were obtained from Ambion® (Thermo Scientific Fisher, Waltham, MA, USA). Lyophilized siRNA nucleotides were resuspended according to the protocol with nuclease-free water to receive a 100µM concentrated stock solution. The stock solution was further diluted to a concentration of 5µM (1:20) as a working solution. To test the optimal siRNA concentration we performed initial experiments with two different concentrations as depicted in the scheme below. HepG2 cells were freshly splitted into 6 well plates and allowed to attach for 4h before transfection was performed (semi-reverse transfection). Each well obtained either 5µl of the 5µM concentrated solution for the wells with 25nM concentration or 10µl for the wells with 50nM concentration. Each well then received 4µl of RNAiMAx Lipofectamine (Invitrogen) and was incubated for 15 minutes. Depending on the amount of siRNA solution in the well, 95µl or 86µl of serum-free Medium was added to each well to reach a volume of 100µl. Finally to yield a total transfection volume of 1000µl, 900µl of normal medium was added. Control cells were treated similarly with the same amount of random scrambled non-sense nucleotides (NT cells). Si experiments were repeated independently at least two times.

Table 4. Scheme of tested siRNA concentrations

NT 25nM	NT 50nM	Pemt 25nM	Pemt 25nM
Pemt 50nM	Pemt 50nM	Pemt 50nM	CTα25nM
CTα 25nM	CTα 50nM	CTα 50nM	CTα 50nM

Statistics:

Data are expresses as mean values and standard deviation of mean. All experiments were repeated at least twice with a minimum of three (*invitro*) and four (*invivo*) replicates. Statistics were performed using Excel and SigmaPlot 11.0. Statistical significance was determined using t-Test or ANOVA-test with Bonferroni post-testing for the comparison of more than two groups. A p-value of <0.05 was regarded as statistical significant.

5. Results

5.1. Expression levels of LRH-1, Pemt and CTα in HepG2 cells and liver tissue.

To evaluate if our chosen cell line systems, i.e. HepG2 cells, express sufficient amounts of our target genes, we first tested expression levels of the NR LRH-1 and the rate limiting enzymes of the Kennedy and PEMT pathway, CTα and PEMT, respectively, in HepG2 cells by RT-qPCR and compared it to expression levels in normal liver tissue. LRH-1, CTα and PEMT were abundantly expressed in both, HepG2 cells and human liver tissue (Figure 5). This indicates that HepG2 cells abundantly express all the necessary rate limiting genes for the proposed experiments including siRNA mediated knockdown of CTα and PEMT.

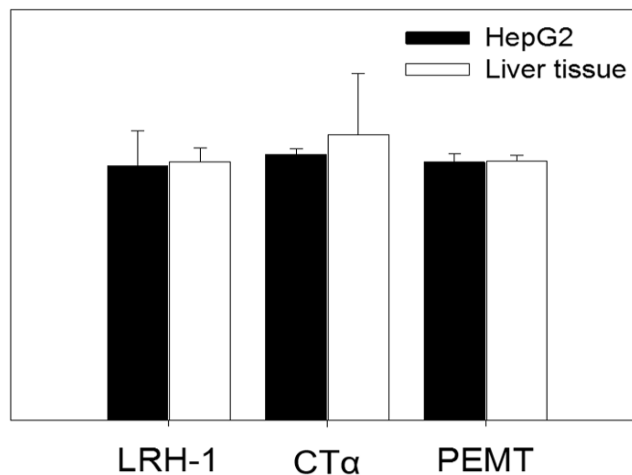

Figure 6. Expression levels of LRH-1, PEMT and CTα in HepG2 cells and liver tissue

mRNA levels of the key enzymes of the Kennedy (CTα) and PEMT (PEMT) pathway of PC synthesis and of the nuclear receptor LRH-1 have been measured by qPCR in HepG2 cells and compared to normal human liver tissue. Experiments have been performed in triplicates.

5.2 Expression of LRH-1 and target genes in Pemt$^{-/-}$ mice

Pemt$^{-/-}$ mice genetically completely lack any Pemt expression and have significantly reduced hepatic and plasma levels of Pemt specific PC molecules (49). To test whether loss of Pemt and subsequently reduction of Pemt specific PC species alter LRH-1 dependent gene expression we analysed gene expression levels in wildtype (WT) and Pemt$^{-/-}$ mice, which were kindly provided by Prof. Dennis Vance. Pemt$^{-/-}$ mice showed significant lower expression levels of the classical LRH-1 target genes Cyp8b1 (6% of WT mice), glucokinase (Gck) (26%) and glycine-n-methyltransferase (Gnmt) (42%) compared to their WT counterparts. Unexpectedly, also LRH-1 mRNA was significantly reduced to 20% in Pemt$^{-/-}$ mice. This experiment therefore suggests that in mice lack of Pemt and Pemt specific PC species significantly affect LRH-1 target genes. However, it remains open if this is due to reduction of the NR and transcription factor LRH-1 itself or due to reduced ligand binding or a combination of both. Unfortunately, liver specific CTα knockout mice, which have a genetically blunted Kennedy pathway of PC synthesis, are currently not available. Therefore, the impact of PC species generated from the Kennedy pathway on LRH-1 signaling could not have been tested in mice but only *invitro* cell line studies.

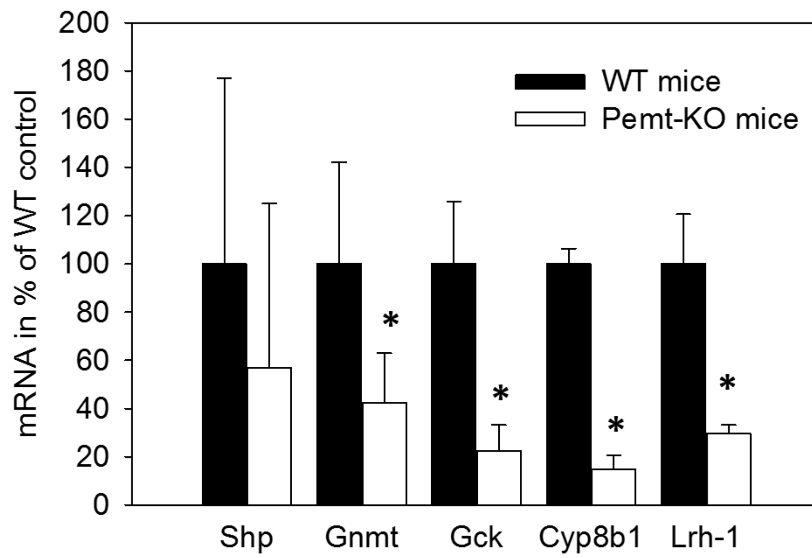

Figure 7. Expression of LRH-1 and target genes in Pemt$^{-/-}$ mice

mRNA expression of LRH-1 dependent genes is measured by qPCR in WT mice (black bar) and Pemt$^{-/-}$ mice (white bar). (N = 4/group; * $p < 0,05$; t-test)

5.3 siRNA mediated knockdown of Pemt and CTα in HepG2 cells

The next experiments were performed in HepG2 cells *invitro* which allowed us to genetically silence not only the PEMT pathway as before in mice *invivo* but also the Kennedy pathway. In addition, since HepG2 cells are a human cell line this allows to perform experiments in and draw conclusions from a human background. In a first set of experiments we determined the optimal concentration of siRNAs by comparing the knockdown efficiency of 25nM and 50nM after 24h of incubation. PEMT silencing was more efficient with the 25nM concentration, whereas CTα silencing was equally efficient with both concentrations (not shown). However, overall knockdown efficacy was only around 40% for both genes. Therefore, we repeated siRNA experiments with each 25nM of siPEMT and siCTα using a semi-reverse transfection protocol to improve knockdown efficacy. Experiments of Figure 5 are performed in semi-reverse transfection using 25nM si-nucleotides for 24h.

siPEMT knockdown resulted in a robust decrease of PEMT mRNA levels to 27% of NT expression levels indicating sufficient knockdown. The LRH-1 target genes GNMT (36%) and GCK (69%) were also robustly decreased, while SHP (70%) and CYP8B1 (78%) were moderately but still significantly decreased (Figure 8). CYP8B1 expression levels in HepG2 cells were overall very low and at the detection limit of the hardware (though technically reliable), which might explain the weak effect in this experiment. Since we reasoned that after 24h PEMT knockdown still high levels of PEMT-specific PC species might exist, we repeated si-knockdown experiments with 48h siPEMT incubation. However, we did not find any further decline in LRH-1 responsive genes after prolonged incubation (data not shown). Similar to experiments in mice, LRH-1 mRNA itself was significantly downregulated to 69% of WT expression (Figure 8), which may contribute to various degrees for the decreased expression of LRH-1 target genes.

siCTα knockdown also led to a significant decline of CTα mRNA levels (31% of NT) confirming robust knockdown results with the semi-reverse transfection method. In contrast to PEMT knockdown, LRH-1 target genes GNMT (68%), GCK (84%), SHP (101%), CYP8B1 (86%) were not or only slightly reduced by CTα knockdown. LRH-1 expression levels remained unaffected (108% of NT) (Figure 9).

Taken together, these set of experiments suggest that knockdown of PEMT, but not knockdown of CTα, has a significant impact on LRH-1 target gene expression and on LRH-1 itself. However, from the experiments so far we are unable to conclude whether reduction of LRH-1 target genes is caused by reduction of a PEMT-dependent LRH-1 ligand as hypothesized or due to reduction of LRH-1 itself. Further experimental strategies to answer these questions are discussed in the discussion section.

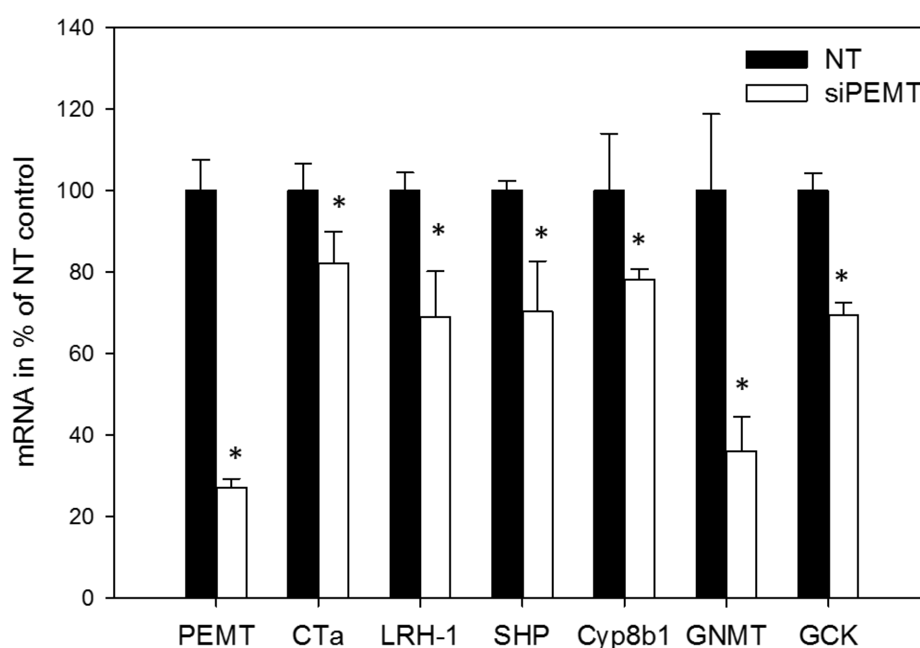

Figure 8. Expression levels of PEMT, CTα, LRH-1, CYP8B1, GNMT and GCK after siRNA mediated knockdown of PEMT in HepG2 cells

mRNA levels of the key enzymes of the Kennedy (CTα) and PEMT (PEMT) pathway of PC synthesis, of the nuclear receptor LRH-1 and LRH-1's target genes have been measured by qPCR in HepG2 cells after treatment with siPEMT and compared to cells treated with non-sense nucleotides (NT cells). Experiments have been performed in triplicates.

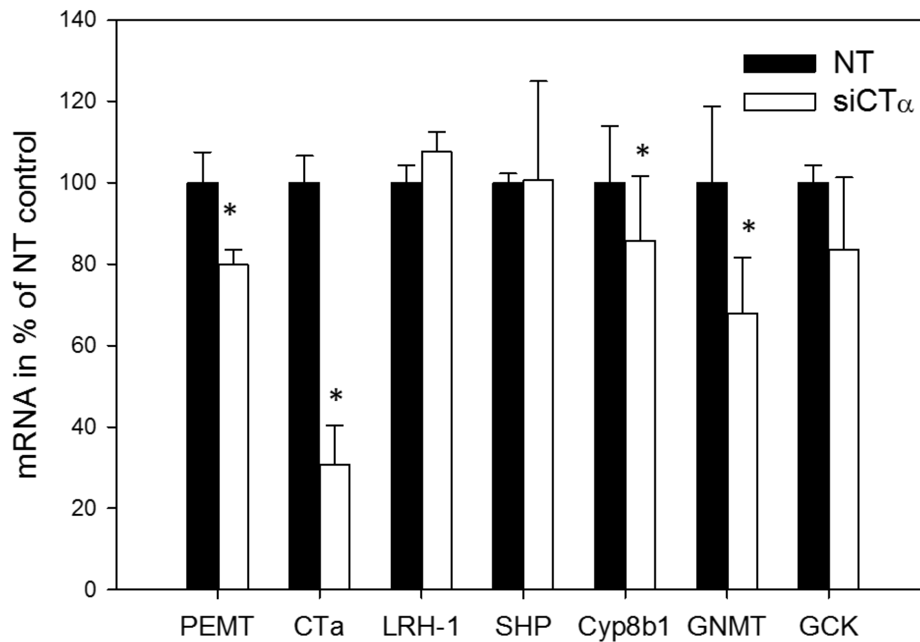

Figure 9. Expression levels of PEMT, CTα, LRH-1, CYP8B1, GNMT and GCK after siRNA mediated knockdown of CTα in HepG2 cells.

mRNA levels of the key enzymes of the Kennedy (CTα) and PEMT (PEMT) pathway of PC synthesis, of the nuclear receptor LRH-1 and LRH-1's target genes have been measured by qPCR in HepG2 cells after treatment with siCTα and compared to cells treated with non-sense nucleotides (NT cells). Experiments have been performed in triplicates.

6. Discussion

Recent studies have shown that specific exogenous phospholipids, in particular PCs, are ligands for LRH-1 (10, 36). However, it has never been examined if endogenously generated PCs are able to bind and regulate the function of LRH-1. There are two metabolic pathways for PC synthesis: the Kennedy pathway via CTα as rate limiting enzyme and the PEMT pathway (48). The aim of this study was to test the hypothesis that endogenous PCs made from either the CTα and/or PEMT pathway are functional ligands for LRH-1 and alter LRH-1 activity. Since both pathways can be modulated via dietary inputs, the results of our studies would provide a dietary strategy to modulate activity of LRH-1, a transcription factor involved in various metabolic diseases, such as fatty liver disease and insulin resistance but also in cancer development.

Our combined results from *invivo* and *invitro* studies indicate that modulation of the PEMT pathway, but not the CTα pathway robustly alters expression levels of LRH-1 target genes. Specifically, our results show that knockout and knockdown of Pemt/PEMT, respectively, reduces LRH-1 target genes. PC-species produced by either the PEMT or the Kennedy pathways are overlapping but also contain pathway specific PC species. In particular, PEMT produces very distinct PC species (i.e. docosahexaenoic acid [22:6(n-3)] and arachidonic acid [20:4(n-6)]) (49). Therefore it is attractive to speculate, that among those distinct PEMT-specific PC species a true LRH-1 ligand may be identified. In our set of experiments we have not yet tested whether one of these distinct PCs species may directly activate LRH-1 nor have we tested whether activation of PEMT can induce LRH-1 target genes *vice versa* (see limitations chapters).

An unexpected finding of our study is that PEMT knockdown not only reduced LRH-1 target genes but also LRH-1 itself. This finding has been consistently observed in both, the *invitro* as well as *invivo* PEMT knockdown experiments but not in the siCTα studies. The consistency between *invitro* and *invivo* results excludes any potential off-target effects by the si-nucleotides. However, this finding implies, that effects of PEMT knockdown on LRH-1 target gene expression may not only be explained by reduction of the putative endogenous ligand (as hypothesized) but additionally by reduction of the transcription factor LRH-1 itself. It is not known if at all and how PEMT activity may regulate LRH-1 on transcriptional levels. Several

possibilities could be envisioned. One possibility would be that LRH-1 is negatively downregulating itself after its putative ligand disappears. Another option would be that with PEMT knockdown a PC-ligand for another transcription factor, most likely PPARα, is decreasing resulting in reduced LRH-1 transcription. However, PPARα and LRH-1 have not been linked experimentally, yet and it is not known if LRH-1 harbors LRH-1 and/or PPARα binding sites in its promotor region. Yet another possibility is, that with the reduction of PEMT S-adenosylmethionine (SAM) accumulates, which has been shown to drive DNA methylation and gene silencing. None of these options have been tested in our study, yet. Thus, from our studies we cannot conclude if reduction of a PEMT-dependent LRH-1 ligand, PEMT-dependent reduction of LRH-1 itself, or a combination of both resulted in the reduction of LRH-1 target genes.

LRH-1 is a key factor for the metabolic balance in the liver due to its important role in bile acid metabolism, glucose and lipid homeostasis. Metabolic diseases such as NAFLD and insulin resistance can clearly be affected by modulations of LRH-1 activity (36). As therapeutic options for hepatic steatosis are still limited, LRH-1 appears to be an attractive target. It has been shown that certain uncommon phospholipids (i.e. DLPC and DUPC) are functional ligands for LRH-1 (10). The administration of these compounds resulted in improved glucose and cholesterol metabolism in diet induced obese mice (36). Another possible way of targeting LRH-1 is through synthetic ligands. Recently RJW101 has been discovered to be a selective agonist for LRH-1 (55). However, when targeting LRH-1 it has to be considered that activating this NR has two sides: First, the wanted effect on metabolism, and secondly, the concerning possibility of tumor development and growth (19). As LRH-1 is involved in pathogenesis of breast, pancreas and colon cancer, activating this NR might come at a high price. Thus, a liver-selective activation of LRH-1 would be most beneficial. Considering the findings of this work, there seems to be a third way to activate LRH-1, apart from synthetic ligands and exogenous phospholipids. Phospholipids derived from the liver-specific PEMT pathway could present an elegant way to achieve an upregulation of LRH-1 target genes and this mechanism of action may be restricted to the liver specifically. Therefore, activation of the PEMT pathway could represent a desirable therapeutic option in NAFLD.

Activity of PEMT is determined by the ratio of SAM to S-adenosylhomocysteine (SAH) (56). In general, SAM, which is the methyl donor for the generation of PCs via the PEMT pathway, induces PEMT activity, whereas SAH, which is the demethylated breakdown product of SAM, strongly inhibits PEMT activity (56). Therefore, we speculate that dietary addition of SAM, which is widely available as an over-the-counter food supplement and induces PEMT activity, may also modulate and induce LRH-1 target genes. In animal models addition of SAM improves non-alcoholic steatohepatitis but human trials using SAM in NAFLD are still lacking (57).

The role of PEMT for NAFLD is supported by findings in human and mice. PEMT polymorphism in human patients has been associated with NAFLD. A certain single nucleotide polymorphism (V175M) has been identified to lead to a dysfunctional PEMT enzyme and was found more often in population with NAFLD (58). It is presumed that loss of PEMT leads to an impaired VLDL secretion, as PEMT-derived PCs are necessary for VLDL formation, and thus triglycerides accumulate in the liver (52). In mice loss of Pemt did not affect liver fat content when mice are put on normal chow diet. However, when mice are fed a high fat diet, $Pemt^{-/-}$ mice develop severe fatty liver (59). It should be mentioned here, that high-fat diet fat mice are, however, protected from obesity and insulin resistance. This can in part be explained by the pivotal role of Pemt for hepatic VLDL export, which protects the extrahepatic organs of $Pemt^{-/-}$ mice for detrimental lipid overload (59). These effects also contribute to the protection from atherosclerosis in $Pemt^{-/-}$ mice (48).

Taken together, our experiments suggest that PEMT activity modulates LRH-1 activity. Since LRH-1 is an important factor and target for liver metabolism and NAFLD, respectively, we speculate that dietary modulations of PEMT activity may be used to support the treatment of metabolic liver diseases such as NAFLD. However, further and more detailed studies as outlined in the limitation section are warranted to draw precise conclusions.

7. Limitations

There are several limitations to our studies, which shall be discussed separately in the following section.

1. We only tested genetic silencing of PEMT. Apart from a genetic *invivo* knockout or si-mediated *invitro* gene silencing there would also be the option of pharmacologically blocking PEMT with 3-deazaadenosine at least in cell line systems (60).

2. The question if *invivo* experiments in mice, which lack CTα and therefore have a compromised Kennedy pathway, also do not show altered LRH-1 target gene expression as in our cell line studies remains unresolved as liver specific $CT\alpha^{-/-}$ mice could not be obtained for these studies.

3. It is not yet clear if reduction in LRH-1 target genes is caused by reduction of LRH-1 ligands as hypothesized or reduction of LRH-1 itself. The proposed PEMT gain of function experiments (i.e. PEMT overexpression and cell treatment with specific PEMT-derived PC molecules) will certainly help to dissect these two possibilities. Yet another planned experiment to answer this question will be to transfect HepG2 cells with a surplus of LRH-1 plasmid which does not undergo transcriptional regulation but remains constantly overexpressed. We would expect that if a LRH-1 ligand is reduced by PEMT knockdown consequently LRH-1 target gene expression should be reduced or the luciferase signal from the LRH-1 response element will be reduced. In the case of transcriptional repression of endogenous LRH-1, gene expression of LRH-1 target genes and luciferase activity should not change.

4. It has been shown that permanent deletion of Pemt in mice results in disappearance of Pemt specific PC species (49). However, we can only speculate that knockdown of PEMT in HepG2 cells will also result in altered PC metabolites. Short term inhibition of PEMT by 3-deazaadenosine did not significantly alter PEMT specific PCs but only PEs (60). It is not known how stable PEMT specific PCs are and if knockdown of PEMT has to be prolonged over longer periods to significantly reduce metabolite levels. To address these questions we could perform stable PEMT knockdown via lentiviral shRNA delivery and measure cellular PC metabolites by mass-spectrometry.

5. The main limitation of this study, however, is that we have not yet tested whether activation of PEMT can induces LRH-1 target genes *vice versa*. PEMT gain of function experiments could be tested genetically via overexpression of PEMT. Pharmacologically, PEMT gain of function could be mimicked via treatment of cells with a very specific PC compound, generated predominately via the PEMT pathway, such as 18:0/20:4 or 18:0/20:6 (49, 50). Moreover, it would also be interesting to test the addition of SAM, which is a known activator of PEMT activity, on expression levels of LRH-1 target genes (56). Both experiments are planned as a completion of our loss of function studies and will be performed in HepG2 cells. For these gain of function experiments we will not only rely on mRNA levels of LRH-1 target genes as main read out but want to use a more sophisticated luciferase assay, where the common LRH-1 response element is fused to a luciferase gene (61). We expect to find an increase of LRH-1 dependent genes and thus confirm our conclusions from the loss of function experiments.

8. References

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