OULU 2021
D 1628

ACTA UNIVERSITATIS OULUENSIS

Mikko Karpale

NUCLEAR RECEPTOR PXR IN OBESITY AND ITS REGULATION BY METABOLIC STATUS

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE;
MEDICAL RESEARCH CENTER OULU;
BIOCENTER OULU

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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium F202 of the Faculty of Medicine (Aapistie 5 B), on 20 August 2021, at 12 noon

UNIVERSITY OF OULU, OULU 2021

Acta Univ. Oul. D 1628, 2021

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ISBN 978-952-62-2997-3 (Paperback)
ISBN 978-952-62-2998-0 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

PUNAMUSTA
TAMPERE 2021

Karpale, Mikko, Nuclear receptor PXR in obesity and its regulation by metabolic status.
University of Oulu Graduate School; University of Oulu, Faculty of Medicine; Medical Research Center Oulu; Biocenter Oulu
Acta Univ. Oul. D 1628, 2021
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Abstract

The prevalence of metabolic diseases is paralleled by increases in exposure to foreign chemicals, e.g. drugs and environmental contaminants. Rather common for these chemicals is their ability to activate Pregnane X receptor (PXR), a master regulator of drug metabolism. PXR has been also implicated to have roles in glucose and lipid metabolism as its activation impairs glucose tolerance, increases liver fat, and modulates cholesterol metabolism. Therefore, PXR activation may in part explain the association of chemical exposure and metabolic diseases. This thesis studies the function of PXR in different metabolic states.

This thesis demonstrates that fasting mitigates transcriptomic PXR activation response and modulates PXR function in bile acid metabolism. In obese and metabolically dysfunctional mice, PXR activation aggravates liver fat accumulation and hepatic insulin resistance and increases cholesterol synthesis. However, these metabolic defects do not reflect to glucose metabolism due to increased hepatic glucose uptake and suppressed fasting response, which together lead to pseudo-improvement of glucose tolerance.

Induction of cholesterol synthesis was due to activation of SREBP2, the main regulator of cholesterol synthesis. Similarly, one-week dosing of rifampicin, a human PXR activator, increased hepatic cholesterol synthesis and circulating atherogenic lipids in humans. Importantly, PCSK9, a drug target to lower LDL, was increased by PXR activation.

This thesis reveals that metabolic status regulates PXR function. In obese mice, PXR activation elicits different and more severe response on glucose and lipid metabolism than in lean mice. Therefore, obesity may sensitize to harmful metabolic effects of PXR activating drugs and environmental chemicals. Further, the thesis defines PXR activation as a possible risk for cardiovascular health as it increases circulating atherogenic lipids and PCSK9.

Keywords: cholesterol, energy metabolism, liver, obesity, Pregnane X receptor

Karpale, Mikko, Tumareseptori PXR lihavuudessa ja sen metabolinen säätely.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Biocenter Oulu
Acta Univ. Oul. D 1628, 2021
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Lihavuuden ja metabolisten sairauksien ilmaantuvuuden on todettu liittyvän vierasaineille, kuten lääkkeille ja ympäristökemikaaleille, altistumiseen. Monet näistä vierasaineista aktivoivat pregnaani X -reseptorin (PXR) johtaen vierasaineiden nopeampaan eliminaatioon. PXR:n aktivaatio vaikuttaa metaboliseen terveyteen haitallisesti: se heikentää sokerinsietoa, rasvoittaa maksaa, ja vaikuttaa kolesterolimetaboliaan. PXR:n aktivaatio saattaakin osaltaan selittää kemikaalialtistuksen ja metabolisten sairauksien yhteyttä. Tässä väitöstyössä tutkittiin PXR:n toimintaa erilaisissa metabolisissa tiloissa.

Työssä havaittiin paaston heikentävän PXR-aktivaation vastetta ja vaikuttavan PXR:n toimintaan sappihappimetabolian säätelyssä. Lihavuuden aiheuttama metabolinen häiriötila taas herkisti hiiriä PXR-välitteiselle maksan rasvoittumiselle, heikensi maksan insuliiniherkkyyttä ja lisäsi kolesterolin synteesiä. Nämä häiriöt eivät kuitenkaan heikentäneet sokerimetaboliaa, sillä PXR:n aktivaatio lisäsi maksassa sokerin soluunottoa ja heikensi paastovastetta.

PXR:n aikaansaama kolesterolisynteesin kiihtyminen johtui SREBP2:n, keskeisen kolesterolisynteesin säätelijän, aktivaatiosta. Sama synteesin kiihtyminen havaittiin terveissä vapaaehtoisissa PXR-aktivaattori rifampisiinin annon jälkeen. Rifampisiini kiihdytti PXR-välitteisesti kolesterolisynteesiä ja lisäsi valtimonkovettumatautia aiheuttavia lipidejä. Lisäksi PXR:n aktivaatio nosti plasman PCSK9-pitoisuutta, mikä lisää LDL-kolesterolin määrää.

Väitöskirja osoittaa aineenvaihdunnallisen tilan, tarkoittakoon se paastoa tai aineenvaihdunnallisia sairauksia, säätelevän PXR:n toimintaa. Lihavissa hiirissä PXR on voimakas maksan sokeri- ja lipidiaineenvaihdunnan säätelijä. Lihavuus saattaakin herkistää PXR:n aktivaattorien, kuten monien lääkkeiden ja ympäristökemikaalien, metabolisille haitoille. Lisäksi väitöstyön osoittama kolesterolisynteesin ja aterogeenisten lipidien ja PCSK9:n lisääntyminen viittaa PXR:n aktivaation olevan haitallista sydän- ja verisuoniterveydelle.

Asiasanat: energia-aineenvaihdunta, kolesteroli, lihavuus, maksa, PXR

"It's a dangerous business, Frodo, going out your door. You step onto the road, and if you don't keep your feet, there's no knowing where you might be swept off to."
-Bilbo Baggins, *The Lord of the Rings*, J.R.R. Tolkien

Acknowledgements

This doctoral thesis was done in the Research Unit of Biomedicine (Pharmacology and Toxicology) of the University of Oulu during the years 2016-2021. The thesis project was supervised by Professor Jukka Hakkola and PhD Outi Kummu. The project was not an effort of an individual, but that of many people, whose continuous support and help I'm honored to acknowledge.

Jukka, thank you for being my principal supervisor, mentor, and the head of the research unit. I am grateful for your guidance, scientific excellence, trust, example you have provided, and especially for always having or making time for whatever problems I have had. Thank you for enabling the research visit to Pittsburgh, which was the definite highlight of my time as a doctoral student. Outi K., thank you for the supervision, help and all the time we spent with the mice. It has been a pleasure to set up and conduct animal experiments and to work with you.

I thank all the collaborators and coauthors of the publications included in the thesis. Especially I acknowledge Professors Janne Hukkanen and Jaana Rysä, our close collaborators, whose previous work with Jukka laid the scientific basis for this thesis.

I thank the pre-examiners of my doctoral thesis, PhD Martine Daujat-Chavanieu and Dr. rer. nat. Oliver Burk, for the critical evaluation of my work and for the valuable comments. I thank my follow-up group members, Professor Peppi Karppinen and Docent Tuire Salonurmi, for your support and suggestions to improve my thesis. Hannu, thank you for the language revision.

My sincere thanks belong to all my colleagues in our research group. Fatemeh, thank you for bringing your character, laughter, and sunshine to the lab. Mahmoud, thank you for all the bold visions and discussions we had. Anja, Heba, Piia and Maria, thank you for these years. I wish to thank Ritva for the excellence in lab, saving my experiments numerous times and for all the support.

My journey wouldn't have been the same without my past and current coworkers in the unit. I thank you for the company, support, and laughter and I wish our paths cross in the future. Outi L., thank you for sharing the office and nearly all the ups and downs of the journey. Long live F105A and our friendship!

I also wish to thank my friends, and especially the group of biochemists, Ryvästys, with whom I can share everything. I thank my mother for all the support throughout the years and for showing what is the most important thing in life, which certainly is not science, but family in all its' forms. I thank my father for setting the example to never lack ambition, curiosity and bold dreams. Asko and Kirsi, thank

you for your continuous support. When I think what sparked my interest in biology when I was a kid, the major contributors must have been my older brother Antti, who showed great enthusiasm towards children's encylopedias, and my grandmother and late grandfather, Mammu ja Pappa, with whom we spent time investigating the wonders of nature. My dear siblings, Antti, Arttu, Lauri and Sara, thank you. I know I can always rely on you.

Kati, my deepest gratitude belongs to you. Thank you for *everything* throughout these years. You have been my biggest supporter, the hardest opponent (sometimes rightfully) and someone who I'm honored to share my life with. I wouldn't be doing any of this without you. Last, I thank Sylvi – and the newcomer – for being something more important than this thesis.

1.6.2021 Mikko Karpale

Abbreviations

ABC	ATP-binding cassette transporter
ACVD	Atherosclerotic cardiovascular disease
AHR	Aryl hydrocarbon receptor
AKR1B10	Aldo-ketoreductase family 1 member B10
AMPK	5' adenosine monophosphate-activated kinase
Apo	Apolipoprotein
AR	Androgen receptor
AUC	Area-under-the-curve
BPA	Bisphenol A
CA	Cholic acid
CAR	Contitutive androstane receptor
CCL2	C-C motic chemokine ligand 2
CD36	Cluster of differentiation 36
CDCA	Chenodeoxycholic acid
CDK	Cyclin-dependent kinase
CES	Carboxylesterase
CETP	Cholesterylester transfer protein
ChREBP	Carbohydrate response element -binding protein
CK2	Casein kinase 2
CPT1A	Carnitine palmitoyltransferase 1A
CREB	Cyclic AMP-response element-binding protein
CYP	Cytochrome P450
DCA	Deoxycholic acid
ED	Endocrine disruptor
ELOVL6	Elongation of very long-chain fatty acids family member 6
ER	Estrogen receptor
ERR	Estrogen-related receptor
FOXO1	Forkhead box protein O1
G6Pc	Glucose-6-phosphatase catalytic subunit
GCK	Glucokinase
GLUT	Glucose transporter
GR	Glucocorticoid receptor
GSK3	Glycogen synthase kinase 3
GST	Glutathione S-transferase
HDL	High-density lipoprotein

HE	Hematoxylin-eosin
HFD	High-fat diet
HL	Hepatic lipase
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2
HNF4A	Hepatic nuclear factor 4 alpha
HOMA-IR	Homeostatic model assessment for insulin resistance
IDL	Intermediate-density lipoprotein
IL1B	Interleukin 1 beta
IL6	Interleukin 6
INSIG1	Insulin-induced gene 1
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LBD	Ligand-binding domain
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MDR1	Multidrug resistance protein 1
MRP	Multidrug resistance-associated protein
mTOR	Mitochondrial target of rapamycin
MTP	Mitochondrial triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alchoholic steatohepatitis
NCor	Nuclear receptor corepressor
NPC1L1	Niemann-Pick C1-Like 1
NR	Nuclear receptor
OATP	Organic anion transporter protein
PCN	Pregnenolone-16α-carbonitrile
PCSK9	Proprotein subtilisin/kexin type 9
PCK1	Phosphoenolpyruvate carboxykinase 1
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B

PKC	Protein kinase C
PPARα	Peroxisome proliferator-activated receptor α
PPARγ	Peroxisome proliferator-activated receptor γ
pPROTEIN	Phosphorylated protein
PXR	Pregnane X receptor
PXR-KO	PXR-knockout
RIN	RNA integrity number
RXR	Retinoid X receptor
SCAP	SREBP cleavage-activating protein
SCD1	Stearoyl-CoA desaturase
SGK2	Serum/glucocorticoid regulated kinase 2
SIRT1	Sirtuin 1
SR-BI	Scavenger receptor class B type 1
SRC	Steroid receptor coactivator
SREBP	Sterol regulatory element-binding protein
SULT	Sulfotransferase
T2D	Type 2 diabetes
T-MCA	Tauro-muricholic acid
TNFα	Tumor necrosis factor α
UGT	UDP-glucuronosyltransferase
WAT	White adipose tissue
WHO	World Health Organization
VDR	Vitamin D receptor
VLDL	Very low-density lipoprotein
XR	Xenobiotic receptor

Original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Hassani-Nezhad-Gashti, F., Kummu, O., Karpale, M., Rysä, J., & Hakkola, J. (2018) Nutritional status modifies pregnane X receptor regulated transcriptome. *Sci Rep, 9*(1),16728. doi: 10.1038/s41598-019-53101-9.

II Karpale, M., Kummu O., Näpänkangas J., Hakkola J. (2021) Dissociation of NAFLD and glucose tolerance by PXR activation in obese mice. *Manuscript*

III Karpale, M., Käräjämäki, A., Kummu O., Gylling H., Hyötyläinen T., Oresic M., Tolonen A., Hautajärvi H., Savolainen M.J., Ala-Korpela M., Hukkanen J. & Hakkola J. (2021) Activation of Pregnane X receptor induces atherogenic lipids and PCSK9 by a SREBP2-mediated mechanism. *Br J Pharmacol*. 178(12):2461-2481. doi: 10.1111/bph.15433

Contents

1 Introduction

Obesity and cardiometabolic diseases have reached pandemic proportions. To establish novel and effective prevention and treatment strategies, there is an urgent need to understand the molecular etiology of these diseases. The prevalence of cardiometabolic diseases is paralleled by increases in the production of synthetic industrial chemicals (Baillie-Hamilton, 2002). We are more frequently exposed to synthetic chemicals than ever before and, indeed, the exposure is an acknowledged risk factor of metabolic diseases in addition to sedentary lifestyle, poor food quality and genetics. To date, the molecular mechanisms that exert the harmful effects of environmental chemicals remain mostly unidentified.

Chemicals foreign to human body, xenobiotics, are sensed by mechanisms which accelerate their elimination. One of the main xenobiotic sensors is pregnane X receptor (PXR), which is activated by a wide array of structurally different chemicals. Worryingly, activation of PXR has been shown to elicit adverse effects on glucose and lipid metabolism (Hakkola, Rysä, & Hukkanen, 2016). Activation of PXR impairs glucose tolerance, induces fat accumulation in the liver and is involved in the regulation of cholesterol metabolism, all key features of cardiometabolic diseases. The dual role of PXR as a sensor of the chemical environment and as a regulator of glucose and lipid metabolism has raised questions whether PXR could mediate the harmful metabolic effects of drugs and environmental chemicals.

PXR is primarily active in the liver, a central hub of glucose and lipid metabolism. Inside the cells, PXR executes its function in complex multifactorial networks constituted by transcription factors and transcriptional coregulators, which are often dysregulated in metabolic diseases and independently contribute to metabolic defects, such as hyperglycemia and dyslipidemia.

This thesis investigates the function of PXR in glucose and lipid metabolism, and how fasting or obesity-induced metabolic dysfunction affects it.

2 Review of the literature

2.1 The ever-increasing obesity epidemic

The World Health Organization (WHO) defines obesity as an excessive fat accumulation that presents a risk to health. In 2014, 10.8% of adult men and 14.9% of adult women were obese globally (Di Cesare et al., 2016). Of concern, the prevalence has constantly increased over the past 50 years (Bentham et al., 2017) and now more people die from obesity rather than malnutrition (Di Cesare et al., 2016). Obesity disrupts metabolism and provokes metabolic syndrome, which is defined by increased waist circumference, blood pressure, insulin resistance and dyslipidemia. Metabolic syndrome predisposes to the development of common metabolic diseases, such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and atherosclerotic cardiovascular disease (ACVD). Despite major efforts, the current prevention and treatment strategies haven't been able to stop the increasing trend in metabolic diseases. In the hopes of novel and effective strategies, molecular mechanisms regarding the etiology of obesity-induced metabolic dysfunction are under scrutinous investigation.

Obesity is fundamentally caused by an imbalance between energy intake and expenditure (Newburgh & Johnston, 1930). Genetics also play an important role in obesity, as body mass index is partly hereditary (Sorensen, Price, Stunkard, & Schulsinger, 1989). Throughout evolution, humans have needed to endure prolonged undernutrition which may have favored a genotype that efficiently stores energy and is physically inactive. In the modern time, when food supply is stable, societal undernutrition reduced and sedentary lifestyle favored, this sophisticated gene set of proto-humans and the predecessors backfires and predisposes modern humans to obesity.

Metabolic dysfunction in metabolic syndrome is caused by functional defects in the central organs of energy metabolism, the adipose tissue, the liver, and the skeletal muscle. Dysregulation of these organs elicits a systemic and multifactorial shift in glucose and lipid metabolism. Pharmacotherapeutic approaches to treat obesity are a few and notoriously many promising drugs have been withdrawn from the market due to adverse side effects (Onakpoya, Heneghan, & Aronson, 2016). Despite the widespread usage of drugs in the fight against the metabolic diseases, the treatment goals are rarely met. Hence, the search continues to identify key

mechanisms and drug targets to prevent, reduce the risk of, and treat obesity and the accompanying metabolic dysfunction.

2.2 Obesity-induced metabolic dysfunction

Obesity is the central risk factor of metabolic syndrome and has been defined as a progressive disease itself (Bray, Kim, & Wilding, 2017). Blood pressure, kidneys, pancreatic β-cells, brown adipose tissue and the central nervous system are important players in metabolic diseases but are out of the scope of this thesis and thus not discussed.

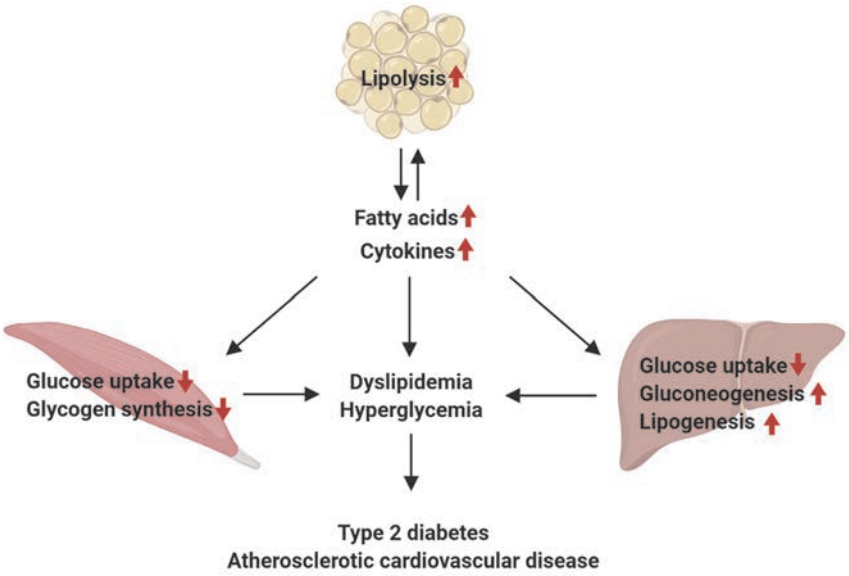

Fig. 1. Development of hyperglycemia and dyslipidemia in insulin resistance. Cytokines secreted from the adipose tissue impair insulin-stimulated suppression of lipolysis increasing fatty acids in the circulation. Cytokines and fatty acids induce insulin resistance in the skeletal muscle and the liver leading to reduced glucose uptake and increased endogenous glucose and lipid production. Hyperglycemia and dyslipidemia may eventually manifest as type 2 diabetes and atherosclerotic cardiovascular disease.

Obesity-induced metabolic dysfunction and insulin resistance is thought to initiate from the adipose tissue, which secretes inflammatory cytokines and fatty acids into circulation (Gregor & Hotamisligil, 2011) impairing the function of liver and

skeletal muscle and provoking hyperglycemia and dyslipidemia and, further, metabolic diseases (Figure 1.).

2.3 Adipose tissue inflammation – initiator of metabolic dysfunction

White adipose tissue (WAT) is the primary tissue to physiologically respond to excess energy. Triglycerides accumulate in the WAT in a state of energy excess. During fasting, the WAT releases fatty acids to the circulation for energy supply. The WAT contains mainly adipocytes, but also macrophages, leukocytes, fibroblasts, progenitor cells and endothelial cells. The presence of multiple secretory cell types underlines the variable WAT functions, and around one hundred adipose-derived signaling molecules have been identified (Hotamisligil, Shargill, & Spiegelman, 1993; Waki & Tontonoz, 2007). Molecules secreted by the adipose tissue, adipokines, include proteins involved in the regulation of energy homeostasis, body weight and inflammation, which comprise a critical aspect of metabolic health. (Longo et al., 2019; Odegaard & Chawla, 2013; Waki & Tontonoz, 2007).

When prolonged energy surplus expands the WAT beyond its normal capacity, adipose vasculature cannot keep up with the nutrient and oxygen demands, and a local hypoxia response is launched. This coupled with the lipotoxicity caused by lipid accumulation leads to an increased expression of proinflammatory chemokines, e.g. C-C motic chemokine ligand 2 (CCL2) and tumor necrosis factor alpha (TNFα) in the adipocytes, which enhances the macrophage presence in the WAT. (Gregor & Hotamisligil, 2011; Longo et al., 2019; Ouchi, Parker, Lugus, & Walsh, 2011; Tilg & Moschen, 2008)

Tissue-resident active macrophages secrete cytokines, major ones being interleukin 6 (IL6), interleukin 1B (IL1B) and TNFα. These cytokines bind to the receptors in the cell surface of adipocytes and activate c-Jun N-terminal kinase (JNK) to impair the insulin signaling and increase lipolysis which manifests as increased serum triglycerides and fatty acids. Cytokines secreted from macrophages also downregulate adipocyte peroxisome proliferator-activated receptor γ (PPARγ), a transcription factor regulating the adipocyte function and triglyceride synthesis, to further increase adipose lipolysis and circulating fatty acids. (Gregor & Hotamisligil, 2011; Longo et al., 2019; Ouchi et al., 2011; Tilg & Moschen, 2008)

Disruption of adipokine secretion in obesity is not limited to cytokines, but include important players of systemic metabolism, such as leptin, resistin and adiponectin (Jung & Choi, 2014). Leptin is produced by the adipocytes and secreted in response to nutrients to reduce appetite and food intake and to increase energy expenditure (Friedman & Halaas, 1998). Leptin also improves insulin sensitivity in the liver and muscle and improves the pancreatic β-cell function (Covey et al., 2006; Lord et al., 1998). Mice deficient in leptin (*ob/ob* mice) are widely used to model obesity for they become profoundly obese and metabolically ill at a young age (Y. Zhang et al., 1994). In obese subjects, circulating leptin is often increased due to leptin resistance in the target tissues (Friedman & Halaas, 1998). Resistin is produced by the adipose tissue and increased resistin secretion in obesity promotes inflammation and insulin resistance (R. R. Banerjee et al., 2004). Mice deficient in resistin are protected from diet-induced hyperglycemia (Qi et al., 2006) and in humans circulating resistin associates with obesity, insulin resistance and diabetes (McTernan et al., 2002). Obesity is also often associated with low adiponectin levels, and treatment of obese and hyperglycaemic mice with adiponectin increase insulin sensitivity (Kern, Di Gregorio, Lu, Rassouli, & Ranganathan, 2003).

In summary, the WAT is the first responder to energy excess and reaching its expansion threshold leads to systemic increases in inflammatory cytokines, altered adipokine profile, and increased fatty acids in the circulation. These defects impair insulin action and secretion and lead to ectopic fat accumulation in the central tissues of energy metabolism, such as liver and muscle, to disrupt their function (Figure 1.). Thus, the WAT is a central mediator of obesity-induced metabolic dysfunction.

2.3.1 Insulin resistance and type 2 diabetes

A stable glucose concentration in the circulation supplies energy to tissues while avoiding glucose-mediated biomolecule glycosylation, glucotoxicity and desensitization of glucose-sensing mechanisms. Blood glucose is mainly regulated by glucagon and insulin secreted from pancreatic α- and β-cells, respectively. Insulin is a growth hormone which stimulates the uptake of glucose from the circulation to the peripheral tissues. Insulin, as well as low blood glucose, increase the secretion of glucagon which signals to the liver to induce glucose release to circulation, forming an insulin-glucagon feedback loop. In metabolic syndrome, effect of insulin on insulin-responsive tissues, such as adipose tissue, liver and skeletal muscle, is impaired. This condition is referred to as insulin resistance,

which leads to elevated blood glucose, hyperglucagonemia, increased hepatic glucose production, impairment of muscle glucose uptake and elevated plasma free fatty acid levels (Figure 1.).

T2D is characterized by high blood glucose due to insulin resistance or impaired insulin secretion or combination of both. Before overt hyperglycemia, prediabetes develops, defined by elevated fasting glucose, impaired glucose tolerance or increased glycated hemoglobin. T2D leads to microvascular complications including retinal, renal and neurological pathologies and to macrovascular complications such as ACVD. T2D is the most common form of diabetes and, due to increased prevalence of obesity, T2D and related comorbidities have become major current and future health concerns. (Defronzo, 2009; DeFronzo et al., 2015; Kahn, 1997)

2.3.2 Molecular determinants of insulin resistance

After a meal, most of the glucose is taken up by skeletal muscles, liver and adipose tissue through glucose transporters (GLUTs). In fasting, hepatic mechanisms which oppose insulin action on liver provide glucose into circulation by increasing glycogen breakdown (glycogenolysis) and glucose production (gluconeogenesis), which is fueled by triglyceride catabolism and gluconeogenic amino acids derived from the adipose tissue and muscles. Resistance to insulin impairs insulin-induced tissue glucose uptake and insulin-stimulated suppression of liver fasting responses which leads to hyperglycaemia.

Insulin acts through its receptors which initiate a complex signalling cascade involving insulin receptor substrates 1 and 2 (IRS1/2), phosphatidylinositide 3 kinase (PI3K), protein kinase B/Akt, mechanistic target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) pathways (Krüger et al., 2008). For example, insulin stimulates Akt to phosphorylate and inactivate forkhead box protein O1 (FOXO1) transcription factor, a central regulator of gluconeogenesis and glycogenolysis, leading to a reduced gluconeogenesis (Farmer, 2003). The insulin signaling pathway stimulates glucose transporters (GLUTs) to uptake glucose into tissues by both translocation of GLUTs to plasma membrane and by increasing GLUT synthesis. Different tissues express different GLUTs, exemplified by GLUT4 mainly expressed in the skeletal muscle and GLUT2 mainly expressed in the liver. Furthermore, insulin promotes glucose storage mechanisms, such as lipid and glycogen synthesis, and inhibits opposing mechanisms, lipolysis and glycogen breakdown (DeFronzo et al., 2015).

Insulin resistance in the liver and skeletal muscle is mainly caused by an inhibitory serine phosphorylation of IRS protein which blocks insulin-stimulated IRS tyrosine phosphorylation (Bouzakri et al., 2006; Copps & White, 2012). The serine phosphorylation of IRS may also lead to increased IRS degradation, which also contributes to insulin resistance (Hiratani et al., 2005). The serine phosphorylation of IRS is catalyzed by the members of a class of protein kinase Cs (PKCs) which are activated by lipids and inflammatory signaling (Krook et al., 2000). Increased ectopic fat in the hepatocytes and the myocytes activate PKC through lipotoxicity, i.e. accumulation of toxic lipid metabolites (Samuel et al., 2004; C. Yu et al., 2002). Circulating free fatty acids have also been shown to bind proinflammatory toll-like receptor 4 to activate the JNK signaling and this way impair insulin-stimulated IRS phosphorylation (Shi et al., 2006). Adipose tissue - derived IL-6 and TNF activate JNK signaling through TNF-receptor (De Alvaro, Teruel, Hernandez, & Lorenzo, 2004).

In skeletal muscle, insulin resistance decreases GLUT4 level in myocyte plasma membranes decreasing glucose uptake (Samuel & Shulman, 2012). In addition, insulin-stimulated glycogen synthesis is diminished (Shulman et al., 1990). The glucose uptake in the muscle is especially important right after a meal, when muscle uptakes most of the glucose.

Proteins are synthesized partly in the ER, whose function is compromised by high fatty acid levels. Conversely, ER stress in tissues is alleviated by weight loss. The activation of mammalian target of rapamycin (mTOR), a regulator of wide array of cellular functions, blocks insulin-stimulated IRS phosphorylation and, interestingly, promotes ER stress (Ricoult & Manning, 2013; Um et al., 2004). mTOR signaling activation associates with obesity, T2D and fatty liver disease (Eizirik, Cardozo, & Cnop, 2008).

Although the causative role is still under debate, mitochondrial dysfunction and endoplasmic reticulum (ER) stress are associated and possibly contribute to insulin resistance in the liver and muscle. The main regulator of mitochondrial biogenesis is peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) which is often downregulated in insulin resistance and when ectopic fat accumulates in the liver. Overexpression of PGC-1α improves fatty acid oxidation and insulin signaling in mouse liver and metabolic function in skeletal muscle of obese rats and myocytes derived from extremely obese individuals (Benton et al., 2010; Consitt et al., 2010; Matthew Morris et al., 2012). However, PGC1α is a well-established positive regulator of gluconeogenesis (Rhee et al., 2003), complicating the picture.

In summary, resistance to the pleiotropic actions of insulin, which is a major growth hormone and a regulator of energy homeostasis, impairs glucose and lipid metabolism in the tissues which reflects to the circulation as hyperglycemia.

Insulin resistance in the liver

The liver is the central organ providing glucose and ketone bodies to the circulation during fasting. In normal physiology, insulin suppresses the fasting response in the liver, decreasing gluconeogenesis and increasing the synthesis of glycogen and triglycerides (Figure 2.). In insulin resistance and T2D, insulin is unable to elicit its normal response to mechanisms which determine hepatic glucose output. Therefore, the impaired insulin response in the liver leads to increased fasting hyperglycemia (Magnusson, Rothman, Katz, Shulman, & Shulman, 1992). Furthermore, metformin, the first line T2D treatment, decreases blood glucose by suppressing hepatic gluconeogenesis.

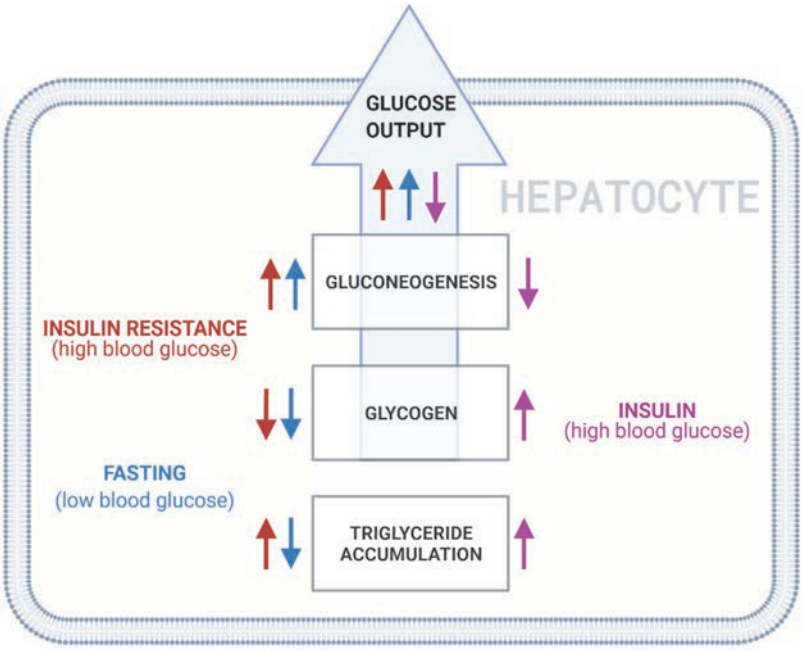

Fig. 2. Mechanisms controlling hepatic glucose output in fasting, under insulin stimulus and in insulin resistance.

Gluconeogenesis is greatly determined by the activity of phosphoenolpyruvate carboxykinase 1 (PCK1) and glucose-6-phosphatase (G6P) (Puigserver et al., 2003) and transcription factors controlling them, FOXO1, cAMP response element binding protein (CREB) and hepatic nuclear factor 4 α (HNF4A). Insulin resistance coupled with hyperglucagonemia and increased substrate flux (fatty acids, lactate, glycerol and amino acids) enhances glucose production in the liver (Baron et al., 1987; Samuel and Shulman, 2012). Amount of glycogen reflects energy surplus or increased energy demand. Insulin effectively increases glycogen synthesis whereas in fasting glycogen reserves are mobilized to supply glucose to extrahepatic tissues (Figure 2.).

Fasting and hepatic insulin resistance both lead to increased hepatic glucose output by increasing glycogenolysis and gluconeogenesis. Hepatic insulin resistance is selective in nature: although insulin fails to suppress gluconeogenesis, it stimulates lipogenesis, leading to the development of fatty liver disease (Brown & Goldstein, 2008) (Figure 2.).

2.3.3 Non-alcoholic fatty liver disease

The trends in global obesity and metabolic syndrome are paralleled by that of NAFLD (Asrani, Devarbhavi, Eaton, & Kamath, 2019). Similar to metabolic syndrome, the prevalence is predicted to rise from the current 30% in the Western countries (Milić & Štimac, 2012; Mokdad et al., 2014). NAFLD and the metabolic syndrome share many similarities, and therefore, NAFLD is considered a local manifestation of the syndrome. Alleviation of NAFLD in mice improves the systemic metabolism, which have probed an interest to target liver for the treatment of metabolic syndrome (Tanaka, Aoyama, Kimura, & Gonzalez, 2017). Pharmaceutical treatments for NAFLD are still scarce, although pioglitazone and Vitamin E hold some promise in NAFLD treatment (Raza, Rajak, Upadhyay, Tewari, & Anthony Sinha, 2021). Currently, the mainstay strategy to alleviate NAFLD is lifestyle intervention therapies. In hopes of novel treatments, attention is being paid to multiple signaling pathways, for example those governed by liver-resident nuclear receptors, central regulators of hepatic functions and systemic metabolism (M. Banerjee, Robbins, & Chen, 2015; Cave et al., 2016; Tanaka et al., 2017).

The most common form of NAFLD is benign isolated steatosis, where fat is accumulating in lipid droplets inside the hepatocytes without a concurrent inflammation. Steatosis may progress to non-alcoholic steatohepatitis (NASH)

which can progress to fibrosis, cirrhosis and hepatocellular carcinoma. Reason for the NAFLD progression to NASH is unknown, but in a widely accepted multiple-hit model, steatosis is the first hit which sensitizes the liver to a second hit caused by an array of factors including adipokines, nutrients, proinflammatory molecules and genetics. (Satapathy, Kuwajima, Nadelson, Atiq, & Sanyal, 2015; Tilg & Moschen, 2010). Circulating factors associated with NAFLD and metabolic syndrome, TNF, IL-6, and adipokines, independently disturb hepatic lipid metabolism and contribute to NASH progression (Kern et al., 2003; Maury et al., 2007).

Mechanisms of NAFLD development

Lipids accumulate in the liver through any disturbances in the lipid acquisition or disposal (Figure 3.) (Ipsen, Lykkesfeldt, & Tveden-Nyborg, 2018). The liver acquires fat whether from increased delivery of fatty acids from the adipose tissue, from the diet or from hepatic *de novo* lipogenesis (Donnelly et al., 2005). Alterations in lipid uptake associate and are one causative factor behind hepatosteatosis. For example, free fatty acid transporter cluster of differentiation (CD36) is upregulated in NAFLD (Greco et al., 2008) and NASH (Miquilena-Colina et al., 2011) and mouse studies have shown hepatic CD36 to drive steatosis by enhancing fatty acid uptake (Koonen et al., 2007; Wilson et al., 2016).

De novo lipogenesis in the liver is mainly controlled by transcription factors sterol regulatory element binding protein 1 (SREBP1) and carbohydrate response element binding protein (ChREBP) in response to insulin or carbohydrates, respectively. SREBP1c and ChREBP control the expression of genes involved in the synthesis of fatty acids from acetyl-CoA derived from glucose through glycolysis or from glucogenic amino acids. SREBP1 has two isoforms, SREBP1a and SREBP1c, of which SREBP1a controls cholesterol and triglyceride synthesis genes whereas gene regulation of SREBP1c is limited to fatty acid synthesis (Horton, Goldstein, & Brown, 2002; Shimano & Sato, 2017).

Insulin stimulates SREBP1 through multiple mechanisms, one being mTOR-mediated activation of p70 S6 kinase (S. Li, Brown, & Goldstein, 2010). Another regulator of SREBP1 is liver X receptors (LXR), a nuclear receptors activated by oxysterols (Repa et al., 2000). ChREBP is activated by glucose, independently of insulin, to promote glycolysis, i.e. glucose catabolism to pyruvate, and lipogenesis (Ortega-Prieto & Postic, 2019). Mice deficient in hepatic ChREBP are protected from obesity-induced hepatic steatosis but develop severe insulin resistance (D.

Zhang et al., 2017) and thus ChREBP plays opposing roles in lipid and glucose metabolism. In NAFLD, SREBP1c is the predominant regulator of lipogenesis whereas ChREBP is often downregulated.

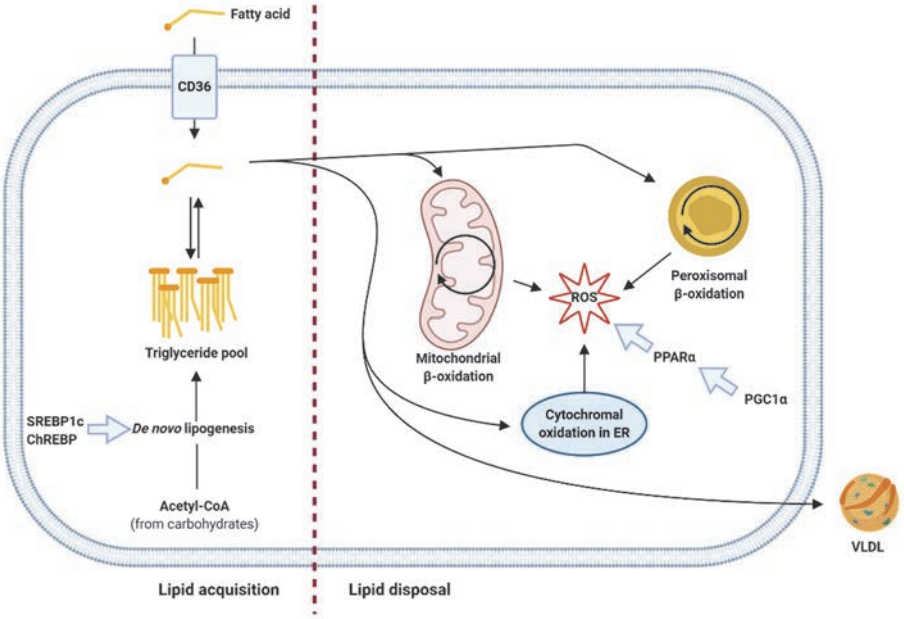

Fig. 3. Overview of hepatocyte fatty acid metabolism. Circulating lipids are transported to hepatocytes through transporters like CD36. Triglyceride pool is determined by *de novo* lipogenesis, regulated by SREBP1c and ChREBP transcription factors, and by consumption and export of lipids. Consumption occurs in oxidation processes regulated by transcription factor PPARα regulated by PGC1α. Main site of lipid oxidation is mitochondria, but peroxisomes and cytochromal processes in the ER also contribute to lipid oxidation which generate ROS and cause cellular damage. Hepatocytes export triglycerides in VLDL particles to meet the demands of extrahepatic tissues. Adapted from Ipsen et al. (2018). CD36 cluster of differentiation 36, ChREBP carbohydrate response element binding protein FABP1 fatty-acid binding protein 1, PGC1α PPARγ coactivator 1α, PPARα peroxisome proliferator-activated receptor α, ROS reactive oxygen species, SREBP1 sterol regulatory element binding protein 1c.

Fatty acids are utilized for energy production in oxidation processes occurring primarily in the mitochondria. Mitochondrial function is often compromised in obesity and insulin resistance and ultrastructural changes in mitochondria precede NAFLD progression to NASH (Sanyal et al., 2001). The expression of multiple genes involved in fatty acid oxidation is controlled by peroxisome proliferator

activated receptor α (PPARα) which again is controlled partly by PGC1α (Figure 3.) (Kersten & Stienstra, 2017). Deficiency of PGC1α leads to impaired fatty acid oxidation through the downregulation of PPARα (Leone et al., 2005). PPARα is also downregulated when NAFLD progresses to NASH and downregulation associates with JNK activation and increased inflammation (Videla & Pettinelli, 2012). Thus, the fatty acid oxidation contributes to the NAFLD progression and severity by increasing oxidative stress and inflammation.

Triglycerides are exported from the liver packed in very low-density lipoprotein (VLDL) particles alongside cholesterol, phospholipids and apolipoprotein B100 (ApoB100) (Kawano & Cohen, 2013). Lipids are attached to apoB100 by microsomal triglyceride transfer protein (MTP) (Wetterau et al., 1992). In NAFLD, VLDL secretion first increases with hepatic steatosis but when >10% of liver volume is fat, VLDL secretion stabilizes (Fabbrini et al., 2008). ER stress, a common finding in NAFLD progression, leads to degradation of ApoB100 and aggravation of steatosis providing a link between steatosis and lipid export (Ota, Gayet, & Ginsberg, 2008). ApoB100 and MTP are both also sensitive to insulin, however, insulin resistance in the liver leads to increased lipogenesis without concomitant inhibition of VLDL secretion (Tessari, Coracina, Cosma, & Tiengo, 2009).

The liver and gut have a bidirectional relationship, and the disruption of one's function reflects on the other. Nutrients absorbed through the intestinal wall first pass the liver via portal vein which predisposes liver to any changes in the gut and diet. Obesogenic diet, frequent meals high in fat, glucose and fructose, modulate intestinal microbial flora and affect the integrity of the intestinal wall through multiple mechanisms (Chakraborti, 2015; Kirpich, Marsano, & McClain, 2015), including increased production of short-chain fatty acids which stimulates hepatic *de novo* lipogenesis, modulation of choline metabolism which affects lipoprotein synthesis and hepatic lipid export, modulation of bile acid homeostasis and increased production of bacteria-derived toxins which trigger proinflammatory cytokine production in the liver (Kirpich et al., 2015). Therefore, obesogenic diet increases nutrient influx to the liver and modifies gut microbiome to impair hepatic metabolism and provoke metabolic alterations.

2.3.4 Dyslipidemia and atherosclerotic cardiovascular disease

Different organs are, to different degrees, dependent on the triglyceride and cholesterol supply. Triglycerides are used for energy production in tissues which

need a lot of energy, such as the heart and other muscle tissues, and cholesterol is an important constituent of biological membranes. Furthermore, cholesterol derivatives like bile acids, steroid hormones and oxysterols, fulfill important biological functions. Lipids derive either from the diet or they are synthesized in tissues, the liver being one of the most central ones. Triglycerides and cholesterol are transported in the circulation in lipoproteins. Obesity disturbs the balance of lipoproteins resulting in dyslipidemia characterized by increased plasma triglycerides and low-density-lipoprotein (LDL) and decreased high-density lipoprotein (HDL).

The function of VLDL and LDL is to deliver triglycerides and cholesterol to extrahepatic tissues whereas HDL handles reverse cholesterol transport from the circulation and the tissues back to the liver. Dyslipidemia, and especially elevated LDL, is the central risk factor of ACVD. Other major risk factors are diabetes, hypertension and smoking (J. L. Goldstein, Hazzard, Schrott, Bierman, & Motulsky, 1973; G. A. Roth et al., 2015). In ACVD, blood flow in arteries is shunted due to plaque formation in the artery wall caused by accumulation of cholesterol, macrophages and connective tissue which predisposes to a thrombus formation, cardiac arrest and stroke (Libby et al., 2019).

LDL is the only atherogenic lipoprotein that can penetrate the endothelium and contribute to plaque formation. Decreasing the circulating cholesterol is the dominant pharmaceutical approach to reduce LDL and the disease risk. For example, statins inhibit cholesterol synthesis, PCSK9 blockers increase hepatic LDL uptake and ezetimibe inhibits cholesterol absorption from the intestine. (Van Der Wulp, Verkade, & Groen, 2013).

Lipoprotein metabolism

Lipoprotein metabolism is summarized in Figure 4. Dietary triglycerides and cholesterol are taken up by enterocytes through specialized transporters such as CD36 and Nieman-Pick C1 like 1 protein (NPC1L1) (Davis et al., 2004; Pan & Hussain, 2012). In the enterocytes, triglycerides and cholesterol together with phospholipids and apolipoprotein B48 (ApoB48; enterocyte-specific lipoprotein) are assembled into chylomicron particles, which enter the circulation (Pan & Hussain, 2012).

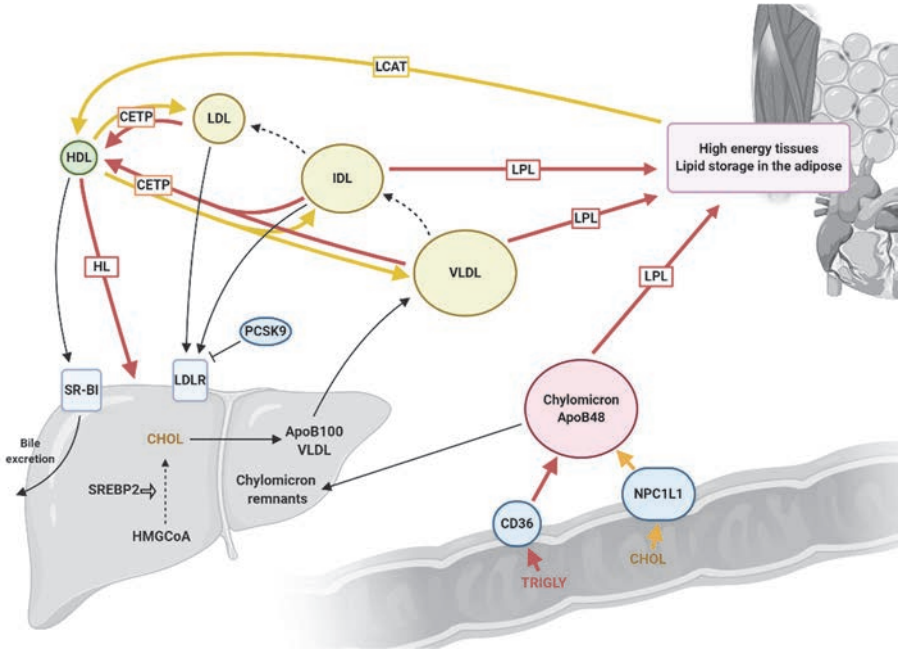

Fig. 4. Overview of lipoprotein metabolism. Dietary lipids are absorbed by CD36 and NPC1L1 and assembled with ApoB48 to chylomicrons which deliver triglycerides to tissues through LPL and form chylomicron remnants, taken up by the liver. The liver assembles VLDL particles from apoB100, cholesterol synthesized by the liver (regulated by SREBP2) and other lipids. VLDL provides triglycerides to tissues and shrinks in size forming LDL, a lipoprotein high in cholesterol and low in triglycerides. LDL and IDL are taken up by the liver through LDLR. HDL sequesters esterified cholesterol (esterification catalysed by LCAT) from the circulation and the tissues and delivers it to liver through SR-BI to be excreted as bile salts or exchanges it to triglycerides with VLDL, IDL and LDL. HDL triglycerides are rapidly taken up by the liver. ApoB48 Apolipoprotein B48, CETP Cholesterylester transfer protein, CD36 Cluster of differentiation, HL Hepatic lipase, LCAT Lecithin-cholesterol acyltransferase, LPL Lipoprotein lipase, NPC1L1 Niemann-Pick C1-Like 1, PCSK9 Proprotein convertase subtilisin/kexin type 9, SR-B1 Scavenger receptor class B type 1, SREBP2 Sterol regulatory element -binding protein 2.

Chylomicrons deliver free fatty acids to the tissues via lipoprotein lipase (LPL) which hydrolyses triglycerides to free fatty acids and serves as a docking station for the lipoproteins in the endothelium. Chylomicrons form chylomicron remnants, which enter the liver to induce formation of apoB100-containing very low-density lipoproteins (VLDL) which have a high triglyceride content. VLDL works like

chylomicrons providing free fatty acids to the tissues. In the process, VLDL particles shrink in size forming intermediate density lipoproteins (IDL) and LDL which are high in cholesterol (Goldberg, Eckel, & Abumrad, 2009). (Nozue, 2017).

IDL and LDL are taken up by the liver through LDL receptors which transport lipoproteins in to the hepatocytes and shuttle back to the plasma membrane (Joseph L. Goldstein & Brown, 2009). Proprotein convertase subtilisin/kexin type 9 (PCSK9), a circulating liver-derived protein, binds to LDL receptor to induce its lysosomal degradation and thus to regulate liver LDL uptake (Raal et al., 2012). In clinical settings, PCSK9 inhibitors are used in combination with statins to lower LDL and cholesterol in the circulation by upregulating the hepatic LDL receptor

Excess cholesterol can be decreased by a reverse cholesterol transport process. The main lipoprotein involved is HDL, which is synthesized in the liver and intestine. In the circulation, HDL picks up free cholesterol which is esterified by lecithin-cholesterol acyltransferase (LCAT) and exchanges cholesterol esters to triglycerides with LDL, IDL and VLDL particles through the action of cholesterylester-transfer-protein (CETP). HDL triglycerides are rapidly hydrolysed by hepatic lipase (HL) and taken up by the liver. Cholesterol esters from the HDL are taken up by the liver through scavenger receptor BI (SR-BI) and can be excreted to the gall bladder as bile salts. Thus, HDL sequesters surplus cholesterol and delivers it to liver and increases VLDL to LDL transition, enhancing the hepatic LDL uptake. (Kosmas et al., 2018)

Obesity decreases LPL in the adipose tissue and muscle, leading to mitigation of chylomicron and VLDL lipolysis and increased circulating triglyceride and free fatty acids (Capell, Zambon, Austin, Brunzell, & Hokanson, 1996; Peterson et al., 1990). Accumulation of large lipoproteins favors the exchange of triglycerides to cholesterol ester with HDL particles, accentuating the role of HDL and the liver in triglyceride clearance. In hypertriglyceridemia, LDL triglyceride content increases and cholesterol content decreases, however, hepatic lipase decreases LDL triglyceride content leading to accumulation of smaller and more dense LDL particles and enhanced hepatic triglyceride accumulation. Small dense LDL are more atherogenic than bigger LDL particles and have extended half-life, increasing the atherogenic effect of these particles (Packard, 2003; Tchernof et al., 1996). Thus, hypertriglyceridemia in obesity drives the formation of atherogenic LDL.

Liver is the main site for endogenous cholesterol synthesis. Transcription factor sterol regulatory element binding protein 2 (SREBP2) is the main regulator of the cholesterol synthesis (Brown & Goldstein, 1997). In its' inactive form, SREBP2 resides in the ER membrane in a complex with sterol regulatory element-binding

protein cleavage-activating protein (SCAP) (Joseph L. Goldstein, DeBose-Boyd, & Brown, 2006; Gong et al., 2015). Low sterol content incites conformational changes to SCAP which leads to translocation of the complex to the golgi where SREBP2 is cleaved off. The cleaved SREBP2 translocates to the nucleus to induce the genes of cholesterol synthesis pathway. Thus, low sterol content launches a feedback response to sustain cholesterol reserves (Brown & Goldstein, 1997).

SCAP-SREBP2 complex is affected by insulin-induced gene 1 (INSIG1), which prohibits the complex from translocating to the golgi (T. Yang et al., 2002). Additionally, INSIG1 downregulates 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), the pharmacological protein target of statins and the rate-limiting enzyme of cholesterol synthesis (Sever et al., 2003). In addition to genes synthesizing cholesterol, SREBP2 also regulates the expression of PCSK9 (Shimano & Sato, 2017). Therefore, usage of drugs that inhibit cholesterol synthesis and lower the sterol content in ER membranes, such as statins, associates dose-dependently with circulating PCSK9, and statins lose some of their effect when the treatment is continued due to PCSK9 upregulation (Dubuc et al., 2004; Welder et al., 2010).

2.3.5 Endocrine disruptors and metabolic diseases

Excess food intake and sedentary lifestyle present the major initiators and modifiable risk factors of obesity and cardiometabolic diseases (Di Cesare et al., 2016; Schwartz et al., 2017). However, as the prevalence of obesity and its' comorbidities continue to rise, a more comprehensive view on the disease etiology and risk factors is demanded. In 2002, Baillie-Hamilton presented a hypothesis that exposure to environmental chemicals contributes to the modern obesity epidemic (Baillie-Hamilton, 2002). Since then, the concept of endocrine disruptors (EDs) has been gaining more and more attention (Gore et al., 2015; Heindel et al., 2015; Küblbeck et al., 2020). Indeed, chemicals that we are ubiquitously exposed to increase obesity and the risk of metabolic diseases.

An ED is defined as *an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action* (Gore et al., 2015). Hormones are secreted by endocrine organs and they act through binding to their receptors in target tissues. Hormonal signals govern essential responses and feedback-loops to regulate and maintain development, physiological processes, and homeostatic functions. The interference of EDs in hormone action often result in adverse effects in the organism or its progeny.

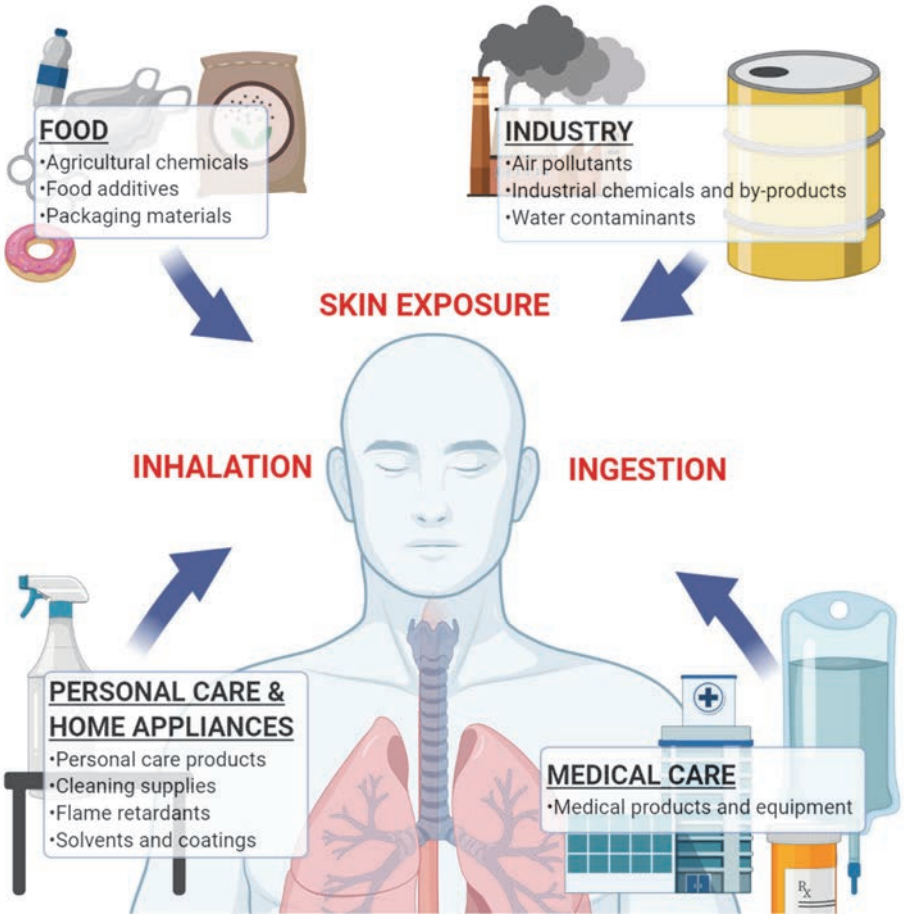

Fig. 5. Major sources of endocrine disruptors and routes of exposure.

There are possibly hundreds or even thousands of EDs in the environment. They are usually synthetic chemicals used in the industry that end up in the food, the air, and the water. For example, pesticides are used to grow crops and plasticizers in plastic packages. Industrial activity creates air pollutants and by-products to the environment. Many household items, such as cleaning and personal care products, contain EDs. Moreover, many drugs and nature-derived compounds disrupt the endocrine system (Gore et al., 2015). Human exposure to EDs occur through exposure to skin, inhalation, or ingestion. Main sources and exposure routes of EDs are summarized in Figure 5. EDs are ubiquitously distributed throughout the globe,

exemplified by a current study showing plastic debris accumulation in the beaches of uninhabited islands (Lavers, Dicks, Dicks, & Finger, 2019).

The constant and long-term exposure to EDs has raised concerns about possible adverse health effects. It's difficult to estimate the net effect of endocrine disruptors as no markers exist for such purpose. Instead, many EDs have been investigated individually. Bisphenol A (BPA) is a prototypical example of an ED and one of the most widely used ones: food packaging, toys, cash receipt paper and other applications utilize BPA (Hormann et al., 2014). As an example of ubiquitous and constant usage, 93% of Americans have BPA in urine (Calafat, Ye, Wong, Reidy, & Needham, 2008).

Epidemiological studies have shown correlation for urinary BPA concentrations and obesity, insulin resistance and T2D (Carwile & Michels, 2011; T. Wang et al., 2012). Further, BPA increases bodyweight and disturbs glucose and lipid metabolism in experimental mouse and rat models (Alonso-Magdalena et al., 2010; Wei et al., 2011). Thus, strong evidence exists to support a link between metabolic diseases and BPA exposure. BPA was first shown to bind estrogen receptor (ER) and have estrogenic properties. Later, the array of receptors which BPA bind has been expanded with PXR, constitutive androstane receptor (CAR), estrogen-related receptor, glucocorticoid receptor, peroxisome proliferator-activated receptors (PPARs) and androgen receptor (summarized in Küblbeck et al., 2020).

The spectrum of receptors bound by BPA substantiate how single ED may influence multiple signaling pathways and physiologic processes. Indeed, foreign chemicals are rarely selective to only one receptor. To aid in risk assessment of a substance, it is of importance to elucidate which receptors are affected and what are the endogenous properties of the receptor.

2.4 Xenobiotic receptors – Potent drug targets for metabolic diseases

ER was the first receptor to be identified as an ED target receptor. Since then, EDs have been described to affect a plethora of receptors (Küblbeck et al., 2020). Nowadays it is acknowledged that EDs exert their adverse events through numerous cellular mechanisms targeting not only nuclear hormone receptors but enzymes, membranes and signaling mediators as well. While some of the receptors have a high sensitivity for their respective endogenous hormones, some accept a wider array of ligands, usually foreign chemicals. These are called xenobiotic

receptors (XRs), prototypical examples being CAR (NR1I3), PXR (NR1I2) and aryl hydrocarbon receptor (AHR) (Timsit & Negishi, 2007). Together, XRs sense the chemical environment with a primary purpose to accelerate the metabolism and excretion of toxic substances. Common for xenobiotic receptors is their wide ligand acceptance, ability to regulate the metabolism of xenobiotics and primary expression in the liver, the main site of xenobiotic metabolism.

PXR and CAR are ligand-activated transcription factors and belong to the superfamily of nuclear receptors (NRs) (Auwerx et al., 1999; Evans, 1988) whereas AHR is a unique ligand-activated basic helix-loop-helix transcription factor (Mulero-Navarro & Fernandez-Salguero, 2016). XRs have larger and more flexible ligand binding pockets compared to other hormone receptors, which explains the spectrum of ligand acceptance. After ligand binding, XRs bind to their respective response elements in the genome and regulate the expression of drug metabolizing, conjugating and transporting phase I-III enzymes and drug transporters. This leads to accelerated detoxification and clearance of possibly harmful chemicals recognized by XRs. This way, XRs constitute a defense system against environmental toxins.

Recently, the role of XRs has been expanded from the regulation of xenobiotic metabolism to cover the regulation of key nodes of endogenous metabolism, such as inflammation and the metabolism of glucose and lipids. It is speculated that, due to their dual role as the sensors of chemical environment and regulators of energy metabolism, they might contribute to the current obesity epidemic and related metabolic dysfunction (M. Banerjee et al., 2015; Hukkanen, Hakkola, & Rysä, 2014).

CAR and PXR share target genes and functions in regulating xenobiotic metabolism but their role in endobiotic metabolism differs. CAR activation has been characterized to be beneficial whereas PXR activation has shown to be detrimental for several aspects of metabolic health. Numerous studies made on mice have established CAR activation to alleviate obesity and related metabolic dysfunction, lower glucose levels by repressing hepatic gluconeogenesis, and to increase basal metabolic rate through thyroid hormone signaling (Dong et al., 2009; Gao, He, Zhai, Wada, & Xie, 2009; Maglich et al., 2004; L. Yu et al., 2016). More conflicting reports have been made about CAR activation and fatty liver. Of importance, human data regarding CAR activation and liver fat is limited.

Activation of PXR, again, has been shown to impair glucose tolerance, and induce accumulation of liver fat, showing a detrimental role in endogenous metabolism (Hassani-Nezhad-Gashti et al., 2018; Rysä et al., 2013; J. Zhou et al.,

2006). Moreover, PXR deficiency protects mice from obesity-induced metabolic dysfunction (He et al., 2013). Despite the detrimental role of PXR activation in endogenous metabolism, PXR activation seems to be beneficial in alleviating inflammatory bowel disease (Shah, Ma, Morimura, Kim, & Gonzalez, 2007; Terc, Hansen, Alston, & Hirota, 2014). Thus, the estimation of harmful effects of a chemical should include determination of which receptors and in which tissue are affected and what specific functions these receptors elicit.

Modulation of NR activity by agonists or antagonists makes them tempting targets for small-molecule drug development. Further, numerous drugs already on the market act through NRs and more are under development for diseases such as asthma, type 2 diabetes, atherosclerosis, osteoporosis and cancer. Defining and distinguishing the function of a single NR in a specific disease and tissue is crucial in validating novel therapeutic targets.

2.5 Pregnane X Receptor

In the 1960s, it was reported that administration of a drug can affect the metabolism of other, structurally unrelated drug (Selye, 1969). Later, this phenomenon of drug-drug interaction was shown to be mediated by cytochrome P450 enzymes (CYPs), inducible oxygenases that are responsible for oxidation of drugs and, thus, their metabolism (Gonzalez, Liu, & Yano, 1993). However, the molecular basis remained unknown. In 1998, a novel murine nuclear receptor, which was shown to be activated by known CYP3A inducers in the liver and intestine, was cloned (Kliewer et al., 1998). Soon after, human ortholog of *Pxr* was cloned by several groups and it was shown to mediate CYP3A4 induction of drugs, and thereby the mechanism of drug-drug interactions was unraveled (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998).

The findings were of special importance since CYP3A4 metabolizes more than half of all prescription drugs. Later, the knowledge has been utilized to develop *in vitro* methods to identify drug-drug interactions and CYP3A4 inducers in the drug discovery process. Simultaneously, natural, environmental and endogenous PXR ligands have been identified. More recently, PXR has been shown to have roles in several diseases. Currently, the role of PXR is studied in a plethora of diseases, spanning from atopic dermatitis to Alzheimer's disease (M. Banerjee et al., 2015). Therefore, the function of PXR seems to be much more complex and pleiotropic than was expected after the initial findings.

2.5.1 Ligands, target genes and gene responses

Before the identification of PXR, multiple CYP3A4 inducers had been characterized, classical ones being pharmaceuticals, such as the glucocorticoid dexamethasone, the antibiotic rifampicin and the antimycotic clotrimazole (Honkakoski, Sueyoshi, & Negishi, 2003; Lehmann et al., 1998). To date, numerous PXR ligands have been characterized involving pharmaceuticals, environmental chemicals and endogenous compounds. One striking feature of PXR ligands is their heterogeneity. The large and flexible ligand binding pocket allows binding of ligands of varying size and chemical features. Some common human PXR ligands are listed in table 1. Identification of a chemical as a PXR ligand has proven to be valuable in understanding the effects of PXR activation in drug-drug interactions but also in systemic metabolism.

For example, rifampicin, a tuberculosis antibiotic and a selective human PXR agonist, was shown to have a detrimental effect on glucose metabolism (Rysä et al., 2013; Takasu et al., 1982). With mouse models, PXR activation was defined to impair glucose tolerance, a finding which possibly explains the rifampicin-associated disturbances in glucose tolerance (Hassani-Nezhad-Gashti et al., 2018). Thus, the initial finding made in rifampicin-treated patients had far-reaching importance in understanding the adverse effects of PXR activation. Some recent reports have characterized ligand-independent roles of PXR, e.g. in regulating platelet function and haemostasis (Flora et al., 2019). Further, PXR splice variants which lack the full-length ligand-binding domain (LBD) have been identified which suggests that PXR has functional relevance beyond regulation by ligands (Lamba et al., 2004). Still, the current knowledge strongly centers in the ligand-dependent features of PXR.

A curious aspect of PXR ligands is their tissue-specificity. Some ligands, such as antibiotic rifaximin, are selective intestinal PXR activators due to poor absorption into circulation (X. Ma, Shah, Guo, et al., 2007). Tributyl citrate and acetyl tributyl citrate, widely used plasticizers, activate rodent and human PXR in the intestine but not in the liver, however, the mechanism is unkown (Sui et al., 2015; Takeshita et al., 2011). Both plasticisers were tested *in vivo* and *in vitro* without a sign of PXR activation in hepatocytes. This interesting occurrence was hypothesized to account for different transcriptional landscapes in the intestine and the liver.

Table 1. Common human PXR ligands.

Source	Type	Ligand	Reference
Endogenic	Steroid hormone	Estradiol	(Xue et al., 2007)
		Pregnane	(Bertilsson et al., 1998)
		Pregnenolone	(Bertilsson et al., 1998)
		Progesterone	(Bertilsson et al., 1998)
	Gut-derived indoles	3-indolepropionic acid	(Venkatesh et al., 2014)
	Bile acid	Litocholic acid	(Staudinger et al., 2001)
Environment	Alkylphenol	Nonylphenol	(Mota, Barfield, Hernandez, & Baldwin, 2011)
	Bisphenol	BPA	(Sui et al., 2012)
	Flame retardant	PBDE	(Pacyniak et al., 2007)
	Persistent organic pollutant	PCBs	(Al-Salman & Plant, 2012)
		DDT	(Lemaire et al., 2006)
	Pesticide	Pretilachlor	(Lemaire et al., 2006)
	Phthalate	DEHP	(Dekeyser, Laurenzana, Peterson, Chen, & Omiecinski, 2011)
	Polyfluorinated chemical	PFOS	(Y. M. Zhang et al., 2017)
Nature	Phytoestrogen	Coumestrol	(H. Wang et al., 2008)
	St. John's Wort extract	Hyperforin	(Watkins et al., 2001)
	Ginkgo Biloba extract	Ginkgolide A	(Lau, Yang, Yap, & Chang, 2012)
Pharmaceuticals	Antibiotic	Clotrimazole	(L. B. Moore et al., 2000)
		Rifampicin	(Bertilsson et al., 1998)
		Ritonavir	(Dussault et al., 2001)
	Barbiturate	Phenobarbital	(L. B. Moore et al., 2000)
	Ca^{2+} channel blocker	Nifedipine	(Drocourt et al., 2001)
	Glucocorticoid	Dexamethasone	(L. B. Moore et al., 2000)
	Hypocholesterolemic	SR12813	(Watkins et al., 2001)
	Statin	Atorvastatin	(Howe, Sanat, Thumser, Coleman, & Plant, 2011)
		Lovastatin	(Howe et al., 2011)

The different functions of PXR in xenobiotic metabolism and different diseases and conditions through gene regulation is summarized in Figure 6. The best characterized function of PXR lies in drug metabolism. PXR regulates genes in all phases of drug metabolism: phase I enzymes, e.g. CYPs and carboxylesterases (CESs), phase II conjugating enzymes, sulfotransferases (SULTs), UDP-glucuronosyl transferases (UGTs), glutathione S-transferases (GSTs) and phase III transporters such as multidrug resistance 1/p-glycoprotein (MDR1), different

multidrug-resistance associated proteins (MRPs), and organic anion transporters (OATPs) (Ihunnah, Jiang, & Xie, 2011). Through PXR activation, drugs and herbs can cause clinically important drug-drug, herb-drug and even food-drug interactions. In some cancer types, PXR-mediated regulation of xenobiotic metabolism renders the cancerous tissue immune to drugs (Qiao et al., 2013).

Multiple PXR ligands have been shown to induce hepatic steatosis and, indeed, liver fat accumulation seems to be an integral part of xenobiotic response orchestrated by hepatic PXR (Hakkola et al., 2016). PXR regulates multiple genes involved in fatty acid uptake, β-oxidation, and *de novo* lipogenesis which induce steatosis. Although the induction of hepatosteatosis is clearly an adverse phenomenon, intact PXR is required to protect the liver from toxic bile acids and cholesterol metabolites through regulation of proteins related to cholesterol and bile acid metabolism, such as CYP7A1 and OATP2 (Sonoda et al., 2005; Staudinger et al., 2001).

In addition to hepatic steatosis, PXR regulates glucose homeostasis by impairing postprandial glucose tolerance (Rysä et al., 2013). On a transcriptional level, PXR regulates gluconeogenic genes *Pck1* and *G6pc* and genes regulating hepatic glucose uptake, *Glut2* and *Gck* (Hassani-Nezhad-Gashti et al., 2018; Kodama, Koike, Negishi, & Yamamoto, 2004).

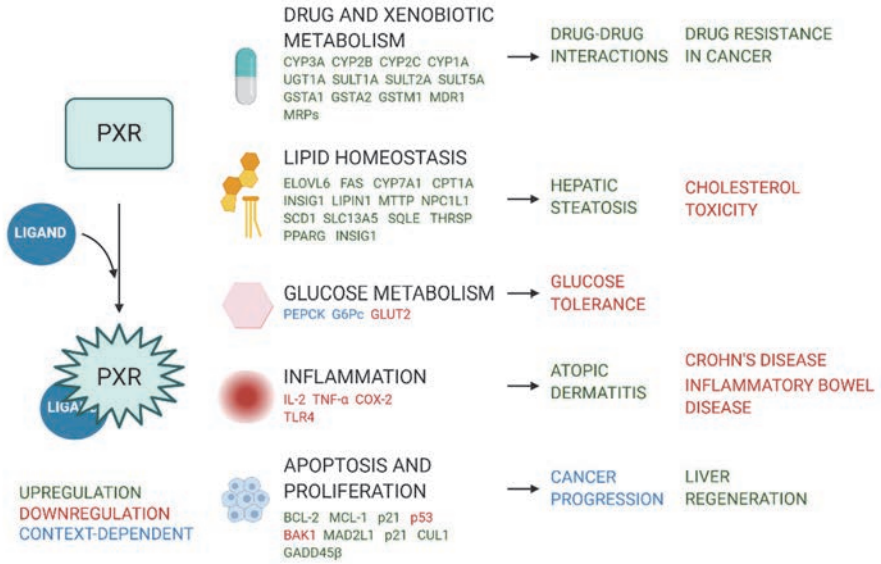

Fig. 6. PXR target genes and their relevance to biological functions and diseases.

In the intestine, PXR activation represses proinflammatory target genes of NF-κB to alleviate inflammation in mouse models of inflammatory bowel disease (IBD). Further, rifaximin, selective intestinal PXR activator, reduces IBD symptoms in humanized PXR mice (X. Ma, Shah, Guo, et al., 2007). In humans, rifaximin has better efficacy in treating IBD than other antibiotics (J. Yang, Lee, Low, Chatterjee, & Pimentel, 2008) possibly due to PXR activation. Albeit the clear role on intestinal inflammation, overexpression of human PXR in the mouse epidermis triggers immune response resembling atopic dermatitis (Elentner et al., 2018). In the liver, PXR additionally regulates hepatocyte cell size and fate and promotes normal liver regeneration after a hepatectomy (Dai, He, Bu, & Wan, 2008; Jiang et al., 2019).

Therapeutic targeting of PXR is disease specific. For instance, despite the anti-inflammatory effect of PXR activation on intestinal epithelium, intestinal PXR activation promotes intestinal cancer growth, chemoresistance and malignancy, and often intestinal cancers have higher PXR activity (R. Pondugula, Pavek, & Mani, 2016; H. Wang et al., 2011).

Adverse effects of PXR activation, like drug-drug interactions, cancer chemoresistance and metabolic perturbations, have probed an interest to identify

and develop selective PXR antagonists which could be utilized to mitigate the effects of PXR activation and inhibit its function. Multiple antagonists have been identified, e.g. fungicide ketoconazole, but they lack in potency and selectivity and relevant *in vivo* concentrations tend to be toxic (Mani, Dou, & Redinbo, 2013). Recently, a novel chemical, SPA70, was found to antagonize human PXR (Lin et al., 2017) but it has not yet been tested in clinically relevant conditions or disease models.

2.5.2 Structure and mode-of-action

PXR, CAR and vitamin D receptor (VDR, NR1I1) constitute the group I of the subfamily 1 of NRs (Auwerx et al., 1999). Like many other nuclear receptors, PXR contains N-terminal DNA-binding domain (DBD), followed by a flexible hinge region and an LBD (Figure 7.). Nuclear receptors have a common ancestor, which can be still seen as shared sequence similarities, especially in the conserved DBD. (D. D. Moore et al., 2006) The human and mouse PXR have a 95% sequence similarity in the DBD and therefore similar DNA binding motifs and target genes.

Importantly, the LBD is less conserved between humans and mice with only 73% similarity in the amino acid level, which is the main reason for species-specific ligand binding. Natural selection has possibly favored diversity in the LBD to adapt to species-specific differences in important ligands (reviewed in Iyer et al., 2006). The differences in PXR ligands makes the relevance of murine models questionable in estimating properties of human PXR ligands. However, this has been overcome by generating a mouse strain where mouse *Pxr* has been replaced by the human *PXR* (Xie et al., 2000). The humanized PXR mouse strain has proved to be a precious tool in assessing CYP induction and related mechanisms of chemicals relevant to humans (*e.g.* Shehu et al., 2019).

Nuclear receptors are divided into five subtypes based on their mode of action (Auwerx et al., 1999). PXR and principally all NR1 subfamily members are type II. Type II NRs heterodimerizes with retinoid X receptor (RXR) after ligand binding to allow DNA binding, which in PXR is facilitated by two C4-type zinc fingers together with P-Box and D-Box motifs. The most common DNA binding site of PXR is AGTTCA-like direct repeat interspaced by four base pair long spacer (Cui, Gunewardena, Rockwell, & Klaassen, 2010). Other binding sites described for PXR include direct repeats interspaced by three or five nucleotides or everted repeats interspaced by six or eight nucleotides. (Chai, Zeng, & Xie, 2013; Pavek, 2016; Smutny, Mani, & Pavek, 2013; Umesono & Evans, 1989)

Fig. 7. The schematic structure of PXR. The N-terminal DNA binding domain (DBD) followed by a flexible hinge region, a ligand-binding domain (LBD) and C-terminal activation function domain (AF-2).

PXR has an unusual ligand binding structure for an NR enabling binding of structurally variable ligands (Watkins et al., 2001). Structurally, the PXR-LBD is an α-helical sandwich consisting of three layers, α1–α3, α4–α5–α8–α9 and α7–α10 and five stranded antiparallel β-sheets. Of note, usually NR LBDs have three β-sheets. The LBD has 28 amino acid residues, of which 20 are hydrophobic which enables binding of lipid-soluble chemicals (Ngan et al., 2009). Importantly, PXR LBD has been shown to cooperatively bind two ligands which had a synergistic effect on PXR activity (Delfosse et al., 2015). The finding shows a possible molecular mechanism for the cocktail effect by which a compound's toxicity might be exacerbated. It also warrants mixtures of compounds to be investigated in addition to single ligands.

The transcriptional activity of PXR is defined by transcriptional coactivators and corepressors. Ligand binding alters the conformation of the PXR LBD to favour binding of coactivators instead of corepressors (Lazar, 2003; Rosenfeld, Lunyak, & Glass, 2006). Recruitment of coactivators is crucial for fixing the ligand into a position inside the LBD. This is mainly due to activation-function domain 2 (AF-2) in the LBD, which binds Leu-Xxx-Xxx-Leu-Leu motifs of transcriptional coactivators, and the Ile/Leu-Xxx-Xxx-Ile/Val-Ile motifs of corepressors (Rosenfeld et al., 2006). Interestingly, the ligand binding hot spots inside the LBD are the same that are bound by the coactivators and corepressors outside the LBD (Xue et al., 2007).

Inactive PXR resides in the cytosol in a complex with CAR retention protein and heat shock protein 90 (Squires, Sueyoshi, & Negishi, 2004). Corepressors involved in the regulation of PXR function include common NR repressors small heterodimer partner and nuclear receptor corepressor 2 (NCor2) (D. D. Moore et al., 2006). Steroid receptor coactivator 1 (SRC-1) was the first identified PXR coactivator (Kliewer et al., 1998; Lehmann et al., 1998). In DNA-bound PXR,

SRC-1 manifests the recruitment of multiple secondary coactivators and histone modifying enzymes which remodel chromatin loose to enable binding of transcriptional RNA polymerase complex to initiate transcription (Pavek, 2016). Curiously, PXR was shown to interact either with SRC-1 or with NR interacting protein 1 in a ligand-dependent manner, showing that the ligand *per se* may affect the LBD conformation to favor one coactivator over another (Masuyama, Hiramatsu, Kunitomi, Kudo, & MacDonald, 2000). Other coactivators PXR is associated with include steroid receptor coactivator 2 (SRC-2) and PGC-1α (D. D. Moore et al., 2006).

Adding to the complexity of gene regulation manifested by PXR, same coregulators are shared by numerous transcription factors and NRs. This is the case, for instance, in PXR-mediated repression of CYP7A1, the rate-limiting enzyme in bile acid synthesis. Ligand activated PXR recruits PGC1α from HNF4A which leads to repression of PGC1α-dependent HNF4A target genes such as *CYP7A1* (Bhalla, Ozalp, Fang, Xiang, & Kemper, 2004). This is a prime example how transcriptional networks are interconnected and in constant communication. Metabolic diseases, for instance, alter these networks but it is not known how modulation of transcriptional networks by a disease state affects the PXR function.

2.5.3 Regulation and protein-protein interactions

Ligand binding and subsequent changes in coregulator binding constitute a central regulatory mechanism for NR-mediated gene regulation (Kliewer et al., 1998; Rosenfeld et al., 2006). Regulation of NRs is further perplexed by post-translational modifications, through which multiple cellular pathways fine-tune NR function (Smutny et al., 2013). In addition to coregulators, NRs are bound by multiple proteins forming multifactorial protein complexes whose composition and modifications define the true gene regulation orchestrated by NRs (P. Oladimeji, Cui, Zhang, & Chen, 2016). This also provides insights to why, in some cases, PXR activation leads to gene downregulation and not induction.

Although this chapter focuses on the regulation of PXR by protein-protein interactions, it is noteworthy that recent reports have shown that different stimuli may affect PXR function by regulating its expression. For instance, insulin suppresses PXR expression which contributes to insulin-induced downregulation of PXR target gene carboxylesterase. Conversely, glucose *per se* was shown to increase PXR expression. (Daujat-Chavanieu & Gerbal-Chaloin, 2020; P. O. Oladimeji, Lin, Brewer, & Chen, 2017; Xuan Yang, Zhang, Liu, Xi, & Xiong, 2019)

Post-translational modifications of PXR

Like many other nuclear receptors, the function of PXR is affected by phosphorylation, ubiquitination, SUMOylation and acetylation. These post-translational modifications interfere with all functions of PXR involving half-life, subcellular localization, dimerization, DNA binding, and coregulator interaction. Generally, intact post-translational modifications inhibit the PXR function. (X. Ma, Shah, Cheung, et al., 2007; Smutny et al., 2013)

The strongest evidence to date lies on site-specific phosphorylation events of PXR and how it affects PXR-mediated CYP regulation. Series of kinases, including p70 S6K, cyclin-dependent kinase 2 (CDK2), protein kinase A (PKA) and protein kinase C (PKC), phosphorylate human PXR in the DBD and LBD affecting coregulator interactions (Lichti-Kaiser, Brobst, Xu, & Staudinger, 2009). Further, immunopurification of human PXR has found PXR to interact with glycogen synthase kinase 3 (GSK3), casein kinase 2 (CK2) and cyclin-dependent kinase 1 (CDK1) kinases (Lichti-Kaiser, Xu, & Staudinger, 2009). These kinases are essential in multiple cellular pathways which mediate the effects of inflammation, cell-cycle progression and growth factors such as hepatocyte growth factor and insulin. Figure 8. summarizes the current knowledge of kinases known to modulate PXR action and how phosphorylation events affect the coregulator interactions.

Activities of PKA and PKC, mediators of the glucagonergic and adrenergic pathways, respectively, are increased in inflammation, when CYP expression is downregulated (Ding & Staudinger, 2005; Kim & Novak, 2007). The effect of PKA activity on PXR function seems to be species specific (potentiation in mice, inhibition in humans) but PKC activity is continuously linked to inhibition of PXR target genes and *in silico* prediction of PXR phosphorylation sites and kinases identify multiple phosphorylation sites for PKA and PKC (Abdel-Razzak et al., 1993; Jover, Bort, Gómez-Lechón, & Castell, 2002; Lichti-Kaiser, Brobst, et al., 2009; Lichti-Kaiser, Xu, et al., 2009).

In addition to inflammation, CYPs are greatly downregulated during liver development and regeneration (Hines, 2007). CDK2, a key regulator of cell-cycle progression, phosphorylates PXR to inhibit its function in the synthesis phase, the S phase, of cell-cycle (Lin et al., 2008; Sugatani et al., 2010). Further, p70 S6K, a downstream kinase of the PI3K-Akt/mTOR pathway, the pathway mediating effects of insulin and growth factors, directly phosphorylates PXR to inhibit its action (Pondugula et al., 2009). Other important kinases phosphorylating PXR are mitogen-activated protein kinases, MAPKs, which govern growth, differentiation

and survival signaling (Smutny et al., 2013). Thus, CYP downregulation during inflammation and different proliferation cues may be mediated by inhibitory phosphorylation of PXR and other nuclear receptors regulating CYP expression.

Not much is known about ubiquitination, SUMOylation and acetylation of PXR, but some reports exist. Polyubiquitination of PXR marks it for degradation by a proteasome complex. PXR interacts with a subunit of the 26S proteasome complex, but only when occupied by progesterone, not in the presence of other PXR ligands, specifically phtalic acid and nonylphenol (Masuyama et al., 2000). This suggests a mechanism by which different ligands may affect the half-life of PXR and shows that PXR protein level is regulated by proteasomal degradation. SUMOylated NRs are often inhibited, and accordingly, PXR is a SUMOylation target after TNFα stimulus indicating another level of inflammatory PXR regulation (Hu, Xu, & Staudinger, 2010). Acetylation also constitutes a common regulatory mechanism of NRs. Rifampicin treatment leads to deacetylation of PXR *in vivo*, possibly partly mediated by histone deacetylase sirtuin 1 (SIRT1) residing in a same complex with PXR (Buler, Aatsinki, Skoumal, & Hakkola, 2011; Pasquel et al., 2016).

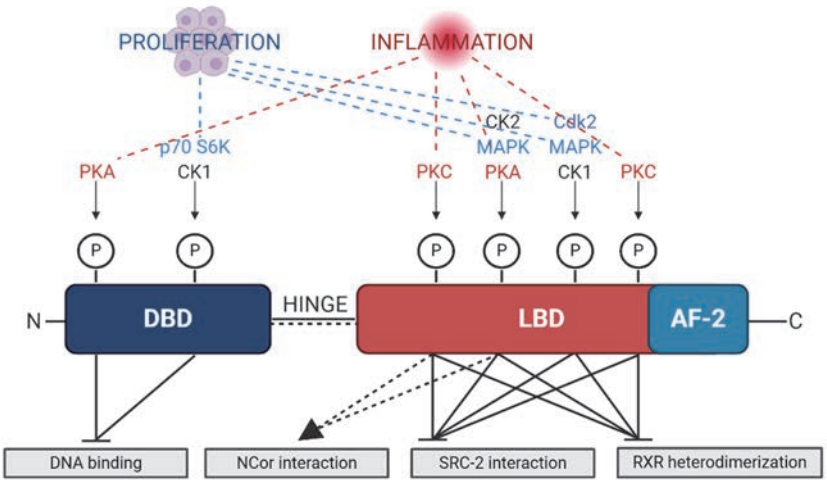

Fig. 8. A model of cellular events leading to phosphorylation of PXR and functional implications through coregulator interaction based on *in silico* predictions and other experimental data. AF-2 Activation function domain 2, CK1 Casein kinase 1, Cdk2

Cyclin-dependent kinase 2, DBD DNA-binding domain, LBD Ligand-binding domain, NCor Nuclear receptor corepressor PKA protein kinase A, PKC Protein kinase C, p70 S6K p70 S6 kinase, RXR Retinoid X receptor, SRC-2 Steroid receptor coactivator 2.

Non-modifying protein-protein interactions of PXR

In addition to enzymes inflicting post-translational modifications to PXR, several intracellular proteins interact with PXR. To date, the data regarding PXR protein-protein interactions is relatively scarce and centers in the regulation of bile acid synthesis and gluconeogenesis, which seem to be downregulated by PXR through interactions with HNF4A, PGC1α, FOXO1 and CREB. Like gluconeogenesis, these factors are highly regulated by the energy status (fasted/fed) and metabolic diseases (Rhee et al., 2003).

In the case of limiting bile acid synthesis, activated PXR represses *CYP7A1* through interactions with HNF4A and its' coactivator PGC1α. Two models have been proposed, the first one suggesting that activated PXR interacts with PGC1α leading to the downregulation of HNF4A transcriptional activity (Bhalla et al., 2004) and the other one suggesting that PXR interacts with HNF4A leading to the PGC1α detachment from the complex (T. Li & Chiang, 2005).

Nevertheless, the functional inhibition of HNF4A by PXR activation extends to the regulation of gluconeogenic genes *PCK1*/*Pck1* and *G6Pc*/*G6pc* (Figure 9.). In the proposed mechanism, activated PXR squelches PGC1α from HNF4A leading to the repression of *Pck1* and *G6pc*. One central regulator of gluconeogenesis and PGC1α expression is CREB, which PXR binds in an inhibitory manner (Kodama, Moore, Yamamoto, & Negishi, 2007). In similar fashion, PXR binds to FOXO1 to downregulate gluconeogenic gene expression (Kodama et al., 2004). Although data regarding mouse *Pck1* and *G6pc* repression by PXR is consistent, a study reported PXR to induce human *PCK1* and *G6Pc* through interaction with serum/glucocorticoid regulated kinase 2 which coactivated PXR (Gotoh & Negishi, 2015). The result indicates that the regulation of gluconeogenic genes by PXR might be specific to species or experimental context.

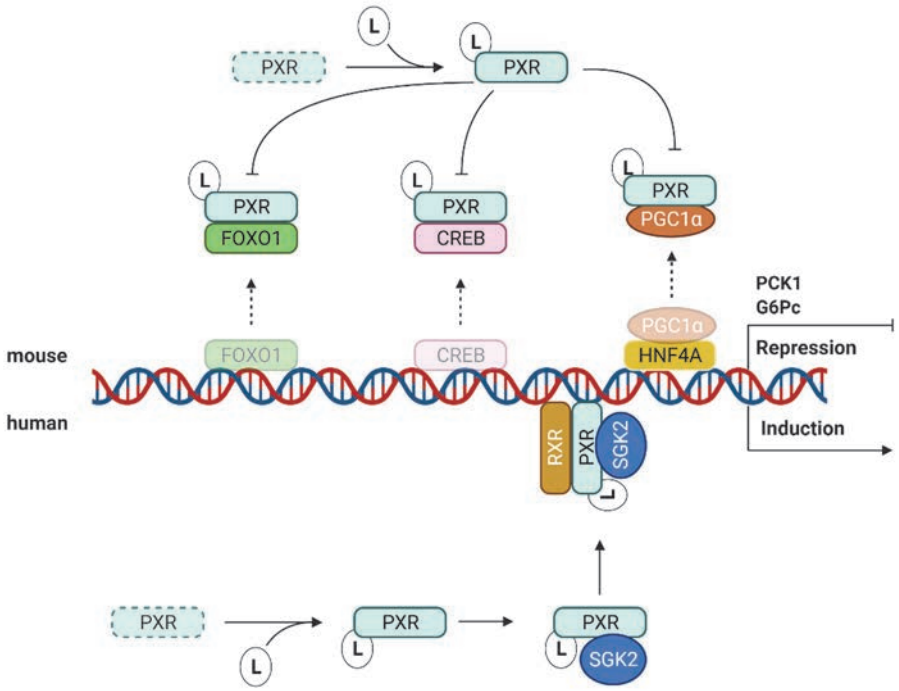

Fig. 9. PXR regulates the expression of gluconeogenetic genes, PCK1 and G6Pc, by protein-protein interactions. Adapted from Hakkola et al. (2016)

HNF4A and FOXO1 are inhibited by PXR interaction, but they have also been described to act as PXR coactivators in addition to PGC1α, which complicates the picture. Interaction of PXR and HNF4A is enhanced by rifampicin and HNF4A augments the PXR mediated transactivation of *CYP3A4* and the mouse equivalent *Cyp3a11* (T. Li & Chiang, 2006; Tirona et al., 2003). In a similar manner, insulin-responsive FOXO1 interacts with PXR to enhance its transactivation function (Kodama et al., 2004). This interaction was also repressively regulated by insulin and PI3K/Akt pathway.

Like PXR activation, insulin represses *CYP7A1*. This is due to SREBP1, which is recruited to HNF4A to inhibit coactivator interaction after insulin stimulus (Ponugoti, Fang, & Kemper, 2007). SREBP1 is a highly insulin-responsive transcription factor responsible for hepatic *de novo* lipogenesis (Horton et al., 2002). Insulin stimulates SREBP1 by affecting mTOR and LXR signaling (Bakan & Laplante, 2012; Oliner, Michael Andresen, Hansen, Zhou, & Tjian, 1996).

Interestingly, overexpression of SREBP1 leads to downregulation of PXR target genes and SREBP1 directly interacts with PXR to inhibit coactivator recruitment. (A. Roth, Looser, Kaufmann, & Meyer, 2008). Although these studies indicate insulin and lipogenesis to possibly inhibit PXR by SREBP1 interaction, untreated diabetes induces drug metabolism (S. Goldstein, Simpson, & Saenger, 1990) and glucose *per se* increases PXR activity (P. O. Oladimeji et al., 2017) indicating insulin and energy surplus to stimulate PXR activity.

Despite some discrepancies in the mechanistic data, it is evident that mechanisms central for glucose and lipid metabolism interfere with PXR function through protein-protein interactions of transcriptional regulators CREB, FOXO1, HNF4A, PGC1α and SREBP1.

2.6 PXR in metabolic diseases and obesity

Multiple PXR activating-drugs (e.g. rifampicin, statins and cyclophosphamide) have been associated with metabolic perturbations, like hyperglycemia, hepatosteatosis and dyslipidemia (Luna & Feinglos, 2001). Quite recently, activation of PXR has been established to affect multiple features of metabolic health: it impairs postprandial glucose tolerance, induces hepatic steatosis and regulates cholesterol homeostasis. (C. Zhou, 2016). Therefore, PXR activation might explain some drug-associated adverse metabolic effects, such as increased type 2 diabetes risk caused by statin therapy (Betteridge & Carmena, 2016).

PXR activation has been shown to affect glucose and lipid metabolism, but PXR function seems to be conversely affected by energy metabolism as well. For instance, obesity and NAFLD have been associated with decreased CYP3A4 activity, possibly indicating PXR to be dysregulated in these conditions (Jamwal et al., 2018; Rodríguez-Morató et al., 2019). Also, insulin-induced suppression of carboxylesterase, a PXR target gene, involves the suppression of PXR expression in insulin-dependent manner, and glucose *per se* has been shown to increase PXR expression (Daujat-Chavanieu & Gerbal-Chaloin, 2020; P. O. Oladimeji et al., 2017). Overall, the links between PXR and glucose and lipid metabolism are well-established but experimentation with mouse have yielded partly elusive results on PXR and metabolic diseases.

2.6.1 Hepatosteatosis

PXR has been shown to induce hepatosteatosis in multiple models, such as in wildtype, atherosclerosis prone and obese mice, and in different human and mouse cell models. PXR has been reported to induce hepatosteatosis by suppressing lipid utilization in β-oxidation and increasing lipid synthesis and fatty acid uptake, covering all aspects of hepatic lipid metabolism (Xiao Yang, Gonzalez, Huang, & Bi, 2020).

Fatty acid uptake due to PXR activation accounts for fatty acid transporter *Cd36*, a direct PXR target gene, which was reported to be upregulated in the livers of transgenic mice expressing constitutively active PXR (J. Zhou et al., 2006). Lipogenetic genes elongation of very long chain fatty acids 6 (*Elovl6*) and stearoyl-CoA desaturase-1 (*Scd-1*) were simultaneously induced independently of SREBP1, the main regulator of lipogenesis.

In another study, treatment with a selective murine PXR agonist, pregnenolone-16α-carbonitrile (PCN), downregulated genes of β-oxidation and ketogenesis, carnitine palmitoyltransferase 1 A (*Cpt1a*) and mitochondrial 3-hydroxy-3-methylglutarate CoA synthase 2 (*Hmgcs2*) (Nakamura, Moore, Negishi, & Sueyoshi, 2007). This was possibly due to direct suppressive PXR interaction with FOXA2, a regulator of *Cpt1a* and *Hmgcs2* involved in hepatic lipid accumulation in hyperinsulinemia (Wolfrum, Asilmaz, Luca, Friedman, & Stoffel, 2004).

In human primary hepatocytes, rifampicin increased lipid accumulation and *SLC13A5*, a gene encoding sodium-dependent citrate transporter, which was shown to be a PXR target gene (L. Li et al., 2015). SLC13A5 facilitates uptake of citrate which is used to synthesize fatty acids and cholesterol. Stressing the functional significance, knockdown of *SLC13A5* decreased lipid content in hepatoma cell line HepG2 (L. Li et al., 2015). By utilizing HepG2 cells with a stable PXR transfection, Bitter et al. showed PXR activation to induce steatosis by upregulating SREBP1 pathway, and specifically the SREBP1a isoform (Bitter et al., 2015). This finding is contradictory to previous findings concerning SREBP1-independent pathway. PXR has been shown to directly interact with SREBP1a isoform suggesting PXR and SREBP1a to constitute a regulatory feedback loop in humans (A. Roth, Looser, Kaufmann, & Meyer, 2008)

Interestingly, PXR deficiency has been shown to increase basal hepatic steatosis in mice (Nakamura et al., 2007). In a similar fashion, knockdown of PXR in HepG2 cells resulted in increased lipid accumulation and upregulation of aldo-

keto reductase 1B10 (*AKR1B10*) which was shown to stimulate *de novo* lipogenesis (Bitter et al., 2015). In human liver samples with non-alcoholic liver disease, *AKR1B10* and target genes of SREBP1 were upregulated and PXR downregulated. Altogether, the findings suggest that PXR regulates hepatic lipid metabolism even in the absence of xenobiotic ligands. PXR deficiency possibly disturbs regulatory feedback loops to induce hepatic steatosis through SREBP1 interaction.

2.6.2 Glucose metabolism

It has been recognized that many PXR-activating drugs can cause hyperglycemia in patients (Luna & Feinglos, 2001). Of special interest have been reports concerning tuberculosis patients treated with rifampicin, which is a rather selective human PXR activator (e.g. Purohit et al., 1984; Takasu et al., 1982b). However, these reports are discrepant showing both impaired and improved glucose tolerance, which is possibly explained by underlying diseases and other medications. To clarify the effect of rifampicin on glucose tolerance, our group performed studies on healthy volunteers. In placebo-controlled and blinded crossover setting, rifampicin impaired postprandial glucose tolerance, and PCN treatment on rats replicated the effect (Rysä et al., 2013). We further replicated the study in wildtype and PXR knockout (PXR-KO) mice and showed the effect of PCN to be PXR-dependent (Hassani-Nezhad-Gashti et al., 2018).

These findings contrasted with the previous reports which indicated PXR to repress hepatic gluconeogenesis. Hepatic gluconeogenesis contributes to hyperglycemia in diabetes and, therefore, it was originally hypothesized that PXR activation would improve fasting glucose and glucose tolerance. However, no one has really tested if the repression of gluconeogenic genes by PXR leads to functional decrease in gluconeogenesis. It is also possible that other, more dramatic, mechanisms overrule the effect on gluconeogenesis which leads to impairment in total glucose tolerance. One such factor is GLUT2, which we established to be downregulated by PXR activation in the liver (Hassani-Nezhad-Gashti et al., 2018). GLUT2 is a major hepatic glucose transporter and thus disturbances in it might lead to decreased glucose uptake and sensing in the liver.

The main expression sites of PXR are liver and intestine. In addition to the liver, intestine has important roles in energy metabolism. In postprandial state, intestine secretes incretin hormones to signal tissues to prepare for upcoming energy and nutrient load. However, rifampicin in humans and PCN in rats did not have an effect

on incretin secretion (Hukkanen, Rysa, et al., 2015) possibly indicating that the liver is the main mediator of harmful metabolic effects of PXR activation.

2.6.3 Atherosclerosis and cholesterol homeostasis

In the search of endogenous PXR ligands, several sterol metabolites, such as bile acid lithocholic acid and bile acid intermediates, were found to activate PXR (Dussault et al., 2003; Staudinger et al., 2001). These bile acids induce a pathway resulting in their enhanced metabolism and decreased toxicity and protection from hepatic injury (Staudinger et al., 2001). It was also shown that PXR-mediated metabolism is critical to protect against cholesterol toxicity as PXR deficiency is lethal if the mice are challenged by a diet rich in cholesterol and cholic acid (Sonoda et al., 2005). Thus, it became apparent that PXR has roles in endogenous cholesterol homeostasis.

The first reports linking PXR and cholesterol homeostasis come from tuberculosis patients treated with rifampicin (Khogali, Chazan, Metcalf, & Ramsay, 1974). Nowadays it is acknowledged that multiple PXR-activating EDs, like BPA and some drugs, increase serum cholesterol (Gore et al., 2015). Further, polymorphisms in PXR, in its interaction partners HNF4A and SHP and in its target genes *CYP7A1* and *CYP27A1* associate with circulating LDL (C. Zhou, 2016). In a small study consisting only of 10 male subjects, six day rifampicin treatment did not increase serum cholesterol and lowered total bile acids, but increased markers of cholesterol and bile acid synthesis (Lütjohann et al., 2004).

In mice, PXR activation has been shown to induce hypercholesterolemia and atherosclerosis by inducing the macrophage CD36 expression and lipid uptake in ApoE deficient mice, a common mouse model to study atherosclerosis (Sui, Xu, Rios-Pilier, & Zhou, 2011; C. Zhou, King, Chen, & Breslow, 2009).

The same group later reported quetiapine, an antipsychotic, to induce hypercholesterolemia by inducing intestinal lipid uptake through upregulation of NPC1L1 and MTP (Meng et al., 2019). By utilizing mice deficient in intestinal PXR, they showed the effect of quetiapine on cholesterol to be dependent on intestinal PXR. In another study, the same group reported that antiviral efavirenz induces hypercholesterolemia by inducing hepatic squalene epoxidase gene by activating PXR (Gwag et al., 2019). In contrast, a different group reported PXR activation to lower plasma LDL in ApoE deficient and in LDL receptor (LDLR) deficient mice (Hoekstra et al., 2009). They showed PCN treatment to decrease hepatic lipase expression and plasma lipolysis rate which possibly caused increased

circulating VLDL and decreased LDL. As reviewed before, hypertriglyceridemia drives increased LDL in obesity due to accentuated roles of HDL and hepatic lipase in triglyceride clearance. Thus, the shift in VLDL and LDL might be a secondary effect to hepatic lipase repression.

Altogether, PXR seems to be associated with cholesterol homeostasis but whether PXR activation is harmful or not remains an open question. Human studies are still few and there is a lack of well-controlled studies. In addition, mechanistic data derived from mouse models is elusive.

2.6.4 Obesity

In 2013, He et al. published the first-ever results showing mice deficient in PXR to be protected from obesity and obesity-induced metabolic dysfunction (He et al., 2013). Same publication provided results showing PXR activation to worsen metabolic dysfunction in obesity. Afterwards, more reports have been published regarding PXR activation and obesity, often with discrepant results.

In their paper, He et al. showed C57BL/6J PXR knockout (PXR-KO) mice to gain less weight on high-fat diet (HFD) and to have less adipose tissue than the wildtype controls (He et al., 2013). Food intake during the diet was the same, but the PXR-KO mice showed increased O^2 consumption and β-oxidation and less hepatic steatosis. Expectedly, the PXR-KO mice had better glucose tolerance and insulin sensitivity. He et al. reported HFD-fed PXR-KO mice to have lower hepatic *Pck1* and *G6P* expression indicating diminished hepatic gluconeogenesis, which was confirmed by hyperinsulinemic-euglycemic clamp method.

To extend the applicability of the findings, the authors crossbred PXR-KO mice with leptin-deficient, obese-prone *ob/ob* mice, termed *obp*. *Obp* mice gained weight similarly than *ob/ob* mice but had less fat, more lean mass and increased energy expenditure. Hepatosteatosis was also alleviated without changes in the liver cholesterol. Replicating the previous results, *obp* mice had better glucose tolerance and improved insulin sensitivity.

To study how PXR activation affects obesity, the authors crossbred *ob/ob* mice with VP-PXR mouse strain (described in Xie et al., 2000), in which PXR gene is fused with viral protein 16 rendering PXR constitutively active, to generate $ob/ob^{VP\text{-}PXR}$ mouse strain. These mice had more triglycerides in the serum and liver probably due to increased lipogenesis and decreased hepatic β-oxidation. Further, $ob/ob^{VP\text{-}PXR}$ showed decreased glucose tolerance and sensitivity to insulin. In conclusion, the paper described PXR deficiency to protect from obesity and

activation to exacerbate obesity-induced metabolic dysfunction. However, the used genetic models were extreme (full knockout or constitutive action) and do not probably resemble human-relevant situations. Of importance, the authors did not exclude the possibility that the difference in weight gain explains the observed metabolic improvements after the HFD in the PXR-KO mice.

A study by Zhao et al. utilized a PXR deficient mouse strain produced in Steven Kliewer's laboratory (described in Staudinger et al., 2001). Similar to He et al., they showed PXR ablation to decrease weight gain and hepatosteatosis on HFD (Zhao, Xu, Shi, Englert, & Zhang, 2017). Interestingly, they also reported PXR-KO mice to have lower total bile acids level in the serum. This was probably caused by induced expression of intestinal FGF15, a regulator of enterohepatic bile acid homeostasis. They further showed alterations in bile acid metabolism caused by PXR deficiency, including differential expression of bile acid synthesizing *Cyp7a1, Cyp8b1* AND *Cyp27a1*. Further, fecal total lipid content was increased possibly indicating increased excretion of lipids.

A third study reported similar findings with PXR-KO mice (from Kliewer's laboratory) to gain less weight on high-fat diet (Spruiell et al., 2014). Interestingly, wildtype and PXR-KO mice had similar amounts of triglycerides in the liver after HFD, but PXR-KO mice had less macrovesicular and more microvesicular steatosis. Macrovesicular steatosis is considered the more harmful form, and in line with this, PXR-KO mice had lower serum ALT activity as a sign of better liver condition. Unlike in the paper by He et al., PXR-KO did not protect against HFD-induced glucose dystolerance and insulin resistance. In line with the He et al. study, PXR-KO mice had less hepatic G6PC and PCK1 proteins after the HFD.

Another study investigated long-term treatment of mice on HFD with PCN (Y. Ma & Liu, 2012). In this study, PCN treatment decreased food intake and weight gain of AKR/J mice during the 7-week treatment period. Reduced weight gain reflected to glucose tolerance and insulin sensitivity, improving both. Surprisingly, PCN treatment also decreased hepatic steatosis. These results are in contradiction with He et al., but, as He et al. mentons, Ma and Liu (2012) do not exclude the possibility that long-term PCN treatment is toxic which might mean the weight loss to be secondary to hepatotoxicity. Further, Ma and Liu (2012) did not use PXR-KO mice to prove the effects of PCN are mediated by PXR.

Taken together, the reports to date indicate that PXR deficiency protects mice from obesity. However, more sensitive approaches are needed to clarify the suitability of PXR as a druggable target to prevent and/or treat obesity. In addition, metabolic consequences of PXR activation in obesity have only been studied in

mice expressing constitutively active PXR (He et al., 2013) and by administering mice a high, possibly toxic, amount of PCN for a long time (Y. Ma & Liu, 2012). Thus, more studies are needed to fill in the gaps regarding the function and regulation of PXR in obesity.

3 Aims of the present study

Accumulating evidence links chemical exposure to increased risk of common metabolic diseases, but the mechanisms are poorly understood. Nuclear receptor PXR, a sensor of xenobiotics and a regulator of their metabolism, has been shown to induce liver fat accumulation, impair postprandial glucose tolerance and modulate cholesterol homeostasis. Thus, PXR may explain some harmful metabolic effects of chemical exposure. PXR interacts with multiple cellular proteins which represent central nodes of energy metabolism, and which also either mediate or are affected by obesity and metabolic diseases. Thus, PXR function might be regulated by metabolic status, such as fasting. Importantly, activation of PXR might impose differential response on glucose and lipid metabolism in obesity. Therefore, this thesis aims to answer the following questions:

1. Does fasting affect PXR function?
2. What are the metabolic consequences of PXR activation in obesity?
3. Does obesity affect the function of PXR in transcriptional regulation?

4 Materials and methods

The materials and methods are described here in brief. The full and detailed descriptions can be found in the original articles and the manuscript.

4.1 Animal experiments

Mouse experiments were performed on male C57BL/6N mice and were approved by the National Animal Experiment Board of Finland (license numbers ESAVI/6357/04.10.07/2014, ESAVI/8240/04.10.07/2017 and ESAVI/23252/2020). During the experiments, mice were housed in individual cages with a 12-hour light cycle at the Oulu Laboratory Animal Centre, University of Oulu, Finland. Mice had *ad libitum* access to food and water unless otherwise stated.

4.1.1 PXR knockout mouse strain

PXR-KO mouse strain was a kind gift from Dr. Wen Xie (University of Pittsburgh School of Pharmacy, Center for Pharmacogenomics, PA, USA). The generation of the mouse strain has been described before (Xie et al., 2000). In short, the second and third exons of the *Pxr* gene in C57BL/6J mice were replaced by a Neo cassette to abolish the gene expression. Before the experiments, the mouse strain was backcrossed in the University of Oulu Laboratory Animal Centre, Oulu, Finland, to C57BL/6N strain for at least six generations to match the wildtype mice.

4.1.2 PCN and vehicle treatments

To activate PXR, mice were treated with intraperitoneal injections of 50 mg/kg PCN or with vehicle (30% dimethyl sulfoxide in corn oil) for four days once a day.

4.1.3 Studies on lean mice

For the animal experiments in I, eight-week-old wildtype mice were treated with PCN or vehicle for four days. Mice were either fasted for five hours and sacrificed or fasted for four hours and then administered glucose (2g/kg) by oral gavage one hour before sacrifice (Figure 10.). After the sacrifice, livers were collected in liquid nitrogen and used for gene expression profiling. Vehicle-treated and fasted group had eight mice and the other groups had seven. The experiment was repeated with

PXR-KO mice with four mice per group. Bile acids were determined from wildtype mice, six mice per group, that were fasted for 12 hours before the one-hour glucose treatment. In addition, the effect of glucose was studied in a separate setting with seven mice per group where mice were either fasted for five hours or fasted for four hours and treated with glucose for one hour before the sacrifice.

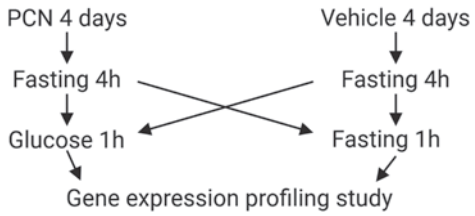

Fig. 10. The experimental setup of I.

In II, hepatic steatosis was evaluated from eight-week old wildtype mice which were treated with PCN or vehicle for four days. Mice were fasted for 12 hours and subjected to oral glucose tolerance test (OGTT), after of which the livers were snap-frozen in liquid nitrogen or fixed with formalin.

4.1.4 High-fat diet and metabolic tests

Obesity was induced by feeding six-week old wildtype or PXR-KO mice a high-fat diet (60% of calories from fat; Envigo td.0644) for 14-18 weeks. The lean control group was kept on a regular chow diet (Envigo td.2018). PCN treatments were performed on four consecutive days, as described before for lean mice. The overview of the setting of HFD studies is presented in Figure 11.

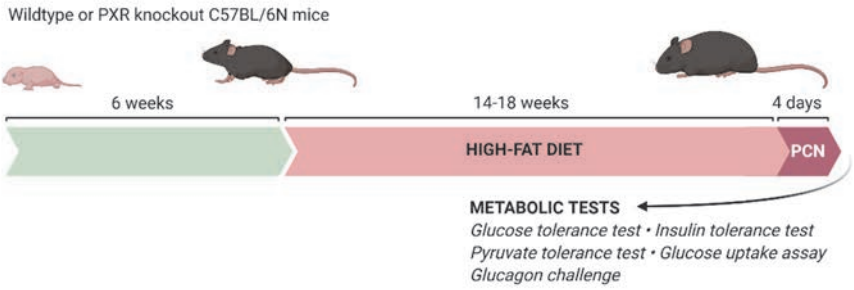

Fig. 11. Overview of the HFD study setup.

Oral glucose tolerance tests

Main laboratory analyses for II and III, such as the transcriptomic analyses of the liver, adipose and muscle, hepatosteatosis determination and analysis of the markers of cholesterol synthesis, were done from samples collected after an oral glucose tolerance test (OGTT). Wildtype and PXR-KO mice were kept on regular chow or on high-fat diet for 15 and 18 weeks, respectively. After 4-day PCN treatments, mice were fasted for 12 hours overnight and wildtype mice were anesthetized with midazolam/fentanyl-fluanisone. PXR-KO mice were not anesthetized. 2 g/kg of glucose was administered to mice orally and blood glucose was measured from hind leg veins in the wildtype mice and from the tail tip in the the PXR-KO mice with a glucometer at 0, 15, 30, 60, 90, 120 minute timepoints. After the last time point, mice were sacrificed via carbon dioxide inhalation and cervical dislocation. Tissues were collected and snap-frozen in liquid nitrogen or fixed with formalin.

Pyruvate and insulin tolerance tests

6-week old male wildtype mice were kept on regular chow or on HFD for 14 weeks and 4-day PCN and vehicle treatments were performed. Pyruvate tolerance test was performed by fasting the mice 12 hours overnight, administering 2g/kg of pyruvate intraperitoneally and measuring blood glucose from the tail tip at 0, 15, 30, 60, 90 and 120 minute timepoints. Mice were not sacrificed, but the diets were continued for two weeks and PCN and vehicle treatments were repeated to the same animals. Then, mice were fasted for six hours and insulin (1U/kg) was administered intraperitoneally. Blood glucose was determined from the tail tip at 0, 15, 30, 60, 90 and 120 minutes timepoints. Then mice were left to recover for two hours, insulin was readministered and after 15 minutes the mice were sacrificed and tissue samples collected to liquid nitrogen or formalin. The samples were used to investigate insulin response in the tissues.

Glucagon challenge

Male wildtype mice were fed HFD for 14 weeks. After a five-hour fast, mice were given somatostatin (7 mg/kg) intraperitoneally 15 minutes prior to subcutaneous administration of glucagon (20 µg/kg). Blood glucose was measured from tail tip at 0, 15, 30, 60, 90 and 120 minutes timepoints.

4.1.5 Glucose uptake assay

6-week old male wildtype mice were fed HFD for 14 weeks and treated with PCN or vehicle for four days. Mice were fasted 12 hours overnight and 2g/kg of glucose 0,7 µCi/kg of ^{3}H-2-deoxyglucose were administered orally. One hour after glucose administration mice were sacrificed and tissues collected. 2-deoxyglucose was extracted by homogenizing the tissues in three-fold volume of chloroform:methanol with TissueLyser II (Qiagen) and centrifuged. The water phase was scintillated for ^{3}H activity. To isolate 2-deoxyglucose-6-phosphate, the water phase was dried, resuspended in water and an aliquot subjected to $Ba(OH)_2/ZnSO_4$ precipitation which separates 2-deoxyglucose from the phosphorylated 2-deoxyglucose.

4.2 Histological analyses

5 µm thick liver or adipose tissue sections were subjected to hematoxylin-eosin (HE) staining with Mayer's hematoxylin. Lipid droplet areas were quantified from HE-stained liver sections with VisioPharm software after scanning the slides with Nanozoomer S60. Adipose tissue cell size was quantified by measuring cell areas of 50 biggest cells per slide with Adobe Photoshop.

10 µm thick frozen liver tissue sections were subjected to Oil Red O (ORO) staining to stain neutral lipids. ORO-stained areas were quantified with quPath program and microvacuolar and macrovacuolar steatosis determined based on sizes of the lipid droplets.

In liver transmission electron microscopy (TEM), thin sections were imaged with Tecnai G2 Spirit 120 kV with Veleta and Quemesa CCD cameras in the Biocenter Oulu Tissue Imaging Center, University of Oulu, Finland, after staining the tissues with potassium ferrocyanide to visualize the glycogen.

4.3 Primary mouse hepatocytes

Primary mouse hepatocytes were isolated from eight-week old C57BL/6N male mice with two-step collagenase method. Mice were terminally anaesthetized with a mixture of medetomidine and ketamine. The first perfusion solution with EDTA passed the liver from the superior *vena cava* to the portal vein. The second perfusion solution contained collagenase. Perfused liver was carefully suspended and centrifuged to separate heavy hepatocytes from other hepatic cells.

Hepatocytes were plated in William's Medium E containing 10% fetal bovine serum supplemented with dexamethasone, gentamycin and insulin-transferrin medium supplement which was changed to serum-free medium after the cells had attached.

In I, cells were treated with 10 µM PCN or 0.1% DMSO for 21 hours. Then, cells were subjected to different concentrations of glucose, insulin or glucagon and PCN and DMSO treatments were continued for 24 hours. Cells were harvested for RNA isolation.

4.4 mRNA analyses

4.4.1 Quantitative PCR

Total RNA was extracted and complementary DNA prepared with RNAzol RT (Sigma) or RNeasy lipid Tissue Mini kit (QIAGEN) and RevertAid Reverse Transcriptase (Thermo Scientific). Quantitative PCR (qPCR) reactions were set up with FastStart universe SYBR Green Master Mix (Roche) or with PowerUp SYBR Green Master Mix (Thermo Scientific). Fold change = $2^{-\Delta Ct\ sample}/2^{-\Delta Ct\ control\ sample}$. Primer sequences and optimized reaction concentrations are listed in table 2.

Table 2. Primers used in quantitative PCR reactions.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Concentration	Used in
18S	cgccgctagaggtgaaattc	ggtatttgctacggctgacc	100 nM	I
Abcb1a	catcagccctgttcttggac	tccccagccttttagcttctt	300 nM	III
Abcg5	gcgagacgttgcgataca	ctgccaatcatttggtcc	300 nM	III
Abcg8	ataccctggaggtctcatagca	acgtcgagtagtgaggctctc	300 nM	III
Acc	atgggcggaatggtctctttc	tggggaccttgtcttcatcat	300 nM	II, III
Acly	acccttttcactggggatcaca	gacagggatcaggatttccttg	300 nM	II, III
Adipoq	agctagctcctgctttggtc	ttcagctcctgtcattccaa	300 nM	II
Alas1	tcgccgatgcccattcttatc	tctactaccttcaaccccgg	100 nM	I
Apob	gctcaactcaggttaccgtga	agggtgtactggcaagtttgg	300 nM	III
Ccl2	gatcccaatgagtaggctgg	ctcttgagcttggtgacaaa	300 nM	II
Cd36	atgggctgtgatcggaactg	tttgccacgtcatctgggttt	100 nM	II
Cpt1a	agatcaatcggaccctagacac	cagcgagtagcgcatagtca	300 nM	II
Cyp2a5	ggacaaagagttcctgtcactgcttc	cctgaagtattggttctttcaccttgtg	300 nM	I
Cyp2b10	aaagtcccgtggcaacttc	aacgacagcaactcggtt	100 nM	I
Cyp3a11	gacaaacaagcagggatggac	ccaagctgattgctaggagca	300 nM	II, III

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Concentration	Used in
Cyp3a13	gacgattcttgcttaccagaagg	caatgagatggaagtgtttggcc	300 nM	I
Cyp4a12a	cctctaatggctgcaaggcta	tctaccctgaagatagtggacc	300 nM	I
Cyp4a12b	ggggagatcagacccaaaagc	taccaaagtcgtggctgctta	100 nM	I
Cyp51	ggagcgaaaagtccaccac	gcatcactccccagaaggta	100 nM	III
Cyp8b1	cctctggacaagggttttgtg	cccctacagaagtgccacg	100 nM	I
Elovl6	gaaaagcagttcaacgagaacg	agatgccgaccaccaaagata	300 nM	II
Fasn	gaggtggtgatagccggtat	tgggtaatccatagagcccag	100 nM	II, III
Fdps	ggaggtcctagagtacaatgcc	aagcctggagcagttctacac	300 nM	III
G6pc	cgactcgctatctccaagtga	gggcgttgtccaaacagaat	100 nM	II
Gapdh	ggtcatcatctccgcccc	ttctcgtggttcacacccatc	100 nM	II, III
Gsta1	tgttgaagagccatggacaa	atccatgggaggctttctct	300 nM	II, III
Hmgcr	agagcgagtgcattagcaaag	gattgccattccacgagcta	300 nM	III
Hmgcs2	agagagcgatgcaggaaactt	aaggatgcccacatcttttgg	300 nM	II
Insig1	tagtgctcttctcatttggcg	agggatacagtaaaccgacaaca	300 nM	III
Insig2	taaatcacgccagtgctaaagt	ggtgacaacggttgctaagaaag	300 nM	III
Insig2a	cctcaatgaatgtactgaaggatt	tgtgaagtgaagcagaccaat	100 nM	III
Lcn2	tggccctgagtgtcatgtg	cgtggtagatactcgatgttctc	100 nM	I
Ldlr	tcagacgaacaaggctgtc	catctaggcaatctcggtctc	300 nM	III
Lep	tggcctgaggagcagg	gggcggaacttagcca	300 nM	II
Lpin1	aagagactgacaacgatcagga	ttccccagagaaccagtggat	300 nM	II
Mttp	aatgcgggtcaacagagag	ctggctcgttttcataggagtag	400 nM	III
Npc1l1	cgcccttctttctacatgggt	gaatctgcgcttacgagggag	300 nM	III
Osgin1	cctccggtatctgcctgtc	ggtcctggatctcatggaaagg	100 nM	I
Pcsk9	cccatcgggagattgag	ttcccttgacagttgagca	300 nM	III
Pck1	agcattcaacgccaggttc	cgagtctgtcagttcaataccaa	100 nM	II
Pln5	cttcctgcccatgactgag	gaccccagacgcacaaagta	300nM	II
Pparg	ctccaagaataccaaagtgcga	cctgatgctttatccccaca	100 nM	II
Pxr	gaagctacagctgcataaggag	gccatgatcttcaggaacagga	100 nM	III
Retn	acaagacttcaactccctgtttc	tttcttcacgaatgtcccac	300 nM	II
Scd1	ttcttgcgatacactctggtgc	cgggattgaatgttcttgtcgt	300 nM	II, III
Socs2	agttcgcattcagactacctact	gattggacgcctaactcatggt	300 nM	I
Srebp1	gcagccaccatctagcctg	cagcagtgagtctgccttgat	300 nM	II, III
Srebp1a	cctgcagaccctggtgagt	agaagaccggtagcgcttct	300 nM	II
Srebp1c	cacagccgtgcagacc	ttgatagaagaccggtagcgc	300 nM	II
Srebp2	tgggcgatgagctgactct	caaatcagggaactctcccac	300 nM	III
Tbp	gaatataatcccaagcgatttg	cacaccatttttccagaactg	200 nM	II, III
Tnf-α	ggtgcctatgtctcagcct	atgagagggaggccatttg	100 nM	II

4.4.2 Microarray

Total RNA from mouse livers was extracted with RNAzol RT (Sigma Aldrich) coupled with RNeasy kit (Qiagen). RNA Integrity Number (RIN) was determined with QIAxcel (Qiagen) and was >7.0 for all samples. RNA was amplified and labelled with Illumina TotalPrep RNA Amplification Kit (Life Technologies) and quality of cRNA was controlled on 21000 Bioanalyzer RNA nano chips (Agilent Technologies). MouseWG-6 v2.0 Expression Beadchips (Illumina) were used in the Direct Hybridization Assay Workflow (Illumina). Chips were scanned with HiScan Instrument (Illumina). Microarray was carried out at the Core Facility of the Estonian Genome Center, University of Tartu, Estonia. Illumina Beadstudio and GeneSpring GX 14.5 (Agilent Technologies) softwares were used to obtain and process the data. Genes were defined as differentially expressed when fold change >1.5 fold and statistically significant ($p < 0.05$, One-Way ANOVA and Tukey's post hoc test). Microarray data sets are available in the NCBI's Gene Expression Omnibus (GEO) database with the accession number GSE125695.

4.4.3 RNA sequencing

Liver, muscle and adipose tissue RNA was extracted with RNAzol RT (Sigma) or with RNeasy Lipid Tissue mini kit (ThermoFisher Scientific). Residual genomic DNA was removed from the samples with RNase-Free DNase Set combined with RNeasy MinElute Cleanup Kit (both QIAGEN). RNA quality and concentration were determined and cDNA libraries were prepared with TruSeq Stranted Total RNA with Ribo-Zero Gold kit (Illumina). Sequencing runs were done with NextSeq550 instrument (Illumina) in the Biocenter Oulu Sequencing Center, University of Oulu, Finland.

Data was analysed with Chipster software (Kallio et al., 2011). Raw reads were aligned to mouse Ensembl reference genome GRCm38 and reads were counted with HISAT2 and HTSeq, respectively. Differentially expressed genes were determined with DESeq2.

Pathway analyses for differentially expressed genes were performed with Ingenuity Pathway Analysis (IPA) software (QIAGEN) which uses Fischer's exact test to score p-values of overlap for pathway enrichment and upstream regulator analyses (Krämer, Green, Pollard, Tugendreich, & Tugendreich, 2014).

Complete RNA sequencing data sets are available in Gene Expression Omnibus (GEO) database with accession numbers GSE136667 (wiltype liver),

GSE162196 (PXR-KO liver), GSE168197 (WAT) and GSE168196 (skeletal muscle).

4.5 Western blot

Antibodies used in western blot are presented in table 3. Mouse liver and muscle total proteins were extracted by homogenizing tissue pieces in lysis buffer supplemented with protease and phosphatase inhibitors with TissueLyzer II (Qiagen). Homogenates were centrifuged and supernatant protein concentration determinated with Bio-Rad Protein Assay.

Table 3. Western blot antibodies.

Antigen	Antibody	Dilution	Producer	Catalog nr	Used in
Akt	Rabbit polyclonal	1/1000	Cell Signaling Technology	9272	II
β-actin	Mouse monoclonal	0.04 µg/ml	Sigma Aldrich	A1978	II, III
DHCR24	Rabbit polyclonal	1/1000	Cell Signaling Technology	2033	III
GAPDH	Mouse monoclonal	1/10 000	Merck Millipore	MAB374	II
HMGCR	Rabbit polyclonal	1 µg/ml	Santa-Cruz	sc-271595	III
INSIG1	Rabbit polyclonal	0.5 µg/ml	Abcam	ab70784	III
IRS1	Rabbit polyclonal	1/900	Thermo Fisher Scientific	PA1-1059	II
Mouse IgG	Goat monoclonal conjugated to IRDye®800CW	0.04 µg/ml	LI-COR	926-32211	II, III
p-Ser235/236-S6	Rabbit polyclonal	1/1000	Cell Signaling Technology	2211S	II
p-Ser1101-IRS1	Rabbit polyclonal	1/500	Thermo Fisher Scientific	PA1-1054	II
p-Ser347-Akt	Rabbit polyclonal	1/1000	Cell Signaling Technology	9271	II
Rabbit IgG	Goat monoclonal conjugated to IRDye®680RD	0.04 µg/ml	LI-COR	926-68080	II, III
S6	Mouse monoclonal	1/1000	Cell Signaling Technology	2317	II
SREBP1	Rabbit polyclonal	0.5 µg/ml	Abcam	ab28481	III
SREBP2	Rabbit polyclonal	0.4 µg/ml	Abcam	ab30682	III

Liver cytosolic and nuclear protein fractions were isolated by homogenizing tissue pieces in phosphate buffer followed by nuclei extraction based on the differential properties of plasma and nuclear membranes. The fraction protein content was determined with Bio-Rad Protein Assay.

Liver microsomal fractions were isolated by homogenizing livers in phosphate buffer followed by sequential centrifugation. Protein content was determined with Bio-Rad Protein Assay.

Protein samples were separated with SDS-PAGE and transferred to nitrocellulose membranes with Trans-Blot Turbo RTA Mini Nitrocellulose Transfer Kit (Bio-Rad) and Trans-Blot Turbo (Bio-Rad). Membranes were blocked with Amersham ECL Prime Blocking Agent (GE Healthcare). Primary antibody incubations were performed overnight in +4°C. Secondary incubations were performed in room temperature for one hour and membranes were imaged with Odyssey Fc (LI-COR). Antibodies used in western blot are presented in table 3.

4.6 Commercial kits

4.6.1 Lipid analyses

Total plasma cholesterol in I was determined with Cholesterol Quantitation Kit (Sigma). Total plasma bile acids in III were determined with Mouse Total Bile Acids Assay kit (Crystal Chem). Liver and plasma triglycerides in II were determined with Triglyceride Quantification Kit (Sigma Aldrich).

4.6.2 Liver glycogen

Glycogen was extracted from mouse livers by dissolving liver pieces in 30% KOH and precipitating glycogen with ethanol. Glycogen was solubilized in water and glycogen content per gram of tissue determined with Glycogen Assay kit (Sigma Aldrich).

4.6.3 Plasma protein analyses

Plasma adiponectin, leptin and resistin were determined with Novex mouse ELISA kits KMP0041, KMC2281 and EMRETN (Invitrogen), respectively. Insulin was determined with Insulin Mouse Serum Assay (Cisbio 62IN3PEF). Plasma ALT activity from wildtype and PXR-KO mouse samples was determined with ALT activity Assay kit (Sigma Aldrich). PCSK9 protein from human and mouse plasma samples was determined with Human PCSK9 ELISA kit (Abcam ab209884) and

Mouse PCSK9 ELISA kit (Abcam ab215538). Circulating ApoB48 was measured with Human ApoB48 ELISA Kit (Elabscience).

4.7 NMR and mass spectrometric analyses

In I, CA, CDCA, DCA and T-MCA acid were quantified from deproteinised plasma samples with Aquity UPLC system (Waters) connected to Synapt G1 Q-TOF mass spectrometer (Waters) in Biocenter Oulu Proteomics and protein analysis core, University of Oulu, Oulu, Finland.

In III, serum samples from Rifa-1 and Rifa-BP (n=34) were subjected to analysis with an NMR metabolomics platform which quantifies routine lipids, lipoprotein subclass distributions, fatty acids and other low-molecular weight metabolites, such as amino acids and ketone bodies in absolute concentrations (Soininen et al., 2009). Serum lathosterol was quantified from Rifa-BP samples with two-dimensional gas chromatography coupled with quantitative time-of-flight mass spectrometry (described in Castillo et al., 2011). From Rifa-1 samples, 4-β-OH-cholesterol was measured with a previously published gas chromatography-mass spectrometry method (Hukkanen, Puurunen, et al., 2015) and from Rifa-BP samples 4-β-OH-cholesterol and 4-α-OH-cholesterol was measured with a liquid chromatography-electrospray-high-resolution mass spectrometry method (Hautajärvi, Hukkanen, Turpeinen, Mattila, & Tolonen, 2018). Mouse plasma and liver cholesterol and non-cholesterol sterols were analysed with a method using gas-liquid chromatography and flame ionization detection (Miettinen, Tilvis, & Kesäniemi, 1989). The concentrations of non-cholesterol sterols were adjusted to cholesterol as ratios to cholesterol to enable their comparison between samples with different cholesterol levels.

4.8 Human studies

In III, human samples from three previously published studies examining metabolic effects of rifampicin were utilised. In these studies, healthy volunteers were administered rifampicin once a day for a week. Rifa-1 (Rysä et al., 2013) studied postprandial glucose tolerance, Rifa-2 (Hukkanen, Rysa, et al., 2015) explored incretin secretion and Rifa-BP (Hassani-Nezhad-Gashti et al., 2020) blood pressure with 12, 12 and 22 participants, respectively. Characteristics of the study subjects are summarized in table 4. By design, Rifa-1 and Rifa-BP were randomized placebo-controlled crossover studies with a four-week washout period between

study arms. Rifa-2 had a one-arm design with no control arm. Rifa-1 and Rifa-2 were not blinded, and Rifa-BP had blinded study personnel. The human studies followed the ethical standards of the Declaration of Helsinki and guidelines of Good Clinical Practice. The Finnish Medicines Agency Fimea and the Ethics Committee of the Northern Ostrobothnia Hospital District (Oulu, Finland) approved the studies (decision numbers 78/2009 for Rifa-1, 73/2010 for Rifa-2, and 6/2012 for Rifa-BP).

Table 4. Characteristics of the study subjects in the human studies. Values are represented as mean ± standard deviation.

Study	Rifa-1	Rifa-2	Rifa-BP
Design	Randomized, crossover, open, placebo-controlled	One-arm study (rifampicin only), open	Randomized, crossover, single-blinded, placebo-controlled
Subjects	Healthy Caucasian volunteers	Healthy Caucasian volunteers	Healthy Caucasian volunteers
Number of subjects	12	12	22
Sex	3 women, 9 men	6 women, 6 men	8 women, 14 men
Age	24 ± 5.2 years	23 ± 3.5	24 ± 3.5 years
Weight	73 ± 10.8 kg	65 ± 7.6 kg	71 ± 10.9
Body mass index	24.0 ± 2.8	22.5 ± 2.2	23.8 ± 2.2

5 Results

5.1 Nutritional status modifies the transcriptomic PXR activation response

Multiple factors that regulate cellular metabolism in response to energy status interact with or are influenced by PXR (Pavek, 2016). To examine the effect of nutritional status on PXR regulated transcriptome, hepatic transcriptome was studied in PCN-treated mice which were either fasted or the fast was broken by giving glucose one hour before sacrificing the mice (Figure 10.).

5.1.1 PCN elicits pronounced transcriptomic response in fed state

Livers of three mice per experimental group were subjected to gene expression profiling by microarray. PCN regulated 189 genes during fasting and 1203 after glucose load (I; Figure 1A. & IB.) Differentially expressed genes were analysed with IPA software to investigate cellular pathway enrichment and activation. In fasted mice, top cellular pathways affected by the PCN treatment were pathways containing drug-metabolising enzymes (I; Figure 1C.). The analyses showed similar results for mice after glucose (I; Figure 1D.) with few exceptions. In mice fed with glucose, top pathways affected by PCN treatment included some pathways involved in bile acid, lipid, and energy homeostasis (I; Figure 2.). Although cholesterol biosynthesis seemed to be activated in glucose-treated mice by PCN, plasma cholesterol level was not affected by PCN in any groups (I; Supplementary Figure 1.).

The top ten upregulated genes by PCN were mainly involved in drug metabolism in both, glucose-fed and fasted mice but fold changes were bigger in glucose-fed mice (I; Figure 3.) suggesting fed state to fortify PCN-mediated gene induction.

5.1.2 PXR activation affects gene expression in a target-gene specific manner in fasted and fed states

The gene response of individual genes to PCN treatment depended on the nutritional status. The most differently affected genes by PCN in fasted and glucose-fed mice were identified and involved several drug metabolizing enzymes

(I, Table 1.). Genes were categorized based on how they respond: genes of which regulation by PCN was potentiated by glucose (e.g. *Cyp3a11, Cyp2b10*), genes which were induced similarly by PCN in fasted and fed state (e.g. *Alas1*) and genes which were regulated in opposite directions by PCN in fasted and fed states (e.g. *Cyp8b1, Cyp4a12, Osgin1*). qPCR analyses of selected genes in bigger data sets and in PXR wildtype and KO animals confirmed the findings of the gene expression profiling and showed the changes to be dependent on PXR (I, Figure 4.).

5.1.3 PCN modulates bile acid synthesis and bile acid pool composition

The most differentially regulated gene by PCN in fasted and fed states was *Cyp8b1*, which was repressed by PCN in the fasting mice and induced in glucose-fed mice (I, Table 1.). Other genes involved in bile acid synthesis did not show similar pattern of regulation. PCN downregulated *Cyp7a1* and *Cyp7b1*, which catalyse the initial steps in the classical and the alternative bile acid synthesis pathways, in fasted and fed states (Chiang, 2017) (I; Supplementary Table 4.). *Cyp27a1*, which is involved in both pathways of bile acid synthesis, was only induced by PCN in the glucose-fed mice. Bile acid synthesis pathways are presented in I, Figure 7A. Bile acid transporters were not majorly regulated by PCN, except efflux transporter *Abcc3*, which was induced by PCN but only in glucose-fed mice.

To clarify, if the regulation of genes involved in bile acid synthesis manifests in bile acid homeostasis, total and individual plasma bile acids and total gallbladder bile acids were measured from mice which were treated with PCN or vehicle for four days, fasted overnight and were given glucose one hour before sacrifice. In line with the repression of *Cyp7a1* and *Cyp7b1*, mice treated with PCN trended to have lower plasma total bile acids and total bile acids in gallbladder were reduced with statistical significance (I; Figure 7.).

Cyp8b1 directs bile acid synthesis to cholic acid (CA), which plasma level trended to be higher in PCN-treated mice despite the lower total bile acids. When CA is formed, less CDCA will form, and accordingly there was less CDCA in the plasma of the PCN-treated mice. In accordance with the lower CDCA, T-MCA and DCA levels were also lower in the PCN-treated mice, although statistical significance was not reached. Altogether, these data show that PCN treatment with glucose feeding suppresses bile acid synthesis and directs the synthesis to create cholic acid instead of chenodeoxycholic acid.

5.2 PXR dissociates hepatosteatosis and insulin resistance from glucose tolerance by increasing hepatic glucose uptake

Obesity-induced metabolic dysfunction associates with a dysregulation of the same pathways that are strictly regulated by fasting (DeFronzo et al., 2015). As fasting affected PXR activation response, the next aim was to investigate the effect of PXR activation on glucose and lipid metabolism in obese and metabolically ill mice.

5.2.1 Aggravated hepatosteatosis in obese mice after PXR activation

Obesity was induced by feeding wildtype and PXR-KO mice with a high-fat diet (HFD) and PXR was activated by a four-day PCN treatment. One experimental group was kept on regular chow diet to control the effects of the HFD. Nontreated HFD-fed mice were compared to mice on regular chow; mice fed HFD and treated with PCN were compared to HFD-fed mice treated with vehicle control.

Wildtype and PXR-KO mice gained weight similarly during the HFD (II; Figure 1A). Vehicle or PCN treatments did not affect the weight, but PCN doubled the liver size of the wildtype mice (II; Figure 1B.). In PXR-KO mice, PCN did not affect the liver size (II; Figure 1B.).

Both, HFD and PXR activation, are known to induce hepatosteatosis (Bitter et al., 2015; He et al., 2013; J. Zhou et al., 2006). In this study, steatosis was determined by quantifying lipid droplet areas from liver sections stained with hematoxylin-eosin (HE) and neutral lipid stain Oil-Red O. The HFD induced steatosis in both genotypes to a similar extent although statistical significance was not reached in the PXR-KO mice due to high group variation (II; Figure 1C., 1D. & 1D.).

PCN treatment dramatically induced hepatosteatosis but only in the HFD-fed wildtype mice (II; Figure 1C., 1D. & 1E). Similar PCN treatment did not induce steatosis in lean wildtype mice (II; Supplementary Figure 1A.) In line with lipid droplet areas, liver triglycerides were induced by the HFD and PCN (II; Figure 1F.). Of interest, the vehicle treatment nearly totally abolished fat from the liver. Although steatosis and triglycerides were reduced by the vehicle treatment, PCN-treated mice had the highest group average (II; Figure 1C., 1D, 1E. & 1F.). As an additional indication of the involvement of PXR in the observed steatosis, *Cyp3a11* expression, a direct target gene of PXR (Kliewer et al., 1998), correlated with the lipid droplet area in the PCN-treated mice (II; Figure 1G.).

Plasma triglycerides were not affected by the HFD or PCN treatment (II; Supplementary Figure 1B.). Plasma ALT activity was increased by the HFD in both genotypes and by PCN in the wildtype mice, showing obesity to be harmful for liver health and PXR activation to aggravate the situation (II; Figure 1H).

PXR-induced liver fat does not stem from the diet or the adipose tissue

Lipids accumulate in the liver by increased acquisition (uptake and synthesis) or by decreased disposal (β-oxidation and VLDL secretion) (Ipsen et al., 2018). To test if the HFD *per se* sensitizes mice to PCN-induced steatosis, HFD was fed to mice only for two weeks, a time in which obesity does not develop, before PCN and vehicle treatments. In this setting, the PCN treatment did not induce visible steatosis (II; Supplementary Figure 1C.) indicating that obesity or the combination of obesity and HFD is needed to sensitize the mice for PXR-induced steatosis.

In obesity-induced metabolic dysfunction, increased secretion of cytokines and fatty acids from the adipose tissue contribute to the development and progression of hepatosteatosis (Donnelly et al., 2005). In this study, the HFD feeding increased adipocyte size, while vehicle or PCN treatments did not further affect adipocyte size (II; Supplementary Figure 2A.). Adipose tissue was further investigated by gene expression profiling, which expectedly suggested the HFD to majorly alter adipose tissue transcriptome compared to chow (~10 000 differentially regulated genes). However, PCN did not alter gene expression with statistical significance due to high variation in samples. If differentially expressed genes were identified based on fold changes only, upstream regulator predictions done with the IPA software suggested PCN to activate inflammatory mediators and increase inflammation (II; Supplementary Figure 2A.). qPCR analysis of full sample sets confirmed HFD and PCN to increase *Tnfa* and *Ccl2* expression (II; Supplementary Figure 2C. and 2D.), indicative of increased inflammation. Of note, HFD increased *Tnfa* and *Ccl2* to the same extent both in the wildtype and in the PXR-KO mice (II; Supplementary Figure 2D). Although the adipose seemed more inflamed, PCN treatment did not alter the expression or secretion of metabolically important adipokines adiponectin, leptin and resistin, like the HFD did (II; Supplementary Figure 2E and 2F).

5.2.2 PXR-induced hepatosteatosis is dissociated from glucose tolerance

Hepatosteatosis associates strongly with disturbed glucose metabolism and PXR activation impairs glucose tolerance in mice and humans (Asrani et al., 2019; Hassani-Nezhad-Gashti et al., 2018; Rysä et al., 2013). To evaluate how PXR activation affects glucose metabolism in obese mice, oral glucose tolerance test was performed. Wildtype and PXR-KO mice fed HFD had higher blood glucose in all time points (II; Figure 2A.) and higher area-under-curve (AUC) values compared to the chow mice (II; Figure 2C.). Despite the severe hepatosteatosis, PCN treatment decreased blood glucose 15 minutes after glucose administration (II; Figure 2B.) and PCN trended to decrease incremental AUC values (II; Figure 2C.) compared to the mice treated with vehicle. In PXR-KO mice, PCN did not affect blood glucose.

In obesity, glucose tolerance is mainly determined by insulin sensitivity. Therefore, the degree of insulin resistance was next determined by HOMA-IR index, based on fasting glucose and insulin, and insulin tolerance test (ITT). HFD-fed wildtype and PXR-KO mice had increased blood glucose and insulin after a 12-hour fast, which also reflected to HOMA-IR values (II; Figure 2D.). PCN treatment did not affect fasting glucose, insulin, or HOMA-IR.

For ITT, mice were fasted for six hours before insulin administration. HFD-fed mice had higher blood glucose 15 minutes after insulin administration, but it did not affect the AUC values of the test. Interestingly, PCN-treated mice had lower blood glucose before the ITT, after a six-hour fast, (II; Figure 2E.) which also caused the AUC values to be decreased by the PCN treatment (II; Figure 2F.). However, if ITT was plotted as a percentual drop from the initial blood glucose, PCN-treated mice did not differ from the vehicle group (II; Figure 2G.).

5.2.3 PCN increases hepatic glucose uptake overcoming impaired hepatic insulin signalling

To gain further insights into the discrepancy between hepatosteatosis and glucose tolerance, activation of insulin signaling pathway in tissues was studied from mice sacrificed 15 minutes after insulin administration. As with the insulin tolerance test, HFD-fed mice had higher blood glucose 15 minutes after insulin, and mice treated with PCN had lower blood glucose (II; Figure 3A).

Phosphorylation of AKT occurs in response to insulin (DeFronzo et al., 2015). Therefore, AKT phosphorylation was used as a marker for the activation of the insulin signaling pathway. The HFD did not affect total or phosphorylated AKT (pAKT), and pAKT/AKT ratio remained stable (II; Figure 3B). PCN treatment also did not affect total AKT but significantly decreased pAKT and further pAKT/AKT implicating decreased AKT activation. Defects in IRS1 phosphorylation and IRS1 degradation associate strongly with insulin resistance (Hiratani et al., 2005). HFD did not affect IRS1, pIRS1 or pIRS1/IRS1 but PCN treatment decreased total IRS1 and pIRS without a change in pIRS/IRS1 (II; Figure 3C), indicative of impaired insulin signaling.

After a meal, skeletal muscles are major organs of glucose disposal. 15 minutes after insulin administration, HFD did not affect AKT, pAKT or pAKT/AKT in skeletal muscles but PCN treatment tended ($p=0.0607$) to increase pAKT/AKT ratio (II; Figure 3D). To clarify if PCN treatment caused any changes to skeletal muscle function, samples collected after oral glucose tolerance test were subjected to gene expression profiling by RNA sequencing. In these samples, the HFD led to differential regulation of 27 genes but PCN treatment did not lead to differential gene regulation, suggesting that PCN treatment does not affect the transcriptional regulation of the skeletal muscle.

To clarify the roles of individual tissues in glucose clearance after a glucose load, a similar experiment with HFD and PCN treatments was done, but after a 12-hour fast, mice were given glucose which was spiked with ^{3}H-labeled 2-deoxyglucose. 2-deoxyglucose may be phosphorylated but it is metabolically inactive and does not go through glycolysis or pentose-phosphate pathway. Thus, tissue 2-deoxyglucose content reflects glucose uptake. PCN did not affect plasma 2-deoxyglucose at the given timepoint. Also, PCN did not affect liver, muscle, WAT or brown adipose tissue (BAT) 2-deoxyglucose content (I; Figure 3E.). A significant proportion of the liver is vasculature and thus the liver results might be distorted by plasma 2-deoxyglucose. Therefore, hepatic 2-deoxyglucose-6-phosphate was isolated. As an indication of increased glucose uptake, PCN increased the liver 2-deoxyglucose-6-phosphate content (II; Figure 3F.).

5.3 PXR activation suppresses fasting response in obesity

5.3.1 PCN-induced hepatic insulin resistance doesn't affect gluconeogenesis

In T2D and insulin resistance, the inability of insulin to suppress gluconeogenesis in the liver contributes to hyperglycemia (Baron et al., 1987; Samuel & Shulman, 2012). Gluconeogenesis is regulated transcriptionally through *Pck1* and *G6pc*. PXR might be involved in the same process, as PXR activation has been shown to repress *Pck1* and *G6pc* (Kodama et al., 2004). Therefore, *Pck1* and *G6pc* expression was investigated and gluconeogenesis assayed.

The HFD induced hepatic *G6pc* in both, wildtype and PXR-KO (II; Figure 4A). *Pck1* was repressed by the HFD in the wildtype but not in the PXR-KO mice. In line with published literature, PCN treatment repressed both, *G6pc* and *Pck1*, but only in the wildtype mice.

Kidney may compensate for defects in hepatic gluconeogenesis (Cappel et al., 2019) and, indeed, the HFD conversely downregulated and upregulated renal *G6pc* and *Pck1,* respectively (II, Supplementary figure 3.). However, PCN did not affect renal *G6pc* and *Pck1* expressions (II; Figure 2B.).

Pyruvate tolerance test was performed to assay gluconeogenesis. Pyruvate, administered to fasted mice, is rapidly converted to glucose in the gluconeogenesis pathway and, therefore, increase in blood glucose is indicative of gluconeogenesis rate after pyruvate administration. In the pyruvate tolerance test, HFD-fed mice had higher blood glucose 15 minutes after pyruvate administration (II; Figure 4C.) but AUC values did not differ between the mice on HFD or on chow (II; Figure 4D.). PCN-treated mice didn't differ from the vehicle-treated controls, suggesting PXR activation to not affect gluconeogenesis rate despite hepatic insulin resistance.

5.3.2 PCN depletes hepatic glycogen and impairs glucagon response

Transmission electron microscopy (TEM) was utilized to gain further insights into liver function. Imaging the periportal regions revealed PCN to induce smooth ER proliferation and glycogen to be almost totally absent in the livers of PCN-treated mice (II, Figure 5.).

Measurement of hepatic glycogen levels and PAS staining confirmed PCN to deplete hepatic glycogen (II; Figure 6A. & 6B.). To examine if PCN decreases glycogen synthesis, liver glycogen was extracted from the mice which had been

given glucose and 3^{H}-labeled 2-deoxyglucose, as 2-deoxyglucose can be incorporated to glycogen and thus presents a measure to estimate glycogenesis (Colwell, Higgins, & Denyer, 1996). PCN-treated mice trended to have less ^{3}H activity in the glycogen fraction one hour after glucose ingestion (II; Figure 6C.) possibly suggesting decreased glycogen synthesis.

In fasting, glucagon stimulates liver to mobilize glycogen reserves to maintain circulating glucose level stable. To test if glycogen depletion affected glucagon response, mice (HFD-fed, PCN or vehicle-treated) were fasted for 5 hours and were then treated with glucagon. Prior to glucagon administration, mice were primed with somatostatin to inhibit glucagon-induced insulin-secretion from the pancreas. Mice treated with PCN had lower fasting glucose but also lower glucose throughout the test (II; Figure 6D.). The statistical significance remained in the incremental analyses (II; Figure 6E.).

5.4 Transcriptomic effects of obesity and PXR activation in the liver

5.4.1 Differential transcriptomic response to high-fat diet in wildtype and PXR-KO mice

For further mechanistic insights, liver transcriptome was studied with RNA sequencing in both, wildtype and PXR-KO mice. In wildtype mice, 796 genes were differentially regulated by the HFD while in PXR-KO mice the HFD led to differential regulation of only 336 genes, of which 197 (59%) were shared with the wildtype mice (II; Figure 7A.). Thus, the HFD regulated less genes in the PXR-KO than in the wildtype mice.

Genes that were regulated in both genotypes or exclusively in only one genotype were identified and pathway enrichment analyses were performed with the IPA software. Multiple collagen genes and CYP 2A and 4A families were regulated similarly by the HFD in both genotypes (II; Supplementary table 1.). Unique to wildtype mice, HFD widely downregulated CYP enzymes which lead to enrichment of multiple pathways containing CYPs (II, Supplementary table 2.). Unique to PXR-KO mice, HFD enriched cholesterol synthesis pathway, since multiple genes involved in cholesterol biosynthesis were downregulated (II, Supplementary table 3.).

qPCR analyses of central fatty acid uptake and lipogenesis genes were performed for full sample sets of experimental groups fed either chow or HFD to investigate in more detail how the PXR-KO affects fatty acid metabolism. In these analyses, the expression profiles were incredibly similar (II, Figure 7B.) suggesting PXR deficiency to not alter fatty acid uptake or triglyceride synthesis.

The expression of PXR-regulated genes, including several involved in lipogenesis, were further studied with qPCR (II; Figure 7F.). Previous studies have shown PXR to upregulate the expression of fatty acid transporter *Cd36*, fatty acid synthetising *Elovl6* and fatty acid regulators *Pparg* and *Lpin1*, and downregulate the expression of *Cpt1a* and *Hmgcs2,* involved in β-oxidation (He et al., 2013; J. Zhou et al., 2006). PXR-KO didn't affect *Cyp3a11* expression in chow-fed mice. Interestingly, it was only repressed by the HFD in the wildtype mice, indicating PXR to be involved in the repression. *Cd36* expression was lower in PXR-KO mice, although its expression was induced in both genotypes by the HFD. *Elovl6*, *Cpt1a* and *Lpin1* expressions were not affected by the PXR-KO in chow-fed mice. However, the expressions of *Elovl6*, *Cpt1a* and *Lpin1* were exclusively induced in the PXR-KO mice by the HFD. Altogether, HFD-feeding elicits partially different transcriptomic response on PXR-KO mice when compared to wildtype mice. These differential regulations hint to the possible differences in the mechanism behind the observed hepatosteatosis between the genotypes, although the extent of hepatosteatosis was similar in both.

5.4.2 PXR activation regulates distinct gene sets in obese mice

In wildtype mice, PCN treatment led to differential regulation of 401 genes, while only 33 genes were affected in PXR-KO mice, showing PCN to be rather selective PXR ligand (II; Fig. 7A.). Interestingly, the HFD and PCN enriched the same cellular pathways containing CYPS, with the difference that the HFD repressed CYPs and PCN induced them (II; Figure 7C). Seven out of ten the most enriched pathways by the HFD were pathways that mostly consisted of CYPs. In samples from PCN-treated mice, the top 10 most enriched pathways included six which mostly consisted of CYPs. Three pathways, namely *EIF2 Signaling, mTOR Signaling* and *Regulation of eIF4 and p70S6K Signaling*, were enriched due to wide induction of ribosomal proteins.

IPA calculates z-scores, an indication of pathway activation or inhibition, based on the direction of gene regulation and experimental evidence. Z-scores predicted the pathways containing ribosomal proteins, such as *mTOR signalling* and *EIF2*

signalling, to be activated (II; Figure 7C.). Ribosomal protein S6 phosphorylation was immunoblotted to clarify the status of mTOR pathway, which is an important regulator of SREBP1, the major regulator of lipogenesis (Bakan & Laplante, 2012). However, PCN treatment reduced total S6 which also reflected to reduced pS6/S6 ratio (II; Figure 7D.), implicating inhibition of mTOR pathway. In TEM imaging, PCN treatment was observed to induce strong proliferation of smooth ER (II; Figure 5.), which possibly links to the upregulation of the ribosomal protein expression. In addition to CYPs and ribosomal proteins, PCN treatment greatly induced genes of cholesterol synthesis.

PXR activation has been shown to induce hepatic steatosis, and several steatogenic target genes and plausible target genes have been described (Hakkola et al., 2016). The expression of these genes was investigated by qPCR analyses of full sample sets in vehicle and PCN groups in both, wildtype and PXR-KO mice (II; Figure 7E). In accordance with previous reports, PCN induced *Elovl6* and repressed ketogenic β-oxidation regulators *Hmgcs2* and *Cpt1a.* Contradictory to previous findings, *Cd36* was not affected by PCN and *Lpin1* was repressed (He et al., 2013; J. Zhou et al., 2006). PCN did not alter the expression of these genes in the PXR-KO mice.

5.4.3 PXR activation induces hepatic cholesterol synthesis in the obese mice

In the pathway analyses of the liver RNAseq data, *Superpathway of cholesterol biosynthesis* was one of the most enriched pathways by PCN treatment (III; Figure 5B.). RNAseq suggested PCN treatment to induce almost all genes involved in the cholesterol biosynthesis pathway (III; Figure 5C & 5D). In qPCR analyses of full sample sets, PCN induced cholesterol synthesis genes *Hmgcr*, *Fdps* and *Cyp51,* and classical PXR target genes in wildtype but not in PXR-KO mouse livers (III; Figure 5E). 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) catalyses the rate-limiting step of cholesterol biosynthesis in the ER , and its microsomal protein level was confirmed to be induced by PCN, which also suggested increased cholesterol synthesis (III; Figure 5F.).

To shed light on cholesterol biosynthesis and the dietary absorption, cholesterol, intermediates of cholesterol synthesis and plant sterols were quantified from plasma and liver samples (Björkhem et al., 1987; Miettinen et al., 1989). Expectedly, the HFD increased plasma and liver cholesterol compared to mice on chow (III; Figure 4A.). PCN did not affect plasma cholesterol but greatly increased

liver cholesterol (III; Figure 4A.). In cholesterol biosynthesis, Acetyl-CoA is converted to squalene and further to lanosterol. After lanosterol the pathway separates to Kandutsch-Russell and Bloch pathways (III; Figure 4B.). Intermediates of the Kandutsch-Russell pathway, lathosterol and zymostenol, were induced by PCN compared to vehicle group in both, plasma and liver (III; Figure 4C.). An intermediate of Bloch pathway, desmosterol, was not affected by PCN. 24-dehydrocholesterol reductase (DHCR24) is the bifurcating enzyme of these two pathways, and in line with the findings, PCN induced DHCR24 protein (III; Figure 5G.).

PXR is mostly expressed in the liver and in the intestine, and PCN treatment induced *Cyp3a11* also in the intestine (III; Supplementary Figure 3A.). However, the levels of cholesterol absorption markers, plant sterols present in the diet, were not affected by the PCN treatment (III; Figure 4D.). Accordingly, PCN treatment did not affect the gene expression of intestinal regulators of cholesterol absorption, *Npc1l1*, *Mttp* and *ApoB*, while cholesterol efflux transporters *Abcb1a* and *Abcg5* were induced by PCN (III; Supplementary Figure 3B. & 3C.). In summary, PCN treatment did not affect cholesterol absorption from the diet.

PXR regulates some genes which synthesize oxysterols and bile acids from cholesterol, and cholesterol metabolism to bile acids present a significant route of cholesterol disposal. However, *Cyp7a1*, the rate-limiting enzyme of bile acid synthesis and a PXR target gene (T. Li & Chiang, 2005), was not repressed by PCN in a statistically significant manner (III; Figure 4F.). Also, plasma total bile acids were not affected by the HFD or the PCN treatment (III; Figure 4E.). Further, target genes of farnesoid X receptor (FXR), the main sensor of bile acids, were not differentially regulated by the PCN treatment (III; Supplementary Figure 5C.) showing that increased cholesterol synthesis did not affect the bile acid homeostasis.

5.4.4 PXR defines a novel pathway stimulating SREBP2

Upstream regulator predictions based on liver RNAseq data suggested PCN treatment to activate PXR but also central regulators of cholesterol synthesis, SCAP and SREBP2, and inhibit INSIG1 (III; Figure 6A.) (Ye & DeBose-Boyd, 2011). INSIG1 is a negative regulator of SREBP2 and its inhibition by PCN treatment agrees with induced cholesterol synthesis. However, INSIG1 inhibition is an unexpected finding, as *Insig1* expression has been shown to be induced by PXR activation (A. Roth, Looser, Kaufmann, Blättler, et al., 2008). qPCR of full sample sets confirmed PCN to induce *Insig1* expression in the livers of wildtype but not

PXR-KO mice (III; Figure 6B.). In line with the SREBP2 activation, *Insig1* induction did not lead to increase in INSIG1 protein (III; Figure 6D.). SCAP senses sterols and is activated by decreasing ER sterol levels. SCAP activation leads to proteolytical cleavage of SREBP2, which then translocates to the nucleus to induce gene expression. This way SCAP and SREBP2 form a regulatory feedback loop to maintain adequate cholesterol supply (Shimano & Sato, 2017). As a sign of SREBP2 activation, SREBP2 accumulated in the nuclear fraction of the livers of PCN-treated obese mice (III; Figure 6C.).

In addition to SREBP2, also SREBP1 may induce cholesterol synthesis genes and compensate for defective SREBP2 function, although SREBP1 is more involved in the regulation of fatty acid synthesis (Shimano & Sato, 2017; Shimomura, Shimano, Horton, Goldstein, & Brown, 1997). Supporting the notion that SREBP1 is not responsible for the induction of cholesterol synthesis, SREBP1 did not accumulate to the hepatic nuclear fraction in immunoblotting (III; Supplementary Figure 5A.) and SREBP1 target genes involved in fatty acid synthesis, *Acc*, *Acly*, *Fasn* and *Scd1* were not affected by PCN treatment (III; Supplementary Figure 5B).

5.4.5 PXR activation induces regulators of circulating cholesterol

SREBP2 target genes are not limited to cholesterol biosynthesis genes but include important mediators of circulating cholesterol, such as *Pcsk9* and *Ldlr.* PCSK9 is a negative regulator of LDLR and thus increase circulating LDL by decreasing hepatic LDL uptake. PCSK9 inhibition has been proven to be an effective pharmaceutical strategy to lower blood cholesterol and LDL (Lagace, 2014). Liver gene expression profiling suggested PCN to upregulate *Pcsk9*. In qPCR analyses of full sample sets, both *Pcsk9* and *Ldlr* were induced by PCN in wildtype but not in PXR-KO mouse livers (III; Figure 6F.). In line with the induction of gene expression, PCN increased circulating PCSK9 in the obese mice (III, Figure 6G.).

5.4.6 SREBP2 target genes are downregulated in obese PXR deficient mice

A previous study showed PXR deficiency to be lethal to mice challenged by a high-cholesterol diet (Sonoda et al., 2005). The authors concluded PXR to play a critical role in the detoxifying metabolism of cholesterol metabolites. To evaluate

endogenous cholesterol metabolism in wildtype and PXR-KO mice, qPCR for full sample sets of chow and HFD-fed mouse liver samples was performed.

In wildtype mice, HFD downregulated *Insig2*, another regulator of SREBPs, and upregulated cholesterogenic genes *Fdps* and *Ldlr (III; Figure 7B.)*. Overall, the effect of HFD on cholesterol metabolism genes was mild in the wildtype mice. In PXR-KO mice, HFD repressed *Srebp2* and *Insig1* and *Insig2a.* Repression of *Srebp2* was paralleled by inhibition of *Srebp2* target genes *Hmgcr*, *Cyp51* and *Pcsk9.*

To clarify the functional significance of differential regulation of *Pcsk9* in HFD-fed wildtype and PXR-KO, circulating PCSK9 was determined. In wildtype mice, HFD increased plasma PCSK9 although *Pcsk9* expression was not altered (III; Figure 7C.). In PXR-KO mice, in which *Pcsk9* was downregulated by HFD feeding, plasma PCSK9 remained unaffected. Although PCSK9 was differentially regulated in the two genotypes, plasma cholesterol was increased in both by HFD-feeding (III; Figure 6D.).

5.4.7 Rifampicin increases atherogenic lipids and PCSK9 in humans

Our group has previously investigated cardiac and metabolic effects of rifampicin, an antibiotic and selective human PXR activator, on healthy volunteers in clinical studies Rifa-1, Rifa-2 and Rifa-BP (Hassani-Nezhad-Gashti et al., 2020; Hukkanen, Rysa, et al., 2015; Rysä et al., 2013). In order to shed light on the translational value of the mouse findings, serum samples of Rifa-1 and Rifa-BP were subjected to metabolomic analysis on an NMR platform which measures lipoprotein fractions and markers of systemic metabolism, such as amino acids and different lipids (Soininen, Kangas, Würtz, Suna, & Ala-Korpela, 2015).

Rifampicin elevated atherogenic intermediate density lipoprotein (IDL) and LDL fractions with a statistical significance and all lipid species of these fractions (III; Figure 1). Other lipoprotein fractions remained generally unaltered, although some marginal differences were observed. In addition to atherogenic lipoproteins, rifampicin increased serum total, esterified, free and LDL cholesterol.

Serum 4-β-OH-cholesterol, a marker of CYP3A4 activity and PXR activation (Hukkanen, Puurunen, et al., 2015), was determined in a separate assay to confirm the compliancy of the study subjects to rifampicin dosing. Rifampicin elevated 4-β-OH-cholesterol in all study subjects (III, Supplementary Figure 1A.). Interestingly, the changes in 4-β-OH-cholesterol correlated with changes in total and LDL cholesterol and IDL and LDL particles (III; Figure 3.) suggesting an

involvement of PXR activation in the observed changes in the lipoprotein and lipid metabolism.

In the metabolomic analysis, rifampicin decreased citrate and acetate, the precursors of cholesterol and fatty acid synthesis (III; Figure 2A.). Circulating lathosterol, a marker of cholesterol synthesis which was induced in obese mice treated with PCN, was also induced after rifampicin dosing in humans (III; Figure 2B.). In accordance with the mouse studies and SREBP2 stimulation, rifampicin also increased circulating PCSK9 (III, Figure 6F.).

In addition to induced synthesis, cholesterol may also stem from increased absorption. However, ApoB48, the intestinal form of ApoB, was not increased but was even slightly decreased by rifampicin dosing (III; Supplementary figure 2B.). This suggests increased atherogenic lipids to stem from increased cholesterol synthesis and not from increased absorption.

In summary, PXR activation by one-week rifampicin dosing in healthy volunteers led to increased cholesterol synthesis and atherogenic lipid profile and elevated circulating PCSK9.

6 Discussion

6.1 Transcriptomic response to PXR activation is modulated by nutritional status

Fasting response in the liver is mediated by multiple transcriptional regulators, of which several interact or are influenced by PXR (I. Goldstein & Hager, 2015; Pavek, 2016). Supported by these observations, this thesis shows PXR function to be affected by nutritional status. Generally, PXR activation displayed hindered transcriptomic response in fasting. Furthermore, PXR activation modified bile acid pool composition depending on the nutritional status due to differential regulation of *Cyp8b1*.

PCN induced six times and repressed seven times more genes one hour after glucose treatment than during fasting. Thus, the transcriptional activity of PXR as an inducer and repressor of gene expression was fortified by glucose or mitigated by fasting. Moreover, less than half of the genes regulated by PCN during fasting were affected after a glucose load, indicating there is significant diversity in the PCN responsive transcriptome.

The most upregulated genes and most affected pathways in fasted and glucose-treated mice were related to xenobiotic metabolism, the most well-established function of PXR (Ihunnah et al., 2011). Importantly, PCN regulated additional pathways after a glucose load, of which several were involved in glucose and lipid metabolism. The role of PXR in glucose and lipid metabolism has been described (Hakkola et al., 2016), and the results further suggest that PXR function in lipid and glucose metabolism adapts to the nutritional status.

Breaking the fast with glucose changed the direction of gene regulation of individual genes. This was especially true for *Cyp8b1*, which was repressed during fasting and induced after glucose by PCN. *Cyp8b1* repression by PCN has been described before (Bhalla et al., 2004).

In addition to *Cyp8b1*, PXR regulates *Cyp7a1* and *Abcc3* (Staudinger et al., 2001; Teng, Jekerle, & Piquette-Miller, 2003). CYP7A1 is the rate-limiting enzyme of bile acid synthesis whereas CYP8B1 directs the synthesis towards CA instead of CDCA, and ABCC3 is a sinusoidal efflux transporter (Chiang, 2017). In agreement with previous results, PCN treatment repressed *Cyp7a1* and decreased biliary bile acids. Furthermore, in agreement with *Cyp8b1* regulation, circulating CDCA was decreased by PCN and there was a tendency for CA elevation one hour after glucose

treatment. Altogether, the results suggest PXR to affect total bile acid level but to also modulate the composition of bile acid pool. Bile acids facilitate lipid absorption but they are also important signalling molecules which regulate physiological functions, including glucose metabolism (Chiang, 2017). Individual bile acids possess different potencies to their target receptors and, hence, modulation of bile acid pool composition may affect the important biological functions regulated by bile acids.

Previous studies have shown glucose to affect the phosphorylation status of PXR and induce PXR activity *in vitro* (Gotoh, Miyauchi, Moore, & Negishi, 2017; P. O. Oladimeji et al., 2017). In this study, glucose feeding *per se* did not induce hepatic *Cyp3a11* and glucose did not induce *Cyp3a11* in primary hepatocytes *in vitro*. The results suggest that glucose feeding after fasting is insufficient to affect PXR function alone.

In summary, these data reveal nutritional status to affect PXR function. The study implicates that nutritional status is a possible confounding factor in animal experiments and should be considered in experimental design and data analysis. Moreover, nutritional status may explain some discrepancies in reports regarding PXR as a regulator of glucose and lipid metabolism.

6.2 PXR activation dissociates hepatic insulin resistance and steatosis from glucose tolerance by increasing hepatic glucose uptake

The ability of nutritional status to modulate PXR response raised an important question of PXR function in obesity-induced metabolic dysfunction. Obesity greatly predisposes to metabolic diseases, making the obese especially vulnerable to metabolic perturbations. PXR activation aggravated hepatic insulin resistance and steatosis caused by the HFD. However, these defects didn't reflect to glucose homeostasis, as insulin-independent hepatic glucose uptake was simultaneously increased.

PXR activation has been described to promote hepatic steatosis in multiple studies and in different models (Bitter et al., 2015; Hakkola et al., 2016; J. Zhou et al., 2006). HFD feeding alone induced hepatic steatosis and increased plasma ALT activity. In line with previous findings, PCN promoted hepatosteatosis in obese wildtype but not in obese PXR-KO mice. Surprisingly, the vehicle, 30% DMSO in corn oil, nearly totally abolished fat from the liver and decreased liver size and plasma ALT activity. Transcriptomic profiling of the liver (data not shown) or TEM

imaging didn't provide insights into vehicle-mediated changes in liver function. Still, DMSO has been shown to affect lipid metabolism possibly through autophagy mechanisms, which may partly explain the strong vehicle effect on steatosis (Deol, Yang, Morisseau, Hammock, & Sladek, 2019). Despite the strong effect of the vehicle treatment on steatosis, PCN-treated mice displayed the most severe hepatosteatosis of all the experimental groups.

The degree of steatosis obtained by a four-day PCN treatment was surprisingly strong. A similar four-day PCN treatment of lean mice did not induce hepatosteatosis. These observations indicate that the obesity-induced metabolic dysfunction or the HFD sensitize mice to PXR-mediated hepatosteatosis.

Induction of CD36, a fatty acid transporter, is one reported mechanism which facilitates PXR-induced hepatosteatosis by increasing lipid uptake (J. Zhou et al., 2006). Lipids in liver steatosis may indeed stem from the diet or, in insulin resistance, from the adipose tissue (Donnelly et al., 2005; Fabbrini et al., 2008). However, feeding the HFD without concomitant obesity did not sensitize mice to PXR-induced steatosis, and adipose tissue function, determined by adipocyte size and adipokine secretion, was not affected by PXR activation. Further, PCN treatment didn't affect circulating triglycerides. In conclusion, PXR-mediated liver steatosis wasn't caused by increased absorption of lipids from the HFD or from the insulin-resistant WAT.

PXR activation did elicit mild proinflammatory effect on the WAT, but considering the severe impairments of hepatic functions, the WAT might nothing but reflect the liver function. In fact, liver-resident macrophages, Kupffer cells, secrete proinflammatory cytokines in parallel with progressing hepatosteatosis which may influence the inflammatory status of the adipose (Baffy, 2009).

Another well-established metabolic consequence associated with PXR activation is the impairment of glucose tolerance (Hakkola et al., 2016; Hassani-Nezhad-Gashti et al., 2018; Rysä et al., 2013). HFD-feeding proved to severely impair glucose tolerance. PXR activation did not further impair glucose tolerance but it even slightly improved it, contradicting the previous studies, which have been performed on healthy, young volunteers and lean, relatively young rodents. Here, obesity reversed the effect of PXR activation on glucose tolerance dissociating hepatosteatosis from glucose tolerance.

One potential cause of improved glucose tolerance would be the improvement of HFD-induced insulin resistance, which largely determines glucose tolerance (DeFronzo et al., 2015). However, PCN treatment did not affect systemic insulin resistance. Interestingly, HFD-fed mice showed increased fasting glucose and

HOMA-IR index but didn't show altered insulin sensitivity in ITT. Insulin was administered to mice in relation to their weight and C57BL/6 mice show significant variation in the increase of WAT mass during HFD (Y. Yang, Smith, Keating, Allison, & Nagy, 2014), and lean mass doesn't increase during HFD as much as the WAT mass does. Hence, the insulin dosing was likely overestimated in the HFD-fed mice when compared to chow-fed control mice. However, the HFD-fed and vehicle or PCN-treated mice were of same weight, excluding the problem.

Immunoblotting of insulin-sensitive AKT and IRS1 phosphorylation from the liver samples of mice sacrificed 15 minutes after insulin dosing revealed PCN to impair hepatic insulin signalling. This agrees with the increased liver steatosis and plasma ALT activity, for NAFLD tightly associates with hepatic insulin resistance (Oh et al., 2006). Skeletal muscle is a major glucose disposal site after a meal and during exercise, and it greatly contributes to insulin resistance. However, PCN treatment didn't affect skeletal muscle AKT phosphorylation with a statistical significance.

To further clarify the role of individual tissues in glucose disposal, tissue glucose uptake was determined. Remarkably, liver glucose uptake was induced by PCN, even without correcting for liver size, which PCN nearly doubled. Other tissues, including skeletal muscles, didn't show alterations in glucose uptake. Thus, it is likely that the dissociation of hepatosteatosis and glucose tolerance is in part due to increased hepatic glucose uptake. Effects of PXR activation in liver glucose and lipid metabolism are summarized in Figure 12.

In a previous study (He et al., 2013), constitutive activation of PXR (achieved by genetic manipulation) in leptin-deficient *ob/ob* mice increased liver triglycerides which associated with poorer glucose tolerance and systemic insulin sensitivity. The model used by He et al. (2013) is rather artificial, as leptin governs biological functions beyond controlling appetite and constitutive PXR activation doesn't resemble real life. In line with previous literature, feeding wildtype mice HFD, the method which was utilised in this thesis, causes obesity, hyperglycemia, hypercholesterolemia and insulin resistance. These are all integral parts of metabolic syndrome seen in humans, and thus HFD-feeding presents a good model to mimic human metabolic syndrome in mice. Nevertheless, despite the different results regarding glucose tolerance, both studies suggest PXR activation to be harmful for liver health in obesity.

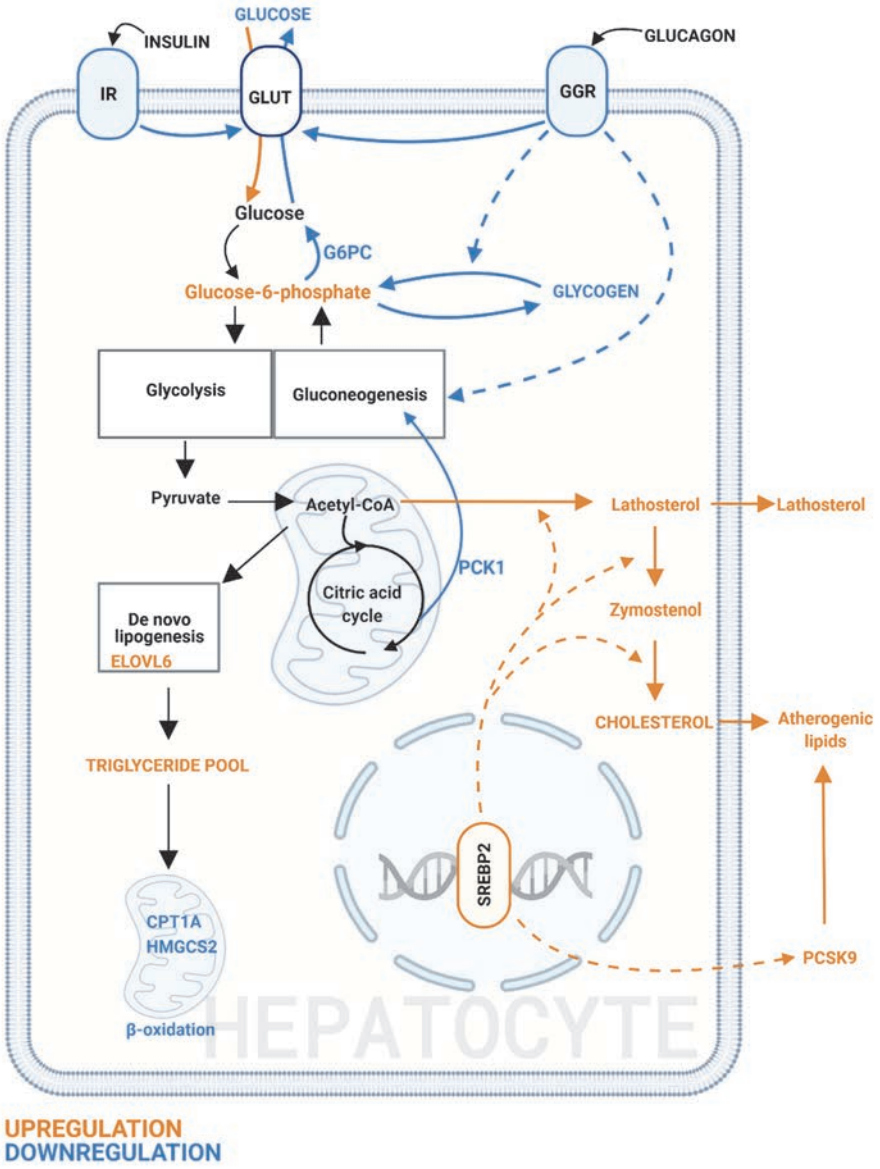

Fig. 12. Schematic overview of changes in hepatocyte glucose and lipid metabolism induced by PXR activation in obese mice. IR Insulin receptor, GGR Glucagon receptor, GLUT Glucose transporter.

The extrahepatic effects of PXR activation in the adipose and skeletal muscles are mild compared to alterations in hepatic metabolism. In addition to liver, PXR is also expressed in the intestine, which has important biological functions in regulating glucose and lipid metabolism. However, PXR activation didn't affect plasma triglycerides or 2-deoxyglucose levels, suggesting that PXR activation does not affect lipid or glucose absorption.

Taken together, PXR activation is detrimental to liver health in obesity, worsening hepatosteatosis and hepatic insulin resistance. However, PXR activation dissociates hepatic dysfunction from glucose tolerance by increasing insulin-independent glucose uptake.

6.3 Suppression of fasting response by PXR activation in obesity

The perturbations of hepatic energy metabolism in obesity occur largely in the same pathways that are regulated by fasting. These pathways control glycogen balance, i.e. glycogenesis and glycogenolysis, and gluconeogenesis to increase hepatic glucose output (DeFronzo et al., 2015; I. Goldstein & Hager, 2015). Previously, PXR has been shown to repress the expression of gluconeogenic genes *G6pc* and *Pck1* (Kodama et al., 2004, 2007). This thesis demonstrates PXR activation to suppress hepatic glucose production during fasting. This effect was caused by inhibiting the effect of insulin resistance on hepatic gluconeogenesis and by depleting hepatic glycogen. The suppression of hepatic fasting response may contribute to the improvement of glucose tolerance despite the aggravation of hepatosteatosis.

In line with the previous studies, PXR activation repressed *G6pc* and *Pck1* in obese mice. HFD feeding alone trended to induce hepatic *G6pc* and repressed *Pck1*. When gluconeogenesis was assayed by pyruvate tolerance test, HFD-fed mice didn't differ from mice fed with regular chow. PCN treatment tended to impair pyruvate conversion to glucose, but statistical significance was not reached.

Central to hepatic insulin resistance and hyperglycaemia is the inability of insulin to suppress gluconeogenesis (Hatting, Tavares, Sharabi, Rines, & Puigserver, 2018), and hepatic glucose output is a measure used to evaluate hepatic insulin resistance. Considering how PCN treatment impaired hepatic insulin resistance, it is possible that PXR activation counteracts the induction of hepatic gluconeogenesis caused by hepatic insulin resistance. This is supported by the notion that PCN didn't cause any changes to the transcriptome of skeletal muscle, which is the main supplier of amino acids for hepatic gluconeogenesis in the Cori

cycle (Katz & Tayek, 1998). However, no clear conclusion can be made without utilising more sensitive methods to investigate the effect of PCN on hepatic gluconeogenesis than pyruvate tolerance test, which is prone to errors caused by different metabolic stimuli and dosing (Hughey, Wasserman, Lee-Young, & Lantier, 2014).

Previously, PCN has been shown to deplete liver glycogen in non-pregnant, pregnant and fetal rats (Tuchweber et al., 1972). Also, in this thesis, PCN treatment depleted liver glycogen. Glycogen is depleted when the glycogenolysis is increased or when glycogenesis is suppressed. The ability of 2-deoxyglucose to incorporate into glycogen (Colwell et al., 1996) was utilised to investigate glycogen synthesis during the first hour after glucose treatment. Interestingly, PCN-treated mice trended to have less 2-deoxyglucose in glycogen than controls suggesting PCN to likely inhibit glycogen synthesis.

Glycogenesis is determined by glycogen synthase activity and glycogenolysis by the activity of glycogen phosphorylase. Both are regulated allosterically and by phosphorylation events. Glycogen synthase is activated by high-energy substrates, and primarily by glucose-6-phosphate, and glycogen phosphorylase primarily by PKA after glucagon stimulus (Roach, 2005; van de Werve & Jeanrenaud, 1987). Although the liver 2-deoxyglucose-6-phosphate content was increased by PCN, less of it was incorporated into glycogen, implicating glycogen synthesis to be indeed impaired. In addition to glucose-6-phosphate, activities of multiple kinases, e.g. GSK3, PKA and CK2 regulate glycogen synthase activity. Interestingly, these kinases have been shown to interact with PXR (Lichti-Kaiser, Brobst, et al., 2009; Lichti-Kaiser, Xu, et al., 2009), and they play roles in NAFLD pathogenesis (Choi et al., 2017; Ibrahim et al., 2011; London et al., 2014), which may provide possible mechanistic explanations.

Glycogenolysis and gluconeogenesis are both effectively induced by glucagon, the main hormone mediating hepatic fasting response. In PCN-treated obese mice, glucagon was unable to increase blood glucose as much as in controls showing that PCN treatment suppresses the fasting response.

Of note, PCN-treated mice demonstrated decreased fasting glucose after a 6-hour fast (in glucagon challenge and in ITT) but not after a 12-hour fast (in OGTT and in pyruvate tolerance test). In a short fast, liver glycogenolysis provides glucose to circulation but when fasting is prolonged, gluconeogenesis plays a bigger role. This implicates that liver is unable to mobilize glycogen after PCN treatment, which is explained either by glycogen being absent or by inhibited glycogenolysis. *G6pc,* which PXR activation repressed, is a liver-specific enzyme which is needed

for gluconeogenesis but also for the final step of glycogenolysis. Therefore, *G6pc* repression by PXR might partly explain both, repression of gluconeogenesis and mobilisation of glycogen to maintain blood glucose.

Altogether, the data shows PXR activation to suppress fasting response in obesity by depleting hepatic glycogen and possibly by affecting gluconeogenesis. Suppression of fasting response may partly counteract the detrimental influence PXR activation had on hepatic insulin resistance.

6.4 PXR activation regulates distinct hepatic gene sets in obese mice

Gene expression profiling of the livers of PCN-treated and obese wildtype and PXR-KO mice revealed PXR activation to affect novel pathways in obesity. In agreement with a previous report (Ann Barretto et al., 2019), PCN regulated only a small fraction of genes in PXR-KO mice when compared to wildtype mice. Importantly, the effect of PCN on the regulation of genes was very weak in the PXR-KO mice, indicating PCN to be highly specific ligand for PXR.

PCN-mediated gene regulation enriched multiple cellular pathways, and not surprisingly many pathways involving enzymes of drug metabolism. Additionally, PCN triggered a wide induction of cholesterol synthesis genes and genes encoding ribosomal proteins. PXR has been shown to induce *Sqle*, a gene of cholesterol synthesis, but no wide induction of cholesterol synthesis genes has been reported (Gwag et al., 2019). Also, induction of ribosomal proteins hasn't been shown in previous gene expression profiling studies performed on lean mice (Bailey, Gibson, Plant, Graham, & Plant, 2011; Cui et al., 2010). However, it may link to PCN-induced proliferation of smooth ER.

Although PXR induced hepatosteatosis, pathway enrichment analyses of PXR-regulated genes didn't suggest enrichment of lipid metabolism pathways. Previous study done on genetically obese *ob/ob* mice showed constitutive hepatic PXR activation to induce liver triglycerides and the expression of *Srebp-1c* and its target genes (He et al., 2013). Another study reported PXR activation to promote lipogenesis in human hepatocytes *in vitro* through SREBP1A (Bitter et al., 2015). Further, the pathway analyses suggested mTOR signaling to be activated by PXR activation due to induction of ribosomal proteins. mTOR is an important regulator of SREBP1, the main regulator of hepatic lipogenesis (S. Li et al., 2010). However, as is described in III, SREBP1 did not show signs of activation, i.e. nuclear accumulation, and classical SREBP1 target genes were not affected by PCN

treatment. Also, mTOR signaling was determined to be suppressed. Therefore, it is concluded that PXR activation does not induce hepatosteatosis through SREBP1 in obese mice.

PXR activation has been shown to induce lipogenic genes *Cd36* and *Elovl6* independently of SREBP1 (J. Zhou et al., 2006). Also, PXR activation has been shown to repress β-oxidation genes *Hmgcs2*, *Cpt1a* and directly induce *Lpin1*, the expression of which strongly associates with hepatic insulin resistance and steatosis (He et al., 2013; J. Zhou et al., 2006). In the experiments described in this thesis, PXR activation induced *Elovl6* and repressed *Hmgcs2* and *Cpt1a* while no effect was seen in *Cd36* expression. Interestingly, PXR activation repressed *Lpin1* in contradiction with the earlier report (He et al., 2013). Altogether, these alterations in gene expression suggest PXR activation to induce hepatic *de novo* lipogenesis and suppress β-oxidation while fatty acid uptake is not altered. The results agree with the metabolic tests, which indicated increased lipogenesis without an effect on fatty acid uptake.

In conclusion, PXR activation regulates novel pathways in obesity which may explain some of the effects seen in metabolic tests. It remains to be solved what causes the SREBP1-independent lipogenesis, although PXR activation regulated several lipogenic genes. Further, the role of mTOR inhibition in PXR-induced changes in liver health require further studies, as mTOR presents a central metabolic hub.

6.5 PXR activation stimulates SREBP2 to induce cholesterol synthesis and atherogenic lipids

The strong induction of cholesterol synthesis genes by PXR activation was shown to associate with activation of SREBP2, a transcription factor that regulates cholesterol synthesis. In addition, PXR activation increased circulating PCSK9, another SREBP2 target. Of great importance, analogical results were obtained in healthy volunteers, in which rifampicin increased cholesterol synthesis, circulating atherogenic lipids and PCSK9.

When inactive, SREBP2 resides in the ER in a complex with SCAP and INSIG1. Low sterol content is sensed by the complex leading to the cleavage of SREBP2, which thereafter translocates to the nucleus to induce cholesterol synthesis genes (Shimano & Sato, 2017).

INSIG1 is a negative regulator of SREBP activation, and *Insig1* has been shown to be a direct PXR target gene (A. Roth, Looser, Kaufmann, Blättler, et al.,

2008). In accordance, *Insig1* mRNA was induced by PXR activation. Surprisingly, INSIG1 protein was not affected, which is in line with increased SREBP2 activation. Additional mechanism by which INSIG1 may suppress cholesterol synthesis is the degradation of HMGCR (Sever et al., 2003). Again, as an indicator of increased cholesterol synthesis, HMGCR protein was induced by PCN. These results suggest INSIG1 stability to be affected by PXR activation which might contribute to SREBP2 activity.

Analysing cholesterol synthesis intermediates showed PCN to increase liver cholesterol without affecting plasma cholesterol. In the liver, PCN treatment increased the intermediates of the Kandutsch-Russell pathway of cholesterol synthesis, which was also reflected on the plasma levels. Intermediate of Bloch pathway and a negative regulator of SREBP2, desmosterol, was not affected by the PCN treatment, which may contribute to SREBP2 activation by PCN (Spann et al., 2012; C. Yang et al., 2006). DHCR24, an enzyme directing cholesterol synthesis to the Kandutsch-Russell pathway instead the Bloch pathway, was also induced by PCN.

HFD-feeding increased plasma and liver cholesterol. HFD also increased liver squalene without affecting the other intermediates. Cholesterol has been shown to negatively regulate SQLE, the enzyme responsible for squalene metabolism, which may explain the accumulation of squalene (Gill, Stevenson, Kristiana, & Brown, 2011). Furthermore, SQLE has been long suggested to be another rate-limiting enzyme of cholesterol synthesis in addition to HMGCR, the statin-targeted enzyme (Hidaka, Satoh, & Kamei, 1990). Previous study reported *Sqle* to be a PXR target gene (Gwag et al., 2019). In agreement, PCN treatment increased liver cholesterol without accumulation of squalene. In fact, there was less squalene in PCN-treated samples than in any controls, although statistical significance was not reached. This likely indicates increased metabolism of squalene by SQLE. This way, PXR activation may overcome cholesterol-mediated inhibition of SQLE activity by inducing its expression. This is another possible mechanism by which PXR activation increases cholesterol metabolism.

PXR activation in obese mice induced *Pcsk9,* a SREBP2 target gene, and circulating PCSK9. PCSK9 induces LDLR degradation, increasing circulating LDL, which has prompted the development of PCSK9 inhibitors, extremely effective drugs in decreasing LDL and cholesterol (Seidah, Awan, Chrétien, & Mbikay, 2014). Importantly, PXR activation in mice led to induction of two mechanisms, cholesterol synthesis and PCSK9, that are both targeted by drugs to decrease morbidity and mortality of atherosclerotic cardiovascular diseases.

The effect of PXR activation on cholesterol metabolism was studied in humans by utilising samples from two clinical studies investigating the effect of rifampicin on cholesterol metabolism in a crossover setting, forming the largest study to date. Serum samples underwent systemic metabolomic screen, which revealed rifampicin to primarily affect cholesterol metabolism. Most of all, rifampicin treatment associated with increase in LDL particles and cholesterol. Changes in 4-β-OH cholesterol, marker of PXR activation, correlated with rifampicin-induced changes, sugggesting PXR dependency.

Cholesterol in the blood originates either from the diet or from the *de novo* cholesterol synthesis occurring in the liver. Rifampicin increased serum lathosterol, marker of cholesterol synthesis (Björkhem et al., 1987), and decreased citrate and acetate, precursors of cholesterol synthesis while intestinal ApoB48 was not affected. This indicated that the cholesterol stemmed from increased cholesterol synthesis. These changes were widely the same which were observed in HFD-fed mice after PXR activation. In mice, PXR activation didn't lead to increase in plasma cholesterol. However, murine and human lipoprotein metabolism are widely different due to lack of CETP enzyme in the mice, which may explain why increased hepatic cholesterol synthesis didn't mirror the plasma cholesterol. Of importance, rifampicin also elevated PCSK9 in humans like PXR activation did in mice.

In PXR-KO mice, HFD feeding led to downregulation of *Sreb2*, its' cholesterogenic target genes and *Pcsk9*, an occurrence which was not observed in in wildtype mice. Further, PCSK9 in circulation was increased by the HFD-feeding in the wildtype but not in the PXR-KO mice, which displayed higher PCSK9 levels already in the chow-fed mice. Despite this difference in PCSK9 expression, wildtype and PXR-KO mice demonstrated similar plasma cholesterol levels in chow- and HFD-fed mice. This suggested that PCSK9 doesn't associate with circulating cholesterol in PXR-KO mice. However, this discrepancy in PCSK9 and circulating cholesterol may be explained by the lack of murine CETP enzyme.

Deficiency of PXR has been shown to be lethal to mice challenged with high-cholesterol diet, possibly through accumulation of toxic cholesterol metabolites (Sonoda et al., 2005). Therefore, the HFD-feeding might lead to accumulation of cholesterol metabolites in PXR-KO mice, which leads to SREBP2 retention in the ER and downregulation of SREBP2 target genes. This notation suggests that PXR is an important endogenous regulator of cholesterol metabolism.

In summary, these data show that PXR activation induces cholesterol synthesis by stimulating SREBP2, which leads to increased circulating atherogenic lipids and

PCSK9. The results establish a molecular mechanism for drug- and chemical-induced hypercholesterolemia and define the long-term exposure to PXR activators as a potential cardiovascular health risk.

6.6 PXR deficiency does not protect from obesity-induced metabolic dysfunction

PXR deficient mice have been shown to be protected from HFD-induced obesity and metabolic dysfunction (He et al., 2013; Spruiell et al., 2014; Zhao et al., 2017). In this thesis, such effect was not observed as PXR-KO mice gained weight and showed similar impairment of metabolic dysfunction like the wildtype mice. However, liver gene expression profiling revealed differences between the genotypes.

Previous studies used C56Bl/6J strain whereas we backcrossed the PXR-KO mouse strain to C57BL/6N strain. These two strains differ by a mutation in *Nnt* gene, which, although controversial, may alter glucose metabolism and cause the mice to respond differently to HFD (Fisher-Wellman et al., 2016). Nevertheless, similar weight gain allowed more sophisticated evaluation of the metabolic effects of PXR deficiency, as the results were not complexed by difference in the weight. Indeed, previous studies didn't exclude the possibility that the difference in weight gain would explain the improvement of energy metabolism.

One limiting factor in this thesis is that the HFD experiments were not originally designed to compare wildtype and PXR-KO mice on HFD. For this reason, the HFD in the wildtype lasted for 15 weeks and in the PXR-KO mice for 18 weeks. Still, during the first 14 weeks of HFD, mice gained weight in a strikingly similar fashion regardless of the genotype, showing the mice to respond to HFD similarly.

HFD regulated fewer and unique hepatic genes in the PXR-KO mice compared to the wildtype mice. PXR, together with few other xenobiotic receptors, is a master regulator of xenobiotic metabolism. CYP enzymes were widely downregulated by the HFD but only in the wildtype mice. Notably, the *Cyp* genes which the HFD repressed were mainly the same which PXR activation induced. Basal *Cyp3a11* expression in chow-fed mice was not affected by PXR deficiency and *Cyp3a11* was strongly repressed by the HFD but only in the wildtype mice. The observations indicate PXR to mediate HFD-induced repression of *Cyp* expression. Whether obesity buffers the exposure to PXR ligands, or NAFLD regulates PXR function through protein-protein interactions remains unanswered. Although the data is

inconclusive, NAFLD in humans has been associated with impaired CYP expression and drug metabolism (Jamwal et al., 2018; Woolsey, Mansell, Kim, Tirona, & Beaton, 2015).

Further investigation showed HFD to affect expression of classical lipogenic genes in both genotypes similarly. Still, examining the basal expression level of lipogenic PXR target genes revealed differences. *Cd36* expression, albeit induced by HFD, was lower in PXR-KO mice when compared to wildtype mice. *Elovl6*, involved in fatty acid synthesis, *Cpt1a*, involved in β-oxidation, and *Lpin1*, showed a higher expression level in the PXR-KO mice after HFD-feeding when compared to wildtype mice. Previous study reported wildtype and PXR-KO mice to gain similar amount of liver fat on HFD but with different histological characteristics of lipid droplets (Spruiell et al., 2014). Such analyses were not performed in this thesis, but it seems that the mechanism of steatosis might be altered by PXR deficiency.

In summary, PXR deficiency doesn't protect C57BL/6N mice from obesity and obesity-induced metabolic dysfunction. Still, hepatic transcriptome responds differently to HFD suggesting PXR to be involved in HFD-induced changes in hepatic transcriptome. Utilization of more sophisticated approaches, such as conditional or tissue-specific knockout mouse models, could shed light on PXR inhibition as a plausible drug target to treat obesity and associated metabolic dysfunction.

7 Conclusions and future prospects

This thesis studied the metabolic function of PXR, a nuclear receptor activated by different drugs and environmental chemicals. PXR is known for regulating the metabolism of drugs, but also that of glucose and lipids, which manifests as fatty liver and impaired glucose tolerance. This thesis shows that PXR function is regulated by metabolic status, and that PXR function in glucose and lipid metabolism is pronounced in obesity-induced metabolic dysfunction.

Fasting inhibited the transcriptional activity of PXR, and its' function in bile acid metabolism was dependent on nutritional status. In obese mice, PXR activation worsened hepatic steatosis and insulin resistance and induced cholesterol synthesis. However, PXR activation increased hepatic glucose uptake and suppressed fasting response, causing pseudo-improvement of glucose metabolism and dissociation of NAFLD and glucose tolerance. Experimentation with obese mice and careful analysis of human samples revealed PXR activation to increase cholesterol synthesis and circulating atherogenic lipids and PCSK9.

Strong induction of cholesterol synthesis after PXR activation associated with activation of SREBP2 transcription factor, the main regulator of cholesterol synthesis. In humans, PXR activation increased cholesterol synthesis in a similar manner, which led to increase in circulating atherogenic lipids. PXR activation in both species also increased PCSK9, a SREBP2 target gene and a central regulator of circulating LDL. Harmful alterations in cholesterol metabolism have been associated with multiple PXR-activating drugs and environmental chemicals. Therefore, this thesis reveals a molecular mechanism for drug- and chemical-induced hypercholesterolemia.

Overall, PXR activation mediated a profound shift in hepatic glucose and lipid metabolism in obesity-induced metabolic dysfunction. It is likely that PXR doesn't directly regulate all these metabolic functions, but some of them are compensatory responses to other alterations. Uptake of glucose and depletion of glycogen both increase intracellular glucose which is likely directed towards energy-demanding processes. PXR activation induced synthesis of cholesterol and triglycerides, which are both high-cost processes that require acetyl-CoA derived from glucose or amino acids. This may indicate induction of lipid synthesis to be the primary effect of PXR activation, and improvement of glucose metabolism to reflect hepatic energy demand. However, future studies are required to define the molecular initiating events to delineate PXR function in NAFLD.

In contradiction to previous studies, PXR deficiency did not protect mice from diet-induced obesity and metabolic dysfunction, although mice deficient in PXR showed alterations in hepatic transcriptome in response to HFD-feeding. Recently, selective human PXR antagonists have been developed, which could be utilized to evaluate PXR as a drug target for metabolic diseases and obesity in a humanized PXR mouse strain. This approach would be more delicate than artificial knock-out models to evaluate PXR as a drug target.

In conclusion, the findings presented in this thesis reveal PXR to be subject for regulation by metabolic status, be it nutritional status or obesity-induced metabolic dysfunction. The findings raise concerns of PXR activation in obese individuals, who might be sensitized to harmful metabolic effects of PXR activators, including many drugs and environmental contaminants. Importantly, the thesis demonstrates PXR activation to be a cardiovascular health risk as it increases atherogenic lipids and PCSK9.

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ISBN 978-952-62-2997-3 (Paperback)
ISBN 978-952-62-2998-0 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)

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