Liver X Receptors
-expression and functions in human placenta

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List of papers

Paper I:

Weedon-Fekjaer MS, Duttaroy AK, Nebb HI. Liver X receptors mediate inhibition of hCG secretion in a human placental trophoblast cell line. *Placenta.* 2005 Nov; 26(10):721-8

Paper II:

Weedon-Fekjaer MS, Dalen KT, Solaas K, Staff AC, Duttaroy AK, Nebb HI. Activation of LXR increases fatty acid uptake through direct regulation of ACSL3 in placental trophoblast cells. *Submitted.*

Paper III:

Weedon-Fekjær MS, Johnsen GM, Sugulle M, Nebb HI, Duttaroy AK, Staff AC. Expression of liver X receptors in pregnancies complicated by preeclampsia. *Under revision for resubmission to Placenta.*

Abbreviations

ABC	ATP-binding cassette
ACC	Acetyl-CoA Carboxylase
ACSBG	Acyl-CoA Aynthetase Bubblegum
ACSL	Acyl-CoA Synthetase Long-chain
AF	Activation Function
ALA	α-Linolenic Acid
ARA	Arachidonic Acid
cDNA	complementary DNA
ChREBP	Carbohydrate Responsive Element-Binding Protein
CoA	Coenzyme A
COX	Cyclooxygenase
DBD	DNA-Binding Domain
DHA	Docosahexaenoic Acid
DR	Direct Repeat
EPA	Eicosapentaenoic Acid
ER	Everted Repeat
FABP	Fatty Acid Binding Protein
FABPpm	plasma membrane FABP
FAO	Fatty Acid Oxidation
FAS	Aatty Acid Synthase
FATP	Fatty Acid Transport Protein
FFA	Free Fatty Acid
FXR	Farnesoid X Receptor
hCG	human Chorionic Gonadotropin
HDL	High Density Lipoprotein
HELLP	Hemolysis, Elevated Liver Enzymes, and Low Platelet count
hPL	human Placental Lactogen
HRE	Hormone Response Element
Idol	Inducible degrader of the LDLR
IR	Inverted Repeat
LA	Linoleic Acid
LBD	Ligand Binding Domain
LCPUFA	Long Chain Polyunsaturated Fatty Acid
LDA	Low Density Array
LDL	Low Density Lipoprotein
LDLR	LDL Receptor
LPL	Lipoprotein Lipase
LXR	Liver X Receptor
LXRE	LXR Responsive Element
NCoR	Nuclear Receptor Corepressor

NR	Nuclear Receptor
OA	Oleic Acid
oxLDL	oxidized LDL
pFABPpm	placenta FABPpm
PGI2	Prostacyclin I_2
PPAR	Peroxisome Proliferator Activated Receptor
PUFA	Poly Unsaturated Fatty Acid
PXR	Pregnane X Receptor
RXR	Retinoid X Receptor
SCD	Stearoyl-CoA Desaturase
SMRT	Silencing Mediator of Retinoic Acid
SR	Scavenger Receptor
SREBP	Sterol Regulatory Element Binding Protein
SUMO	Small Ubiquitin-like Modifier
TAG	Triglyceride
VLDL	Very Low Density Lipoprotein

1. Introduction

1.1 Introduction to placenta

The placenta is a highly specialized organ with a limited life span. It is unique to mammals, and in concert with fetal membranes and amniotic fluid it supports the normal growth and development of the fetus (1). Placenta is quite remarkable as it performs multiple functions, which in the adult are associated with several individual organs. The transfer of respiratory gases and nutrients between mother and fetus are functions analoguous to those of the lung and intestine respectively, while the handling and transport of fetal waste products is analogue to the kidney. Substances transported to the fetus include amino acids, carbohydrates, lipids, vitamins, minerals, water and oxygen. Reverse transport of metabolic waste products to the maternal circulation include carbon dioxide and urea (reviewed in (2)). In addition it is an important endocrine gland, which biosynthesis hormones that are essential to the maintenance of pregnancy (3). These placental functions serve as substitutes for the developing fetal organs until they are mature enough to fulfil the functions on their own. Other placental functions include energy metabolism to support placental needs, metabolic modifications of maternal nutrients destined for the fetus, maintenance of an immunological barrier, transfer of heat, and detoxification of xenobiotics.

There are large species variations in placental architecture. Histologically humans and rodents have a placenta where the maternal blood comes into direct contact with the trophoblast cells. Still the human placenta differs from that of rodents in that only a single layer of trophoblast cells separate the fetal endothelial cells from direct contact with the maternal blood. At term, the average normal human placenta is ~22 cm in diameter, 2-2.5 cm thick and with a weight of ~500 grams (Figure 1).

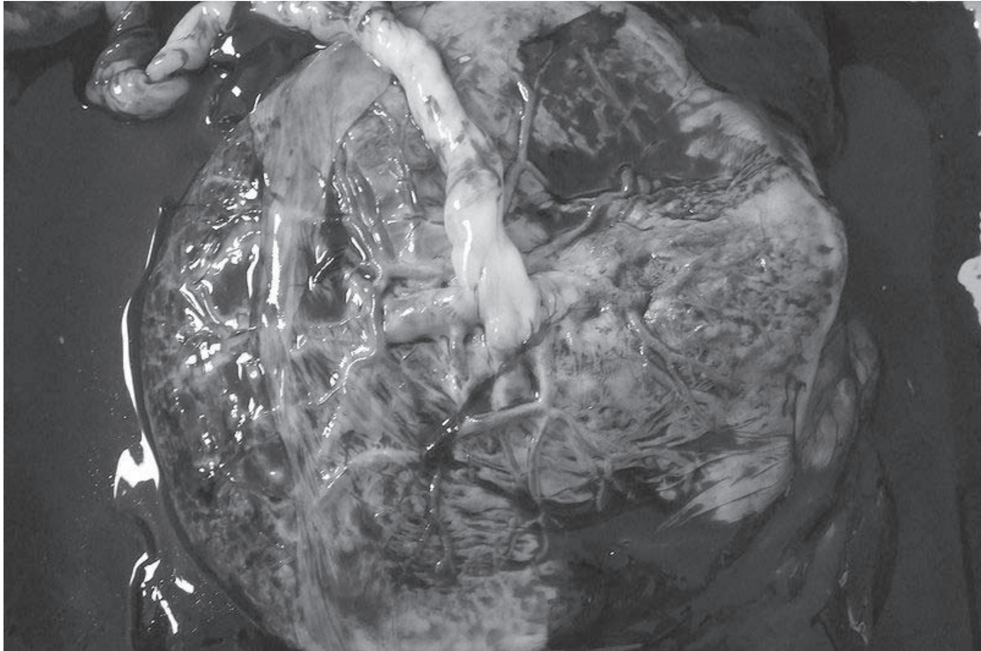


Figure 1: Human third trimester placenta; seen from the fetal side including the umbilical cord. From www.wikipedia.org

1.1.1 Development of the placenta

The fertilized egg develops into a blastocyst with an inner cell mass that becomes the fetus. The outer layer of cells consist of trophoblast cells. They invade the uterine wall (endometrium) in a highly regulated process and have a crucial role both in implantation and placentation (Figure 2, (1)). They develop into the placenta (which is fetally derived) and the fetal membranes (Figure 2). Failure to control the invasion of the trophoblasts results in a very aggressive cancer, named choriocarcinoma (4). The maternal part of the placenta, the *decidua basalis*, is the term for the uterine lining during pregnancy that is developed from the endometrium (Figure 3, (5)). The trophoblasts from the outer layer of the blastocyst eventually differentiate into either villous or extravillous trophoblasts. The extravillous trophoblasts invade the decidua and remodel the uterine spinal arteries to accommodate the increased blood flow

needed during gestation. Insufficient trophoblast invasion with altered remodeling of the spiral arteries is a common feature of preeclampsia (6).

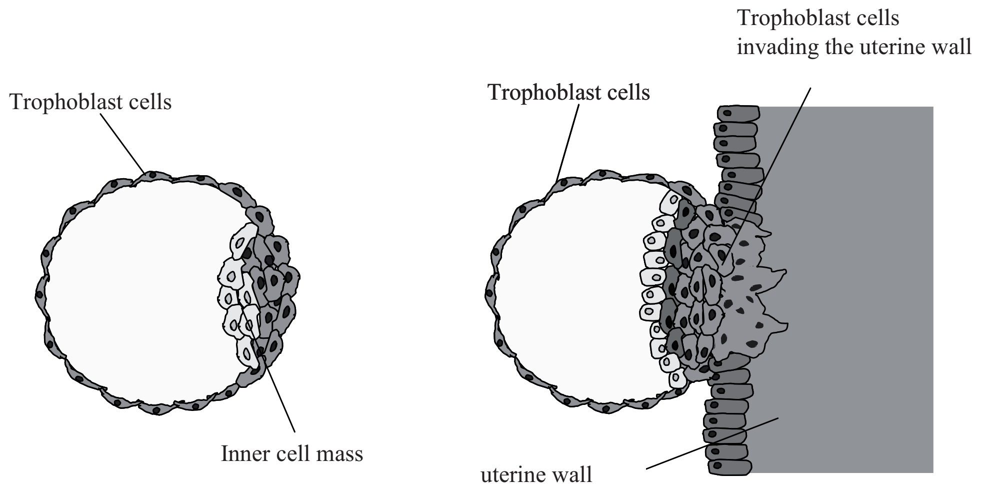


Figure 2: Implantation of the blastocyst. The trophoblast outer layer of the blastocyst forms an attachment with the endometrium about 6 days after fertilization in humans.

The villous trophoblasts consist of two cell populations, the cyto- and the syncytiotrophoblasts. The cytotrophoblasts proliferate, differentiate and subsequently fuse to multinuclear syncytiotrophoblasts (Figure 3). The finger-like chorionic villi are the functional units of the placenta and are surrounded by maternal blood in the intervillous space provided by the transformed uterine spiral arteries (Figure 3). The villi bring the maternal and fetal side in close proximity, and are constructed to give maximal area of contact between the fetal and maternal blood for efficient feto-maternal exchange (Figure 3). In the beginning of pregnancy, the villi consist of both a cyto- and a syncytiotrophoblast layer, but the cytotrophoblasts gradually disappear throughout gestation (reviewed in (1;2)). Despite having a key role in active

transport, hormone biosynthesis and metabolic regulation, the syncytiotrophoblasts have been thought to be transcriptionally inactive. Very recently this hypothesis was challenged by the finding of nucleoside incorporation and histone modifications associated with active chromatin and potential transcriptional activity in these cells (7).

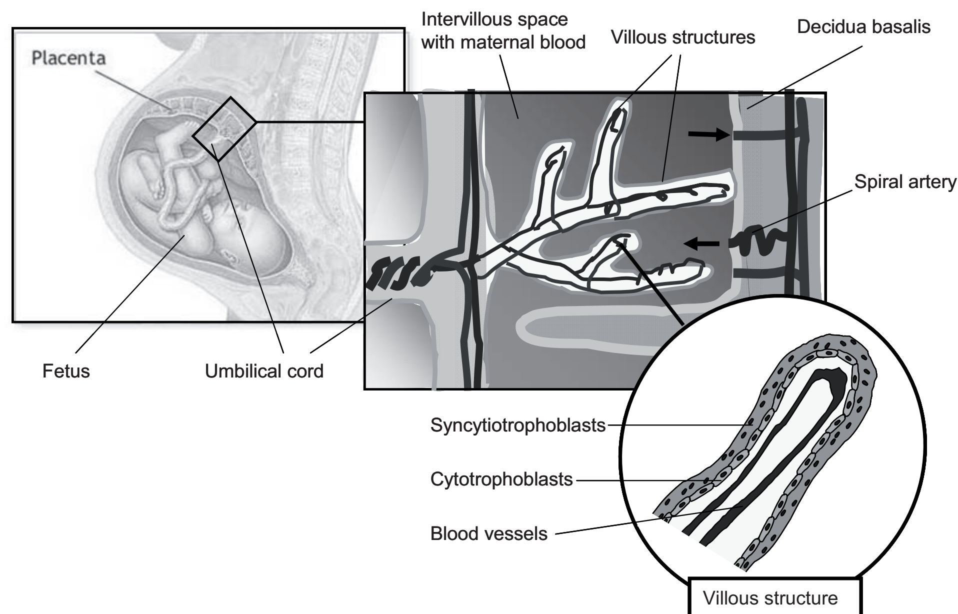


Figure 3: The villous structure of placenta. The villi, the functional units of the placenta are surrounded by maternal blood from the transformed spiral arteries of the uterine wall. The villi consist of an outer layer of syncytiotrophoblasts and an inner layer of cytotrophoblasts. Modified from www.wikipedia.com

1.1.2 Placenta endocrine functions - biosynthesis of human chorionic gonadotropin

Because the placenta is not innervated, communication with the mother and fetus is mainly through the biosynthesis and secretion of hormones and other substances into the circulations. The human placenta produces estrogens, progesterone, human chorionic gonadotropin (hCG), human placental lactogen (hPL), placental growth

hormone, as well as a number of growth factors, cytokines, chemokines, eicosanoids and many others (reviewed in (2)). The two main steroid hormones biosynthesized in placenta are estrogen and progesterone, while hCG and hPL are two of the main peptide/protein hormones biosynthesized by the placenta (1).

hCG is a glycoprotein composed of two subunits (α and β) non-covalently joined together (8;9). It is produced by the syncytiotrophoblasts mainly in early pregnancy with peak plasma levels at about 8 weeks of gestation, and secreted into mainly the maternal circulation (10). It promotes the corpus luteum progesterone production until the placenta develops sufficient production of progesterone to maintain pregnancy (10). hCG increases cytotrophoblast differentiation and placental angiogenesis, functions that are critical for efficient placentation in humans (11-13).

1.1.3 Maternal and fetal lipid changes in healthy pregnancies

Pregnancy is a metabolically dynamic situation that can be divided into two metabolically different periods, an anabolic period in early and mid pregnancy followed by a catabolic period at the end of pregnancy. During the anabolic period there is limited fetal growth and maternal hyperphagia which promote the accumulation of maternal body fat (14;15). The catabolic period is simultaneous with the period of maximal fetal growth, when most of the fat deposition in the fetus occurs (Figure 4). At this stage there is an increased maternal adipose tissue lipolysis and increased circulating free fatty acids (FFAs, Figure 4 (15;16)). This is thought to be secondary to the development of insulin resistance (17), which is a normal feature of a healthy pregnancy (reviewed in (18)). Much of the FFA are taken up by the liver, activated to acyl-Coenzyme A (CoA) and re-esterified to triglycerides (TAGs, Figure 4). These TAGs are further released into the circulation as part of very low-density lipoproteins (VLDL, Figure 4). As a result, there is an approximately 3-fold increase in circulating TAGs during late gestation (19). The circulating TAGs are also partly increased due to inhibition of adipose tissue lipoprotein lipase (LPL) as a result of insulin resistance and elevated estrogen concentrations (20). A positive correlation

between maternal TAG and newborn weight is reported (21). Circulating maternal total cholesterol is increased by 50% in late pregnancy, and is present mostly in low density lipoproteins (LDL) (19). A small increase in phospholipids is also observed (22). 6-10 weeks after delivery the gestational hyperlipidemia is essentially completely reversed to pre-pregnant levels (19).

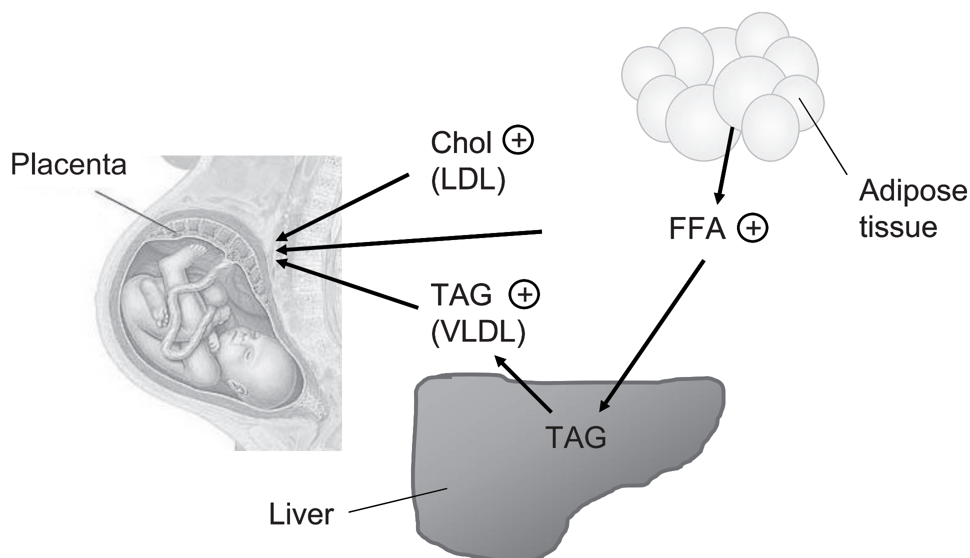


Figure 4: Maternal circulating lipid changes at the end of pregnancy. Modified from www.wikipedia.org

1.1.4 Placental β-oxidation, fatty acids and TAG biosynthesis

De novo fatty acid biosynthesis is the metabolic pathway converting dietary carbohydrates to fatty acids for incorporation into various macromolecules, including membrane lipids and lipid storage in lipid droplets. Acetyl-CoA carboxylase α (ACC) and fatty acid synthase (FAS) are the principal enzymes required for *de novo* fatty acid biosynthesis (23;24). FAS is a large multifunctional enzyme complex, which converts acetyl-CoA and malonyl-CoA to fatty acids. ACC produces malonyl CoA, which is substrate for FAS. *De novo* fatty acid synthesis occurs primarily in the liver, with some activity in adipose tissue. These hepatic fatty acids are further exported as

VLDL to other organs through the circulation. In addition to the synthesis in liver and adipose tissue, endogenous fatty acid synthesis is observed in rapidly proliferating tissues such as the human placenta. Although little information is available on placenta *de novo* fatty acid synthesis, this information suggests that placenta has a high *de novo* fatty acid capacity. It has been reported that human placental fatty acid synthesis is more then 100-fold greater than that observed in cultured rat hepatocytes (25;26). These fatty acids can be further activated to acyl-CoA and thereafter esterified and stored as TAG in placenta. Both TAG synthesis and lipid droplets are reported in placental trophoblast cells (27;28). The fatty acids may also be transported directly to the fetus. Alternatively, the fatty acids may be used as energy for the placenta itself since high capacity for placental β-oxidation is reported (29-31). The importance of a functional β-oxidation in fetal tissues is evident as fetal fatty acid oxidation (FAO) defects are potentially causes of maternal and fetal morbidity and mortality (32;33). Because the placenta is of fetal origin, the FAO defects are also present in the placenta. FAO defects of the fetus/placenta have recently been associated with hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome, placental floor infarct and acute fatty liver of pregnancy (32;33). Several reports suggest that not only the fetus, but maybe rather the placenta may be involved in these maternal toxic effects of FAO defects. However, more research is needed for a definite clinical conclusion (30;34;35).

1.1.5 Transport of lipids across the placenta

The transport of a molecule from the maternal to the fetal circulation includes traversing the syncytiotrophoblast cell layer, connective tissue, and the fetal capillary endothelium. Every one of these layers can contribute to the transport properties of the placenta. In addition, the placenta is not merely a transport organ; placental metabolism itself also contributes to the net transfer of nutrients to the fetal side.

Materno-fetal nutrient transfer across the placenta may proceed by different mechanisms, including facilitated diffusion, simple diffusion and active transport (2).

The proportion of transport across the placenta varies with the period of gestation and nutritional status of the mother.

Most maternal fatty acids are transported as TAG in TAG-rich lipoprotein particles, Therefore these lipoproteins are important sources of fetal fatty acids and need to be transported to the fetus (36). TAG cannot directly cross the placental barrier. Thus, in order to accomplish this transport, an elaborate placental transport system has been developed. It includes LDL receptor (LDLR) and VLDL/apoprotein E receptors, placental LPL, placental phospholipase A_2 and intracellular placental lipases (36-43). Alteration in placental LPL activity, and placental protein levels of LDLR and scavenger receptor (SR)-B1 were associated with intrauterine growth restriction (IUGR) (44-47). These alterations suggest a clinical importance of placental lipid transport for fetal growth.

FFA and glycerol can cross the placental membrane by either diffusion or fatty acid binding/transport proteins. These proteins are involved in regulating the direction and amount of net flux of fatty acids across the placenta. CD36/fatty acid transporter is one such fatty acid transporter. It is a multifunctional transmembrane glycoprotein that is involved in long chain fatty acid uptake and is expressed in human primary trophoblast cells (48;49). Interestingly it also function as a class B scavenger receptor for oxidized LDL (oxLDL, (48;50-52)).

1.1.6 Transport of cholesterol to the fetus

Cholesterol is an important structural component of cellular membranes. It is also the precursor of steroid hormones, synthesized by the syncytiotrophoblasts. Maternal hypercholesterolemia correlate to the fatty streak formation in the fetal aorta (53), indicating the existence of materno-fetal cholesterol transfer. The ATP-binding cassette (ABC) cholesterol transporter proteins ABCA1 and ABCG1 are probably important for this trans-placental transport. Stefulj *et al* (54) recently demonstrated that inhibition of ATP-binding cassette (ABC) A1 and silencing of ABCG1 resulted

in approximately 60 to 70% reduction in cholesterol efflux from placental third trimester endothelial cells. Interestingly, both transporters were predominantly located in the apical membrane of placental endothelial cells. Both the SR-B1 and the oxLDL (lectin-like) receptor 1 and the lipoprotein receptor LDLR are also present in the placenta and could potentially contribute to the transfer of cholesterol from the maternal to the fetal side (55-57). Even if recent publications indicate a number of proteins involved in transport of cholesterol across the placenta, more studies are needed to clarify if placental cholesterol transfer is required to support the fetal needs and placental growth and biosynthesis of steroid hormones.

1.1.7 Placental transfer and fetal needs for long chain polyunsaturated fatty acids

Long chain polyunsaturated fatty acids (LCPUFA)s are of critical importance for the development of the fetus. Data from large cohort studies and randomized controlled trials highlight the importance of adequate amount of n-3 LCPUFAs for optimal cognitive and visual development and reducing the risk of preterm delivery (58-63). Docosahexaenoic acid (DHA, 22:6, n-3) is selectively accumulated in the retina and brain during development (64;65). Arachidonic acid (ARA, 20:4, n-6) serves as a precursor of bioactive eicosanoids and is reported to be associated with infant birth weight and preterm infant growth (66;67).

The human body cannot introduce double bonds distal to the ninth carbon from the methyl end (ω- or n-end) of fatty acids. Thus, n-3 and n-6 poly unsaturated fatty acids (PUFA)s are essential fatty acids that must be obtained from the diet, either as linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3), or their LCPUFA derivatives; ARA, eicosapentaenoic acid (EPA, 20:5, n-3) and DHA. If obtained as LA and ALA in the diet, the fatty acids must be elongated and desaturated to provide the biologically active LCPUFAs (Figure 5). Basal expression of delta 5 and delta 6 desaturase and elongase has been detected both in placenta and fetal liver (68-70). However, their enzyme activities are low. Studies in baboons show that DHA from

the maternal diet is much more efficient than ALA as a source for fetal DHA accretion (71;72). Furthermore, supplementation of pregnant women with ALA does not result in higher umbilical cord blood levels of DHA (73). Taken together, the high needs of fetal LCPUFAs are difficult to meet by metabolism of their precursor PUFAs alone. Therefore, dietary intake of LCPUFAs and subsequent transport by the placenta to the fetus is important for optimal fetal development.

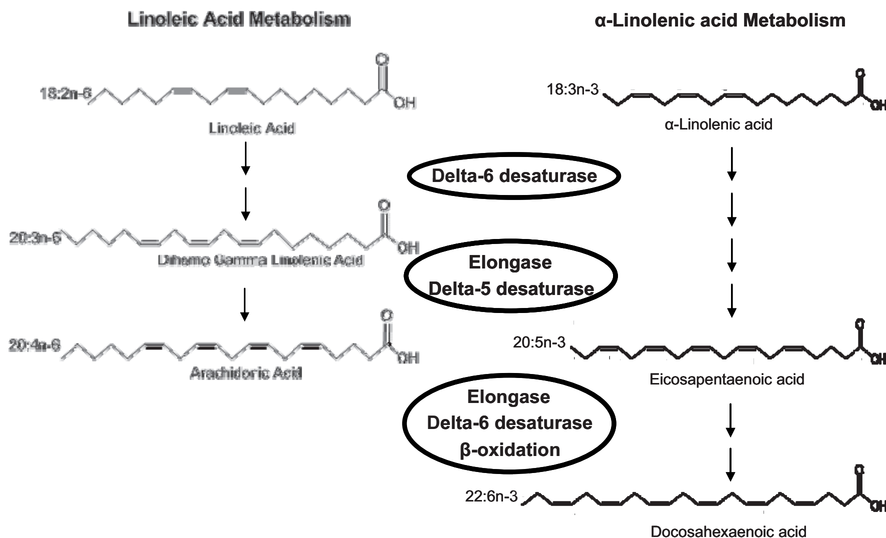


Figure 5: LA and ALA elongation and desaturation to ARA and DHA respectively in placenta. Picture modified from www.wikipedia.org

A number of studies have shown significantly higher ARA and DHA concentration in the fetal blood (the cord blood) compared to the maternal circulation, while LA and ALA concentrations were lower (22;74;75). This process, described as biomagnification, results in the preferential accumulation of DHA and ARA in the fetus during pregnancy. It represents an important mechanism to secure a sufficient supply of these critically important fatty acids to meet the increasing fetal demands. The transport of PUFAs across the placenta is selective in the order of preference, DHA > ARA > ALA > LA (76;77). Recently, the sophisticated use of ^{13}C-DHA

demonstrated *in vivo* the preferred human placental transfer of DHA relative to LA, oleic acid (OA) and palmitic acid (78).

1.1.8 Preferential uptake of LCPUFAs in placenta

It has been reported that fatty acids can flip flop across membranes, but protein-mediated transport is essential for the preferential transport of LCPUFAs (79). Placental transfer of LCPUFAs is probably a multi-step process involving both uptake and intracellular translocation. It is facilitated by several placental fatty acid uptake proteins, including CD36, fatty acid transport proteins (FATPs), fatty acid binding proteins (FABPs) and plasma membrane fatty acid binding protein (FABPpm) (Figure 6, reviewed in (80)). FABPpm is a peripheral membrane protein which, by binding to and increasing the concentration of fatty acids extracellulary, could contribute to placental transfer of fatty acids (81). The placenta-specific FABPpm (pFABPpm) resembles the ubiquitously expressed FABPpm, but is located exclusively on the maternal facing syncytiotrophoblast membranes and is involved in the preferential uptake of LCPUFAs in the placenta (Figure 6 (77;82;83)). FATP1 to 4 and FATP6 are fatty acid transporters reported to be expressed in primary human term trophoblasts (84). The expression of particularly FATP1 and FATP4 could also be important for the materno-placental transport of LCPUFAs, as their placental expression were positively correlated to DHA levels in the placental phospholipid fraction (85). Once taken up by the cell, the fatty acids can be transported in the cytosol by FABPs. FABP1, 3, 4 and 5 are expressed in primary human trophoblast cells, and they are able to bind long chain fatty acids (48;86;87). Fatty acids can also be activated to acyl-CoA by acyl-co A synthetases and the activated protein can further be transported in the cytosol bound to acyl-CoA binding protein (ACBP, reviewed in (88-90). Several proteins containing acyl-CoA synthetase activity and ACBP are detected in placenta but further studies are needed to clarify their roles in placenta (84;91;92).

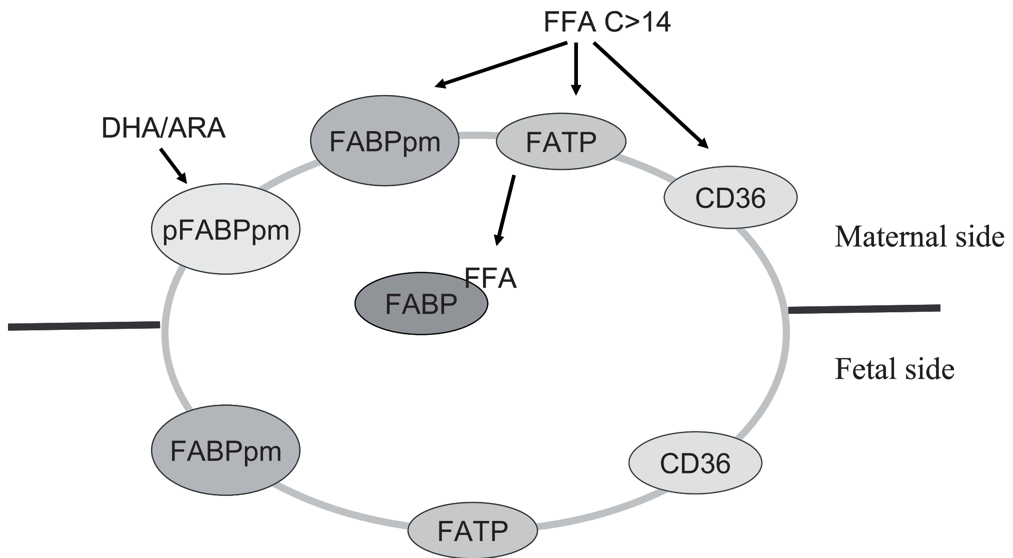


Figure 6: Proteins involved in lipid transport in placental trophoblast cells (modified from AK Duttaroy (80))

1.2 Preeclampsia

1.2.1 Introduction to preeclampsia

Preeclampsia is a pregnancy-specific syndrome, defined clinically as *de novo* hypertension and proteinuria occurring after 20 weeks of gestation. In Norway it affects 3.7% of all pregnancies, and is a leading cause of maternal death (93;94). World-wide preeclampsia is the cause of 50.000 maternal deaths annually (95). The severe forms of preeclampsia typically results in preterm delivery, low-birth weight and increased risk of fetal morbidity and mortality (96).

The definition of preeclampsia has been debated, and there is no global consensus. According to the National High Blood Pressures Education Program Working Group and High Blood Pressure in Pregnancy the following criteria for diagnosis of preeclampsia is recommended (97). These criteria have also been used to define the preeclamptic patient group in this thesis:

1) "Blood pressure at 140 mm Hg systolic or higher or 90 mm Hg diastolic or higher that occurs after 20 weeks of gestation in a woman with previously normal blood pressure".

2) "Proteinuria, defined as urinary excretion of 0.3g protein or higher in a 24 hour urine specimen (or protein dipstick reading equal to or higher than 1+ on more than one midstream urine sample six hours apart)".

When pre-existing hypertension is present in the women, preeclampsia is defined as superimposed on the chronic hypertension. Eclampsia is an end stage of the disease involving the occurrence of seizures in a preeclamptic woman, where the seizures cannot be attributed to other causes. The HELLP syndrome is a variant of preeclampsia (98).

Preeclampsia is unpredictable in its onset, progression and severity. It is sometimes divided into severe, early onset preeclampsia occurring prior to week 34 of gestation, and a mild, late onset preeclampsia occurring at or after 34 weeks of gestation (99;100).

Despite decades of research on the etiology and mechanism of preeclampsia, the pathogenesis is not fully understood, but increasing evidence support the concept that it is a multifactorial syndrome involving both the mother and the fetus. The pathogenesis is generally recognized to include an abnormal placental implantation and endothelial dysfunction resulting from oxidative stress and excessive inflammatory response, but numerous other factors are also suggested to be involved (reviewed in (6;101)). Several lines of evidence point to a key role of the placenta in the aetiology of the disease as delivery resolves the syndrome, it occurs without the presence of a fetus (in molar pregnancies) and the risk of preeclampsia is increased by greater placental mass (102).

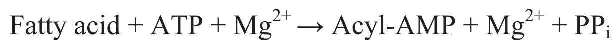
1.2.2 Hyperlipidemia in preeclampsia

Hyperlipidemia of pregnancy develops in every pregnant woman, but is significantly increased in women with preeclampsia relative to healthy pregnancies. The lipid abnormalities of preeclampsia include hypertriglyceridemia, increased circulating FFAs, reduced high density lipoproteins (HDL) and increased concentrations of small LDL which leads to the presence of oxLDL in maternal circulation (103-108), while total and LDL cholesterol levels are not substantially different (104;105). This exaggerated lipid adaptation of preeclamptic pregnancy is strikingly similar to abnormalities associated with cardiovascular disease (109-112). Indeed, preeclampsia is related to an increased risk of developing cardiovascular disease later in life (systematically reviewed in (113)). The two disease entities also have several risk factors in common including obesity, diabetes mellitus, insulin resistance and lipid abnormalities (111;114). Both cardiovascular disease and preeclampsia include presence of endothelial dysfunction, partly due to increased lipid peroxidation. “Acute atherosis” of the decidual/uterine spiral arteries in preeclampsia closely resembles the early stages of atherosclerotic lesions found in cardiovascular disease (115). In addition, recent research indicates increased risk of cardiovascular disease in the offspring of preeclamptic mothers, but whether this is attributed to genetic or epigenetic factors or only an adverse pro-atherogenic lipid profile *in utero* is not clear (reviewed in (116)).

A rise in circulating TAG is also shown to be present prior to clinical onset of preeclampsia (117), as early as 10 weeks of gestation (118). There seems to be a dose-response effect, with a four-fold higher adjusted risk of developing preeclampsia in women with the highest circulating levels of TAG compared to normal TAG levels (108). Although hypertriglyceridemia may contribute to the development of preeclampsia, therapeutic intervention is probably not a good alternative, as severe correction of maternal hypertriglyceridemia in rodents has been shown to mediate negative effects on fetal growth and development (119).

1.3 Long chain acyl-CoA synthetases

The activation of free fatty acids by the addition of a CoASH group is essential for the participation of fatty acids in most metabolic reactions, including β-oxidation, biosynthesis pathways and phospholipid remodelling. Acyl-CoA synthetases have been found in all organisms investigated, testifying to the evolutionary conservation and essential role of this activity (89). The activation is a two-step reaction where the first step is the biosynthesis of an acyl-AMP intermediate from fatty acid and ATP, and the second step is the exchange of AMP with CoASH to produce the activated acyl-CoA:



$$\text{Fatty acid} + \text{ATP} + \text{Mg}^{2+} \rightarrow \text{Acyl-AMP} + \text{Mg}^{2+} + \text{PP}_i$$

$$\text{Acyl-AMP} + \text{CoASH} \rightarrow \text{Acyl-CoA} + \text{AMP}$$

All proteins containing acyl-CoA synthetase activity have two conserved amino acid (aa) sequence domains: a 10 aa, highly conserved AMP-binding domain and a 35 aa domain originally proposed to be important for fatty acid-binding properties (120;121). Later it has been found that the fatty acid binding is located between these two domains (122).

The family of acyl-CoA synthetases can be divided into five sub-families based on fatty acid chain length preference; acyl-CoA synthetase short-chain (C2 to C4), medium chain (C4 to C12), long chain (ACSL, C12 to C20), bubblegum (ACSBG, C14 to C24) and very long chain (FATP, C18 to C26) (reviewed in (89;123)). Thus, there are three related families of proteins able to activate long chain fatty acids, ACSL, ACSBG and FATP (124-129).

The ACSBG1 and ACSBG2 were only recently discovered and little information is available on their function (129;130). Both ACSBGs have acyl-CoA synthetase activity with preference for long chain fatty acids more than very long chain fatty acids, and expression restricted to brain, ovary and testis (128-131).

The FATPs are the most thoroughly investigated of the long chain and very long chain acyl-CoA synthetase families. Six members of the FATP family are known, FATP1 to FATP6, which differ in their tissue expression, subcellular localizations and substrate specificities (132-137). They are documented to have intrinsic acyl-CoA synthetase activity and to increase uptake of fatty acids when overexpressed. Thus, it has been an ongoing controversy as to whether they are fatty acid transporters, acyl-CoA synthetases or both (reviewed in (90;138)). Studies on FATP4 have debated whether plasma membrane location is necessary for cellular fatty acid uptake. Despite the exclusive localisation to endoplasmic reticulum, FATP4 over-expression significantly increased the cellular uptake of fatty acids in one study, but not in another study (139;140). Mutated FATP4, containing a non-functional acyl-CoA synthetase domain, did not increase the uptake of fatty acid when overexpressed, in contrast to wt FATP4, indicating that the acyl-CoA synthetase function could be necessary for the fatty acid uptake (140). Evidence for a role of acyl-CoA synthetase in uptake of fatty acids is supported by the fact that the acyl-CoA synthetase inhibitor Triacsin C strongly reduces the fatty acid uptake (141). Co-immunoprecipitation of ACSL1 and FATP1 in adipocytes further suggests that acyl-CoA synthetase is important for fatty acid uptake (141). On the other hand murine FATPs transfected into FATP mutated yeast strains did not show correlation between acyl-CoA synthetase activity and long chain fatty acid uptake (142). Furthermore, FATP3 was found to have acyl-CoA synthetase activity without increasing fatty acid uptake (134). It appears that acyl-CoA synthetase in certain situations is necessary for fatty acid uptake, but is alone not sufficient to ensure fatty acid uptake. Thus, clearly more studies are needed for a definitive conclusion on the role of acyl-CoA synthetase activity on fatty acid uptake.

Five genes in the ACSL family have been identified based on sequence homology (143;144). They are named ACSL1 and ACSL3 to 6 and differ in their tissue distribution, intracellular locations and regulation, suggesting distinct functions (143;145;146). The ACSL family can be further divided into two subfamilies where

the ACSL1, 5 and 6 constitute one subfamily and ACSL3 and 4 constitute another subfamily (143). Coleman *et al* have suggested that instead of being redundant, the different ACSLs channel fatty acids into distinct metabolic pathways (147). ACSL5 was initially thought to be involved in β-oxidation (148), but has later also been shown to increase TAG biosynthesis in hepatoma cells (149). Overexpression studies reveal that ACSL6 in the brain is important for uptake and incorporation of DHA and ARA into phospholipids, but not TAG (150;151). Although increasing evidence supports the hypothesis, many questions regarding the "channelling hypothesis" still remain to be answered, including the subcellular localization of the different ACSLs in different cells and environments, which could be important for the channelling mechanism (89).

Besides activation of fatty acids, a function similar to that reported on FATPs in the transport of fatty acids has been indicated for members of the ACSL family. Expression of mammalian ACSL1, ACSL4 and ACSL6 in yeast cells lacking native long-chain acyl-CoA activity leads to enhanced fatty acid uptake (146). Overexpression of ACSL5 and ACSL6 in rat hepatoma cells and neuronal cells respectively increased the uptake of fatty acids in these cells (149;150). The hypothesis is that FFAs cross the plasma membrane, where it is esterified to the acyl-CoA product that is no longer lipid permeable and thus is trapped inside the cell. Interestingly, Lobo *et al* (152) recently showed that ACSL1 was involved in fatty acid efflux rather then uptake in an adipocyte cell line, opening the possibility for a novel role of the ACSLs.

ACSL3 was cloned from rat brain in 1996 and in human placenta the year after. It was shown to have substrate preference in the order of myristic acid (14:0): ARA : EPA > OA >> DHA (92;124). The ACSL3 is highly expressed in prostate, skeletal muscle, testis, heart and placenta (153). It consist of 17 exons, spanning a domain of more than 80 kb (153). A difference in translation start gives rise to two different ACSL3 isoforms with similar cellular and tissue localization (154). ACSL3 is one of the most abundant proteins associated with lipid droplets in huh7 hepatocytes, but it

was also found in the endoplasmatic reticulum and plasma membrane fraction in these cells (155;156). ACSL3 was associated with the biosynthesis of neutral lipids in Huh7 cells in one study (156). In another study in 3T3-L1 adipocytes ACSL3 was detected on lipid droplets only during lipolysis (157). Hence, the role of ACSL3 in channelling fatty acids to anabolic compared to catabolic pathways needs more investigation.

1.4 Nuclear Receptors

It took the scientific world approximately 10 years from the human genome sequencing project was initiated until the entire human genome was sequenced, and it was found to consist of 20.000-25.000 protein coding genes (158). The same genetic material is present in almost all cells of the body, and the variability among cells and tissues depend on the regulation of these genes. The importance of an accurate transcriptional control is emphasized by the fact that almost 10% of all human genes are estimated to be transcription factors (159). Hence, transcription factors constitute the single largest family of human genes. The comprehension of this transcriptional regulation is fundamental for the molecular understanding of human biology in health and disease

The Nuclear receptor (NR) superfamily is a diverse group of evolutionary related DNA binding transcription factors of which 48 are identified in humans (reviewed in (160)). Many of these NRs are ligand dependent transcription factors, but a significant number are still considered as orphan receptors, since no physiological relevant ligand has yet been identified (161;162). Their ligands are hydrophobic and small size, but except from these common features, they consist of a great variety of molecules. They include several fatty acids, cholesterol derivatives (steroid hormones, vitamin D, bile acids, oxysterols and other cholesterol metabolites), retinoids, thyroid hormone, prostaglandins, leukotriens, and xenobiotics (163). The ligand-dependent property permits NR to serve essential functions as communicators

between the intracellular or body environment and the genome. They play critical roles in a variety of biological processes including development, reproduction, homeostasis, inflammation, and metabolism, by altering target-gene expression (161;163;164).

1.4.1 Subfamilies of the NRs

The NRs can be divided into subfamilies in many different ways, based on properties such as phylogenetic analysis of sequence homology, ligand sources and physiological functions (160;165). A classical way of dividing the NRs is according to DNA-binding and dimerization properties, as presented in table 1 (162). This classification divides the NRs into four groups (Table 1). Class 1 receptors include the classical steroid hormone receptors, which bind to regulatory sequences on DNA as homodimers. Class 2 receptors are heterodimers with RXR and function in a ligand-dependent manner. Examples of these receptors include several NRs that are known to regulate lipid biosynthesis, flux, storage and utilization, of which some of the most important are the peroxisome proliferator-activated receptors (PPAR)s and the liver X receptor (LXR)s. The next two classes contain orphan nuclear receptors, which are so named because their ligands were unknown, at least at the time when the receptor was identified. Class 3 are orphan receptors, which function as homodimers, and class 4 are orphan receptors, which function as monomers.

Recently the crystallization of the first full-length NR pair, PPARγ and RXRα was identified after many years of effort. It showed that the PPAR ligand binding domain (LBD) dominates the entire RXR molecule and seems to play the major role of the two (166).

Steroid Receptors		**RXR heterodimers**	
NR	*agonist*	*NR*	*agonist*
GR	glucocorticoid	LXR	oxysterols
MR	mineralocorticoid	PPARa	fatty acids/
PR	progesterone	PPARg	15d-delta^{12,14}-PGJ2
AR	androgen	FXR	bile acids
ER	estrogen	PXR/SXR	xenobiotics
		T_3R	tyroid hormone
		RAR	all-trans RA
		VDR	1,25-(OH)2-VD
		CAR	androstane
		EcR	ecdysone
Dimeric Orphan Receptors		**Monomeric/Tethered Orphan Receptors**	
RXR	9-cis RA	NGFI-B	
COUP		SF-1	
HNF-4		Rev-erb	
TR2		ROR	
TLX		ERR	
GCNF			

Table 1: The nuclear receptor superfamily. The table shows the subdivision of NRs into four classes according to their DNA-binding and dimerization properties (162).

1.4.2 Structure of the NRs

NRs share a characteristic structure that consists of five to six homologousdomains (Figure 7, reviewed in (160;163)). The amino-terminal domain (often referred to as the A/B domain or the modulator domain) does highly vary in sequence between different NRs. This domain is structurally flexible and contains surfaces for both activation and repression of gene transcription (reviewed in (167)). The A/B domain usually contains a transcriptional activation functional domain, termed activation function 1 (AF-1). The AF-1 domain may be involved in cofactor interaction, and it is the target of post-translational modifications (168). Next to the A/B domain is the C domain also called the DNA-binding domain (DBD). It is a highly conserved domain containing two zinc fingers, which bind to specific sequences of DNA called hormone response elements (HRE)s. These HREs consist of one or two consensus core half sites in the promoter or enhancer region of the target genes, but the exact

HRE nucleotide sequence in each target gene can differ considerably from the consensus sequence (169). The HRE of dimeric NRs are organized as direct (DR), inverted (IR) or everted repeats (ER) of a six-nucleotide half site DNA consensus sequence. The D region serves as a hinge between the DBD and the E domain, which is also called the ligand-binding domain (LBD) This flexibility permits the DBD and the LBD to adopt different conformations. The LBD is located in the carboxy-terminaly part of the protein, which is the hallmark of NRs. Within this C-terminal domain is another AF domain, which is termed AF-2 (Figure 7, reviewed in (170)). The AF-2 is important for the NR LBD to activate gene transcription and it mediates ligand dependent transactivation and cofactor recruitment.

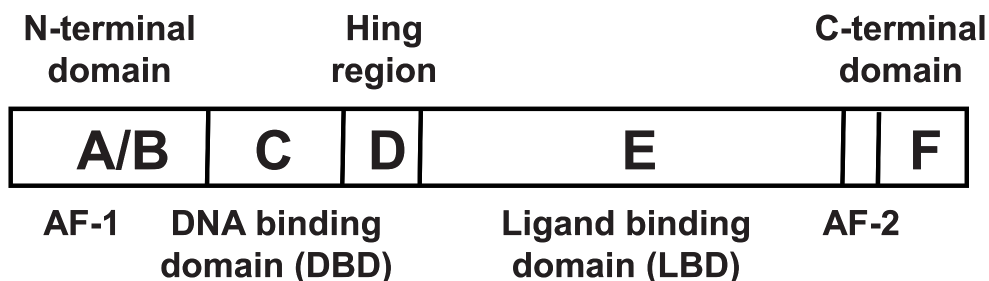


Figure 7: The protein structure of NRs. The NRs share a characteristic structure that consists of five to six homologous domains. Modified from www.wikipedia.org

1.4.3 NR cofactors

In addition to site-specific DNA binding and binding of ligand, the NR transcriptional activity depends on interaction with coactivators and corepressors. More than 300 NR coregulators have been characterized, and they can broadly be divided into coactivators that increase transcription and corepressors which silence gene expression (reviewed in (171;172)). The coactivators are recruited upon NR ligand binding due to a conformational change in the LBD of the receptor. Most coactivator complexes stimulate transcription through direct interaction with the basal transcription machinery or by inducing histone protein modifications that enhances

the access to the DNA (172). Some unliganded nuclear receptors are bound to DNA in association with corepressors, repressing transcription of target genes. Examples of such corepressors are nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid (SMRT, (173)). Upon NR ligand binding, conformation changes in the NR LBD releases the corepressors in an active mechanism clearing the corepressors from the NRs and recruiting the coactivators.

In addition to ligand and cofactor binding the NRs are reported to be modified in many other ways such as by; phosphorylation, glycosylation, methylation, acetylation, ubiquitinylation and small ubiquitin-like modifier (SUMO)ylation (172).

1.4.4 RXRs and PPARs

The RXRs consist of three members RXRα, β and γ ((174-176)). They can form transcriptionally active homodimers on DR-1 elements, but are also the heterodimeric partner of many NRs (177;178). The RXR are activated by the endogenous agonist 9-cis retinoic acid and several synthetic agonists (179-181).

The PPARs consist of three distinct members, PPARα, δ and γ (182-184). The array of genes regulated by each PPAR is divergent, and include genes involved in cellular differentiation, development and metabolism (185). The PPARs are important regulators of lipid metabolism. PPARα and PPARδ predominantly enhance energy expenditure in metabolic tissues, and PPARγ promotes lipid storage (186). The PPARs heterodimerize with RXRs and preferentially bind to DR-1 elements. PPARα and PPARγ are the molecular targets of a number of marketed drugs. Fibrates are PPARα activators and used in humans as a class of hypolipidemic drugs (187). The thiazolidinediones are selective PPARγ agonists used in humans to increase insulin sensitivity (188). Fatty acids and eicosanoids have also been identified as natural ligands for the PPARs (186).

1.5 LXRs

The LXRs were cloned in 1994-1995 (189-194). Subsequently until today, more than 1200 publications have established a role for the LXRs in cholesterol homoeostasis, lipogenesis, glucose metabolism, atherosclerosis, diabetes, Alzheimer's disease, dermatitis, immunology, and inflammation (195-199). The LXR subfamily of NRs consists of two members; LXRα (NR1H3) and LXRβ (NR1H2), which share considerable sequence homology and are activated by the same agonists (200). LXRβ is ubiquitously expressed, whereas LXRα has a more restricted expression pattern predominantly in tissues known to be important in lipid metabolism (189;193;194;201).

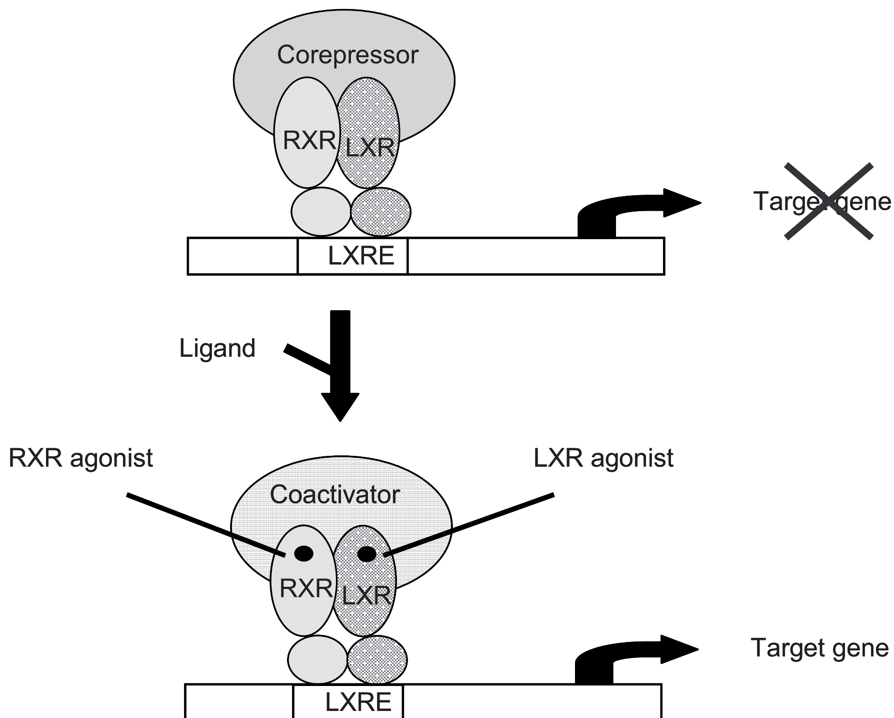


Figure 8: Binding of RXR/LXR heterodimer to LXRE with and without activators

Both LXRs form heterodimers with RXR, and the heterodimer bind to LXR responsive elements (LXREs) in the promoter of the target genes (202). The LXREs are classified as DR-4 elements because they consist of two direct repeat hexamers separated by four nucleotides (189;193). In the absence of ligands, the heterodimer functions as an inhibitor bound to the LXRE, together with corepressor complexes. Upon ligand binding, a conformational change of the protein complex induces the release of corepressors and the recruitment of coactivators resulting in transcription of the target genes (Figure 8 (203;204)).

1.5.1 LXR ligands

A major breakthrough in the understanding of LXR was the identification of oxysterols as endogenous LXR agonists (205;206). Oxysterols are oxidized derivatives of cholesterol and are present for example in oxLDL (207). Some of the most potent of these endogenous LXR activators are 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol, 24(S), 25-epoxycholesterol, and 27-hydroxycholesterol (200;205;206). In addition LXR can be activated by non-steroidal synthetic agonists, of which T0901317 and GW3965 are the most commonly used (208;209).

LXR activation by synthetic agonists leads to reverse cholesterol transport (returning of peripheral cholesterol to the liver) resulting in decreased atherosclerosis (210). Despite this promising effect by these agonists on atherosclerosis, the undesirable side effects of increased hepatic lipogenesis leading to liver steatosis and hypertriglyceridemia has made it unlikely that this first generation of LXR ligands will be used therapeutically (209). Still, these synthetic LXR agonists have been valuable tools for the identification and characterization of LXR target genes and the understanding of LXR regulated physiological processes. Along this line, there is an ongoing search for tissue selective agonists or agonists selective for only one of the LXRs (reviewed in (211)). An ideal agonist would have preserved the beneficial effects of inducing ABCA1 transporters in macrophages and liver, without the sterol regulatory element binding protein (SREBP)-1 regulatory effects leading to

hypertriglyceridemia and liver steatosis (212). Recently, the first human trial of an LXR agonist on healthy subjects was performed, but many side effects, mainly in the central nervous system were observed (213).

In vivo studies with either LXRα$^{-/-}$ or LXRβ $^{-/-}$ mice indicate a more prominent role of LXRα than LXRβ in controlling hepatic lipogenesis. Hence, selective LXRβ agonists are candidates for such beneficial effects and several LXRβ agonists have been tested (214). Still, the question remains to be answered whether the activation of LXRβ alone is sufficient to ameliorate the atherosclerosis process.

In contrast to many oxysterol and synthetic LXR agonists that activate LXR, several compounds with antagonising properties have been reported, including PUFAs. These fatty acids inhibit LXR activation by competing with LXR agonist binding in the order of ARA > EPA > DHA > ALA, whereas saturated and monounsaturated fatty acids have very little effect on LXR activation in Human Embryonic Kidney 293 cells (215).

1.5.2 LXR in cholesterol homeostasis

The LXRs were initially described as regulators of cholesterol metabolism through the identification of the first LXR target gene, cholesterol 7 alpha-hydroxylase, in rodent liver, which is the rate-limiting enzyme in conversion of cholesterol into bile acids (200). Later, numerous studies have established the LXRs as cholesterol sensors that regulate both cellular and systemic cholesterol homeostasis in a tissue-specific manner. In the liver, LXR regulates genes leading to increased biliary cholesterol excretion (216). In peripheral tissues like macrophages, the LXRs control the expression of genes involved in reverse cholesterol transport (217-222). Recently, a new regulatory pathway of LXR in cholesterol homeostasis was reported by Zelcer *et al* (223). LXR was shown to increase the expression of the gene inducible degrader of the LDLR (Idol), which subsequently target LDLR for degradation, resulting in a

suppression of LDL uptake in a number of tissues. Taken together, the systemic activation of LXRs leads to net cholesterol excretion and reduced atherosclerosis.

1.5.3 LXR in lipogenesis

Analysis of mice deficient in LXRα and LXRβ reveal the involvement of the LXRs in fatty acid biosynthesis (lipogenesis) and secretion of TAG in the liver (224). These mice were deficient in hepatic expression of a number of lipogenic genes including SREBP-1, stearoyl-CoA desaturase (SCD)-1, FAS and ACC. Accordingly, administration of LXR agonist to wild-type mice induced the expression of these lipogenic genes and results in elevated plasma and hepatic triglyceride levels (209). LXR activation was also shown to increase expression of SREBP-1 and FAS and give lipid accumulation in cultured adipocytes (225). The effect of LXRs on lipogenesis is largely due to the direct LXR-mediated induction of the transcription factor SREBP-1c, which subsequently upregulates FAS, SCD-1 and ACC. SREBP-1c is a master regulator of genes involved in fatty acid biosynthesis, through the binding to SREBP responsive elements in the promoter of target genes (reviewed in (226)). However, the treatment of SREBP-1c$^{-/-}$ animals with LXR agonist indicates the ability of LXR to induce the expression of some lipogenic genes independently of SREBP-1c (227). Furthermore, the recognition of the known SREBP-1c target genes FAS, SCD-1 and ACC as direct LXR target genes supports this observation. Peroxisome proliferator activated receptor (PPAR)γ, Carbohydrate responsive element-binding protein (ChREBP), LPL, Angiopoietin-like 3 and CD36 are also direct LXR target genes involved in lipid metabolism (228-232). Hence, systemic activation of LXR induces a whole collection of genes involved in lipid metabolism, with the net effect of increasing hepatic lipogenesis, circulating TAG and hepatic steatosis.

In addition to hepatic *de novo* fatty acid biosynthesis, circulating FFAs are also a source of hepatic lipids. LPL is an important enzyme in lipid metabolism hydrolyzing TAG in circulating lipoproteins to release FFA to muscle, adipose tissue, and

macrophages. The direct LXR-mediated regulation of LPL is shown in several tissues to increase fatty acid uptake and further TAG biosynthesis and lipid accumulation (233). The involvement of LXR in lipid uptake is further demonstrated by the recent identification of CD36 as a direct LXR target gene in liver (234). CD36 is an important gene in lipid homeostasis, and is tightly controlled by a number of NRs. In addition to the regulation by LXR, CD36 is shown to be a direct pregnane X receptor (PXR) and PPARγ target gene, and an additive increase in CD36 expression is observed with both PXR and LXR agonist together in liver (234). Not only is CD36 regulated by a number of NRs, but the NRs themselves are also reported to regulate each other in different tissues, which again could potentially influence the regulation of CD36. PPARγ is induced by PXR activation in liver, and PPARγ is a direct target gene for LXR in adipocytes (234;235). Furthermore, LXR is a target gene for PPARγ in macrophages and adipocytes (225;236). Such a complex regulation of genes in the lipid metabolism including a whole network of transcription factors is not unique to CD36 (229;237-240). It permits the fine-tuning of mRNA concentrations of important metabolic genes to meet the shifting metabolic needs of the body, by integrating the information from nutritional, hormonal and cell signalling.

1.5.4 LXR, glucose, insulin and diabetes mellitus

Increasing evidence has placed LXR as regulator of glucose homeostasis and insulin action. LXR agonists improve glucose tolerance in diabetic rodent models mainly through hepatic LXRα (241). In liver, LXR activation represses phosphoenolpyruvate carboxykinase and glucose-6-phosphatase expression, whereas in adipose tissue, LXR mediate direct upregulation of glucose transporter 4 (199;242;243). Furthermore, high concentrations of glucose was shown to bind and activate LXR, although these findings have been debated (244;245). Tobin *et al* (246) show that hepatic LXRα is involved in insulin regulation of both cholesterol homeostasis and triglyceride metabolism in liver as insulin increases the expression of hepatic LXRα and LXR target genes. Finally, LXRβ was shown to increase glucose dependent

insulin secretion in pancreatic β-cells (247). However, LXR-mediated activation does not always lead to increased insulin-sensitivity. Activation of LXRs increase TAG accumulation in human skeletal muscle and pancreatic β-cells, and increase pancreatic β-cell apoptosis, conditions usually associated with insulin resistance and type 2 diabetes mellitus (247-249). Thus, even if several studies support a beneficial role of LXR activation, further studies are needed to clarify the net effect of LXR agonists on type 2 diabetes mellitus.

1.6 PPARs and RXRs in placenta

Of the NRs involved in lipid metabolism, most research in placenta has been done on the PPARs and the RXRs. As RXR is a heterodimeric partner of both LXR and PPAR, and numberous studies show the cross talk between PPARs and LXRs, the regulation of these NRs in placenta could be relevant for the understanding of LXRs in placenta. An overview of the literature on RXRs and PPARs in placenta is given in this thesis introduction, Also, the role of LXR in placenta and other reproductive organs will be addressed, but limited information is available on LXR in placenta.

All three PPARs and two RXRs (RXRα and RXRγ) were detected in human and rat placenta and placental trophoblast cells, while RXRβ was only detected in rat placenta (250). Both PPARs and RXRs have been reported to be involved in several aspects of pregnancy development including implantation, placentation, trophoblast invasion and fatty acid uptake (reviewed in (251)).

Studies of PPARγ $^{-/-}$ mice revealed abnormal placental development resulting in embryonic death at mid gestation (252). Detailed analysis of these mice revealed that the PPARγ/RXR heterodimers are essential for differentiation of trophoblast cells and the formation of a functional placenta (252). Both PPARγ and RXRα agonists also increase differentiation of human primary cytotrophoblast (253). Increased hCG production is a hallmark for cytotrophoblast differentiation, and this reproductive hormone was also increased by PPARγ, and shown to be a direct PPARγ target gene

(253;254). Even though several PPARγ target genes have been identified in placenta, this cannot fully explain the lethal phenotype observed in PPARγ $^{-/-}$ mice (255;256). Hence, there is a continuous research going on to decipher the gene regulation responsible for this PPARγ $^{-/-}$ phenotype (251)

A role for PPARγ in trophoblast invasion has also been demonstrated. Both synthetic and natural PPARγ agonists were shown to inhibit extravillous cytotrophoblast cell invasion in *in vitro* invasion assays (257;258).

PPARγ is involved in differentiation, fatty acid transport and accumulation in adipocytes (259). Likewise, PPARγ seems to play a role in fatty acid uptake and accumulation in placenta. PPARγ $^{-/-}$ and RXR $^{-/-}$ mice both lack lipid droplets normally present in wt mice, and PPARγ and RXR agonist increase fatty acid uptake and accumulation in primary human trophoblasts (84;252;260;261). In line with these observations, both the fatty acid transporters FATP1 and FATP4, and the lipid droplet protein adipophilin are upregulated by PPARγ activation in human trophoblast cells (84;262).

An essential role for PPARδ in placental development has been demonstrated in implantation and trophoblast differentiation. PPARδ $^{-/-}$ mice develop placental abnormalities, with compromised size of trophoblast giant cell and increased embryonic lethality (263). In line with this observation, PPARδ was later shown to be crucial for giant cell differentiation *in vitro* (264). PPARδ regulate the production of cyclooxygenase (COX)-2 derived prostacyclin I_2 (PGI_2), and COX-2 $^{-/-}$ female mice display decreased fertility, partly due to impaired blastocyst implantation and decidualization (265;266). Treatment of these mice with PPARδ selective agonist restored the implantation (266). PPARδ was also shown to be essential for the effect of PGI_2 to enhance mouse blastocyst invasion (267;268).

PPARα activation is reported to inhibit secretion of hCG and increase secretion of progesterone from immortalized human extravillous trophoblast cells (269). PPARα $^{-/-}$ mice had increased abortion rate, which was further increased in diabetic PPARα $^{-/-}$

mice (270). The increased abortion rate was suggested to involve a compromised materno-fetal lipid exchange, but no placental abnormalities were observed. Hence, further studies are needed to clarify the role of PPARα in embryonic development.

RXRα is the main RXR isoform expressed in the placenta (250). As the PPARs heterodimerize with RXR and both PPARγ and PPARδ are essential for placental development it would be expected that RXR $^{-/-}$ mice display a similar abnormal placental development. As expected RXRα $^{-/-}$ mice die *in utero* from day E12.5 to E16.5 and exhibit a similar placental phenotype as PPARγ (260;271). The RXRα $^{-/-}$/RXRβ $^{-/-}$ mice exhibited a much more severe phenotype resembling both PPARγ $^{-/-}$ and PPARδ $^{-/-}$ mice, indicating that RXRβ to some degree could compensate for the loss of RXRα (272).

1.7 LXR in reproductive tissues

The major part of research on LXRs has focused on their role in liver and macrophages. However, recent studies indicate putative roles for LXRs in many other tissues including placenta and other reproductive tissues. Both male and female LXRα $^{-/-}$, LXRβ $^{-/-}$ and LXRα $^{-/-}$/β $^{-/-}$ mice are less fertile then their wild type (wt) controls (273-276). Male LXRβ -/- mice were essentially infertile by 5 months of age, while male LXRα -/- mice had reduced testicular testosterone and higher apoptotic rate of germ cells, indicating that LXRα and LXRβ collaborate to maintain both integrity and function of the testis (273). It appears that LXR regulation of cholesterol homeostasis during the maturation of sperm cells is crucial for male fertility (273;274;276). In female LXRα $^{-/-}$/β $^{-/-}$ mice, oocyte maturation is impaired due to a reduced effect of follicle-stimulating hormone, while LXR agonist stimulates resumption of meiosis in isolated oocytes (275). Further, female LXRα $^{-/-}$/β $^{-/-}$ mice were recently identified to have many of the same features of complications as animals with ovarian hyperstimulation syndrome, which is a common complication of fertility treatment when inducing ovulation (277). The complications included

enlarged ovaries with increased vascularity, and elevated circulating estradiol. Isolated uteri from LXRβ $^{-/-}$ mice had reduced contractile response to oxytocin, probably due to an abnormal increase in cholesterol content, indicating a role in uterine contraction during labour (278).

A function for LXR in placental cells was first described by Pavan *et al* in 2004 (279). They showed that oxLDL inhibited cultured extravillous cytotrophoblast invasion *in vitro*. Only oxLDL containing a high amount of oxysterols contained these properties. These results were confirmed with a synthetic LXR agonist, suggesting that LXR activation could interfere with implantation. Later the identification of endoglin as a direct LXR agonist in JAR cells further suggest inhibitory activity of LXRs in trophoblast invasion (280). Endoglin is an antiangiogenic protein and an inhibitor of trophoblast invasion, with increased maternal circulating levels in preeclampsia (281).

Several recent publications have investigated the role of LXR on cholesterol transport in the placenta. Stefulj *et al* (54) showed that isolated endothelial cells from human term placenta had a 2.5-fold higher cholesterol release to apoprotein A-I and increased cholesterol efflux following LXR activation, as compared to human umbilical vein endothelial cells. Fetuses with Smith-Lemli-Opitz syndrome are incapable of *de novo* cholesterol biosynthesis (282). *In utero* treatment of a Smith-Lemli-Opitz syndrome mouse model with LXR agonist resulted in increased cholesterol content, probably due to increased expression of Abca1 (57). This opens for the possibility that activation of LXR *in utero* could attenuate the irreversible congenital malformations present in Smith-Lemli-Opitz syndrome patients already at birth.

2. Aims of the study

At the start of this thesis, very little information was available on the role of LXR in placenta. The importance of LXR in lipid homeostasis in other tissues prompted us to explore the transcriptional activity and physiological roles of LXRα and LXRβ in placental lipid transport and metabolism. This knowledge could be important in order to increase the understanding of molecular mechanisms involved in pathological pregnancies associated with hyperlipidemia.

The following projects were addressed in Paper I, II and III:

1. Gene expression of LXRs in human trophoblast cell lines and placental tissue

2. The role of LXRα and LXRβ in lipid metabolism and other functions in placental trophoblast cells

> Are the LXRs involved in *de novo* fatty acid biosynthesis in trophoblasts?
>
> Are the LXRs involved in activation of fatty acids in trophoblasts?
>
> Are the LXRs involved in fatty acid uptake by trophoblasts?
>
> Are the LXRs involved in regulation of hCG expression and secretion in trophoblasts?

3. The search for new LXR target genes in human placental trophoblast cells.

During the work of this thesis, we observed increased expression of ACSL3 on microarray in human primary trophoblast cells treated with LXR agonist. Based on this finding the following additional questions were addressed:

> Is ACSL3 a direct LXR target gene in trophoblasts?
>
> Is ACSL3 involved in LXR-mediated fatty acid activation and uptake in trophoblasts?

4. The expression of LXRα and LXRβ in relation to changes in physiological lipid parameters, in placental tissue in preeclampsia

Is LXRα, LXRβ and LXR target genes differentially expressed in preeclamptic placentas compared to controls?

3. Summary of papers

3.1 Paper I:

Liver X receptors mediate inhibition of hCG secretion in a human placental trophoblast cell line

In the first paper, we investigated whether LXR has similar lipid metabolic effects in placental trophoblast BeWo cells as observed in liver and adipose tissues. We further investigated the LXR-mediated regulation of hCG secretion from BeWo cells.

We identified the expression of LXRs in placenta trophoblastic BeWo and JAR cells. We further indirectly identified the presence of functional LXR protein, by induced expression of a minimal LXRE containing promoter when stimulating with LXR agonist.

Next, we investigated the effects of LXR activation on *de novo* fatty acid synthesis and further metabolism into complex lipids. We observed increased biosynthesis of all classes of lipids investigated (FFA, PL/MG, DG and TAG) in BeWo cells prestimulated with LXR agonist compared to controls. The known LXR target genes SREBP-1 and FAS were also highly induced upon LXR activation, and therefore suggest to be involved in the observed increased *de novo* fatty acid biosynthesis.

We finally examined if activation of LXRs could affect expression and secretion of the glycoprotein hormone hCG during BeWo cell differentiation. Simultaneous incubation with LXR agonist and a differentiation agent (forskolin) throughout cell differentiation, produced a dose dependent reduction in expression and secretion of hCG.

In conclusion, these results indicate that LXR has similar effects in placenta as in liver and adipose tissue on *de novo* fatty acid metabolism and further metabolism into complex lipids. Furthermore, the results suggest a role for the LXRs in regulation of trophoblast differentiation through the regulation of hCG.

3.2 Paper II:

Activation of LXR increases fatty acid uptake through direct regulation of ACSL3 in placental trophoblast cells

Our results in Paper I encouraged us to further explore the role of LXRs in placental trophoblast cells. We therefore performed a microarray on human primary third trimester trophoblast cells incubated with LXR agonist (T0901317) or vehicle. Analysis of the microarray identified ACSL3 as a hitherto unknown LXR target gene, which was highly induced upon LXR agonist stimulation. Theoretical promoter analysis of the ACSL3 promoter identified a very promising LXRE. EMSA and transfection studies of the full length ACSL3 promoter and promoters without a functional LXRE identified ACSL3 as a direct LXR target gene.

After establishing ACSL3 as a direct LXR target gene, we examined whether acyl-CoA synthetase activity and fatty acid uptake was increased following LXR activation in BeWo cells. Incubation of BeWo cells with LXR agonist increased the activation of OA and the uptake of OA, EPA, DHA and ARA. To further test the importance of ACSL3 on fatty acid uptake and activation, we silenced ACSL3 in BeWo cells and incubated the cells with LXR agonist. The LXR-mediated induction of fatty acid uptake and activation was strongly inhibited in these cells. Interestingly, silencing of ACSL3 also reduced the basal fatty acid uptake by 30% and the basal acyl-CoA synthetase activity by 50%.

In conclusion, our results show that LXR increase the uptake of LCPUFA. Furthermore, LXR increase the uptake and activation of OA through the direct induction of ACSL3 expression. Our results also identify ACSL3 as the main long chain acyl-CoA synthetase and a major fatty acid uptake protein in BeWo cells.

3.3 Paper III:

Expression of liver X receptors in pregnancies complicated by preeclampsia

Preeclampsia is a pregnancy specific disorder associated with hyperlipidemia. Results from Paper I and II suggest that LXRs could be involved in regulation of lipid metabolism and uptake in human placenta. In Paper III we therefore wanted to investigate the expression of LXRα and LXRβ in human term placental tissue in preeclampsia and uncomplicated pregnancies. We also analyzed placental tissue concentrations of lipids and the fatty acid profile in a subpopulation of these samples.

Expression of LXRα, LXRβ and fatty acid transporter CD36 was significantly decreased in placental tissues while increased expression was observed for LXRα in adipose tissue from pregnancies complicated by preeclampsia. There was a positive correlation between placental LXRβ expression and placental free fatty acids in preeclampsia. Our results suggest a possible role for LXRα and LXRβ as transcriptional regulators in the preeclamptic situation.

4. Discussion

4.1 Methodological considerations

4.1.1 Model systems

In the papers included in this thesis, a human placental cell line, BeWo cells, and human term placental tissue were used to identify functions of the human placenta (Paper I, II and III). Supplemental information was obtained from human primary third trimester trophoblasts and other trophoblast cell lines (Paper I and II). We further used BeWo cells as a model system for trophoblast cells in several functional studies (Paper I and II).

Cultured cell lines permit a high degree of control of the experimental settings. In addition, many cultured cell lines have retained properties of their origin, and therefore represent useful model systems for studies of cellular molecular biology. Primary human cells can be obtained from patients with different genetic, nutritional and metabolic background. Larger standard error is therefore expected in experiments using these cells than in cell line experiments. On the other hand, primary cells in culture are expected to represent a more physiologically "natural" model system.

The BeWo cell line was the first commercial human trophoblast cell line to be developed (283). It was established from a malignant gestational trophoblast cancer (a choriocarcinoma), and has maintained many of the properties of primary cytotrophoblasts, such as the differentiation to syncytiotrophoblasts and secretion of hCG (284). Later, the cell line was also shown to have retained many of the properties of lipid transport and metabolism characteristic of human primary cyto- and syncytiotrophoblast cells. BeWo cells for example, have similar transport properties as primary trophoblasts, including a polarized transport of LCPUFAs and expression of many of the same genes involved in lipid metabolism (48;285;286). We

therefore believe that the BeWo cell line represents a good model system to study LXR-mediated regulation of lipid metabolism.

The isolation of primary cytotrophoblast cells is more time-consuming and expensive, and primary cells are more difficult to keep in culture for longer periods compared to BeWo cells. We have therefore chosen to use BeWo cells as our principal cell model system in Paper I and II. Primary trophoblast cells were also used to supplement and confirm the results observed in BeWo cells (Paper II).

Cell culture experiments are often supplemented with *in vivo* experiments. For these experiments, rodents are commonly used to obtain results from a more physiological, but still controllable laboratory setting. The access to the use of most human tissues for research purposes is limited. In placental research, the situation is quite different. There is an abundant access to human tissues, and although several general features of fetal nutrient uptake and utilisation are similar among mammalian species, major differences are present, especially concerning placental permeability. This is particularly important for research regarding lipid uptake and metabolism where permeability properties play a major role. These properties therefore limit the use of animal models in this field of human placental research. Species differences in lipid uptake and metabolism are reflected by a large species variation in fetal lipid accumulation during pregnancy. While fat constitutes 16% of body mass at birth in humans, it constitutes 10% in guinea pigs and only 2-4% in ruminants (reviewed in (2)). The net flux of fatty acid across the placenta also differs among species. In placentas having both maternal and fetal layers, such as sheep, pig and cat, the maternal fatty acid transfer is small (287-290), while in species in which the placenta is formed by layers of fetal origin, such as rabbit, rat, guinea pig and primates, the placental transport is much higher (290-295). It also appears that transport of individual FFAs across the placenta is determined largely by the placental characteristics of the species. While perfusion studies revealed little selectivity in the rate of transfer between different fatty acids in guinea pig and rabbits, similar studies in humans resulted in a preference for transfer of ARA and DHA (75;76;292;296).

On the basis of this information, we have therefore chosen not to use animal models for placenta studies in this thesis.

4.1.2 LXR agonists

At the initial stage of this work, the most common synthetic LXR agonist of that time, T0901317 (Paper I) was used. Later, when it was published that T0901317 could interfere with farnesoid X receptor (FXR) and PXR signalling pathways, we replaced the use of T0901317 with that of the selective LXRα/LXRβ agonist, GW3965 in our experiments (Paper II) (297;298). However, we cannot rule out the possibility that some of our findings in Paper I are not due to activation of PXR or FXR. Nevertheless, the confirmation of SREBP-1 and FAS induction by GW3965 in BeWo cells indicate reproducibility of some of the data with a new agonist (unpublished data and Paper II).

4.1.3 The use of qRT-PCR

During the last few years, quantitative real-time-polymerase chain reaction (qRT-PCR) has essentially taken over from the traditional Northern blotting method for quantitative measurement of mRNA expression levels (Paper II and III as compared to Paper I). The combination of real time quantification with high levels of amplification permits fast, precise and accurate results. Modern qRT-PCR reactions produce a nearly exact doubling of product at every cycle in the exponential face of the reaction, in order to obtain a quantitative relationship between the amount of starting target sample and amount of PCR product produced per cycle. The amplification permits the detection of down to one copy of mRNA, but any experimental biases such as differences in RNA yields and PCR inhibition will effect the amplification. To compensate for such differences, there is a general agreement on normalizing the data to endogenous controls. The ideal endogenous control should be expressed at a constant level within the entire experiment, but unfortunately, no such universal endogenous control exists. If the endogenous controls are regulated it

could in the worst cases produce results reflecting the regulation of the endogenous control, not that of the target gene (Figure 9). Each new cell and tissue experiment should therefore be accompanied by the test of endogenous controls to ensure the use of a control with constant expression levels throughout the experiment.

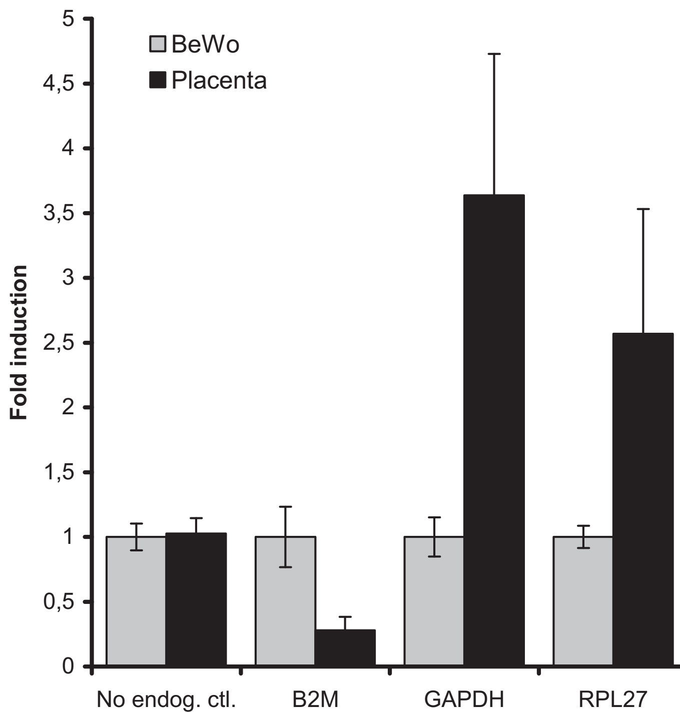


Figure 9: Expression of ACSL1 in untreated BeWo cells and human term placenta using three different endogenous controls, or no endogenous control. cDNA was synthesized from BeWo cell and human placenta total RNA. qRT-PCR was run using cDNA from equal amount of measured total RNA. The following assays were used: ACSL1, Hs00960561_m1; Beta-2-microglobulin (B2M), Hs99999907; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Hs99999905_m1; ribosomal protein L27 (RPL27), Hs01594520_m1. All materials were from Applied Biosystems, and the methods were run according to the manufacturer's instruction. Results were quantified using the ΔCt and ΔΔCt method. n = 3. Mean ± SEM is shown.

Unfortunately, more commonly than not, information on the testing of endogenous controls are not presented in published papers. In this thesis, each new experimental

setting has been accompanied by thorough testing of several endogenous controls to ensure the use of a non-regulated one (Paper II and III). When comparing basal expression in different tissues or cell lines, we were not able to find non-regulated endogenous controls (Figure 9). By assuring linearity between mRNA to complementary DNA (cDNA) and cDNA to PCR conversion, we ended up not using any endogenous control when comparing the expression between different tissues, assuming that equal amount of total RNA added to each well, represented equal amplification (Paper III). Although unusual to do, we believe this method represents a more correct normalization than using a regulated endogenous control, in our setting.

4.1.4 Problems with gene correlation studies using LDA cards

Low density arrays (LDA) have recently become commercially accessible and thus efficient new tools for detection of multiple genes simultaneously using qRT-PCR technology have become available. The access to the transcriptional information of dozens of genes when studying clinical materials as in Paper III, provides a good possibility to run correlations between the genes to search for statistically significant and possibly biological relevant correlations between genes. We were interested in running correlations between placental LXRα or LXRβ expression and placental expression of genes that are known LXR target genes in other tissues. Our intention when running the LXR-target gene correlation was to look for possible biological functions of the LXRs in placenta. Unfortunately, we came to the conclusion that we could not trust such a statistical use of our expression data. We observed a surprising number of biologically plausible significant correlations. As a control we further tested the correlation between genes that we did not believe had any biological correlation (e.g. endogenous controls), and the same surprising number of significant correlations was found between these genes. Our material was found to have linearity between total RNA, cDNA and PCR synthesis and potential PCR inhibition was not detected (Paper III). However, as with the run of any LDA cards, all the gene

expression data from every patient came from the same sample. Therefore, there was not a random distribution of technical differences between the genes analyzed in each sample. These systematic biases were probably so strong that whatever biological correlation present was impossible to distinguish from the technical differences in each sample.

However, the data are excellent for analysis of average gene expression, because in this case the technical variation is not systematic. Confounding factors are present in any laboratory experiment, but are not usually of any significance for the results because they are not systematically distributed. If separate sample handling all the way from taking the biopsy to the running of the qRT-PCR and single genes were performed, the use of correlation between genes could have been possible, but very time-consuming.

4.1.5 Gestational age

There is a significant difference in gestational age between our two study groups in Paper III, with the preeclamptic group delivering earlier than the control group. However, avoiding a gestational difference between prematurely delivered PE and term-delivered uncomplicated pregnancy is clinically not feasible when sampling placental tissue. This is due to the fact that premature deliveries of uncomplicated pregnancies are ethically unacceptable and therefore not available as a control group. If a baby and placenta is delivered prematurely, it is normally due to pathological conditions, such as inflammation/infection and therefore not suitable as a control. Correcting for gestational age is mathematically possible but it is not necessarily biologically correct, as premature delivered women with preeclampsia will generally have a more severe form of the disease than women delivered at term (100). Still we cannot exclude that differences in gestational length between the study groups could potentially affect our results and conclusions in Paper III.

4.2 Discussion of results

4.2.1 Roles for the LXRs in lipid metabolism in placenta

Numerous LXR target genes have been identified in tissues involved in lipid metabolism, such as liver, muscle and adipose tissue. When considering that the human placenta is secreting 50-200 times more fatty acids than rat hepatocytes, we expected LXRs to have a regulatory role in controlling lipid metabolism in placenta (26). Our initial analyses of SREBP-1c, FAS and LXR suggested a role of LXR in lipogenesis in human placental BeWo cells (Paper I). To further understand the role of LXRs in placenta, we performed microarray and qRT-PCR on total RNA isolated from human term placental trophoblast cells stimulated with the synthetic LXR agonist, T0901317 (unpublished data and Paper II). These data confirmed the induction of SREBP-1 and FAS gene expression in primary trophoblast (Paper II and unpublished data) observed in BeWo cells (Paper I). In addition, the expression of other known direct LXR target genes was induced (~6-fold induction of SCD-1, ~4-fold induction of ABCG1, ~3-fold induction of ABCA1 and an ~2.5-fold induction of ACC). This demonstrates a major regulatory role for LXRs in placental trophoblast cells apart from other metabolic tissues, such as macrophages, adipose tissue and liver (unpublished data, (198)). The microarray analysis further revealed several hitherto unknown potential LXR target. One of these genes, lipin-1, was investigated further. However, theoretical promoter analysis and gene expression studies in BeWo cells and LXR$\alpha^{-/-}$/$\beta^{-/-}$ mice suggested that it is not a direct LXR target gene (unpublished data). Then, ACSL3 was also identified as a potential LXR target gene, which was induced by the LXR agonist T0901317, on the microarray. Theoretical analysis of the ACSL3 promoter, using a consensus LXRE element, identified a potential theoretical LXRE, which was shown subsequently to be functional (Paper II). Further analysis in liver, muscle and adipose tissue in mice suggested that this regulation was not restricted to placenta (Figure 10).

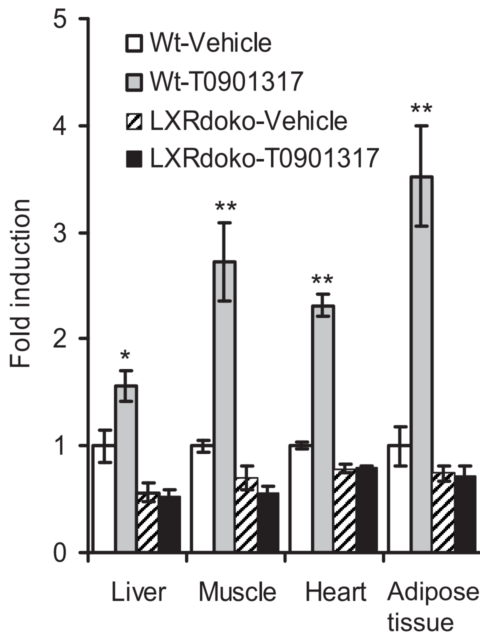


Figure 10: LXR mediate regulation of ACSL3 in different tissues in mice. Male LXRα $^{+/+}$/β $^{+/+}$ and LXRα $^{-/-}$β $^{-/-}$ mice were gavaged with vehicle (1% CMC) or T0901317 (30 mg/kg) 24 hours prior to scarification. Total RNA (Trizol, Invitrogen) from liver, skeletal muscle, heart and white adipose tissue were analyzed for expression of Acsl3 (Mm01255804_m1) and Fas (Mm00662319_m1) by qRT-PCR (all from Applied Biosystems, according to the manufacturers instructions) normalized to Tbp (Mm00446973_m1). The results are presented as mean ±SEM, relative to control (n = 4-6 for each group). P-values * < 0.05 and ** < 0.01 C).

In addition to differential expression of target genes in both primary trophoblasts and BeWo cells, we identified an LXR-mediated increased *de novo* fatty acid biosynthesis, increased uptake of several types of fatty acids (OA and LCPUFAs) and increased concentrations of FFA and complex lipids such as TAG, phospholipids and cholesteryl esters in BeWo cells (Figure 11, Paper I and II). These results are in line with the observed increased LXR-mediated expression of SREBP-1, FAS and ACSL3 in BeWo cells and primary trophoblasts (Figure 11, Paper I and II). We did not observe increased β-oxidation of fatty acids when incubating BeWo cells with LXR agonist (unpublished data). The expression of genes leading to increased uptake and lipogenesis and lack of regulation of β-oxidation, suggests LXR as a regulator of anabolic pathways of lipid metabolism in placental trophoblast cells. All these above

multiple roles of LXR suggest a coordinating role of LXR in lipid metabolism in placental trophoblast cells and perhaps in placental tissues (figure 11).

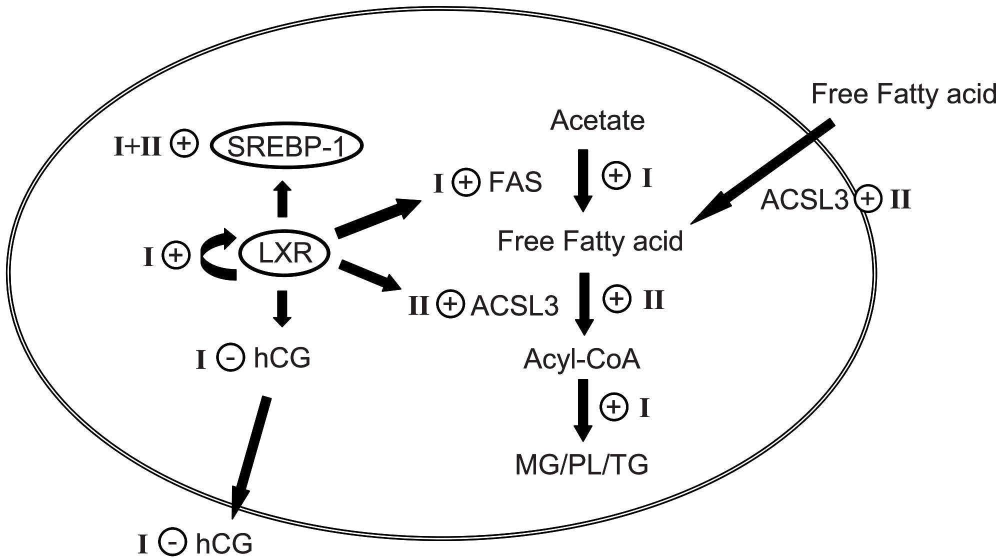


Figure 11: Effects of LXR on human placental trophoblast cells. Summery of Paper I and II. The influence of LXR is marked with +. The results obtained from each paper is indicated (I = Paper I, and II = Paper II).

Numerous observations in this thesis demonstrate overlapping functions between the regulatory roles for LXR in placenta and other lipid metabolizing tissues. Although the regulation in different tissues appears to be similar, the physiological role could be quite different. ACSL3 is highly expressed in placenta, brain and intestine and in inguinal adipose tissue (153). Based on the LXR-mediated ACSL3 induction observed in several tissues (Figure 10), we suggest that a similar LXR-mediated regulation of ACSL3 could also have important roles in tissues with high levels of ACSL3 expression. The physiological consequences of such a regulation could differ depending on the function of the tissue. The ACSL3-mediated uptake of LCPUFAs in brain could be important for the high need for LCPUFAs in this organ during fetal development (60). In the intestine, the regulation could be important for LCPUFAs

uptake from the diet. In lymph node-containing adipose tissues such as the inguinal adipose tissue, LCPUFA uptake play a role in the interaction with the immune system (reviewed in (299)). Increased PUFA accumulation has been reported in node-containing tissues, and is thought to be important for the production of prostaglandins and leukotriens (300).

Insulin has a major effect on lipid metabolism in liver, muscle and adipose tissue, and LXR is upregulated by insulin. LXR is involved in insulin-mediated upregulation of SREBP-1, and so it would have been interesting if LXR-insulin stimulation had any influence on placental gene expression (246). However, when performing microarray on primary human trophoblasts incubated with insulin alone or insulin and T0901317 together, no insulin-mediated regulation of any genes was observed compared to the respective controls (cells incubated with T0901317 alone, or cells incubated without T0901317 or insulin added; unpublished data). Limited impact of insulin on placental lipid metabolism function has also been reported previously (301). The lack of response to insulin also indicates major differences between placenta and other lipid metabolizing and insulin sensitive tissues such as adipose tissue, muscle and liver in terms of LXR regulation.

4.2.2 LXR and ACSL3 functions on lipid droplets

Our research group has previously observed an increase in lipid droplet size when incubating preadipocytes with LXR agonist during differentiation to adipocytes, but the mechanisms behind this increase are not fully understood (225). ACSL3 is one of the main proteins associated with lipid droplets in hepatocytes, and the concentration of ACSL3 on lipid droplets correlated with the TAG biosynthesis in Huh7 hepatocytes (155;156). The identification of ACSL3 as a direct LXR target gene (Paper II) and the presence of this regulation in adipose tissue (Figure 10) therefore suggests a possible mechanism for the observed LXR-mediated increase in lipid droplet size in adipocytes, and perhaps also in other tissues. The localisation of ACSL3 on the lipid droplet would then perhaps increase the uptake and activation of

fatty acids and thereby increasing the production of TAG and the size of lipid droplets. A plasma membrane location of ACSL3 is possibly not necessary for the fatty acid uptake function of this gene as fatty acid uptake mediated by FATP4 has been reported to occur by FATP4 proteins exclusively located on intracellular organelles (140). ACSL3 could be involved in the channelling of fatty acids for TAG biosynthesis and further storage in lipid droplets. This suggests that ACSL3 together with LXR could have an important role in protecting cells from toxic effects of FFA and subsequent insulin resistance in tissues such as liver, muscle or adipose tissue.

4.2.3 LXR in preeclampsia and atherosclerosis

To the best of our knowledge, there is no information available on the levels of endogenous LXR agonists (oxysterol) in placenta in preeclampsia. In Paper III we observed a highly significant downregulation of both LXRs in preeclamptic placentas as compared to controls. An autoregulation of LXRα has been described in some tissues including BeWo cells (Paper I, (302;303)), suggesting that this autoregulation is present in placenta. Dyslipidemia, including increased oxLDL is a common feature of preeclampsia (110). It could perhaps result in increased placental concentrations of endogenous LXR agonists. However, the reduced placental expression of both LXRs in preeclampsia compared to uncomplicated pregnancies in Paper III suggests that there is not an increased placental concentration of LXR agonists in preeclampsia. Combined with reduced LXR expression, this suggests a reduced LXR activity in the preeclamptic placenta. However, ligand-independent factors such as cofactor-interaction, and post-translational modifications of LXRs could also be important for the regulation of placental LXR expression and activity in preeclampsia.

Preeclampsia and cardiovascular disease have many risk factors in common including endothelial dysfunction, obesity, insulin resistance, hypertension and dyslipidemia (110;112;113;304). There are strong indications for a protective role of LXR in atherosclerosis by the reverse cholesterol transport of endothelial cells and macrophages. The reduced expression of both LXRs in preeclamptic placentas (Paper

III) could result in a reduced reverse cholesterol transport from placental macrophages and endothelial cells. Endothelial dysfunction is a common feature of preeclampsia and plaque formation is observed in acute atherosis in Preeclampsia (115). Therefore, we speculate that the reduced expression of LXR may exacerbate these "atherogenic" characteristics of preeclampsia. We did not however observe a correlation between total placental cholesterol levels and LXRα or LXRβ expression in placenta (Paper III). Separate analysis of cholesterol and cholesteryl ester levels in placental macrophages and endothelial cells would be necessary to clarify this issue.

Our gene expression data from preeclamptic and control placentas suggest that LXR is not the main regulator of many known LXR target genes in placenta in Preeclampsia (Paper II). Although not anticipated, the results are not surprising, as complex mechanisms involving many unknown factors are involved in the regulation of genes. The identification of a gene as an LXR target gene in one tissue under certain conditions is therefore just an indication of a functional relationship between these genes in other tissues and conditions. Especially in a clinical situation, with many unpredictable potential regulatory factors, it is often difficult to predict the regulation of any gene based on such knowledge.

4.2.4 Is there a lipid metabolism independent role for the LXRs in placenta?

Regulatory roles for LXRs apart from lipid metabolism have been reported in macrophages (196;305;306). Pavan *et al* (279) and our results on hCG in Paper I also indicate a non-lipid metabolic role for LXR in placenta. Pavan *et al* showed that LXR activation inhibits a cell model of first trimester trophoblast invasion. This is in line with our observation of an inhibitory LXR-mediated regulation of hCG, as hCG is important for the retaining of the implanted blastocyst in the uterine wall (Paper I). Furthermore, in Paper I we observed increased expression of LXRβ during BeWo cell differentiation from cyto- to syncytiotrophoblasts. hCG is reported to increase

differentiation of trophoblasts (12). These findings suggest that LXRβ could be involved in inhibiting trophoblast differentiation from cyto- to syncytiotrophoblasts.

Many genes involved in inflammation are reported to be increased in preeclampsia and many of these genes are down regulated by LXRs. These genes include inducible nitric oxide synthase, COX-2, matrix metallopeptidase-9, interleukin 6 and tumour necrosis factor α (196;305;306). Thus, it is possible that LXR has hitherto unrecognized roles in placenta in preeclampsia.

4.2.5 ACSL3 in placenta

ACSL3 appears to have a central position in fatty acid activation and transport in placental trophoblast cells, but also ACSL1 and ACSL4 were highly expressed in these cells in our study (Paper II). However, our data suggest that ACSL3 is the main ACSL involved in fatty acid activation in placental trophoblast cells, as silencing of ACSL3 reduced the total oleoyl-CoA synthetase activity by 50% in BeWo cells (Paper II). Silencing of ACSL3 further reduced the basal fatty acid uptake by 30% in BeWo cells (Paper II). Silencing of ACSL3 could be accompanied by a compensatory increase in one or more of the other ACSLs, as is often observed between homologous genes in lipid metabolism. We did however not observe any alternations in the mRNA expression of any members of the ACSL family when ACSL3 was silenced in BeWo cells (unpublished data). Thus, ACSL3 appears to have a unique role in trophoblast cells that cannot be compensated by other members of the ACSL family, and this suggests a predominant role of ACSL3 in placental fatty acid uptake and metabolism.

Activation of PPARα, δ and γ, that are key regulators of numerous genes involved in fatty acid transport and lipid metabolism, did not have any effect on *ACSL3* expression in BeWo cells (unpublished data). These results agree with another study which showed that activation of PPARα had no effect on ACSL3, but increased the expression of ACSL1 in rodent heart (307;307).

Although insulin, fatty acids, oncostatine and Vitamin D_3-mediated regulation of ACSL3 has been reported (307-309), to the best of our knowledge our analysis is the first to demonstrate that ACSL3 is being directly regulated through any identified cis-regulatory element in the promoter (an LXRE, Paper II).

5. Conclusions

The conclusions from the present study can be summarized by listing the following main findings:

1. LXRα and LXRβ are expressed in placenta and BeWo cells (Paper I and III).
2. LXR activation increases *de novo* fatty acid synthesis, fatty acid activation and fatty acid metabolism into complex lipids in BeWo cells (Paper I and II).
3. LXR activation decreases the expression and secretion of hCG from BeWo cells (Paper I).
4. LXR activation increases the uptake of OA and LCPUFAs in BeWo cells (Paper II).
5. ACSL3 is a direct LXR target gene in BeWo cells (Paper II).
6. LXR-mediated increased fatty acid uptake and activation are dependent on ACSL3 (Paper II).
7. LXRα and LXRβ are downregulated in preeclamptic placentas compared to placentas from uneventful pregnancies (Paper III).

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