

Dissertation

Maternal-to-fetal transfer of fatty acids across the human placenta

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Acknowledgement has been made in the text to all other material used. Parts of this thesis are published in the *International Journal of Obesity* (Hirschmugl et al. Int J Obes (Lond). 2017 Feb;41(2):317-323. doi: 10.1038/ijo.2016.188). Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

Date.....

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Abbreviations

17-HDHA	17-hydroxydocosahexaenoic acid
18-HEPE	18-hydroxyeicosapentaenoic acid
AA	Arachidonic acid
ATGL	Adipose triglyceride lipase
BM	Basal membrane
BMI	Body mass index
BSA	Bovine serum albumin
CD36/FAT	Fatty acid translocase
CE	Cholesteol ester
CGI-58	Comparative gene identification-58
CRP	C-reactive protein
DG	Diglycerides
DHA	Docosahexaenoic acid
EL	Endothelial lipase
EPA	Ecosapentaenoic acid
ER	Endoplasmic rediculum
FA	Fatty acid
FABP	Fatty acid binding protein
FABPpm	Plasma membrane fatty acid binding protein
FATP	Fatty acid transport protein
FFA	Free fatty acid
FM ratio	Fetal-maternal ratio
GC	Gas chromatography
GC-MS	Gas chromatography - mass spectrometry
GDM	Gestational diabetes mellitus
GWG	Gestational weight gain
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HOMA-IR	Homeostatic model assessment for insulin resistance
HSL	Hormone-sensitive lipase

IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-6	Interleukin-6
LA	Linoleic acid
LCFA	Long-chain fatty acid
LCPUFA	Long-chain polyunsaturated fatty acid
LD	Lipid droplet
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LPL	Lipoprotein lipase
MCP1	Monocyte chemoattractant protein1
MGL	Monoglyceride lipase
MVM	Microvillous plasma membrane
OA	Oleic acid
n3 FA	Omega-3 fatty acid
n6 FA	Omega-6 fatty acid
PA	Palmitic acid
PL	Phospholipid
PLIN	Perilipin
qRT-PCR	Quantitative real time polymerase chain reaction
TG	Triglyceride
TLC	Thin layer chromatography
TLR4	Toll like receptor 4
TNF- α	Tumor necrosis factor- α
VLDL	Very low density lipoprotein
WAT	White adipose tissue

Zusammenfassung

Adipositas bei Neugeborenen steht in direktem Zusammenhang mit einem erhöhten Risiko auch im späteren Lebensalter an Adipositas und Folgeerscheinungen zu erkranken. Darüber hinaus besteht ein vermehrtes Risiko bei adipösen Müttern, Kinder mit erhöhtem Anteil an Fettgewebe zu gebären. Die zu Grunde liegenden Mechanismen und auch die diesbezügliche Funktion der Plazenta in der späten Schwangerschaft werden noch nicht gut verstanden.

Das Hauptziel dieser Dissertation war, den Einfluss von präexistierender mütterlicher Adipositas auf den Transport von Fettsäuren (FS) über die humane Plazenta zu untersuchen. Ein weiteres Ziel war, die Verteilung von exogen angebotenen FS zwischen der Plazenta und der fetalen Zirkulation zu verstehen. Diese Studie setzte sich auch zum Ziel, den Effekt von bestehender mütterlicher Adipositas auf Gen- und Proteinexpression, involviert in Lipid- und FS- Aufnahme, Metabolismus und Transport sind, festzustellen.

Der Transport von freien FS (FFS) von der Mutter zum Fetus, wurde an Plazenten von normalgewichtigen (Body Mass Index vor der Schwangerschaft (BMI) $\leq 25 \text{ kg/m}^2$) und adipösen Müttern (BMI $\geq 30 \text{ kg/m}^2$) mittels der *ex-vivo* Perfusion untersucht. ^{13}C -markierte FFS wurden im mütterlichen Kreislauf angeboten und deren Konzentration nach Transport über die Plazenta in fetalen Proben mittels Gaschromatographie (GC) – Massenspektrometrie analysiert. Die Verteilung der FS in die einzelnen Lipidklassen wurde mittels Dünnschichtchromatographie und GC ermittelt. Spezifische Gene wurden mittels nCounter Technologie untersucht, und Protein-Expression und -Lokalisation in Plazentagewebe von Müttern mit normalem und adipösem BMI festgestellt.

In der fetalen Zirkulation wurde der Hauptanteil an übergetretenen Palmitin-, Öl- und Linolsäure in Phospholipiden (PL) und FFS detektiert. Geringe Mengen Palmitinsäure befanden sich auch in den neutralen Lipidklassen Cholesterinester und Triglyzeride (TG). In Plazenten von adipösen Müttern ist der Transfer von Palmitin-, Öl- und Linolsäure leicht erhöht, und jener von Docosahexaensäure (DHA) war signifikant ($P=0.040$) um 44% höher als bei normalgewichtigen Müttern. Es besteht ein deutlicher Zusammenhang ($R=0.697$; $P<0.003$) von DHA Transfer über die Plazenta mit der DHA Konzentration, die auf der mütterlichen Seite angeboten wurde. Es wurden

1,7 – 58,6 mal mehr endogene FS aus plazentaren Lipidspeichern mobilisiert und in die fetale Zirkulation freigesetzt, als exogen angebotene FS transportiert wurden. In dieser Studie wurden 73 Ziel-Gene untersucht und 6 davon zeigten eine positive Korrelation ($P < 0.05$) mit mütterlichem BMI, nämlich ATGL, FATP1, FATP3, PLIN2, PPARG und CGI-58. Die Protein-Expression von CGI-58 in Plazenten von Müttern mit adipösem BMI waren zweifach erhöht ($P < 0.001$) und korrelierten mit mütterlichem Plasma-Insulin zum Zeitpunkt der Geburt ($R = 0.61$; $P < 0.001$).

Die Ergebnisse meiner Dissertation lassen darauf schließen, dass der FFS Transport über die Plazenta sehr komplex ist und mehrere voneinander unterscheidbare Routen einschließt. Mindestens drei wechselwirkende Mechanismen werden vermutet um den Fetus optimal mit FS zu versorgen. Erstens, ein effizienter direkter Transfer von FFS aus dem mütterlichen Plasma zum Fetus. Zweitens, die Mobilisierung von FFS aus plazentaren Lipidspeichern und drittens, die Freisetzung von PL aus der Plazenta in die fetale Zirkulation. Mütterliche Adipositas führt zur signifikanten Erhöhung von plazentaren Genen und Proteinen, die wichtig sind für den Transport und die Speicherung von Neutrallipiden. Insbesondere reguliert CGI-58 die intrazelluläre TG-Hydrolyse und vermutlich ist damit eine vermehrte Mobilisierung von FS in der Plazenta verbunden. Zusammengefasst stellt der erhöhte direkte FFS Transfer von der adipösen Mutter zum Fetus und der überproportionale Lipid-Umsatz in der Plazenta vermutlich einen Überschuss an FFS für die fetale Versorgung bereit, der in der Folge in fetalem Fettgewebe gespeichert werden kann.

Abstract

Infant and childhood obesity is known to be a risk factor for obesity and related diseases later in life. Obese women are prone to deliver more likely infants with elevated adipose tissue mass. The underlying mechanisms and the functional role of the placenta during late pregnancy are not well understood yet.

The main objective of this thesis was to investigate whether maternal pre-pregnancy obesity impacts fatty acid (FA) transfer across the human placenta and to understand the distribution of exogenously provided FAs between placental and fetal compartment. This study aimed also to examine the effect of maternal pre-pregnancy obesity on placental genes and proteins, which are involved in lipid and FA uptake, metabolism, and transport.

Maternal-to-fetal free FA (FFA) transfer was examined in placentas of lean (pre-pregnancy body mass index (BMI) ≤ 25 kg/m²) and obese women (BMI ≥ 30 kg/m²) by *ex-vivo* perfusion approach. ¹³C-labelled FFAs bound to albumin were offered to the maternal compartment and transfer of ¹³C FFAs to the fetal compartment was followed by gas chromatography (GC) – mass spectrometry. Distribution of FAs to different lipid classes of maternal and fetal perfusates was measured by thin-layer chromatography and GC. Target specific gene expression, determined by nCounter technology, as well as protein expression and localization were performed in placental tissue of lean and obese women.

Results from perfusion experiments showed that transferred palmitic, oleic, and linoleic acid are mainly restored in phospholipids (PL) and as FFAs in fetal perfusates. Minor amounts of palmitic acid were also detectable in cholesterol esters and triglycerides (TG). Maternal-to-fetal transfer of palmitic, oleic, and linoleic acid is slightly higher in obese compared to lean placentas. In placentas of obese women the transfer of docosahexaenoic acid (DHA) is significantly ($P=0.040$) elevated by 44% compared to lean placentas. By stratifying the cohort according to fetal sex, comparable absolute transfer of all FFAs was observed in obese male and lean female placentas. Again, DHA transfer was significantly ($P<0.05$) increased in obese compared to lean female placentas. Maternal-to-fetal DHA transfer correlated positively ($R=0.697$; $P<0.003$) with maternally offered DHA concentrations, independently of maternal BMI. Additionally, endogenously

stored FAs were mobilized from intracellular lipid pools and were released to the fetal compartment, which was 1.7 to 58.6-fold higher than direct FFA transfer.

In this study 73 placental target genes were examined and six showed positive correlation ($P < 0.05$) with maternal pre-pregnancy BMI, which were ATGL, FATP1, FATP3, PLIN2, PPARG and CGI-58. CGI-58 protein level was 2-fold higher ($P < 0.001$) in placentas of obese compared to lean women. Furthermore, CGI-58 protein amounts correlated positively with maternal plasma insulin levels at the time of delivery ($R = 0.63$, $P < 0.001$).

In conclusion, the results of my thesis suggest that FFA transport across the placenta is a complex process including more than one distinct route. To guarantee sufficient FFA supply to the fetus at least three related mechanisms are proposed. Direct efficient transfer of maternally derived FFAs takes place and is corroborated by mobilization of FFAs from placental lipid pools, if required. In addition the placenta releases phospholipids to the fetal compartment, which provides a second lipid species for fetal requirements. Maternal pre-pregnancy obesity leads to significantly elevated expression of placental genes and proteins related to transport and storage of neutral lipids. In particular CGI-58, important for initiation and regulation of TG hydrolysis, may contribute to elevated intracellular lipid turnover in placentas of obese women. Taken together the increased direct transfer of maternally derived FFAs and the enhanced lipid turnover may lead to elevated FFA supply to fetus and subsequently accretion in adipose tissue.

1 Introduction

1.1 Maternal obesity in pregnancy

Obesity has become a major public health problem in western countries, but also in emerging nations over the last two decades. The prevalence among adults for being overweight which is defined by a body mass index (BMI) between 25 and 29.9 kg/m² or being obese (BMI $\geq$ 30 kg/m²) increases since the 1980s. In the United States 67.3% of the adult population is overweight or obese. Moreover, in the United States obesity among adults has risen from 1980 to 2014 from 14.7% to 33.9%, respectively. But also in central Europe the proportion of overweight or obese people gains alarming numbers, as seen exemplarily in the United Kingdom where 63.4% and 28.1% of the people are overweight and obese, respectively. In comparison to UK, in Austria the proportion of overweight adults is 53.1% and 18.4% of the population lives with a BMI $\geq$ 30 kg/m² (1). Particularly dramatic is the global increase in numbers of overweight or obese children (aged 0-5 years) from 32 million in 1990 to 42 million in 2013 (2) demonstrating very impressive the social burden for the next years.

Obesity is characterized among others by elevated lipid deposition in adipose tissue which is accompanied by insulin resistance, increased leptin levels and systemic dyslipidemia. Obesity in adults is also considered as a state of low-grade inflammation, due to many metabolic derailments and cellular responses, like macrophage infiltration in adipose tissue followed by secretion of pro-inflammatory cytokines (3-5). These changes in the obese body may result in health problems, like type 2 diabetes or cardio vascular diseases (6). During pregnancy, elevated maternal pre-pregnancy BMI is associated with a higher risk for endothelial dysfunction (7), hypertension, pre-eclampsia, gestational diabetes mellitus (GDM) (8), and complications during parturition (9).

Maternal pre-pregnancy obesity has become of interest since several studies demonstrated a strong relation with adiposity in the offspring as well. Whitaker (10) described a 24.1% prevalence for obesity among children until the age of 4 years when they were born by obese mothers in a large cohort retrospectively (10). This finding was confirmed in recent studies where numerous associations between maternal and

newborn anthropometric, metabolic and inflammatory parameters were reported which underline the impact of maternal pre-pregnancy BMI on fetal and neonatal health. In addition, maternal total and between 12 and 28 weeks of gestation weight gain (GWG) correlates with birth weight. This relation is independent of maternal pre-pregnancy BMI (11) suggesting that maternal pre-pregnancy BMI does not directly affect neonatal birth weight. However, body fat mass of infants from overweight or obese mothers is significantly increased in comparison to infants born by lean mothers (12). Furthermore, the percentage of body fat in offspring correlates with fetal insulin resistance. (13). The metabolic disarrangement in the obese mother is reflected also in the unborn by an increased ability of the fetal pancreas to produce insulin during gestation (14) together with the direct respond to maternal factors which also enhances fetal insulin secretion (15,16).

Plasma levels of inflammatory markers are elevated in obese pregnant women. C-reactive protein (CRP) (13,17), and interleukin-6 (IL-6) (13,18) levels are higher in plasma of obese mothers compared to lean mothers at the end of gestation. These studies demonstrated that pre-pregnant obesity exaggerates the degree of inflammation, although pregnancy itself is considered already as a state of low-grade inflammation (19). Additionally, elevated levels of IL-6 were detected in umbilical cord plasma of infants born by obese mothers, which indicates that not only the obese mother but also the fetus is exposed to an inflammatory environment (13).

Imbalance in lipid metabolism is characteristic for obese patients and was also observed in obese pregnant women during pregnancy and at the time of delivery (term) (Figure 1). In first trimester of pregnancy total cholesterol, low density lipoprotein cholesterol (LDL-C) and triglycerides (TG) were significantly higher and high density lipoprotein cholesterol (HDL-C) was significantly lower in overweight/obese women when compared to normal weight women (20). Maternal serum total cholesterol, TG, LDL-C, and HDL-C increase significantly throughout gestation in lean and obese pregnancies (21). In the second trimester total cholesterol was significantly lower in obese and HDL-C showed a tendency to be reduced in obese in comparison to lean women. LDL-C and TG were similar in lean and overweight/obese in second trimester (20). The rate of change for total cholesterol and LDL-C is lower in overweight/obese women later in pregnancy

and resulted in significantly lower levels in the last trimester of pregnancy. The trajectories of HDL-C and TG did not differ in lean and obese women and HDL-C is lower in obese women around term (7,21,22). However, plasma TG levels are elevated in obese women in the third trimester of pregnancy around term (7,22,23). Some studies also examined lipid levels in cord blood of neonates born by obese mothers and found that cholesterol was decreased in cord blood when the mother was obese (24). Among other lipid parameters such as free fatty acids (FFA) and TG no differences in cord blood were found between lean and obese women (24,25) and there is no correlation between maternal plasma and cord blood FFA (25).

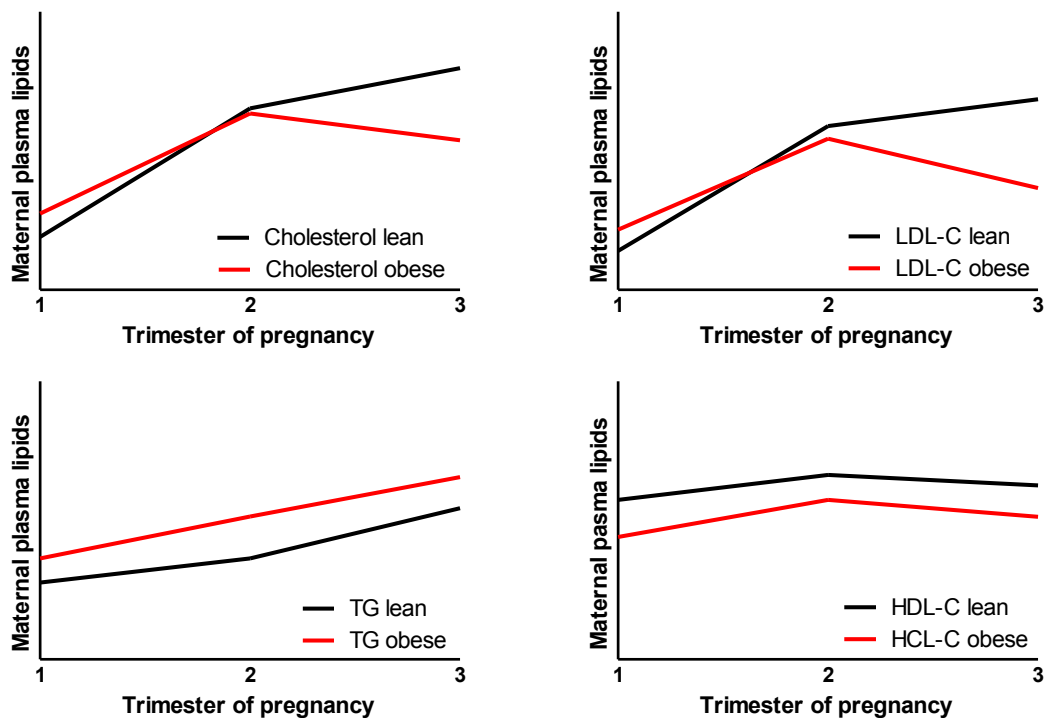


Figure 1: Changes in maternal plasma lipids throughout pregnancy in lean and obese women. Trajectories of changes in maternal plasma cholesterol, low density lipoprotein cholesterol (LDL-C), triglycerides (TG) and high density lipoprotein cholesterol (HDL-C) during lean and obese pregnancies are shown schematically.

In summary, the metabolic changes and distinct inflammatory environment of obese pregnancies impact the fetal development already *in utero*, as seen by elevated fat mass of the offspring at birth and is a continued later in life since these children are at a higher risk to become obese. These findings support the hypothesis of fetal programming which assumes that changes in maternal metabolism influences infants outcome already *in utero* (13,26). However, the growing fetus is highly dependent on the temporal changes of maternal physiology e.g. glucose, amino acid, and fatty acid supply which represents an additional regulative factor for the nutritional demand of the unborn. In particular, alterations in maternal lipid metabolism are specifically pronounced in pregnancy and therefore probably of particular importance for fetal growth (27).

1.2 Lipid metabolism in uncomplicated pregnancy

1.2.1 Lipids and fatty acids and their importance for fetal development

Fatty acids (FAs) are chemically composed of a carbon chain with a carboxyl group on one end. Depending on the number of carbon atoms, FAs are grouped into short-chain (≤ 5 carbons), medium-chain (6 – 12 carbons), long-chain (13 - 21 carbons), and very-long-chain fatty acids (≥ 22 carbons). Saturated FAs carry only single bounds, unsaturated FAs can have one or multiple double bonds between the carbon atoms. Two essential fatty acids exist, which cannot be synthesized by the human body and have to be taken up by the diet. Essential FAs are α -linolenic acid (ALA, 18:3 n3) with the first double bond starting at the omega-3 or third carbon from the methyl end of the carbon chain and linoleic acid (LA, 18:2 n6) with the first double bond staring at the omega-6 or sixth carbon of the carbon chain. Fatty acids are crucial for cellular integrity, and function. Fatty acids are part of phospholipids (PLs) and therefore integral components of all cellular membranes. Membrane expansion with the aid of PLs takes place during cell growth and division.

For fetal development, PLs are important for synthesis of lipoproteins, bile, and surfactant, respectively (28). Esterification of FAs to glycerol generates TGs, which serve as energy source and are the primary storage form of lipids in human adipose tissue. Lipid content in fetal adipose tissue increases from 0.5 – 16% starting at 12 weeks of gestation to term. Moreover, the FA content in fetal adipose tissue processes, from

gestational week 25 to term. For instance, saturated and monounsaturated FAs increase significantly from approximately 80 to 300 mg/g and 80 to 230 mg/g, respectively (mg FA/g wet weight adipose tissue). The amount of omega-3 and omega-6 FAs per gram wet weight of adipose tissue is constant between week 25 and term. However, concomitant with fetal growth the increase in adipose tissue results also in accretion of omega-3 and omega-6 FAs (29). Saturated and monounsaturated FAs primarily accumulate in fetal adipose tissue. Whereas, omega-6 and omega-3 FAs, mainly arachidonic and docosahexaenoic acid (DHA), play a prominent role also in brain and liver lipids (30,31). In particular, for fetal brain development sufficient amounts of DHA are crucial (32).

1.2.2 Fat accumulation in maternal depots

In the first and second trimester of pregnancy, maternal elevated food intake is combined with higher insulin levels and normal (33) or slightly decreased (34) insulin sensitivity. As a result excessive weight gain and body fat accumulation takes place during this period. Fatty acids and break-down products, originating of lipid enriched meals, are absorbed by the intestine and TG-rich lipoproteins, such as very low density lipoproteins (VLDL), are assembled by the liver of the mother. Triglyceride-rich lipoproteins are transported within the systemic circulation to adipose tissues, where the lipoprotein lipase (LPL) hydrolyses TG. In response to high levels of insulin, LPL hydrolyses FFA from TG and enables FFA uptake and accumulation of excessive lipids in adipose tissue in the mother (33) (Figure 2).

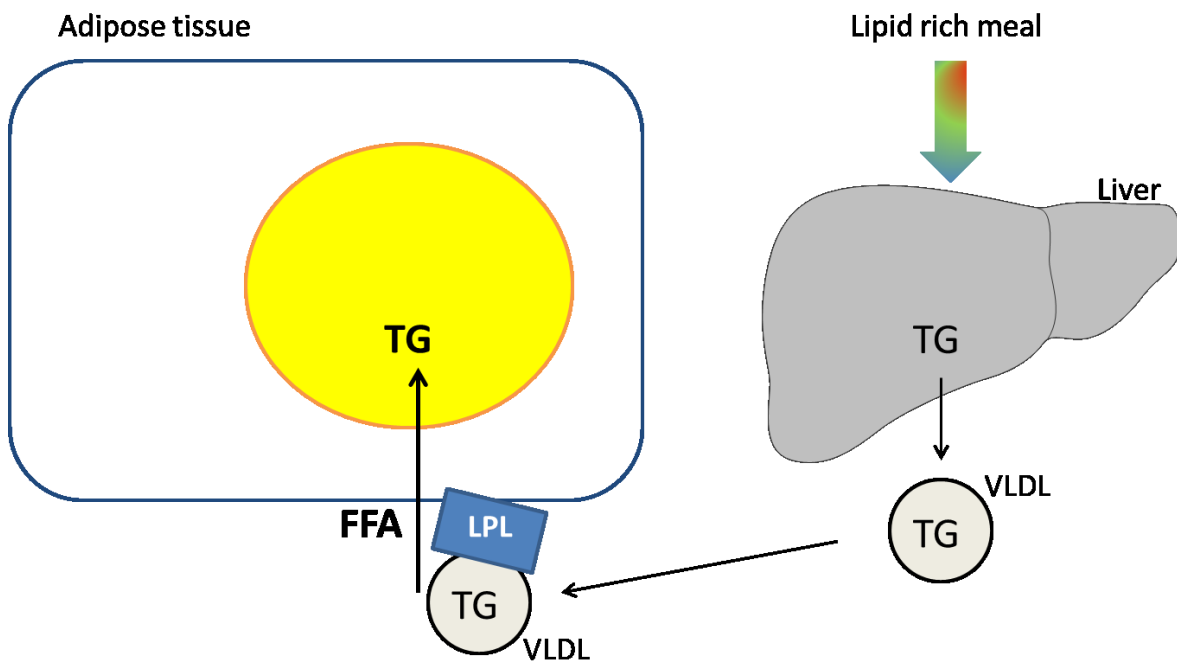


Figure 2: Fat accumulation in maternal adipose tissue in first and second trimester. Very low density lipoproteins (VLDL) are assembled in the liver after food intake. VLDL particles reach maternal adipose tissue with the blood stream. Lipoprotein lipase (LPL) hydrolyses triglycerides (TG) from VLDL particles and free fatty acids (FFA) are taken up. In adipocytes FFA are re-esterified into TG and stored.

1.2.3 Adipose tissue and lipid droplets

Adipose tissue is specialised to store lipids in large lipid droplets (LD). LDs are cell organelles responsible for the dynamic intracellular storage and hydrolysis of neutral lipids such as TG and cholesterol ester (CE) which both make up the core of LDs (35). Beside adipose tissue, LDs are found in most of the organs, like liver, heart, and muscles where they have functional roles in metabolism. Sub-cellular location sides of LDs are in close proximity to endoplasmic reticulum (ER) and mitochondria (36).

The LD surface is covered by a phospholipid (PL) monolayer and core lipids are protected from lipolytic enzymes by several regulatory proteins (37). These proteins belong to the perilipin protein family consisting of five members, perilipin 1-5 (PLIN1-5) in human and mice, and are involved in the maintenance and control of LD size (37,38). In white adipose tissue (WAT), PLIN1 serves as a scaffolding protein at the LD surface mediating protein/protein interactions (39). If lipolysis is suppressed by insulin (basal conditions), comparative gene identification-58 (CGI-58) preferentially binds to un-phosphorylated PLIN1 (Figure 3A). Upon lipolytic stimulation such as fasting, PLIN1 is phosphorylated by protein kinase A which leads to the dissociation of CGI-58. Free CGI-58 protein interacts with the close to the LDs located adipose triglyceride lipase (ATGL) (40). CGI-58 itself is unable to hydrolyse TG but is necessary for maximal activation of TG lipolysis (41). This process initiates the first step in hydrolysis of TG, which are temporary stored in LDs (40) (Figure 3B). The products of the first step in TG lipolysis are FFA and diglycerides (DG) which are further hydrolysed by the hormone-sensitive lipase (HSL). Finally, the monoglyceride lipase (MGL) is involved in the last step of the lipolytic cascade, by producing FFA and glycerol (42). The lipolytic process in WAT is terminated when food intake leads to insulin secretion. Insulin signalling in WAT results in reduction of PLIN1 phosphorylation and ATGL mediated lipolysis (40).

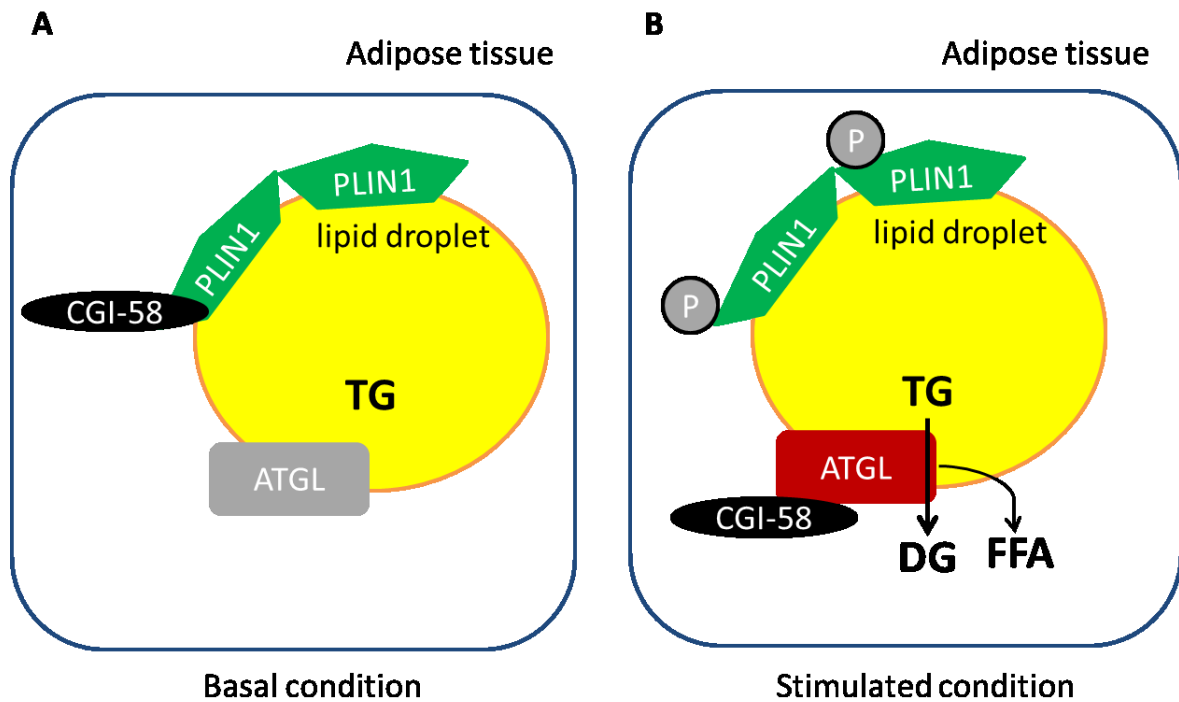


Figure 3: Lipid droplets and their crucial role in lipid storage. **A:** Perilipin 1 (PLIN1) sequesters comparative gene identification-58 (CGI-58) under basal conditions. Adipose triglyceride lipase (ATGL) mediated hydrolysis of triglycerides (TG) is prevented. **B:** Fasting stimulates PLIN1 phosphorylation (P) which further induces release of CGI-58. ATGL is activated by CGI-58 and hydrolysis of TG into free fatty acids (FFA) and diglycerides (DG) takes place.

1.2.4 Mobilization of fatty acids from maternal fat depots in the third trimester of pregnancy

The last trimester of pregnancy is characterized by increased maternal insulin resistance and hyperlipidemia, as a result of elevated lipid breakdown in the mother (43). Free fatty acids are released from LD in adipocytes into maternal plasma by the above described mechanism (1.2.2) and transported to the liver. In maternal liver re-esterification of FFA into TG takes place, followed by incorporation into VLDL. Triglyceride enriched lipoproteins, such as VLDL and LDL particles, and remaining FFA (1-3%) reach the placenta with the maternal blood stream (33).

1.3 The role of the placenta in lipid handling

1.3.1 Transport barriers in the human placenta

The human placenta is the physical barrier that separates the maternal and fetal blood circulation and simultaneously enables an exchange of nutrients and gases between mother and fetus. The human placenta is organized in 10 - 40 cotyledons, each cotyledon acts as a separate functional unit (44) (Figure 4).

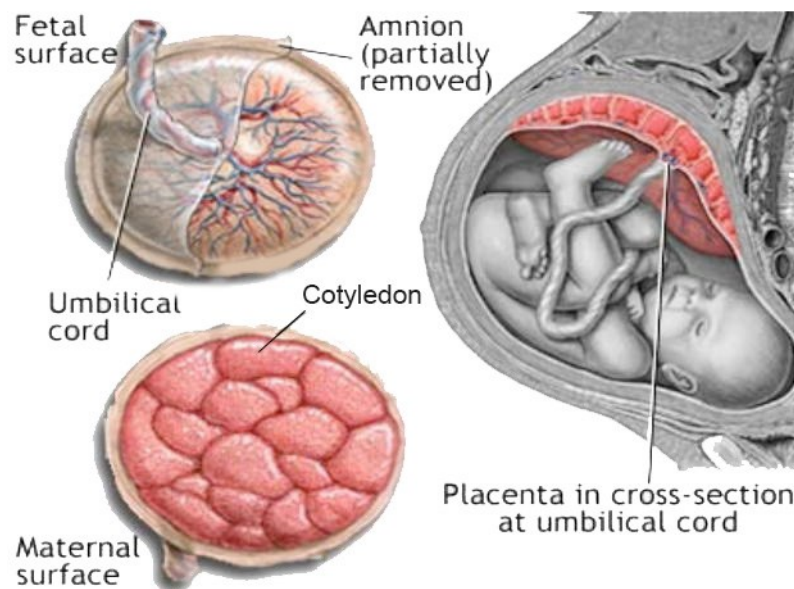


Figure 4: The anatomy of the human placenta. Chorionic arteries and veins, which are unified in the umbilical cord, cover the fetal surface of the human placenta. The amnion, an integral part of the placenta, covers closely the fetus and contains the amniotic fluid. The maternal side of the tissue is grouped in functional units (cotyledons). This picture was reproduced with permission from Barcena et al., *Transfusion* (2011) and modified (45).

The maternal blood circulation is in direct contact with the fetal-derived trophoblast cell layer. The trophoblasts resolve their lateral cell membrane between individual cells and form the continuous syncytiotrophoblast as pregnancy progresses (46). The syncytiotrophoblast layer is structured in the microvillous plasma membrane (MVM), facing the maternal blood, and the basal membrane (BM) which orientates towards the

fetal endothelial capillaries. The fetal endothelial capillaries have wide spaces between cells, which allow diffusion of substances with a molecular weight smaller than 1500 Da. The exchange of nutrients, gases, and waste products between mother and fetus takes place at the border of MVM, BM, and placental fetal endothelium (Figure 5). Small lipophilic molecules are able to rapidly pass the placental cell barriers by simple diffusion which is dependent on the concentration gradient of the molecule and the blood flow rate in maternal and fetal circulation. The transport of hydrophilic molecules is more complex and involves carrier mediated transport routes across the MVM and BM. Carrier proteins can facilitate diffusion, work as exchange transporters, or actively transport substances. These transport mechanisms depend on number, affinity, activity and localisation of the receptors or carrier proteins in the plasma membrane (47).

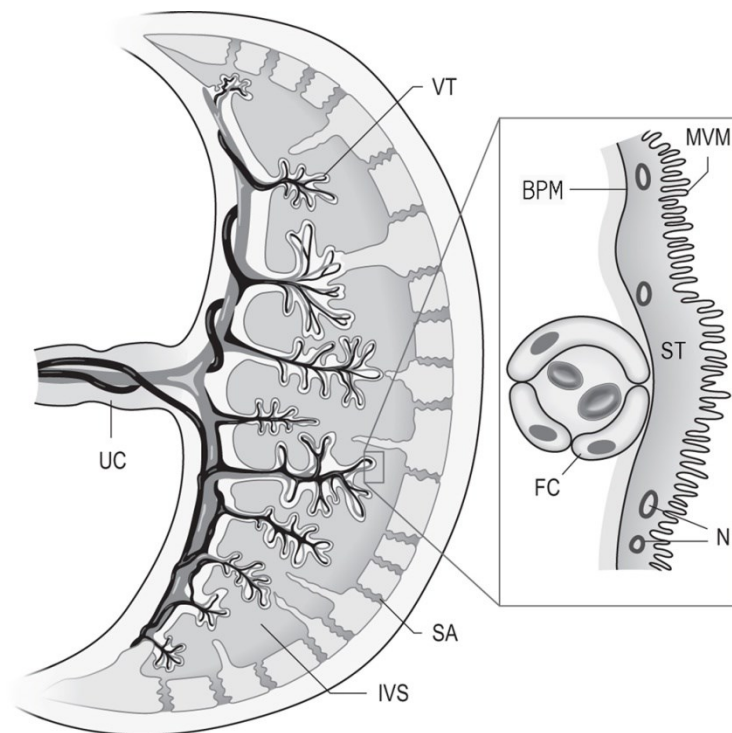


Figure 5: Cross-section of the human placenta. Maternal blood enters the intervillous space (IVS) through the spiral artery (SA). The villous tree (VT) bathes in maternal blood and is served by fetal capillaries which reunite in the umbilical cord (UC). The insert shows the placental barrier at term, which consists of the fused syncytiotrophoblast (ST) and its nuclei (N). Endothelial cells form the fetal capillary (FC). The maternal circulation is in contact with the microvillous membrane (MVM) of the syncytiotrophoblast. The basal membrane (BPM) of the syncytiotrophoblast is in close proximity to the fetal capillaries.

This figure was reproduced with permission from Gaccioli et al., *J Dev Orig Health Dis.* (2013) (48).

1.3.2 Models for studying nutrient and substance transfer across the human placenta

The human placenta is a multicellular and complex organ, which fulfils a variety of essential transport functions in order to guarantee adequate fetal organ development and growth. In the past, numerous *in vitro* and *ex vivo* models to study placental transport mechanisms were established and tested. Classical uptake and efflux studies were performed in primary trophoblast cells (23,49), primary endothelial cells (50), and tissue explants (49) all isolated from term placentas. Characterized cell lines, like the choriochorinoma cell line BeWo, were often used in uptake (51) and transport studies (52) as a model for placental cells.

Recently, the importance of a preferably intact cellular structure closest to placental tissue for transport studies receives more and more attention. Thus, a number of different co-culture systems using placental derived cell lines (53-55) were introduced in order to mimic the multicellular placental tissue. However, the most convincing approach for examining placental transport mechanisms from the mother to the fetus and *vice versa* represents the *ex-vivo* placental perfusion method, because the intact and vital tissue is used. The *ex-vivo* perfusion method was first described by Panigel in 1962 (56) and was further developed and adapted by several research groups all over the world. With the re-establishment of the fetal and maternal systemic circulations right after delivery of the placenta – the transfer of liquid soluble substances from the maternal to the fetal circulation (or fetal to maternal) can be studied. A variety of substance classes, gases and specific molecules, all of which are important for fetal growth and development, like oxygen (57), glucose (58), lactate (59), fatty acids (60), amino acids (61), and immunoglobulin G (IgG) (62) were examined. Furthermore, the materno-fetal transfer of drugs (63,64), chemicals in convenience goods (65,66), and nano-materials (67,68) were investigated by the *ex-vivo* perfusion method. This sophisticated but powerful *ex-vivo* method allows to study transport processes in the intact and viable

human placenta under experimental conditions as close as possible to the *in vivo* situation.

1.3.3 Current understanding of cellular fatty acid transport mechanisms

In the past decades numerous studies in a variety of model systems were performed in order to understand cellular FA uptake and efflux mechanisms. The fatty acid transport protein family (FATP) consists of six members, FATP1 – 6 (69) and facilitates cellular long chain fatty acid (LCFA) uptake. In humans FATPs are expressed in a tissue specific manner (70). These integral membrane proteins possess a single trans-membrane domain and multiple domains connected with the inner plasma membrane (69). The first family-member identified was FATP1 and is the main FATP expressed in adipose tissue (71). On cellular level FATP1 is also present in small vesicles in the cytoplasm of adipocytes (69). Upon insulin stimulation, FATP1 containing vesicles move towards the plasma membrane, thereby FATP1 is integrated into the bilayer. This change in the localization of FATP1 simultaneously increases FA uptake in adipocytes (72). Moreover, FATPs are able to form acyl-FAs concomitantly with FA uptake (69). This was demonstrated by heterologous over-expression of FATP1 in COS cells which resulted in elevated FA-acyl-CoA synthase activity (73). When FATP1 was knocked out in adipocytes of mice, FA uptake was reduced even in insulin stimulated cells (74,75). In contrast to FATP1, FATP4 did not show any similar function as seen in the FA-uptake of mouse adipocytes, suggesting that FATP4 has a different role than FATP1. FATP4 is supposed to act as an intracellular acyl-CoA synthase rather than being directly involved in FA translocation across the plasma membrane (74). However, the exact mechanism by which FATPs facilitate FA uptake is unknown. One theory is that LCFA are incorporated into specialized membrane domains, also known as lipid rafts. Thereafter, FATPs facilitate acyl-fatty acid formation, thereby enabling FA release from lipid rafts (69).

Lipid rafts together with the structure protein caveolin-1 form microdomains in the plasma membrane, which are termed caveolae. In the region of caveolae another protein important for FA uptake, the fatty acid translocase (FAT or CD36), is present and contributes to LCFA uptake (76). In caveolin-1 knock-out mice no caveolae are found in a variety of tissues apart from muscles and these mice had reduced capability for FFA

uptake into adipose tissue (77). Caveolin-1 seems to be important for the translocation of CD36 to the plasma membrane, and therefore for FA uptake (76).

The efflux mechanisms by which FA are released from cells is poorly understood. In mouse adipocytes, lipolysis of intracellular lipids and FA-release was not altered when FATP1 and FATP4 were knocked down. These results suggest that FA efflux is independent of FATP1 and FATP4 in this model (74). Recently, Henkin et al. (78) inhibited FA efflux from mouse adipocytes without affecting intracellular lipolysis or glycerol release. This study discussed two possible candidates for FA-efflux in adipocytes. First, the organic anion transport protein (OATP/SLC21), which mediates uptake and efflux of hydrophilic compounds such as bile acid, hormones, drugs or eicosanoids. Second, the ATP-binding cassette (ABC)-transporters are well known to be involved in lipid efflux for instance of phospholipids or cholesterol and therefore likely candidate proteins for cellular FA-efflux. It has been stated that FATPs only work unidirectional, and it is very unlikely that the same FA-transporters also enable FA-efflux. Henkin and colleagues found that inhibition of FA-efflux in adipocytes does not influence glycerol release and leads to intracellular FA-accumulation suggesting a protein mediated FA-efflux in by a not yet identified transporter (78).

1.3.4 One model for fatty acid handling in the human placenta

Fetal growth is highest in last trimester of pregnancy and accompanied by an enhanced demand of nutrients. Hence, the mother provides elevated levels of serum lipids and other nutrients for the supply of the fetus. The current knowledge about FA-transfer from the mother to the fetus is summarized in Figure 6 and was recently reviewed (27,79). In maternal plasma, lipoprotein particles enriched in TGs are the main source for FAs. Only 1-3% of FAs in plasma are not esterified and contribute to the free fatty acid (FFA) lipid fraction. Fatty acids can pass the placenta only in their free or non-esterified form (FFA) (27). Accordingly, two possible mechanisms for FA-uptake into the placenta were discussed in the past. First, TG enriched lipoprotein particles are taken up via VLDL-receptor (80) or the LDL-receptor (81), followed by further processing and hydrolysis of lipoproteins. Second, at the MVM hydrolysis of lipoproteins with the aid of lipases such as lipoprotein lipase (LPL) (22), endothelial lipase (EL) (82), or phospholipase A2 (83) takes place. The hydrolysed FFAs are subsequently taken up by the

syncytiotrophoblast via a receptor/transporter facilitated mechanism (22,84-86) In placental tissue FATP1, FATP6, FATP4, CD36 and plasma membrane fatty acid binding protein (FABPpm) mRNA was detected (86). In addition, CD36 and FATP4 protein expression was found in placental term tissue (22). The G protein-coupled receptor GPR120, as an omega-3 FA receptor, mediating potent anti-inflammatory and insulin sensitizing effects, was identified on the MVM of the placenta (87). Although, several transporters responsible for placental FA-uptake are described, the exact mechanisms by which specifically FAs are taken up, are still under debate.

However, FATPs, for instance FATP1, facilitate FA uptake and simultaneously act as very long chain acyl-CoA synthetase (73). The acyl-CoA synthetase activity of FATP1 or the cytoplasmic acyl-CoA synthetases (88) activate FA by formation of FA-acyl-CoA. Activated FA-acyl-CoA can bind to fatty acid binding proteins (FABP) which guide them from the MVM of the trophoblast cell towards the BM. Several members of the FABP family, like FABP1, FABP3, FABP4, FABP5, and FABP7 were detected in the human placenta on mRNA (22,23,86) and protein (22) level. FA-efflux, at the BM of the syncytiotrophoblast, might be also facilitated by membrane transporters in order to provide FAs for the fetus (discussed in chapter 1.3.3). In isolated BM preparations from placental tissue, FATP4 protein was found to be expressed (22) but it is uncertain whether FATPs are involved in cellular FA-efflux or solely in FA-uptake (78).

Beside the transfer towards the fetus, FAs can also be further processed by the placenta for generating energy by β -oxidation. Two enzymes, involved in the process of β -oxidation, long chain 3-hydroxyacyl-CoA dehydrogenase and short chain 3-hydroxyacyl-CoA dehydrogenase were shown to be active in the human placenta (89). Excessive FAs can be esterified into TGs and further stored in LDs (23,84,90). In addition to FA-uptake and storage, placental trophoblast cells are also active in synthesis of FAs up to a chain length of 18 carbons. Furthermore, the newly synthesized FAs as well as previously stored FAs can be released from the trophoblast cells (91). Moreover, the placenta has the capability to convert FAs, in particular arachidonic acid, into generating signalling lipids like the vasodilator prostacyclin PGI_2 and the vasoconstrictor thromboxane A_2 (92). Prostacyclins and thromboxanes are involved in regulation of the vascular tone of the placenta (92,93) and for example in the onset of labour (94).

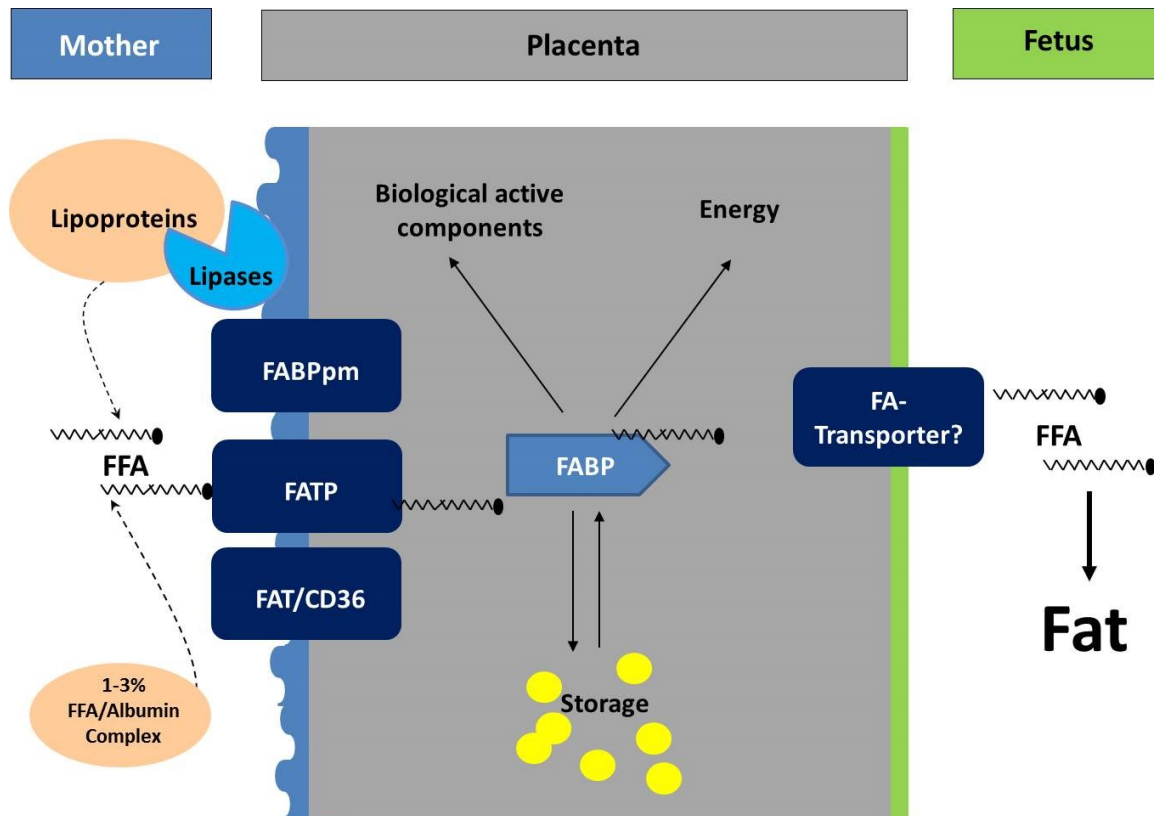


Figure 6: Current model of FA transfer across the human placenta. In the maternal circulation FFA are either complexed to albumin (1-3%) or incorporated into lipoproteins (97-99%). Placental lipases hydrolyse FFA from lipoproteins thereby enabling their uptake into placental tissue via different FA transport proteins, such as FA transport protein (FATP), plasma membrane FA binding protein (FABPpm) or FA translocase (FAT/CD36). Intracellular FFAs can be stored in placental tissue, used as energy source or signalling molecules. Within the placenta FFA are bound to FABP for their further transport towards the fetal circulation. This picture was adapted from Herrera and Desoye, *Horm. Mol. Biol. Clin. Investig.* (2015) (27).

1.3.5 Fatty acids originating from the mother and their fate in the placenta

In pregnancy near term, the plasma concentrations of FFAs are markedly increased compared to non-pregnant women. In maternal plasma and blood in intervillous space concentrations of saturated, monounsaturated and the two essential FA, α -linolenic (n3

or omega-3 series of FA) and linoleic acid (n6 or omega-6 series) are similar. However, cord blood concentrations of these FFAs are two to five -fold lower than in the mother. In contrast, concentrations of non-esterified arachidonic acid and DHA are comparable in cord blood and maternal blood. Interestingly, the concentrations of these two long-chain polyunsaturated fatty acids (LCPUFAs) are threefold higher in the placental intervillous space compared to maternal blood. One possible explanation is local activity of phospholipase A2 at the syncytiotrophoblast layer which preferentially hydrolyses PL and releases arachidonic acid (92). High levels of arachidonic acid and DHA in the intervillous space might build up a concentration gradient for these FAs which would drive their transfer from the mother/placenta compartment towards the fetus. However, knowledge about the fate of FA in the placenta is still poor although several groups investigated FA-uptake, metabolism, or transfer *in vivo* and *in vitro* in placental tissue and on cellular level. Since DHA is crucial for fetal brain and retinal development in the last trimester of pregnancy (95), many studies focused on DHA and other LCPUFAs.

In the *in vivo* study of Larque et al. (96), mothers were supplemented with DHA and eicosapentaenoic acid (EPA) in 2nd half of pregnancy. Supplementation of the mother with DHA did not change FA transporters (FATPs) expression, but placental total phospholipids were enriched in the DHA receiving group (96). In another *in vivo* study, women were supplemented with a combination of DHA and EPA in 2nd half of pregnancy. DHA levels in placentas of the DHA and EPA group were significantly increased compared to the control group. DHA levels in cord blood erythrocytes correlate positively with placental DHA levels (97). When women received stable isotope (¹³C) labelled FFAs 12 hours prior caesarean section, the majority of the labelled FAs were esterified into placental PL fraction. Some ¹³C tracer was also found in the TG fraction but not in CE in the tissue. Interestingly, the ratio between cord blood and maternal plasma for tracer concentrations of DHA was three times higher than for palmitic, oleic or linoleic acid suggesting an enrichment of DHA in fetal cord blood (98). The prevalence of DHA transfer from the mother to the fetus is in line with previously published *ex-vivo* perfusion experiments where DHA transfer across the placenta was higher than the transfer for linoleic, oleic or arachidonic acid (99,100).

Recently, a class of lipid mediators, originating from n3 LCPUFAs, was described. Lipid mediators, for instance resolvins, protectins and maresines are involved in promotion of inflammatory homeostasis. Two precursors of these lipid mediators, are 18-hydroxyeicosapentaenoic acid (18-HEPE) modified EPA and 17-hydroxydocosahexaenoic acid (17-HDHA) originated from DHA. When women were supplemented with n3 LCPUFAs during pregnancy, the concentrations of 17-HDHA and 18-HEPE were significantly increased in placenta tissue compared to the control group. Furthermore, 17-HDHA concentrations correlated positively with their corresponding resolvins and protectins in the placenta (97). Beside its crucial role for fetal development, DHA might have additional function in placental inflammatory homeostasis. However, a direct effect of 17-HDHA or 18-HEPE driven resolvins and protectins on placental cytokine expression in normal pregnancies was not documented until now.

1.4 Maternal obesity and its impact on the placenta and the unborn

1.4.1 Maternal obesity impacts placental lipid metabolism

Numerous studies provided evidence that the imbalance in maternal lipid metabolism in obese pregnancies affects the fetuses already *in utero*. Thus the role of the placenta, which is a fetal organ, in providing lipids and other nutrients is of particular interest. Recently, Saben and colleagues (24) examined placental tissue and found that total lipids were increased by 50% in placentas of obese women in comparison to placentas of lean women. Furthermore, a lipid droplet-associated protein (CIDE-A) was significantly increased in placentas of these obese women. Moreover, one important inhibitor of the LPL, termed ANGPTL4, was significantly decreased in placentas of obese women (24). In line with this result, increased LPL activity was found in placentas of obese women (22). These results suggest that maternal obesity leads to increased placental LPL activity which would provide more FFAs for placental uptake. Thus may result in first line in increased lipid accumulation in the placenta of obese women.

As previously discussed (1.3.4), a bundle of receptors and transporters are considered to be involved in the FA-uptake process and several studies tried to disclose the impact of maternal obesity on these factors. Two studies did not find altered FABP4 and FABP5 mRNA and protein expression (23,101) or FATP4 and FABPpm mRNA expression (101) in

obese placentas. In contrast, in placentas of obese women accompanied by GDM, FABP4 and FABP5 mRNA and FABP4 protein expression was higher (23). In MVM and BM, isolated from term placentas, the FATP2 protein expression correlated with maternal pre-pregnancy BMI in the BM fraction but not in the MVM fraction. However, neither CD36 nor FATP4 protein expression in MVM or BM correlated with maternal BMI (25). In another study, placental mRNA and protein expression of FATP4, FABP1 and protein levels of FABP3 were reduced when the mother was obese (22). Moreover, in placentas of male offspring FABP5 mRNA was lower in obese compared to lean pregnancies (101). Expression of CD36 was found to be reduced in male offspring of obese mothers (101), but CD36 was up-regulated on mRNA and protein level in placentas of obese women not taken into account fetal sex (22). Due to the diverse results regarding FA-transport and binding proteins the impact of maternal obesity alone on these molecules is unclear. It cannot be excluded that obesity in combination with GDM alters expression levels FA-transport or binding proteins. However, expression levels of FA-transporters alone cannot explain the consequences of maternal obesity for placental FA transfer. Therefore, tracer experiments examining direct FA transfer are needed, in order to identify physiological relevant changes in placentas of obese women.

1.4.2 Maternal obesity and associations with placental inflammation

In different hosts and with different approaches like in total placental tissue and different isolated primary cell type models, metabolic and inflammatory pathways have been investigated which are all known to be altered by obesity in humans. In placentas of obese ewes, as one used animal model, enhanced levels of inflammatory cytokines and toll like receptors were determined (102). Inflammatory markers, for instance IL-6, TNF- α , IL-1, and MCP1 (monocyte chemoattractant protein1, facilitating monocyte adhesion and infiltration of inflamed tissue), were increased on mRNA levels in macrophages originating from placentas of obese women (16). Accordingly, mRNA of IL-6, IL-8 and TNF- α increased in a dose dependent manner in isolated trophoblast cells treated with palmitic acid. Furthermore, the palmitic acid treatment increased toll like receptor 4 (TLR4) mRNA and stimulated cytokine release into culture medium (103). Saturated FAs are able to elevate moderately inflammatory cytokines in the placenta (103), a condition

that was previously described as low-grade chronic inflammation in pregnancies of obese women (16).

When obese women received n3 FA (DHA and EPA) supplementation during pregnancy (10 week – term), inflammation determined by plasma CRP levels was reduced at time of delivery. Furthermore, mRNA expression of pro-inflammatory cytokines, IL-6, IL-8, and TNF- α as well as TLR4 was reduced in placental tissue and biopsies of adipose tissue obtained from the mother during caesarean section. In trophoblasts isolated from placentas of obese women mRNA expression of TLR4 and the pro-inflammatory cytokines IL-6 and IL-8 was stimulated by palmitic acid. The up-regulation was diminished when trophoblast cells were treated with a combination of palmitic acid and EPA or DHA, accounting for the anti-inflammatory properties of EPA and DHA in trophoblast cells (104).

In summary, imbalance of the lipid metabolism in the obese mother is accompanied by systemic low-grade inflammation. This low-grade inflammatory environment transfers also on the placenta where pro-inflammatory cytokine expression is increased when the mother was obese before pregnancy. Furthermore, the saturated palmitic acid is able to trigger an inflammatory response in placental cells, which might be diminished by long-chain polyunsaturated FAs such as DHA. Therefore, the imbalance of maternal and placental lipids such as saturated and polyunsaturated FAs may contribute to the unfavourable *in utero* environment of obese pregnancies.

1.4.3 Consequences for the unborn and offspring later in life

Although many recent studies demonstrating that maternal obesity is relevant to a variety of adaptations in the placenta and many lead to impairments in fetal growth and development, a direct causality is still missing. However, a broad range of associations are known today, connecting offspring health with its *in utero* environment of overweight or obese mothers.

Children, at 4 years of age, who were born to obese mothers have a 2.7 times higher risk of being obese than children who were born to lean mothers (10). More particularly, maternal obesity is associated with increased neonatal fat mass (12). Importantly, significant sexual dimorphisms during pregnancy are seen in girls and boys of obese

mothers. Boys, born from obese mothers have elevated body fat starting at 2 years of age which becomes significantly increased between 4 and 6 years. In contrast, girls of obese mothers have higher percentage of body fat than girls from lean mothers in the first year of life. Between 2 and 5 years of age there is no difference in body fat in girls from lean and obese mothers. However, at 6 years of age the percentage of body fat is significantly increased in girls of obese mothers (105). It is obvious that maternal obesity carries a high level of risk for childhood obesity with different trajectories for boys and girls. Additionally, these sexual dimorphisms are prolonged also later in life, shown in pubertal development of obese girls and boys (106).

Elevated fat depots in the fetus are likely to be associated with metabolic changes as well. For instance, neutral lipids such as cholesterol and LDL cholesterol were found to be significantly elevated in cord blood of obese pregnancies (22). Cord blood levels of the inflammatory markers, IL-6 (13,24) and TNF- α (24), were found to be increased in obese pregnancies. Furthermore, cord blood leptin was significantly higher in obese than in normal weight pregnancies (13,24). In pregnancies accompanied by gestational diabetes mellitus, cord blood leptin levels were positive correlated with fetal birth weight and fat mass (107). In addition, fetuses of obese mothers are more resistant to insulin and the insulin resistance is associated with fetal adiposity (13). Among children and adolescent who are obese the prevalence for impaired glucose tolerance is 24 and 21%, respectively (108).

All together, these results strengthen the concept that maternal pre-pregnancy obesity is strongly associated with infant and childhood adiposity including metabolic changes towards an obese phenotype. Furthermore, infant and childhood obesity is a risk factor for adult adiposity (109) which is known to be related to insulin resistance, type 2 diabetes, cardiovascular disease or non-alcoholic fatty liver disease (110).

2 Hypothesis and Objectives

Maternal pre-pregnant obesity has been shown to be related to several metabolic changes in the placenta and the fetus. The aim of this thesis was to examine the FA-uptake in placentas from obese compared to lean women and whether maternal dyslipidemia, hyperinsulinemia and inflammatory conditions impact placental FA-metabolism. The hypothesis was that dysregulated placental FA-uptake may also lead to altered FA-transport across the placenta thereby affecting fetal development and growth. These metabolic changes during pregnancy may provoke long-term health consequences for the offspring later in life already *in utero*.

Objectives

To establish a protocol for *ex-vivo* placental perfusion experiments, in order to study maternal to fetal transfer of ^{13}C labelled fatty acids.

To determine maternal to fetal free fatty acids transfer in placentas of obese women ($\text{BMI} \geq 30 \text{ kg/m}^2$) compared to lean women ($\text{BMI} \leq 25 \text{ kg/m}^2$).

To understand how exogenously provided fatty acids are distributed within the placental tissue, and if or how, these fatty acids are forwarded to the fetal circulation.

To examine the impact of maternal pre-pregnancy obesity on placental genes and proteins all of which are involved in fatty acid and lipid uptake, transport, and metabolism.

3 Materials and Methods

3.1 Study population and ethics

3.1.1 Subjects for the maternal to fetal fatty acid transfer study

The ethics committee of the Medical University of Graz approved this placenta perfusion study (study number: 24-529 ex 11/12). Pregnant women, undergoing elective caesarean section at the Department of Obstetrics and Gynecology of the Medical University of Graz, were asked by the responsible study nurse to participate in the placenta perfusion study and signed a written informed consent. Subjects with pre-eclampsia, existing diabetes and gestational diabetes were excluded from the study. The study population was designed to collect placentas from lean women with a pre-pregnancy BMI 18.5 - 25 kg/m² and obese women with a pre-pregnancy BMI $\geq$ 30 kg/m².

3.1.2 Cohort of the placental lipid metabolism study

Women with uncomplicated, singleton pregnancy, who gave birth in the Metrohealth Medical Center, Cleveland, US, were recruited prior to elective caesarean section between 38 and 40 week of gestation. This study was approved by the Institutional Review Board of Metrohealth Medical Center, Case Western Reserve University. All women signed a written informed consent prior placental tissue and blood was collected. Obesity was defined by maternal pre-pregnancy BMI $\geq$ 30 kg/m². In the control group the lean pre-pregnancy BMI was 19.4 - 25 kg/m². Cases with gestational diabetes or existing diabetes were excluded. Following double-clamping of the umbilical cord, approximately 1 cm³ fragments of placental villous tissue were collected and snap-frozen in liquid nitrogen. Maternal clinical and metabolic characteristics were obtained from medical records. Placental biopsies and data collected in Metrohealth Medical Center, Case Western Reserve University Cleveland, US, were kindly provided by Prof. Sylvie Hauguel-de Mouzon. The ethics committee of the Medical University of Graz approved the analysis of the placenta biopsies and data under the study number 25-401 ex 12/13.

3.2 Maternal to fetal fatty acid transfer

3.2.1 *Ex-vivo* perfusion of a single cotyledon of the human placenta

The *ex-vivo* perfusion system used in this study was adapted from the model previously described by Schneider et al. (111). A schematic diagram of the perfusion setting used in this study is presented in Figure 7.

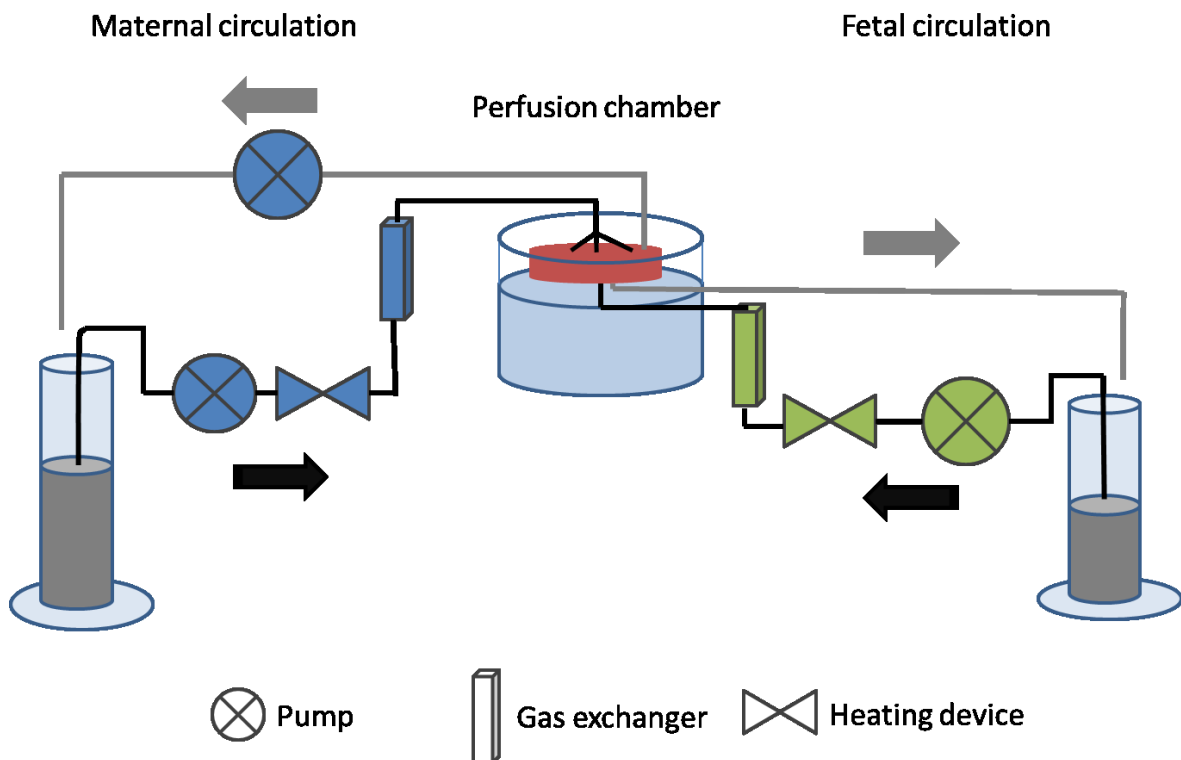


Figure 7: Schematic illustration of the human placenta *ex-vivo* perfusion system. The cannulated placenta cotyledon (in red) is placed in the pre-warmed (37°C) perfusion chamber. Medium from the fetal reservoir (right side, devices in green) is pumped through the heating device and gas exchanger (the fetal perfusate is gassed with 5% CO₂ in 95% N₂) and enters the placenta by the cannulated fetal artery on the chorionic plate (black arrow indicates flow direction). The backflow to the reservoir in the fetal circulation occurs passively via the corresponding fetal vein on the chorionic plate (grey arrow indicates flow direction). Medium from the maternal reservoir (left side, devices in blue) is pumped through the heating device and gas exchanger (the maternal perfusate is gassed with 5% CO₂, 20% O₂, 75% N₂) and enters the placental intervillous space through three cannulas which mimic the spiral arteries (black arrow indicates flow direction). The

maternal perfusate is pumped back into the maternal reservoir from the placenta (grey arrow indicates flow direction).

For all experiments perfusion medium, containing phenol red free DMEM (Gibco, UK) and Earl's buffer (6.8 g/l NaCl, 0.4 g/l KCl, 0.14 g/l NaH_2PO_4 , 0.2 g/l $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.2 g/l CaCl_2 , 2.2 g/l NaHCO_3 , all from Merck, Darmstadt, Germany) mixed in a 3:1 ratio was used. Amoxicillin (250 mg/l, Sigma-Aldrich, Steinheim, Germany), 10 g/l dextran FP40 (Serva, Heidelberg, Germany), a total concentration of 2 g/l glucose (Merck, Darmstadt, Germany) and 5 g/l bovine serum albumin essentially fatty acid free (BSA) (Sigma-Aldrich, Steinheim, Germany) were added.

The placenta was brought to the laboratory within 15 min after delivery of the placenta. A corresponding chorionic artery and vein pair supplying one intact cotyledon was cannulated and immediately flushed with pre-warmed (37°C) perfusion medium. The cannulated cotyledon and surrounding tissue was placed in the pre-warmed perfusion chamber within 40 min after delivery of placenta. The fetal artery cannula was connected to the fetal reservoir containing perfusion medium kept on 37°C in a water bath. The fetal circulation was conducted open and was gassed with 5% CO_2 in 95% N_2 through the gas exchanger (Living Systems, St. Albans, VT, US) during the whole experiment. A constant fetal artery inflow of 4 ml/min was generated by using a magnetic pump (Codan, Salzburg, Austria). Volume loss was monitored within the first 30 min, then every hour on fetal venous outflow. Only perfusion experiments with at least 95% fetal flow recovery, passed the pre-perfusion period checkpoint and were pursued. Fetal arterial pressure was recorded in line during the whole experiment by inserting a micro catheter pressure sensor (Millar, Houston, US) into fetal arterial cannula. Only pressure values < 65 mbar, in fetal artery, meets the quality criteria for continuing the experiment.

The maternal circulation was established within 30 min by inserting three cannulas into the intervillous space of the cotyledon. The flow rate in maternal circulation was set to 8 ml/min, and perfusion media was gassed with 5% CO_2 , 20% O_2 in 75% N_2 during the entire experiment as described above, unless otherwise stated. Antipyrine (Sigma-Aldrich, Steinheim, Germany) (100 µg/ml), a small lipophilic drug, was added to the maternal

reservoir in order to determine the overlapping exchange tissue area of the maternal and fetal circulation in each experiment. During the perfusion phase with antipyrine, both circulations were conducted in an open manner, and samples were collected at time point 0 min and after 10, 20 and 30 min from maternal artery and fetal vein outflow antipyrine measurements.

After establishing both circuits and perfusion of antipyrine, maternal reservoir was changed to perfusion medium (200 ml) containing ^{13}C labelled FFAs in a mixture reflecting maternal plasma TG composition as described by Haggarty et al. (99) (Table 1). FFAs were bound to 0.5% BSA which leads to a molar ratio of BSA to FFAs of 0.78. The maternal perfusate was recirculated (closed approach) during the entire experiment. Samples were collected at distinct time points 0, 10, 20, 30, 60, 90, 120, 150, and 180 min during perfusion from maternal arterial and venous cannula and from fetal venous outflow. All samples were stored at -80°C for further analysis. Figure 8 shows a schematic presentation of a representative perfusion experiment. At the end of the experiment, wet weight of the perfused cotyledon was determined and tissue samples were collected, snap frozen in liquid N_2 and stored at -80°C for further analysis.

Table 1: Composition of FFA mixture used for perfusion experiments

	Fatty acid	Concentration
^{13}C PA	16:0	37.2 $\mu\text{mol/l}$
^{13}C OA	18:1 n9	40.5 $\mu\text{mol/l}$
^{13}C LA	18:2 n6	16.9 $\mu\text{mol/l}$
^{13}C DHA	22:6 n3	0.3 $\mu\text{mol/l}$
AA	20:4 n6	0.8 $\mu\text{mol/l}$
EPA	20:5 n3	0.2 $\mu\text{mol/l}$
DH- γ -LNA	20:3 n6	0.9 $\mu\text{mol/l}$
α LNA	18:3 n3	1.0 $\mu\text{mol/l}$

Perfusion medium (200 ml), containing 0.5% BSA, was incubated with FFA over night at 37°C . PA: palmitic acid, OA: oleic acid, LA: linoleic acid, DHA: docosahexaenoic acid, AA: arachidonic acid, EPA: ecosapentaenoic acid, DH- γ -LNA: dihomo- γ -linolenic acid, α LNA: α -linolenic acid.

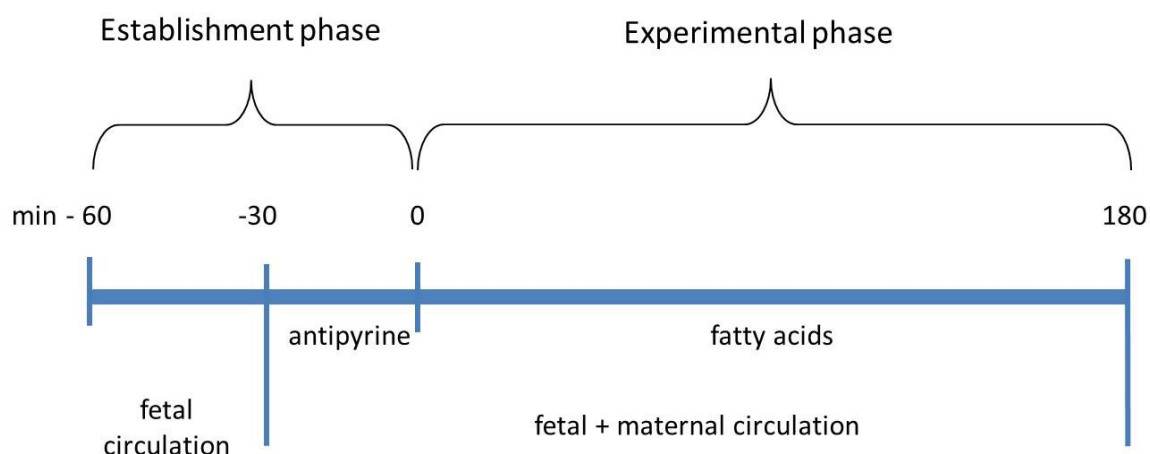


Figure 8: Schematic presentation of performed perfusion experiments. Representative perfusion experiment consists of an establishment phase in which the fetal and maternal circulation of the placenta are re-established after delivery. Transfer rate of antipyrine serves as a quality and normalization value of overlapping area of maternal and fetal circulations. In the experimental phase, FFAs were offered in the maternal circulation and FFA-transfer to the fetal circulation was determined by measuring FFA concentrations in fetal perfusates by GC-MS.

3.2.2 Antipyrine measurement by HPLC

Antipyrine concentrations were determined in maternal and fetal perfusates by HPLC in accordance with the protocol published by Annola et al. (112). Briefly, 100 μ l of perfusate was mixed with 100 μ l methanol (Sigma-Aldrich, Schnelldorf, Germany), vortexed and centrifuged at 12000 rpm for 15 min. Upper phase (150 μ l) was transferred to a new tube and mixed with 150 μ l acetonitrile (Sigma-Aldrich, Schnelldorf, Germany), vortexed and centrifuged. A standard curve with antipyrine concentrations from 5 μ mol/l to 1 mmol/l was prepared as described above. The clear supernatant was used for determination of antipyrine concentration by HPLC. The HPLC (Knauer, Berlin, Germany) was equipped with an aquasil 150x2.1 5 μ column (Thermo scientific, Waltham, MA, USA) and UV detector. 10 μ l sample or standard were injected and run at isocratic flow (0.2 ml/min) with 20 mM KH_2PO_4 (Merck, Darmstadt, Germany), mixed 1:1 with acetonitrile. Antipyrine peaks were detected in UV light at 255 nm and concentrations in maternal and

fetal perfusates were calculated. Antipyrine FM ratio for each experiment was calculated and only perfusion experiments with FM ratio ≥ 0.30 were accepted as successful.

3.2.3 Gas chromatography and mass spectrometry

All reagents were purchased from Sigma-Aldrich, Steinheim, Germany, unless otherwise stated. Total lipids were extracted as described by Matyash et al. (113). Briefly, internal standard C15:0 was added to 1 ml perfusion media. Total lipids were extracted in methanol/tert-butyl-methyl-ether/water (1.5/5/1.25). The organic phase was dried in a vacuum centrifuge. Lipids were dissolved in 1 ml CHCl_3 /methanol (1/1) and used for further analysis.

In the first set of experiments, free fatty acids were determined in total lipid extracts of perfusion media, by a Trace-DSQ system for gas chromatography – mass spectrometry (GC-MS) (Thermo Scientific, Waltham, US) on electron impact MS mode, as previously published by Fuchs et al. (114). FFA species, including ^{13}C labelled FFA, were detected in full mass scan and identified by retention time and mass. Areas under the curve were calculated by Xcalibur QuanBrowser. The ratio between the internal standard C15:0 (3.75 nmol/ml) and the FFA species in each sample were determined and concentrations were calculated.

In the second set of experiments, FFA in lipid extracts obtained from perfusion samples were determined by GC-MS (Agilent Technologies, Santa Clara, CA, US) on negative ion chemical ionization in selected-ion monitoring (SIM) mode. Areas under the curve were determined by Agilent Mass Hunter Software (Agilent Technologies, Santa Clara, CA, US) and FFA concentrations were quantified as described above.

3.2.4 Separation of lipid classes by thin layer chromatography followed by total fatty acid analysis

Thin layer chromatography (TLC) and gas chromatography (GC) were performed as described by Amusquivar et al. (115). Briefly, 100 μl internal standard mix ((2 mg/ml of 1,2 diheptadecanoyl-sn-glycero-3-phosphatidyl-choline (17:0, Lordan Fine Chemicals, Malmö, Sweden); 2 mg/ml triheptadecanoin (17:0, Lordan Fine Chemicals, Malmö, Sweden); 2 mg/ml heneicosanoic acid (21:0); cis-10-nonadecenoic acid (19:1); 2 mg/ml

cholesteryl heptadecanoate (17:0, Santa Cruz Biotechnologies, Dallas, USA) solved in chloroform/methanol (1/1)) was added to 2 ml of maternal or fetal perfusates and lipid extraction was performed as described above. After extraction, lipids were dissolved in 1 ml chloroform/methanol (1/1). Lipid extracts (0.6 ml) were used for separation on silicagel plates (Merck, Darmstadt, Germany) which were developed with heptane/diisopropyl ether/acetic acid (60/40/3). Separated lipids were visualised with 2',7'-dichlorofluorescein (Merck, Darmstadt, Germany) by using UV light. Bands corresponding to phospholipids (PL), non-esterified fatty acids (FFA), triglycerides (TG) and cholesterol esters (CE) were scraped from the plate and transferred into glass tubes containing 2 ml methanol/toluene (4/1) (Merck, Darmstadt, Germany). FA methyl esters were generated by adding 0.2 ml acetyl chloride (Merck, Darmstadt, Germany) and incubation for 2.5 hours at 80°C in a water bath. Tubes were brought to room temperature and 0.5 ml toluene and 5 ml 6% K₂CO₃ (Merck, Darmstadt, Germany) were added. After centrifugation, the upper phase was transferred into a glass vial and dried under nitrogen. FA methyl esters were dissolved in 40 µl toluene and analysis was performed on a Perkin-Elmer gas chromatograph (Autosystem, Norwalk, USA) with a flame ionization detector and a 30 m x 0.25 mm Omegawax capillary column (Sigma-Aldrich, Steinheim, Germany). Total FA in four lipid classes were identified and compared to known standards based on retention times. The ratio between the areas under the curve of internal standards and identified total FAs were computed, and FA concentrations were calculated.

3.3 Placental lipid uptake, metabolism and transfer

All methods of this chapter are published in the *International Journal of Obesity* (116).

3.3.1 Plasma assays

Plasma glucose and insulin concentrations were determined by glucose oxidase method (Yellow Springs, OH) and ELISA (EMD Millipore Corporation, Billerica, MA), respectively by the Metrohealth Medical Center (Case Western Reserve University, Cleveland, US). The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated by multiplying fasting plasma insulin (milliunits/l) by fasting plasma glucose ([mg/dl]/405) (117). Total cholesterol, non-esterified cholesterol, TGs, PLs and FFA were

measured using enzymatic reagents from Diasys (Holzheim, Germany) on an Olympus AU640 analyzer. Secondary standards from Roche Diagnostics (Mannheim, Germany) and Diasys (Holzheim, Germany), respectively were used for daily calibration. Esterified cholesterol was calculated as the difference between total and non-esterified cholesterol. The variation between days (coefficient of variation) was < 5% (118).

3.3.2 Triglyceride levels in placental tissue

Frozen placental tissue (50 mg) was mixed with 1 ml acetone to generate tissue homogenates. Homogenates were incubated on a shaking platform overnight in a tightly closed tube to extract total lipids. Lipid extracts were centrifuged at 13.000 rpm for 15 min and 5 µl supernatant was used for TG determination by an enzymatic kit (Diasys Diagnostic Systems, Holzheim, Germany) following the manufacturer's protocol.

3.3.3 RNA isolation

Placental tissue (50 – 100 mg) was homogenized in RLT lysis buffer (Qiagen, Hilden, Germany) by using precellys ceramic kit (PepLab, Erlangen, Germany) in the MagNA lyser system (Roche, Basel, Switzerland). Tissue homogenates were used to isolate RNA by RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

The quality of the RNA was assessed by Bioanalyzer (Agilent Technologies, Santa Clara, USA). Samples with RNA integrity number (RIN) above 7.0 were further processed for gene expression analysis.

3.3.4 mRNA analysis by nCounter system

For genomic conformation and analysis of small amounts of placental tissue a custom Code Set was designed at NanoString Technologies (NanoString Technologies, Seattle, WA). Hybridization of 100 ng total RNA of term placenta was performed in a proof of principle study at NanoString Headquarters in Seattle according to manufacturer's instructions on a NanoString nCounter Analysis system. Expression data were normalized using the nSolver 2.0 Analysis Software (NanoString Technologies, Seattle, WA, US).

3.3.5 Quantitative real time polymerase chain reaction

RNA (2 µg) was transcribed into cDNA by using Super Script II reverse transcriptase (Thermo scientific, Waltham, USA) and random hexamer primer (Thermo Scientific,

scientific, Waltham, USA) following the manufacturer's protocol. Quantitative real time polymerase chain reaction (qRT-PCR) was performed, using Taq man gene expression assays (Applied Biosystem, Darmstadt, Germany) as indicated in Table 2, according the manufacturer's instructions and a final cDNA concentration of 10 ng/assay (measured in triplicates). For the qRT-PCR expanding steps subsequent protocol was used on the AB7900 Cyciler (Applied Biosystem, Darmstadt, Germany): 1 cycle at 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. Target gene expression was normalized to TATA box binding protein (TBP, housekeeping gene) expression and $2^{-\Delta CT}$ values were used for data presentation and statistical analysis.

Table 2: Taq man gene expression assays (Applied Biosystem, Darmstadt, Germany)

Gene Symbol	Gene	Gene ID	Taq man assay
ABHD5	Abhydrolase domain containing 5, CGI-58	51099	Hs01104373_m1
PLIN1	Perilipin 1	5346	Hs00160173_m1
PLIN2	Perilipin 2, ADRP	123	Hs00605340_m1
PLIN3	Perilipin 3, TIP47	10226	Hs00998416_m1
PLIN4	Perilipin 4	729359	Hs00287411_m1
PLIN5	Perilipin 5, OXPAT	440503	Hs00965990_m1
TBP	TATA box binding protein	6908	Hs00427620_m1

3.3.6 Immunohistochemistry

Cryostat sections (5 µm) were used for expression and localisation of PLIN2 (1:500) and PLIN3 (1:5000). Paraffin embedded tissue was used for detection of ATLG (1:5000) by immunohistochemistry. Briefly, cryostat sections were thawed, dried for 30 min at room temperature and fixed with 4% formaldehyde. Four washing cycles using 88 mM tris-boric acid-EDTA buffer (TBE) containing 0.1% tween (Sigma-Aldrich, Steinheim, Germany) were performed between each incubation step. Sections were incubated with hydrogen peroxidase and UV-block (Thermo Scientific, Fremont, USA) in order to avoid unspecific antibody binding. Thereafter, the primary antibody was added for one hour followed by incubation with primary antibody enhancer (Thermo Scientific, Fremont, USA) for 20 min. HRP polymer (Thermo Scientific, Fremont, US) was used for 30 min in the dark. AEC single solution (Thermo Scientific, Fremont, US) served as a substrate (10 min). Counterstaining with haemalaun was performed.

Paraffin embedded tissue sections (5 µm) were trimmed and a Xylol-ethanol line was used to remove the paraffin. Antigen retrieval in 1 mM EDTA, pH 8.0 was performed for 20 min in the microwave. Staining was performed as described above.

3.3.7 Protein expression analysis by Western blot

Protein was extracted from placental tissue by homogenizing 80 – 150 mg tissue in ice cold RIPA buffer (Sigma-Aldrich, Steinheim, Germany) containing protease inhibitor (Roche, Basel, Switzerland). Samples were centrifuged at 13.000 rpm and 4°C for 10 min. Protein concentration of the supernatant was measured by BCA protein assay kit (Thermo Scientific, Rockford, US) following the manufacturer's protocol. Tissue protein (10 µg) was mixed with laemmli buffer (Sigma-Aldrich, Vienna, Austria) boiled for 5 min at 95°C and loaded on 10% Tris/Glycine/SDS gels (Bio-Rad Laboratories, Vienna, Austria). Proteins were semi-dry blotted on nitrocellulose membrane (Bio-Rad Laboratories, Vienna, Austria). Membranes were blocked with 5% dry milk (Bio-Rad Laboratories, Vienna, Austria) and incubated with primary antibodies overnight followed by incubation with secondary antibodies (Table 3). Densidometric analysis was performed using AlphaDigiDoc 1000 (Alpha Innotech Corporation, San Leandro, US). Protein signals were normalized to β-actin or GAPDH. One sample was loaded on each blot and used for adjustment for inter blot variations.

Table 3: Antibodies used for western blotting

Antibody	Dilution	Company	Catalog No.
Rabbit anti-ATGL (#)	1:1000	Abcam, Cambridge, UK	ab109251
Mouse anti-PLIN2 (ADRP) (#)	1:500	Progen, Heidelberg, DE	AP 125
Mouse anti-PLIN3 (#)	1:2000	R&D Systems, Minneapolis, US	MAB 76641
Mouse anti-CGI-58 (ABHD5)	1:250	Abnova, Taipei, Taiwan	H00051099-M01
Mouse anti-β-actin	1:20000	Abcam, Cambridge, UK	ab6276
Mouse anti-GAPDH	1:20000	Novus, Cambridge, UK	NB100-79955
Goat anti-rabbit-HRP	1:2000	Bio-Rad Lab., Vienna, AT	170-6515
Goat anti-mouse-HRP	1:2000	Bio-Rad Lab., Vienna, AT	170-6516

(#) antibodies were used for IHC in dilutions as discribed in 3.3.6

3.4 Calculations and statistics

For statistical analysis and figure presentation, SPSS 22.0 (IBM Corporation, Armonk, New York, US) and GraphPat prism 5.0 (GraphPat Software inc., La Jolla, CA, US) were used.

Antipyrine transfer was calculated as followed.

$$FM\ ratio = \frac{fv}{ma}$$

fv = concentration in fetal vein

ma = concentration in maternal artery

Maternal to fetal FFA transfer was normalized to perfused cotyledon mass (25 g) and calculated as the FM ratio.

$$FM\ ratio = \frac{\frac{fv}{pt\ g} * 25}{ma}$$

fv = concentration in fetal vein

ma = concentration in maternal artery

pt g = perfused tissue mass (g)

Total FFA transfer was normalized to perfused cotyledon mass (25 g) and calculated as followed.

$$Total\ FFA\ transfer\ (\%) = \frac{Rm\ t0 * 0.2l}{\frac{Rf\ t90 * fV * 25}{pt\ g}} * 100$$

Rm t0 = concentration in maternal reservoir at time point 0 min

Rf t90 = concentration in fetal (vein) reservoir at time point 90 min

fV = volume in fetal (vein) reservoir at time point 90 min

pt g = perfused tissue mass (g)

All data are expressed as mean ($\pm$ SD) or if stated mean ($\pm$ SEM). For statistical testing between groups the non-parametric Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's post hoc test was performed as appropriate. Validation of housekeeping genes (HKG) was performed by one-way ANOVA. Correlation analysis was performed by Spearman correlation. P-values < 0.05 were defined as significant.

4 Results

4.1 Maternal to fetal fatty acid transfer

4.1.1 Placental transfer of FFAs depends on its double bonds

The first set of perfusion experiments was designed in order to evaluate first, the influence of differences in saturation of FFAs on direct transfer from the mother to the fetus and second, to determine the lipid classes in which FAs might be integrated if they are transferred to the fetal circulation. For this purpose, equimolar concentrations (17 $\mu\text{mol/l}$) of ^{13}C -labelled saturated palmitic acid (16:0), ^{13}C -labelled monounsaturated oleic acid (18:1) and ^{13}C -labelled polyunsaturated, essential linoleic acid (18:2n6) were bound to 0.5% BSA. These BSA-FFA complexes were used for three independent perfusion experiments over 180 min.

Direct FFA transfer was determined by measuring ^{13}C -labelled FFA concentrations in maternal inflow (maternal artery) and fetal venous outflow (fetal vein). The FFA-ratio between fetal and maternal concentration (FM ratio) was calculated for each sampled time point (0, 10, 20, 30, 60, 90, 120, 150, and 180 min of the experiment Figure 9). The FM ratio of oleic acid was lowest among the investigated FAs with a maximum of 0.014 (± 0.005) after 30 min, followed by a decline between 30 and 90 min (FM ratio 0.007) and a second increase of the FM ratio between 120 and 180 min (max 0.014). For palmitic and linoleic acid the FM ratio followed a similar pattern with the maximum after 20 min, which was 0.020 (± 0.005) and 0.023 (± 0.006) for palmitic and linoleic acid, respectively. The second FM ratio peak was equal for palmitic and linoleic acid (0.018) after 120 min again. In this first set of experiments the preference for maternal to fetal transfer followed the order palmitic acid = linoleic acid > oleic acid.

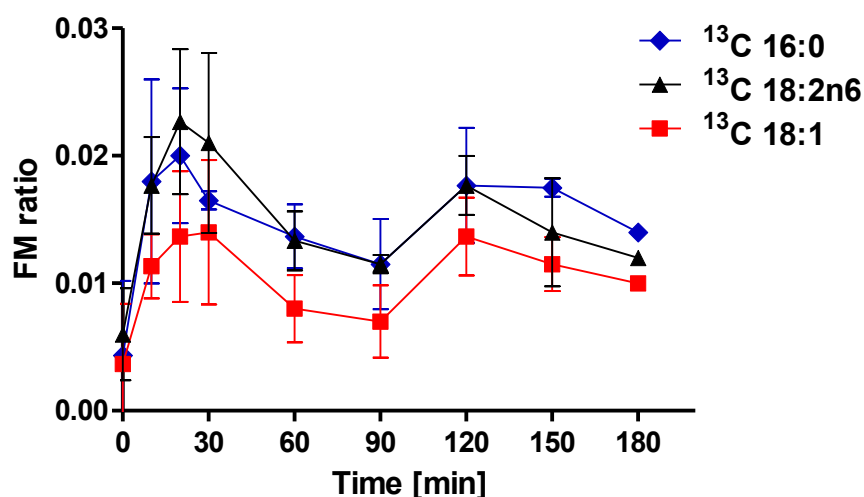


Figure 9: Maternal to fetal transfer of FFA. Direct transfer of ^{13}C -labelled palmitic acid (16:0; 17 $\mu\text{mol/l}$; 4.6 mg/l), oleic acid (18:1; 17 $\mu\text{mol/l}$; 5.1 mg/l), and linoleic acid (18:2n6; 17 $\mu\text{mol/l}$; 5.0 mg/l) all bound to 0.5% BSA was examined by *ex-vivo* placental perfusion. Each ^{13}C FFA (equimolar concentrations) was added to the maternal circulation and each labelled ^{13}C FFA was analysed in the fetal compartment. Means ($\pm$ SD) of fetal-maternal ratio (FM ratio) out of three experiments were calculated at distinct time points.

4.1.2 The placenta releases mainly FFA and PL to the fetal circulation

Lipid extracts of fetal perfusates obtained from previous experiments were separated by thin layer chromatography into PL, FFA, TG, and CE lipid classes followed by total palmitic, oleic, and linoleic acid concentration measurements. The fetal circulation was conducted as an open circuit experiment, therefore reflecting lipid class distribution at distinct time spans (10, 30, 60, and 120 min) and not accumulation of FAs over the perfusion time. Results of the fetal perfusates were normalized to 25 g tissue mass since the average weight of the perfused cotyledons of all three experiments was 24.5 g but the individual mass ranged between 16.3 – 38.5 g. As depicted in Figure 10, the majority of released lipid species containing palmitic, oleic, and linoleic acid in the fetal compartment are PLs and FFAs with a concentration peak after 60 min perfusion time. In this set of experiments both exogenously provided FAs and placental endogenous FAs were determined. Within the PL fraction (Figure 10A), palmitic acid was detectable already after 10 min and 8.4 mg/l (SEM, $\pm$ 3.7) was the highest measured PL

concentration after 60 min. Oleic acid and linoleic acid levels in PLs reached 3.9 mg/l (SEM, $\pm$ 2.9) and 2.8 mg/l (SEM, $\pm$ 2.7), respectively, after 60 min.

Under the chosen experimental condition the placenta releases palmitic, oleic, and linoleic acid as FFAs into the fetal circulation already after 10 min perfusion time (Figure 10B). The maximal released FFA concentrations were determined after 60 min, with 6.2 mg/l (SEM, $\pm$ 1.6), 2.4 mg/l (SEM, $\pm$ 1.0), and 1.0 mg/l (SEM, $\pm$ 0.8) for palmitic, oleic, and linoleic acid, respectively. Interestingly, negligible amounts of palmitic acid (0.9 mg/l) and oleic acid (0.4 mg/l) were detectable and incorporated in TGs as shown in Figure 10C. Palmitic acid was the only detectable FA within released placental CE (60 min; 2.9 mg/l; SEM, $\pm$ 0.9; Figure 10D).

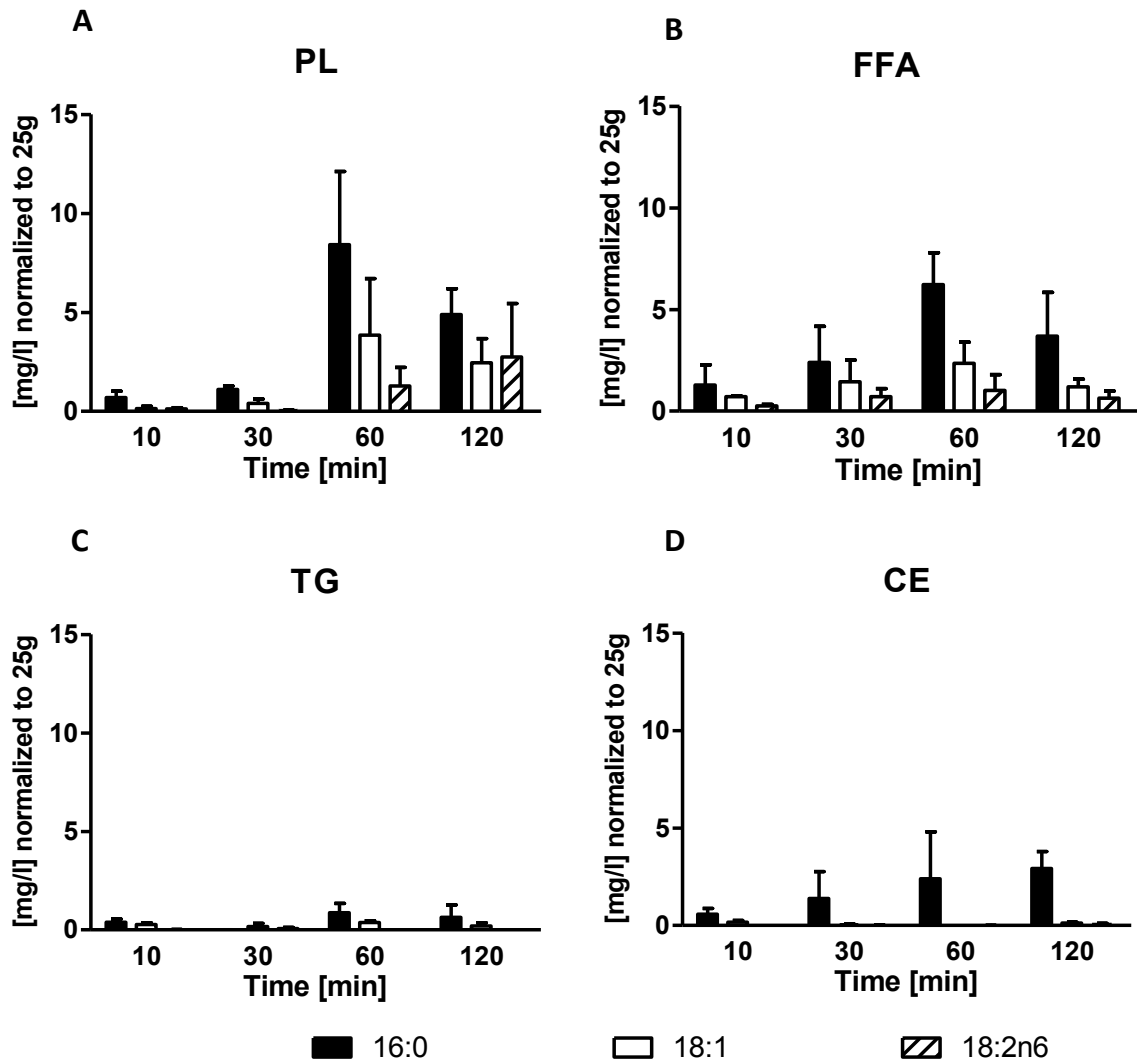


Figure 10: Release of placental lipid species containing 16:0, 18:1 and 18:2n6 FAs, into fetal perfusates. Maternal perfusate contained palmitic acid (16:0; 4.6 mg/l), oleic acid

(18:1; 5.1 mg/l), linoleic acid (18:2n6; 5.0 mg/l) bound to 0.5% BSA and was re-circulated during the perfusion experiment. Fetal circulation was conducted open. Total lipids in fetal perfusates were extracted and separated by TLC. FA concentrations in the phospholipid (PL), free fatty acid (FFA), cholesterol ester (CE) and triglyceride (TG) fraction were determined by GC and are represented as means ($\pm$ SEM) of three experiments, normalized to 25 g perfused tissue.

The maternal perfusate was re-circulated during the experiment (closed circuit) and palmitic, oleic, and linoleic acid were added in equimolar concentrations as described above. However, an accumulation of PL was observed and the concentrations of all three FAs in the PL fraction increased over time (Figure 11A), with the most pronounced increase for palmitic acid. In contrast, concentrations of palmitic, oleic, and linoleic acid in the FFA lipid fraction remain unchanged during the experimental period (Figure 11B). In the TG fraction (Figure 11C), accumulation of palmitic and oleic acid was equal (10.0 mg/l, SEM, $\pm$ 4.5), whereas only 2.6 mg/l (SEM, $\pm$ 1.4) of linoleic acid was detectable after 120 min. But linoleic acid gained its highest concentration (19.1 mg/l; SEM, $\pm$ 7.9) in the CE fraction which was even higher than for palmitic and oleic acid within the CEs (Figure 11D). In the maternal perfusate, enrichment of PL, TG and CE, containing distinct FAs, was observed over the duration of the experiment. Individual distribution of the examined fatty acids in the four lipid classes was identified.

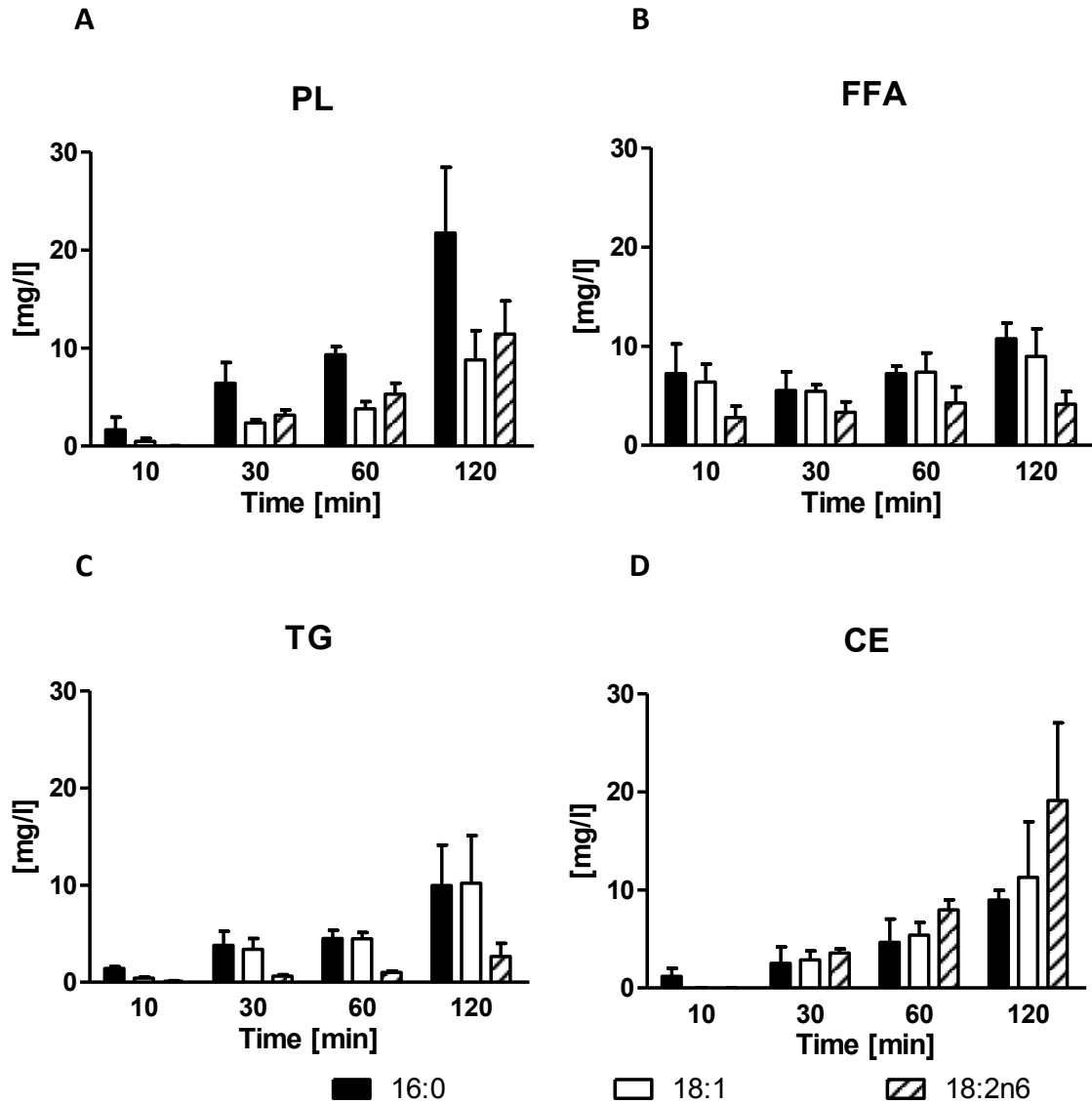


Figure 11: Placental FA release into different lipid classes and enrichment in maternal perfusates. Maternal perfusate contained palmitic acid (16:0; 4.6 mg/l), oleic acid (18:1; 5.1 mg/l), linoleic acid (18:2n6; 5.0 mg/l) bound to 0.5% BSA and was re-circulated during the perfusion experiment. Total lipids in maternal perfusates were extracted and separated by TLC. FA concentration in phospholipid (PL), free fatty acids (FFA), cholesterol ester (CE) and triglyceride (TG) fraction were determined by GC and are presented as means ($\pm$ SEM) of three experiments.

4.1.3 Testing of the adapted placental perfusion set-up

In order to test our hypothesis that maternal to fetal FFA transfer is affected by maternal pre-pregnancy obesity, *ex-vivo* perfusion experiments of the human placenta were designed. The composition of FFAs, in this set of experiments reflects the proportion and concentration of selected FAs in plasma TGs of term pregnant women. Physiological albumin concentration in human plasma ranges between 30 and 55 g/l. However, for the perfusion experiments, a bovine serum albumin concentration (BSA) of 5 g/l was chosen, secondary dextran (10 g/l) was added to compensate for the reduced concentration of plasma macromolecules. In all experiments a total FFA concentration of 97 $\mu\text{mol/l}$ bound to 5 g/l BSA was used which reflects the molar ratio (0.78) of albumin to FFA in plasma of pregnant women at term (99). Free fatty acids, labelled with ^{13}C , were used in order to follow direct maternal to fetal FFA transfer.

Out of all experiments, in five experiments aeration of perfusates was exclusively performed on fetal side (set-up 1). A gas mixture of 5% CO_2 in 95% N_2 was used in order to reduce oxygen levels and to mimic physiological oxygen levels in fetal circulation. The other set of fifteen perfusion experiments was performed after implementing a second gas exchange device for the maternal perfusate (set-up 2). For the maternal side a gas mixture of 5% CO_2 , 20% O_2 and 75% N_2 was used to sustain appropriate oxygen levels during the experiments. Table 4 shows summarized subject characteristics and differences between the two experimental set-ups.

Table 4: Maternal, placental and fetal subject characteristics.

	Perfusate not gassed (set-up 1, n=5)	Perfusate gassed (set-up 2, n=15)	Statistics
Maternal age [years]	30.8 (± 3.6)	31.0 (± 4.8)	n.s.
Pre-pregnancy BMI [kg/m²]	29.9 (± 11.1)	27.3 (± 7.7)	n.s.
Gestational age [weeks]	38.6 (± 0.55)	38.7 (± 0.46)	n.s.
Placental weight [g]	658 (± 91)	692 (± 141)	n.s.
Birth weight [g]	3599 (± 256)	3508 (± 525)	n.s.
Fetal sex (n female/n male)	(3/2)	(9/6)	

In the first set of experiments (n=5) medium in maternal reservoir was not fumigated. After modified experimental set-up the second set of experiments (n=15) were performed using maternal perfusates gassed with 5% CO₂, 20% O₂ and 75% N₂. Differences in subject characteristics between the two experimental set-ups were tested by the non-parametric Mann-Whitney U test. P-values < 0.05 were considered as significant different. n.s.: not significant.

During all perfusion experiments, defined quality control parameters were obtained and compared. The perfused cotyledon size was 26 g (± 8) and 27 g (± 6) in first and second experimental set-up, respectively. The transfer of the small lipophilic substance antipyrine, the pH in fetal artery, and fetal lactate production were determined and did not change in both sets of experiments. However, the pH in the maternal reservoir (maternal artery), and the fetal arterial back-pressure raised considerable in experiments, when the maternal perfusate was not fumigated. In contrast, lactate production in the maternal circulation was significantly lower when the maternal perfusate was not gassed (Table 5).

Table 5: Placental perfusion parameters for quality check were determined during 90 min perfusion time.

	Perfusate not gassed (set-up 1, n = 5)	Perfusate gassed (set-up 2, n = 15)	Statistics
Cotyledon [g]	26.0 (± 8.0)	27.0 (± 6.0)	n.s.
Pressure [mmHg]	57.3 (± 18.8)	35.8 (± 10.2)	P = 0.015
Antipyrine FM ratio	0.46 (± 0.01)	0.55 (± 0.08)	n.s.
pH fetal artery	7.47 (± 0.04)	7.48 (± 0.05)	n.s.
pH maternal artery	7.88 (± 0.11)	7.56 (± 0.05)	P = 0.001
Lactate fetal vein [mmol/l]	0.82 (± 0.16)	1.00 (± 0.28)	n.s.
Lactate maternal artery [mmol/l]	2.83 (± 0.56)	4.70 (± 0.82)	P = 0.003

Differences between the two set-ups were statistically tested by the non-parametric Mann-Whitney U test. P-values < 0.05 were considered as significant different. n.s.: not significant.

Concentrations of ¹³C-labelled FFAs were determined in samples of maternal artery and fetal vein by GC-MS at distinct time points. Fetal to maternal FFA-ratio (FM ratio) was calculated for both experimental set-ups. Experiments with set-up 1 showed maximal FM ratio after 20 min with the highest FM ratio for DHA, which was 0.035 (± 0.014). Palmitic, linoleic, and oleic acid followed with a FM ratio of 0.023 (± 0.008), 0.017 (± 0.005), and 0.011 (± 0.002), respectively (Figure 12A). For set-up 2 experiments perfusion time was reduced to 90 min, as the highest FM ratio for all FFAs was also detected after 20 min perfusion time. FM ratio for DHA (0.04 ± 0.02) was again highest among the examined ¹³C FFAs, followed by palmitic acid (0.024 ± 0.012), linoleic acid (0.023 ± 0.011), and oleic acid (0.015 ± 0.007) (Figure 12B). In summary experiments with set-up 2 revealed reduced fetal arterial pressure, stable pH in the maternal reservoir and slightly increased maternal to fetal transfer for DHA and linoleic acid, probably caused by the used gas mixture for the maternal perfusates. On the basis of outcome and comparison of these two set-ups only experiments with set-up 2 were further analysed.

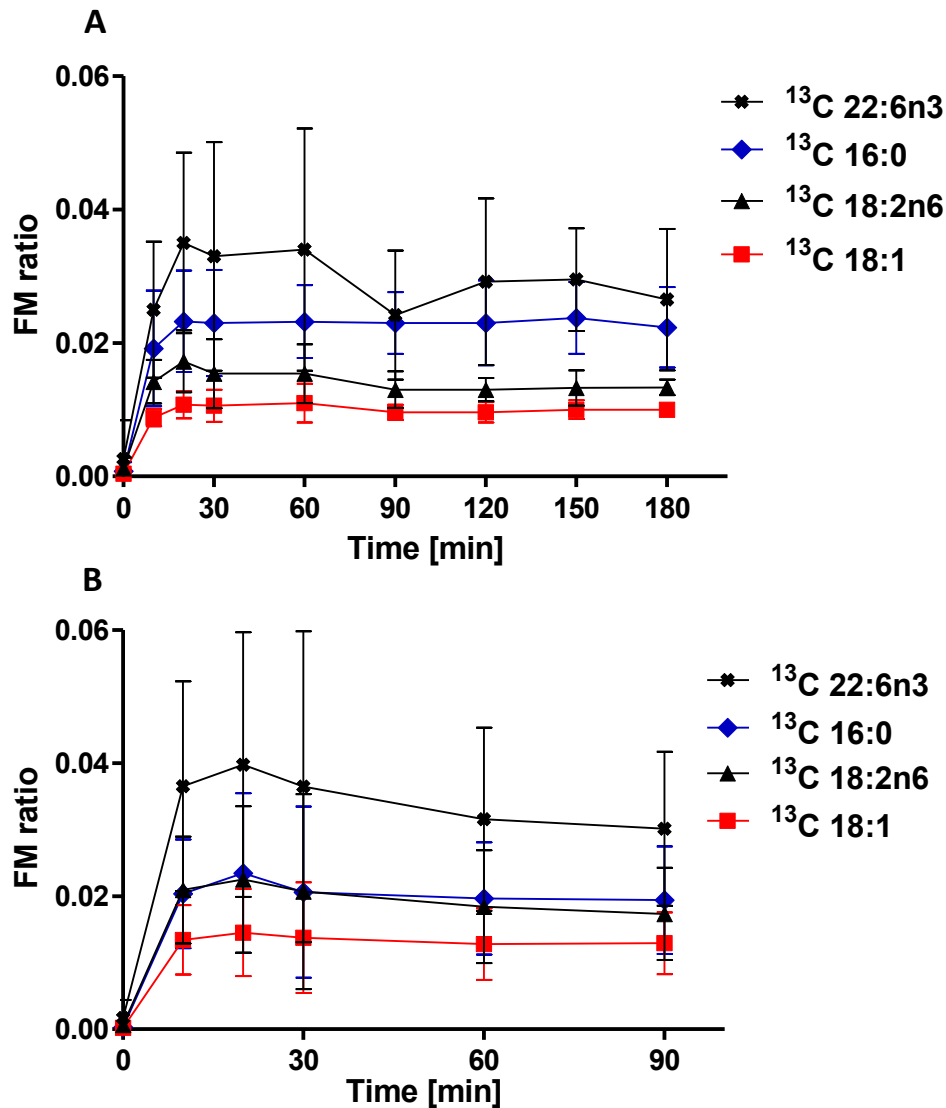


Figure 12: Time dependent maternal to fetal transfer of ^{13}C -labelled FFAs. Transfer of ^{13}C -labelled palmitic acid (16:0; 37.2 $\mu\text{mol/l}$), oleic acid (18:1; 40.52 $\mu\text{mol/l}$), linoleic acid (18:2n6; 16.79 $\mu\text{mol/l}$) and docosahexaenoic acid (DHA, 22:6n3; 0.29 $\mu\text{mol/l}$) in addition to non-labelled FFAs (Table 1) bound to 0.5% BSA was examined by *ex-vivo* placental perfusion. **A:** Mean ($\pm$ SD) fetal-maternal ratio (FM ratio) was estimated at 0, 10, 20, 30, 60, 90, 120, 150, 180 min. Five experiments were performed prior to gas exchange in maternal circulation (set-up 1). **B:** FM ratio (mean $\pm$ SD) out of 15 experiments was calculated at time points 0, 10, 20, 30, 60, 90 min upon maternal perfusates were conditioned with 5% CO_2 , 20% O_2 and 75% N_2 (set-up 2).

4.1.4 Maternal to fetal DHA transfer is elevated in obese pregnancies

FFA transfer of placentas from lean (n=8) and obese (n=7) women were compared by perfusion experiments. Subject characteristics between the two groups were similar (Table 6). Maternal pre-pregnancy BMI was significantly higher in the obese group as expected. The sex ratio of born females and males was 6 to 2 and 3 to 4 in lean compared to obese women, respectively.

Table 6: Characteristics of mothers and offspring.

	Lean (n = 8)	Obese (n = 7)	Statistics
Maternal age [years]	32.0 (± 4.8)	30.0 (± 4.9)	n.s.
Pre-pregnancy BMI [kg/m²]	21.2 (± 1.8)	34.2 (± 5.2)	P < 0.001
Gestational age [weeks]	38.98 (± 0.44)	39.0 (± 0.41)	n.s.
Placental weight [g]	660 (± 142)	729 (± 143)	n.s.
Birth weight [g]	3367 (± 314)	3669 (± 686)	n.s.
Fetal sex (n female/n male)	(6/2)	(3/4)	

Study subjects were differentiated according to maternal pre-pregnancy BMI which resulted in a lean (BMI ≤ 25 kg/m²) and obese (BMI ≥ 30 kg/m²) subject group. Values are expressed as mean (± SD). Differences between lean and obese group were tested by the non-parametric Mann-Whitney U test. P-values < 0.05 were defined as statistical significant. n.s.: not significant.

In order to investigate the impact of maternal pre-pregnancy obesity on FA supply to the fetus the FM ratios for ¹³C-labelled palmitic, oleic, linoleic acid, and DHA were compared between placentas from lean (n=8) and obese (n=7) women. For all labelled FFAs the transfer rate in the obese group was elevated in comparison to the leans, but absolute concentrations of transferred ¹³C-FFAs to the fetal compartment was extremely low. In particular, FM ratio of palmitic acid was higher in the obese (0.028 ± 0.014) compared to the lean group (0.020 ± 0.009) after 20 min perfusion time (Figure 13A). Oleic and linoleic acid transfer was increased in obese placentas 0.017 (± 0.007) and 0.026 (± 0.013) compared to lean placentas 0.013 (± 0.006) and 0.019 (± 0.009), respectively (Figure 13B, C). Strikingly, FM ratio for DHA of obese placentas (0.046 ± 0.013) was

highest among all examined FFAs and was even elevated in comparison to lean placentas (FM ratio; 0.034 ± 0.018 ; Figure 13D).

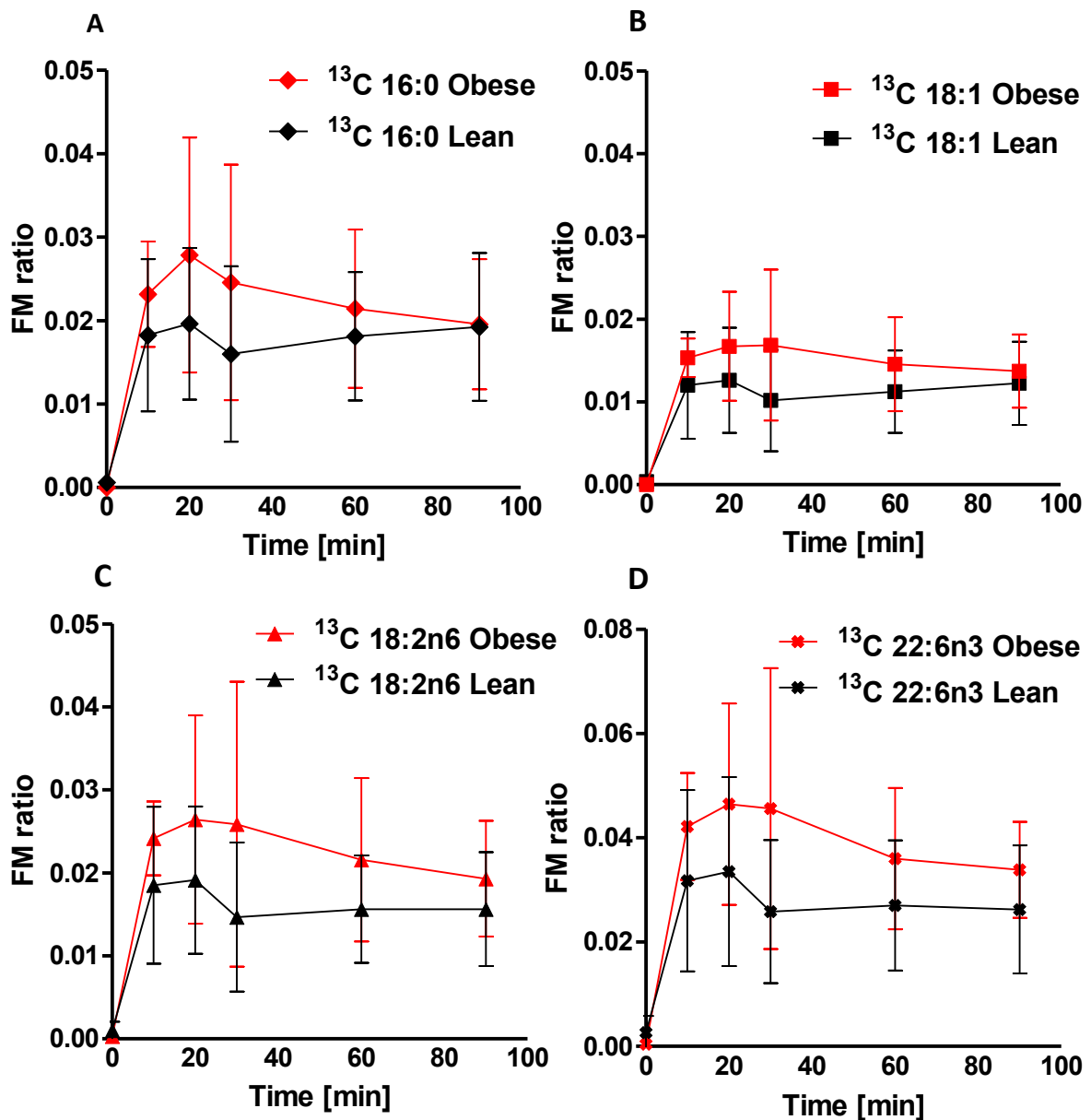


Figure 13: Maternal to fetal transfer of ^{13}C labelled FFA in placentas of lean and obese women. Direct transfer of ^{13}C labelled **A:** palmitic acid (16:0; $37.2 \mu\text{mol/l}$), **B:** oleic acid (18:1; $40.52 \mu\text{mol/l}$), **C:** linoleic acid (18:2n6; $16.79 \mu\text{mol/l}$) and **D:** docosahexaenoic acid (DHA, 22:6n3; $0.29 \mu\text{mol/l}$) in addition to non-labelled FFAs bound to 0.5% BSA (Table 1) was measured at distinct time points. Means ($\pm$ SD) of lean ($n=8$) and obese ($n=7$) perfusion experiments are shown.

The percentage of maternally offered ^{13}C FFA, transferred to the fetal circulation within 90 min of the perfusion was observed more in detail. The calculated total FFA-transfer, shown as percentage of initial maternal concentration, was 2.3% for palmitic acid, 1.6% for oleic acid, 2.4% for linoleic acid, and 3.4% for DHA in lean placentas (Figure 14A). The ^{13}C FFA transfer capacity of placentas from obese women was 2.7%, 1.9%, 2.9% and 4.9% for palmitic, oleic, linoleic acid, and DHA, respectively. DHA transfer was significantly higher compared to palmitic and oleic acid (Figure 14B). Interestingly, the placental release of endogenous, not labelled FFA was 1.7 – 58.5 fold higher compared to offered ^{13}C -labelled FA which was statistically significant for all FFAs in lean placentas (Table 7). In obese placentas, endogenous FFA release into the fetal circulation was 1.1 - 26.6 fold higher in comparison to ^{13}C FFA which was statistically significant except for oleic acid (Table 8).

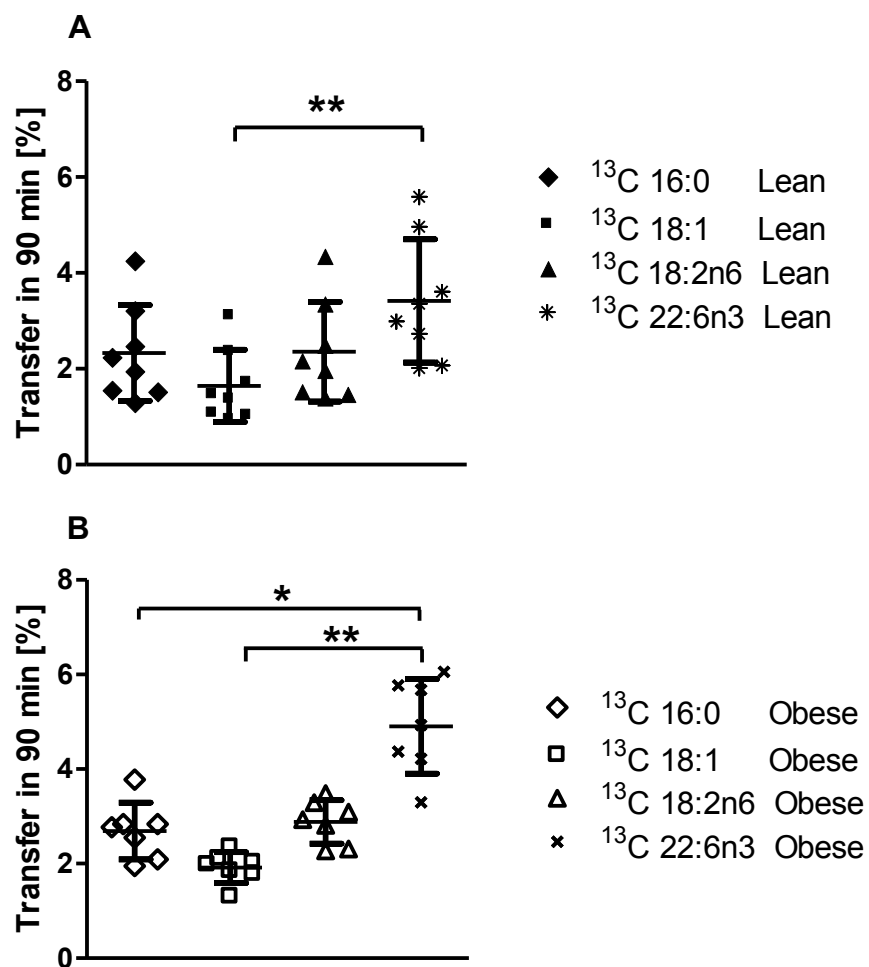


Figure 14: DHA maternal to fetal transfer is highest among examined FFAs. Initial ^{13}C FFA concentrations in maternal reservoir and in fetal outflow after 90 min perfusion

were determined by GC-MS. FFA transfer was normalized to 25 g tissue mass and results are expressed as % of initial maternal FFA concentration (mean $\pm$ SD) of (A) perfused lean (n=8) and (B) obese (n=7) placentas. Differences in total FA transfer between individual ^{13}C FFA were tested by Kruskal-Wallis followed by Dunn's multiple comparison test. * $p < 0.05$, ** $p < 0.01$.

Table 7: Release of endogenous and ^{13}C -labelled FFAs into fetal circulation in lean placentas.

Lean (n=6)	Ratio	endogenous FFA	^{13}C FFA	Statistic
16:0 vs ^{13}C 16:0	5.4	0.6415 ($\pm$ 0.2221)	0.1179 ($\pm$ 0.0507)	**
18:1n9 vs ^{13}C 18:1n9	1.7	0.2627 ($\pm$ 0.0979)	0.1566 ($\pm$ 0.0561)	*
18:2n6 vs ^{13}C 18:2n6	4.5	0.3779 ($\pm$ 0.1786)	0.0848 ($\pm$ 0.0283)	**
22:6n3 vs ^{13}C 22:6n3	58.6	0.0721 ($\pm$ 0.0291)	0.0012 ($\pm$ 0.0005)	**

Endogenously derived FFA and ^{13}C -labelled FFA concentrations were determined in maternal and fetal reservoirs, total transfer was calculated for 90 min perfusion experiments and adjusted to 25 g tissue mass. Results are expressed as mean ($\pm$ SD) $\mu\text{mol}/90$ min of perfused lean (n=6) placentas. Ratio between endogenous FFAs and ^{13}C FFAs was calculated. Differences between individual endogenous and corresponding ^{13}C FFAs were tested by Kruskal-Wallis. * $p < 0.05$, ** $p < 0.01$.

Table 8: Release of endogenous and ^{13}C -labelled FFAs into fetal circulation in obese placentas.

Obese (n=6)	Ratio	endogenous FFA	^{13}C FFA	Statistic
16:0 vs ^{13}C 16:0	3.2	0.6273 ($\pm$ 0.2952)	0.1946 ($\pm$ 0.0547)	**
18:1n9 vs ^{13}C 18:1n9	1.1	0.2027 ($\pm$ 0.0371)	0.1874 ($\pm$ 0.0574)	n.s.
18:2n6 vs ^{13}C 18:2n6	2.8	0.3004 ($\pm$ 0.0661)	0.1077 ($\pm$ 0.0330)	**
22:6n3 vs ^{13}C 22:6n3	26.6	0.0448 ($\pm$ 0.0126)	0.0017 ($\pm$ 0.0005)	**

Endogenously derived FFA and ^{13}C -labelled FFA concentrations were determined in maternal and fetal reservoirs, total transfer was calculated for 90 min perfusion experiments and adjusted to 25 g tissue mass. Results are expressed as mean ($\pm$ SD)

$\mu\text{mol}/90\text{ min}$ of perfused obese ($n=6$) placentas. Ratio between endogenous FFAs and ^{13}C FFAs was calculated. Differences between individual endogenous and corresponding ^{13}C FFAs were tested by Kruskal-Wallis. * $p<0.05$, ** $p<0.01$.

By comparing the total transfer across obese and lean placentas no differences for palmitic, oleic, and linoleic acid were observed and ranged between 2 - 6% (Figure 15A), despite slightly elevated FM ratios (Figure 13A-C). However, DHA transfer was significantly increased by 44% ($P = 0.040$) in placentas of obese women in comparison to placentas of lean women (Figure 15A). This specific increased transfer of DHA in obesity was not mirrored by the FFA-uptake into the placental lipid pool. Approximately 45% of offered DHA, 37% of palmitic acid, and 27% of oleic and linoleic acid were transferred to the placental lipid pool. No difference in placental FFA-uptake between lean and obese groups was observed (Figure 15B).

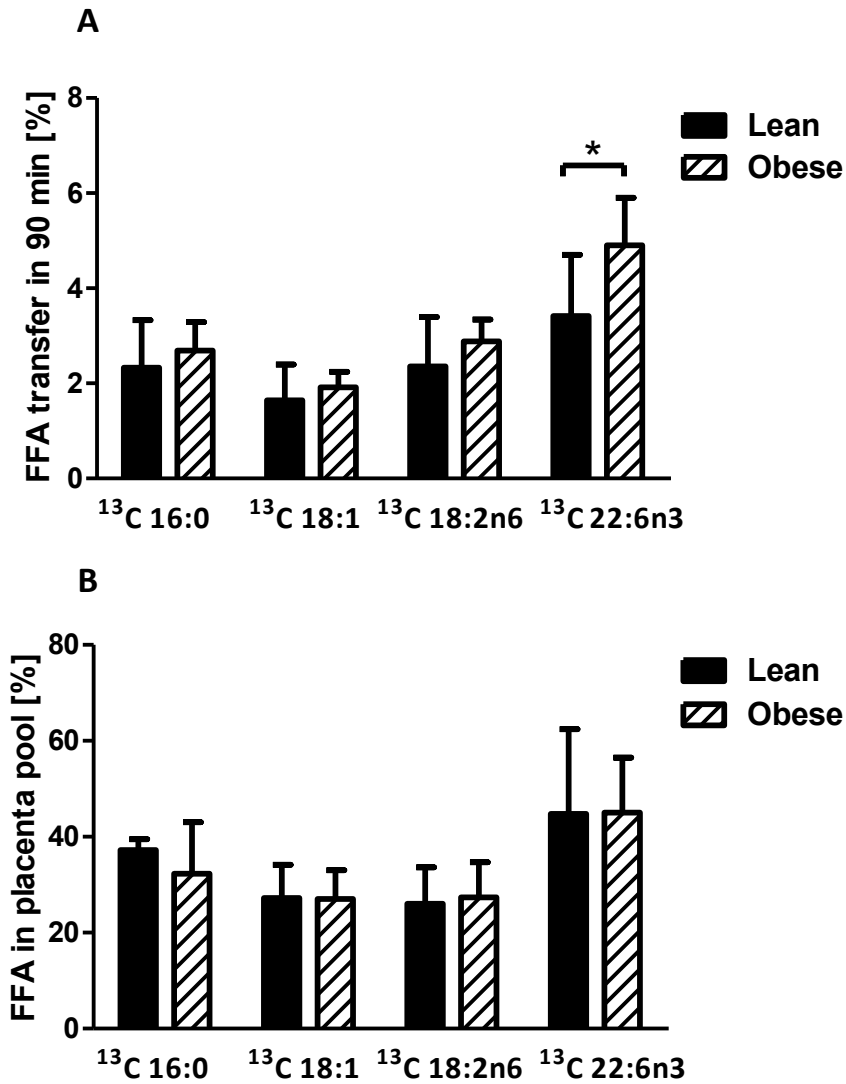


Figure 15: Maternal to fetal FFA transfer and FFA-uptake into the placenta. Maternal ^{13}C FFA concentrations before and after experiments, and in fetal outflow after 90 min were determined by GC-MS. Results are expressed as % of initial maternal FFA concentration (mean $\pm$ SD) of lean (n=8) and obese (n=7) perfused placentas. **A:** Maternal to fetal ^{13}C FFA transfer was calculated and related to 25 g tissue mass. **B:** ^{13}C FFA-uptake into the placental tissue was determined and is expressed as % of initial maternal FFA concentration (mean $\pm$ SD) of lean (n=4) and obese (n=3) perfused placentas. Non-parametric group comparison (Mann-Whitney U) was performed. * P < 0.05.

Obesity is associated with increased risk for development of type 2 diabetes and cardiovascular disease. Importantly, men have a higher amount of visceral adipose tissue

than premenopausal women and are more prone to develop pathological alterations in glucose and lipid metabolism. Thus, understanding how sex influences placental lipid metabolism already *in utero* might give answers to, how obesity related diseases can be prevented. Independent of maternal pre-pregnancy weight, placentas from female infants tend to transfer higher levels of FFAs following the order DHA > linoleic acid ≥ palmitic acid > oleic acid (Figure 16).

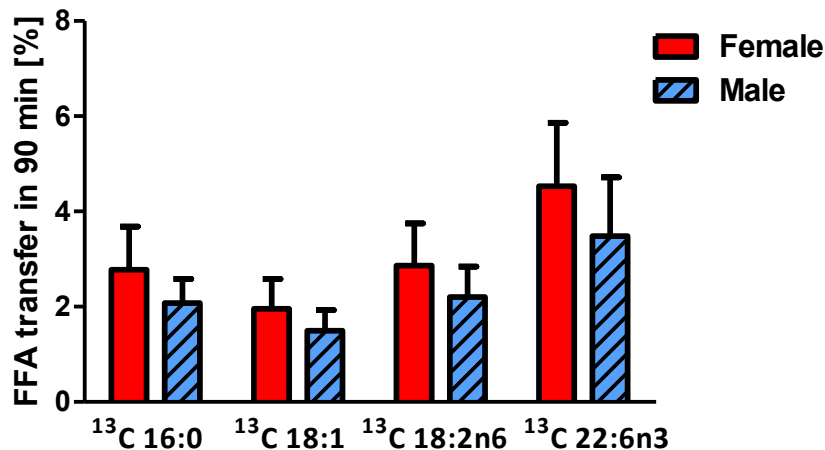


Figure 16: Maternal to fetal FFA transfer related to sex. To test the sexual dimorphism on placental FFA transfer, obtained results were associated to sex of the newborn. Results are expressed as % of initial maternal FFA concentration (mean ± SD) of female (n=9) and male (n=6) perfused placentas. Non-parametric group comparison (Mann-Whitney U) was performed. P-values < 0.05 were considered as significant different.

In a more detailed analysis, the experiments were stratified according to sex and obesity. Transfer of all FFAs was similar between the male obese group and the lean female group. In the female obese group transfer of palmitic, oleic and linoleic acid was enlarged compared to the female lean group (Figure 17A-C). DHA transfer was significantly higher in female obese placentas (5.8% ± 0.2) compared to the lean group (3.9% ± 1.1, Figure 17D). However, statistical testing was not performed in the male group due to the low numbers within the stratified groups (Figure 17).

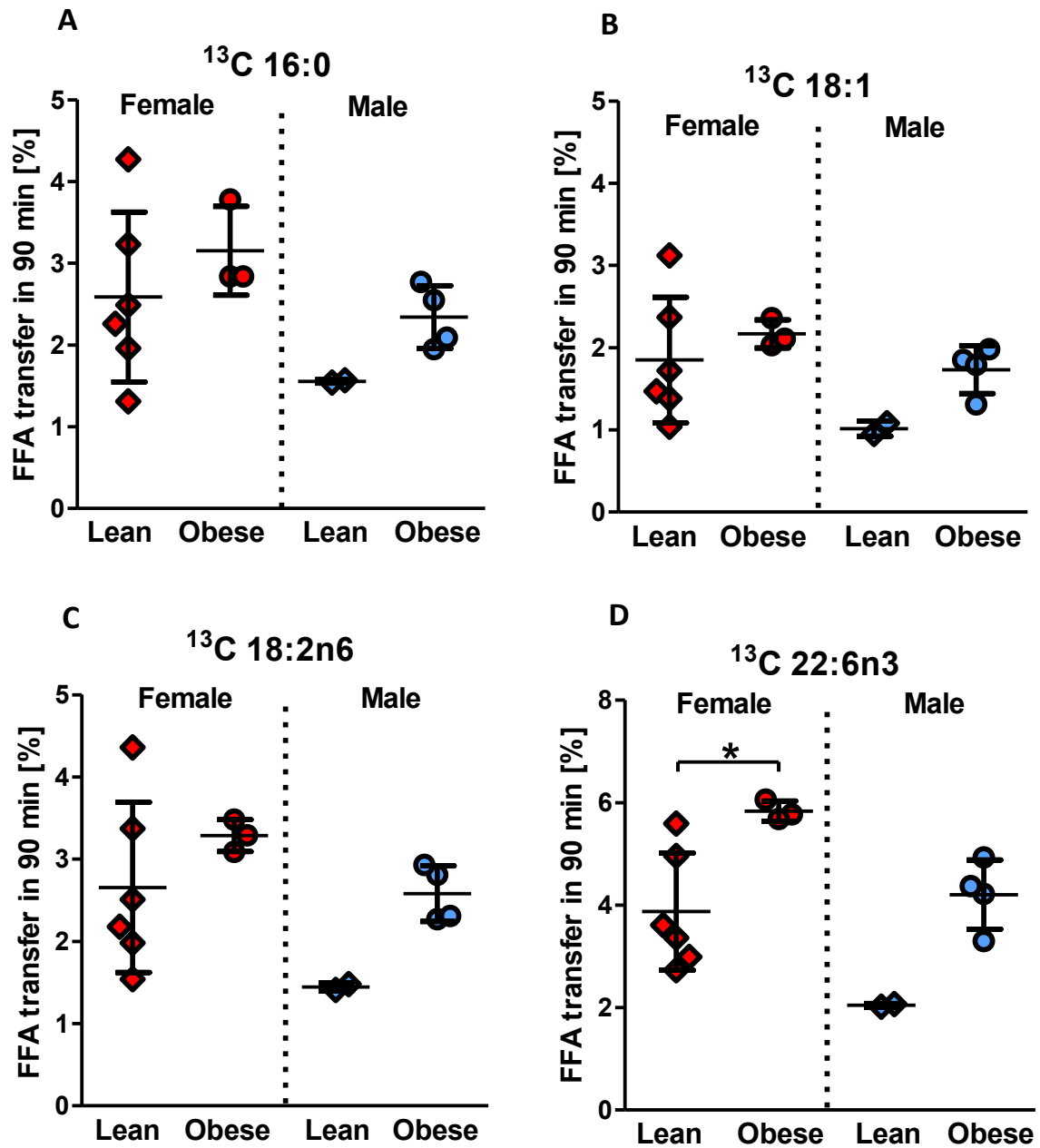


Figure 17: Maternal to fetal FFA transfer stratified according to sex and obesity. ^{13}C FFA transfer was compared between female lean (n=6) and obese (n=3) to male lean (n=2) and obese (n=4). Differences between female lean and female obese group were tested by Mann-Whitney U test. * P < 0.05.

4.1.5 Maternal to fetal DHA transfer rate depends on FA concentration

In order to test if direct maternal to fetal FFA transfer is dependent on offered FFA concentration in the maternal circulation, two concentrations of ^{13}C -labelled DHA were tested subsequently in the same experiment. DHA concentration started with $0.3\ \mu\text{mol/l}$ during the first 90 min of perfusion time, followed by a 30 min wash out phase and a second perfusion phase with $1.5\ \mu\text{mol/l}$ of DHA in maternal circulation. All other FFAs were applied in equal concentrations (Table 1). In addition, to ^{13}C -labelled FFAs, the endogenous release of FFAs from the placenta to the fetal compartment was measured.

In the first phase the concentration of ^{13}C palmitic acid offered in maternal perfusate was $37.2\ \mu\text{mol/l}$ and decreased in the closed system to $24.2\ \mu\text{mol/l}$. During the wash out phase no ^{13}C palmitic acid was detectable. In the second phase of the experiment, the starting concentration for ^{13}C palmitic acid was $36.4\ \mu\text{mol/l}$ which decreased to $25\ \mu\text{mol/l}$ after 90 min. The placental release of endogenous palmitic acid was between 2.2 and $6.9\ \mu\text{mol/l}$ (Figure 18A). The direct transfer of ^{13}C palmitic acid from the maternal to the fetal reservoir was constant between 0.51 and $0.72\ \mu\text{mol/l}$ in both experimental phases and decreased during the wash out phase to $0.06\ \mu\text{mol/l}$. Palmitic acid release from placental endogenous lipid pools into the fetal compartment was between 1.7 and $3.6\ \mu\text{mol/l}$. The endogenous release was approximately 5-fold higher than maternal to fetal ^{13}C palmitic acid transfer (Figure 18B).

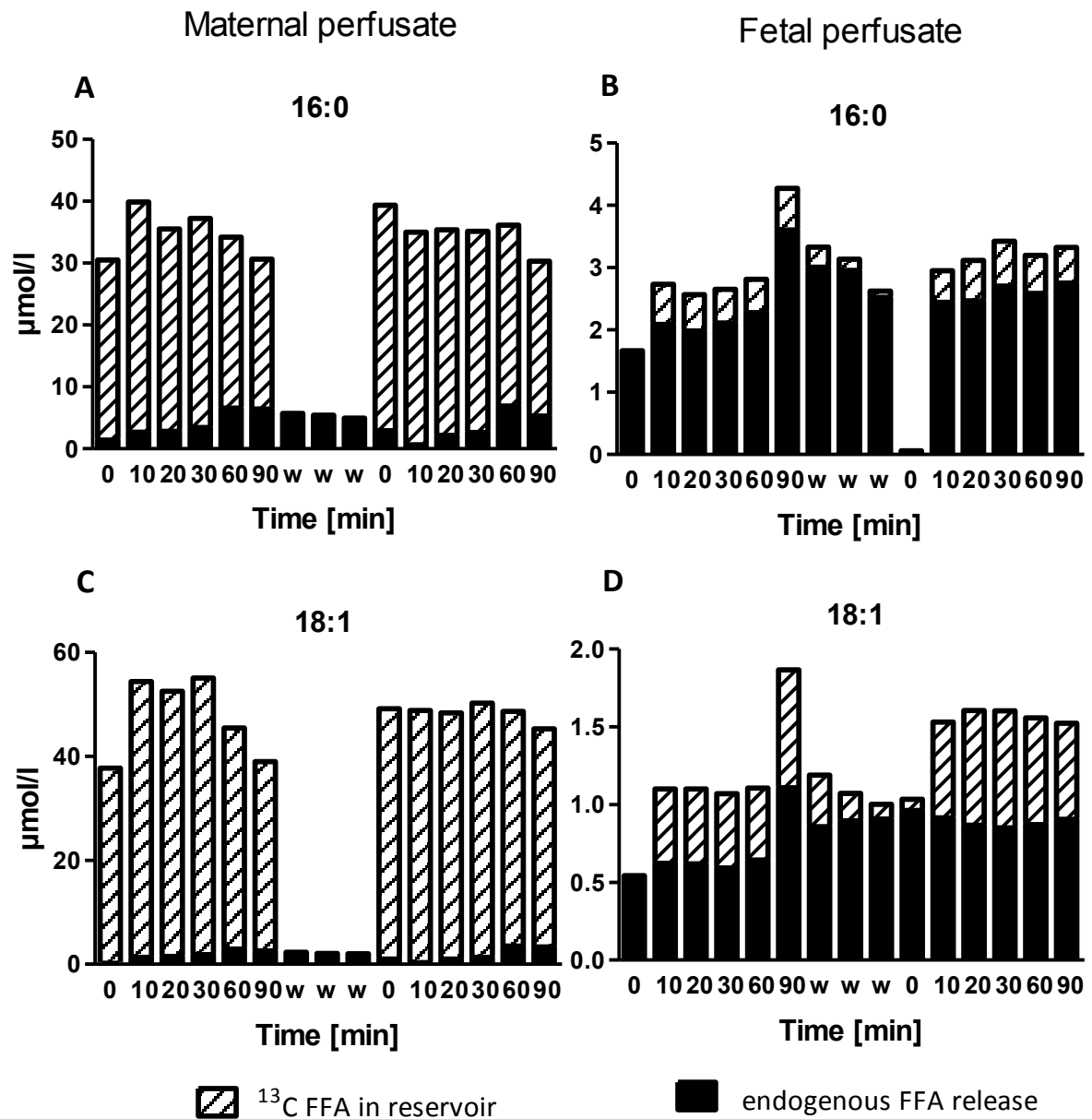
^{13}C oleic acid concentrations in the maternal reservoir decreased from 53 to $36\ \mu\text{mol/l}$ and from 48.5 to $42\ \mu\text{mol/l}$ in the first and second experimental phase, respectively. The endogenous oleic acid release into the maternal compartment was on average $1.8\ \mu\text{mol/l}$ (Figure 18C). Maternal-to-fetal transfer of ^{13}C oleic acid was $0.53 (\pm 0.06)$ and $0.68 (\pm 0.06)\ \mu\text{mol/l}$ in the first in the second phase, respectively. The placental release of endogenous oleic acid into the fetal circulation was between 0.54 to $1.1\ \mu\text{mol/l}$ and was about 30% higher than directly transfer of ^{13}C labelled oleic acid (Figure 18D).

The initial concentration in the maternal reservoir of ^{13}C linoleic acid was 21.2 and $19.8\ \mu\text{mol/l}$ in the first and second phase and decreased in both experimental phases. The endogenous release of non-esterified linoleic acid into the maternal compartment was $2.9 (\pm 0.9)$ and $2.6 (\pm 1.9)\ \mu\text{mol/l}$ (Figure 18E). The transfer of ^{13}C linoleic acid into the

fetal circulation was $0.28 (\pm 0.04)$ and $0.36 (\pm 0.04)$ $\mu\text{mol/l}$ in the first and second experimental phase. The placental release of endogenous linoleic acid into fetal compartment was 3.5-fold higher than the maternal-to-fetal transfer of ^{13}C -labelled linoleic acid (Figure 18F).

The initial ^{13}C DHA concentration in the maternal perfusate declined from $0.25 \mu\text{mol/l}$ to $0.1 \mu\text{mol/l}$ in the first 90 min. In the second phase of the experiment a ~5 fold higher starting concentration of ^{13}C DHA ($1.4 \mu\text{mol/l}$) was used. A continuous release of endogenous DHA into the maternal reservoir was detected throughout the experiment and was on average $0.34 (\pm 0.17)$ and $0.40 (\pm 0.31)$ $\mu\text{mol/l}$ in the first and second 90 min of the experiment (Figure 18G). In the fetal compartment the concentration of ^{13}C DHA was $0.004 (\pm 0.001)$ $\mu\text{mol/l}$, when lower DHA amounts were offered in the maternal compartment. The increase of ^{13}C DHA in the maternal circulation, resulted in a 6.8-fold increase of ^{13}C DHA in the fetal perfusate. Placental release of endogenous DHA into the fetal compartment was $0.17 (\pm 0.05)$ and $0.24 (\pm 0.01)$ $\mu\text{mol/l}$ in the first and second 90 min of the experiment, respectively (Figure 18H).

In summary direct transfer of ^{13}C DHA from maternal to fetal circulation is dependent on maternal concentration under the chosen experimental conditions. In the second phase of the experiment, the mean concentrations of ^{13}C FFAs were higher in the fetal perfusate, suggesting additional release of accumulated ^{13}C FFAs from the placental pool. This is further supported by the observation that the maternal initial concentrations were equal or even lower in the second experimental phase. In addition to ^{13}C FFAs, the placenta continuously releases FFA from an endogenous pool into the fetal circulation. Interestingly, the amounts of placental endogenous FFAs are between 1.3 and 9-fold higher compared to ^{13}C FFAs in the fetal perfusate depending on the FFA species.



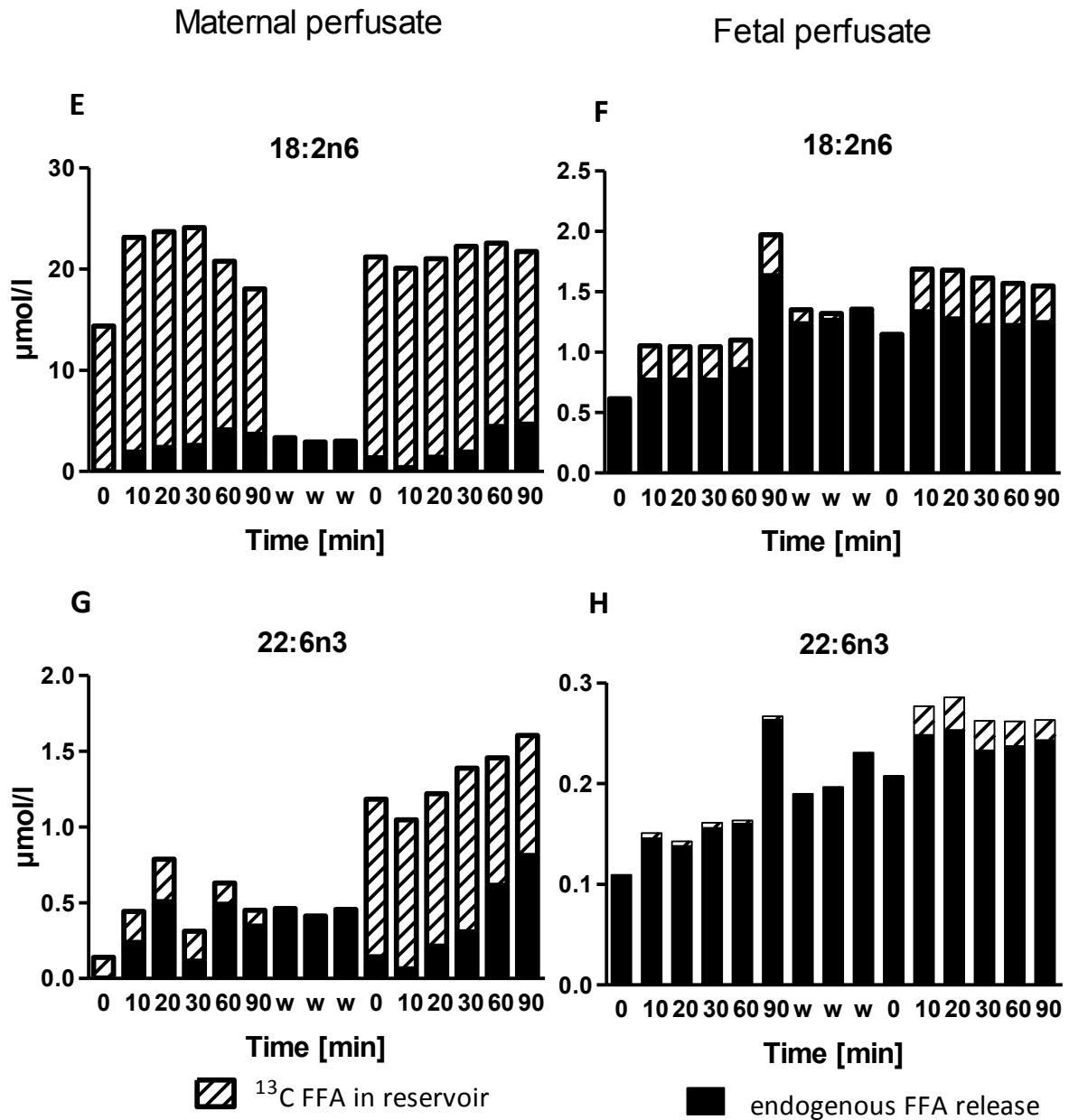


Figure 18: Placental endogenous FFA release and concentration dependent maternal-to-fetal DHA transfer. Perfusion experiment was performed with FFA mix (containing ^{13}C 0.3 μM DHA) for 90 min followed by 30 min wash out and a second perfusion period with FFA mix (containing 1.5 μM ^{13}C DHA). **A** and **B**: Palmitic acid (16:0; 37.2 $\mu\text{mol/l}$), **C** and **D**: oleic acid (18:1; 40.52 $\mu\text{mol/l}$), **E** and **F**: linoleic acid (18:2n6; 16.79 $\mu\text{mol/l}$) and **G** and **H**: docosahexaenoic acid (22:6n3; 0.3 $\mu\text{mol/l}$ and 1.5 $\mu\text{mol/l}$) in combination with non-labelled FFAs bound to 0.5% BSA (Table 1). FFA concentrations were determined in maternal (left panel) and fetal perfusates (right panel). FFA concentrations in maternal reservoir are expressed as delta to previous time point.

The relationship between maternal initial ^{13}C FFA concentration and total maternal-to-fetal transfer within 90 min was examined in a secondary analysis of 16 perfusion experiments. In experiment Ob105 (green symbols in Figure 19) two experimental phases were performed, therefore this perfusion was processed as two single experiments (for detailed description see also above). In three experiments, the initial maternal ^{13}C DHA concentration was increased to approximately 1 $\mu\text{mol/l}$. The initial concentration in the maternal reservoir for ^{13}C palmitic acid was unexpectedly low in three experiments and this resulted in lower absolute transfer to the fetal compartment. However, the values of all experiments were close to each other, thus the correlation did not reach significance (Figure 19A). There was no significant correlation between maternal and fetal absolute concentrations for oleic acid (Figure 19B) and linoleic acid (Figure 19C), indicating that the starting maternal concentrations and total fetal concentrations were equal in all 16 experiments. In case of ^{13}C DHA, the initial concentration in 12 experiments was 0.3 $\mu\text{mol/l}$, in three experiments 1 $\mu\text{mol/l}$ and in one experiment 1.5 $\mu\text{mol/l}$. Absolute concentrations of ^{13}C DHA in fetal reservoir and maternal initial concentrations correlated significantly (Spearman, $R = 0.697$, $P < 0.003$) as indicated in Figure 19D.

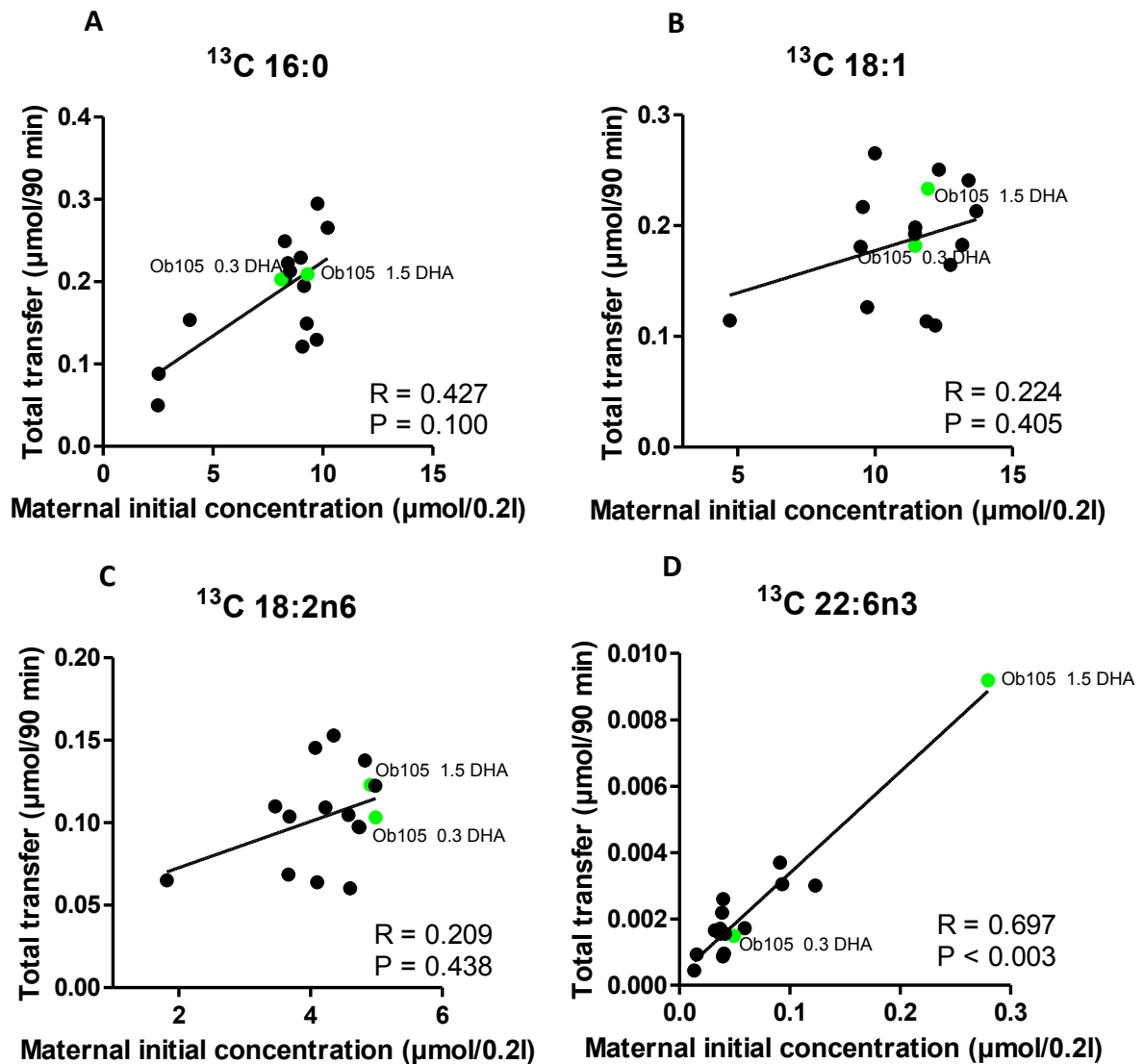


Figure 19: Maternal to fetal ^{13}C FFA transfer related to initial ^{13}C FFA concentration in maternal compartment. Concentrations of ^{13}C -labelled **A:** palmitic acid (16:0), **B:** oleic acid (18:1), **C:** linoleic acid (18:2n6) and **D:** docosahexaenoic acid (22:6n3) were determined in maternal and fetal perfusates after 90 min. Absolute ^{13}C FFA transfer was calculated as $\mu\text{mol}/90 \text{ min}$. Correlations between ^{13}C -labelled FFA absolute amounts were tested by non-parametric Spearman correlation in 16 experiments. P-values < 0.05 were conducted as significant. Green circles (●) indicate experiment Ob105 with ^{13}C DHA in two different concentrations (0.3 $\mu\text{mol}/\text{l}$ and 1.5 $\mu\text{mol}/\text{l}$, other FFAs were equal in both periods).

4.2 Genes sensitive to maternal obesity in placental lipid metabolism

All results within this chapter are published in the *International Journal of Obesity* (116){Ref Hirschmugl 2016}.

4.2.1 Cohort characteristics

Placental samples of a subject cohort collected in Cleveland, US, were examined. The subjects were separated into a lean and an obese group, with a maternal pre-pregnancy BMI ≤ 25 kg/m² and BMI ≥ 30 kg/m², respectively. The range of maternal pre-pregnancy BMI was 19.4 kg/m² - 24.9 kg/m² in the lean and 30.2 kg/m² - 64.3 kg/m² in the obese group. The subjects in both groups matched in terms of maternal age, gestational age at delivery, and blood pressure. However, maternal pre-pregnancy BMI, maternal plasma insulin levels and homeostatic model assessment for insulin resistance (HOMA-IR) factor were significantly increased in the obese group whereas maternal weight gain during pregnancy was significantly lower (Table 9). Plasma TG and FFA levels were similar in obese and lean mothers. In contrast, plasma CE, PL, and HDL cholesterol (HDL-C) was significantly lower or tend to be lower in case of CE in the obese compared to the lean group (Table 10). Neonatal characteristics were very similar between lean and obese groups (Table 11).

Table 9: Cohort characteristics of mothers.

	BMI $\leq$ 25 kg/m² (n = 34)	BMI $\geq$ 30 kg/m² (n = 55)	Statistics
Pre-pregnancy BMI [kg/m²]	21.6 ($\pm$ 2.1)	37.9 ($\pm$ 6.8)	P < 0.0001
Weight gain [kg]	16.5 ($\pm$ 5.9)	12.2 ($\pm$ 8.1)	P < 0.01
Maternal age [years]	29.4 ($\pm$ 6.6)	27.6 ($\pm$ 6.4)	n.s.
GA at delivery [weeks]	39.2 ($\pm$ 0.4)	39.2 ($\pm$ 0.5)	n.s.
Insulin [mU/l]	9.4 ($\pm$ 3.1)	22.7 ($\pm$ 8.2)	P < 0.0001
Insulin Resistance [HOMA-IR]	1.7 ($\pm$ 0.7)	4.6 ($\pm$ 2.2)	P < 0.0001
Blood pressure systolic [mmHg]	118 ($\pm$ 13)	118 ($\pm$ 11)	n.s.

Maternal clinical and systemic parameters were displayed according to patient records at term. Insulin and glucose levels were measured in maternal plasma at term. Data are expressed as mean ($\pm$ SD). Differences between the lean (BMI $\leq$ 25kg/m²) and the obese group were tested by non-parametric Mann-Whitney U test, P-values < 0.05 were conducted as significant. n.s.: not significant.

Table 10: Maternal plasma lipids at term.

	BMI $\leq$ 25 kg/m² (n = 17)	BMI $\geq$ 30 kg/m² (n = 18)	Statistics
Cholesterol [mg/dl]	214 ($\pm$ 50)	179 ($\pm$ 59)	P = 0.067
TG [mg/dl]	167 ($\pm$ 79)	149 ($\pm$ 78)	n.s.
Free Cholesterol [mg/dl]	71 ($\pm$ 16)	62 ($\pm$ 16)	P = 0.089
CE [mg/dl]	143 ($\pm$ 35)	117 ($\pm$ 39)	P = 0.045
PL [mg/dl]	247 ($\pm$ 50)	208 ($\pm$ 55)	P = 0.032
FFA [mmol/l]	0.86 ($\pm$ 0.24)	0.93 ($\pm$ 0.20)	n.s.
HDL-C [mg/dl]	44 ($\pm$ 15)	27 ($\pm$ 5)	P = 0.001

Lipid species were determined in maternal plasma at term. Differences between the lean (BMI $\leq$ 25 kg/m²) and the obese group (BMI $\geq$ 30 kg/m²) were tested by non-parametric Mann-Whitney U test, P-values < 0.05 were conducted as significant different. n.s.: not significant

Table 11: Characteristics of the neonates.

	BMI ≤ 25 kg/m² (n = 34)	BMI ≥ 30 kg/m² (n = 55)	Statistics
Birth weight [kg]	3.26 (± 0.45)	3.28 (± 0.45)	n.s.
Birth length [cm]	49.6 (± 2.0)	48.9 (± 1.7)	n.s.
Body fat mass [%]	11.7 (± 3.3)	12.6 (± 3.4)	n.s.
Placental weight [g]	597 (± 159)	668 (± 187)	n.s.
Infant/placental weight ratio	5.7 (± 1)	5.1 (± 1)	n.s.

Pregnant women (n=89), with singleton pregnancies and gestational age (GA) ≥ 38 weeks were included. Neonatal characteristics are expressed as mean ($\pm$ SD). Mann-Whitney U non-parametric group comparison test was performed and P-values < 0.05 were defined as significantly different. n.s.: not significant.

4.2.2 Placental triglyceride levels are elevated by maternal obesity

First of all, the impact of maternal obesity on placental neutral lipid content was examined. As shown in Figure 20, TG-levels in placental tissue biopsies of obese women were significantly higher in comparison to the lean group, although maternal plasma TG-levels were unaffected by obesity, suggesting a higher lipid storage capacity of obese placentas (Table 10).

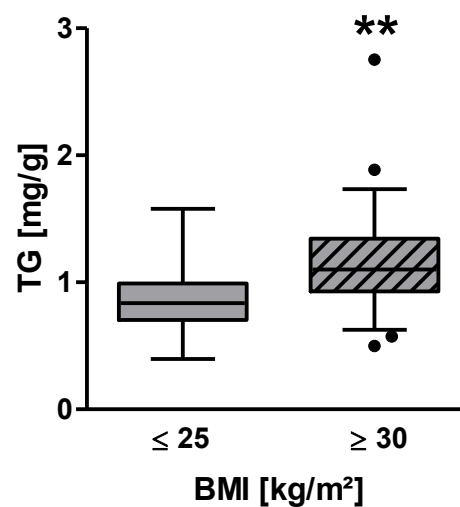


Figure 20: Placental triglycerides are elevated in pregnancies of obese mothers. TG concentration was measured by an enzymatic colorimetric method in placental biopsies

of lean ($\text{BMI} \leq 25 \text{ kg/m}^2$, $n=18$) and obese women ($\text{BMI} \geq 30 \text{ kg/m}^2$, $n=55$). Mann-Whitney U test was performed, ** $P < 0.01$.

4.2.3 Genes involved in lipid and FA-uptake and storage are affected by maternal obesity

In order to examine influence of maternal obesity on expression of genes related to uptake, storage, metabolism, and transfer of lipids and FAs, a target specific, quantitative gene expression assay (nCounter technology) was performed. Genes were selected based on literature search and are listed in Supplemental Table 1 (Appendix section). In total, 34 housekeeping genes were validated for their adequacy to study the influence of maternal pre-pregnancy obesity on placental gene expression. Therefore, the study population was divided into four groups according to maternal pre-pregnancy BMI ($\leq 25 \text{ kg/m}^2$, $30\text{-}34.9 \text{ kg/m}^2$, $35\text{-}39.9 \text{ kg/m}^2$, $\geq 40 \text{ kg/m}^2$) and one-way ANOVA analysis was performed. Six housekeeping genes were identified to be regulated by maternal obesity and were excluded from further analysis. In total, target gene expression was normalized to a panel of 28 not by obesity regulated housekeeping genes, as listed in the Supplemental Table 2 (Appendix section), by using the nSolver software. Among the examined target genes, ATGL, CGI-58, FATP1, FATP3 and PLIN2, PPARG and S1PR1 showed significant positive correlations with maternal pre-pregnancy BMI. In contrast, APOE correlated negatively with maternal pre-pregnancy BMI (Table 12). Since ATGL, CGI-58 and PLIN2 are directly associated with intracellular lipid droplets (LD) and therefore TG metabolism, mRNA abundance of the main other members of the PLIN family were tested. As demonstrated in Figure 21, PLIN2 and PLIN3 were exclusively expressed in placental villous tissue among all five family members (PLIN1-5). An increased PLIN2 gene expression was confirmed by qRT-PCR in the obese group ($\text{BMI} \geq 30 \text{ kg/m}^2$).

Table 12: Placental genes involved in lipid metabolism are related to maternal pre-pregnancy BMI.

Gene	Spearman correlation	Statistics
ATGL	0.248	P = 0.034
CGI-58	0.326	P = 0.005 #
FATP1	0.238	P = 0.042
FATP3	0.245	P = 0.037
PLIN2	0.258	P = 0.028
PPARG	0.278	P = 0.017
S1PR1	0.240	P = 0.041
APOE	-0.243	P = 0.028

Target specific gene expression analysis in placental tissue biopsies (n=73) was performed by nCounter technology or qRT-PCR (#). Maternal pre-pregnancy BMI ranged between 20 – 64 kg/m². Spearman correlation was performed between maternal pre-pregnancy BMI and targeted genes. P-values < 0.05 were defined as statistical significant. This table was adapted from Hirschmugl et al. Int J Obes (Lond). 2017 Feb;41(2):317-323; (116).

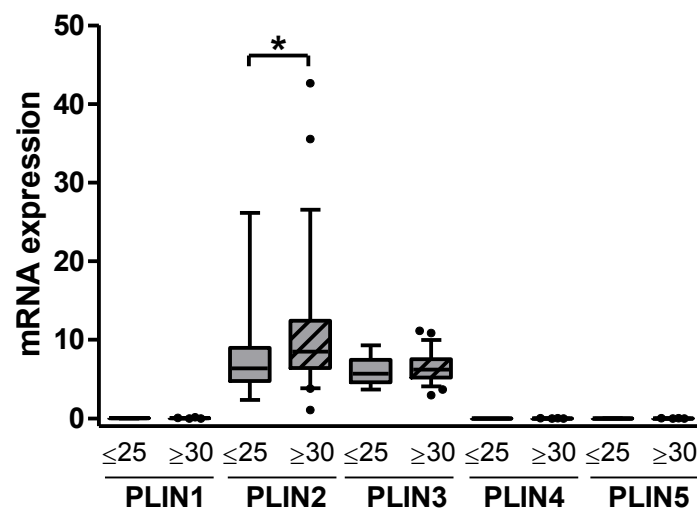


Figure 21: PLIN2 and PLIN3 are exclusively expressed in the placenta. qRT-PCR was performed in order to determine mRNA expression of LD associated members of the PLIN family in placenta tissue biopsies. mRNA expression was normalized to TBP (housekeeping gene) and displayed as non-logarithmic values. Mann-Whitney U sum rank

test was performed, differences between the lean ($\text{BMI} \leq 25 \text{ kg/m}^2$, $n=18$) and obese group ($\text{BMI} \geq 30 \text{ kg/m}^2$, $n=55$) were defined as significant if p-values were < 0.05 (*). This picture was adapted from Hirschmugl et al. *Int J Obes (Lond)*. 2017 Feb;41(2):317-323; (116).

4.2.4 Maternal obesity is associated with an up-regulation of the ATGL co-activator CGI-58

Localisation of the LD-associated proteins PLIN2, PLIN3, ATGL and GCI-58, were determined in placental term tissue. Furthermore, PLIN2, PLIN3, ATGL and GCI-58 protein abundance was examined by using immune blotting method. The two members of the LD covering protein-family, PLIN2 and PLIN3, are localised mainly in the syncytiotrophoblast of the human placenta. PLIN2 showed a punctate staining (Figure 22A), whereas staining for PLIN3 was distributed across the cytoplasm (Figure 22B). The triglyceride lipase ATGL was dispersed localized within the placental syncytium (Figure 22C). In order to visualize the syncytiotrophoblast layer, cytokeratin 7 was used (Figure 22D). Equal concentrations of rabbit IgG (Figure 22E) and mouse IgG (Figure 22F) were used as a negative control. Despite intensive efforts of testing different antibodies, detection of CGI-58 by IHC was not successful.

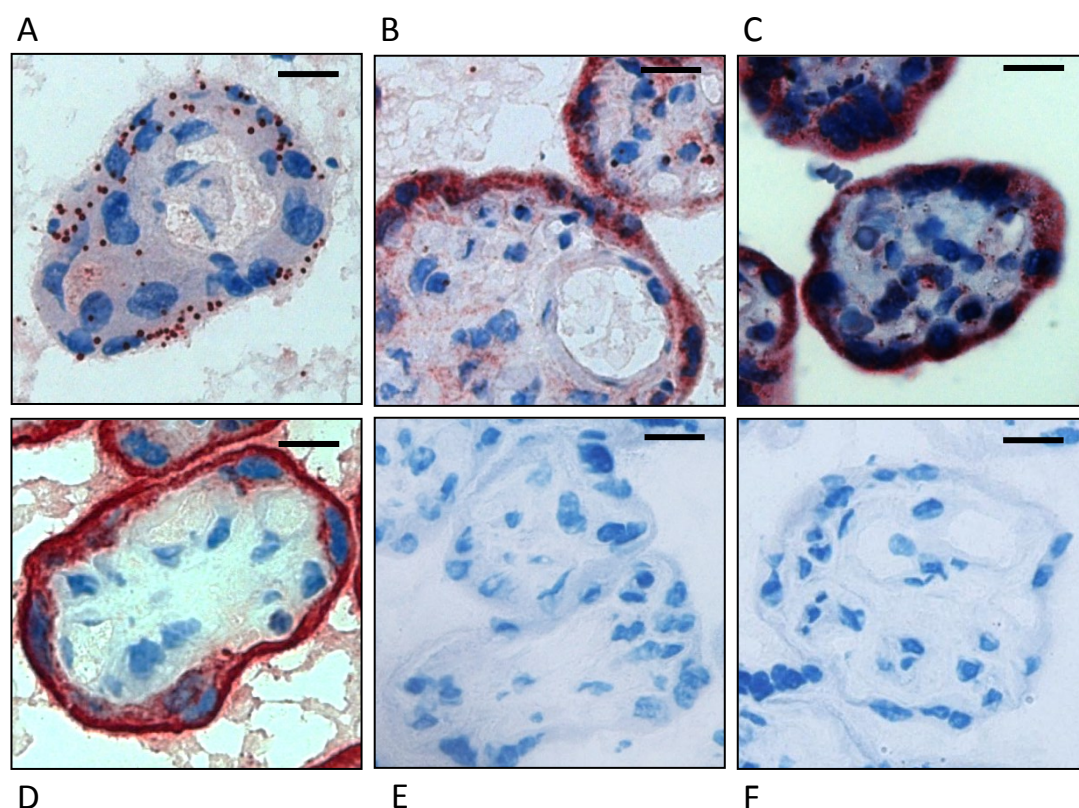


Figure 22: Localization of PLIN2, PLIN3 and ATGL in term placental tissue. **A:** PLIN2 was found in the syncytiotrophoblast by a punctate staining. **B, C:** PLIN3 and ATGL were mainly detected and equally distributed in the syncytium. **D:** Cytokeratin 7 served as positive control for localization of placental syncytium. Negative staining, with equal IgG concentrations, for rabbit IgG (**E**) and mouse IgG (**F**) was performed. Bar scale 50 μ m. This picture was adapted from Hirschmugl et al. *Int J Obes (Lond)*. 2017 Feb;41(2):317-323; (116).

The protein content of PLIN2 and PLIN3 in the obese group was unchanged in comparison to the lean group (Figure 23A and 23B). Although, placental PLIN2 mRNA correlated positively with maternal pre-pregnancy BMI and plasma insulin determined around time of delivery, PLIN2 protein did not (Figure 24A and 24B). Furthermore, the protein content of ATGL was comparable in placentas of lean and obese women (Figure 23C). In contrast, CGI-58 protein was significantly higher expressed in the obese compared to the lean group (Figure 23D). This was in line with significantly positive correlation of CGI-58 mRNA and protein with maternal pre-pregnancy BMI as well as maternal insulin levels at term (Figure 24C and 24D).

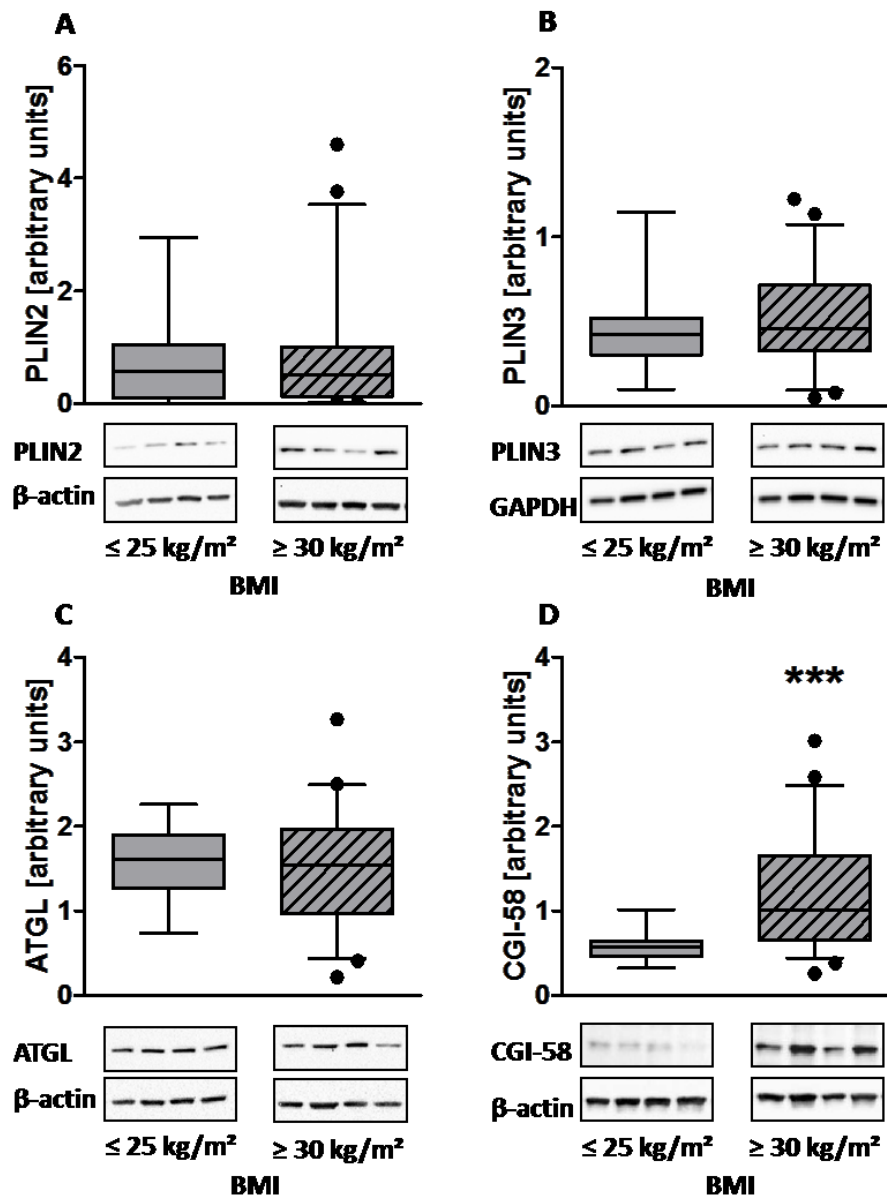


Figure 23: Lipid droplet associated relative protein expression in placental tissue of lean compared to obese mothers. **A:** PLIN2 protein expression in placentas of normal and obese women **B:** PLIN3 protein expression in placentas of normal and obese women. **C:** Abundance of ATGL protein expression in lean compared to obese placentas. **D:** Different CGI-58 protein expression between BMI-groups. Lower panels: Representative Western blots (n=4 per group) of term placental tissue. All protein signals were quantified by densitometry, normalized to β -actin or GAPDH. Mann-Whitney U sum rank test was performed, differences between the lean (BMI $\leq 25 \text{ kg/m}^2$, n=18) and obese (BMI $\geq 30 \text{ kg/m}^2$; n=45) group were defined as significant if P-values were < 0.05 . *** P < 0.001 . This picture was adapted from Hirschmugl et al. *Int J Obes (Lond)* 2017 Feb;41(2):317-323; (116).

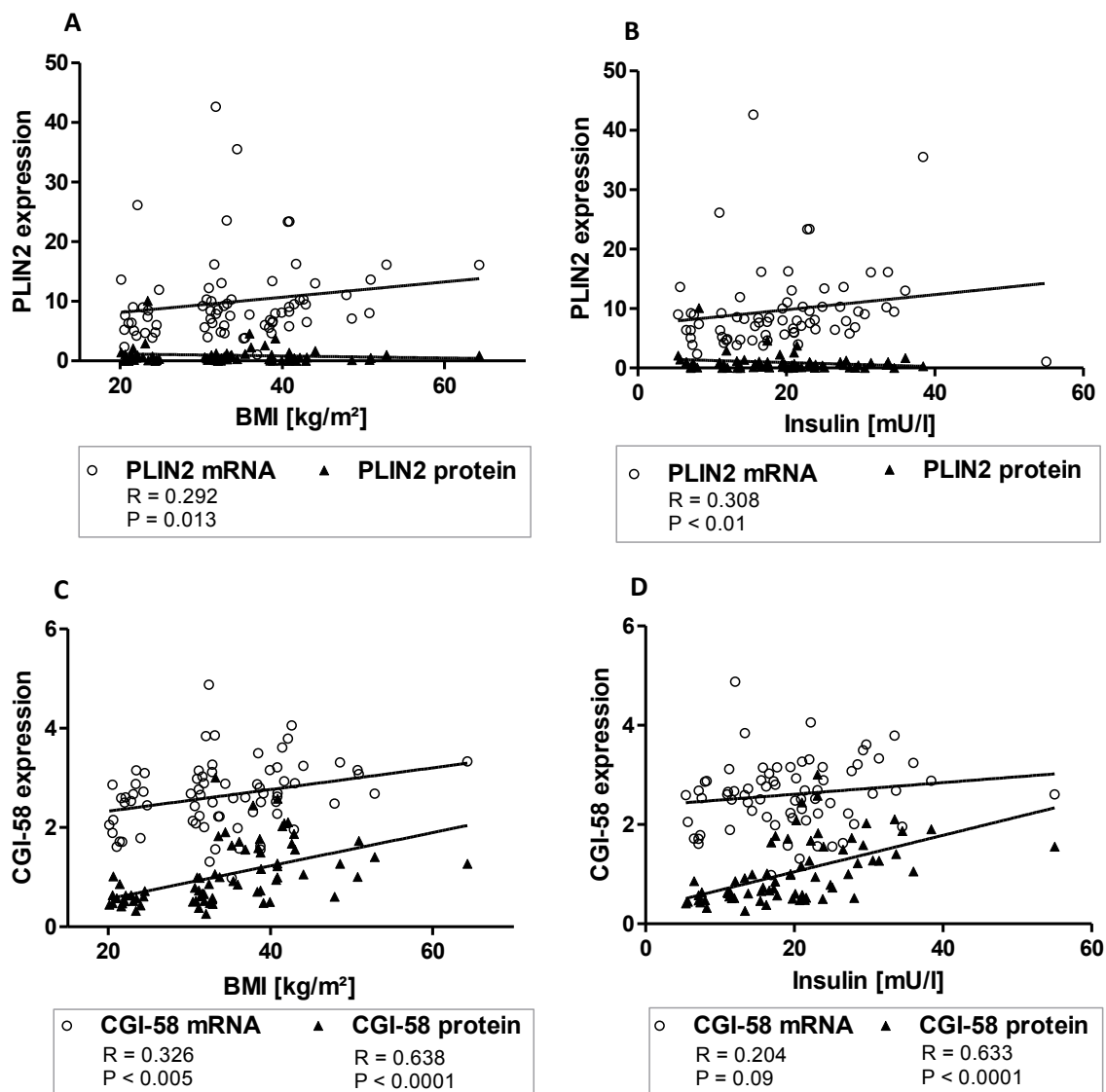


Figure 24: Association of placental PLIN2 and CGI-58 mRNA and protein with maternal insulin levels at term and maternal pre-pregnancy BMI. Correlation analysis was performed between PLIN2 mRNA or protein expression in placental tissue and maternal pre-pregnancy BMI (A) or maternal plasma insulin (B). CGI-58 mRNA and protein levels were correlated with maternal pre-pregnancy BMI (C) and maternal plasma insulin levels (D). Black triangles (▲) protein expression, open circles (○) mRNA expression, n=73. Spearman correlation was defined as significant if P-values were < 0.05. This picture was adapted from Hirschmugl et al. *Int J Obes (Lond)*. 2017 Feb;41(2):317-323; (116).

5 Discussion

The *ex-vivo* perfusion of the human placenta is a powerful method for studying maternal-to-fetal transport of numerous synthetic and biological substances. While findings of such experiments on an intact organ are most significant, this sophisticated method is also linked to several experimental challenges. Therefore, before starting with specific experiments it is mandatory to define and validate criteria and checkpoints in order to guarantee reproducibility of such experiments. The time between delivery of the placenta and first flushing of the selected fetal capillary is the first critical phase. Therefore, a time period of 20 min must be strictly adhered to for establishing fetal circulation and to eliminate the risk of vessel blood clotting. Once the placenta is fixed in the perfusion chamber the recirculated and recovered volume of the fetal perfusate is also one measurable parameter reflecting the integrity and tightness of the fetal circulation. A recovery of >95% of the initial volume over all experiments was determined which is in accordance to published results (95 - 98%) of other laboratories (119,120).

After establishing the maternal circulation by inserting blunted needles, oxygen supply to the fetal circulation is initiated. Thus, oxygen levels in fetal vein start to increase and exceed that of fetal artery or at least should be equal. Otherwise, it is necessary to rearrange the catheters on the maternal side of the tissue (120). Sufficient maternal to fetal oxygen supply is a marker for a good overlap of the maternal and fetal circulation. In addition, transfer of antipyrine, a small lipophilic substance, gives further information about the degree of overlap of maternal and fetal exchange area. Antipyrine easily passes membranes passively and thus transfer rate and steady state concentration provides reliable information of the quality and success of the experiment. In the present study the transfer of antipyrine was examined by using maternal and fetal open circulation set-up for 30 min and the calculated FM ratio of 0.30 was achieved in all experiments. This observed mean FM value is in the range of previously published antipyrine FM ratios (0.26 – 0.42) (121).

Integrity of perfused fetal capillaries and an efficient oxygenation of the tissue as well as a proper FM antipyrine transfer are crucial checkpoints for a reproducible perfusion

experiment. In addition, further systemic parameters of perfusion media like lactate or glucose levels provide data sets on the metabolic condition of the perfused tissue. These parameters were also determined by several groups working in the field (67,120,121). In particular important is the consecutive monitoring of the pH in the maternal and fetal perfusates and its adjustment to physiological ranges (pH 7.2 - 7.5). Adjustments are achieved by either adding hydrochloric acid or sodium hydroxide to the perfusion media during ongoing perfusion experiment (67,120,121). Moreover, in our study adjustment of pH was conducted by introduction of a gas mixture containing CO₂ in the maternal circulation. In general, a physiological pH is indispensable for a variety of cellular uptake and transport mechanisms in order to guarantee sufficient and efficient binding capacity of ligands to transport proteins or receptors. In human primary trophoblasts LCPUFA uptake was tested by using cell culture medium with different pH values. The increase from pH 7.5 to pH 8.5 reduced the uptake capability of arachidonic acid and DHA by 40% and 17%, respectively (85). In line with this outcome, the perfusion experiments in present study showed also reduced direct transfer of unsaturated FAs, in particular of DHA suggesting rising pH values when the maternal perfusates were not fumigated with CO₂ and thereby the bicarbonate buffer system collapsed. As a consequence, the first five perfusion experiments were excluded from further analysis with respect to maternal obesity and FFA transfer.

The main and novel finding of this study is that DHA-transfer from the maternal to the fetal circulation was significantly elevated in obese compared to lean placentas. The total transfer of palmitic, linoleic, and oleic acid tends to be higher in placentas of obese women, but not significantly which is probably due to the high biological variances between different placentas and hence low number of subjects to be included. Comparable results were found for arachidonic acid transfer across the placenta in pregnancies accompanied by insulin dependent diabetes mellitus. Although, the women had well controlled glucose levels at term, the transfer for arachidonic acid was significantly increased compared to control placentas by using also *ex-vivo* perfusion approach (122). Recently, Pagan and colleagues (123) supplemented pregnant women 12 h prior to caesarean section with a single dosage of ¹³C-labelled FA (palmitic, oleic,

linoleic acid, and DHA). Tracer enrichment and distribution in women with GDM in comparison to normal glucose tolerant women was investigated. Enrichment of oleic and linoleic acid after 12 h was similar in maternal plasma, placental tissue, and cord blood. Interestingly, GDM affected ^{13}C palmitic acid enrichment which was significantly higher in placental tissue and arterial cord blood compared to normal glucose responders. In contrast, ^{13}C DHA enrichment was significantly reduced in maternal plasma, placental tissue and cord blood by GDM compared to controls (123). One explanation of these findings might be that fetuses of GDM mothers receive less DHA, but it is also likely that fetal adipose tissue is more active in fatty acid storage and this leads to lower cord blood DHA levels. Furthermore, depending on the metabolic condition of the mother and the unborn the capability to store or to metabolize FAs actively changes *in vivo*, which indicates the complexity of lipid metabolism in the mother, placenta and fetus during pregnancy. Additionally, this all points to the complexity of such studies in which direct transfer of FAs from the mother to the fetus were determined.

In our study, the placental transfer of DHA is elevated by obesity, which is explainable by higher demands of LCPUFA to the fetus in order to balance the pro-inflammatory and unfavourable *in utero* environment. Recently, Haghiac et al. (104) demonstrated that obese pregnant women, which received n3 FAs (DHA and eicosapentaenoic acid) supplementation during pregnancy resulted in diminished expression of inflammatory genes in maternal adipose tissue, and in the placenta as well as in decreased maternal CRP-levels (104). In addition, the total lipid content in placental tissue was significantly lower because of reduced FA ester production within placental tissue (124). While saturated FAs induce pro-inflammatory gene expression in isolated trophoblast and adipose tissue cells, n3 FAs are able to diminish this effect (104). In order to balance inflammatory responses within placental tissue DHA and eicosapentaenoic acid facilitates the production of lipid mediator precursors like resolvins (97). In line, DHA and other n3 FAs are able to reduce the secretion of pro-inflammatory cytokines at least in the mother and the placenta (104,125). Therefore, it is very likely that the anti-inflammatory action of DHA persists also in the fetus. The general preference of DHA transfer across the placenta and the even elevated transfer in placentas of obese women, may mainly support fetal brain and retinal development, but also has the capability to regulate inflammatory

processes in the fetus. If the positive and exclusive effect of DHA is potent enough to protect the fetus from the lipotoxic and pro-inflammatory environment in obese pregnancies is still questionable and needs further investigation.

One very interesting result of the present study is that there is a difference in direct FFA transfer related to fetal sex. In general, there is a trend of higher transfer of the examined FFAs in placentas of girls compared to boys. In particular, boys of obese mothers tend to receive less DHA and other FAs than girls. In accordance with our observation Brass et al. (101) published that the placental uptake of oleic and arachidonic acid in male placentas was reduced compared to female placentas if the mother was obese. In contrast, the uptake rates for oleic and arachidonic acid were higher in female placentas of obese mothers. The group did not find any differences with respect to DHA uptake in placental explants (101). Since the used concentration of these experiments exceeds plasma levels for DHA in FFA fraction by the factor 45 - 170 (126,127), it is likely that involved transport mechanisms are overwhelmed by offered FFAs, thereby generating a non-physiological environment to study transport mechanisms. However, male fetuses receive lower amounts of FAs, in particular LCPUFA, than female fetuses and this phenomenon is even more pronounced if pregnancy is complicated by maternal obesity.

Recently, it was shown that maternal pre-pregnancy weight and BMI are strong predictors for neonatal adiposity in boys but not in girls. However, adiposity in female infants is associated with maternal inflammatory markers (128) suggesting that male and female fetuses respond differently to changes in maternal physiology. Obese mothers have altered plasma FA-profiles with elevated saturated, monounsaturated and n6 FAs and reduced n3 FAs (129), very likely due to imbalanced dietary habits. It is supposed, that boys are more sensitive to changes of maternal diet because the placental preserve capacity is more restricted than that of female placentas and thus male fetuses are more directly dependent on maternal nutrient supply (130).

Under the chosen experimental condition, direct transfer of DHA from the maternal to the fetal circulation is dependent on the DHA concentration offered on the maternal side. This has been demonstrated by using different DHA concentrations within one placenta perfusion experiment, but also among different individual placentas. There is evidence in

literature that LCPUFA distribution in PLs and TGs of maternal plasma correlates with cord blood plasma levels (131), which would support my results. In the present study, the offered FFA concentrations were relatively low (97.8 μ M) in comparison to previously determined maternal plasma FFA concentrations (320 – 670 μ M) (22,132,133). Given the fact that these used FFA concentrations exceed the K_d -values of several binding proteins at the maternal-fetal interface manifold, this approach does not allow to study concentration dependent FA-uptake to the placenta. However, at least at low concentrations, DHA-transport across the human placenta is highly concentration dependent which argues for a specific protein or receptor dependent tightly regulated uptake mechanism. The FFA concentrations were chosen in proportion to the FFA levels of maternal plasma and plasma albumin as important FA carrier protein in the third trimester of pregnancy. The molar ratio of albumin to FFA used in this study (0.76) was in the range of previously published results, in particular between 0.72 (22) and 0.78 (99). The selected composition of FFAs reflects the FA composition of plasma TG (99). Triglycerides are transported in plasma as VLDL and LDL particles and are the main source of FAs for the placenta. Plasma TGs are composed of approximately 40% saturated FAs, 44% monounsaturated FAs, 1% trans-FAs (origin from hydrated lipids in dietary lipids), 14% n6 FAs, 0.3% DHA and 0.7% other n3 FAs (127). The here chosen DHA concentration reflects 0.3% of total offered FFAs (97.8 μ M) and the other FAs were offered in similar values as Berghaus et al. (127) published for maternal plasma TGs at term.

Among the examined FAs, DHA has the highest FM ratio followed by linoleic, palmitic, and oleic acid irrespective of maternal pre-pregnancy obesity. In line with the FM ratio the total transfer of DHA is also the highest among all FAs and in particular significantly higher compared to oleic acid in the lean and obese group. This finding is in agreement with previous perfusion experiments by Haggarty et al. (99) in which the transfer of DHA was higher than for α -linolenic, linoleic and arachidonic acid normalized to oleic acid transfer results (99). The phenomenon of LCPUFA preferentially transferred to the human placenta is known as 'biomagnification' in the literature. Kuhn and Crawford (134) demonstrated that the transfer rate for linoleic acid is double- to threefold of that for arachidonic acid by using the *ex-vivo* perfusion model. Interestingly, linoleic acid cumulates in the fetal circulation in the free fatty acid form whereas 60% of the

transferred arachidonic acid incorporates into phospholipids. Moreover, arachidonic acid serves as an important substrate for prostaglandin production (PGE_2 , PGI_2) which are mainly released into the maternal circulation. It is likely, that the human placenta has different routes to provide the fetus with FAs. In particular, FFAs are able to cross via facilitated transport the placenta whereas FAs, incorporated into PLs, CE or TGs are restricted to the placenta and are further utilized. However, PL can also reach the fetal circulation (134), which was also shown in the present study by a yet unknown mechanism.

In the present study, palmitic, oleic and linoleic acid were mainly transferred to the fetal circulation as PLs and FFAs. Interestingly, only palmitic acid was incorporated and detectable in the CE fraction. This is in agreement with the findings of Kuhn and Crawford (134) and accounts for individual compartmentalization of different FAs with varied options for further utilization by the fetus and the placenta. The higher levels of FAs in PLs compared to FFAs presented here, are understandable by the fact that endogenously derived placental FAs and offered FFAs were simultaneous determined. In opposite, Kuhn and Crawford measured radioactivity of the tracer in the different lipid species without taking into account the endogenous placental release of FAs.

However, the percentage of directly transferred FFAs to the fetal compartment was unexpected low for all examined FFAs in the lean and obese group related to the offered concentrations. Similar results were obtained by Dancis and colleagues (60) when they examined palmitic acid transfer of the perfused placenta with transfer rates comparable to antipyrine diffusion between 1.6 and 6.5%. The authors estimated that placental FA-transfer contributes only 20% to FA levels in fetal fat depots at term, and about 80% are generated by endogenous FA synthesis in the fetal liver from glucose (60). In this presented study, the placental uptake of all examined FAs fluctuated between 27 and 45% of the initially offered FA concentration in the maternal circulation after 90 min perfusion time. The fact, that direct transfer was about 10-fold lower (1.8 and 4.9% of the labelled FFAs offered in maternal circulation) than placental uptake, leads to the conclusion that the majority of FAs in the fetal circulation either is provided by a lipid pool composed of maternally or endogenously derived FAs within the placenta or directly utilized by the placenta itself. This is also supported by the finding that the placenta

releases endogenously derived FAs towards the fetal circulation, indicating that the placental lipid stores are purged during the perfusion experiment. The overall FA transfer to the fetus is probably a product of constant endogenous release of FAs from placental lipid pools and additionally direct transfer from the mother.

The study has some limitations. First of all, to determine the ^{13}C -tracer in different lipid species of fetal effluents and placenta tissue was not achievable. Although technically feasible, these measurements would have exceeded the scope of this PhD thesis. Furthermore, only one distinct FFA mixture bound to albumin was offered to the maternal circulation. It would have been interesting to examine the transfer of labelled FA incorporated into reconstituted lipoproteins such as very low density lipoprotein or low density lipoproteins. Again, the analytical methods to detect FA as tracer in other lipid species than FFA were not available at the core facility for mass spectrometry together with the fact that such an additional objective would have exceeded the focus of my thesis. For reasons to calculate associations between subject data and FA results the cohort was stratified into lean and obese group and further by fetal sex. But stratification leads to low subject number of one distinct group e.g. fetal sex; the lean group comprised only two placentas of boys which made it impossible to perform comparable statistics for male subjects.

All results discussed in this part of the thesis are published by Hirschmugl et al. (116).

Lipid metabolism of the mother changes during pregnancy in order to guarantee placental and fetal development and growth. The present study demonstrated that placental TGs are elevated when the mother is obese although maternal plasma TG levels are unchanged. In the literature conflicting data exists whether maternal plasma TG levels are elevated (22) or similar (23) in obese women close to delivery. In contrast, data about elevated TG levels in the placenta are corroborated by previous published results on increased total lipids in placentas of obese women (22,24). Therefore, maternal plasma TG levels can be excluded as a causative factor accounting for elevated lipid deposit in the placenta. Thus, maternal obesity itself, or other physiological changes than plasma TG,

facilitates placental lipid uptake by elevated lipase activity (22) or adapted assignments of intracellular lipid metabolism.

In this study six genes, associated with TG metabolism (FATP1, FATP3, PPARG, CGI-58, ATGL, PLIN2), were found to be positively correlated with maternal pre-pregnancy BMI. Fatty acid transport protein 1 and 3 belong to the solute carrier family 27 (SLC27A), which is important for FA uptake (135). Several members of the SLC27A, including FATP1, were found to be expressed in the human placenta (22,84-86). The mechanism of FA uptake by FATPs and their specificity for different FA species is still under debate.

In order to get new insights into placental lipid storage the present study focused on CGI-58, ATGL, and PLIN2 which expressions all positively correlated with maternal pre-pregnancy BMI. All three proteins are involved in intracellular lipid metabolism and mobilization of FAs in LDs. The importance of LDs in the human placenta is supported by *in vitro* experiments in isolated trophoblasts. Conditions mimicking the obese environment of the mother resulted in elevated TG levels and LD formation (23,136). The present study examined for the first time CGI-58 mRNA and protein expression in the human placenta under metabolic conditions of maternal obesity. The most important finding is that maternal pre-pregnancy BMI and maternal plasma insulin levels at term strongly correlate with placental CGI-58 protein but also mRNA levels. CGI-58 is an important co-activator of ATGL which is involved in hydrolysis of intracellular lipid storage pools. Almost all cell types in the human body are able to remove excessive intracellular lipids from the cytoplasm and capable to store temporary these lipids in LDs which are specialized organelles. The process of cellular lipid storage and remobilization of FFAs from LDs was extensively examined in adipocytes. In particular, the first step of TG hydrolysis in LDs is tightly regulated and involves the TG-lipase ATGL (137). ATGL exerts its maximal lipolytic activity if CGI-58 is in close proximity to ATGL under insulin stimulated conditions, but CGI-58 itself does not have functional TG-lipase activity (41).

Only little knowledge exists about the expression and function of CGI-58 and ATGL in human placenta to date. One study demonstrated that ATGL is expressed in placenta tissue by Western blot, confirmed by positive ATGL-staining of the syncytiotrophoblast and fetal capillaries (138). The results of the present study partly confirmed that the

syncytiotrophoblast is the major site of ATGL protein synthesis within the human placenta. Furthermore, PLIN2 and PLIN3, two members of the LD associated perilipin protein-family, are localized in the syncytiotrophoblast layer. In previous studies, PLIN2 was found in placental fetal membranes (139) and the amnion epithelium (140), which let suggest that PLIN2 play a role in placental lipid metabolism. In adipose tissue PLIN1 is the major LD covering protein but other tissues express alternative members of the perilipin protein family (PLIN 2-5). Patel et al. (141) demonstrated that PLIN2 and PLIN3 exhibit differences in the C-terminal phosphorylation side compared to PLIN1. Therefore, PLIN2 and PLIN3 are only partly able to sequestrate CGI-58. Thus suppression of basal lipolytic activity of ATGL is un-effective and TG hydrolysis in LDs is constantly active (141).

In summary, the significant higher CGI-58 protein levels detected in obese placentas suggest that the lipid mobilization in obese placentas is elevated. This is further supported by the fact that PLIN2 and PLIN3 are expressed in the human placenta and both have reduced protective properties in respect to LD hydrolysis compared to PLIN1. Maternal pre-pregnancy BMI and in particular increased systemic insulin levels are associated with placental CGI-58 protein suggesting a direct effect on placental lipid metabolism.

There are some limitations of the study worthwhile mentioning. Pregnancy is a state of continuous metabolic changes during 9 months, but to investigate placental metabolism at the end of pregnancy does not reflect spatiotemporal changes in the 1st and 2nd trimester of pregnancy. This observational part of the study showed that maternal pre-pregnancy BMI and maternal insulin levels at birth are related to placental lipid metabolism, but further investigations are needed to proof direct causality.

5.1 Conclusion

In conclusion, the results of my thesis suggest that FFA transport across the human placenta is a complex, highly dynamic and regulated process, which depends on more than one distinct route (Figure 25). To guarantee sufficient FFA supply of the fetus at least three interconnected mechanisms contribute to this process. First, a direct efficient transfer of FFA from the mother to the fetus across the placenta takes place. Second, an uptake of maternally derived FFA into a placental intracellular lipid pool may occur concomitantly, out of which FFA can be mobilized again, and thereby providing FFAs to the fetus. In addition, the placenta itself is able to release mainly PLs into the fetal circulation.

Notably, direct placental FFA transfer is tightly associated with pre-pregnant BMI of the mother. Among the investigated FFAs, only DHA-transfer is significantly increased. Across placentas of female fetuses this DHA transport is higher compared to males in late pregnancy, indicating that already *in utero* sexual dimorphism is verifiable. Not only FFA transfer across the placenta is altered, but also placental lipid metabolism itself is affected by maternal obesity. Placentas of obese women store more lipids, which is reflected by elevated TG levels, due to excessive lipid supply by the mother and/or enhanced uptake capacity/expression by the placental FA-transporters such as FATP1 and FATP3. More importantly, stored lipids (TG) in placental lipid pools can be mobilized by ATGL which unfolds its maximal lipolytic activity in close cooperation with CGI-58. CGI-58 is directly associated with maternal insulin levels in the obese state. Thereby, high placental levels of CGI-58 cause activation of ATGL and enhanced FFA mobilization from placental lipid pools in placentas of obese mothers. If the mother is obese, this process would enhance FFA supply to the fetus *in utero* and further might result in elevated adipose tissue of the newborn.

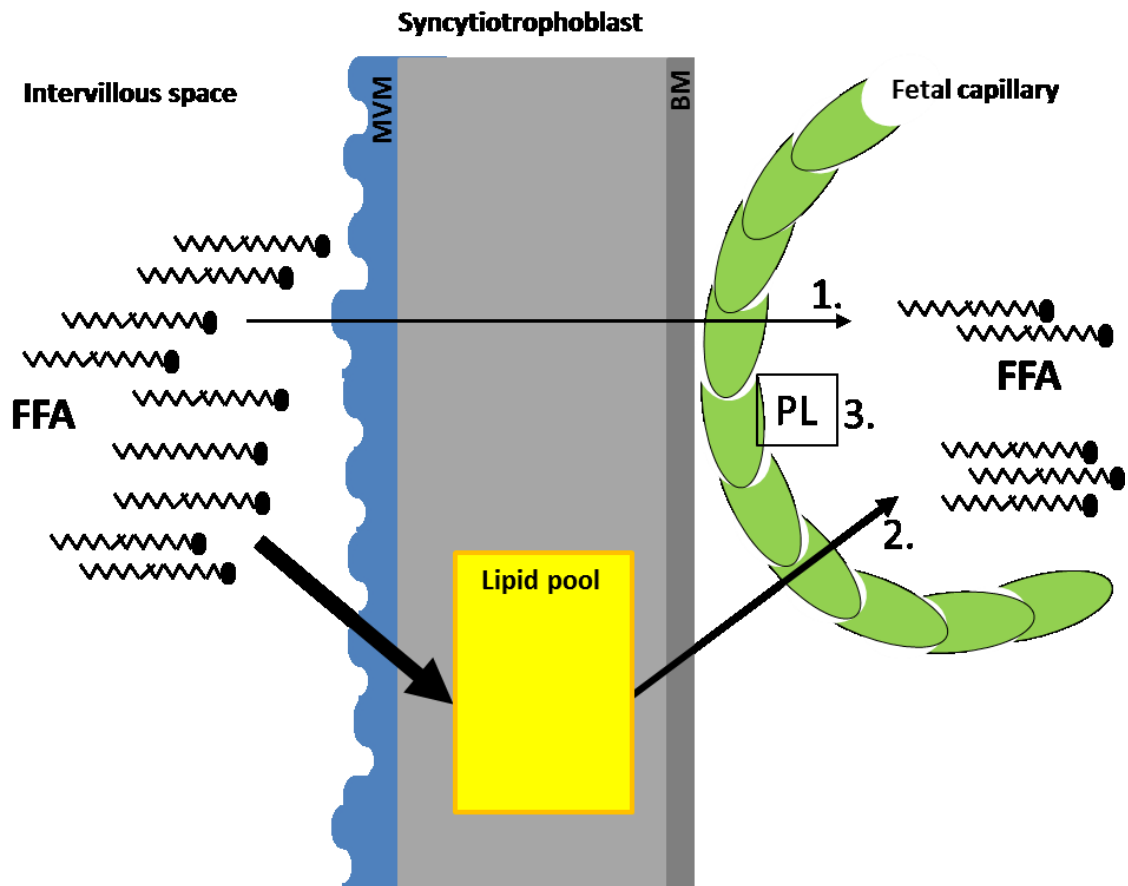


Figure 25: FFA transfer across the placenta follows multiple routes. FFAs are transferred directly after hydrolysis of maternal lipoproteins across the syncytiotrophoblast layer to fetal capillaries (1.). Alternatively, maternally derived FFAs are taken up and stored into a lipid storage pool (2. lipid pool, yellow) within the syncytium. FFAs from the placental lipid storage pool are either used for placental metabolism or released and transported to the fetal circulation by a still unknown pathway. Additionally, placental phospholipids (PL) are released into the fetal capillary (3.). MVM: microvillous membrane, BM: basal membrane, FFA: free fatty acids.

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7 Appendix

Supplemental Table 1: Gene expression analysis

	Lipid, FA binding/uptake; lipoproteins	Gene	Gene ID	Spearman	P-value
1	LDL receptor	LDL-R	3949	-0.025	0.837
2	VLVL receptor	VLDL-R	7436	-0.049	0.680
3	Scavenger receptor class B, member 1 (SRB1)	SCARB1	949	0.198	0.094
4	Fatty acid transport protein 1	FATP1	376497	0.238	0.042
5	Fatty acid transport protein 2	FATP2	11001	0.005	0.965
6	Fatty acid transport protein 3	FATP3	11000	0.245	0.037
7	Fatty acid transport protein 4	FATP4	10999	0.144	0.225
8	Fatty acid transport protein 5	FATP5	10998	n.d.	n.d.
9	Fatty acid transport protein 6	FATP6	28965	-0.001	0.996
10	CD36 molecule, fatty acid translocase, FAT	CD36	948	-0.115	0.333
11	Glutamic-oxaloacetic transaminase 2, mitochondrial	GOT2	2806	0.202	0.087
12	Fatty acid binding protein 1, liver	FABP1	2168	n.d.	n.d.
13	Fatty acid binding protein 3, heart	FABP3	2170	n.d.	n.d.
14	Fatty acid binding protein 4, adipocyte	FABP4	2167	n.d.	n.d.
15	Fatty acid binding protein 7, brain	FABP7	2173	n.d.	n.d.
16	Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1	GPIHBP 1	338328	n.d.	n.d.
17	Apoprotein A1	ApoA1	335	n.d.	n.d.
18	Apoprotein C2	ApoC2	344	n.d.	n.d.
19	Apoprotein C3	ApoC3	345	n.d.	n.d.
20	Apoprotein A4	ApoA4	337	n.d.	n.d.
21	Apoprotein A5	ApoA5	116519	n.d.	n.d.
22	Apoprotein E	ApoE	348	-0.243	0.038
23	Apoprotein M	ApoM	55937	n.d.	n.d.
24	Sphingosine-1-phosphate receptor 1	S1PR1	1901	0.240	0.041
25	Sphingosine-1-phosphate receptor 3	S1PR3	1903	-0.039	0.741
26	ATP-binding cassette transporter A1	ABCA1	19	0.067	0.572
27	ATP-binding cassette transporter G1	ABCG1	9619	-0.072	0.545
	Lipid storage/esterification	Gene	Gene ID	Spearman	P-value
28	Perilipin 2, adipophilin, ADRP	PLIN2	123	0.258	0.028
29	Perilipin 5, OXPAT	PLIN5	440503	n.d.	n.d.
30	Perilipin 1	PLIN1	5346	n.d.	n.d.

	Lipid storage/esterification	Gene	Gene ID	Spearman	P-value
31	Abhydrolase domain containing5, CGI-58	ABHD5	51099	-0.167	0.158
32	Fat mass and obesity associated	FTO	79068	-0.014	0.906
33	Lipoprotein lipase	LPL	4023	-0.088	0.458
34	Endothelial lipase	LIPG	9388	0.074	0.531
35	Hepatic lipase	LIPC	3990	n.d.	n.d.
36	Adipose triglyceride lipase	ATGL	57104	0.248	0.034
37	Monoacylglycerol lipase	MGLL	11343	0.065	0.583
38	Diacylglycerol lipase –alpha	DAGLA	747	n.d.	n.d.
39	Diacylglycerol lipase –beta	DAGLB	221955	0.218	0.064
40	Hormone sensitive lipase	LIPE	3991	n.d.	n.d.
41	Phospholipase A2 group 7	PLA2G7	7941	n.d.	n.d.
42	Phospholipase A2 group 2A	PLA2G2A	5320	-0.161	0.175
43	PAF receptor	PAFR	5724	-0.161	0.174
44	Cholesterol ester hydrolase	CEH	1066	-0.085	0.475
	FA elongation/desaturation/oxidation	Gene	Gene ID	Spearman	P-value
45	Elongase 1	ELOVL1	64834	-0.159	0.178
46	Elongase 2	ELOVL2	54898	-0.041	0.729
47	Elongase 3	ELOVL3	83401	n.d.	n.d.
48	Elongase 4	ELOVL4	6785	n.d.	n.d.
49	Elongase 5	ELOVL5	60481	n.d.	n.d.
50	Elongase 6	ELOVL6	79071	n.d.	n.d.
51	Elongase 7	ELOVL7	79993	0.031	0.795
52	Stearoyl-CoA desaturase (delta-9-desaturase)	SCD	6319	-0.070	0.559
53	Fatty acid desaturase 1	FADS1	3992	-0.048	0.685
54	Carnitine palmitoyltransferase 1A (liver)	CPT1A	1374	-0.119	0.316
55	Carnitine palmitoyltransferase 1B (muscle)	CTP1B	1375	n.d.	n.d.
56	Carnitine palmitoyltransferase 1C (brain)	CTP1C	126129	n.d.	n.d.
57	Carnitine palmitoyltransferase 2	CPT II	1376	0.085	0.476
58	Acyl-CoA-Synthase long-chain family member 3	ACSL3	2181	-0.008	0.949
59	Acyl-CoA-Synthase long-chain family member 5	ACSL5	51703	0.035	0.766
60	Acetyl-CoA acyltransferase 2	ACAA2	10449	0.068	0.568

	Hormons; Transcription factors	Gene	Gene ID	Spearman	P-value
61	Leptin	LEP	3952	n.d.	n.d.
62	Leptin receptor	LEPR	3953	-0.007	0.950
63	Adiponectin	ADIPOQ	9370	n.d.	n.d.
64	Adiponectin receptor1	ADIPOR1	51094	0.033	0.783
65	Adiponectin receptor2	ADIPOR2	79602	-0.112	0.347
66	Peroxisome proliferator-activated receptor alpha	PPARA	5465	-0.061	0.609
67	Peroxisome proliferator-activated receptor gama	PPARG	5468	0.277	0.018
68	Peroxisome proliferator-activated receptor delta (beta)	PPARD	5467	-0.025	0.833
69	Sterol regulatory element binding transcription factor 1	SREBP1	6720	0.204	0.084
70	Sterol regulatory element binding transcription factor 2	SREBP2	6721	0.188	0.112
71	cAMP responsive element binding protein 1	CREB1	1385	-0.047	0.692
72	cAMP responsive element binding protein 3	CREB3	10488	-0.066	0.577
73	cAMP responsive element binding protein 5	CREB5	9586	0.014	0.905

In total expression of 73 genes was examined in placental tissue specimens from women (n=73) with pre-pregnancy BMI between 20 – 64 kg/m². Spearman correlation between gene expression and maternal BMI was performed and P-values <0.05 were defined as statistical significant. n.d.: not detected. This table was adapted from Hirschmugl et al. Int J Obes (Lond). 2017 Feb;41(2):317-323; (116).

Supplemental Table 2: Housekeeping genes for NanoString analysis

Housekeeping genes suitable for analysis of obesity related genes			
Housekeeping gene (HKG)		Gene ID	ANOVA P-value
1	ANGEL1	23357	0.447
2	ATG4B	23192	0.093
3	C7orf26	79034	0.844
4	CLTC	NM_004859.2	0.212
5	CYC1	1537	0.143
6	GAPDH	NM_002046.3	0.559
7	HAUS2	55142	0.694
8	KCTD2	23510	0.137
9	KIAA2013	90231	0.782
10	NEK9	91754	0.379
11	OAZ1	4946	0.385
12	PAIP1	10605	0.872
13	PGK1	NM_000291.2	0.796
14	PPIA	5478	0.875
15	PPP1R10	5514	0.906
16	RING1	6015	0.100
17	RNF220	55182	0.626
18	RPL30	6156	0.205
19	SMARCD1	6602	0.274
20	TBP	6908	0.667
21	TOP1	7150	0.559
22	TRADD	8717	0.389
23	TUBB	NM_178014.2	0.446
24	UNC45A	55898	0.334
25	VPS18	57617	0.318
26	WDR45L	56270	0.621
27	YWHAZ	7534	0.557
28	ZNF101	94039	0.615

Housekeeping genes excluded from analysis of obesity related genes			
Housekeeping gene (HKG)		Gene ID	ANOVA P-value
29	GUSB	NM_000181.1	0.007
30	HPRT1	3251	0.048
31	POLDIP3	84271	0.055
32	SDHA	6389	0.000
33	TNIP2	79155	0.081
34	ZC3H10	84872	0.082

In total 34 housekeeping genes were examined in placental tissue specimens from women with pre-pregnancy BMI between 20 – 64 kg/m². The study population was divided into one lean and three obese groups based on maternal pre-pregnancy BMI. One way ANOVA was performed. HKGs with significantly different or close significance P-values (0.05 – 0.09) were excluded from the normalization process. P-values <0.05 were defined as statistical significant. This table was adapted from Hirschmugl et al. Int J Obes (Lond). 2017 Feb;41(2):317-323; (116).