CYTOKINES AND LIPIDS IN PREGNANCY

EFFECTS ON DEVELOPMENTAL PROGRAMMING AND PLACENTAL NUTRIENT TRANSPORT

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ABSTRACT

Metabolic disturbances, in particular those associated with nutritional challenges, that take place during development, both *in utero* and early postnatal life, have long-lasting health consequences on an individual. The most pronounced evidence of these challenges is a deviation in birth weight. This is a process recognized as developmental programming of adult health and disease. The etiologies of metabolic health disorders such as insulin resistance and obesity are complex; and developmental programming may be a factor contributing to the increased worldwide prevalence. Women who are overweight or diabetic have a higher risk for delivering large infants, and such infants are themselves at an increased risk of developing metabolic disturbances. Fetal growth is intimately linked to placental nutrient transport capacity. We hypothesized that the altered nutritional, hormonal, and metabolic environment of overweight or diabetic women (hyperlipidemia, pro-inflammatory status) modifies placental nutrient transport and contributes to altering the adult phenotype of these children. The aim of this thesis was to investigate the importance of maternal interleukin-6 during development for offspring adiposity and insulin sensitivity at an adult age in mice, examine the effects of cytokines and lipids on human placental nutrient transport functions and to describe mechanisms underlying these changes.

The main findings of this thesis were:
Interleukin-6 deficient mice weighed more and had a more pronounced adiposity which developed at a younger age if born of interleukin-6 deficient dams compared to dams with a heterozygote interleukin-6 genotype. At an older age (6 to 7 months of age) both groups had enlarged adipocytes and reduced insulin sensitivity. Wild-type mice fostered by interleukin-6 deficient dams also weighed more, had an augmented adiposity and larger adipocytes, and higher systemic leptin levels at an adult age compared to wild-type mice fostered by wild-type dams. Milk from interleukin-6 deficient dams contained twofold higher leptin concentrations compared to milk from wild-type dams. These observations suggest that lack of maternal interleukin-6 or, alternatively, factors modified by this cytokine have developmental programming effects that contribute to the development of adipose tissue and obesity.
Using primary cell cultures of human trophoblast cells, we demonstrated the production site of placental lipoprotein lipase to be cytotrophoblast cells and syncytiotrophoblast. We also observed that elevated levels of free fatty acids and triglycerides reduce trophoblast lipoprotein lipase activity; while insulin, interleukin-6, and tumor necrosis factor-α had no regulatory effect on lipoprotein lipase. Interleukin-6 did however increase placental lipid accumulation. Free fatty acids changed the release of cytokines from trophoblast cells and stimulated amino acid uptake through the System A transporter. Using RNA interference techniques, we demonstrated that toll-like receptor 4 is required for fatty acids to stimulate placental amino acid uptake.

In summary, we found that an altered maternal hormonal or metabolic environment can affect the developing fetus, causing long-term programming effects on adult phenotype. The effects of cytokines on placental lipid transport were moderate; however, there was a pronounced effect of fatty acids upon amino acid uptake. Therefore maternal circulating factors known to be altered in obesity may augment placental nutrient transport and contribute to an accelerated fetal growth.

LIST OF PUBLICATIONS

This thesis is based on the following papers, published or in manuscript, which will be referred to in the text by their roman numerals:

I. **Perinatal Lack of Maternal Interleukin-6 Promotes Development of Adiposity in Adult Mice**
S. Lager, I. Wernstedt Asterholm, E. Schéle, N. Jansson, S. Nilsson, J.-O. Jansson, M. Lönn, and A. Holmäng
Submitted

II. **The Effect of Maternal Triglycerides and Free Fatty Acids on Placental LPL in Cultured Primary Trophoblast Cells and in a Case of Maternal LPL Deficiency**
A.L. Magnusson-Olsson & S. Lager, B. Jacobsson, T. Jansson, and T.L. Powell
Am J Physiol Endocrinol Metab, 2007

III. **Effect of Cytokines on Fatty Acid Uptake in Cultured Human Primary Trophoblast Cells**
S. Lager, N. Jansson, A.L. Olsson, M. Wennergren, T. Jansson, and T.L. Powell
Submitted

IV. **Oleic Acid Stimulates System A Amino Acid Transport in Primary Human Trophoblast Cells Mediated by Activation of Toll-Like Receptor 4**
S. Lager, H.N. Jones, M. Wennergren, T. Jansson, and T.L. Powell
Submitted

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ABBREVIATIONS

ANOVA	analysis of variance
BMI	body mass index
cDNA	complementary deoxyribonucleic acid
C_T	cycle threshold
DEXA	dual energy X-ray absorptiometry
DMEM	Dulbecco's modified eagle's medium
FABP	fatty acid binding protein
FATP	fatty acid transport protein
FFA	free fatty acid
hCG	human chorionic gonadotropin
IL-$6^{-/-}$	interleukin-6 deficient
ITT	insulin tolerance test
JAK	janus kinase
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MeAIB	methylaminoisobuturyric acid
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
PCR	polymerase chain reaction
pFABPpm	placenta specific membrane bound fatty acid binding protein
PPAR	peroxisome proliferator-activated receptor
RT	reverse transcriptase
SDHA	succinate dehydrogenase complex, subunit A
SDS	sodium dodecyl sulphate
SEM	standard error of mean
siRNA	short interfering ribonucleic acid
SNAT	sodium coupled neutral amino acid transporter
STAT	signal transducer and activator of transcription
TBP	TATA box binding protein
TG	triglycerides
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
WT	wild-type

INTRODUCTION

Throughout the world today many women are obese or overweight when entering pregnancy. Such women are more likely to develop gestational diabetes mellitus and are at increased risk for delivering infants with high birth weight. It is believed that the global trend of increased birth weight is an effect, at least in part, due to increasing maternal weights. Delivery of a large infant is associated with increased occurrence of serious medical complications for both mother and child. Moreover, an accelerated growth *in utero* is connected with a higher prevalence of multiple health disorders during adulthood. The causative mechanisms underlying accelerated fetal growth in these pregnancies have not been well established. A possible contributing factor may be an altered maternal metabolic environment. The fetus is wholly dependent upon the placenta's ability to transfer nutrients for development and growth. Adjustments of placental nutrient transport capacity have been theorized as a primary mechanism linking placental nutrient transfer with maternal nutrient availability.

Pregnancy

The length of normal human pregnancy is approximately forty weeks. During this time a new being will structure from a single celled zygote. Approximately six days after fertilization, the zygote has developed into a blastocyst which hatches from the zona pellucida and begins implanting itself into the uterine wall. The blastocyst's inner cell mass will develop into a fetus, while the outer cell layer will form a placenta. The main phase of organogenesis occurs during the first twelve weeks of pregnancy, or the first trimester, when the zygote transforms into an embryo, and latter a fetus. By the end of the first trimester maternal blood begins to flow continuously into the placenta's intervillous space, bathing the villous trees. From this point until delivery, the fetus is dependent upon maternal/placental supply of nutrients, and oxygen, as well as removing waste products for a successful pregnancy (1).

Maternal adaptations to pregnancy – lipid metabolism

Upon pregnancy, the expectant mother's metabolism changes in order to sustain her and the developing fetus successfully throughout pregnancy. The source behind these metabolic changes is believed to be an altered endocrine environment. Early pregnancy can be characterized as an anabolic state, where maternal fat stores are built up. These fat stores can be utilized during late gestation, when fetal demand for nutrients and lipids is high (2).

During late gestation, maternal hyperlipidemia is accomplished through increasing adipose tissue lipolysis (3, 4). The elevated rate of lipolysis is thought to result from a combination of increasing circulatory levels of placental lactogen and insulin resistance. It has been shown that placental lactogen has somewhat stimulatory effects on adipocyte lipolysis (3). In late gestation insulin, which normally inhibits lipolysis, loses some of this inhibitory ability (5).

Maternal hyperlipidemia is not only an effect of increased lipolysis but also an enhanced hepatic production of very low-density lipoproteins. The increased production of very low-density lipoproteins is believed to be caused by enhanced estrogen levels in late pregnancy (6). A systemic lipid profile change occurs during gestation; circulating triglyceride levels increase approximately threefold (7, 8), reflected in the enrichment of triglycerides in some lipoprotein particles (7, 9). Furthermore, the triglyceride levels remain more stable during the day compared to the non-pregnant state, hence reducing the normal variation between fasting and feeding (2). Cholesterol, needed for steroid hormone synthesis, increases by approximately 50 % in maternal circulation (7). By late gestation there is an increase in circulating free fatty acids as well (10).

A third contributing factor to maternal hyperlipidemia is reduced activity of hepatic lipase and lipoprotein lipase (8, 9). These lowered enzymatic activities will result in a decreased clearance of triglyceride-rich lipoproteins in maternal circulation (11). Together, all these changes contribute to maternal hyperlipidemia, with increased plasma levels of triglycerides in particular, but also of cholesterol, lipoproteins, and free fatty acids (8, 10). Maternal hyperlipidemia together with progressing insulin resistance also promotes maternal use of lipids as a source of energy, consequently preserving other nutrients for placental transfer to the fetus (11).

Pregnancies complicated by gestational diabetes mellitus and obesity

According to the World Health Organization's definition a person is overweight when having a body mass index (BMI) greater than 25 kg/m^2 and obese when greater than 30 kg/m^2. During the past few decades the prevalence of obesity and overweight has increased in various parts of the world (12, 13). However, recent data suggests that this trend may be interrupted, at least among women (14, 15). Today, many women worldwide are obese or overweight. In Sweden approximately one quarter of the women of reproductive age have a BMI exceeding 25 kg/m^2 (15), compared to approximately half of the women in the US (14). Having a high BMI is associated with fertility issues (16), which may explain the lower estimated frequency of 40 % of US mothers being obese/overweight when entering pregnancy (17).

Obesity is not only related to higher rates of infertility, but such pregnancies are also at greater risk for pathological or medical complications. These complications include an increased risk of developing gestational diabetes mellitus, gestational hypertension, preeclampsia, cesarean delivery, late fetal death, congenital malformations, or giving birth to a large infant (18). Hence, maternal pre-pregnancy obesity is associated with numerous potential pregnancy complications for both mother and child.

Women who are obese or overweight are at increased risk of developing gestational diabetes. Gestational diabetes is defined as glucose intolerance which either appears during pregnancy or glucose intolerance first being recognized during pregnancy. Occurrence of gestational diabetes differs between populations (19). In Sweden the prevalence is low with less than 2 % in the general population (20), however among obese women the prevalence is approximately 17 % (21). Gestational diabetes is, just as excessive maternal weight, associated with an elevated risk of delivering a large infant (22).

Obesity may be regarded as a systemic low-grade inflammatory condition. This low-grade inflammation is characterized by increased circulatory levels of factors such as C-reactive protein, interleukin-6, serum amyloid A, and tumor necrosis factor-α (23). The importance of fat distribution should be noted, such as in women with a more central body fat distribution having further elevated levels of the above mentioned parameters (23). Circulatory lipids are altered in obesity and overweight as well, in particular an elevation of plasma triglycerides and free fatty acids (23, 24). These deviations are present also during pregnancy. Both circulatory lipids and pro-inflammatory cytokines are accentuated further in pregnant women with a high BMI or gestational diabetes compared to lean pregnant women (7, 25-27).

Determinants of fetal growth

There are several definitions of fetal overgrowth. Macrosomia often refers to a birth weight over a specific threshold; common limits are a weight over 4 or 4½ kilograms. Large-for-gestational age combines gestation length with birth weight, comparing it with the expected weights of the population studied. Large-for-gestational age can be defined as a weight over the 90th percentile or two standard deviations above the mean weight for gestational age. In Sweden, as in many other parts of the world, the prevalence of large infant births is increasing (28). More than 20 % of infants delivered in Sweden weigh over 4 kilograms (29), with 4.6 % more than 4½ kilograms (28).

Delivering a large infant is associated with medical complications for both mother and child. There is an increased risk of prolonged labor, shoulder dystocia, and cesarean delivery. The maternal complications include genital tract injuries and uterine atonia. The infant is at increased risk for asphyxia, brachial plexus injury, hypoglycemia, and fractures. The large infant is also more likely to need care in a neonatal intensive care unit (30, 31).

Fetal growth is ultimately dependent upon maternal nutrient supply and placental capacity to transport these nutrients. Hormones, such as insulin and insulin-like growth factors, also regulate fetal growth. Fetal insulin is recognized at one of the most important hormones promoting intrauterine growth (32). Pancreatic secretion of this hormone is stimulated by amino acids as well as glucose (32). In humans cord blood insulin levels are highest in infants born large-for-gestational age, intermediate in appropriate-for-gestational age and lowest in the infants born small-for-gestational age (33). Krew and coworkers have shown that fetal insulin production correlates well with infant fat mass, but not lean body mass (34). Multiple animal studies have drawn further attention to the effects of insulin during development where experimentally induced low insulin levels resulted in reduced body weight, with high insulin having the opposing effect (35).

Throughout gestation the insulin-like growth factors I and II also have an important role in fetal growth regulation. Plasma concentrations of these genes correlate with birth weight in humans, mice, and several other species. The importance of insulin-like growth factor I and II has been demonstrated in animal studies. Mice deficient in either insulin-like growth factor I or II, or their

receptor, are growth restricted. Over-expressing insulin-like growth factor II leads to excessive fetal growth, with these mice pups being born large (36).

The capacity of the placenta to transport nutrients is closely linked to fetal growth. Changes in placental nutrient transporter activity have been suggested as representing a primary mechanism by which fetal nutrient supply and growth are altered in response to maternal nutrient availability (37). This hypothesis is based upon observations that small infants have placentas with reduced capacity to transport nutrients (38-40), while the placentas of large infants have a greater nutrient transporting capacity (40-42).

Fetal fat accumulation and growth have been associated with several maternal factors, such as pre-pregnancy BMI (43-45), circulatory levels of free fatty acids, triglycerides (45-47), and interleukin-6 (48). Obesity and gestational diabetes are associated with increased risk of fetal overgrowth (22, 28), but it has been reported that these maternal conditions also alter the body composition of the infant by increasing adiposity (44, 49). Therefore the infant of an obese mother or mother with gestational diabetes may have more body fat and less lean mass than an infant of similar birth weight but delivered by a normal weight/normal glucose tolerance mother. Children born large-for-gestational age of diabetic mothers display more adverse health symptoms than children born large-for-gestational age of mothers with normal glucose tolerance (50). It has been suggested that alterations in body composition may represent a better predictor of developmental programming consequences than birth weight alone (44).

Developmental programming

The hypothesis of 'developmental programming' suggests that events occurring during critical periods of development may have long-term significant effects on function and structure for that individual (51). The nature of these possible events is multifaceted, including exposure to issues such as deficiency or excess of hormones and nutrients, maternal infections, as well as xenobiotics. The effects on the developing fetus depend upon timing of exposure together with duration and magnitude. Adaptations to the event may subsequently result in a permanent change of the individual's physiology. One possible outcome of such developmental adaptations renders an individual more susceptible to various health disorders later in life. Supporting the idea of developmental programming are epidemiological observations of humans, as well as results from experimental animal studies. In humans, associations have been shown between altered fetal growth and a higher risk of developing diabetes, heart disease, hypertension, and obesity (50, 52-55). Of course, the etiologies of these conditions are multifactorial and depend upon genetic as well as environmental factors.

The importance of nutrition in developmental programming has been emphasized (56). A nutritional restriction during different time-points of pregnancy has diverse effects on adulthood disease risks, as exemplified with the Dutch famine. Epidemiological studies of this limited famine period have shown that *in utero* exposure to undernutrition during early gestation has more adverse health consequences than exposure during late gestation (57). An increased prevalence of reduced glucose tolerance was found among individuals exposed to famine *in utero*

independent of time of gestation, while heart disease, obesity, and an artherogenic lipid profile was higher only among the individuals exposed to famine during early gestation (57).

The importance of nutrition has been revealed in experimental animal studies. Undernutrition, manipulation in content of macronutrients (fat and protein) as well as micronutrients (vitamins and minerals) in the maternal diet has long-lasting health consequences in the offspring (58). Not only manipulation of maternal nutritional status has effects on developmental programming, but also exposure to single factors. Experimental animal studies have demonstrated that exposure to elevated levels of cytokines can result in developmental programming. In rats, elevated perinatal interleukin-6 exposure affects the central nervous system, results in decreased insulin sensitivity, hypertension, as well as increased fat mass (59-61). In mice, continuous maternal infusion of tumor necrosis factor-α during the second half of pregnancy results in an accelerated development of adipose tissue in offspring (62).

One proposed potential mechanism behind developmental programming is epigenetic alterations (63). Epigenetics have been described as a change in gene expression occurring without a change in the DNA sequence. Importantly, these changes are heritable and transmitted during cell division (64). Main epigenetic modulators are DNA methylation and histone modifications. DNA methylation is limited to cytosine nucleotides which are followed by a guanine nucleotide (CpG) in the genomic sequence. Methylation of CpG islands (short stretches rich in CpG dinucleotides) is linked with silencing of transcription of the associated gene. Histone modifications result in chromatin remodeling. These modifications include acetylation, methylation, and phosphorylation. Acetylation of histones generally relaxes the chromatin structure, allowing gene transcription, whereas histone methylation is associated with gene silencing. Histone phosphorylation is connected to chromosome condensation and seen during cell division (64). In animal models nutritional changes, such as folate deficiency, has been shown to effect DNA methylation (63). As the placenta constitutes the interface between maternal and fetal circulations, its importance or role in developmental programming has been highlighted (65, 66).

The human placenta

The placenta constitutes an interface between maternal and fetal circulations. This organ has to provide the fetus with all requirements for normal development and growth. The placenta is concurrently responsible for transport of ions, minerals, nutrients, and vitamins, as well as respiratory gas exchange and waste product removal. It produces and responds to an extensive variety of hormones and signaling molecules. Furthermore, the placenta also forms an immunological barrier between mother and fetus (67). Exchanges between maternal and fetal circulations are dependent upon several factors such as blood flow in umbilical cord and placenta, concentration gradients, placental metabolism, as well as transporter proteins. Activity and number of specific transporter proteins seems to be a primary factor subject to regulation (37).

The placenta develops from the blastocyst's outer cell layer, the trophoblast cells. These progenitor cells proliferate and differentiate into three separate cell types: extravillous trophoblast cells, villous cytotrophoblast cells, and syncytiotrophoblast. The extravillous trophoblast cells

invade and migrate into the decidua, remodel the uterine arteries. The cytotrophoblast cells proliferate and fuse to form the syncytiotrophoblast. The syncytiotrophoblast forms the epithelial layer of the villous tree structures. The syncytiotrophoblast is a true, multinucleated syncytium formed by fusion of underlying cytotrophoblast cells. This cell has two polarized plasma membranes, a basal plasma membrane directed towards the fetal capillary, and a microvillous plasma membrane facing the intervillous space (67).

Maternal blood is in direct contact with the syncytiotrophoblast layer and enters the intervillous space of the placenta through the spinal arteries. In the term human placenta, maternal and fetal circulations are separated by only two cell layers: the fetal capillary endothelium and the syncytiotrophoblast. The syncytiotrophoblast forms the transporting epithelium of the placenta, as the endothelial cells of the fetal capillaries do not structure a continuous barrier. Hence, transfer across the syncytiotrophoblast's two plasma membranes is the rate limiting step of placental nutrient transport (66).

Placental endocrine functions

The placenta produces several different hormones and signaling molecules which can be released into maternal and/or fetal circulations (67). These factors perform multiple functions, such as contributing to maternal adaptations and sustaining pregnancy, affecting placental nutrient transport as well as fetal growth and development.

Human chorionic gonadotrophin (hCG) is produced by the syncytiotrophoblast and released predominantly into the maternal circulation. The levels of hCG production peak between 8 and 12 weeks of pregnancy, followed by lower levels until end of pregnancy (68). This dimeric hormone consists of a α-subunit, shared with other glycoprotein hormones, and a unique β-subunit. Functions of hCG include stimulating trophoblast differentiation and maintaining the corpus luteum until the placenta predominates in progesterone production. Another important function of hCG is sustaining the myometrial and decidual spiral arteries, hence the maternal blood supply to the placenta as well (69).

The placenta produces the steroid hormones, progesterone and estrogen (estriol, estradiol, and estrone). By the end of first trimester, the placenta produces progesterone after the corpus luteum has atrophied. Progesterone is released into fetal and maternal circulations. The primary function of progesterone is maintaining pregnancy by sustaining the endometrium and quiescence of the myometrium (70). Estrogens act on maternal reproductive organs (67) and are believed to have effects on maternal metabolism, contributing to adaptations in lipid metabolism such as the increased hepatic production of very low-density lipoproteins (6).

Maternal metabolism is adjusted by several hormones produced by the placenta, such as placental lactogen and placental growth hormone. Production of these hormones increases with gestation, promoting maternal insulin resistance and lipolysis (3, 71). Consequently, these hormones contribute to the maternal catabolic state during the second half of pregnancy, subsequently increasing nutrient supply in the maternal circulation available for placental transport.

The human placenta produces and secretes many other factors (67). Some of these have been shown to affect placental nutrient transport, including insulin-like growth factor I and II, interleukin-6, and leptin. Interestingly, placentas from pregnancies complicated by gestational diabetes express higher levels of interleukin-1β and tumor necrosis factor-α (72). Furthermore, placentas from obese pregnancies are infiltrated with macrophages, expressing higher amounts of cytokines such as interleukin-6 (25) potentially contributing to the chronic maternal pro-inflammatory status.

Placental nutrient transport

Fetal growth is largely dependent on the availability of nutrients (73), which in turn is related to the placenta's capacity for nutrient transport. Since the polarized plasma membranes of the syncytiotrophoblast represent the primary barrier limiting transport across the placenta, transport characteristics of these two membranes will have major influence upon net nutrient transport and consequently fetal growth. Numerous factors influence transport across the placenta such as blood flow, concentration gradients, placental surface area, transport mechanisms, expression and activity of transporters, but also the consumption of nutrients by the placenta.

Amino acids

The fetus uses amino acids for protein synthesis; amino acids are a potent stimulus of fetal pancreatic insulin secretion (74) and amino acids may also be metabolized for energy (75). It is estimated that one third of the required energy for fetal growth and development derives from amino acids (75). Importance of amino acids may be further emphasized as maternal protein restriction leads to reduced fetal growth in rodent gestation (76).

Transfer of amino acids is an active process, resulting in concentrations higher or much higher in the fetal circulation compared to maternal circulation (77, 78). However, the highest amino acid concentrations are found within the placenta (79-81). The human placenta expresses several different amino acid transporters which are classified into 'systems' depending on their characteristics such as substrate specificity, sodium dependence, and transport mechanism (75, 82).

System A

The System A is a sodium dependent amino acid transporter, mediating uptake of small non-essential neutral amino acids such as alanine, serine, and glycine (82). This transporter system accomplishes transport of amino acids against their concentration gradient by co-transporting sodium. The human placenta expresses three different isoforms of System A, which are sodium dependent neutral amino acid transporters (SNAT) 1, 2, and 4. All three isoforms are present both in first trimester as well as in term placenta (83), however their relative contribution to System A amino acid uptake has been suggested to differ between the trimesters (83). System A activity has been observed in both plasma membranes of the syncytiotrophoblast, though highly polarized to the microvillous membrane (84). Further, increased System A activity has been measured in microvillous membrane vesicles isolated from term placentas compared to first

trimester placentas (83, 85). This finding has been confirmed in placental villous fragments by some (83), but not others (86).

The activity of placental System A has been found to be regulated by several cytokines and hormones *in vitro*. Among the factors stimulating System A activity are cortisol, epidermal growth factor, globular adiponectin, interleukin-6, insulin, insulin-like growth factor I, leptin, and tumor necrosis factor-α (87-93); as measured in cultured primary trophoblast cells, primary villous fragments, or in the BeWo trophoblast cell model. Jones *et al.* recently showed that the stimulatory effect of insulin on System A activity can be brought to an end by full-length adiponectin in primary trophoblast cells (91). Furthermore, System A activity and expression increases in response to substrate deprivation, a phenomenon called adaptive regulation. This effect on System A has been shown in BeWo cells (94), as well as in other cell types (95). Factors reducing placental System A amino acid transporter activity include hypoxia (96) and interleukin-1β (97). In primary trophoblast cells Roos *et al.* have shown that System A can be regulated through the mammalian target of rapamycin (mTOR) signaling pathway (98).

Recently Lewis *et al.* reported a correlation between maternal muscle mass and placental System A activity (99). They suggest that the lower System A activity of a mother with less muscle mass may represent an adaptive response in order to prevent excessive amino acid transport to the fetus unsustainable by maternal reserves. In their cohort no correlation was found between birth weight and placental System A activity. However, it has been shown that in cases of altered fetal growth the activity of System A is altered as well. For instance, the activity of this amino acid transporter is markedly reduced in microvillous membranes isolated from placentas where the infant was growth restricted *in utero* (100, 101). The effects on placental System A activity in accelerated fetal growth accompanied with maternal diabetes is inconclusive, as increased (41) as well as decreased (102) amino acid transport has been reported.

System L

The System L amino acid transporter is sodium independent and consists of a heterodimer formed from a light chain protein together with a heavy chain transmembrane protein. This transporter is an obligatory exchanger system, transporting neutral amino acids with either aromatic or branched side chains, such as leucine and phenylalanine, against their concentration gradient, in exchange for non-essential amino acids (103).

Activity of System L has been shown in both plasma membranes of the syncytiotrophoblast, although with a higher activity level in the microvillous membrane (104). The regulation of System L is less understood in comparison to the known mechanisms regulating placental System A activity. However, in cultured primary trophoblast cells System L activity increases with higher glucose concentrations (92) and activity is stimulated by insulin (92) as well. In the BeWo trophoblast cell model a combination of PMA (an activator of protein kinase C) with calcium ionophore increases System L activity (105). Further, the mTOR signaling pathway was shown to regulate the activity of System L in both cultured primary trophoblast cells and in villous fragments (98, 106). System L activity has been found to be reduced in cases of fetal growth restriction (38), while increased in accelerated fetal growth accompanied by maternal gestational diabetes (41).

Glucose

The major energy substrate for both fetus and placenta is glucose. The fetus depends upon placental supply of glucose from the maternal circulation, as fetal glucose production is minimal. Approximately 30 % of the glucose taken up by the placenta is metabolized, with the rest transferred to the fetus (107). The transport of glucose across the syncytiotrophoblast plasma membranes occurs through facilitated carrier-mediated diffusion. Hence, specific glucose transporter proteins are required. The transporting systems are energy-independent and transport of glucose only occurs down its concentration gradient. Therefore the higher maternal glucose concentrations, when compared to fetal, drive a net glucose transport from mother to fetus (108).

Placental glucose uptake increases with gestation (86); the transport is regulated by several hormones such as estrogen, progesterone, and resistin (109, 110). The effect of insulin is unclear, as reported effects are not conclusive (109, 111, 112). Accelerated fetal growth in pregnancies complicated by type-1 diabetes (42, 113), but not gestational diabetes (114), is associated with an up-regulation of placental glucose transporter expression as well as activity. It has been suggested that this alteration may contribute to the occurrence of large infants in women with pre-gestational diabetes despite good metabolic control (42).

Lipids

The fetus requires an adequate supply of fatty acids for normal development and especially during the last trimester of pregnancy fetal demand for fatty acids is high. At this time, fat begins to accumulate in adipose tissue depots (2, 115), with approximately 14 % of the newborn infant's weight consisting of body fat (116). The brain, which has a high fat content, grows rapidly and increases approximately fivefold in weight during the last trimester (117). Additionally, fatty acids are a source of energy, constitute an important part of cellular membranes, and are precursors for important bioactive compounds.

The fetus has the ability to synthesize fatty acids (118-120), but depends entirely upon maternal and placental supply for essential fatty acids (linoleic acid and α-linolenic acid) (121). The fetus also depends upon maternal supply for long-chain polyunsaturated fatty acids (such as docosahexaenoic acid and arachidonic acid), as fetal and placental ability to convert the essential fatty acids are limited (122-127).

Docosahexaenoic acid and arachidonic acid are central for cell membrane fluidity and function; larger quantities are incorporated in brain and adipose tissue (128). Deficiencies of these fatty acid have been associated with learning disabilities (129) and improper development of the retina (130). Docosahexaenoic acid supplementation in cell culture models has shown positive effects on cell survival of retinal photoreceptors (131), neurite growth and synaptogenesis in hippocampal neuronal cells (132). Preterm infants feed formula supplemented with arachidonic and docosahexaenoic acids have improved development of the visual system (133, 134). The importance of such fatty acids for fetal growth is also apparent in that a low maternal intake of essential fatty acids correlates with lower birth weight of the infant (135).

Placental transport of fatty acids

Maternal fatty acids available for transfer to the fetus are either bound to albumin as free fatty acids or in the form of triglycerides incorporated into lipoprotein particles (chylomicrons and very low-density lipoproteins). As pregnancy progresses these sources become increasingly available as maternal circulatory triglyceride levels increase approximately threefold (7, 8), but free fatty acid levels also increase, by late gestation (10). It has been suggested that free fatty acid transfer to the fetus is driven by maternal-fetal gradient and thereby dependent upon maternal concentrations. In pregnant guinea pigs preferential placental transfer of free fatty acid deriving from triglycerides over free fatty acids bound to albumin (136), suggests hydrolysis of maternally originating triglycerides as an important source of fatty acids for the fetus. In figure 1 a schematic representation of placental fatty acid transport is presented, details will discussed further below.

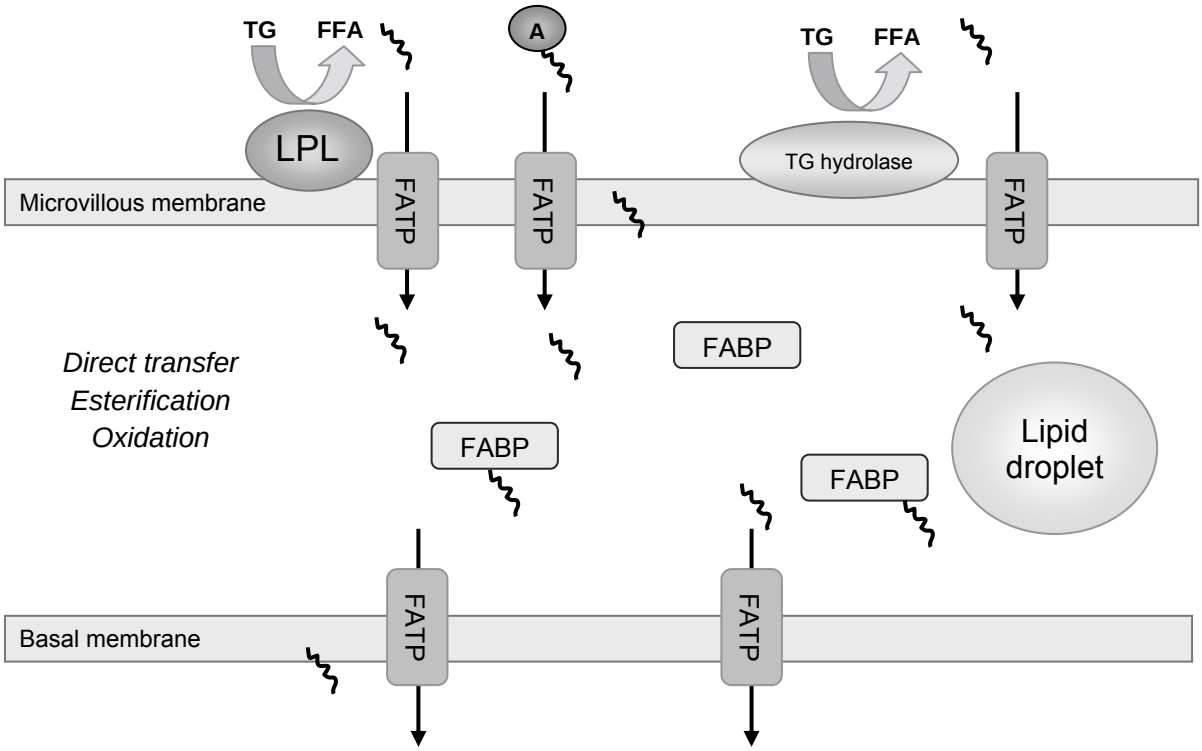

Figure 1. *Schematic illustration of placental free fatty acid transport. In the maternal circulations lipids are transported either as free fatty acids (**FFA**) bound to albumin (**A**) or as triglycerides (**TG**) incorporated into lipoproteins or chylomicrons. Associated with the microvillous membrane are lipoprotein lipase (**LPL**) and other TG hydrolases which release fatty acids from lipoproteins and chylomicrons. The FFA can subsequently be transported across the microvillous membrane. Within this membrane as well as in the cytosol of the syncytiotrophoblast several different fatty acid transport proteins (**FATP**) and fatty acid binding proteins (**FABP**) direct the FFA to different sites for esterification, oxidation, or direct transfer to the fetus. Lipids can be stored in lipid droplets. These droplets are coated with proteins, such as adipophilin, perilipin, and Tip47. The fatty acids are believed to cross the basal membrane by either simple diffusion or assisted by FATPs.*

Intact triglycerides are not transferred across the placenta (136). Therefore maternally derived triglycerides must be hydrolyzed into free fatty acids by lipases located in the microvillous membrane. The placenta expresses several triglyceride lipases (137). Waterman *et al.* has reported activity from four different lipases in human placenta (138). One of these lipases was identified by the authors as lipoprotein lipase based on its pH optima, together with its ability to be stimulated by serum and inhibited by high salt concentrations (138). In addition to observed activity of lipoprotein lipase in the microvillous membrane, expression at both mRNA and/or protein level have been reported by several groups (40, 137, 139, 140). Lindegaard and coworkers found lipoprotein lipase mRNA expressed in the syncytiotrophoblast by *in situ* hybridization and confirming the protein's cellular localization by immunohistochemistry (139).

Lipoprotein lipase

Lipoprotein lipase is an enzyme hydrolyzing preferably chylomicron or very low-density lipoproteins incorporated triglycerides into free fatty acids and monoacylglycerol. This lipase is produced by many tissues, including adipose tissues, cardiac and skeletal muscle. Upon synthesis in parenchymal cells of these tissues, lipoprotein lipase is secreted and transported to the luminal surface of vascular endothelial cells where it is anchored to heparan sulphate-proteoglycans (141-143). Lipoprotein lipase can be released from this anchoring by heparin.

Functionally, lipoprotein lipase is a homodimer, with its subunits arranged in a head-to-tail orientation (144). For full enzymatic activation a specific co-factor is required: apolipoprotein C2 (145). In addition to hydrolysis of triglycerides, lipoprotein lipase has been reported to interact/anchor lipoproteins to vessel walls and facilitate lipoprotein uptake, promote exchange of lipids between lipoproteins, as well as mediate selective uptake of lipoprotein-associated lipids and lipophilic vitamins (143).

Regulation of lipoprotein lipase activity and expression is intricate, ranging from transcriptional to post-translational level. The regulatory region of the lipoprotein lipase gene has several binding sites for different transcription factors, such as peroxisome proliferator-activated receptor (PPARs) and nuclear factor-1. Furthermore, mRNA stability and translational efficiency are other means of regulating this lipase. The posttranslational modifications include glycosylation and translocation to an active site (142, 143). Lipoprotein lipase is also modified in a tissue-specific manner; however it is unclear what the responsible mechanisms are for such differential regulation.

Some factors known to affect activity of lipoprotein lipase include: *estrogen*, which reduces the lipase activity in adipose tissue (146, 147); *free fatty acids and triglycerides*, which decrease the activity in endothelial cell by displacing lipoprotein lipase from its anchoring site (148), in contrast to macrophages where some fatty acids increase lipoprotein lipase activity (149); *insulin*, stimulates lipoprotein lipase activity in adipocytes (150), but not muscle (151); *interleukin-6* lowers lipoprotein lipase activity in adipocytes (152, 153) while having no effect on macrophages (154); finally, *tumor necrosis factor-a,* which lessens lipoprotein lipase activity in macrophages (154).

The information concerning regulatory mechanisms of placental lipoprotein lipase is limited. Magnusson-Olsson and coworkers have shown that lipoprotein lipase activity is higher in term placenta compared to first trimester placenta (155). Further, they have also shown that longer

exposure of primary villous fragments to cortisol, estradiol, insulin-like growth factor I, and insulin decrease the activity of lipoprotein lipase (155). In contrast, shorter exposure to a physiological concentration of estradiol increased the activity, as did insulin in combination with hyperglycemia (155).

The mRNA expression of lipoprotein lipase has been reported to be increased in placenta from pregnancies complicated by intrauterine growth restriction (140, 156). However, the enzymatic activity is not increased in such pregnancies, but rather reduced in microvillous membrane vesicles isolated from preterm placenta (40). Further, in pregnancies complicated by maternal insulin-dependent diabetes, a condition often resulting in a larger infant, the activity of placental lipoprotein lipase was found to be increased (40). Combined, these results suggest post-translational regulation has a major role in activity regulation of placental lipoprotein lipase.

Transporters of fatty acids

The released free fatty acid can traverse the placenta's cellular membranes by simple diffusion. The transfer of fatty acids from mother to fetus is believed to be driven by relative concentrations of free fatty acids. This gradient is created by the higher fetal concentration of albumin and the fetus has a third of the free fatty acids to albumin ratio compared to mother (2).

Within the fetal compartment there is an enrichment or 'biomagnification' of certain fatty acids (2, 157, 158), suggesting an active or selective transport of these fatty acids. It should be noted that the blood in the intervillous space has a higher fraction of arachidonic and docosahexaenoic acids than maternal peripheral blood (159). Therefore it has been suggested that this increase in long-chain polyunsaturated fatty acids may be enough to account for fetal accretion of these fatty acids without a placental specific transport (2). Nevertheless, there is evidence for selective fatty acid transport, both in cell culture models (160, 161) as well as in perfused placenta (122, 162). Furthermore, Larqué and coworkers (158) using stable isotopes demonstrated a preferential transport of docosahexaenoic acid *in vivo* across human placenta.

Fatty acid transport proteins (FATPs) are integral membrane proteins with a cytosolic C-terminal and an extracellular N-terminal domain (163). The FATPs family consists of six related proteins, of which five are expressed in human placenta (FATP1-4, and 6) (164-166). However, it has been questioned whether or not FATPs function as true transporters or merely assist in accumulation of cellular fatty acids by preventing efflux (167). FATP1 and FATP4 are the most extensively studied isoforms (163). FATP1 is a transporter of long-chained fatty acids (164) and has been shown to be insulin-sensitive, translocating to the cellular membrane upon stimulation (168). The expression of FATP1 is regulated by tumor necrosis factor-α, interleukin-1, and endotoxin (169). FATP4 is believed to be important for transport of docosahexaenoic acid (170). In contrast to FATP1, FATP4 is not sensitive to insulin (168). Both isoforms have been shown to be regulated by PPARγ in human placenta (164). Maternal plasma docosahexaenoic acid correlates with placental expression of both FATP1 and FATP4 (165).

Other membrane-associated proteins with the ability to transport fatty acids expressed in human placenta are fatty acid translocase/CD68 (164) and a placenta specific membrane bound fatty acid binding protein (pFABPpm) (160). Fatty acid translocase/CD68 does not display apparent preferential transport of certain fatty acids and its importance in selective transport of long-

chained fatty acids across the placenta has been questioned (171). On the other hand, pFABPpm is believed to have a high affinity for transporting arachidonic and docosahexaenoic acids, suggesting that this transporter may be involved in a preferential uptake of these important fatty acids (160).

Once within the cytosol, the free fatty acids are bound to and transported by fatty acid binding proteins (FABPs). The FABPs direct the fatty acids to various sites within the syncytiotrophoblast or guide them for direct transfer over to the fetus. The expression of four isoforms has been shown in placenta FABP1 (also known as liver(L)-FABP), FABP3 (cardiac (C)), FABP4 (adipose (A)), and FABP5 (epidermal) (172). The FABPs seems to have no particular preference for specific fatty acids, however they do display a greater affinity for fatty acids with longer chain length (173). Biron-Shental and coworkers have shown that exposing primary human trophoblast cells to hypoxia increases the expression of A-FABP, C-FABP, and L-FABP (172). They further observed an increased expression of A-FABP and L-FABP after exposing the cells to a PPARγ agonist (172). The protein expression of L-FABP is increased in placentas from pregnancies complicated by maternal diabetes, but not altered in intrauterine growth restriction (40). The increased expression of L-FABP together with higher lipoprotein lipase activity may contribute to the increased fetal fat accumulation in pregnancies complicated by maternal diabetes (40).

AIM OF THESIS

Overall aim and central hypothesis

The overall aim of this thesis was the investigation of maternal factors that may alter placental nutrient transport, as well as study of the influence of maternal interleukin-6 on the offspring's adult phenotype. We are particularly interested in the role of maternal hyperlipidemia and pro-inflammatory status on alterations in fetal growth as these are common metabolic disturbances in overweight, obese and gestational diabetic pregnancies. The central hypothesis was that an altered maternal metabolic environment in pregnancies complicated by maternal overweight, obesity or gestational diabetes contributes to a stimulation of placental nutrient transport. Such an environment with increased placental nutrient transfer potentially contributes to the accelerated fetal growth often seen in these pregnancies. Furthermore, altered levels of interleukin-6 during pregnancy may be of importance in shaping the adult metabolic phenotype of the children born of women with chronic elevated pro-inflammatory cytokines.

Specific aims and hypotheses

I. **To study effects of maternal interleukin-6 deficiency for adult phenotype in mice.**
   Hypothesis: Absence of maternal interleukin-6 during pregnancy and/or lactation augments the phenotypic development of interleukin-6 deficient or wild-type mice, with respect to adiposity, adipocyte size, and insulin sensitivity.

II. **To analyze regulation of lipoprotein lipase in human placenta.**
   Hypothesis: Placental lipoprotein lipase activity and expression are regulated by alterations in circulating triglycerides and/or free fatty acids.

III. **To investigate effects of pro-inflammatory cytokines on placental fatty acid transport mechanisms in human placenta.**
   Hypothesis: Placental lipid uptake is regulated by pro-inflammatory cytokines, which have been reported to be elevated in maternal plasma of pregnancies complicated by overweight, obesity or gestational diabetes.

IV. **To examine effects of elevated free fatty acids on placental cytokine release and amino acid transport in human placenta.**
   Hypothesis: Placental pro-inflammatory cytokine release is stimulated by circulating free fatty acids via toll-like receptor 4 (TLR4); the rise in local cytokine concentration in the intervillous space stimulates amino acid transport via a Signal Transducer and Activator of Transcription 3 (STAT3) mediated mechanism.

METHODOLOGICAL CONSIDERATIONS

Ethics

All animal experimental procedures were approved by the Animal Ethics Committee at the University of Gothenburg. The mice were housed under standard conditions with access to food and water *ad libitum* (*paper I*). The collection of placental tissue was conducted with informed consent and approved by the Committee for Research Ethics at University of Gothenburg (*paper II – IV*).

Animal study (paper I)

Experimental animals

Interleukin-6 deficient (IL-$6^{-/-}$) mice were created by Kopf and coworkers through disruption of the interleukin-6 gene via insertion of a neo fragment into the first coding exon (exon 2) (174). These mice have previously been described as prone to develop mature-onset obesity with disturbed glucose metabolism, hyperleptinemia, and altered plasma lipids during adulthood (175). This phenotype can be partially reversed by interleukin-6 replacement therapy (175), suggesting that the observed phenotype is caused by disruption of the interleukin-6 gene and not genetic flanking regions (176). In rats, centrally administrated interleukin-6 treatment decreases their adipose tissue and body weight, as well as increases energy expenditure (175, 177). Consequently, this indicates that the phenotype of IL-$6^{-/-}$ mice could be dependent upon interleukin-6 deficiency in the central nervous system rather than peripherally (175). However, the metabolic phenotype of IL-$6^{-/-}$ mice has not always been apparent (178), implying that additional factors or processes may contribute to the overall phenotype. We were interested in investigating whether or not maternally derived interleukin-6 could be one of these factors.

Study design

All mice were reared in groups of 7 to 9 pups. In *experiment I* IL-$6^{-/-}$ mice were bred from IL-$6^{-/-}$ dams and IL-$6^{+/-}$ males or IL-$6^{+/-}$ dams and IL-$6^{-/-}$ males. In *experiment II* IL-$6^{-/-}$ mice were bred from IL-$6^{-/-}$ parents. In both experiments wild-type (WT) mice were bred from WT parents. Figure 2 presents an outline of the study design.

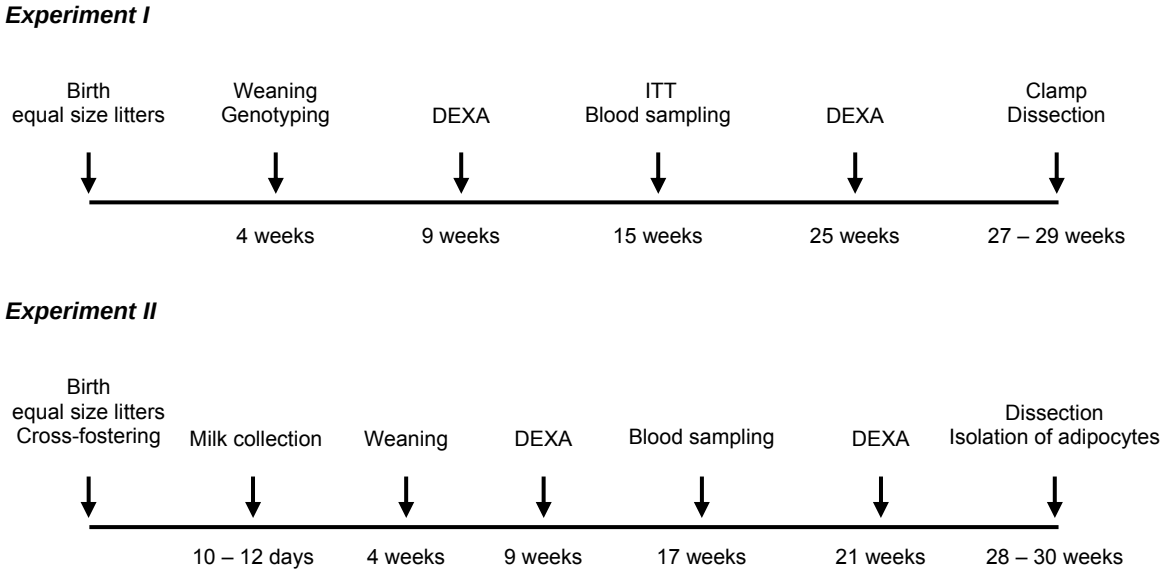

Figure 2. *Overview of the study design: within twenty-four hours of birth the pups were moved to create equal sized litters, and in the case of pups included in experiment II to a foster dam of a different interleukin-6 genotype. Pups remained with the dam until four weeks of age. DEXA, dual-energy X-ray absorptiometry; ITT, insulin tolerance test.*

Milk collection

In preliminary experiments the milk collection method was optimized with respect to amount of oxytocin given, as well as timing between oxytocin administration and starting milk collection. Care was taken to minimize separation time between dam and pups as well. Milk was collected by gentle massaging of the mammary glands. By mild movements of the teflon tube an intermittent suction was obtained. It is of utmost importance to avoid forceful suction as this may damage the milk ducts (A. Oskarsson, personal communication). Presented in figure 3 is an illustration of the milking device.

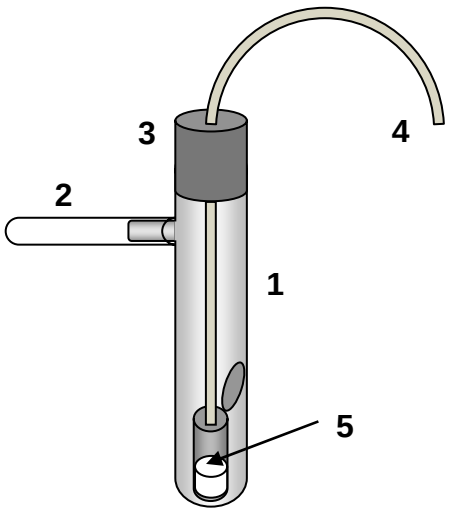

Figure 3. *The milking device consists of a glass test tube with a side outlet (1). In order to create a vacuum within the test tube, the side outlet is connected to a water aspirator via tubing (2) and tube sealed with a rubber stopper (3). Through a central hole in the rubber stopper, a 1 mm inner diameter teflon tubing (4) is used to stimulate the mammary glands. Placed inside the test tube is an eppendorf tube (5) where the milk is collected. Approximately 200 µl milk was typically collected during the 15 minute milking procedure.*

In mice, maximal breast milk production occurs approximately 8 to 10 days after delivery (179). The mice were milked on lactation day 10 (with day of delivery set as lactation day 0). If milk collection was unsuccessful, an additional attempt was made on lactation day 12. The breast milk composition may change during the lactation period. However, the parameters analyzed in our study did not apparently differ between lactation day 10 and 12. Hence, results from the two milking occasions were pooled for the statistical analysis.

Dual energy X-ray absorptiometry

The principle mechanism behind dual energy X-ray absorptiometry (DEXA) is that X-rays are attenuated differently depending on tissue composition. DEXA distinguishes between skeletal and non-skeletal tissue, where the non-skeletal tissue is designated either as fat or lean mass. The composition of the tissue is calculated by a ratio between attenuation of two different X-ray energies (180). The use of DEXA for estimation of body fat has been validated in mice and correlates well with dissected adipose tissue depots (181), even though total fat is assessed, rather than fat in adipose tissues. However, the PIXImus2 DEXA has a reported tendency to overestimate the amount of total body fat (182). Nevertheless, DEXA is a rapid, non-invasive method for estimation of body composition.

Measurement of insulin sensitivity

Euglycemic hyperinsulinemic clamp evaluates tissue sensitivity to insulin and considered to be the reference standard for measuring insulin sensitivity in humans (183). This technique is also used in mice (184). During the euglycemic hyperinsulinemic clamp, insulin is continuously infused in a high, determined concentration (hyperinsulinemic) while maintaining blood glucose levels within the physiological range (euglycemic). The amount of glucose metabolized, reflected in the glucose infusion rate needed to sustain euglycemia, adjusted for body weight, provides an index of tissue insulin sensitivity. Combined with radioactively labeled glucose it is possible to measure glucose uptake by specific tissues.

Insulin tolerance test provides a cheap, fast, and less technically advanced alternative to the euglycemic hyperinsulinemic clamp. With this test, insulin sensitivity was assessed by an intraperitoneal insulin injection followed by measurements of blood glucose levels for a predetermined length of time. From the obtained glucose values collected before as well as 15, 30, and 60 minutes after the insulin injection an area under the curve was estimated. However this method is less sensitive than the euglycemic hyperinsulinemic clamp, but in contrast the insulin tolerance test allows for repeated measurements.

Determination of adipocyte size

In *paper I,* computerized image analysis has been used for determination of adipocyte size in adipose tissue sections and in fat cell suspensions, respectively. Both applications have strengths as well as limitations. In *experiment I*, adipocyte size was assessed using images of histological preparations of parametrial adipose tissue. With this technique adipocyte cell contours were manually delineated and corresponding areas automatically calculated. All images were analyzed by one person following a meticulous protocol; all enclosed fat cell contours in each image were delineated to avoid subjective evaluation. This technique is limited by possible distortion of the adipocytes during sectioning as well as lengthy analysis. The method is advantageous because it only requires fixation of the tissue in formalin at the time of dissection, while preparation and analysis can be performed later.

In *experiment II*, adipocyte size was determined using images of adipocytes isolated from parametrial adipose tissue incubated in the presence of collagenase. The surface of the relevant areas is measured automatically, and the diameter of the corresponding circles calculated. This technique permits size assessment of a large number of adipocytes along with possible use as a reliable evaluator of cell size distribution (185). During the isolation procedure some adipocytes may rupture, resulting in lipid droplets within the preparation. These lipid droplets can visually be discriminated from adipocytes and must be excluded manually from the analysis of each image. Discrimination between small adipocytes and small lipid droplets may be difficult at times. Further, contours of the rare, extremely large adipocytes may be blurred and therefore excluded in the analysis - a common focal point is difficult to achieve for both extremely large and small cells.

Human placenta studies (paper II – IV)

Patient selection and tissue collection

Placental tissue was collected at the Sahlgrenska University Hospital after either cesarean or vaginal delivery, collection was conducted with informed consent. Only placentas from singleton, term pregnancies of healthy women were collected, with the exception of the lipoprotein lipase deficient patient (discussed further below). Characteristics of the cohort of lean to obese pregnant women represent a subset of women previously described by Jansson N. *et al.* 2008 (186).

Trophoblast cell culture

The method for trophoblast cell isolation from human term placenta was originally described in 1986 by Kliman and coworkers (187). Placental tissue was digested by DNase/trypsin, yielding a single cell solution. The digestion buffer contains DNase to avoid formation of cell aggregates, caused by DNA release from damaged cells, as it cleaves single and double-stranded DNA.

Trypsin has stronger digestive properties than DNase. Trypsin used in this protocol contains a crude mixture of lipases, nucleases, polysaccharidases, and proteases. Using a Percoll gradient, which separates cells according to density, cytotrophoblast cells were isolated from the natural mixture of placental cells in the digestion buffer. Trophoblast cells were grown up to four days in a humidified incubator with 5 % CO_2, 95 % air at 37°C, along with daily changes of cell culture media. The cell media contain equal volumes of DMEM (high glucose) and Hams F-12, supplemented with L-glutamine, penicillin, streptomycin, gentamicin, and 10 % fetal bovine serum.

When isolated and cultured, cytotrophoblast cells will, under the right conditions, differentiate and fuse to form multinucleated syncytiotrophoblast-like islands of cells. This mimics the placenta's transporting epithelium and provides an efficacious model for nutrient uptake studies. In addition, using cell culture as an experimental model allows for precision control of milieu and manipulation of the incubation media. Formation of the multinucleated syncytiotrophoblast-like cell islands is accompanied by increased production and release of hCG into the cell culture media. The amount of hCG in the cell culture media can be measured and used as a biochemical marker of differentiation.

Establishment of primary cell cultures has advantages in comparison to cell lines, such as no transformation and hence likely a better reproduction of the *in vivo* situation. However, this cytotrophoblast isolation method yields a limited number of cells. Furthermore, by isolating one cell type possible important interactions *in vivo* between different cells are lost. Using a single cell type can also be advantageous as effects upon that particular cell type are studied.

Listed in table 1 are the different effectors used in this thesis for amino acid transporter activity, lipid accumulation, lipoprotein lipase activity, mRNA and protein expression measurements. Concentrations of the effectors were selected to mimic physiological levels (in maternal circulation) or slightly higher than physiological. Exact concentrations at the maternal-fetal interface *in vivo* are unknown for these effectors. It is feasible to speculate that the local contractions are higher for substances produced and released by the placenta, such as in the case for interleukin-6.

Table 1. *Effectors used in trophoblast cell cultures*

Effectors under study	Working concentration
Arachidonic, linoleic, and oleic acid combination	200 – 400 μM
Interleukin-6	0.02 – 20 ng/ml
Insulin	36.25 – 290 ng/ml
Intralipid	4 – 400 mg/dl
Linoleic acid	100 – 400 μM
Oleic acid	100 – 400 μM
Tumor Necrosis Factor-α	0.02 – 20 ng/ml

RNA interference

The method for silencing expression of a specific gene, through introduction of a short interfering RNA (siRNA), is based upon the inherent cellular mechanism of degrading double-stranded RNA (188). The protocol for siRNA transfection in primary human trophoblast cells was originally described by Forbes *et al.* (189). With this protocol siRNA was introduced into trophoblast cells with a lipid-based transfection reagent (DharmaFECT siRNA transfection reagent, Thermo Fisher Scientific), prior to differentiation into multinuclear syncytiotrophoblast-like aggregates. Silencing a gene, in a cell as in a whole animal, may have multiple and unforeseen consequences as most genes likely participate in numerous networks. However, silencing expression of TLR4 did not affect basal System A or System L activities (Figure 6 in *paper IV*). Furthermore, exposure to the transfection reagent may also have diverse effects on trophoblast cells. Nevertheless, it did not compromise their ability to produce hCG (88) or their response to fatty acids with respect to amino acid transportation (Figure 6 in *paper IV*).

Lipoprotein lipase deficient patient

The condition of the lipoprotein lipase deficient woman has previously been described (190). The patient lacks significant extracellular lipoprotein lipase activity, resulting in severe chylomicronemia. The lipoprotein lipase deficiency results from two different mutations: one allele not producing lipoprotein lipase mRNA and the other allele resulting in catalytically active but defectively transported lipoprotein lipase (190). During both pregnancies the patient was on a fat restricted diet, supplemented with omega-3 fish oil tables. Additionally, during the first pregnancy the patient was treated with plasmapheresis every third week (triglyceride values between 800 and 4000 mg/dl). In the second pregnancy the patient was on the restricted diet only (triglyceride values between 2000 and 9000 mg/dl). Both infants were of normal size and delivered by cesarean section near or at term (36 weeks and 38 weeks). The placentas appeared normal anatomically, with the exception of milky colored maternal blood in the intervillous space.

Microvillous plasma membrane preparation

The protocol for isolation of syncytiotrophoblast membranes was first described by Illsley *et al.* (191). With this method both microvillous plasma membrane and basal membrane can be isolated from the same placenta. Concisely, low-speed centrifugation was used to separate cell membranes and tissue debris. This centrifugation step will leave all membranes in the supernatant. After high-speed centrifugation, the microvillous plasma membrane was purified from other membranes by addition of magnesium chloride. Magnesium forms aggregates with all membranes but brush-border membranes. Subsequently, the basal membrane can be isolated from other non-brush border membranes by a sucrose step gradient. By measuring alkaline phosphatase activity, the purity of the microvillous plasma membrane extraction can be assessed, with a tenfold enrichment compared to placental homogenate being considered acceptable.

Isolated microvillous plasma membranes present a helpful model when studying uptakes across the membrane or activities of enzymes associated with this membrane (such as lipoprotein lipase).

Amino acid transporter activity

The protocol for measuring System A and System L amino acid transporter activities simultaneously in cultured human trophoblast cells was originally established by Roos and coworkers (98). The System A amino acid transporter activity was measured as sodium dependent uptake of ^{14}C-methylaminoisobuturyric acid (MeAIB). MeAIB is a non-metabolizable amino acid analogue, which the System A has a unique ability to transport. This amino acid analogue can be transported by all the SNAT isoforms, however by SNAT4 to a lesser extent than SNAT1 and SNAT2 (192). Activity of the System L amino acid transporter was assessed by 2-amino-2 norbornanecarboxylic acid (BCH)-inhibitable uptake of ^{3}H-leucine. The linear portion of leucine and MeAIB uptakes have previously been established in our lab; chosen incubation time (8 minutes) is within this linear portion (98). With a cell culture model, intracellular mechanisms involved in amino acid transport regulation can be explored. A limitation of this method is that only uptake of amino acids is measured and not net transfer across the trophoblast cells. However, transport of amino acids across the microvillous membrane is considered to be the rate limiting step in transplacental transfer (82).

Lipid accumulation assay

The method for measuring placental lipid accumulation was staining neutral lipids with BODIPY and has been described by Biron-Shental and coworkers (172). With this technique placental trophoblast cells were initially fixed with formalin, followed by membrane permeabilization and staining of accumulated lipids, then finally cell lysis and measurement of lysate fluorescence. Exact concentrations of lipids are not acquired, but treatment groups are expressed relative to a control. The source or mechanism behind a potential lipid accumulation is not evaluated with this method, but rather provides a rapid description of the cellular lipid stores.

Lipoprotein lipase activity

Lipoprotein lipase activity was measured according to a protocol developed by Waterman *et al.* (193), later established in our laboratory by Magnusson and coworkers (40). Activity of lipoprotein lipase was measured by hydrolysis of ^{3}H-trioleate in a sodium phosphate assay buffer with pH adjusted to 8.0 for optimal enzymatic activity. The assay buffer contains fetal calf serum and bovine serum albumin, which will function as a carrier of the released free fatty acids. Fetal calf serum is a source of apolipoprotein C2, the co-activator of lipoprotein lipase. The addition of serum also inhibits activity of the previously described placenta specific lipase (193).

Lipoprotein lipase activity was assessed in cultured primary trophoblast cells, isolated microvillous plasma membrane vesicles, and in fresh villous tissue. To optimize enzymatic activity, different incubation times and protein concentrations were tested. For cultured trophoblast cells 60 minutes with 150 μg total protein was optimum. The optimal assay conditions for microvillous plasma membrane vesicles (30 minutes, 150 μg total protein) and fresh villous tissue (30 minutes, 50 μg) have previously been established in our lab (40, 155).

Measurement of lipoprotein lipase activity will not provide information on placental fatty acid uptake or transfer, but rather represents an indication of free fatty acid availability for uptake and transport to the fetus. This means with higher lipoprotein lipase activity, more triglycerides will be hydrolyzed into free fatty acids which subsequently can be taken up and transferred across the placenta. As placental transport of intact triglycerides is virtually nonexistent (136), activities of lipases associated with the microvillous membrane are essential for making fatty acids of the triglyceride source accessible for transfer.

Quantitative RT-PCR

Quantitative reverse transcription - polymerase chain reaction (RT-PCR) allows for sensitive and specific quantification of nucleic acids. This technique combines three steps: 1) conversion of RNA to complementary (c)DNA via reverse transcription, 2) cDNA amplification with PCR, and 3) detection and quantification of amplified products in real time. The fidelity of the method is dependent on several factors, such as RNA quality (purity and integrity), primer design, and assay optimization (194). Assessing mRNA expression by quantitative RT-PCR may be a very sensitive method, but lacks in its ability to provide specific information of where these genes are expressed in the tissue analyzed.

In *paper I* 384-well micro fluid TaqMan low-density array cards were used. These cards allow for simultaneous analysis of several genes, requiring only a small sample size. The cards were designed in a 48-format, with reaction wells preloaded with primers and probes for 48 target genes, including seven potential endogenous controls or reference genes.

In *paper II – IV* quantitative RT-PCR was carried out on a LightCycler using SYBR Green I. Primers used in the PCR amplification are listed in table 2. Since SYBR Green I binds to all double-stranded DNA it makes primer design important when using this method. Primers should span an intron, thereby avoiding amplification of potential contaminating genomic DNA. The purity of the RNA preparations was assessed by the A260/A280 ratio. All samples had a ratio greater than 1.8, meaning the samples were pure of protein or DNA contaminations.

Two different methods were used for quantifying amplification transcripts: comparison of cycle thresholds (C_T) (*paper I*) and relative to a standard curve (*paper II – IV*). With the comparative C_T method, quantity of a target gene was standardized against two selected reference genes, expressed relative to a calibrator (in this case the sample with highest expression) and given as fold change ($2^{-\Delta\Delta C_T}$) (195). With the relative standard curve method, a standard curve for each gene product was made from a cDNA dilution series. The standard curve was acquired by plotting log concentration on the x-axis and C_T on the y-axis. The relative amount of an

amplification transcript was determined by the formula $10^{(\text{Intercept} - C_T)/\text{slope}}$, where intercept and slope values were obtained from the standard curve. In *paper II – IV* the amplification efficiencies were between -3.0 and -3.7 (the slope of the standard curve) and the linear regression of the curves were between 0.98 and 1.

Table 2. *Primers used for PCR amplification (paper II – IV).*

Gene name	Oligonucleotide sequence or Primer	Assession no
Adipophilin	QT00001911; QuantiTect Primer Assay	NM_001122
FATP1	QT00049063; QuantiTect Primer Assay	NM_198580
FATP4	QT01015728; QuantiTect Primer Assay	NM_005094
LPL*	5´-GAGATTTCTCTGTATGGCACC-3´ (upper primer) 5´-CTGCAAATGAGACACTTTCTC-3´ (lower primer)	NM_000237
LPL**	QT00036771; QuantiTect Primer Assay	NM_000237
SDHA	5'-TACAAGGTGCGGATTGATGA-3' (upper primer) 5'-AGGTGATAGTTCCCGAAGTC-3' (lower primer)	NM_004168
TBP*	5´-CACCACAGCTCTTCCACTCA-3´ (upper primer) 5´-GCGGTACAATCCCAGAACTC-3´ (lower primer)	NM_003194
TBP**	5'-GTTCTGGGAAAATGGTGTGC-3' (upper primer) 5'-GCTGGAAAACCCAACTTCTG-3' (lower primer)	NM_003194
TLR4	QT01670123; QuantiTect Primer Assay	NM_003266

** Paper II, ** Paper III. FATP, fatty acid transport protein; LPL, lipoprotein lipase; SDHA, succinate dehydrogenase complex, subunit A; TBP, TATA box binding protein; TLR4, toll-like receptor 4; QuantiTect Primer Assay was purchased from Qiagen, Hilden, Germany.*

Western blotting

Protein expression analysis by Western blotting can provide qualitative and semiquantitative information about a specific protein. This technique was used in *paper II – IV* for analysis of protein expression levels and post-translational modifications. The initial step for Western blotting is separation of proteins with gel electrophoresis, followed by transfer (blotting) onto a second matrix (in this case a nitrocellulose membrane). Blotted membranes should be blocked to avoid nonspecific binding of antibodies to the membrane surface (blocking was performed with a non-fat dry milk solution). Listed in table 3 are target proteins and antibodies used. It is the interaction specificity between antigen and antibody that permits detection of a target protein amid a complex protein mixture. Bands were detected after incubation with horseradish peroxidase-labeled secondary antibodies by chemiluminescence in a CCD-camera or on film. The relative densities of detected bands were evaluated with densitometry. Each step of the Western blotting procedure may be subject to optimization, including sample collection/preparation, to choice of gel, blocking concentration and time, antibody concentrations, incubation times and temperatures, along with detection method.

Table 3. *Antibodies used in Western blotting.*

Primary antibody	Dilution	Reference/Company	Incubation time	Secondary antibody
β-actin	1:2000	A2228; Sigma-Aldrich	1 hour, RT	anti-Mouse (1:5000)
FATP4	1:200	sc-101271; Santa Cruz Biotechnology Inc.	Overnight, 4˚C	anti-Mouse (1:1000)
L-FABP	1:100* 1:1000 **	Ab7366; Abcam	Overnight, 4˚C	anti-Mouse (1:1000)
LPL**	1:500	Ab21356; Abcam	Overnight, 4˚C	anti-Mouse (1:1000)
LPL (5D2)*	1:250	Gift from Dr. J. Brunzell	1 hour, RT	anti-Mouse (1:1000)
(p)-p70 S6 Kinase (Thr389)	1:250	#9205; Cell Signaling Technology (CST)	Overnight, 4˚C	anti-Rabbit (1:3000)
(p)STAT3 (Tyr705)	1:500	#9145; CST	Overnight, 4˚C	anti-Rabbit (1:3000)
p70 S6 Kinase	1:250	#9202; CST	Overnight, 4˚C	anti-Rabbit (1:3000)
STAT3	1:2000	#9139; CST	Overnight, 4˚C	anti-Mouse (1:1000)
Vimentin	1:500	Ab20346; Abcam	1 hour, RT	anti-Mouse (1:5000)

** Paper II; ** Paper III; RT, room temperature; All secondary antibodies were incubated with membranes for one hour at room temperature*

The lipoprotein lipase protein has protease sensitive regions, and under the reducing conditions of sodium dodecyl sulphate (SDS) gel electrophoresis a 37 kDa protein may sometimes be detected, as in the case of the LPL (5D2) antibody. This 37 kDa protein part is the C-terminal region of lipoprotein lipase, which has been suggested as important in bridge formation between lipoproteins and cell surface receptors (196).

Statistics

Results are presented as mean ± SEM. The number of experiments (n) represents the number of placentas studied (*paper II – IV*), the number of mice (*paper I*), or number of litters (*paper I*). A *P*-value below 0.05 was considered significant. Generally in *paper I* pair wise comparisons were evaluated by independent samples t-test. For multiple comparisons, one-way ANOVA followed by Tukey's *post hoc* test was used. The body weight development of mice in *experiment I* were analyzed with a linear mixed effects model with individual weight residuals modeled as an AR(1) process, assuming the growth from 5 to 20 weeks of age was linear. In *paper II* differences

between multiple groups were evaluated by one-way ANOVA or repeated-measures ANOVA, followed by Dunnett's post hoc test; a difference between two groups was evaluated by paired t-test. In comparing data from the single lipoprotein lipase negative patient with controls, the results from the control group were expressed as mean and 95 % confidence intervals. In *paper III* and *IV*, generally the non-parametrical statistical Friedman's test (followed by Wilcoxon signed ranks test) was used for comparisons between multiple groups, and Wilcoxon signed ranks test was used when comparing two groups.

SUMMARY OF RESULTS AND DISCUSSION

Maternal interleukin-6 affects offspring's adult adiposity (paper I)

A number of studies suggest interleukin-6 has consequences upon developmental programming (59-61). For example, rats exposed to high interleukin-6 levels during early life have increased fat mass, decreased insulin sensitivity (61), and are hypertensive (60). The programming effects of interleukin-6 have also been reported to manifest in the central nervous system (59, 60). Since interleukin-6 can cross the placenta (197), it is conceivable that maternally produced interleukin-6 could affect the developing fetus or the placenta.

In *paper I* we studied an interleukin-6 deficient mouse model. Deletion of a gene may affect multiple systems. Interleukin-6 is often induced together with pro-inflammatory cytokines (*e.g.*, interleukin-1β and tumor necrosis factor-α), having a role in the induction of the acute-phase response. For example, absence of endogenous interleukin-6 has been reported to cause a further increase in pro-inflammatory cytokines in response to lipopolysaccharide (LPS) stimulation (198). Pregnancy alters several factors in the maternal circulation, including cytokine levels (199). In fact, during pregnancy, the level of acute-phase reactants and activation of maternal leukocytes are both increased (200, 201). This natural inflammatory state may be altered in the absence of interleukin-6. Altered maternal cytokine levels may have consequences for developmental programming of the fetus, as chronic maternal infusion of tumor necrosis factor-α during pregnancy accelerates adipose tissue development in offspring (62).

In humans, a low birth weight (just as a high birth weight) is associated with an increased risk of adverse metabolic health disorders later in life (54). In fetal growth restriction, systemic levels of inflammatory markers, such as C-reactive protein, interleukin-6, and tumor necrosis factor-α are elevated (202). In fetal macrosomia in combination with maternal gestational diabetes, systemic levels of interleukin-6 and tumor necrosis factor-α are also affected. With maternal levels of these cytokines increased, whilst the newborns levels are decreased (203). Hence, altered systemic cytokines are found in both accelerated and reduced fetal growth, two conditions associated with adverse metabolic health disorders. Our model reduces interleukin-6 during pregnancy and allows us to study the role of this cytokine in mediating developmental effects and long-term outcomes. The compensatory alterations of other cytokines have not been determined. We predict there may be potentially elevated levels of other pro-inflammatory cytokines during development (due to absence of functional interleukin-6), affecting the expected adverse metabolic outcome later in life (obesity and disturbed glucose tolerance).

This study was designed to investigate the potential importance of maternal interleukin-6 deficiency during pregnancy and lactation for the adult phenotype of offspring mice. In *experiment I* three groups of mice were followed: 1) WT mice, 2) IL-$6^{-/-}$ offspring of IL-$6^{-/-}$ dams and 3) IL-$6^{-/-}$ offspring of IL-$6^{+/-}$ dams (dams with a heterozygote interleukin-6 genotype). Mice with a heterozygote interleukin-6 genotype have a phenotype comparable to WT mice (unpublished observations). Hence, significance of maternal milieu during development is tested in a model

expected to accumulate excessive body fat and disturbed glucose metabolism during adulthood. The aim of *experiment II* was to investigate the importance of the lactation period for the adult phenotype, which was studied by cross-fostering. Four groups of mice were followed: WT mice fostered by WT dams were compared with WT mice fostered by IL-$6^{-/-}$ dams; and IL-$6^{-/-}$ mice fostered by IL-$6^{-/-}$ dams were compared with IL-$6^{-/-}$ mice fostered by WT dams.

Interleukin-6 deficiency does not affect fetal or early growth

Maternal and fetal deficiency in interleukin-6 did not affect fetal growth or placental weight (*paper I*). In human placenta interleukin-6 can stimulate nutrient uptake, as shown by Jones *et al.* (88) and in *paper III* of this thesis. However, since no difference in birth weight was observed between IL-$6^{-/-}$ and WT mice, a major change in placental nutrient transfer is unlikely to have occurred. Growth during the lactation period was not affected (first three weeks of life), with IL-$6^{-/-}$ mice and WT mice having similar body weights.

Long-term effects of maternal genotype - pregnancy and lactation (experiment I)

Maternal genotype affected time of onset for excessive fat accumulation in IL-$6^{-/-}$ mice. At nine weeks of age the IL-$6^{-/-}$ offspring of IL-$6^{-/-}$ dams had more body fat compared to IL-$6^{-/-}$ offspring of IL-$6^{+/-}$ dams (measured by DEXA; Figure 4 in *paper I*). As expected with increased body fat, these mice also had higher levels of circulating leptin (15 weeks of age; Figure 3 in *paper I*). An increased amount of body fat at this age in IL-$6^{-/-}$ mice has not been observed previously (175, 204). At nine weeks of age there was no difference between WT mice and IL-$6^{-/-}$ offspring of IL-$6^{+/-}$ dams with respect to body weight or body composition (Figure 4 in *paper I*).

At an adult age (20 weeks and older) the phenotype of IL-$6^{-/-}$ mice were independent of maternal genotype. Both groups of IL-$6^{-/-}$ mice weighed more than WT mice (Figure 2 and 4 in *paper I*). This increase in body weight was caused by an excessive body fat accumulation, as there was no difference in lean body mass (measured by DEXA; Figure 4 in *paper I*). The IL-$6^{-/-}$ mice, again independent of maternal genotype, had enlarged adipocytes in the parametrial depot. The larger adipocytes and increased body fat may both contribute to the decreased insulin sensitivity of IL-$6^{-/-}$ mice compared to WT mice, as measured by the euglycemic hyperinsulinemic clamp (Figure 6 in *paper I*).

For humans, increased fat mass in visceral depots has been associated with insulin resistance (205). Suggested mechanisms for the adverse effects of visceral fat depots include location (with direct connection to the liver through the portal circulation) (206) and high metabolic activity (visceral adipocytes show higher catecholamine-induced lipolytic activity and lower sensitivity to the antilipolytic action of insulin than subcutaneous adipocytes) (207, 208). Also, in mice the visceral fat depot proves to have more undesirable metabolic effects than subcutaneous depot (209). In our IL-$6^{-/-}$ mice the inguinal, parametrial, and retroperitoneal depots, but not the mesenteric depot, were heavier than in WT mice (Table 2 in *paper I*).

The enlarged adipocyte size may also contribute to the reduced insulin sensitivity of IL-6$^{-/-}$ mice. Larger adipocytes are associated with reduced insulin sensitivity (210) and an increased abdominal adipocyte size is an independent predictor of type-2 diabetes (211). In *paper I* we show that the parametrial adipocytes are enlarged in IL-6$^{-/-}$ mice, this finding complements the previously reported larger subcutaneous adipocytes in these mice (212). Furthermore, cell size impacts adipocyte function, with larger adipocytes having an increased expression and secretion of genes associated with insulin resistance or inhibiting insulin signaling (213, 214), such as serum amyloid A (215) and interleukin-8 (216).

A certain variability regarding time of onset for fat accumulation has been observed in our colony of IL-6$^{-/-}$ mice (unpublished observations). Do differences in maternal interleukin-6 genotype explain the phenotypic variability we have observed? It may be one of several contributing factors to the variability, as phenotypic differences were only observed at a younger age. However, in older IL-6$^{-/-}$ mice (20 to 30 weeks of age) only body weight development (Figure 2 in *paper I*) and inguinal adipose tissue weight (increased approximately 40 %; Table 2 in *paper I*) differed depending on maternal genotype. Hence, the IL-6$^{-/-}$ mice accumulate excessive body fat at an older age independent of maternal genotype. But the accumulation starts earlier in the mice exposed to an altered maternal hormonal milieu.

In summation, the phenotype of younger IL-6$^{-/-}$ mice differs, seemingly affected by maternal genotype. This result is in accordance with observations done with other transgenic mice (217-219), together providing evidence of developmental programming by maternal genotype causing phenotypic differences in genetically similar offspring. The causative mechanism(s) behind the phenotypic differences in IL-6$^{-/-}$ mice may include differences in intrauterine environment or in maternal milk.

Long-term effects of maternal interleukin-6 deficiency during lactation (experiment II)

In order to determine whether the observed time difference in excessive body fat accumulation depends upon pre- or postnatal environment, IL-6$^{-/-}$ and WT mice were cross-fostered. Cross-fostering IL-6$^{-/-}$ mice with WT dams did not affect the phenotype (observations made from birth to 30 weeks of age), hence the observed differences in *experiment I* are likely caused by differences in the intrauterine environment. Interestingly, WT mice fostered by IL-6$^{-/-}$ dams developed increased adiposity (Figure 5 in *paper I*), larger adipocytes, and fourfold higher circulatory levels of leptin compared to WT mice fostered by WT dams (17 weeks of age; *paper I*).

IL-6$^{-/-}$ mice have elevated circulatory leptin and triglyceride levels (175). These differences could be reflected in maternal milk. We detected a twofold increase in milk leptin concentrations in milk from IL-6$^{-/-}$ dams, but no difference in lipid content (Figure 1 in *paper I*). Among its functions adipocyte-derived circulatory leptin affects energy homeostasis (220). During the lactation period leptin can influence the development of hypothalamic feeding circuits, as shown in leptin deficient mice where peripheral administration of leptin partly restores their lacking hypothalamic innervation (221, 222). Although differences in milk leptin concentrations are one potential mediator of programming, breast milk does contain a mixture of many hormones,

nutrients, etc. Support for the possibility that leptin from maternal milk might be a programming factor behind the differences between our WT mice are the observations made in rats. Firstly, an early exposure to high levels of leptin, administered by subcutaneous injections, increases their adult body weight (223). Secondly, the immature stomach of a rat pup can absorb orally provided leptin (224). In these rat pups administration of leptin resulted in increased serum levels as well as affecting gastric content (224), therefore displaying functional effects. These results suggest that leptin ingested through maternal milk is functional and biologically active.

We did detect an altered hypothalamic gene expression between WT mice, depending upon foster dams' genotype, such as genes regulating food intake (Figure 7 in *paper I*). Anticipated as an indicator of potential mechanisms as well as a future direction for continuing our studies, these results were not conclusive. However, if the hypothalamic feeding circuits have been affected, as suggested by Bouret *et al.* (221, 222), similar expressions of a gene may have different effects due to altered hypothalamic circuits or 'wiring'.

It is interesting that WT mice were affected by cross-fostering, but IL-$6^{-/-}$ mice were not. The underlying mechanism(s) may be numerous. One possibility could be that factor(s) in milk with developmental programming effects work synergistically with/are dependent upon endogenous interleukin-6 in order to have an effect. Possibly, the factor(s) may share signaling pathways, such as in the case with interleukin-6 and leptin which both can utilize the Janus kinase (JAK)-STAT signaling pathway (220, 225). Hence, lack of endogenous interleukin-6 may alter the response of the 'programming' factor, leading to a different outcome. Or, as in this case no effect upon IL-$6^{-/-}$ offspring.

To summarize, we show that an altered maternal cytokine(s) status during pregnancy and/or lactation has long-term adverse health consequences for the offspring, without affecting perinatal growth. Previous studies have demonstrated the long-term health effects of elevated temporary exposure to cytokines during development (61, 62). Our study complements these previous studies by illustrating effects of an altered maternal milieu throughout gestation as well as lactation. Pregnancies complicated by maternal obesity or gestational diabetes are characterized by elevated maternal systemic levels of interleukin-6, leptin, and tumor necrosis factor-α (7, 25, 26). These factors can stimulate placental nutrient transport (88, 226), hence have potential to affect fetal growth. However, even in circumstances that do not result in altered prenatal growth, these factors can have consequences upon long-term health of the offspring. These findings are important as they may contribute to an understanding of why children born of mothers with gestational diabetes have an increased risk of adverse health outcomes as compared to similar sized children born of healthy mothers (50).

Cytokines and lipids affecting placental nutrient transport (paper II – IV)

Women who are obese are at increased risk of developing gestational diabetes (227, 228), as well as delivering a large infant (22, 28, 227-229). A high maternal pre-pregnancy BMI is also associated with potential medical complications during delivery for both mother and child (30, 31). Furthermore, being large at birth is associated with metabolic health disorders later in life,

such as an increased risk of type-2 diabetes and obesity (52, 54). However, not only birth weight but also body composition may be affected by maternal obesity and gestational diabetes, with infants having an increased adiposity (44, 49). Moreover, children born of mothers with gestational diabetes are at increased risk of adverse health disorders already in childhood, as compared to children of similar birth weight born of healthy mothers (50). This observation suggests that exposure to the maternal diabetic environment has effects upon developmental programming, even without affecting fetal growth.

These observations are especially pertinent because they lead us to question how maternal environment differs in obesity/gestational diabetes and what effects these differences may have upon the fetus. Maternal circulatory levels of cytokines and lipids rise during normal pregnancy (7, 199). However, in pregnancies complicated by maternal obesity or gestational diabetes there are additional changes, as compared to lean pregnancies without diabetes. These deviations include further elevated maternal circulatory triglycerides and free fatty acids (7, 10, 26). Systemic concentrations of C-reactive protein, interleukin-6, insulin, leptin, and tumor necrosis factor-α are also higher in pregnant women with gestational diabetes or high BMI, compared to lean pregnant women (25-27).

Alterations in placental nutrient transport capacity have been reported in association with maternal obesity and/or diabetes. These alterations include an increased activity of the amino acid transporters System A and System L in the microvillous membrane (41), and increased glucose transport across the basal membrane (42, 113). The expression of L-FABP is increased in placenta exposed to maternal diabetes (40). Furthermore, the activity of lipoprotein lipase is elevated in placenta from pregnancies complicated by maternal type-1 diabetes (40). Hence, transporting systems for amino acids, glucose, and lipids are all affected, displaying elevated activity in such pregnancy complications. Together these observations support the hypothesis of an increased placental nutrient transfer leading to an accelerated fetal growth in pregnancies complicated by maternal obesity or gestational diabetes. However, the underlying mechanisms for the increased activity of placental nutrient transporters are not well established.

The studies presented in *paper II – IV* were designed to evaluate the potential influence of elevated maternal circulatory levels of cytokines (interleukin-6 and tumor necrosis factor-α), hormones (insulin) and lipids (triglycerides and free fatty acids) on placental nutrient uptake. Primary cultures of placental trophoblast cells, exposed to varying levels of these factors, were used as model for the altered hormonal and metabolic environment of the mother with obesity or gestational diabetes.

Placental lipoprotein lipase

Lipoprotein lipase is a key enzyme involved in lipoprotein metabolism. This lipase is involved in the initial step of providing free fatty acids available for transfer to the developing fetus. The aims of *paper II* and *III* were to investigate the effects of elevated maternal cytokines, insulin, and lipids upon this lipase.

Maternal circulatory levels of triglycerides and free fatty acids increase during late pregnancy (8, 10). Free fatty acids can cross the placental barrier without prior modification, but intact triglycerides cannot (136). As long-chained polyunsaturated fatty acids are considered to be particularly important during development (128-130), and these fatty acids are mainly incorporated into triglyceride-rich lipoproteins (230), lipases associated with the microvillous membrane are critical for ensuring adequate fetal access to these fatty acids. The activities of several lipases in human placenta have been reported previously (138, 139, 193). In other tissues lipoprotein lipase is produced and secreted by parenchymal cells and subsequently transported to its active site (141-143), raising the question of the cellular source for placental lipoprotein lipase production. It was previously unknown whether mesenchymal cells in the villous interior or trophoblast cells that form the epithelial barrier of the villous were the source of placental lipoprotein lipase. This is critical since the syncytiotrophoblast are in direct contact with maternal blood and have some characteristics of endothelial cells. However, other endothelial cells are not known to produce lipoprotein lipase (141).

In *paper II* we show that isolated cytotrophoblast cells have measurable lipoprotein lipase activity as well as expression (Figure 1 in *paper II*). The expression (mRNA and protein) as well as the activity of lipoprotein lipase was measurable up to four days in culture. Hence, both cytotrophoblast cells and differentiated syncytiotrophoblast-like islets have the ability to produce this lipase. However, since cytotrophoblast cells become reduced in number by late pregnancy (67), the syncytiotrophoblast itself seems more likely as the production source of placental lipoprotein lipase. Our observations combined with the results of Lindegaard *et al.* (139), indicate that syncytiotrophoblast cells are the production source of placental lipoprotein lipase.

In further support of the placental origin of lipoprotein lipase, and excluding the possibility of maternal origin for placental lipoprotein lipase, is our observation of placenta from a lipoprotein lipase deficient mother. Albeit reduced (to approximately 25 to 50 %), these placentas had measurable activity of lipoprotein lipase (Table 1 in *paper II*). The measured reduction in lipase activity may result from a displacement of lipoprotein lipase from its anchoring site (148) by the pathologically elevated maternal triglyceride levels. In support of this possibility was that lipoprotein lipase mRNA expression was increased, suggesting that the site of regulation is post-translational, potentially by substrate inhibition.

Lipoprotein lipase is regulated in a complex, tissue-specific manner. For instance, activity of lipoprotein lipase in muscle and adipose tissue is regulated by nutritional status (231, 232). In rat adipose tissue lipoprotein lipase activity is reduced by fasting, but quickly restored in response to re-feeding (231). However, the response to feeding depends upon the tissue studied as well as meal composition. For example, rats fed a high carbohydrate meal have a reduced skeletal muscle lipoprotein lipase activity while having increased activity in adipose tissue (232). The effect of a high fat meal differs, insomuch as it does not reduce skeletal muscle lipoprotein lipase activity (232). Further contributing to the differential regulation, in rats insulin stimulates adipose tissue lipoprotein lipase activity but not in muscle (151). This stimulatory effect of insulin has been confirmed in adipocyte cell cultures (150). The mechanisms behind this differential regulation of lipoprotein lipase in different tissues are unclear. However, it is apparent that in order to understand how placental lipoprotein lipase functions, it must be studied in this tissue.

We observed no change in placental lipoprotein lipase activity or expression after incubation with insulin (three or twenty-four hour exposure with up to 290 ng/ml insulin), neither under normoglycemic nor hyperglycemic conditions (Table 2 in *paper II*) in cultured trophoblast cells. This observation is in contrast to our previous report of isolated villous fragments where a combination of hyperglycemia and insulin for three hours stimulated lipoprotein lipase activity (155). The reason for this discrepancy in results is unclear. It may be due to effects of culture conditions on the regulation of lipoprotein lipase, or reflect the different models used (single cell type in culture *vs.* tissue fragment). One consideration related to unresponsiveness of placental lipoprotein lipase to insulin is that a continuous functional lipoprotein lipase activity would ensure a continuous lipid transfer to the fetus, unaffected by changes in maternal nutritional state. Another indication of the importance of a continuous lipid flow is the observation that maternal circulatory lipids change little over the course of a day, seemingly less dependent on fasting/feeding than in the non-pregnant state (2). Consequently, this provides a steady source of triglycerides for generation of free fatty acids by placental lipases, creating a continuous lipid transfer to the fetus, independent of maternal metabolic status.

We tested the effect of elevated triglycerides and free fatty acids on placental lipoprotein lipase activity and expression. We found that placental lipoprotein lipase activity was reduced by elevated triglycerides and free fatty acids, without a major effect on either mRNA or protein expression (Figure 2 and 3 in *paper II*). This observation is in agreement with reported effects in endothelial cells (148); this reduction in activity is believed to result from displaced of lipoprotein lipase from its anchoring site by fatty acids. Such a reduction in lipoprotein lipase activity by triglycerides and free fatty acids has been suggested as a protective mechanism, thereby avoiding formation of free fatty acids at rates surpassing the tissues' uptake ability (148). Hence, the reduced placental lipoprotein lipase activity may be a mechanism to prevent excessive placental fatty acid uptake and transfer to the fetus when local free fatty acid concentrations rise. It has been shown that maternal circulatory levels of triglycerides are higher in pregnancies with maternal obesity and fetal macrosomia, compared to an obese mother delivering an appropriate-for-gestational age infant (233). Furthermore, the circulatory level of triglycerides is elevated in the macrosomic infant of an obese mother (233). A potential placental preventative mechanism appears to have been insufficient in averting fetal overgrowth, or is counteracted by the freely permeable free fatty acids that are also elevated in these pregnancies. Since infants of mothers with obesity or gestational diabetes have an augmented adiposity (25, 44, 49), it is clear that an increase in placental fatty acid transfer is critical for these fetuses to accelerate their fat deposition during late gestation. We investigated other regulatory mechanisms that might contribute to alterations in lipoprotein lipase activity in obese and diabetic women.

In addition to elevated triglycerides and free fatty acids, maternal systemic concentrations of pro-inflammatory cytokines, such as interleukin-6 and tumor necrosis factor-α, are also higher in obese and gestational diabetes pregnancies (25-27). Both interleukin-6 and tumor necrosis factor-α regulate lipoprotein lipase activity in other tissues (152-154), suggesting that placental lipoprotein lipase could be affected by elevations in cytokines either from the mother or from cytokines produced within the placental tissue. In a small cohort of lean to obese women we observed that maternal levels of interleukin-6 in third trimester plasma inversely correlated with

placental lipoprotein lipase activity (Figure 5 in *paper III*), suggesting that placental lipoprotein lipase might be inhibited by interleukin-6. However, since interleukin-6 did not directly alter placental lipoprotein lipase activity *in vitro*, the *in vivo* correlation may not reflect a cause-and-effect relationship (Figure 5 in *paper III*). Likewise, a cause-and-effect relationship is not apparent for adipose tissue lipoprotein lipase activity. In adipose tissue, lipoprotein lipase activity shows no correlation with plasma interleukin-6 (234), while *in vitro* experiments indicated a reduced enzyme activity in cultured adipocytes after exposure to this cytokine (152, 153).

Tumor necrosis factor-α has been suggested as an important regulator of lipid metabolism (235). This cytokine has been shown to reduce lipoprotein lipase activity in several tissues, such as macrophages (154), adipocytes (236), heart muscle (237), and in a osteosarcoma cell line (238). However, in our model of cultured trophoblast cells tumor necrosis factor-α did not modulate placental lipoprotein lipase activity (Figure 5 in *paper III*).

An adequate supply of fatty acids during development is important for the fetus. Especially, the long-chained polyunsaturated fatty acids are considered particularly important (128-130) for brain and eye development. These fatty acids are primarily incorporated into triglyceride-rich lipoproteins (230). Therefore the activity of lipases directed towards the maternal blood is important for acquiring adequate fetal access to these fatty acids. In human placenta, activities of at least four lipases have been reported (138, 139, 193). Activity of acid cholesterol ester hydrolase and hormone sensitive lipase have been measured in the cytosol (138), and consequently do not contribute to the lipid hydrolysis that enables uptake of fatty acids. Associated with the microvillous membrane are lipoprotein lipase as well as a placenta-specific lipase (138). Due to their location these two lipases can exert enzymatic activity on maternally derived lipoproteins. However, the placenta specific lipase is inhibited by serum (138). Hence, it appears to be of limited importance for hydrolysis of lipoprotein in maternal circulation. Endothelial lipase is also expressed in human placenta and mainly hydrolyzes phospholipids incorporated into high-density lipoproteins (139). However, the main location of placental endothelial lipase appears to be with endothelial cells (that is towards the fetal circulation), with only a weaker expression in syncytiotrophoblast (towards maternal circulation) (139). Together these observations suggest that lipoprotein lipase has an important role in enabling placental transfer of maternally derived triglycerides.

In summation, maternally elevated circulatory levels of free fatty acids and triglycerides, such as seen in obese and gestational diabetic pregnancies, has an inhibitory effect on placental lipoprotein lipase activity. For this reason, they may protect the fetus from excessive lipid transfer rather than stimulate such. We did not measure any effect on lipoprotein lipase after prolonged exposure to interleukin-6 or tumor necrosis factor-α. Nevertheless, it is important to note that these cytokines are not inhibitory in placenta, as reported in other tissues with respect to lipoprotein lipase activity (152-154). The role of insulin is unclear. In our cell culture model insulin did not affect lipoprotein lipase activity. However, the combination of hyperglycemia and insulin stimulates lipoprotein lipase activity in isolated villous fragments (155), thereby increasing the availability of free fatty acids accessible for transfer to the fetus. Together (with the possible exception of insulin combined with hyperglycemia) these observations do not provide a mechanistic explanation for increased fetal adiposity often seen in pregnancies complicated by

maternal obesity or gestational diabetes. Hydrolysis of triglycerides into free fatty acids constitute the initial step in placental transfer, we extended our studies focusing on the subsequent steps of lipid transfer and possible effects of cytokines.

Cytokines and placental lipid accumulation

Maternal systemic concentrations of interleukin-6 and tumor necrosis factor-α are elevated in pregnancies complicated by obesity or gestational diabetes (25-27) and placental interleukin-6 production is elevated in obese women (25). Interleukin-6 and tumor necrosis factor-α have been revealed as metabolically active cytokines, affecting both glucose as well as lipid metabolism (235, 239). It has been proposed that elevated levels of interleukin-6, in the absence of energy expenditure, could be a contributing factor to ectopic lipid accumulation in skeletal muscle (239). In *paper III* we examined whether or not interleukin-6, as well as tumor necrosis factor-α, could have a similar effect in placental cells.

In contrast to the lack of effect on trophoblast lipoprotein lipase activity, we observed an increased accumulation of lipids in trophoblast cells exposed to high levels of interleukin-6 (increased lipid accumulation approximately 30 %), but not tumor necrosis factor-α (Figure 2 in *paper III*). This lipid accumulation occurred at a concentration exceeding maternal circulatory levels. However, both systemic and placental cytokine production are elevated in obese women (25). Since placentally produced interleukin-6 is primarily released into the intervillous space (240), it is plausible that the concentrations of interleukin-6 at the microvillous membrane surpass levels found in maternal systemic circulation. With an increased placental lipid accumulation there is potentially a greater availability of lipids for transfer to the fetus. This idea is supported by the reported observations of increased lipid accumulation in placenta exposed to maternal type-1 diabetes in humans and diabetic rats (137, 241); a condition also associated with accelerated fetal growth (242). We suggest that this data supports the hypothesis that elevated interleukin-6 levels in pregnancies complicated by maternal obesity and/or diabetes stimulate placental fatty acid uptake, increasing placental lipid transfer and resulting in an increased fetal adiposity. There is a strong correlation between maternal circulatory interleukin-6 and fetal growth/adiposity (48), supporting the importance of this cytokine for fetal growth.

The underlying mechanism by which interleukin-6 stimulates trophoblast fatty acid accumulation remains to be established. Our data suggests that this effect is not mediated by increased mRNA or protein expression of adipophilin, FATP1, FATP4, L-FABP or lipoprotein lipase; all of which constitute key genes mediating cellular fatty acid uptake or lipid storage (Figure 3 and 4 in *paper III*). Yet, these are just a few of the genes involved in placental lipid uptake/transfer and the increased lipid accumulation may involve other important genes, such as endothelial lipase, fatty acid translocase/CD68, or pFABPpm that were not studied.

We show that in cultured trophoblast cells insulin does not affect lipoprotein lipase activity or expression (*paper II*), and tumor necrosis factor-α does not modulate lipoprotein lipase activity or lipid accumulation (*paper III*). However, it has been established in other studies that both insulin and tumor necrosis factor-α stimulate placental amino acid uptake (88, 93). This suggests that

these factors may be involved in excessive nutrient transport in women who are hyperinsulinemic and have elevated cytokines, potentially contributing to accelerated fetal growth. However, their effect upon placental lipid transfer appears limited. The observation by Duttaroy and Jørgensen (243) using the BeWo trophoblast cell model that neither insulin nor leptin (which also stimulates placental amino acid uptake (93)) have any effect on fatty acid uptake further supports our data that insulin has limited effects on placental lipid transfer. Furthermore, in *paper III* we demonstrate that interleukin-6 has no effect on lipoprotein lipase activity but increases placental lipid accumulation. Interleukin-6 also increases placental System A amino acid transporter activity (88), suggesting that this cytokine regulates multiple placental nutrient transport systems.

When comparing the effects of maternal cytokines and hormones on placental nutrient transport, it becomes clear that the lesser effect on lipid transport, compared to amino acid transport, may be connected to differences in the nature of these two transporting systems. In contrast to amino acids, which depend upon active transport against their concentration gradient in order to cross the microvillous membrane, free fatty acids can diffuse across this barrier with ease. Furthermore, the lower fetal fatty acid levels as compared to maternal levels create a concentration gradient, driving the transfer of fatty acid towards the developing fetus. The regulatory mechanisms for passive diffusion processes are likely to be quite different from those involved in regulating active transporting mechanisms.

To summarize, insulin, interleukin-6, and tumor necrosis factor-α have stimulatory effects upon placental nutrient transport, predominantly on amino acid transport. Their effect on fatty acid uptake and transport appears limited, though interleukin-6 does have a moderate stimulatory effect on lipid accumulation. The effects of these maternal factors on glucose uptake have been less studied. The effects of insulin on placental glucose uptake are inconclusive, as both stimulatory effects and an absence of effect have been reported (109, 111, 112). However, insulin, interleukin-6, and tumor necrosis factor-α, which all are elevated in pregnancies complicated by maternal obesity or gestational diabetes, stimulate placental nutrient transport, in particular amino acid uptake. Increased nutrient availability stimulates the fetal pancreas to secrete insulin which is a powerful growth factor in fetal life. This could contribute to the accelerated fetal growth often seen in these pregnancies.

Oleic acid, toll-like receptor 4, and placental amino acid uptake

Toll-like receptors (TLRs) are an important part of our innate immune system; involved in the first line of defense against pathogens, such as bacteria, fungi, and viruses. TLR4 recognizes a variety of pathogen-associated molecular patterns, including LPS of gram-negative bacteria (244), mouse mammary tumor virus (245), and respiratory syncytial virus (246). Upon activation, TLR4 signaling results in downstream activation of transcription factors and subsequent transcription of inflammatory related genes. This response can be modified depending on which adaptor proteins are involved in the downstream signaling pathway (247, 248). Presented in figure 4 is a schematic overview of TLR4 signaling.

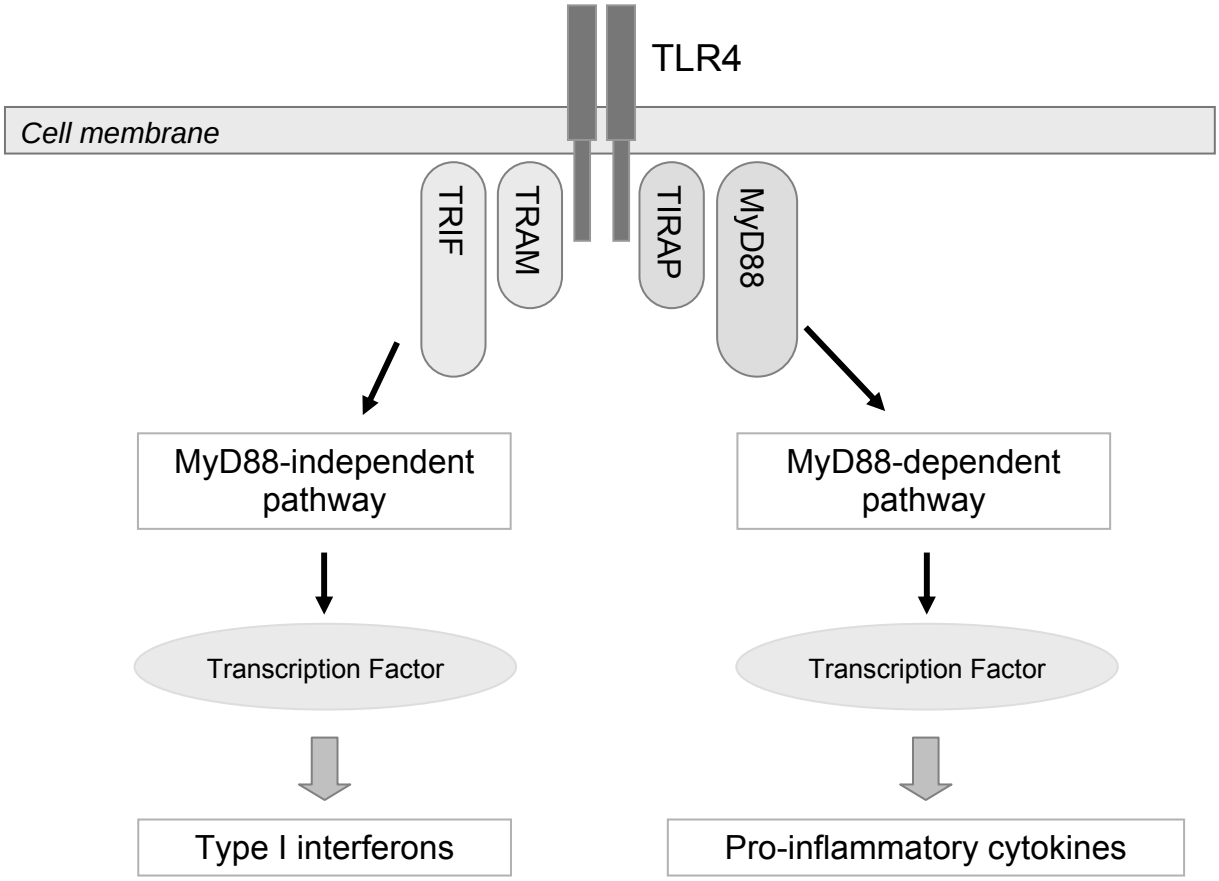

Figure 4. *Schematic illustration of TLR4 signaling. Upon activation of TLR4 intracellular signal transduction is initiated and occurs through recruitment of adaptor proteins, interacting with the TIR (Toll-interleukin-1 receptor) domains of the TLR4 receptor. There are five different adaptor proteins: MyD88, TIRAP, TRIF, TRAM, and SARM. After activation by LPS, the signaling response can be divided into an early MyD88-dependent response and a delayed MyD88-independent response. Signal transduction results in activation of transcription factors, such as AP-1, nuclear factor-κB and interferons response factors, and then gene expression (247, 248).*

In addition to pathogens, several endogenous ligands have been proposed as activating TLR4, including fatty acids (249-255). Fatty acids have been demonstrated to interact with TLRs in a wide variety of cell types, such as B-cells, dendritic cells, hypothalamus, kidney cells, as well as monocytes/macrophages (249-252, 254, 255). The fatty acids (in particular saturated and monounsaturated fatty acids) seemingly provoke a similar response on TLR4 as stimulation by LPS. Fatty acids have been shown to stimulate expression or release of pro-inflammatory cytokines, such as interleukin-1β and interleukin-6, as well as tumor necrosis factor-α (249, 252, 254). However, the physiological relevance of fatty acid stimulated TLR4 signaling and subsequent cytokine release with respect to cellular function remains to be established.

The mechanism by which fatty acids activate TLR4 signaling is unclear. It has been suggested that fatty acids might interact with the same binding site on the TLR4 complex as the lipid A moiety of LPS (256). A direct binding has however been questioned (257). Nevertheless, several

groups have shown the effects on TLR4 signaling by fatty acids, but despite this the precise mechanism remains to be established. Wong *et al.* (249) have shown that lauric acid enhances the formation of lipid rafts in the cell membrane, bringing the TLR4s in close proximity of each other leading to dimerization, which results in signaling activation. Interestingly, long-chained polyunsaturated fatty acids also affect TLR4 signaling. However, they reduce activation and display inhibitory effects on TLR4 signaling initiated by saturated fatty acids or LPS (249-251). An elevation in circulating lipids is associated with inflammation and insulin resistance (258, 259). Activation of TLR4 by saturated and monounsaturated fatty acids provide a potential mechanistic link between chronic inflammation, hyperlipidemia, and conditions such as diabetes and obesity (256, 260).

The human placenta expresses TLR4 in the microvillous plasma membrane of the syncytiotrophoblast (261-263). It has been demonstrated that the human placenta responds to LPS stimuli by augmenting cytokine release, including interleukin-1β, -6, -8, and -10, as well as tumor necrosis factor-α (264-267). High maternal circulatory lipid levels are a hallmark of late pregnancy and are further elevated in obese and gestational diabetic pregnancies. The recent suggestion that fatty acids also can modulate TLR4 signaling further broadens their biological effects beyond a nutritional or energy source and towards additional functions as signaling molecules. We tested the hypothesis that elevated levels of oleic acid alter placental release of cytokines, mediated by activation of TLR4. Furthermore, we speculated that the cytokine release after TLR4 activation would affect the placenta in an autocrine/paracrine manner and stimulate placental nutrient transporting functions. The effects of oleic acid were studied since it is one of the physiologically most predominant fatty acids. During pregnancy oleic constitutes approximately 30 % of plasma total free fatty acids (268). Furthermore, oleic fatty acid is also a major component of adipose tissue (269), therefore likely to be readily available for release and placental exposure during late pregnancy as maternal lipolysis increases (4).

We observed an altered release of some cytokines after a twenty-four hour exposure to oleic acid, including a threefold higher release of interleukin-6 (Figure 2 in *paper IV*). Such a release would elevate the local concentrations of interleukin-6 at the microvillous membrane, since placental interleukin-6 has been shown to be predominately released into the maternal circulation (240). It has recently been reported that trophoblast cells exposed to a palmitic acid and oleic acid mixture increase their release of tumor necrosis factor-α (270). We did not detect any measurable amounts of this cytokine after oleic acid exposure in our trophoblast cells. Consequently, not only alterations in maternal circulatory fatty acid levels but also composition may affect placental cytokine release. Furthermore, if released in sufficient amounts, these cytokines could possibly affect maternal metabolism. Interleukin-6 has been linked to glucose as well as lipid metabolism (239). It has been shown that plasma interleukin-6 correlates with reduced insulin sensitivity (271) and has been proposed as a predictor of type-2 diabetes (272).

Consequently, alterations in maternal circulatory fatty acid levels or composition may affect placental cytokine release, which in turn may modify placental function in a paracrine/autocrine manner. As shown in *paper III* interleukin-6 increases placental lipid accumulation and oleic acid increases placental release of this cytokine (*paper IV*). Tobin *et al.* (161) have shown that trophoblast cells exposed to oleic acid have an increased intracellular triglyceride accumulation.

When combining our results, a potential mechanism for Tobin's observation emerges. That is, oleic acid exposure stimulates placental interleukin-6 release which then in turn stimulates the accumulation of lipids. We speculate that activation of TLR4 by oleic acid is the mediator resulting in increased cytokine release. Indirectly supporting this idea of TLR4 involvement are further observations by Tobin and coworkers. They demonstrated that trophoblast cells exposed to docosahexaenoic acid had a much lower intracellular triglyceride accumulation compared to the cells exposed to oleic acid (161). This difference could potentially depend upon differential modulation of TLR4 signaling by unique species of fatty acids.

Since fatty acids can activate trophoblast TLR4 signaling and interleukin-6 stimulates System A activity (88), we hypothesized that elevated oleic acid levels would stimulate placental amino acid uptake. The increased amino acid uptake would be accomplished through an increased interleukin-6 release mediated via activation of TLR4 and phosphorylation of STAT3. Indeed, after an exposure to oleic acid placental System A amino acid transporter activity was twofold higher than control (Figure 3 in *paper IV*). We further investigated the cellular signaling mechanisms that might be responsible for the alteration in amino acid uptake after stimulation with oleic acid.

Interleukin-6 signaling is initiated by the binding of this cytokine to its membrane-bound receptor. This cytokine-receptor association forms a complex with the signaling transducing receptor subunits, glycoprotein 130, which initiate a signal transduction involving the activation of JAK. Signal transduction and activation of JAKs will lead to an activation of the transcription factor STATs. Dimers of activated STATs translocate into the cell nucleus and initiate gene transcription. In addition to activating the JAK-STAT signaling pathway, interleukin-6 can also activate the mitogen-activated protein kinase pathway. Both signaling pathways lead to activation of transcription factors and gene expression (225). Interleukin-6 stimulation of System A activity has been previously shown to be dependent upon STAT3 (88). In *paper IV* we demonstrate that phosphorylation of STAT3, at an activating site Tyr705, was increased in oleic acid stimulated trophoblast cells (increased 70 %; Figure 4 in *paper IV*). This observation suggests that interleukin-6 and STAT3 are likely to be involved in oleic acid stimulation of placental amino acid uptake.

The stimulatory effect of oleic acid on System A activity in trophoblast cells was completely suppressed when TLR4 expression was silenced (Figure 6 in *paper IV*). This observation is, to the best of our knowledge, the first report that fatty acids stimulate amino acid uptake through activation of TLR4, in any tissue. This could represent a mechanism which contributes to accelerated fetal growth in pregnancies complicated by elevated maternal systemic levels of cytokines and lipids. An increased placental cytokine release after fatty acid exposure which results in elevated amino acid uptake and lipid accumulation may also affect other functions. mTOR has been suggested as an important determinant of fetal growth (273) and amino acids are a major regulator of this signaling pathway (273). In placenta, mTOR has been shown to regulate amino acid uptake (98). However, in our oleic acid exposed trophoblast cells, no significant difference in mTOR activity was observed (measured as S6 Kinase phosphorylation, Figure 4 in *paper IV*). Increased intracellular levels of lipids may affect placental function, as lipids are ligands

for the PPARs (274, 275). Along with several other functions (276), PPARγ has been shown to regulate expression of several fatty acid transport proteins in human placenta (166).

The mechanism of fatty acids stimulating TLR4 which results in cytokine release and the following nutrient uptake could be applicable in other tissues as well. For example, obesity and type-2 diabetes is associated with an increased ectopic lipid accumulation. In skeletal muscle lipid accumulation correlates with reduced insulin sensitivity; elevated interleukin-6 levels have been suggested as an underlying mechanism for the increased fat deposition (239). Hence, the elevated systemic levels of lipids under such conditions may stimulate its own uptake, via activation of TLR4 and subsequent cytokine release.

Could placental amino acid uptake, mediated by fatty acid activation of TLR4, be of importance for accelerated fetal growth in obese or gestational diabetes pregnancies? We speculate the following:

1) Maternal circulatory fatty acid levels are elevated in these pregnancy complications. In normal pregnancies maternal fatty acids levels increase with progressing gestation, activation of TLR4 by the elevated fatty acids may function as a natural stimulus to increase placental amino acid uptake in late gestation. However, in the obese mothers or mothers with gestational diabetes the pathological elevations in fatty acid levels may cause excess TLR4 activation, release of interleukin-6 and subsequent stimulation of amino acid transport.

2) Obese individuals have lower circulatory levels of long-chained polyunsaturated fatty acids (277). These fatty acids can inhibit TLR4 signaling initiated by saturated fatty acids (249, 251, 254). Consequently, a naturally occurring mechanism to interrupt TLR4 signaling may be reduced in obese pregnancies and therefore a maximal stimulation of System A amino acid transporter activity would occur.

3) Placentas from obese pregnancies are infiltrated with macrophages (25). Likewise, adipose tissue of obese individuals is infiltrated with macrophages (278). It has been shown that free fatty acids released from adipocytes can activate macrophages, causing them to secrete cytokines; this response is mediated though TLR4 (253). It has been suggested that these cytokine-secreting macrophages substantially contribute to total release of cytokines from adipose tissue (278, 279). Assuming placental macrophages are activated by fatty acids resulting in increased cytokine release and that increased System A activity by oleic acid depends upon interleukin-6, an increased number of macrophages in the obese placenta may contribute to increased trophoblast amino acid uptake. We have however not excluded the possibility of a direct connection between the System A amino acid transporter and the TLR4 signaling cascade; nor have we definitively confirmed the importance of interleukin-6 release in the signaling pathway for oleic acid stimulation of trophoblast amino acid uptake.

4) In placentas from obese ewes the expression of TLR4 is increased, compared to normal weight ewes (280). Hence, these placentas have an increased number of TLR4s available for activation by the fatty acids, potentially leading to excessive activation of this receptor.

These factors (*i.e.*, elevated maternal fatty acids, reduced long-chained polyunsaturated fatty acid concentrations, placentas infiltrated with macrophages, as well as increased TLR4 expression)

may all contribute to an augmented amino acid uptake. In addition, they possibly contribute to accelerated fetal growth in pregnancies complicated by maternal obesity or gestational diabetes. Summarized in figure 5 are the results presented in *paper II – IV* from the cultured primary human trophoblast cells after exposure to free fatty acids, insulin, interleukin-6, triglycerides, or tumor necrosis factor-α.

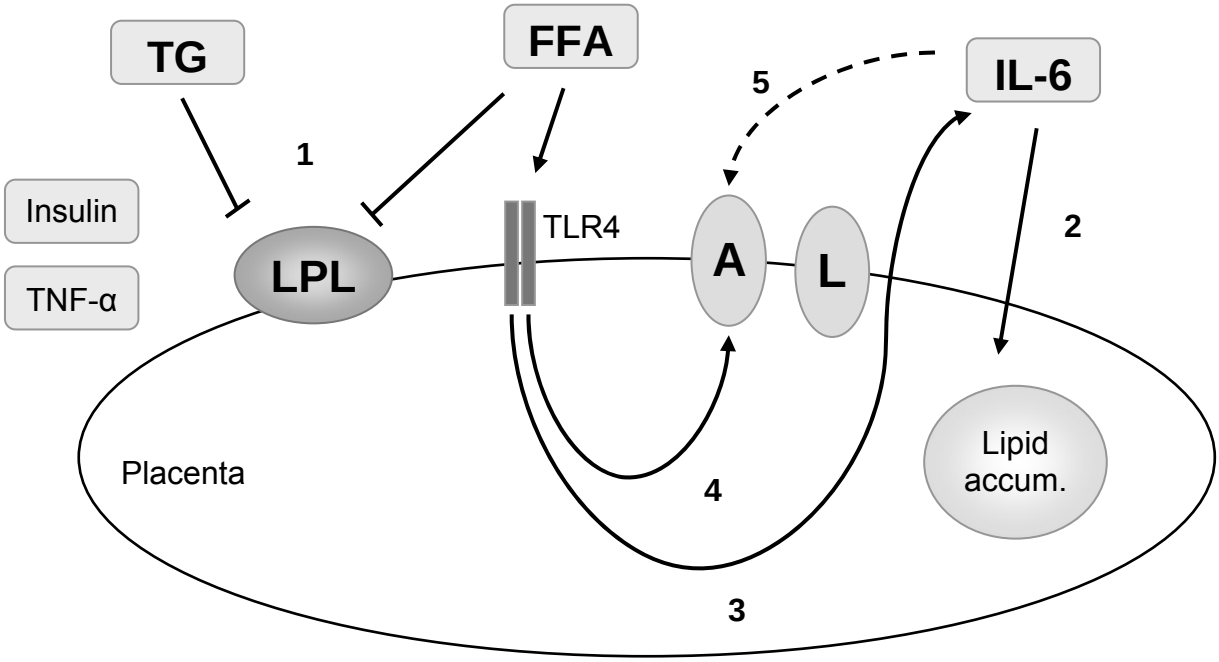

Figure 5. *An overview of results from cultured trophoblast cells presented in paper II – IV. Paper II: Elevated levels of triglycerides (**TG**) and free fatty acids (**FFA**) reduced the activity of lipoprotein lipase (**LPL**) (**1**). Triglyceride exposure also resulted in a small increase in lipoprotein lipase protein expression. No effect was observed upon lipoprotein lipase mRNA levels after exposure to either free fatty acids or triglycerides. Insulin had no effect on placental lipoprotein lipase. Paper III: Interleukin-6 (**IL-6**) exposure increased placental lipid accumulation (**2**), but did not affect protein (FATP4, L-FABP, lipoprotein lipase) or mRNA (Adipophilin, FATP1) expression. Lower mRNA expressions of FATP4 and lipoprotein lipase were observed after interleukin-6 incubations. Tumor necrosis factor-a (**TNF-α**) did not affect lipid accumulation. Neither interleukin-6 nor tumor necrosis factor-a altered activity of lipoprotein lipase. Paper IV: After incubating the trophoblast cells with free fatty acids (oleic acid) the release of cytokines was altered, including increased release of interleukin-6 (**3**). Further, System A (**A**) activity was increased twofold (**4**), without affecting System L (**L**). The stimulatory effect of oleic acid on System A was mediated through toll-like receptor 4 (**TLR4**), as silencing this receptor completely abolished the effect. The increased release of interleukin-6 may be responsible for elevating System A activity after oleic acid incubation (**5**; dashed arrow), as it has been previously shown that interleukin-6 stimulates amino acid uptake via the System A transporter (88). All effects studied after a twenty-four hour exposure, except the effect of insulin which was evaluated after three and twenty-four hours. Lipid accum., lipid accumulation.*

CONCLUDING REMARKS

The significance of the maternal metabolic environment, during pregnancy as well as early postnatal life, has been highlighted in this thesis. We have investigated some possible mechanisms contributing to a vicious circle: obese or diabetic pregnant women delivering large infants, infants that are at increased risk of emergent metabolic health disorders later in life, which then affects their pregnancies and the next generation.

We have shown that maternal interleukin-6 deficiency increases adiposity and body weight in offspring. Furthermore, being fostered by an interleukin-6 deficient dam also increases body weight, adiposity and adipocyte size. These findings suggest that absence of maternal interleukin-6 or, alternatively, factors modified by this cytokine, have consequences on developmental programming. These studies have been performed using a mouse model lacking interleukin-6. In the human obesity or overweight conditions peripheral interleukin-6 is increased. Our *in vivo* studies of interleukin-6 deficiency highlight the importance of this cytokine, illustrating that altered maternal levels can have developmental programming consequences.

We have also observed alterations in placental nutrient transport capacity in response to metabolic factors known to be altered in pregnancies complicated by maternal obesity or gestational diabetes, namely pro-inflammatory status and hyperlipidemia. Infants of these mothers often have an increased adiposity and/or birth weight. We identified two effects which potentially contribute to accelerated fetal growth: 1) interleukin-6 increases placental lipid accumulation and 2) fatty acids enhance placental amino acid uptake. The increased lipid accumulation suggests that more fatty acids are available for transfer to the fetus. However, the most pronounced effects on trophoblast cells were detected after exposure to free fatty acids. These effects were a decreased lipoprotein lipase activity, an increased interleukin-6 release, activation of the STAT3 signaling pathway, and increased amino acid uptake by System A but not System L. The changes in System A activity appear to be mediated through TLR4 and may be a result of the increased interleukin-6 secretion as we have recently shown that interleukin-6 has a stimulatory effect on System A (88). We have previously suggested that the placenta to functions as a nutrient sensor during pregnancy (37). In cases of obesity and diabetes the altered maternal metabolic environment signals an abundant nutritional status. Consequently the placenta responds by increasing nutrient transport to the fetus to sustain the development of a larger infant. Our data indicates there may be 'brakes' in this nutrient sensing system. The reduction in lipoprotein lipase activity by free fatty acids and triglycerides may be one of these inhibitory functions that could protect the feto-placenta unit, not only from excessive lipid transfer, but perhaps also from excessive release of free fatty acids at the level of the placental epithelia which effectively stimulates amino acid uptake. Amino acids are a known potent stimulator of fetal pancreatic insulin secretion, which in turn functions to promote fetal growth.

FUTURE PERSPECTIVES

The studies within this thesis shed light on some important aspects of developmental programming as well as regulation of placental nutrient transport. A number of questions arise as well. The causative or contributing mechanisms behind the observations remain to be further established, such as:

What are the underlying mechanisms causing developmental programming in the interleukin-6 deficient mice? We have demonstrated phenotype differences between genetically identical mice depending on maternal differences *in utero* or in the postnatal period. What maternal differences (in addition to genetic) are responsible for the observed programming effects remains to be established. Furthermore, how are the phenotypic dissimilarities accomplished and maintained over the long-term growth and development of the individual. Are there differences in epigenetic modification to the genome or in neuronal circuits?

What are the mechanisms behind the increased lipid accumulation in trophoblast cells after exposure to interleukin-6 treatment? Additional studies are required to elucidate whether or not the increased lipid accumulation is generated by an enhanced uptake or altered metabolism. Further, if the uptake is modulated which systems are involved and how are they regulated?

Are more amino acids and fatty acids transferred to the fetus? We have shown an increased accumulation or uptake of lipids and amino acids in a cell culture model system of the placental epithelial barrier. Demonstration of an actual increased net transfer has not been accomplished. This could be further explored with trophoblast cell lines using a trans-well system or in an animal model of obesity.

What signaling pathways are involved that link cellular fatty acid exposure and increased System A activity? We have identified TLR4 as an obligatory mediator of the fatty acid effect on System A activity. But questions remain concerning whether this receptor is directly activated by the fatty acids or a fatty acid metabolite? What downstream signaling pathways are involved? We speculate that the increased release of interleukin-6 is involved in stimulating the amino acid uptake, though there could be direct effect of TLR4 signaling cascade on System A. These questions could be addressed with additional targeted gene silencing.

Do fatty acids have distinct effects upon placental amino acid uptake? We hypothesize that the increased System A activity after oleic acid exposure is connected to the increased release of interleukin-6. However, trophoblast cells' secretion of cytokines differs and this depends upon which types of fatty acid(s) are exposed to the cells. The effects on amino acid uptake may differ as well. In addition, long-chained polyunsaturated fatty acids have inhibitory effects on TLR4 signaling, thereby presenting an additional mechanism for differential trophoblast responses to fatty acid species and to combinations of fatty acids that occurs in vivo.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Summary in Swedish

Den miljö vi utsätts för under vår tidiga utveckling, det vill säga miljön vi exponeras för under graviditeten och strax efter födelsen, har visat sig kunna påverka risken att utveckla sjukdomar senare i livet. Även om genetiska faktorer och livsstil är viktiga bakomliggande orsaker till folksjukdomar såsom typ-2 diabetes, hjärtkärlsjukdom och fetma, så kan en ogynnsam miljö under vår tidiga utveckling ytterligare öka risken för dessa hälsoproblem. Kvinnor som är överviktiga eller utvecklar graviditetsdiabetes får ofta stora barn, barn som väger mycket vid födelsen. Att föda ett stort barn är förknippat med en större risk för komplikationer under förlossningen, för både mamman och barnet. Barnet själv har även en större risk att utveckla diabetes och fetma senare i livet. Det skapas på så sätt en ond cirkel med överviktiga kvinnor och kvinnor med graviditetsdiabetes som föder stora barn, barn som i sin tur har en ökad risk att utveckla diabetes och fetma i vuxen ålder. Är barnet en flicka kan mönstret återupprepas och föras vidare till nästa generation.

Vår kunskap och förståelse är begränsad om varför barn födda av överviktiga kvinnor och kvinnor med graviditetsdiabetes har en förhöjd sjukdomsrisk och vad som gör att dessa barn ofta växer för mycket innan födelsen. Man vet att fostrets tillväxt är nära förknippat med moderkakans transport av näringsämnen. Personer med övervikt eller diabetes har förändrade blodnivåer av cytokiner (signalmolekyler), fetter och hormoner. I den här avhandlingen har vi undersökt om några av dessa förändringar kan påverka moderkakans näringstransport hos människor. Vi har även studerat betydelsen av när interleukin-6 (en cytokin) saknas under graviditeten och amningen hos möss.

De viktigaste fynden som presenteras i denna avhandling är att möss som saknar interleukin-6 och är födda av en moder som också saknar interleukin-6 är yngre när de blir överviktiga än mössen som är födda av en moder som har interleukin-6. Även de möss som har interleukin-6 men som ammats av en musmamma som saknar interleukin-6 blir tjockare samt får större fettceller.

Hur cytokiner, fetter och insulin påverkar den humana moderkakans transport av näringsämnen undersöktes i cellodlingar. Vi fann att moderkaksceller som exponeras för höga nivåer av interleukin-6 tar upp mer fett, fett som sedan skulle kunna transporteras vidare till fostret. Vi fann att förhöjda nivåer av fetter reducerar aktiviteten av ett enzym (lipoprotein lipas) som är viktigt för att fett skall kunnas tas upp i moderkakan, möjligtvis för att förhindra ett allt för omfattande fettupptag. Vidare påverkar fetterna hur mycket cytokiner cellerna själva utsöndrar, samt stimulerar cellerna att ta upp mer aminosyror.

Sammanfattningsvis har vi funnit att förändrade nivåer av cytokinen interleukin-6 och fetter stimulerar moderkakans näringsupptag, detta skulle kunna bidra till en accelererad fostertillväxt. Vi har även funnit att förändrade nivåer av interleukin-6 hos modern kan påverka hennes avkommas framtida hälsa. Dessa fynd bidrar med några pusselbitar i förståelsen om varför överviktiga kvinnor får stora barn, barn som har förhöjd risk att utveckla metabola sjukdomar senare i livet.

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