Fatty acid uptake, transport and storage in placenta in normal and dyslipidemic pregnancies

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List of Papers

Paper 1:

Tobin KAR, Johnsen GM, Staff AC, Duttaroy AK.

Long-chain polyunsaturated fatty acid transport across human placental choriocarcinoma (BeWo) cells.

Placenta. 2009 Jan; 30(1):41-7. Epub 2008 Nov 17.

Paper 2:

Johnsen GM, Weedon-Fekjær MS, Tobin KAR, Staff AC, Duttaroy AK.

Long-chain polyunsaturated fatty acids stimulate cellular fatty acid uptake in human placental choriocarcinoma (BeWo) cells.

Placenta. 2009 Dec; 30(12):1037-1044. Epub 2009 Oct 31.

Paper 3:

Weedon-Fekjær MS, Johnsen GM, Sugulle M, Anthonisen EH, Nebb HI, Duttaroy AK, Staff AC.

Expression of liver X receptors in pregnancies complicated by PE.

In revision.

Paper 4:

Johnsen GM, Weedon-Fekjær MS, Tobin KAR, Sugulle M, Kliman HJ, Duttaroy AK, Staff AC.

Increased Adipose Differentiation Related Protein (ADRP) expression in preeclamptic placenta.

Submitted.

Abbreviations

AA	Arachidonic acid
ACS	Acyl-CoA synthetase
ACSBG	Acyl-CoA synthetase, bubblegum
ACSL	Acyl-CoA synthetase, long-chain
ADRP	Adipose differentiation-related protein
ALA	A-linolenic acid
APOE	Apolipoprotein E
CAV1	Caveolin-1
CD36/FAT	CD 36 molecule (thrombospondin receptor)/fatty acid translocase
cDNA	Complementary DNA
ChREBP	Carbohydrate responsive element-binding protein
CoA	Coenzyme A
DHA	Docosahexaenoic acid
DM	Diabetes mellitus
EPA	Eicosapentaenoic acid
FABP	Fatty acid binding protein
FABPpm	Plasma membrane-associated FABP
FATP	Fatty acid transport protein
FFA	Free fatty acid
FGR	Fetal growth restriction
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
hCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HELLP	Hemolysis, elevated liver enzymes, and low platelet count
IL-6	Interleukin 6
IUGR	Intrauterine growth restriction
LA	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid
LDA	Low density array
LDL	Low density lipoprotein
LDLR	LDL receptor
LPIN1	Lipin-1
LPL	Lipoprotein lipase
LSDP5/PLIN5	Lipid storage droplet protein 5/perilipin 5
LXR	Liver X receptor
NR	Nuclear receptor
OA	Oleic acid
oxLDL	Oxidized low density lipoprotein
PA	Palmitic acid
PCR	Polymerase chain reaction
pFABPpm	Placenta FABPpm
PLIN1	Perilipin 1

PPAR	Peroxisome proliferator activated receptor
PUFA	Poly unsaturated fatty acid
qRT-PCR	Quantitative real-time reverse transcriptase PCR
ROS	Reactive oxygen species
RXR	Retinoid X receptor
S3-12/PLIN4	S3-12/perilipin 4
SGA	Small for gestational age
SPE	Superimposed preeclampsia on diabetes mellitus
SREBP	Sterol regulatory element binding protein
TAG	Triacylglyceride
TBP	TATA binding protein
TIP47/PLIN3	Tail-interacting protein, 47 kDa/perilipin 3
TNF-α	Tumor necrosis factor alpha
VLDL	Very low density lipoprotein
YWHAZ	Tyrosine 3/tryptophan 5 -monooxygenase activation protein, zeta polypeptide
18S	18S ribosomal RNA

1. Introduction

1.1. Placenta

The placenta is a transient organ that supports the growth and development of the fetus (Figure 1). It provides exchange of oxygen, nutrients and waste products between mother and fetus. It functions as a substitute for the lungs, intestines and kidneys of the fetus until these organs are fully developed and can perform these functions on their own. The nutrients transported across the placenta to the fetus include amino acids, carbohydrates, lipids, vitamins, minerals and water. The waste products transported from the fetus to the mother are carbon dioxide and urea [1]. Placenta is also an important endocrine gland responsible for the production of many hormones important for the maintenance of pregnancy. Other placental functions include energy metabolism to support the placentas own needs, modification of nutrients destined for the fetus, maintenance of a immunological barrier, transfer of heat and detoxification of xenobiotics.

Placentation

After conception the fertilized egg develops into a blastocyst. The inner cell mass of the blastocyst develops to become the fetus while the outer cell mass becomes the placenta and the fetal membranes. The outer cell layer consists of highly specialized placental cells called trophoblasts (Figure 2). The trophoblasts invade the endometrium in a tightly controlled manner that is important for the implantation and placentation process. Failure to control the invasion process results in a very aggressive cancer, choriocarcinoma [2].

Placentation occurs from about weeks 6 to 18 of pregnancy [4]. The maternal and fetal circulations are separated by the chorionic villi. The chorionic villi are finger-like structures covered with an outer layer of syncytiotrophoblast, surrounding a cell layer of cytotrophoblasts. Underneath the cytotrophoblasts there are stromal cells and fetal endothelial cells that line the fetal vessels in the chorionic villi. The villi are divided in floating and anchoring villi; the floating villi are bathed in maternal blood and function as the place of exchange of gases and nutrients between mother and fetus, whereas the anchoring villi attach the placenta to the endometrium.

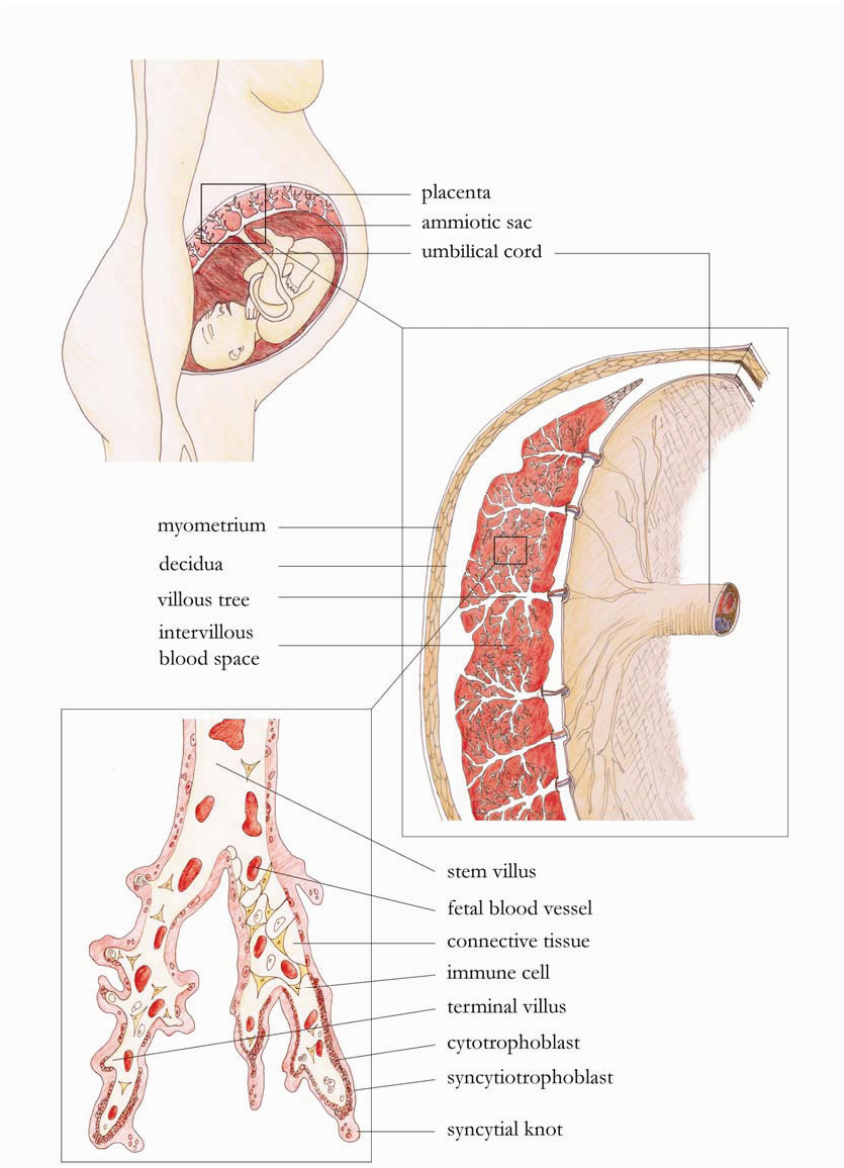

Figure 1: Schematic overview of the placenta, the placental structure and a placental terminal villus. Modified from Benirschke & Kaufmann [3].

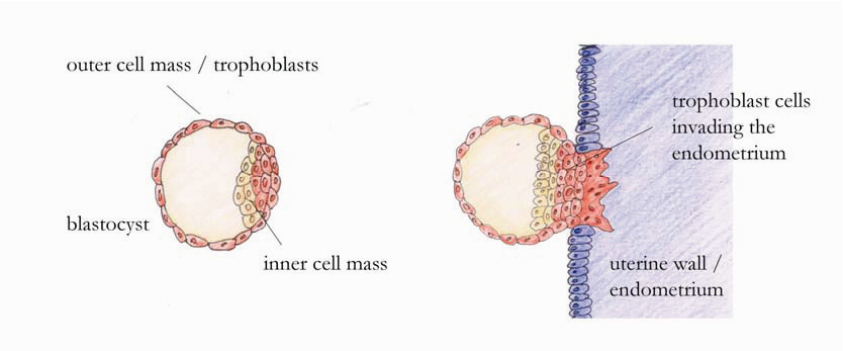

Figure 2: Implantation of the blastocyst. Within 4-5 days after fertilization the embryo develops into a blastocyst, a spherical structure composed on the outside of trophoblasts and on the inside the inner cell mass. The inner cell mass will develop into the fetus and the trophoblasts will develop into the placenta and the fetal membranes. At about day 6 the blastocyst will attach to the uterine wall (endometrium) and the trophoblast cells will start invading the endometrium and by that the process of placentation begins.

In the first trimester of gestation the cytotrophoblasts within the floating villi proliferate and differentiate into the multinucleate syncytiotrophoblast by fusion. The syncytiotrophoblast is subject to continuous renewal and the aged nuclei form syncytial knots that buds out from the villi and can be released into the maternal circulation as cellular debris. The cytotrophoblasts within the anchoring villi can fuse to form syncytiotrophoblast or they form columns of extravillous trophoblasts. The extravillous trophoblasts invade the decidua (the gestationally altered maternal uterine endometrium during pregnancy) and are believed to migrate either interstitially (through the decidual tissue) or retrogradely (through the spiral arteries) into the maternal decidua. They transform the narrow spiral arteries into wide, thin-walled and dilated vessels, which transport the arterial blood from the maternal side to the placenta (Figure 3).

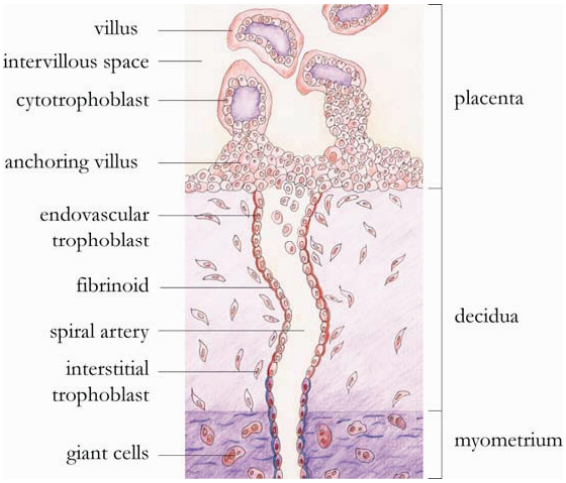

Figure 3: Trophoblast invasion. The villous cytotrophoblasts are trophoblast stem cells that can differentiate into two major cell lineages; the syncytiotrophoblast and the invasive extravillous trophoblasts. In the first trimester the cytotrophoblasts proliferate and form cell columns that anchor the placenta to the uterus. Two types of extravillous trophoblasts are derived from the cell columns; the interstitial and the endovascular invasive trophoblasts. The interstitial invasive trophoblast migrates through and invades the uterine tissue, whereas the endovascular invasive trophoblast migrates to the maternal uterine spiral arteries. There the trophoblast displaces and replaces the endothelial cell lining of the spiral arteries and plays a role in the degradation of the muscle and elastic coat which is replaced with fibrinoid tissue. The trophoblasts migrate deep into the uterine myometrium where they fuse to form giant cells. Modified from Moffet-King [5].

The blood flow to the placenta changes dramatically during early pregnancy. In the first trimester the spiral arteries are in the process of being transformed and are essentially blocked by a column of trophoblast cells so the maternal blood flow to the placenta is at a minimum. Ultrasound measurements show that the uteroplacental blood flow increase significantly at week 12 and reaches maximum at week 14 of gestation [6]. Burton *et al* found by studying hysterectomy samples that the maternal spiral arteries are blocked by trophoblasts at week 6-8 and that this blockage gradually disappears between week 8-12 of gestation so that a substantial blood flow to the placenta is not established until week 12 [7]. The oxygen tension in the intervillous space increases gradually from

week 8-12, and also coincides with an increase in anti-oxidant systems in placental tissue [8].

This means that before week 12 the invading cytotrophoblasts are subjected to an oxygen gradient, with increasing oxygen tension from the intervillous space to the maternal blood in the myometrium. This gradient could be important in the regulation of trophoblast invasion [9]. At the end of the first trimester with full onset of the uteroplacental circulation, the oxygen concentration in the placenta rises three-fold and poses new challenges for the trophoblast cells [10]. The syncytiotrophoblast mitochondria are particularly sensitive to high oxygen levels, as they have low levels of antioxidants [10]. It has been found that excessive levels of antioxidants in cytotrophoblasts inhibit syncytialization and therefore the oxygen level is also important for the differentiation of cytotrophoblasts [10].

The area of the placenta that is available for exchange of nutrients is increasing rapidly until week 26 of gestation, when the villous surface area exceeds 4 m^2 [3]. At the end of the second trimester the mature intermediate villi appear and a few weeks later the terminal villi appear and increase rapidly in numbers from this point onwards. The fetal blood flow to the placenta also increases exponentially with gestational age and the increase in exchange area. Increased blood flow from the maternal side and the appearance of terminal villi coincide with a marked increase in fetal fat deposition.

1.2. Fatty acids and their importance in fetal nutrition

Placental uptake of maternal fatty acids (FAs) is essential for growth and development of the feto-placental unit. During the last trimester of pregnancy the fetus accumulates large amounts of fat, and free fatty acids (FFAs) are the main class of naturally occurring lipids transferred across the placenta [11]. The fetal circulation is enriched with long-chained polyunsaturated fatty acids (LCPUFAs) compared to the maternal circulation.

Fatty acids

FAs are a normal constituent of the human diet and they are derived from animal or vegetable fats. They serve as building blocks of phospholipids and glycolipids, they

are fuel molecules stored intracellularly as triacylglycerides (TAG) (predominantly in adipocytes), and they are precursors for hormones and intracellular messenger molecules. FAs are carboxylic acids with hydrocarbon chains of varying lengths and a carboxyl group at the terminal end. Natural FAs normally have between 4 to 28 carbons in their chain, and most have an even number of carbon atoms because their biosynthesis involves acetyl-CoA, a coenzyme carrying a two-carbon-atom group. The FAs are classified by the numbers of double bonds in their hydrocarbon chain or the degree of saturation. A saturated FA has no double bonds; a mono saturated FA has one double bond, while polyunsaturated FAs (PUFAs) have 2 or more double bonds. The location of the first double bond in the hydrocarbon chain relative to the methyl end (termed n or ω) is of importance for the physiological function of the FA (Figure 4).

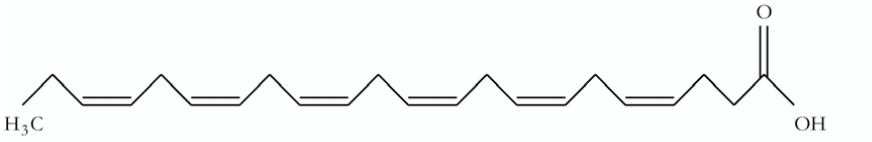

Figure 4: Structure of the n-3 long-chain polyunsaturated fatty acid docosahexaenoic acid (DHA, 22:6n-3), with 22 carbon atoms in the hydrocarbon chain and 6 double bonds. The first double bond relative to the methyl (H_3C) end is located in the n-3 position.

Essential fatty acids

The FAs linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) are called essential FAs because they cannot be synthesized by the body itself, because humans lack the enzymes necessary for introducing a double bond below the n-9 position. Consequently these FAs must be derived from the diet. The primary producers of these FAs are plants and marine microalgae, and together with oils from vegetables, seeds and nuts they are the main source for the essentials FAs in our diet. The long chained n-3 and n-6 PUFAs may be synthesized in the body from the essential FAs (Figure 5), however this conversion is not very efficient and therefore it is important to obtain these from the diet as well. The main dietary source of n-3 PUFAs, such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), is oily

fish. The main source of n-6 PUFA, such as arachidonic acid (AA, 20:4n-6), is animal fat and eggs.

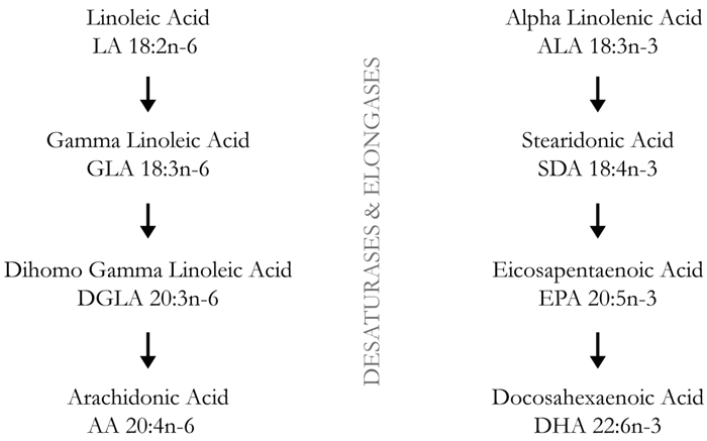

Figure 5: Conversion of the essential fatty acids linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) by desaturase and elongase enzymes to form the long-chain polyunsaturated fatty acids arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3), respectively.

Physiological functions of LCPUFAs

The LCPUFAs are important constituents of cellular membranes. They increase the fluidity of membranes due to their high degree of unsaturation and may reduce the cholesterol content of membranes and thus alter their structure and organization [12]. LCPUFAs are also important in signal transduction and eicosanoid production. AA is a precursor of several classes of signaling molecules called eicosanoids, including prostaglandins, prostacyclins, thromboxanes and leukotrienes. These eicosanoids are important for the regulation of several cellular processes such as immune response, inflammation, coagulation and vasoconstriction [13]. LA is a constituent of complex lipids in the permeability barrier of the skin [14]. EPA is also a precursor for eicosanoids but it is a poorer substrate than AA and the resulting eicosanoids are less potent [15,16]. High levels of n-3 PUFAs may reduce AA-induced signaling because they replace AA in the incorporation of membrane phospholipids and also compete for enzymes involved

in the eicosanoid synthesis, and thereby contribute to an anti-inflammatory effect [17]. DHA is the most abundant n-3 PUFA in tissues, especially in brain and retina [14] and it is important in fetal development.

The PUFAs may also regulate gene expression due to their effect on several transcription factors. Both n-3 and n-6 PUFAs are natural ligands for the transcription factors PPARs and SREBP-1 which control various genes of inflammatory signaling and lipid metabolism [17].

Importance of LCPUFAs in fetal nutrition

The fetus depends on maternal supply of essential FAs and LCPUFAs. Even if the essential FAs (LA and ALA) are obtained from the maternal circulation, they must be elongated and desaturated to be converted into LCPUFAs. Basal expression of Δ-5 and -6 desaturase and elongase have been reported both in fetal liver and in the placenta [18,19], however their enzyme activities are low. Both AA and DHA are important structural components of the nervous system and adequate supply of these could be critical at the time of embryonic organogenesis as well as during the growth of the fetal brain, which is at its peak in the last trimester [20].

Supplementation of pregnant women with ALA did not lead to increased DHA levels in the umbilical cord [21]. On the other hand, several studies report that intake of EPA and DHA by pregnant women raises the content of these FAs in fetal tissues [22]. Increased consumption of n-3 LCPUFAs from fish and fish oils during pregnancy has also been suggested to be beneficial to the fetus and to lower the risk of PE [23,24]. Hence, an adequate maternal dietary intake of LCPUFAs and subsequent adequate transport across the placenta is critical for the development for the fetus.

Fatty acid metabolism in the placenta

During the first trimester of pregnancy there is an accumulation of maternal body fat that allows accumulation of LCPUFAs in adipose tissue, which can be mobilized in the latter half of the pregnancy and transferred to the fetus. FAs are mainly stored in the body as TAGs. In order to be utilized by the cells as fuel, the FAs are processed in three steps. First the TAGs are degraded to FAs and glycerol by lipase enzymes in a process called lipolysis, which takes place on the outer plasma membrane

of the cell. The FFAs are then bound to serum albumin which transports them across the membrane. Inside the cell, on the outer membrane of the mitochondria, the FAs must be activated by the attachment of coenzyme A (CoA) before they can be transported into the mitochondria where they are degraded. This activation is catalyzed by the enzyme acyl-CoA synthetase (ACS). In the degradation process the FA is oxidized to introduce a double bond. This double bond is subsequently hydrated to introduce an oxygen atom and thereby the FA is converted to an alcohol. The alcohol is then oxidized to a ketone and finally a two-carbon unit is cleaved off by CoA to yield acyl-CoA and a FA, which is two carbon atoms shorter than the original FA. This process can be repeated until the FA is completely converted in to acyl-CoA. FA degradation and synthesis are reverse processes regulated in response to diet by a host of different hormones and enzymes.

Cholesterol

Cholesterol is an important component of structural membranes and a precursor of steroid hormones and oxysterols. Cholesterol is distributed in cells as free cholesterol in the plasma membrane and internal membranes and as cholesteryl esters stored in lipid droplets [25,26]. The plasma membrane is highly enriched in cholesterol which constitutes about 30 mole percent of the lipids [27] and contributes to the rigidity of the membrane and the organization of specialized membrane domains called lipid rafts. Cells need a constant supply of cholesterol in order to maintain their membranes [28], but on the other hand accumulation of excessive free cholesterol is toxic to the cell [26,29]. Correlation between maternal hypercholesterolemia and fatty streak formation in fetal aorta [30] suggests the existence of a placental cholesterol transport system.

1.3. Fatty acid transport in the placenta

Fetal lipid deposition increases exponentially during gestation and 90% of the fat is deposited in the last 10 weeks of pregnancy (reaching 7 g/day) [31]. The fetus is capable of synthesizing FAs, however it obtains most of its FAs from the maternal circulation via the placenta [32]. The barrier between the maternal and fetal circulation consists of trophoblast cells connective tissue and fetal endothelial cells. All these cell

layers may contribute to the transport across the placenta; in addition the placental metabolism also contributes to transfer of nutrients from the maternal to the fetal side. There are different mechanisms involved in placental transport such as simple diffusion, facilitated diffusion and active transport [1].

Most maternal FAs are transported to the fetus as TAGs in lipoprotein particles [33]. TAGs cannot directly cross the placental barrier and consequently a complex system of placental transport has developed. This system involves several receptors and enzymes such as LDL receptor, VLDL/apoprotein E receptors, placental lipoprotein lipase, placental phospholipase A_2 and intracellular lipases [33,34,35,36,37,38,39,40]. Alterations in placental LPL activity and LDL receptor protein expression are associated with IUGR [41,42,43,44], and imply that the placental lipid transport system is important for adequate fetal growth.

Maternal FAs, as well as FAs newly synthesized in the placenta, are transported across the trophoblasts by diffusion or by active transport [45,46]. The existence of a complex FA transport system comprising multiple membrane and cytoplasmic proteins responsible for FA transport and metabolism in human placenta has been demonstrated [47], including fatty acid binding proteins (FABPs) and CD36 (also named FAT for fatty acid transporter), and fatty acid transport proteins (FATPs) (Figure 6).

Preferential uptake of LCPUFAs

Fetal blood is especially enriched in LCPUFAs compared to the maternal circulation at the time of birth [48,49,50,51], but how this selective enrichment occurs is largely unknown. Active transfer of FAs from the maternal circulation to the fetus has been demonstrated by *in vitro* and *in vivo* experiments. Pregnant women were administered [^{13}C]-labeled FAs four hours previous to elective cesarean section, and the [^{13}C]-labeled FAs were detected in both placental tissue and cord blood at the time of delivery [48]. Preferential transfer of LCPUFAs has also been demonstrated using perfused human placenta [52,53].

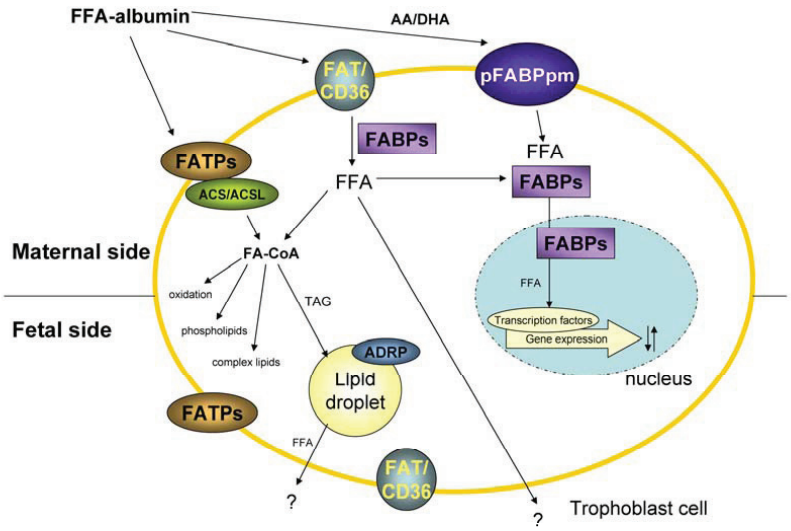

Figure 6: Schematic overview of proteins involved in fatty acid transport in trophoblast cells. Modified from Duttaroy [20].

Fatty acid binding and transport proteins

FABPs are cytoplasmic proteins involved in the intracellular trafficking of FAs in different tissues, and they all bind long chained FAs [54,55]. Several isoforms have been detected in human placental trophoblasts, and the expression of FABP1 and 4 was enhanced by hypoxia and PPARγ agonists [55].

The plasma membrane FABP (FABPpm), the FATPs and FAT/CD36 are the main trophoblast membrane proteins. FABPpm is a peripheral membrane protein thought to act as an extracellular FA receptor that facilitates the diffusion of FAs through the membrane [31]. The placenta specific protein p-FABPpm has been found exclusively on the maternal facing microvillous membrane [47], and has been shown to preferentially bind DHA and AA [56]. The p-FABPpm is similar in size (~40 kDa) to the ubiquitous version of the same protein, which is found in most mammalian cells, but the amino acid composition is different [46]. A specific placental version of

FABPpm has been described [57] located on the microvillous membrane of the placenta facing the fetal circulation [58].

FATPs are integral proteins with membrane spanning regions and are thought to function as FAs transporters. There are six members of this family identified so far and they differ in tissue expression, subcellular location and substrate specificity [59,60,61,62,63,64]. FATP1, 2, 3, 4 and 6 have been shown to be expressed in placenta [65,66]. FATP1 and 4 have also been found expressed in trophoblast cells [66]. The FATPs have inherent ACS activity, which enables catalyzation and conversion of FFAs into acyl-CoAs [67]. It has also been reported that FATP1 and 4 is upregulated by PPARγ/RXR agonists in primary human trophoblasts [68].

FAT/CD36 is a transmembrane FA transporter and scavenger receptor for oxidized LDL [47,69,70,71]. It is expressed in human trophoblast cells and is involved in the uptake of long chain FAs in placenta [47,72]. FAT/CD36 was also shown to be associated with lipid rafts (which are microdomains of the plasmamembrane rich in cholesterol and sphingolipids and important in cell signaling) in adipocytes [73]. Lipid rafts are important for virus-induced syncytium formation [74], but it is not known whether they are involved in comparable processes in syncytiotrophoblast formation in placenta. Their presence in syncytiotrophoblast was demonstrated for the first time in work by Linton and colleagues [75,76].

Caveolin-1 is another protein involved in cellular uptake of FAs and cholesterol. It is the main structural element of caveolae [77], a subclass of lipid rafts forming characteristic flask shaped invaginations that can be distinguished by electron microscopy. Caveolae are dynamic structures that can bud from the plasma membrane, forming cytoplasmic vesicles involved both in receptor-mediated uptake of solutes into the cell [78] and in transcytosis (vesicular transport through the cell membrane) [79]. The unique lipid raft environment attracts key signaling proteins, such as G proteins, protein kinase C, protein kinase A, prostacyclin synthase and endothelial nitric oxide synthase (eNOS) [80,81]. Recent studies have shown that caveolin-1 can associate with lipid bodies in a reversible and lipid regulated fashion [82,83]. Caveolin-1 can also bind FAs [84], and some data imply that lipid rafts might be involved in regulating LCPUFA uptake [85,86,87,88,89].

Long chain acyl-CoA synthetases (ACSLs)

The acyl-CoA synthetases are enzymes necessary for *de novo* lipid synthesis, FA degradation and remodeling of membranes. They activate FAs by converting them into membrane impermeable acyl-CoAs, this facilitates FA transport by trapping the FAs inside cells. The acyl-CoAs have numerous metabolic fates within cells, including incorporation into triacylglycerols (TAG) and membrane phospholipids, as substrates for ß-oxidation and protein acylation, and as ligands for transcription factors. They have been found in all organisms investigated and have an essential role that has been conserved through evolution [90]. The ACSLs can be divided into five subfamilies based on their FA chain length preference; acyl-CoA synthetase short chain (ACSS, C2-C4), medium chain (ACSM, C4-C12), long chain (ACSL, C12-C20), bubblegum (ACSBG, C14-C24) and very long chain (FATPs, C18-C26) [90,91].

Five genes have been identified in the ACSL family. They are named ACSL1 and 3 to 6 and vary in tissue distribution, intracellular locations and regulation, implying that the different isoforms have distinct functions [92,93,94]. It has been suggested that the different ACSLs direct the FAs into distinct metabolic pathways [95]. Presently, there is little knowledge about the role and function of these proteins in placental FA uptake.

Lipid droplets and associated proteins

Most mammalian cells are able to store neutral lipids in intracellular lipid droplets. In addition to serving as lipid storage depots, lipid droplets appear to participate in lipid homeostasis, cell signaling, intracellular vesicle trafficking, and disease processes [96,97,98,99,100]. The structure of the lipid droplets is similar to lipoproteins; a neutral lipid core surrounded by a phospholipid and cholesterol monolayer onto which the lipid droplet associated proteins (LDAPs) are attached [98]. The LDAPs (recently named perilipins [101]) consist of a group of 5 proteins with sequence homology that are associated with lipid droplets and include S3-12, LDAP5, TIP-47, perilipin and ADRP. Many other proteins have also been found associated with lipid droplets, including caveolin-1. ADRP (also called perlipin 2/ADFP/adipophilin) belongs to a group of PAT family proteins (perilipin, ADRP and tail-interacting protein of 47 kDa (TIP-47)) [102]. Perilipin expression is mainly confined to adipocytes while ADRP and TIP-47 expression is widely distributed in

different tissues [103]. Perilipin has a well-defined role as a regulator of TAG lipolysis in adipocytes, where it stimulates translocation of hormone sensitive lipase. The roles of ADRP and TIP-47 are, on the other hand, still unclear.

ADRP (Adipose Differentiation Related Protein)

ADRP is a 48-50 kDa lipid droplet associated protein expressed widely in different cells and tissues that store or synthesize lipids and is extensively used as a marker for lipid droplets. The protein co-localizes with the surface of neutral lipid droplets inside the cell and is assumed to play a role in uptake, transport and storage of lipids [104]. ADRP is highly expressed in adipose tissue and is induced early during adipocyte differentiation [105,106]. ADRP mRNA expression is increased at the transcriptional level in the presence of FAs in preadipocytes [107]. Furthermore, ADRP binds to FAs [108] and cholesterol [109]. ADRP is highly expressed in placenta tissue on mRNA level [110]. It is expressed in human villous trophoblasts and both mRNA and protein expression is enhanced during differentiation of cytotrophoblasts into syncytiotrophoblast [111].

Regulation of ADRP expression suggests it to be a PPAR/RXR target gene, both in adipose cells and placental trophoblasts [111]. Primary trophoblasts in culture spontaneously differentiate, and accumulate lipids in the form of small lipid droplets [112]. PPAR activators, including oxidized lipids, have been shown to promote trophoblast differentiation [112,113]. ADRP is expressed in human villous trophoblasts and in concordance with the accumulation of lipid droplets, the mRNA and protein expression is enhanced during differentiation of cytotrophoblasts into syncytiotrophoblasts [111]. Recently it was reported that ADRP is a direct LXR target gene with several LXRE response elements, and both mRNA and protein expression of ADRP is increased in hepatocytes treated with the synthetic LXR agonist GW3965 [114].

1.4. Nuclear receptors

Transcription factors are proteins involved in the regulation of gene transcription; they include about 10% of all human genes and are the largest family of human genes [115]. The nuclear receptor (NR) super family is a diverse group of evolutionary related DNA binding transcription factors, and 48 different types are identified in humans [116]. Most of the NRs are ligand-dependent but there is still a large number of orphan receptors, meaning NRs without any known ligands [117]. The ligands are small hydrophobic molecules that include FAs, cholesterol derivatives, retinoids, thyroid hormone, prostaglandins, leukotrienes and xenobiotics [118]. Because the NRs are dependent on ligands for activation, they have essential roles in communication between the cell/body environment and the genome. They have vital roles in a variety of biological processes such as development, reproduction, homeostasis, inflammation and metabolism [118,119,120].

NRs share a characteristic structure that consist of five to six homologous domains [116], with different functions that ensures site-specific binding to DNA and binding of ligands and cofactors [121]. Over 300 cofactors that increase or repress the transcription of genes have been identified [122,123]. In addition to regulation by ligands and cofactors, NRs can also be modified by phosphorylation, glycosylation, methylation, acetylation, ubiquitinylation and small ubiquitin-like modulation [122].

The NRs can be divided into several subfamilies depending on sequence homology, ligand sources or physiological functions [116,124]. They are usually classified according to the DNA-binding and dimerization properties. This classification consists of four different groups [117]. Class 1 receptors include homodimeric steroid hormone receptors. Class 2 receptors are ligand-dependent and form heterodimers with retinoid X receptors (RXRs). Class 3 and 4 include orphan receptors, homodimers and monomers respectively. In this study we were interested in the Class 2 receptors peroxisome proliferator activated receptors (PPARs) and liver X receptors (LXRs).

Peroxisome Proliferator Activated Receptors (PPARs)

The PPARs have distinct tissue distribution and control a vast array of genes involved mainly in the lipid metabolism, but are also involved in other cellular processes such as inflammation and cellular differentiation [125,126,127,128]. The PPARs have

three distinct members PPARα, δ and γ [129,130]. PPARα is expressed in metabolically active tissues including the liver, heart, kidney and skeletal muscle [131] where it controls lipid catabolism and transport. PPARγ is mainly expressed in adipose tissue, macrophages, colon and placenta [131]. It is essential for adipocyte differentiation [132,133] and activates genes that promotes fat storage and reduces serum lipid levels. Other effects of PPARγ are improved glucose homeostasis and decreased inflammation [131]. PPARδ is expressed throughout the body [131] and is involved in many biological processes such as cholesterol transport.

Natural activators for PPARs include medium and long-chained FAs [134,135], oxidized metabolites of linoleic acid from oxLDL [136] and eicosanoids [137,138,139]. Synthetic ligands for PPARα are fibrates [137] that are used therapeutically in humans for lowering hepatic production of triglycerides by increasing FA oxidation [131], and as anti-inflammatory drugs [140]. Thiazolidinediones are synthetic ligands for PPARγ that have been used therapeutically in humans to increase insulin sensitivity [141,142].

Stimulation of PPARγ activity by these ligands, can among other functions, enhance the transcription of CD36/FAT, leading to further uptake of oxLDL and differentiation of monocytes into foam cells [136,143,144].

Liver X Receptors (LXRs)

LXRs play key roles as regulators of lipid and glucose metabolism [145]. In the lipid metabolism they regulate *de novo* FA synthesis, TAG synthesis, LDL synthesis and metabolism, and cholesterol homeostasis [146,147,148]. They are also involved in the pathogenesis of many diseases including atherosclerosis, diabetes and inflammation [149].

LXRs consist of two isomers, termed alpha and beta, which share considerable sequence homology and are activated by the same ligands [150]. LXRβ is ubiquitously expressed, while LXRα expression is restricted mainly to tissues involved in lipid metabolism such as the liver and adipose tissue [151].

The natural ligands that activate LXRs are oxysterols [152,153]. Oxysterols are oxidized derivatives of cholesterol that are present in oxLDL [154], and maternal plasma oxLDL has been associated with increased in maternal circulation in PE [155]. LXRs can also be induced by non-steroidal synthetic ligands such as T0901317

(Tularik) and GW3965 [135,156]. Activation of LXR by synthetic ligands results in a reverse transport of cholesterol from peripheral tissues to the liver, and has been shown to inhibit the development of atherosclerosis in mice [157]. This has made LXR a promising target for treatment against atherosclerosis; however, there are undesirable side-effects such as increased hepatic lipogenesis leading to hepatic steatosis [156].

There are also natural ligands with antagonizing effects against LXRs, such as PUFAs. These FAs inhibit the activation of LXR by competing with the activating ligands in the order ARA>EPA>DHA>ALA, whereas saturated and monounsaturated FAs have very little effect on the activation of LXR [158].

PPARs and LXRs in placenta

All three PPAR isoforms have been detected in human placenta and in placental trophoblast cells [159]. Both the PPARs and the RXRs are involved in several aspects of pregnancy development such as implantation, placentation, trophoblast invasion and FA uptake [160]. Studies of PPARγ knock-out mice have shown that abnormal development of the placenta results in embryonic death at mid-gestation [161], and that PPARγ/RXR heterodimers are essential for differentiation of trophoblast cells. PPARγ and RXR agonists also increase the differentiation of isolated primary human cytotrophoblast cells [162]. Cytotrophoblast differentiation is characterized by increased hCG production and hCG has been shown to be a direct PPARγ target gene and its expression is increased by PPARγ [112,162]. It has also been shown that PPARγ has a role in trophoblast invasion. In an *in vitro* invasion assay, using extravillous cytotrophoblast cells, it was shown that both natural and synthetic PPAR ligands inhibited trophoblast invasion [163].

LXRs have also been shown to be involved in placentation and trophoblast invasion. Both oxysterols and synthetic LXR agonists inhibited invasion of extravillous cytotrophoblasts *in vitro* [164]. The anti-angiogenic protein endoglin, which is increased in maternal circulation in PE [165,166], was identified as a direct LXRα target gene in the placental cell line JAR [167]. Endoglin is highly expressed in syncytiotrophoblast and has been shown to inhibit trophoblast invasion [167]. Weedon-Fekjaer *et al* found that LXR increased the synthesis of FAs and inhibited the secretion of hCG in placental BeWo cells [168]. The role of LXRs in cholesterol transport in the placenta has been

investigated in human placental endothelial cells and it was found that these cells had increased cholesterol efflux after LXR activation compared to human umbilical vein endothelial cells (HUVEC) [169].

1.5. Preeclampsia

Preeclampsia (PE) is a major complication of pregnancy characterized by hypertension and proteinuria [170] developing in the second half of pregnancy [171]. PE affects at least 3-4% of all pregnancies, representing a major threat to maternal and fetal health, and responsible for approximately 50 000 maternal deaths annually [172]. The severe forms of PE typically results in preterm delivery, low-birth weight and increased risk of fetal morbidity and mortality [173].

Definitions

There are several definitions of PE in use, and in the papers included in this thesis we have used a widely accepted definition from the American College of Obstetrics and Gynecologists [170]. The criteria for diagnosis of PE are as follows:

1) Hypertension is defined as "blood pressure of 140 mmHg systolic or higher or 90 mmHg diastolic or higher that occurs after 20 weeks of gestation in a woman with previous normal blood pressure".
2) Proteinuria is defined as "urinary excretion of 0.3 g protein or higher in a 24 h urine specimen". This corresponds to a protein dipstick reading of +1 or higher.

PE is unpredictable in its onset, progression and severity. It is sometimes divided into early onset PE occurring prior to week 34 of gestation, and a late onset PE occurring at or after 34 weeks of gestation [174,175]. PE is considered to be severe if the blood pressure ≥ 160/110, or proteinuria at 5 g/24 h (≥ +3 on dipstick) is present. Eclampsia is a severe variant 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 another variant of PE [176], which includes hemolysis, elevated liver enzymes and low platelet counts.

Intrauterine growth restriction (IUGR) or fetal growth restriction (FGR) is defined as the failure of a fetus to reach its expected growth potential at any gestational age [177]. The newborn birth weight percentiles were calculated according to national birth registry data [178] or an ultrasound based weight percentile [179].

Gestational hypertension is defined as new onset hypertension ≥ 140/90 after week 20 of gestation but without proteinuria [170]. In cases of superimposed PE on hypertension, the women have developed hypertension before week 20 (or pregestationally), with new-onset proteinuria after week 20. Also, we defined superimposed PE on diabetes mellitus (pregestational diabetes type 1 or 2, or gestational diabetes mellitus) as PE developing in a pregnant woman already diagnosed with diabetes mellitus (pregestational or gestational diabetes mellitus) according to WHO criteria [180].

Pathophysiology

The exact pathophysiology of PE remains unknown. A "two-stage model" was proposed by CW Redman in 1991 [181]. In the first stage there is poor placentation while the second stage is the maternal syndrome diagnosed by hypertension and proteinuria. Poor placentation includes abnormal implantation, inadequate remodeling of the spiral arteries and thereby reduced or altered placental perfusion (Figure 7).

This altered blood flow to the placenta leads to oxidative stress, which in some cases may be due to hypoxia. It is proposed that the oxidatively stressed placenta releases different pro-inflammatory factors into the maternal circulation and thereby causes the maternal syndrome. These suggested placental factors are pro-inflammatory cytokines (TNF-α, IL-6 [182,183]), anti-angiogenic factors (sFLT1 and sEng [165,184,185,186]), placental debris (such as syncytiotrophoblast microparticles: STBMs) [187] and activated immune cells [188,189]. In the second stage it is thought that the maternal circulating factors produced by the stressed placenta cause an excessive systemic inflammatory maternal response [188] with generalized maternal endothelial dysfunction [190], contributing to the maternal clinical features of PE [191,171].

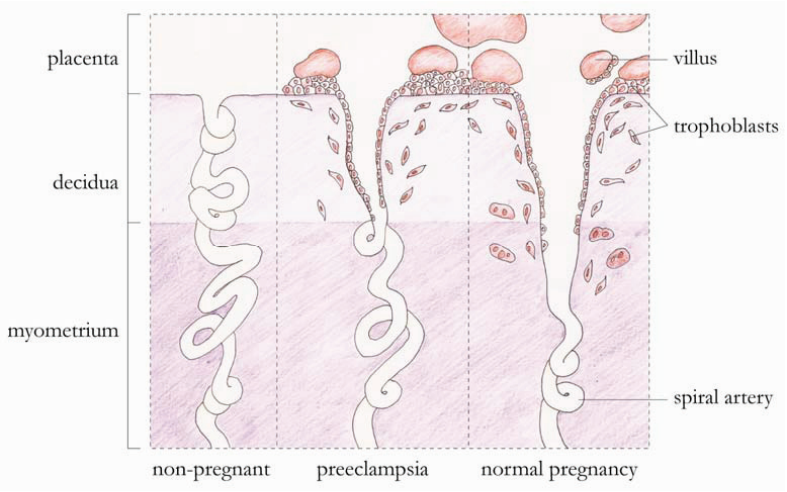

Figure 7: Illustration of the uterine spiral arteries in a non-pregnant woman, in preeclampsia and in normal pregnancy. In preeclampsia the trophoblast invasion and physiological transformation of the spiral arteries are incomplete, resulting in an abnormal blood supply to the placenta. Modified from Moffet-King [5].

Recently it has been proposed that the development of PE starts even earlier than at the stage of spiral artery remodeling, with an increased release of proteins already in the first trimester [192]. It has also been suggested that the blood volume to the placenta is not reduced, as previously suggested [193]. Instead, the narrower, non-transformed spiral arteries in PE cause the blood to enter the intervillous space at a velocity much greater than normal. This, in turn, causes damage to the placental villi both on a micro- and macroscopic level and may alter the placental morphology and function [194].

Risk factors and treatment

Risk factors for PE include previous history of PE and a family history of PE, primiparity, multiple pregnancy, obesity and chronic medical conditions such as preexisting hypertension and diabetes [191].

Presently PE cannot be prevented and the only "treatment" is delivery of the fetus with removal of placental tissue. If placenta tissue is retained after termination of the pregnancy, PE can persist [195]. Also, removal of decidua after delivery with

curettage has shown beneficial clinical effects [196,197]. Treatment with antihypertensive medication may serve to prolong the pregnancy [198], due to improved maternal blood pressure control and reduced need for premature delivery. There have been a number of treatment and primary as well as secondary prophylactic intervention studies. Antioxidant supplementation using vitamin E and C did not reduce the incidence in a large randomized controlled trial (RCT) [199], whereas a previous smaller RCT was promising [200]. Calcium supplementation has also proven ineffective, but could be useful in developing countries where nutritional levels are insufficient [201]. Also, n-3 FAs have been suggested to play a role in the prevention of PE, but the evidence for this is not conclusive. There have been three randomized trials where fish oil supplementation was given to high risk women, but none these trials reported any reduction in PE [202,203]. Although acetyl salicylic acid (aspirin) has shown to reduce the incidence of PE in some studies, without augmenting complications such as placental abruption, there is hitherto no conclusive evidence as to which group of pregnant women that would benefit from such an intervention [204].

Inflammation

Normal pregnancy is a state of mild systemic inflammatory response, but the physiological basis for this is not known except that the phenomenon arises from the placenta itself [205]. PE is associated with a more extreme maternal inflammatory response than occurs in normal pregnancy [188]. How the problems of abnormal placentation generates the systemic inflammatory problems of PE remains to be explained, but it is thought that release of pro-inflammatory factors from the syncytial surface of the placenta into the maternal circulation could be important, and could possible be the link between stage 1 and 2 of PE [205]. Syncytiotrophoblast microparticles (STBMs) are vesicles shed from the placenta into the maternal circulation during pregnancy. In PE there is a significant increase in the amounts of particles that are shed [187]. These particles have an anti-endothelial effect and stimulate release of pro-inflammatory substances form the endothelium [187]. The pro-inflammatory cytokines tissue necrosis factor (TNF)-α and interleukin (IL)-6 are both elevated in preeclamptic circulation [182]. It is suggested that the placenta contributes to the elevated plasma cytokine levels, but the dysfunctional maternal endothelium, peripheral

blood mononuclear cells and other tissues, such as the adipose tissue, are also likely to be involved [206].

Oxidative stress

Reactive oxygen species (ROS) are highly reactive molecules that contain an oxygen atom. ROS can be free radicals with unpaired electrons such as superoxide ($O_2^{\cdot-}$) and hydroxyl anions ($OH^{\cdot}$), or non-radical intermediates such as hydrogen peroxide (H_2O_2). ROS are natural byproducts of oxygen metabolism and have important physiological roles in the regulation of cell signaling, for instance in the regulation of nitric oxides and vascular tone. However, if the concentration of ROS becomes excessive, due to different environmental stresses, and an imbalance between the level of ROS and the level of antioxidants arises, then ROS can start attacking lipids, proteins or DNA. These attacks cause chain reactions that lead to widespread damage and loss of function in cells, and this situation is referred to as oxidative stress.

Pregnancy is a state of excessive oxidative stress arising from increased placental mitochondrial activity and production of reactive oxygen species and decreased expression and activity of antioxidants [207]. In PE there is an increased level of oxidative stress, which results from ischemia-reperfusion injury that in turn is caused by the altered perfusion of the placenta due to the abnormal placentation [208]. The oxidative stress of PE is not restricted to the placenta but is dispersed in the maternal circulation and is a part of the systemic inflammatory response [205].

In placenta the evidence for excessive oxidative stress in PE includes finding of increased generation of lipid peroxides and isoprostanes [207,209,210,211], xanthine oxidase [212] and nitro tyrosine residues [213], which indicates an excessive production of superoxide. In the maternal circulation the evidence include increased superoxide production from circulating neutrophils [214]. It is also known that TNF-α, which is increased in the circulation in PE [182], can induce oxidative stress directly [215] or indirectly by enhancing the levels of oxLDL [216] or through the xanthine oxidase pathway [217]. Placental oxidative stress is viewed as a key intermediary step in the pathophysiology of PE, as a mediator of the endothelial cell dysfunction [191,218].

Hypoxia is a situation where the oxygen tension is too low and this may result in oxidative stress. It has been suggested that hypoxia is the mediator of the pathological

changes observed in pregnancy complications such as PE, however there is limited evidence to support this [10]. Burton *et al* proposed instead that it may be fluctuations in the oxygen level in the intervillous space that creates the pathological changes due to ischemia-reperfusion stress in PE [219].

Dyslipidemia

The dyslipidemia of PE is an amplification of the lipid changes observed in normal pregnancies. It includes elevated cholesterol and triglycerides, increased circulating FFAs, reduced high density lipoproteins (HDL) and increased concentrations of small LDL which leads to the presence of oxLDL in maternal circulation [220,221,222,223,224,225], while total and LDL cholesterol levels are not considerably different [221,222]. Augmented circulating maternal concentrations of the oxidized lipid 8-isoprostanes are also reported in PE [226,227].

The maternal dyslipidemia is present already in the first and second trimester of gestation and is evident before the clinical detection of PE [228,229,230,231]. A rise in circulating TAG concentrations is present [232] as early as 10 weeks of gestation [233]. A dose-response effect of TAG has been observed, with a four-fold elevated risk of developing PE in women with the highest circulating levels of TAG compared to normal levels [234]. Even though hypertriglyceridemia may contribute to the development of PE, therapeutic intervention is probably not a good alternative, as strict correction of maternal hypertriglyceridemia in rodents has been shown to have negative effects on fetal growth and development [235].

The lipid abnormalities in PE are similar to abnormalities as observed in patients with cardiovascular disease [236,237]. The two diseases also have several other risk factors in common, including obesity, diabetes mellitus, insulin resistance and endothelial dysfunction [236,238]. Also, the phenomenon of "acute atherosis" of the decidual/uterine spiral arteries, which is more often seen in PE than in uneventful pregnancies [239], closely resembles the early stages of atherosclerotic lesions found in cardiovascular disease [240].

Lipotoxicity

Accumulation of excess lipids in non-adipose tissues is termed lipotoxicity and can lead to cell dysfunction and death [241]. Lipotoxicity has been suggested to play a role in insulin resistance and hyperlipidemia [242], which are also features of PE and diabetes mellitus. The combination of hypoxia, oxidative stress and increased lipid concentrations observed in PE may result in excess lipid peroxidation products. Lipid peroxides and oxygen free radicals stimulate peroxidation reactions that damage cells and cell membranes. These effects include alterations in membrane fluidity and permeability and endothelial cell injury and dysfunction [243]. Oxidized lipids, such as the endogenously produced 9S-hydroxy-octadecadienoic acid (9-HODE), 13S-hydroxy-octadecadienoic acid (13-HODE), and 15S-hydroxy-eicosatetraenoic acid (15-HETE), are particularly relevant to trophoblast biology, in which they are implicated in trophoblast injury [32,244]. PE has been associated with enhanced lipid peroxidation in trophoblasts [245,246] and there has been demonstrated an increased production of 15-HETE *in vitro* from trophoblasts derived from preeclamptic women [247,248]. 8-iso-PGF2α, a lipid peroxidation product, is a well-known marker of oxidative stress and is elevated in the maternal circulation as well as in placental/decidual tissue in PE [226,209,210]. Staff/Halvorsen *et al* demonstrated an accumulation of fat in the trophoblast cell line JAR, when incubated with 8-iso-PGF2α, as well as reduced trophoblast invasion [249], which could suggest a possible *in vivo* effect of this oxidized lipid in PE.

Acute atherosis

Acute atherosis is defined as accumulation of CD68 positive foamy macrophages in the uteroplacental spiral arteries, including areas of fibrinoid necrosis [250,251]. The name acute atherosis is derived from atherosclerosis because the phenomenon resembles early atherosclerotic changes of other systemic blood vessels. These areas of lipid deposition are found in non-transformed spiral arteries and have been associated with PE, although it is not specific for this pregnancy complication [252,253,254]. Acute atherosis in spiral arteries are associated with augmented risk for local thrombosis and thereby necrosis in the placental tissue underlying the plugged arteries, adding to the reduced placenta function more common in PE than in uneventful pregnancies.

Presently there is little knowledge of the molecular mechanisms behind the formation of acute atherosis, however it has been suggested that the hyperlipidemia in the maternal circulation in PE could participate in the lipid changes in the spiral arteries [223]. Also, it is probable that reduced transformation of the spiral arteries could contribute to the lipid deposition in the narrow parts of untransformed spiral arteries [255].

Diabetes mellitus in pregnancy

Diabetes mellitus and PE share many pathophysiological features, including insulin resistance, endothelial dysfunction, oxidative stress, and inflammation [256,257,258]. Both pregestational diabetes and gestational diabetes mellitus (GDM) are associated with a two- to four-time increased risk of developing PE in pregnancy [259,260,261]. Gestational diabetes and pre-pregnancy obesity is associated with large babies, while PE is associated with growth restricted babies [262]. Superimposed PE on diabetes mellitus (SPE) includes women with diabetes mellitus (preexisiting or gestationally induced) that develop PE in the present pregnancy. SPE present a higher risk for poor perinatal outcome and placental abruption than PE alone [263].

The development of insulin resistance [264] together with adipose tissue accumulation [265] in the third trimester of pregnancy is a possible adaptation of the maternal metabolism to optimize fetal nutrition. Insulin inhibits hormone sensitive lipase and thereby decreases triglyceride hydrolysis in the adipose tissue resulting in reduced circulation of FFA and glycerol. Insulin resistance thus increases the activity of hormone sensitive lipase and results in increased levels of lipoproteins and FFA in the circulation. Gestational insulin resistance is accentuated in PE [266], and can be observed weeks before the clinical onset of PE [267,268]. Furthermore, placenta secretes a variety of hormones that is suggested to play a role in gestational insulin resistance [269]. However the role of adipose tissue could also be important. Adipose tissue has an endocrine function, secreting several metabolically active proteins such as leptin, resistin, adiponectin, TNF-α and IL-6, termed adipokines [270]. During pregnancy, the placenta is an additional source of adipokines, such as leptin and resistin [271,272].

2. Aims of present study

The main aim of this study was to increase the understanding of the role of trophoblasts and placenta in lipid transport and storage in general, and in dyslipidemic pregnancies specifically. The first objective was to study the transport of FAs across the trophoblast cells, in order to gain a better understanding of how the selective enrichment of LCPUFAs in the fetal circulation occurs. The second objective was to explore the role of lipid transport and storage associated proteins in the placenta in dyslipidemic pregnancies, such as pregnancies complicated by PE and/or diabetes mellitus, with a particular focus on the lipid droplet associated protein ADRP.

Specifically, the following questions were addressed:

1. Is there any difference in the transport of LCPUFAs compared to that of non-essential FAs across trophoblast cells?
2. Is there any difference in the uptake and storage of LCPUFAs compared to that of non-essential FAs in trophoblast cells?
3. Does LCPUFAs influence the uptake of FAs in trophoblast cells?
4. If so, which lipid metabolism genes are involved in the LCPUFA influenced uptake of FAs in BeWo cells?
5. Are the transcription factors LXR and PPAR dysregulated in PE in placenta, decidua and adipose tissue?
6. Is there any dysregulation of the expression of lipid droplet associated proteins such as ADRP and FA transport/binding proteins such as FATPs and FABPs in placenta in pregnancies complicated by PE or diabetes mellitus?
7. Where in the placenta is ADRP protein expression localized?
8. Is ADRP expression in trophoblast cells regulated by FAs and/or oxidative stress?

3. Summary of Papers

Paper 1: Long-chain polyunsaturated fatty acid transport across human placental choriocarcinoma (BeWo) cells

LCPUFAs are critical for the growth and development of the fetus. In the first paper our aim was to investigate the mechanism behind the differential transport of LCPUFAS and non-essential FAs assumed to take place in trophoblast cells. We used the BeWo cell line as model of placental trophoblasts and a transwell cell system to study the transport of radiolabeled FAs across these cells.

Results:

- BeWo cells incubated with OA contain more TAGs, more lipid droplets and have higher ADRP (lipid droplet marker protein) expression than cells incubated with DHA.
- Incubation of the FAs together with triacsin C (an inhibitor of the esterification of FAs into acyl-CoA) abolished the TAG accumulation and the expression of ADRP.
- Caveolin-1, a structural protein of lipid rafts in the plasma membrane, and also believed to partake in FA uptake, was induced by OA, but expression was not affected by triacsin C.
- Radiolabeled DHA and AA was more efficiently transported across the cell layer than OA and PA, the concentration of DHA and AA in the basolateral chamber was ~4 and ~2.5 fold higher than for OA, respectively. OA was similar to PA.
- A physiological FA mix was used to mimic the plasma concentration of FAs in the third trimester of pregnancy, the concentrations of PA and OA was ~20-fold and ~10-fold higher than for DHA and AA, and we found that the relative transport of LCPUFAS was more efficient.
- Triacsin C inhibited the uptake of radiolabeled PA and OA by ~70%, whereas uptake of LCPUFAs was inhibited by only 20%.
- Triacsin C increased the efflux of radiolabeled OA compared with DHA in a transwell system.

Paper 2: Long-chain polyunsaturated fatty acids stimulate cellular fatty acid uptake in human placental choriocarcinoma (BeWo) cells

In light of our findings in Paper 1 and the importance of LCPUFAs for fetal nutrition, our aim was to study the effect of LCPUFAs on the uptake of FAs in placental trophoblast cells. Further we wanted to study the effect of LCPUFAs on the expression of genes involved in FA uptake and lipid metabolism in trophoblasts. We used BeWo cells and studied the uptake of radiolabeled FAs, as well as gene expression by quantitative real-time RT-PCR.

Results:

- Preincubation of BeWo cells for 24 h with LCPUFAs increased the uptake of FAs by ~20-50%. Preincubation with OA on the other hand did not significantly change the FA uptake as compared with the uptake in untreated cells (controls).
- After preincubation with LCPUFAs, radiolabeled FAs were incorporated into phospholipid fractions to a greater extent compared to cells preeincubated with OA or controls, simultaneously there was a decreased incorporation of FAs into the TAG fraction.
- The gene expression of long-chain acyl-CoA synthetases ACSL1 and ACSL5 were increased when BeWo cells were incubated with the LCPUFAs AA and DHA compared to both OA and control. Incubation with EPA also increased the expression of these genes compared to the control.
- The gene expression of the lipid droplet associated protein ADRP was increased by AA, EPA and DHA compared with the control.
- The gene expression of ACSL3 and LPIN1 was decreased after incubation with all the FAs including OA compared with the control.

Paper 3: Expression of liver X receptors in pregnancies complicated by PE

We wanted to move from the placental trophoblast cell model to explore the expression of placental genes involved in lipid metabolism in the placenta, and explore whether any of these genes were dysregulated in PE. In the third paper we focused on transcription factors LXRs (α and β) and PPARs (δ and γ) and their target genes. We examined tissue samples from uncomplicated and preeclamptic pregnancies and studied the gene expression in placenta, decidua and subcutaneous fat tissue. The placental lipid classes and FA profile were also analyzed.

Results:

- The expression of LXRα and β were similar in all three gestational tissues.
- PPARδ had a higher expression in placenta than in decidua and fat (~20 fold higher) while PPARγ was more highly expressed in fat tissue than the other tissues.
- The expression of LXRα and β and PPARγ was significantly decreased in preeclamptic placenta, while PPARδ was not differentially expressed as compared to uneventful pregnancies.
- The expression of the target genes CD36/FAT, and APOE was significantly decreased in preeclamptic placenta, while the expression of LDL receptor was increased.
- Protein expression of LXR β was decreased in preeclamptic placenta, while LXR α was similarly expressed in PE and controls.
- The mean concentration of FFA in placenta with PE was significantly lower than in control placenta.
- The total concentration of n-6 PUFAs (LA, DGLA and AA) was increased in preeclamptic placenta.
- There was a positive correlation between the gene expression of LXRβ and the concentration of FFA in preeclamptic placentas.

Paper 4: Increased Adipose Differentiation Related Protein (ADRP) expression in preeclamptic placenta

In the fourth paper we explored the expression of genes involved in lipid metabolism, focusing on genes involved in FA transport in the placenta and exploring whether there is any dysregulation of these in PE. We examined placenta tissue samples from uncomplicated (n=33) and preeclamptic pregnancies (n=30), as well as pregnancies complicated by DM (n=10) and SPE (n=6). We also incubated BeWo cells with FAs, oxidative stress and inflammatory agents in order to mimic the preeclamptic situation.

Results:

- The gene expression of the lipid droplet associated protein ADRP was increased in preeclamptic placenta.
- The gene expression of FATP1 and CAV1 was decreased in PE placenta.
- In SPE (superimposed PE on top of diabetes mellitus) the expression of PLIN, S3-12, LSDP5, FABP3 and 4 and FATP1 and 4 was increased.
- The protein expression of ADRP was increased in PE, while the expression of caveolin-1 was unaltered.
- ADRP protein expression was localized to vacuoles within fibrinoid tissue in placental tissue sections, and also in vesicles in trophoblast cells.
- Caveolin-1 protein expression was mainly localized to endothelial cells in placental tissue sections and in some specimens in cytotrophoblast cells.
- The FAs OA and LA that are increased in maternal circulation in preeclamptic pregnancies increased the expression of ADRP in BeWo cells on both gene and protein level, while PA did not.
- Oxidative stress induced by hydrogen peroxide increased the expression of ADRP in BeWo cells on both gene and protein level.

4. Methodological considerations

4.1. Advantages of combining clinical research and *in vitro* experiments

The work presented in this thesis is based on analyses of blood and tissue samples from pregnant women and on *in vitro* experiments in cell culture. Both approaches have advantages and restrictions, but in general they complement each other. Clinical material gives an overview of the actual situation in the patient population, and screening of target genes or proteins in patient populations can be useful both for generating hypotheses and for confirming them. Data obtained from clinical material can pose interpretation challenges, because there can be huge individual differences from one patient to another, and also there are many variables that cannot be controlled. In addition, when studying a whole organ, such as the placenta, it is important to remember that it consists of a number of different cell types that may have very different roles and functions. In a tissue/organ, the different cell types communicate with each in a paracrine manner by secreting messenger molecules, and will also be subject to endocrine communication by hormones or other circulating molecules from other organs. In cell experiments, on the other hand, it is possible to control the experimental settings so that the influence of confounding factors will be limited. However, cell experiments have the disadvantage of being pure model systems, where the settings are usually far from the complex physiological situation that we try to mimic. By combining the two systems it is possible to test hypotheses generated from the complex *in vivo* situation in the purer *in vitro* situation, facilitating a more comprehensive understanding of physiological processes, such as FA uptake and transport in the placenta.

4.2. Cell system

The BeWo choriocarcinoma trophoblast cell line is used as a model of placental trophoblast in this study. BeWo was the first commercially human trophoblast cell line available, developed in 1968 [273]. It was prepared from a malignant gestational

trophoblast cancer (choriocarcinoma), which is composed entirely of syncytiotrophoblast and cytotrophoblast cells [274]. BeWo cells grow as undifferentiated cytotrophoblast but can be induced to differentiate into syncytiotrophoblast, and they also secrete hCG [275]. BeWo cells also have similar properties of lipid transport and metabolism as isolated primary cytotrophoblasts and syncytiotrophoblast for instance polarized transport of LCPUFAs and expression of genes involved in lipid metabolism [47,274,276]. BeWo cells have been widely used to study the uptake and transport of various biological compounds in the placenta, such as amino acids [277,278,279,280], glucose [281], cholesterol [282] and FAs [283,284]. BeWo cells can form confluent monolayers when grown on permeable membranes, and demonstrate polarized membrane expression of apical and basolateral protein markers and tight junction formation [283]. Therefore we believe that BeWo is a good model for studying FA uptake and transport in the placenta.

4.3. Patient selection

In this study we have used clinical information and biological samples from an ongoing biobank inclusion of patients with complicated or uncomplicated pregnancies at Oslo University Hospital, Ullevål, Norway. This hospital has the largest delivery unit in Norway, presently with over 7000 deliveries annually. The biobank collection was started by our research group in 2001 and includes women with uncomplicated pregnancies and women with pregnancies complicated by PE or diabetes mellitus. For the papers included in this thesis, a total of 79 women with singleton pregnancies were included from the biobank study. We chose to include patient samples where sufficient tissue samples from placenta, decidua and subcutaneous fat were available, as well as maternal plasma and serum. We had originally intended to measure the concentration of lipid associated proteins in the maternal circulation; however, in the end we did not include any such analyses in the papers included in this thesis.

All patients were delivered by cesarean section. None of the women were in active labor, had ruptured membranes or clinical signs of infection at the time of delivery. No women with chronic hypertension or renal disease were included. The cesarean section was clinically indicated independently of the research study.

Uncomplicated pregnancies were used as a control group (n=33), and included normotensive and otherwise healthy women undergoing elective cesarean section due to breach presentation or psychosocial reasons.

The women diagnosed with PE (n=30) were previously healthy and normotensive prior to pregnancy. The reasons for delivery by cesarean route in these cases were that vaginal delivery was not appropriate due to disease progression or severity and/or unfavorable cervical ripening. Of the 30 patients with PE three had HELLP syndrome and two had both PE and clinically demonstrated IUGR.

The patients with superimposed PE on diabetes mellitus (n=6) were delivered by cesarean route with the same clinical indications as the PE group. The women with diabetes mellitus (n=10) were delivered by cesarean route with the same clinical indications as the control group. Therefore the number of patients in this group is small, as patients with DM are normally delivered vaginally.

Cases of superimposed PE on hypertension were not included in this Ph.D. project because these cases are probably distinct from PE and may have different pathophysiology [285].

All patients that agreed to participate in the study signed a voluntary informed consent. They have the possibility to withdraw from the study at any time without giving any reason and without any consequences regarding the relationship to Oslo University Hospital, Ullevål. The cesarean section biobank study was approved by the Regional Committee of Medical Research Ethics in Eastern Norway (REK Øst), with permission from the Norwegian Data Inspectorate (Datatilsynet) and final approval by the Ministry of Health (for the biobank).

4.4. Delivery mode

A major strength of our study is the relatively large placental sample size from a clinically well-characterized study population. In addition, only pregnancies delivered by cesarean section without any indications of labor were included in the study. Hence, unpredictable effects of vaginal delivery such as oxidative stress can be avoided and will not influence the blood samples or tissue material. We believe that this is an advantage of our study protocol, since vaginal delivery is associated with oxidative stress in

maternal and fetal circulation [286,287,288]. Uterine contractions during labor leads to intermittent utero-placenta blood that causes hypoxia-reperfusion injury and increased oxidative stress [212,219]. Xanthine oxidase activity was increased in placental tissue of laboring women [212]. Markers of oxidative stress and apoptosis were increased in placental tissue after labor, and the expression of several genes and proteins were altered due to vaginal delivery [219]. On the other hand, some authors suggest that labor does not affect gene expression as compared to vaginal delivery [289,290] but our view is that one cannot safely rule out some degree of increased oxidative stress in a preeclamptic delivery as compared to deliveries in uneventful pregnancies.

Another advantage associated with delivery by cesarean section is the possibility to obtain decidua and fat tissue. It would be difficult to obtain sufficient decidual suction tissue samples after a vaginal delivery. Also, subcutaneous fat biopsies would also be more difficult to obtain in vaginal deliveries, demanding additional local anesthesia and extra skin incision. The alternative method for obtaining decidual tissue is blind curettage, but we consider this to be unethical as it is a clinically unnecessary procedure after an uncomplicated delivery, with a small risk of uterine perforation and infection. The decidual tissue sampled for this project was collected by the vacuum suction method designed by Staff and coworkers [291,292], and evaluated as superior to classic placental bed biopsies and basal membrane biopsies in collecting decidual spiral arteries, but inferior to placental bed biopsies in collecting myometrial tissue and topographically adequate decidual tissue. The quality of our tissue material, with rapid nitrogen freezing after collection, is a major advantage of our study protocol, combined with extensive clinical information and a thoroughly clinically selected study population.

Due to the mode of delivery of the patient population included in our biobank, we have a subgroup of patients with more severe PE than in the general Norwegian preeclamptic population. Of the 30 women with PE included in this study, 20 had severe PE according to ACOG criteria. Women with clinically less severe PE are more often delivered vaginally. Therefore the patients with PE delivered by cesarean section tend to have a more severe form of the disease than the total group of women with PE delivered at our hospital. Our PE group is therefore not representative for all women with PE. However, it is possibly a more interesting group to study as early and severe

PE has the most severe consequences for the infant and maternal health, as well as for their health later in life.

4.5. Gestational age

A limitation of our study is that there is a significant difference in gestational age between our study groups, the PE group delivering earlier than the control group. It is ethically unacceptable and impractical to have gestationally age matched controls to the cesarean delivered preeclamptic patients, as women will not have an early cesarean section if the pregnancy is normal. Premature deliveries are generally due to pathological conditions, such as inflammation or infection and therefore not suitable as controls. Correcting for gestational age is mathematically possible but it is not necessarily biologically correct, as premature delivered women with PE will generally have a more severe form of the disease than women delivered at term. Still we cannot exclude that differences in gestational length between the study groups could potentially affect our results and conclusions. It is possible that the differences we report between PE and controls could be over- or underestimated, depending on how placental gene expression varies with pregnancies or PE severity. On the other hand, in a longitudinal study of gene expression during pregnancy [293] none of the genes found to be regulated by gestational length correspond to the genes that we found dysregulated in PE or diabetes mellitus in the present study (Paper 3 and 4).

4.6. Protein expression in placenta samples

Immunoblotting of whole placenta proteins lysates is not optimal because the placenta tissue consists of a number of different cell types such as trophoblasts, immune cells endothelial cells and stromal cells. In Paper 3 and 4 we did immunoblotting on placental lysates in order to confirm our findings on the mRNA level. This is not always possible because proteins can be regulated differently on the mRNA and protein level. The regulation of protein expression is complex and involves several steps, including initiation of transcription, possible modifications of the mRNA by alternative splicing, translation of mRNA into protein, and numerous posttranslational modifications of the

protein. For example, ADRP protein is degraded when lipids are not present [294]. This is clearly observed in BeWo cells where protein expression of ADRP is almost absent in control cells grown in regular medium, while cells incubated with OA express high amounts of ADRP (Paper 1 and 4). We have observed that the mRNA expression of ADRP in placenta tissue is high while the protein expression in the tissue is very low (Paper 4).

In Paper 4 we performed immunohistochemistry of ADRP and caveolin-1 protein expression in placental sections to be able to study the localization of these proteins in the tissue. Both proteins are expressed highly in the placenta on the mRNA level [110] and the proteins are expressed in primary term trophoblasts [276,111]. However, when studying these proteins with IHC in cesarean delivered samples, we found that ADRP was mainly expressed in clusters of vacuoles in the fibrinoid tissue in the extracellular matrix, and we only found occasional staining of ADRP in syncytiotrophoblast. The explanation for this may be that IHC is a rough visual technique for detecting protein expression in tissue.

4.7. Quantitative real-time PCR

Selection of genes for gene expression analysis

In Paper 3 and 4 we performed quantitative real-time RT-PCR (qRT-PCR), using custom-made 384-well microfluid cards (TaqMan Low Density Array; LDA). In Paper 3 we selected the NRs LXRα and β, RXRα and PPARγ and δ and some of their target genes. In Paper 4 we selected genes involved in FA uptake, transport and storage in the placenta. We selected four endogenous controls, 18S rRNA [293], GAPDH [295], TBP and YWHAZ [296], all commonly used for this purpose and previously used as endogenous controls in placenta gene expression analyses. We found that YWHAZ was the most stable control in placental and decidual tissue, while TBP was the most stable in adipose tissue. Cleal *et al* recently did a test of different endogenous control genes in placenta tissue using real-time PCR and found that YWHAZ was the most stable control in placenta [297]. In Paper 2 we also analyzed gene expression using qRT-PCR,

totally we selected 47 genes involved in the lipid metabolism and five endogenous controls listed in Table 1 (Paper 2, 3 and 4).

Calculation of gene expression

In Paper 2, 3, and 4 we used qRT-PCR and microfluid cards to investigate the expression of several genes both in BeWo cells and in pregnancy tissues. To calculate the gene expression we used the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgens [298].

In Paper 3, we used a modified version of this method in order to be able to compare the gene expression of LXRα and LXRβ in the different tissues investigated. Because we found that all the four endogenous controls were in some way regulated in one or more of the tissues, we could not find a single common endogenous control to use for all tissues. Therefore we chose to compare the $2^{(-Ct)}$ values directly. Briefly, the arbitrary values were calculated using the Ct value for each qRT-PCR reaction according to the equation: $(2^{(-Ct)}) \times 10^9$. We multiplied with a factor of 10^9 in order to obtain a number close to 1 instead of a tiny number that would be difficult to read and comprehend. Our reason for doing the calculation this way is that it is often difficult to find a good endogenous control (a control which it self is not regulated, or regulated very little) when comparing gene expression between tissues [299]. And using a regulated "endogenous control" could in the worst cases produce results reflecting the regulation of the endogenous control, and not that of the target gene.

The qRT-PCR system we use is producing a doubling of product in every cycle as long as there are not any inhibitors present that could interfere with the reaction. We did thorough testing of the material to assure that we included equal amounts of RNA in each sample and that there was no inhibition of either the reverse transcription of RNA to cDNA or in the qRT-PCR. Hence, the amount of total RNA initially measured directly represents the level of expression for each gene. We therefore believe that the use of Ct values, not normalized to a regulated endogenous control, is the most correct way of calculating the gene expression between the tissues in our experiment.

Table 1: Lipid metabolism genes analyzed in Paper 2, 3 and 4

Lipid droplet associated protein		**Paper**
ADRP	Adipose differentiation-related protein	2,4
LSDP5	Lipid storage droplet protein 5	4
PLIN1	Perilipin 1	4
S3-12	Perilipin 4	4
TIP47	Tail-interacting protein, 47 kDa	4
Fatty acid transport/binding proteins		
FABP1	Fatty acid binding protein 1	2,4
FABP3	Fatty acid binding protein 3	2,4
FABP4	Fatty acid binding protein 4	2,4
FABP5	Fatty acid binding protein 5	2,4
FABPpm	Plasma membrane-associated FABP	2,4
FATP1	Fatty acid transport protein 1	2,4
FATP2	Fatty acid transport protein 2	2,4
FATP3	Fatty acid transport protein 3	2,4
FATP4	Fatty acid transport protein 4	2,4
FATP6	Fatty acid transport protein 6	2,4
Long chain acyl-CoA synthetases		
ACSL1	Long chain acyl-CoA synthetase 1	2
ACSL3	Long chain acyl-CoA synthetase 3	2
ACSL4	Long chain acyl-CoA synthetase 4	2
ACSL5	Long chain acyl-CoA synthetase 5	2
ACSL6	Long chain acyl-CoA synthetase 6	2
Transcription factors		
LXRα	Liver X receptor alpha	3
LXRβ	Liver X receptor beta	3
PPARγ	Peroxisome proliferator activated receptor gamma	3
PPARδ	Peroxisome proliferator activated receptor delta	3
RXRα	Retinoid X receptor alpha	3
SREBP-1c	Sterol regulatory element binding protein 1c	3
LXR target genes		
ABCA1	ATP-binding cassette, sub-family A, member 1	3
ABCG1	ATP-binding cassette, sub-family G, member 1	3
APOE	Apolipoprotein E	3
CD36/FAT	CD 36 molecule thrombospondin receptor/fatty acid translocase	2,3
ELOVL5	Fatty acid elongase 5	3
FAS	Fatty acid syntethase	3
LDLR	Low density lipoprotein receptor	3
LPL	Lipoprotein lipase	3
Other genes involved in lipid metabolism		
AGPAT	1-acylglycerol-3-phosphate-0-acyltransferase 1	2
CAV-1	Caveolin-1	2,4
CD36/FAT	CD 36 molecule thrombospondin receptor/fatty acid translocase	2,3
DGAT	Diacylglycerol-0-acyltransferase	2
GPAT	Glycerol-3-phosphate acyltransferase	2
HSL	Hormone sensitive lipase	2
LPIN1	Lipin-1	2
PPARGC1A	PPAR gamma, coactivator 1 alpha	2
Positive control		
LEP	Leptin	3,4
Endogenous controls		
18S	18S ribosomal RNA	3,4
B2M	Beta-2-microglobulin	2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	3,4
TBP	TATA binding protein	2,3,4
YWHAZ	Tyrosine 3/tryptophan 5 -monooxygenase activation protein, zeta polypeptide	3,4

Relative versus absolute quantification of gene expression

Relative quantification is based on analyzing the changes in gene expression in a specific sample compared to a control sample (in our case normal pregnancy samples or untreated cells). The $2^{-\Delta\Delta Ct}$ method, or comparative Ct method, is based on a relative quantification of the gene expression. It is also possible to do relative quantification based on a standard curve method where the gene expression in the unknown samples is determined by comparison to the standard curve and extrapolating the value.

However, if an absolute quantification of the gene expression is needed, none of these two methods are sufficient. Then a standard curve, where the absolute quantities of the standards must be known, is needed. This can be done by using plasmid DNA and *in vitro* transcribed RNA. The RNA concentration is measured at 260 nm and the RNA copy number is found by using the molecular weight of the DNA or RNA. However, a plasmid is not behaving the same way as an endogenous mRNA molecule, and using this approach will not identify any potential inhibitors in the samples, which are usually the most important confounders of the expression level. Also, plasmid DNA can often be contaminated with RNA, which can make the reading at 260 nm inaccurate and thereby inflate the copy number of the plasmid. Therefore DNA should not be used directly to determine the copy number because it will not be possible to know the efficiency of the reverse transcription step [300]. Furthermore, we believe that going through the procedure of creating plasmid vectors and cloning them into a cell line would be very time consuming and costly and would make the use of PCR methods such as LDA cards difficult. Therefore, we believe that the method of relative quantification of gene expression is the most useful approach for the gene expression analyses performed in Paper 2, 3 and 4 in the current study.

5. General discussion

5.1. Transport and uptake of LCPUFAs in placenta

As described in the Introduction, there is a selective enrichment of LCPUFAs from the maternal to the fetal circulation. This indicates that there is a system of preferential transport of LCPUFA in the placenta that is responsible for this biomagnification. FA transport and binding proteins are involved in the placental FA uptake [20]. However, the cellular FA uptake is also dependent on several intracellular metabolic processes such as the formation of acyl-CoA, complex lipids, lipid droplets and oxidation [20]. We therefore studied several mechanisms for FA transport and uptake in a trophoblast cell line (BeWo). Using a transwell cell culture system we found that OA induces lipid droplets and ADRP expression, and raises the intracellular TAG level, to a greater extent than DHA (Paper 1). This indicates that different transport mechanisms are involved in the transport of LCPUFAs compared to OA.

Furthermore, we found that LCPUFA and OA differentially regulate the expression of ACSL1 and 5 (Paper 2). These enzymes are responsible for the conversion of FFAs into acylCoAs, which is an obligatory step for further metabolism of FFAs once they are taken up by the cell. Inhibiting this step using triacsin C inhibited the uptake of radiolabelled OA and PA by ~70%, while the uptake of AA and DHA was only inhibited by ~20% in standard cell culture plates (Paper 1). When the same incubations were carried out in a transwell system we found that the addition of triacsin C increased the efflux of OA to the basolateral chamber, and had no significant effect on DHA (Paper 1).

Our results in Paper 1 led us to believe that LCPUFAs could somehow circumvent the acetylation step and be directly transported across the cell. However, the results in Paper 2 do not fully support this proposal. Instead, it points towards different roles of individual enzymes with ACS activity in regulating the uptake and intracellular fates of different FAs. The "channeling hypothesis" by Coleman *et al* suggests that the variety of ACSL isoforms channel FAs into different metabolic pathways [95]. Triacsin C inhibits both phospholipid and TAG synthesis in fibroblasts, TAG synthesis in hepatocytes, and B-oxidation but only to a lesser extent [92]. Triacsin C selectively

inhibits ACSL1, 3 and 4, but not 5 and 6 [90]. Both ACSL1 and ACSL5 have a preference for saturated and unsaturated FAs of 16-20 carbons as substrates [301], as shown in epithelial cells and adipocytes. However not much is known about ACSLs in the placenta and trophoblasts, and their function have not been studied in BeWo cells previously.

We found that enhanced uptake of radiolabeled FAs after pre-incubation of BeWo cells with LCPUFAs was associated with an increased incorporation of the FAs in phospholipid fractions and reduced incorporation in TAG fractions, while pre-incubation with OA had the opposite effect (Paper 2). The ACSL enzymes could possibly be involved in this process, directing the FAs towards different intracellular lipid pools. In the brain it has been shown that ACSL6 have an important role in uptake and incorporation of AA and DHA into phospholipids [302,303].

Previous results from our group indicate that LCPUFAs can modulate the uptake of AA, EPA and DHA, but not OA in BeWo cells [276]. In Paper 2 we report that the LCPUFAs can modulate the uptake of both OA and LCPUFAs, and that FAs are distributed in different lipid classes depending on whether the uptake was mediated by LCPUFA or OA. We showed that LCPUFAs, but not OA upregulate the mRNA expression of the ACSLs, ACSL1 and 5, and we therefore suggest that these genes are involved in the uptake of LCPUFAS.

Over-expression of ACSL1 [304,305] and ACSL5 [306] lead to enhanced cellular FA uptake in different cell types. Co-immunoprecipitation of ACSL1 and FATP1 in adipocytes further suggests that acyl-CoA synthetase is important for FA uptake [307]. ACSL proteins are predicted to be localized to membranes, but not much information about their actual localization is available, and they have been identified in association both with membranes and in the cytosol [90]. ACSL1 is associated with the plasma membrane [307], mitochondria and in lipid droplets in adipocytes [308]. ACSL5 has been detected in the plasma membrane [309,310,311], in mitochondria [312] and in lipid rafts [313]. We did not study the intracellular localization of these enzymes in the BeWo cells.

We did not observe any regulation of the FATPs by any of the FAs in BeWo cells (Paper 2). Possibly, incubating BeWo cells with FAs could have an effect on the intracellular location of the FATPs and not their expression level. In adipocytes,

incubation with insulin did not regulate the expression of FATP1 but instead mediated a translocation of the protein from the cytosol to the plasma membrane, and by that the transport of long chain FAs was increased [314]. Elchalal *et al* reported that incubating primary term trophoblast with a combination of OA, LA and insulin did not alter the mRNA expression of any of the FATPs [315].

5.2. Role of ADRP and lipid droplets in placenta

When this project was started, the knowledge about lipid droplet proteins and ADRP was limited, especially in the placenta. In the past few years the lipid droplet research field has increased greatly, with more detailed knowledge about the role of lipid droplets. Formerly they were recognized as organelles for lipid storage, but recent research has shown that they have many roles in lipid metabolism and in disease situations.

The background for this project was the previous finding by our research group that the lipid droplet associated protein ADRP was up-regulated by FAs both on the mRNA and protein level in BeWo cells and primary trophoblast [276]. We hypothesized that ADRP might be involved in the uptake and transport of FA in the placenta, and we wanted to further study the role of ADRP in placenta and whether ADRP could have a role in PE. We have therefore investigated the ADRP expression in BeWo cells in relation to FA transport and also the effect of FFA and oxidative stress, that are found to be increased in PE, on both gene and protein expression of ADRP in BeWo cells.

The placenta is not a lipid storing organ, as it does not contain large lipid storage areas, in contrast to the acute atherosis phenomenon of decidual spiral arteries. Hence, the role of LDs in placenta could be something different than storing lipids as an energy source. The transport of lipids from the mother to the fetus is extensive in the latter half of pregnancy and there is an increasing accumulation of lipid droplets in the placenta with gestational age [316]. It has been speculated that enhanced ADRP expression may contribute to FA uptake by the placenta [111]. In a healthy pregnancy, ADRP could be important in regulation of FA signaling pathways. Furthermore, in pathological pregnancies, dysregulation of ADRP could possibly lead to alterations in FA signaling pathways, which are necessary for a healthy pregnancy. We hypothesized

that the lipid droplet associated protein ADRP could be involved in the preferential transport of FAs from the maternal circulation to the fetus, and that it could possible translocate from the lipid droplet to lipid rafts and caveolae in the plasma membrane and be involved in the intracellular transport of FAs.

There is increasing evidence that lipid droplets are involved in intracellular lipid trafficking between different organelles [317]. A variety of proteins that regulate membrane traffic have been found associated with lipid droplets by proteomic studies, including proteins involved in vesicle formation and motility, motor proteins involved in movement along the cytoskeleton, vesicular trafficking and others [317]. Lipid droplets have been found to interact with different organelles such as the endoplasmic reticulum, endosomes, peroxisomes and mitochondria [317]. The association with mitochondria has been reported in adipocytes, liver cells, and skeletal muscle and this interaction is suggested to be involved in beta oxidation of FAs [317]. However, ADRP and the other lipid associated proteins are mainly thought to act as structural proteins for lipid droplets, stabilizing the droplets [318].

We found that the expression of ADRP in BeWo cells was increased at mRNA level by LCPUFAs but not by OA (Paper 2). The protein level was induced by OA to a greater extent than DHA (Paper 1), and also by LA but only slightly by PA (Paper 4). Using immunofluorescence cytochemistry (with antibodies against ADRP as a marker for lipid droplets) we observed that OA, more than DHA, induced an increase in the accumulation of lipid droplets (Paper 1). We found no evidence of co-localization of caveolin-1 (a structural protein of caveolae/lipid rafts) and ADRP in BeWo cells (Paper 1). We also isolated lipid rafts (detergent resistant membranes) by the method of Lygren *et al* [319], and we detected caveolin-1 in the raft fractions, but not ADRP (data not shown).

5.3. Role of ADRP and lipid droplets in preeclampsia

Accumulation of excess neutral lipids in lipid droplets is associated with metabolic diseases, such as obesity, DM2, hepatic steatosis and atherosclerosis [320]. In Paper 4 we show that this may also be the case in PE. We found that the lipid droplet marker protein ADRP is increased ~2 fold in preeclamptic placenta tissue.

Dyslipidemia and lipotoxicity

The lipid abnormalities of PE are described in the Introduction. These metabolic alterations include hyperlipidemia, a feature of normal pregnancy but further increased in PE. In our patient selection, however, we did not demonstrate elevated total cholesterol or TAG in the maternal serum as compared with the control group (Table 2). The explanation for this discrepancy to other PE population findings where TAG concentrations are elevated [232,321,322] could be the patient selection. There is a large biological variation between the patients, and we may have included by chance women with less hyperlipidemia than other cohorts, and also our population was not very large (n=79), implying study power challenges. We did not measure the FFA concentration in maternal blood, or other lipid fractions that previously have been shown to be dysregulated in PE such as HDL cholesterol. HDL cholesterol is found to be decreased in PE [322,323], while total cholesterol and LDL cholesterol is not altered in PE compared to normal pregnancies [324]. In previous studies from our group, using the same patient material from our biobank, it was found that the oxidized lipid 8-isoprostane was elevated in maternal plasma in PE [226] and also in decidual tissue [209]. However, the elevated isoprostane could be a marker for oxidative stress [325] and not hyperlipidemia. In Paper 3 we measured FFAs in placental tissue and found that n-6 PUFAs levels were increased in PE compared with control. On the other hand, not all women with PE become dyslipidemic and not all women with gestational dyslipidemia develop PE [263].

Table 2: Concentrations of total cholesterol and TAG in maternal serum in our study

	Controls (n=27)	**Preeclampsia (PE) (n=28/29)**	**Controls vs. PE**
	Median (min-max)	**Median (min-max)**	**P-value**
Totalcholesterol (mmol/L)	6.69 (4.93-9.15)	6.36 (3.72-14.26)	0.36
Triacylglyceride (mmol/L)	2.78 (1.34-7.42)	3.43 (0.81-6.73)	0.17

Values shown are (number of patients included) median serum concentrations and minimum and maximum values. In the PE group total cholesterol was measured in 28 patients, while TAG was measured in 29 patients. The concentrations were determined using routine enzymatic methods. The *P*-value is given for each diagnosis group compared to the control group and was calculated using non-parametric Mann-Whitney test.

Accumulation of excess lipids in non-adipose tissues is termed lipotoxicity and can lead to impaired cell signaling, cell dysfunction and cell death [241]. Lipid accumulation in heart, skeletal muscle, pancreas, liver and kidney is associated with diseases such as heart failure, obesity and diabetes [320]. Lipotoxicity has also been suggested to play a role in insulin resistance, and hyperlipidemia [242], conditions associated with both DM and PE.

In women who later develop PE, the concentrations of OA, LA and PA in the maternal circulation is elevated already at week 16-20 of gestation [228,232]. We incubated BeWo cells with these FAs and found both mRNA and protein expression of ADRP increased with OA and LA, but only slightly with PA (Paper 4). In cell culture, OA supplementation leads to TAG accumulation and is well tolerated, whereas PA is poorly incorporated into triglyceride and causes apoptosis [326]. In Paper 1 we found that OA induced the protein expression of ADRP, formation of lipid droplets and accumulation of intracellular TAG and to a greater extent than DHA. Hence, the increased ADRP expression in PE could be due to the higher concentrations of circulating OA and LA found in the maternal circulation. It would be useful to measure the concentration of these FAs in maternal blood in the samples from our biobank in order to support this proposition. However, oxidative stress in the placenta or the maternal circulation could also be responsible for the increased ADRP expression in preeclamptic placenta.

Some studies suggest that lipid droplets may have protective effects in cells against lipotoxicity [326,327]. In cardiomyocytes lipid droplets have a protective effect against ischemia-reperfusion injury by sequestering FFAs [328]. In muscle cells lipid droplets are reported to have a protective effect against the damaging effects of FAs on insulin action and glucose tolerance [329,330]. Recently, Gubern *et al* studied stress induced lipid droplet biogenesis without any external source of FAs and found that lipid droplet biogenesis was not dependent on *de novo* FA synthesis, instead structural membrane phospholipids were directed to the lipid droplets. The authors suggest that this represents a survival strategy whereby the cell recycles membrane lipids to use as energy generating substrates as a response to stress such as glucose depravation [331]. This is supported by the view that lipid droplets have a role in the management of membrane lipids. The composition of lipids in the lipid droplets is complex and

includes a variety of different neutral lipids and phospholipids, and the large amount of lysophospholids in the lipid droplets implies a role for them in the recycling of phospholipids [332,333]. Possibly, recycling of lipids from cell membranes does not occur in situations where the cell is in an environment with excess lipids.

The actual role of the alterations in the lipid metabolism in pregnancy and PE is not fully understood. In normal pregnancy the hyperlipidemia might be important for adequate fetal nutrition. In PE it might contribute to mobilize extra energy from the mother that can be taken up and stored by the placenta as a support against the stressful PE environment. Another way of looking at the maternal hyperlipidemia is that it is harmful for the placenta, and therefore the placenta sequesters these extra lipids as a protective mechanism to save the feto-placental unit from lipotoxicity.

Oxidative stress and inflammation

Oxidative stress is a feature of PE and is believed to be important in the pathophysiology of the syndrome. We induced oxidative stress in BeWo cells using different concentrations of H_2O_2, and found that the number and size of ADRP coated lipid droplets increased with increasing doses and incubation times with H_2O_2 (Paper 4). This finding is supported by several studies where different types of oxidative stress induced accumulation of lipid droplets in different cell types and species [334,335,336]. Hence, we speculate that the increased ADRP gene expression in PE could be due to increased oxidative stress in the placenta. Possibly, the combination of hyperlipidemia and oxidative stress is even more potent in inducing accumulation of lipid droplets. ROS could be involved in oxidation of the excess lipids in the maternal circulation or in oxidative attack on lipids in cell membranes in the placental tissue.

Lipid droplets are normally sparse in normal cells (except adipocytes), but increase in number and size in cells associated with inflammation [337]. The accumulation of lipid droplets is induced by different inflammatory agents such as LPS or cytokines in different cell types [338,339,340], and is also induced in inflammatory and infectious diseases [339,341]. In placenta, the number of lipid droplets in amnion epithelium increases during labor, indicating that lipid droplets are induced by stress [342]. PE is associated with a more extreme maternal inflammatory response than occurs in normal pregnancy. The systemic inflammatory response involves an increase

in circulating cytokines. We measured the cytokine concentrations of TNF-α and IL-6 in maternal plasma in order to confirm the presence of inflammation in our PE group. We found that both TNF-α and IL-6 concentrations were increased in plasma from our patients with PE as compared to controls (Table 3). It is possible that these pro-inflammatory cytokines also could be involved in inducing lipid droplet accumulation in preeclamptic placenta; however, presently we have no evidence for this. Over-expression of ADRP increase the expression of cytokines in macrophages and this is suggested to be important in the development of atherosclerosis [343]. Hence, it would be interesting to study the effect of TNF-α and IL-6 on ADRP expression and lipid droplet accumulation in trophoblast cells and vice versa.

Table 3: Concentrations of cytokines in maternal plasma in our study

	Controls (n=30)	**Preeclampsia (PE) (n=30)**	**Controls vs. PE**
	Median (min-max)	**Median (min-max)**	**P-value**
IL-6 (pg/mL)	0.7 (0.1-3.5)	1.3 (0.8-13.1)	0.023*
sTNF R1 (pg/mL)	1184 (762-2383)	1535 (793-3438)	0.008*

Values shown are (number of patients included) median plasma concentrations and minimum and maximum values. Enzyme linked immunosorbent assays (ELISA) for IL-6 (HS600B, Quantikine HS) and sTNF R1 (DRT100, Quantikine) both from R&D Systems Europe (UK) was performed in duplicates on EDTA-plasma samples according to the manufacturer's instructions. The sTNF R1 ELISA measures the total amount of free soluble receptor plus the total amount of soluble receptor bound to TNF present in the samples. The circulating levels of the receptor increase in response to TNF-α production, and because the half-life of the receptor in plasma is much longer than the half-life of TNF, the receptor concentration is considered to be a reflection of the TNF-α activity [344]. The *P*-value is given for each diagnosis group compared to the control group (*P <0.05), and was calculated using non-parametric Mann-Whitney test.

There is also increasing evidence that lipid droplets are important regulators of inflammatory processes. Several eicosanoid-forming enzymes, involved in the conversion of AA into eicosanoids, have been detected both *in vitro* and *in vivo*, and accumulation of lipid droplets correlates with the capacity of cells to produce eicosanoids [345].

In the preeclamptic situation, there is both a dysregulation of the lipid metabolism with excess availability of lipids from the maternal circulation, and also

excess inflammation and oxidative stress. Possibly lipid droplets are accumulated in placenta during pregnancy due to excess FFA in the circulation and oxidative stress in the placenta. We speculate that lipid droplets could have a protective role in placenta, whereby excess lipids are stored in the droplets in order to prevent them to exert harmful effects intracellularly. Another hypothesis is that the lipid droplet could serve as a survival mechanism for the placental cells, whereby it is storing lipids as an energy reserve. Possibly, the increased maternal circulating FAs could serve as an adaptive protective measure against the oxidative stress and inflammation in the placenta during pregnancy.

Fibrinoid tissue

In Paper 4, ADRP immunostaining was mainly found to stain clusters of droplets in the fibrinoid tissue of the placental extracellular matrix. This result is in agreement with Meadows *et al* who also found that ADRP was localized to fibrinoid tissue in the placenta, in a punctuate pattern corresponding to the size and location of lipid droplets [346]. The fibrinoid tissue we observed with IHC is most probably of fibrin-type fibrinoid, derived from the coagulation cascade. Fibrin-type fibrinoid is more prevalent in PE placentas [347], and is associated with apoptosis in trophoblast cells in PE [348]. In cultured primary term trophoblasts fibrin protects against hypoxic injury [349].

Many functions for the fibrin-type-fibrinoid in the placenta have been suggested, such as mechanical stability for anchoring the stem villi, as a regulator of intervillous circulation, a barrier to limit the invasiveness of the trophoblasts and as a facilitator of materno-fetal transport of macromolecules [3]. Alphafetoprotein has been shown to pass through fibrin-type fibrinoid gaps in the syncytiotrophoblast layer [350]. Ackerman *et al* identified numerous large lipid droplets in trophoblasts, which increased in size and numbers the closer they were in proximity to the maternal interface, and they were also associated to regions of necrotic cellular debris [351]. Possibly the lipid droplets could be transported out of the placenta via areas of fibrinoid tissue and subsequently shed to the maternal circulation in the same manner as STBMs. There is also the possibility that the lipid droplets are transported from the placenta to the decidua where they are engulfed by macrophages associated with acute atherosis. Katabuchi *et al* observed areas

of fibrin deposition and accumulation of foam cells in the spiral arteries of the decidua, predominantly in patients with PE [352]. These foam cells were filled with lipid droplets of various sizes.

5.4. Dysregulation of FA transport genes in preeclampsia

In Paper 3 and 4 we studied the expression of genes involved in lipid metabolism in preeclamptic placenta. We found that several genes were dysregulated. Here we will discuss the regulation of three of these that are involved in the uptake of FAs. FATP1, CD36, and CAV1 were all significantly decreased in preeclamptic placenta (Paper 3 and 4).

FATP1 is a 63 kDa transport protein that mediates FA uptake and is expressed in a wide variety of cell types and tissues. It is involved in transport of long chained FAs with a preference for very long chained FAs and is linked with acyl-CoA synthetase activity [353]. Characterization of purified mouse FATP1 protein conveyed that FATP1 has ACS activity [354]. The protein has a transmembrane domain and several membrane associated domains [353]. FATP1 was first identified in placental membranes [47] and the expression is induced by PPARγ and RXR agonists in primary trophoblasts [162].

We found the FATP1 gene expression to be decreased in PE, unfortunately we were unable to quantify the protein expression and to observe the intracellular location by immunohistochemistry in term placenta. Previously FATP1 has been found localized to the plasma membrane and to small vesicles in the cytoplasm [62]. Over-expression of FATP1 and CD36 in muscle cells resulted in increased transport of OA and PA [355]. FATP1 was localized throughout the cytosol in a reticular pattern, partly co-localized with the Golgi system, and CD36 was localized to the extracellular membrane, neither of the proteins were co-localized with lipid droplets [355].

FATP1 is downregulated by TNF-α in the liver [314]. In line with this we speculate that inflammation could be involved in down-regulating FATP1 in placenta in PE, as we did not observe any regulation of FATPs with FAs in Paper 2. Also, FATP1 and CAV1 were not regulated by the FAs (PA, OA and LA) that are found up-regulated in the maternal circulation in PE, and neither by LCPUFAs (AA and DHA) (Figure 9).

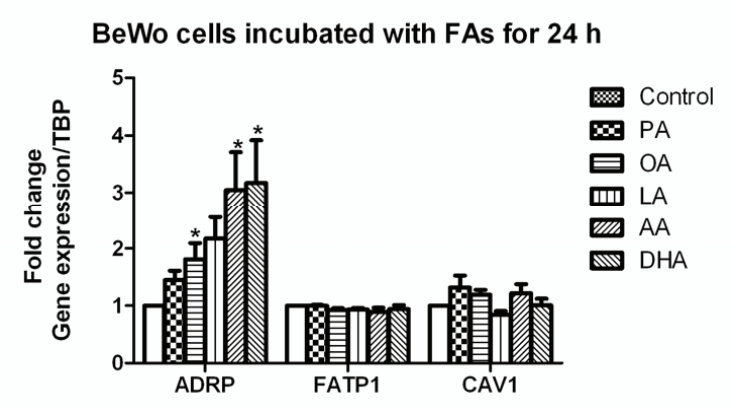

Figure 9: Gene expression of ADRP, FATP1 and CAV1 in BeWo cells after 24 h incubation with 100 μM of different fatty acids (FAs). Gene expression was analyzed using quantitative real-time RT-PCR. TBP was used as endogenous control. The *P*-value is given for each FA compared to the control (*$P < 0.05$) and was calculated using Students T-test.

CD36 is reported to be localized to lipid rafts (detergent resistant membranes) [73] and in caveolae [86]. Pohl *et al* showed that caveolin-1 targets CD36 to the plasma membrane in mice embryonic fibroblasts, and that in caveolin-1 knock-out mice CD36 was not localized to the plasma membrane [356]. Interestingly, we found that mRNA expression of CD36 and caveolin-1, both associated with caveolae, were decreased in PE. In Paper 4 we found caveolin-1 localized mainly to endothelial cells in placental sections, but we also observed some occasional staining of cytotrophoblasts. However, we found no difference between the sections from preeclamptic pregnancies compared to controls (Paper 4). Vandré *et al* (2007) found caveolin-1 protein expression associated with endothelial cells and stromal cells in placenta [357], and Byrne *et al* found by immuno-gold labeling that caveolin-1 in placenta was mostly expressed in endothelial cells, and only infrequently in trophoblast cells [358]. The gold particles were localized to the basal and apical membrane of the endothelium, mostly on the basal side, suggesting a role in active lipid transport from the maternal side to the fetal side. We speculate that the reason for the decreased expression of these genes is connected somehow to the FA transport system of the placenta, and could be a response to excess FFA in the maternal circulation in PE. However, regulation of gene expression is

complex and may involve many other factors. The down-regulation could be a protective mechanism against further lipid accumulation in the placenta, or it could be a reduction of lipid transport from the placenta to the fetus either because the excess lipids can be harmful for the fetus or because the placenta needs to accumulate lipids to protect itself under the stressful PE environment. Our observation that caveolin-1 is mainly expressed in fetal endothelial cells of the placenta (Paper 4) supports this notion.

6. Conclusions

The main conclusions of this study are as follows:

1. LCPUFAs (AA and DHA) are more efficiently transported across BeWo cells compared to non-essential FAs (PA and OA).
2. The non-essential FA (OA) is incorporated into TAG fractions and induces accumulation of lipid droplets in BeWo cells to a greater extent than LCPUFAs (DHA).
3. LCPUFAs modulate the uptake of both LCPUFAs and OA in BeWo cells. The uptake of all FAs increase after preincubation with LCPUFAs and the FAs are preferentially incorporated into phospholipids.
4. Incubation of BeWo cells with LCPUFAs increased the gene expression of long-chain acyl-CoA synthetases, ACSL1 and ACSL5.
5. There is a dysregulation of LXRα, LXRβ and PPARγ in preeclamptic placenta, the gene expression is significantly decreased for all three transcription factors.
6. ADRP gene expression is increased, while FATP1 and CAV1 expression is decreased in preeclamptic placenta, and ADRP is also increased on the protein level.
7. The protein expression of ADRP in placenta tissue is localized to clusters of vacuoles in the fibrinoid tissue and also to small vesicles inside trophoblast cells.
8. ADRP expression is regulated by FAs and oxidative stress (H_2O_2) in BeWo cells.

In conclusion, this study supports the previous observations that LCPUFAs are selectively enriched in the fetal circulation. Our results suggest that there are different transport mechanisms for LCPUFAS (DHA) and non-essential FAs (OA) in the trophoblasts of the placenta. The transport mechanism for OA may involve accumulation of TAG in lipid droplets, while DHA possibly to some extent circumvents this step. Our results support the concept of a special system for active transport of LCPUFA across the placenta. We also found that LCPUFAs influence the uptake of FAs in trophoblast cells and this could have important implications for fetal nutrition and for dietary advice given to pregnant women. Our results suggest that ACSL1 and

ACSL5 could be involved in FA uptake and that conversion of FFAs to acyl-CoA is an important step in the LCPUFA influenced uptake of FAs in the placenta.

We also aimed at exploring the dysregulation of the lipid metabolism in pregnancies complicated by PE and/or DM and we show that both PPARs and LXRs are dysregulated in placenta in PE. We suggest that these transcription factors could be involved in the PE situation, but further research is required for definite conclusions. We also show that the lipid droplet associated protein ADRP is dysregulated in PE. The increased expression of ADRP is possibly induced by oxidative stress in the placenta, even though dyslipidemia may also play a role in this regard.

7. Future perspectives

At the end of the road to a Ph.D. project, there are many questions surfacing that would be interesting to study further. Here are some thoughts:

- What happens at earlier time points in the pregnancy? It would be interesting to perform a longitudinal study of placenta tissue samples, both gene expression patterns of our proteins of interest and immunohistochemistry analysis could provide valuable insights into the metabolic development and adaptations of the placenta during the pregnancy. Possibly, excess chorionic villi sampling tissue used for prenatal diagnosis could be used for such longitudinal sampling, although this material is very difficult to obtain in reasonable amounts.
- Do LCPUFAs modulate increase in the uptake of all FAs? In this study we analyzed the effects on LCPUFA uptake and OA, but what about PA or other short chained FAs that are not considered to beneficial in the diet? Is it correct to recommend dietary supplementation of n-3 LCPUFA for pregnant women if these FAs enhance the uptake of other FAs in the placenta?
- How is the FA uptake and transport in primary trophoblast cells and differentiated BeWo cells? Could there be other cells in the placenta, in addition to trophoblasts, involved in the preferential LCPUFA transport? It would be interesting to isolate primary endothelial cells from placenta to compare the FA transport in these with other endothelial cells, for example from the umbilical cord and aorta.
- Are lipid droplet associated proteins involved in the development of acute atherosis? Decidual tissue with areas of acute atherosis can be used as a model for studying the process of atherosclerosis in general, because of similarities in the development of lipid deposition in decidua and other tissues. It has been shown that ADRP is involved in the formation of plaque in atherosclerosis [359]. Hence, it would be interesting to investigate the possible involvement of ADRP and other LDAPs in decidua in areas of acute atherosis.

Placenta remains the key factor to a successful pregnancy and is also an important predictor of future maternal and offspring health. Further placental research will be essential for solving the riddle of PE and other pregnancy complications, important for the wellbeing of both the mother and the fetus. The similarities in the pathophysiology of PE with other diseases, such as diabetes and atherosclerosis, makes further research on PE important also when the focus is on development of other diseases.

8. References

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Paper 1

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Paper 2

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Paper 3

Expression of liver X receptors in pregnancies complicated by preeclampsia

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Running title: Expression of LXRs in preeclamptic pregnancies

Abstract

Preeclampsia is a pregnancy specific disorder associated with hyperlipidemia. Liver X receptor (LXR) α and LXRβ are key regulators of lipid homeostasis. In the current study, we investigated expression of LXRα, LXRβ and their target genes in human term placenta, decidua and subcutaneous adipose tissue from pregnancies complicated by preeclampsia. Furthermore, we analyzed the protein levels of LXRα and LXRβ in placenta. We also analyzed lipid concentrations in term placental tissue. Gene 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. The placental protein level of LXRβ was reduced, and there was a positive correlation between placental LXRβ mRNA expression and placental free fatty acids in preeclampsia. Our results suggest a possible role for LXRβ as a transcriptional regulator in the preeclamptic situation.

1. Introduction

Preeclampsia is a pregnancy-specific disorder affecting 3-10% of all pregnancies and a significant cause of maternal and neonatal morbidity and mortality. It is clinically defined by hypertension and proteinuria developing after week 20 of gestation. The pathogenesis of preeclampsia is still not fully understood and is most likely multifactorial. However, a key role for the placenta in the etiology of the disease is widely acknowledged (reviewed in [1]).

Hyperlipidemia of pregnancy develops in every pregnant woman, but is significantly increased in women with preeclampsia relative to healthy pregnancies, also prior to clinical onset of the disease [2]. The lipid abnormalities of preeclampsia include hypertriglyceridemia, increased circulating free fatty acids (FFA), increased concentration of small low density lipoproteins and the presence of oxidized low density lipoproteins in maternal circulation [3-5], lipids that could add to the endothelial dysfunction observed in preeclampsia. However, the regulation of lipid metabolism in preeclamptic placentas has not been studied extensively.

Liver X receptors (LXR) are ligand-activated transcription factors belonging to the nuclear receptor superfamily. Two isoforms are known; LXRα and LXRβ, and both are activated by oxidized cholesterol

derivatives, oxysterols [6]. They form obligate heterodimers with the nuclear receptors retinoid X receptors (RXRs). The LXRs have been identified as key regulators of lipid metabolism through the transcriptional regulation of genes involved in *de novo* fatty acid metabolism, triacylglycerol (TAG) synthesis and cholesterol homeostasis (reviewed in [7,8]). Other transcription factors that are important regulators of lipid metabolism include nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) family and the sterol regulatory element binding protein (SREBP) family [9,10]. We previously found that LXR increased the synthesis of fatty acids and inhibited secretion of human chorionic gonadotropin in human placental BeWo cells [11]. A role for the LXRs in placentation and trophoblast invasion has also recently been described [12], as well as in regulation of placental cholesterol transport [13,14]. These findings suggest that the LXRs may be important in human placentation and feto-placental lipid transport and metabolism.

The LXRs have been extensively studied in rodents *in vivo*, while clinical data in humans are limited. In order to explore the role of LXR in preeclampsia, we investigated the mRNA and protein expression in placenta, decidua and subcutaneous adipose tissue of LXRα and LXRβ, their target genes, and other transcription factors involved in regulation of lipid metabolism. We found a statistically significant lower placental mRNA expression of LXRα and LXRβ, as well as lower LXRβ protein levels and lower concentrations of placental FFAs in preeclampsia compared to controls, and a correlation between placental mRNA LXRβ expression and placental FFAs in the preeclamptic group. Based on these findings we speculate that placental LXRβ may have a role in regulating FFA levels in the preeclamptic placenta.

2. Materials and Methods

2.1. Patient selection

Samples were obtained from an ongoing biobank collection of patient samples from complicated and uncomplicated pregnancies at Oslo University Hospital, Ulleval. Women with singleton pregnancy undergoing caesarean section (n=61) were included in this study; including 33 women with uncomplicated pregnancy (controls) and 28 with preeclamptic pregnancy. No women with chronic hypertension or renal disease were included. All patients were fasted for a minimum of 6 hours; none were in active labor, had ruptured membranes or clinical signs of infection. Preeclampsia was defined as blood pressure augmentation after 20 weeks' gestation to >140/90 on ≥ two occasions six hours apart in a previously normotensive woman, combined with proteinuria. Proteinuria was defined as protein dip stick ≥ 1+ on ≥ two midstream urine samples six hours apart or a 24-hour urine excretion of ≥ 0.3 g protein, in the absence of urinary infection. Severe preeclampsia was defined by the American College of Obstetricians and Gynecologists criteria (ACOG) [15], including women with blood pressure of 160 mmHg systolic or higher. The newborn birth weight percentiles were calculated according to national birth registry data [16] or a ultrasound based weight percentile [17]. The study protocol was approved by the Regional Committee of Medical Research Ethics in Eastern Norway, and informed written consent was obtained from each patient.

2.3. Tissue samples

All tissue samples were obtained during cesarean section. Subcutaneous adipose tissue biopsies were sampled adjacent to the lower abdominal incision. Placenta biopsies from a macroscopically normal looking,

centrally located cotyledon were collected, omitting the decidual layer. Decidual tissue was collected through vacuum suctioning of the uterine wall underlying the placenta (corresponding to the superficial layer of the placental bed, including minimal myometrial tissue), as described previously [18]. All tissues were snap-frozen in liquid nitrogen and stored at –80°C.

2.4. RNA isolation from tissue and cDNA synthesis

The tissues were pulverized in liquid nitrogen and ~15 mg of tissue was homogenized in 800 µl of RNA lysis buffer using an Ultra-Thurrax homogenizer for 30 sec. Total RNA was extracted from placental and decidual tissues using ABI6100 (Applied Biosystems, Foster City, CA) and adipose tissue using RNeasy Lipid Tissue Mini kit (Qiagen, Netherland) according to the manufacturers' instructions. The quality and quantity of the RNA was determined using spectrophotometer (NanoDrop 1000, NanoDrop Technologies, Boston, MA) and capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA) according to manufacturer's protocol, and was found to be sufficient for the gene expression analysis with 260/280 and 260/230 ratios above 2 and RNA integrity numbers above 7. cDNA was synthesized (20 µl) from extracted total RNA (400 ng) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions.

2.6. mRNA gene analyses by quantitative reverse transcriptase polymerase chain reaction

Quantitative real-time PCR (qRT-PCR) was performed using custom-made 384-well microfluid cards (TaqMan Low Density Array) and Gene Expression Master Mix (both from Applied Biosystems). The RT-PCR was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems), and data acquisition and analysis were done according to the manufacturer's instructions, using the ΔΔCt method except for Figure 1, where a slightly different ΔCt method version was used. Briefly, the arbitrary values were calculated using the Ct value for each qPCR reaction according to the following equation $((2^{(-Ct)}) \times 10^9$. The multiplication with 10^9 was done to get a number close to 1 instead of a very small number that would be difficult to read. The following TaqMan gene expression assays were employed:

LXRα (Hs00172885_m1), LXRβ (Hs00173195_m1), RXRα (Hs01067640_m1), PPARδ (Hs00602622_m1), PPARγ (Hs01115513_m1), SREBP-1 (Hs01088691), fatty acid synthase (FAS) (Hs00188012_m1), fatty acid elongase 5 (Elovl5) (Hs01094711_m1), CD36 (Hs00169627_m1), lipoprotein lipase (LPL) (Hs00173425_m1), low density lipoprotein receptor (LDLR) (Hs01092525_m1), apolipoprotein E (ApoE) (Hs00171168_m1), ATP-binding cassette (ABC), sub-family A, member 1 (ABCA1) (Hs00194045_m1), ABC, sub-family G, member 1 (ABCG1) (Hs01555189_m1), leptin (Hs00174877_m1). Four genes, all commonly used as endogenous controls; 18S (Hs99999901-s1), glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1), TATA box binding protein (TBP) (Hs99999910_m1), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) (Hs00237047_m1) were included in the expression analysis and the expression values of each investigated gene in all tissue samples were normalized against the most stabile endogenous control in each tissue, which was YWHAZ for placenta and decidual tissue and TBP for adipose tissue. For the comparison of LXRα and LXRβ between the tissues, all four endogenous controls were found to be differentially expressed between the tissues. If the chosen endogenous controls are regulated, in worst case the choice of controls could produce results merely reflecting the regulation of the endogenous control, not that of the target gene. Our thorough testing of the material assured us that we included equal amount of RNA in each sample and that there was no

inhibition of the qRT-PCR reaction, hence, that the amount of total RNA initially measured would directly represent the level of expression for each gene. We therefore believe that the use of Ct values, not normalized to the regulated endogenous control, when calculating the difference in expression between the tissues, is the most correct way of measuring the gene expression between the tissues in our experiment (Figure 1).

2.7. Western blots: Protein analysis of LXRα and LXRβ

A ~5 mg piece of placental tissue was added to a tube containing 1 spoon of glass beads and 300 µl of lysis buffer on ice (1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS and 15 µl/ml of proteinase inhibitor cocktail). The tissue was homogenized using a Precellys24 homogenizer (Bertin Technologies, France) at 5000 rpm for 2 x 20 seconds. Protein concentration was quantified using the Bio-Rad colorimetric assay system with BSA as protein standard (Bio-Rad Laboratories, Hercules, USA). Fifty µg of total proteins were separated on SDS polyacrylamide gels (Bio-Rad Laboratories, Inc.), and LXRα and LXRβ levels were determined by blotting with mouse monoclonal anti-human LXR antibodies (R&D Systems, Perseus Proteomics, Inc., Japan: PP-K8607-00 (LXRα) or PP-K8917-00 (LXRβ), 1: 500). Levels of β-actin were determined using mouse monoclonal anti-β-actin antibody (Sigma-Aldrich Inc.: A5441, 1: 10000). Secondary anti-mouse IgG antibody (Abcam plc, UK: ab6728, 1: 10000).

2.8. Measurement of lipids and the fatty acid profile in placental tissues

Lipid class analysis

Homogenized placenta tissue (100 - 200 mg), that was initially crushed under liquid nitrogen, was removed from -80°C storage, transferred to a frozen vial with a frozen spatel and immediately added 1.5 ml methanol and vortex-mixed to avoid metabolism of free fatty acids. Lipids were then extracted by Folch, evaporated to dryness by vacuum centrifugation and the lipids dissolved in 200 µl hexane. The hexane containing the lipids was aliquoted into two vials, one for polar lipid analysis and one for apolar lipids analysis. Lipid classes were separated by normal phase HPLC and detected with Evaporative Light Scattering Detector. Apolar lipids were separated with 0.5 % acetic acid in heptane delivered at 2.0 ml /min on a Merck Purospher Si, 100 mm x 4.6 mm, while polar lipids were analyzed in separate method using the same column but with a mobile phase consisting of 0.5 % acetic acid and 5 % methyl tert butyl ether in heptane.

Fatty acid analysis

Approximately 40 mg placenta tissue powder, that was initially crushed under liquid nitrogen, was removed from -80°C storage, transferred to a frozen vial with a frozen spatel and immediately added 0.9 ml HCl Methanol and vortex-mixed. Transmethylation was performed in an ultrasound bath held at 70°C for 30 min, then for additional 120 min at 80°C without ultrasound. After cooling and neutralization by KOH, fatty acid methyl esters were extracted with 500 µl hexane.

Gas chromatographic analysis

Analyses were performed using a 6890N GC with a split/splitless injector, a 7683B automatic liquid sampler, and flame ionization detection (Agilent Technologies, Palo Alto, CA). Separation was performed with a Supelco SP2380 (30 m × 0.25 mm i.d. × 0.25 µm film thickness) GC column. Temperature program, initial: 90ºC with 0.5 min hold, ramp 50ºC/min to 150ºC, 10ºC/min to 225ºC, 120 ºC/min to 245ºC with hold 3 minutes. Carrier gas was H_2 with a flow of 2.2 ml/min. Fatty acid analysis was performed by auto injection of 0.5 µl of each sample at a split ratio of 0.1:1, constant flow mode, injector temperature 250°C. The flame ionization detector

temperature was 270ºC. The sampling frequency was 10 Hz. The run time for a single sample was 12.37 min. Theoretical response factors were used.

2.9. Statistical analysis

Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS-PC), version 16.0. For gene expression data and lipid concentration data, the results were normally distributed and the significance was calculated using Student's t-test and correlations using linear regression. For the patient characteristics, differences in continuous variables between the control and preeclamptic group were tested by non-parametric Mann-Whitney tests. A probability level of <0.05 was considered statistically significant.

3. Results

3.1. Clinical characteristics

Clinical characteristics of the 28 preeclamptic and 33 control pregnant women included in the study on placenta are shown in Table 1. Not all samples were available or used for all analyses (decidua: 30 controls and 27 preeclampsia; adipose tissue: 29 controls and 28 with preeclampsia; placenta tissue lipid analysis: 12 controls and 12 with preeclampsia, western blot analysis: 6 controls and 6 with preeclampsia). In the patient groups used for decidual and placental lipid analyses, there were no significant differences in diastolic blood pressure <week 20 between the preeclamptic group and the control group. In the placental lipid analysis group, there was also no difference in body mass index (BMI) prior to pregnancy or at delivery between preeclamptic and controls. The remaining clinical variations between the patient groups were not significantly different from the results presented in Table 1. All preeclamptic patients had severe preeclampsia according to ACOG criteria [15]. In the preeclampsia group, 20 patients delivered prematurely, before week 37, and 17 of these delivered prior to week 34. In the uncomplicated pregnancy group, there were no premature deliveries. Further, three of the preeclamptic patients had evidence of HELLP (hemolysis, elevated liver enzymes and low platelets) syndrome [15]. There was a significantly higher median BMI (weight (kg)/height (meter)^2) in the preeclamptic group compared to controls, while birth weight, neonatal weight percentile and gestational age at delivery were significantly lower in the preeclamptic group as compared to controls.

3.2. mRNA expression of transcription factors in placenta, decidua and adipose tissue in healthy subjects

mRNA expression of LXRα, LXRβ, PPARγ, PPARδ, RXRα and SREBP-1 in placenta, decidua and adipose tissue from healthy controls are shown in Figure 1. Judged by Ct values, LXRα and LXRβ were similarly expressed in the gestational tissues, with an approximately equal expression in placenta and decidua, and an equal or lower expression than for the rest of the transcription factors investigated. In adipose tissue, LXRβ was similarly expressed, whereas higher expression of LXRα was observed compared to placenta and decidua.

3.3. Expression of genes in placenta, decidua and adipose tissue in pregnancies complicated by preeclampsia

The results from the placental transcription factor expression analysis according to patient groups are shown in Figure 2a. We found a statistically significant lower mean level of expression of LXRα, LXRβ, RXRα and PPARγ in the placentas of the preeclamptic group compared to those of the control group. However, a significantly higher expression of SREBP-1 was observed. There was no difference in the expression of PPARδ. The results from the placental expression

analysis of genes involved in lipid metabolism are shown in Figure 2b. There was a significantly lower expression of both CD36/FAT and ApoE, and a significantly higher expression of LDLR in preeclamptic placentas as compared with the controls (Figure 2b). We included leptin as a positive control because it is upregulated in preeclampsia in both serum and placental tissue [19] and found a 12-times higher expression in preeclamptic placentas compared to control placentas (P=.0001) (data not shown). The results from the decidua expression analysis according to patient groups are shown in Figure 2c and 2d. We found a statistically significant lower mean level of expression of ABCG1 in the decidua of the preeclamptic group compared to the control group. There were no differences in the expression of any of the other genes that were analyzed. The results from the adipose tissue expression analysis according to patient groups are shown in Figure 2e and 2f. We found a statistically significant higher mean level of expression of LXRα, RXRα, PPARδ and ABCA1 in the preeclamptic group compared with those of control group. There were no differences in the expression of any of the other genes that were analyzed.

3.4. Placental protein levels of LXRα and LXRβ in pregnancies complicated by preeclampsia

To dissect the biological implications of LXR in placenta, we next investigated the protein levels of LXRα and LXRβ in both healthy controls and pregnancies complicated by preeclampsia. Total proteins (50 μg) from 6 patients in each group were analysed for LXRα and LXRβ expression using specific LXR antibodies (Figure 3). Both LXRα and LXRβ were detected in the control group. The protein level of LXRβ was clearly down-regulated in the preeclamptic group compared to the control group. In the case of LXRα, no difference in the expression level was seen between the controls and the preeclamptic group. Comparing mean LXRα and LXRβ mRNA levels for the whole patient groups to the mean LXRα and LXRβ mRNA levels for the selected patient groups used for protein analyses (data not shown) did not reveal any selection bias for the group used for protein analysis, indicating that this was a representative selection of the total study group.

3.5. Placenta concentrations of lipids and fatty acid profiles in pregnancies complicated by preeclampsia

The results from the analysis of placental lipid classes are shown in Table 2a. A statistically significant lower mean concentration of FFAs was observed in placenta tissues from the preeclampsia group compared to the control group. No statistically significant difference in concentration was found for any of the other classes of placental lipids between the patient groups.

We further analysed the total fatty acid profile (esterified and non-esterified fatty acids together) in placenta, which included all saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids (PUFAs). We found a small but significantly higher concentration of total n-6 PUFAs (the total of linoleic acid, 18:2n-6 (LA), dihomo gamma linolenic acid, 20:3n-6 (DGLA) and arachidonic acid, 20:4n-6) (ARA) in placenta tissues from pregnancies complicated by preeclampsia compared to controls (Table 2b). No statistically significant difference in concentration was observed for any of the other fatty acids or groups of fatty acids in preeclamptic placentas compared to controls (data not shown).

3.6. Correlation between LXR expression and concentrations of lipids

There was a significant positive correlation between LXRβ expression in preeclamptic placentas and FFA

concentration in preeclamptic placentas (Figure 3). The same, but statistically non-significant trend, was observed between placental concentrations of FFA and placental expression of LXRα and CD36 in preeclampsia (data not shown). There was no correlation between placental expression of LXRα, LXRβ or CD36 and placental FFA concentration in the control group (data not shown).

4. Discussion

There is an increasing interest regarding the role of LXRα and LXRβ in placental biology [11,14,20-23], however, their regulation in placenta in pathological pregnancy has to the best of our knowledge not previously been addressed. In this paper, we show a statistically significant lower mRNA expression of LXRα, LXRβ and their heterodimeric partner RXRα in preeclamptic placentas as compared to controls. Furthermore, we detected both LXRα and LXRβ proteins expression in healthy controls, and we found the protein level of LXRβ to be down-regulated in the preeclamptic placentas compared to controls. However, we did not find any regulation of LXRα protein expression in preeclamptic placentas as compared to controls. The observed discrepancies between the transcript and protein level could suggest mRNA processing or post-translational modifications of LXRα in placenta dissimilar for that of LXRβ. LXRα has previously been reported to be differentially expressed between mRNA and protein levels in macrophages [24], and recently the amount of LXRα protein was shown to be highly regulated by LXRα agonists, preventing the protein from degradation by ubiquitination [25]. The clear difference between expression of LXRβ in controls and in preeclamptic placentas, point towards a role of LXRβ in normal placenta function.

Rodie *et al* have previously reported no consistent difference in placental mRNA and protein levels of RXRα, PPARδ or PPARγ compared to controls [26], while we detected a statistically significant lower mRNA expression of PPARγ in preeclamptic placentas compared to controls. The discrepancy could be due to differences in the two study populations. We did not investigate the expression of PPARα in our study as it has lower level of expression in placenta compared to PPARδ [27]. We also analyzed the expression of SREBP-1 (a master regulator of genes in *de novo* fatty acid biosynthesis). There was a higher expression of SREBP-1 in preeclamptic placentas as compared to controls. Although SREBP-1 is a direct LXR target gene in many tissues, the two transcription factors have also previously been shown to independently regulate *de novo* lipidogenesis [28]. Taken together, our data suggest that the LXRβ, PPARγ, their heterodimeric partner RXRα, and SREBP-1c might play regulatory roles in lipid metabolism during preeclampsia as these genes have a dysregulated or altered mRNA expression, and protein level (LXRβ), in preeclamptic placentas compared to controls, in contrast to the other lipid regulating transcription factors investigated in this study. Small changes in gene expression, especially of transcription factors, can have major differences in vivo as they can alter the expression of many different genes [29].

We observed a decreased expression of CD36 in preeclamptic placentas compared to controls. CD36 is a direct LXR target gene in liver and a fatty acid transport protein [30]. However, Laivuori et al did not find any difference in expression of CD36 in preeclamptic placentas compared with those of controls [31]. This contrasting data may be due to study differences in mode of delivery, differences in gestational age or other differences between the study populations. CD36 is important for the LXR mediated increase in liver FFA concentrations in mice [30]. We demonstrated a statistically significant decreased concentration of FFA in preeclamptic placentas compared to

controls. LXR activation is previously reported to increase FFA levels in pancreatic β cells and liver tissue [30,32], in our study we observed a positive correlation in placenta between expression of LXRβ and levels of FFA in preeclamptic subjects. Taken together, these data suggest that LXRβ could play a role in the control of preeclamptic placental concentrations of FFA.

We found significantly higher concentrations of total n-6 PUFAs (esterified and non-esterified fatty acids together) in preeclamptic placentas as compared to controls. This could indicate a selective uptake or reduced oxidation of n-6 PUFAs by the placenta in order to meet the fetal demands of long-chain PUFAs. The elevated PUFAs in preeclamptic placentas could also reflect differences in food intake between the preeclamptic and control mothers. Circulating free fatty acid levels of linoleic acid has been reported to be increased in preeclampsia in one study [33]. Wang et al found lower concentrations of total non-esterified n-6 and n-3 PUFAs mainly due to lower concentrations of ARA and docosahexaenoic acid (DHA) in preeclamptic placentas compared to controls [34]. This discrepancy between our findings is probably due to the difference in measuring non-esterified fatty acids alone or esterified and non-esterified fatty acids together.

The expression of LXRα and LXRβ is influenced both by the fasting-refeeding situation [35,36] and by oxidative stress in different tissues [37]. A strength of our study is that our results were not confounded by the influence of variations in fasting-feeding as all women were fasted for a minimum of 6 hours. In addition, elective cesarean section secured no variation in labor duration and thereby possible variations in oxidative stress and stretching of fetal membranes.

There is a significant difference in gestational age between our two study groups with the preeclamptic group delivering earlier than the control group. Premature deliveries of uncomplicated pregnancies are ethically unacceptable and not available as a control group. Similarly, premature deliveries are normally due to pathological conditions, such as inflammation/infection and therefore not suitable as controls. 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 [38]. Still we cannot exclude that differences in gestational length between the study groups could potentially affect the conclusions.

Placenta is composed of a number of different cell types. LXRα and LXRβ are reported to be expressed in both trophoblasts, macrophages and endothelial cells, which are also present in the placenta [11,39,40]. To explore which placental cells are important for the differences in gene expression and protein levels observed between the preeclamptic and control group, it would be necessary to investigate the protein expression of LXRα and LXRβ in the different placental cell types, e.g. using immunohistochemistry (IHC). However, due to the relative high background detecting LXRs by western blotting, and the less controlled antibody-specificity in IHC (due to lack of size controls), better LXR antibodies need to be developed. These experiments are ongoing in our laboratory.

In summary, the present study shows a down-regulation of LXRα and LXRβ mRNA levels and LXRβ protein levels in preeclamptic placentas compared to controls. Further, it suggests a role for placental LXRβ in regulating placental FFA concentrations in third trimester preeclamptic pregnancy.

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Figure and Table legends

Figure 1. Gene *expression of transcription factors in placenta, decidua and adipose tissue in healthy subjects.* Gene expression was analyzed on total RNA extracted from placenta, decidua and adipose tissue (controls only) using qRT-PCR. The arbitrary values on the y-axis were calculated as follows: $(2^{(-Ct)}) \cdot 10^9$. The results are presented as mean fold change ± standard error of the mean (SEM).

Figure 2. Gene *expression of transcription factors and LXR target genes in preeclampsia in placenta, decidua and fat compared to controls.* Gene expression was analyzed in total RNA extracted from preeclamptic (PE) and control tissues, using qRT-PCR normalized to YWHAZ for placenta and decidua and TBP for fat tissue. A) Transcription factors in placenta B) LXR target genes in placenta C) Transcription factors in decidua D) LXR target genes in decidua E) Transcription factors in fat tissue F) LXR target genes in fat tissue. The results are presented as mean fold change ±SEM relative to controls. P-values * < 0.05 and ** < 0.01.

Figure 3. *Placental protein levels of LXRα and LXRβ in preeclampsia compared to controls.* Fifty µg total proteins isolated from six patients in the control group (C1-C6) and six patients in the preeclampsia group (PE1-PE6) were subjected to SDS-PAGE and blotted using anti-LXRα, anti-LXRβ or anti-β-actin antibodies as indicated. The control lane (C) contains exogenously expressed human LXRα / LXRβ in a human hepatoma cell line and is used for positive control. Arrow points at LXRα, LXRβ or β-actin proteins. * = unspecific band.

Figure 4. *Correlation between expression of LXRβ and concentrations of FFA*. Linear regression between placental LXRβ expression and placental concentrations of FFA. Preeclamptic group alone, $r^2 = 0.237$, p-value <0.05

Table 1. *Clinical characteristics of the control and preeclamptic (PE) patient groups included in the placental gene expression analysis (n=61).* Values shown are median (and minimum-maximum). The p-values are given for PE compared to control. P-values * < 0.05 and ** < 0.01. n.s. = not statistically significant.

Table 2. *Concentration of different classes of lipids and PUFAs in placenta and maternal serum lipids from preeclamptic (PE) compared to control pregnancies.* The results are shown as mean and 95% confidence interval concentrations. A) Placental lipids (mg/g placental wet weight) B) Placental PUFAs (esterified and non-esterified together, g/100 g fatty acid methyl ester (FAME)). The p-values are given for PE compared to controls. P-values * < 0.05. n.s. = not statistically significant.

Figures and Tables
Figure 1.

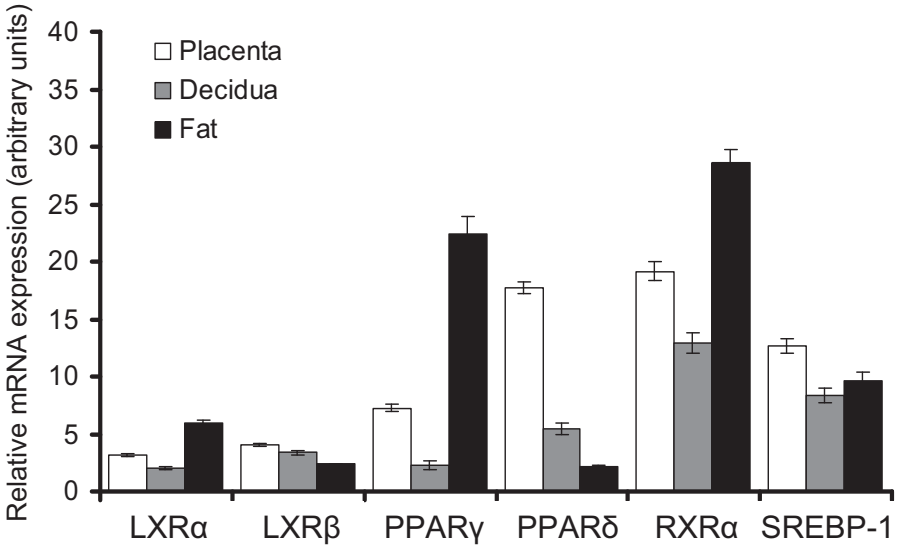

Figure 2a. Placenta

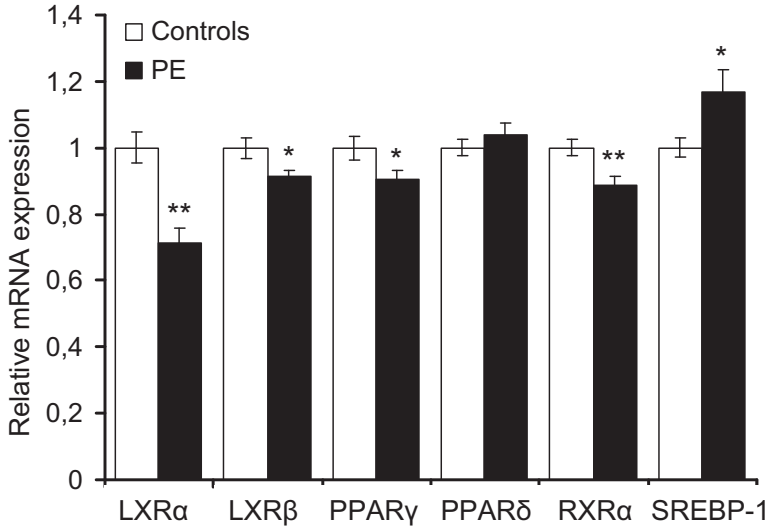

Figure 2b. Placenta

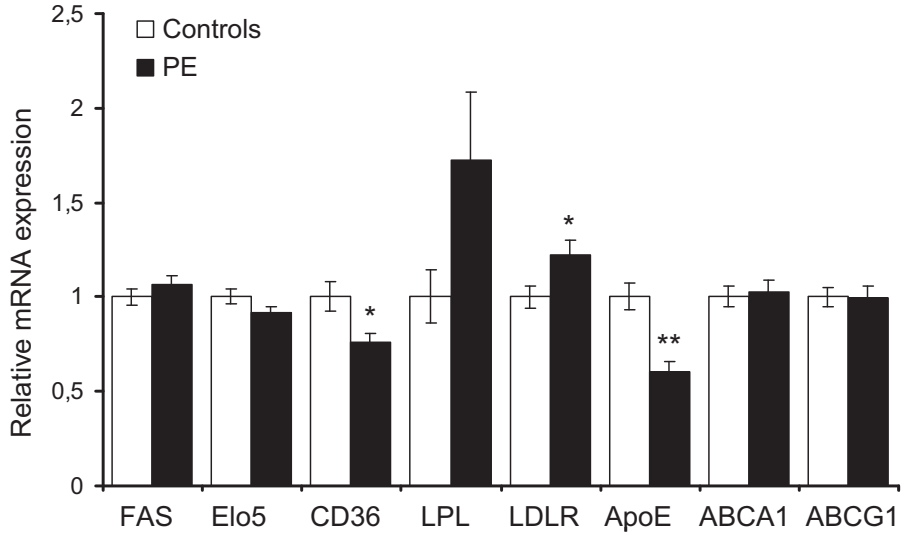

Figure 2c. Decidua

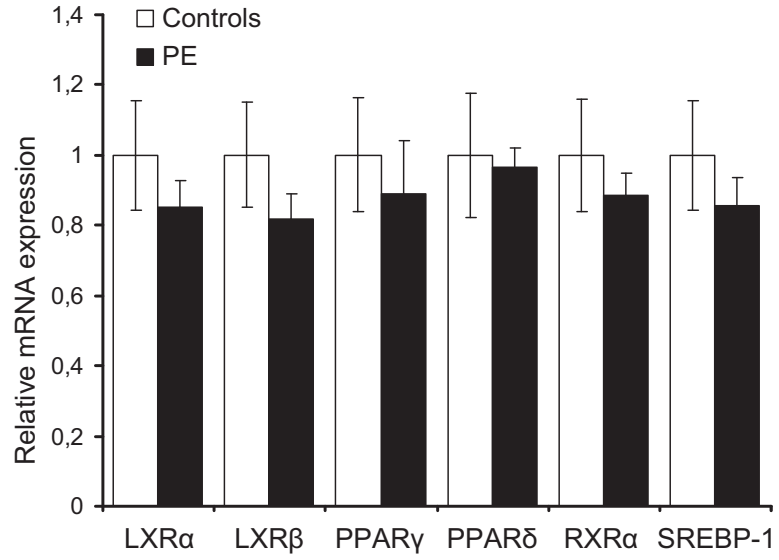

Figure 2d. Decidua

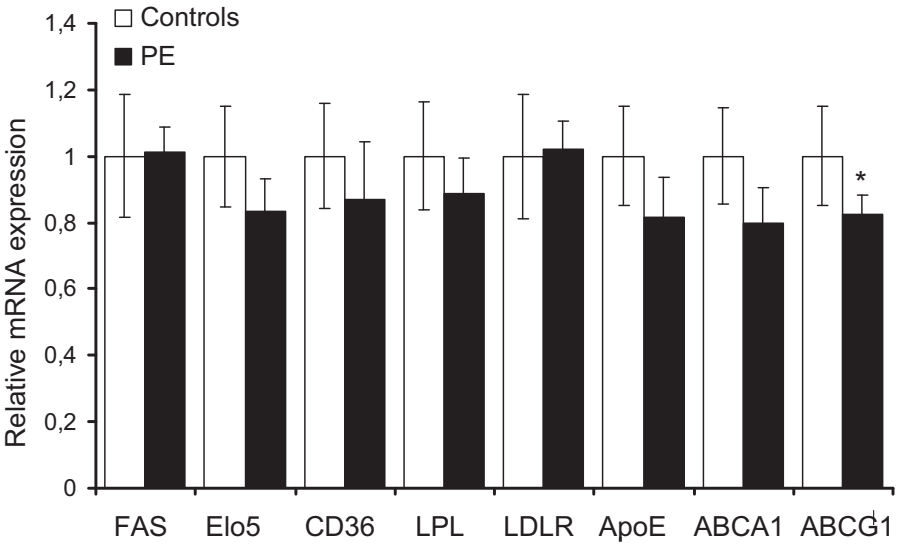

Figure 2e. Fat

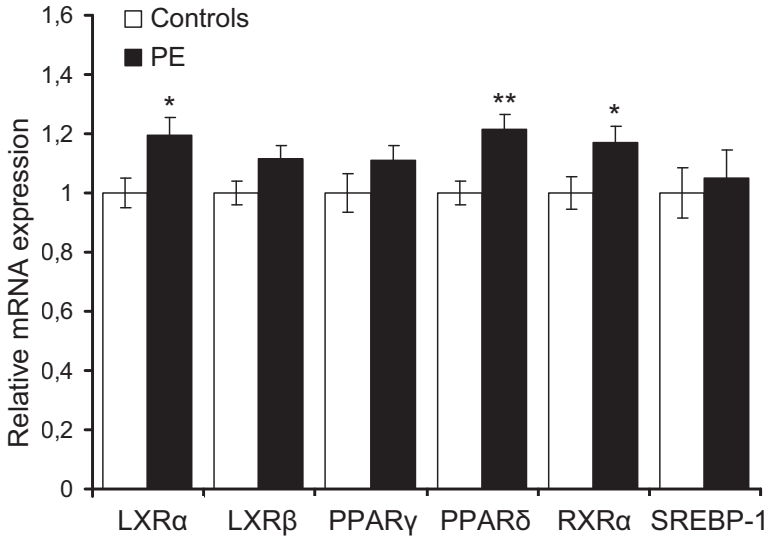

Figure 2f. Fat

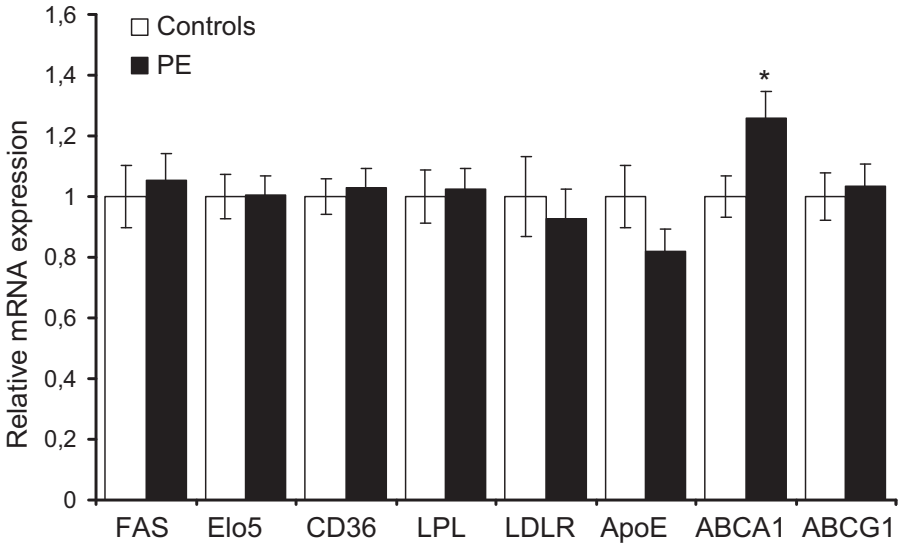

Figure 3.

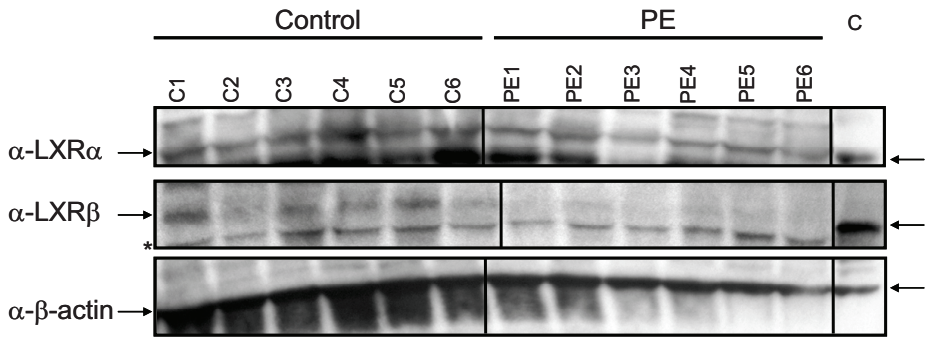

Figure 4. *Correlation between placental expression of LXRβ and placental concentrations of FFA.*

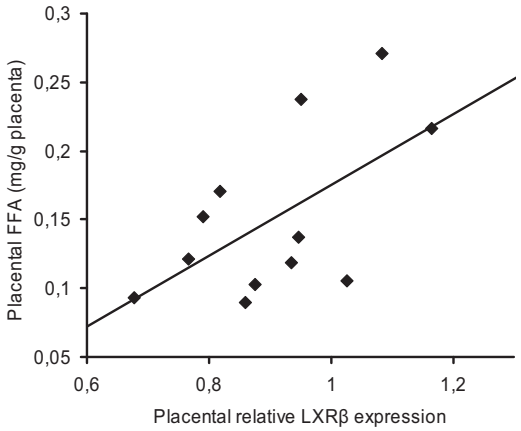

Table 1. *Clinical characteristics of subjects*

	Control (n=33)	PE (n=28)	PE vs. control
	Median (min.-max.)	Median (min.-max.)	p-value
Patient age at delivery (years)	33 (25-40)	31 (18-42)	n.s.
BMI before pregnancy (kg/m^2)	21.7 (18-30.7)	24.9 (19.4-41.1)	<0.05*
BMI at delivery (kg/m^2)	27.9 (22.8-37.5)	31.3 (24.0-49.6)	<0.05*
Parity	0 (0-3)	0 (0-3)	n.s.
Gestational age at delivery (weeks)	38.7 (37-41.7)	32.6 (24.9-39.3)	<0.001**
Systolic BP <week 20 (mmHg)	110 (90-135)	117 (95-135)	<0.05*
Diastolic BP <week 20 (mmHg)	65 (55-93)	73 (50-89)	<0.05*
Systolic BP at delivery (mmHg)	120 (100-153)	161 (140-220)	<0.001*
Diastolic BP at delivery (mmHg)	70 (55-92)	100 (90-119)	<0.001*
Neonatal weight (g)	3600 (2800-4376)	1710 (540-5036)	<0.001*
Neonatal weight percentile[1]	62.0 (13.6-99.5)	34.8 (1.0-99.5)	<0.001*
Neonatal weight percentile[2]	77.7 (9.0-99.5)	7.2 (0.5-99.5)	<0.001*

[1] Neonatal weight percentile according to national birth registry data [16]
[2] Neonatal weight percentile according to ultrasound based weight percentile [17]

Table 2a. Placental lipids

	Control (n = 12) Mean (95% confidence interval) Lower	Upper	PE (n = 12) Mean (95% confidence interval) Lower	Upper	PE vs. control p-value
PC mg/g placenta	5.51		6.06		n.s.
	4.89	6.14	4.9	7.22	
PE mg/g placenta	2.79		2.8		n.s.
	2.42	3.15	2.25	3.34	
FFA mg/g	0.24		0.15		<0.05*
placenta	0.18	0.31	0.11	0.19	
TAG mg/g	0.17		0.31		n.s.
placenta	0.07	0.27	0.06	0.55	
CE mg/g placenta	0.05		0.05		n.s.
	0.03	0.06	0.03	0.06	
Cholesterol mg/g	3.05		3.03		n.s.
placenta	2.72	3.38	2.42	3.63	

Table 2b. Placental PUFAs

g/100 g FAME	Control (n = 12) Mean (95% confidence interval) Lower	Upper	PE (n = 12) Mean (95% confidence interval) Lower	Upper	PE vs. control p-value
	Omega-3 PUFAs				
α-linolenic acid	0.24		0.24		n.s.
(18:3 n-6)	0.22	0.25	0.23	0.25	
EPA (20:5 n-3	0.32		0.25		n.s.
	0.23	0.41	0.19	0.31	
DPA (22:5 n-3)	0.84		0.8		n.s.
	0.73	0.94	0.72	0.89	
DHA (22:6 n-3	4.83		4.56		n.s.
	4.29	5.38	3.97	5.16	
Total n-3 PUFAs	6.23		5.85		n.s.
	5.53	6.92	5.18	6.52	
	Omega-6 PUFAs				
Linoleic acid	8.31		9.04		n.s.
(18:2 n-6)	7.80	8.81	8.44	9.64	
DGLA	4.5		4.99		n.s.
(20:3 n-6)	3.95	5.04	4.58	5.39	
ARA	15.57		15.66		n.s.
(20:4 n-6)	14.76	16.38	14.71	16.61	
Total n-6 PUFAs	28.37		29.69		<0.05*
	27.48	29.26	28.85	30.53	
	Total PUFAs (n-3 and n-6 together)				
	34.6		35.54		n.s.
	33.94	35.26	34.71	36.37	

Paper 4

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