Diploma Thesis

Regulation of lipolysis in the human term placenta by maternal obesity

submitted by

Sabine Payr

for the Academic Degree

Doktor(in) der gesamten Heilkunde

(Dr.^{in} univ. med.)

at the

Medical University of Graz

carried out at the

Department of Obstetrics and Gynaecology

under guidance of

Assoc. Prof. Christian Wadsack, PhD

and

Birgit Hirschmugl, MSc

Graz, 4 October 2016

Eidesstattliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne Fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, 4. Oktober 16 Sabine Payr e.h.

Declaration

I, hereby, declare that the following diploma thesis has been written only by the undersigned and without any assistance from third parties. Furthermore I confirm that no sources have been used in preparation other than those indicated in the thesis.

Graz, 4 October 16 Sabine Payr m.p.

1 Abstract

Background:

Knowledge of consequences of maternal obesity in human placental fatty acid (FA) transport is limited. Animal studies suggest that placental FA-uptake is altered by maternal over-nutrition. Catabolism of FA storage depots and mobilization of free fatty acids (FFA) in the placenta depends on lipases. Placental FA metabolism is tightly regulated by maternal lipolytic activities.

Hypothesis:

Maternal pre-pregnancy body mass index (BMI) affects human placental FA transport by modifying expression of key genes. The impact of maternal over-nutrition on functionality of the feto-placental unit was investigated.

Methods:

Term placentas from normal (BMI 18.5–24.9 kg/m2) and obese (BMI > 30 kg/m2) women were analysed. Based on NanoString® generated results from placental tissues, adipose triglyceride lipase (ATGL) and its co-factors comparative gene identification 58 (CGI-58) and G0/G1-switch gene 2 (G0S2) were identified as key genes in lipolysis together with perilipin 3 (PLIN3), angiopoetin-like protein 4 (ANGPTL4), sterol O-acetyltransferase 1 and 2 (SOAT1/SOAT2).

Correlations of obesity dependent placental lipid associated genes with maternal and neonatal available clinical parameters were calculated. Additionally, using placental tissue, qRT-PCR and Western blot were employed, to confirm global genomic data on RNA and protein levels in placentas from highly obese mothers compared to controls.

Results:

Degree of maternal obesity was not related to ATGL mRNA levels in the studied placentas. The ATGL co-activator and lipid droplet binding protein CGI-58 however, correlated positively with maternal BMI. No association could be detected between CGI-58- and ATGL-levels. Influence of CGI-58 on ATGL enzyme activity was not tested in this study.

Conclusions:

Placental lipolysis appears to be altered by alternated CGI-58 levels in maternal obesity.

2 Zusammenfassung

Hintergrund:

Das Wissen über die Konsequenzen von mütterlicher Adipositas in der Schwangerschaft auf den Fettsäuretransport über die Plazenta ist immer noch sehr eingeschränkt. In Tiermodellstudien wurden Hinweise darauf gefunden, dass Überernährung der Mutter zur vermehrten Aufnahme von Fettsäuren in der Plazenta führt.

Der Abbau von mütterlichen Fettdepots und die Mobilisierung von freien Fettsäuren für die Aufnahme in die Plazenta sind von Enzymen, wie Lipasen abhängig. Der gesamte Fettstoffwechsel der Plazenta ist über den mütterlichen Fettstoffwechsel und sehr spezifisch über die lipolytische Aktivität reguliert.

Hypothese:

Der Body Mass Index (BMI) einer Frau vor der Schwangerschaft beeinflusst die Aufnahme, den Transport und die Speicherung von Fettsäuren durch Expressionsveränderungen der in der Lipolyse relevanten Gene in der humanen Plazenta. Untersucht wurde der Einfluss von mütterlicher Überernährung auf die Funktion der feto-plazentaren Einheit.

Methoden:

Plazentas von termingerechten Geburten von 18 normalgewichtigen (BMI 18.5–24.9 kg/m^2) und 55 adipösen (BMI > 30 kg/m^2) Müttern wurden analysiert. Basierend auf Ergebnissen von NanoString® Auswertungen aus Plazentagewebe, wurden Adipose Triglyceride Lipase (ATGL) und ihre Co-Faktoren Comparative Gene Identification 58 (CGI-58) and G0/G1-switch Gene 2 (G0S2) als Schlüsselgene identifiziert, und gemeinsam mit Perilipin 3 (PLIN3), Angiopoetin-like Protein 4 (ANGPTL4), Sterol O-acetyltransferase 1 und 2 (SOAT1/SOAT2) zur weiteren Untersuchung ausgewählt.

Das Plazentagewebe der Probandinnen wurde mittels quantitativer RT-PCR und Western Blot untersucht. Diese Ergebnissen und den erhobenen mütterlichen und kindlichen klinischen Parametern wurden Korrelationen errechnet, um

Zusammenhänge zwischen normalgewichtigen und adipösen Müttern und dem Fettsäuremetabolismus der humanen Plazenta bzw. ihrer Funktion aufzuzeigen.

Ergebnisse:

Mütterliche Fettleibigkeit vor der Schwangerschaft steht nicht in Zusammenhang mit der mRNA Expression von ATGL (adipose triglyceride lipase), einem Schlüsselenzym im Fettabbau. Allerdings, das ATGL Ko-Aktivator-Protein CGI-58 korreliert auf mRNA- und Proteinebene direkt mit dem mütterlichen BMI vor der Schwangerschaft. Zusammenhänge zwischen CGI-58 und ATGL konnten aber auf Expressionsebene nicht gefunden werden, ob Unterschiede in der ATGL Enzymaktivität durch Veränderung von CGI-58 Expression bewirkt konnte in dieser Studie nicht gezeigt werden.

Zusammenfassung:

Der Abbau von Triglyceriden in der humanen Plazenta durch Lipolyse wird bei mütterlicher Adipositas durch CGI-58 reguliert.

3 Introduction

3.1 Obesity

The WHO defines obesity by using the Body Mass Index (BMI). The BMI puts body weight in relation to the square of the body height (kg/m^2). Obesity is per definition a BMI equal or higher than 30. In 2014 worldwide 13 % of all adults are considered to be obese (1). Focusing on Europe the problem is even bigger. In 2008 23 % of all women and 20 % of men living in the WHO European Region were per definition obese (2).

The characteristic of obesity is an excess of adipose tissue, mostly white adipose tissue (WAT), that is very important for energy metabolism and storage of energy reserves (3). Reason for this expansion of WAT is a persistent positive energy balance that mediates via peroxisome proliferator-activated receptor-γ (PPAR-γ) hyperplasia and hypertrophy of the adipocytes (4,5).

Massive expansion of WAT, as it is the case in obesity, is resulting in dysfunction of the adipose tissue, with less insulin sensitivity, heightened immune cell infiltration, fibrosis and hypoxia (6). This process of adipose tissue dysfunction is going hand in hand with lipid accumulation in other tissues, for example liver tissue or skeletal muscle, and metabolic dysregulation (4,6).

Once this process is getting chronic as it is the case in obesity the way is paved for development of metabolic syndrome (6). This syndrome is a conglomerate of diseases caused by metabolic dysregulation including heightened blood pressure, dyslipidaemia, impaired glucose tolerance and central obesity (7).

Obesity is seen as a big health issue, mostly because it is related to lot of co-morbidities like cardiovascular disease, hypertension and type 2 diabetes mellitus (8). The pathogenesis of these co-morbidities is tightly connected to metabolic deregulations, inflammatory processes and oxidative stress (9).

3.1.1 Clinical problems with maternal obesity

As obesity is a big health problem world wide, it is also an important issue in women of childbearing potential. Maternal pre-pregnancy obesity is associated with many short- and long-term risks for mothers and especially their offspring (10). Short-term risks include higher risk for maternal diseases in pregnancy like preeclampsia, gestational diabetes mellitus (GDM) and gestational hypertension. Reasons for this gestational diseases and their risk factors are mostly not yet defined in detail, however (epi)genetic background and impaired metabolic processes of the women may explain to a certain degree the entry of these diseases (11).

Preeclampsia is a syndrome defined by the coexistence of hypertension after the 20th week of gestation, proteinuria and oedema. It is clearly associated with maternal obesity and also with the extent of obesity (12). This syndrome is induced by placental ischemia and might be exaggerated by obesity related inflammation (13).

GDM is the occurrence of hyperglycaemia and impaired glucose tolerance for the first time in pregnancy (14). During gravidity insulin sensitivity is increased by 50-60 % in every woman, no matter if she is obese or not. But obese women have a higher risk for GDM because of possible pre-existing subclinical metabolic dysfunctions due to the high body weight (15).

Next to the problems during pregnancy also obstetrical complications as caesarean section, tearing and manual placenta extraction are more likely in obese collectives, leading to higher mortality and morbidity of mother and child in the peripartal period (10,11).

Peripartal problems also accrue for their offspring. Neonates from obese mothers are more often born large for gestational age (LGA), compared to neonates from lean mothers, and are more often transferred to special care units (11). Transfers to neonatal special care in that group is caused firstly by intrapartal problems and birth injuries, additionally complicated postpartal periods in offspring of obese

mothers are triggered by the heightened risk for hypoglycaemia and respiratory problems (10,16).

3.1.2 Long-term consequences of maternal obesity in offspring

In the long run offspring of obese mothers are predisposed to develop metabolic disease and obesity in later live. This is not only shown in epidemiological studies, there are also animal studies addressing *in utero* programming of obesity and metabolic disease (17). Mice models for example showed that maternal diet-induced obesity leads to hypertension, insulin resistance, hyperphagia and adiposity in their offspring (18).

Studies on siblings born before and after bariatric surgery of their mother showed a significant difference in their cardiovascular risk profile even in long-term follow-up studies (19,20). Smith et. al. compared in 2009 children born from obese mothers to children born after successful surgical weight loss to prove the impact of the intrauterine environment on the offspring's lipid profile und inflammation markers. To reduce the influence of genetics and familiar environment as many siblings as possible were included in this study. The outcome of this study was that a better intrauterine environment could significantly improve the cardiovascular risk profile of the offspring (19).

Potential mechanism that conduct the susceptibility for future mainly metabolic diseases in offspring of obese mothers are endocrine changes, epigenetic modification, increased inflammation, changes in the immune system, vascular differences, ectopic fat accumulation, nutritional modifications and changes in energy metabolism. All this conducted via an malignant intrauterine environment (21).

3.2 Lipid metabolism in pregnancy

During gravidity there are two phases of changes in lipid metabolism of the mothers. In the first two trimesters there is an acceleration of fat accumulation in the maternal fat depots caused by hyperphagia and lipogenesis. In the third

trimester deprivation of this depots by upregulated lipolysis is more relevant to deliver of fatty acids, glycerol and ketone bodies via the placenta to the fetus (22) (Figure 1). This acceleration of lipolysis in the last weeks of gestation is necessary to ensure the lipid supply for the final growth phase of the fetus. Transport of fatty acids through the placenta is crucial for the fetal supply with essential fatty acids and long chain polyunsaturated fatty acids (LCPUFA) (23). The placenta itself also uses oxidation of FFA for energy supply (23–25).

During gestation mild hyperlipidaemia occurs with higher plasma levels of cholesterol (Chol), triglycerides (TG) and phospholipids (PL). Higher levels of very low-density lipoproteins (VLDL) in the circulating blood of the mother due to stimulation of VLDL production in the liver and elevated TG levels in low-density lipoproteins (LDL) and high-density lipoproteins (HDL) have also been described (22,26,27).

These changes are typically occurring in every healthy pregnancy but are exaggerated in pre-gestational obese women because of metabolic dysregulation prior to pregnancy (24). Several studies show that pre-gestational obese women have higher levels of TG, total cholesterol and LDL combined with lower levels of HDL when entering pregnancy, a so called atherogenic profile that might lead to early negative pregnancy outcomes and other pregnancy complications (28). Throughout pregnancy these women have lower levels of TG and LDL acceleration compared to prior normal weight women, so that the prior normal weight population had higher LDL and TG levels by beginning of the 3rd trimester (29–31).

Lipids in form of FFAs and cholesterol are essential for fetal development and growth (32). Speaking about FFA metabolism in pregnancy there is also to mention the lipid metabolism of the unborn child. The fetus is on one hand able to produce its own fatty acids but on the other hand also dependent on FFA supply from the mother especially LCPUFA (32). In addition, the fetus is also able to use FFAs from fatty acid oxidation for energy production (33).

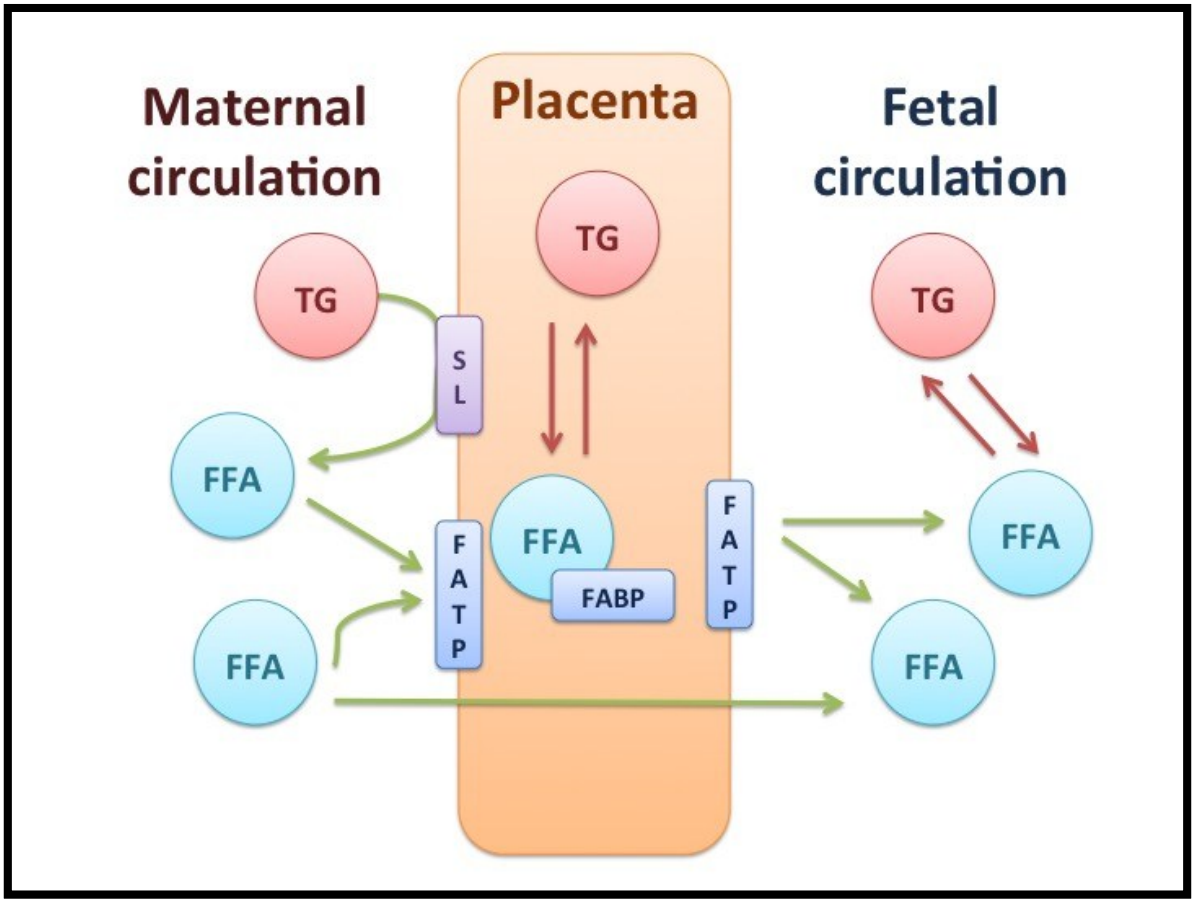


Figure 1: Scheme of lipid transport from the materal to fetal circulation *via* the placenta. TG (triglycerides), FFA (free fatty acids), SL (surface lipase), FATP (fatty acid transport protein), FABP (fatty acid binding protein).

3.3 Function of the placenta

The placenta is the connection between maternal and fetal circulation. The placenta and the umbilical cord supply the fetus with nutrients and oxygen. Also the placenta is crucial for evacuation of waste products from the fetus (34) (Figure 1). But the placenta is not only a passive transmitter of substrates, it is also an important endocrine organ that reacts to maternal and fetal signals and is able to modulate placental transport and metabolic function (35).

There are multiple parameters that might be responsible for alternated nutrient transport to the fetus in maternal obesity. Possible factors are nutrient availability, placental size, exchange barrier surface and thickness, altered function of transport mechanisms or changes in blood flow and vasculature (36). As

connective tissue between mother and child the placenta of obese mothers is on one side the target of pathogenic metabolites on the other side the source of potential negative factors (24).

Pre-gestational obese women enter pregnancy in a pro-inflammatory state with dysregulated lipid metabolism, defective endothelial function and alternated insulin resistance. This negative environment might lead to modifications in the placental structure and function (24,37).

Free fatty acids - if chronically elevated - are accumulating in the body organs. There they cause lipotoxic damage and promote metabolic syndrome (38). Connected to the maternal circulation the placenta is prone to this changes (39). Augmented fat accumulation in combination with elevated oxidative stress and inflammation leads even to lipotoxicity in the placenta of obese mothers (40).

Lipotoxicity is a multifactorial incident based on many dysregulations in the lipid metabolism. A crucial role has the dysregulation of lipolysis, because exaggerated lipolysis causes high levels of FFAs (38).

3.4 Lipolysis

The sequential hydrolysis of TGs to FFAs and glycerol is called lipolysis. TGs are conserved in lipid droplets (LD) within the cytoplasma of cells. The liberation of FFAs is physiologically needed in times of fasting or exercise (41).

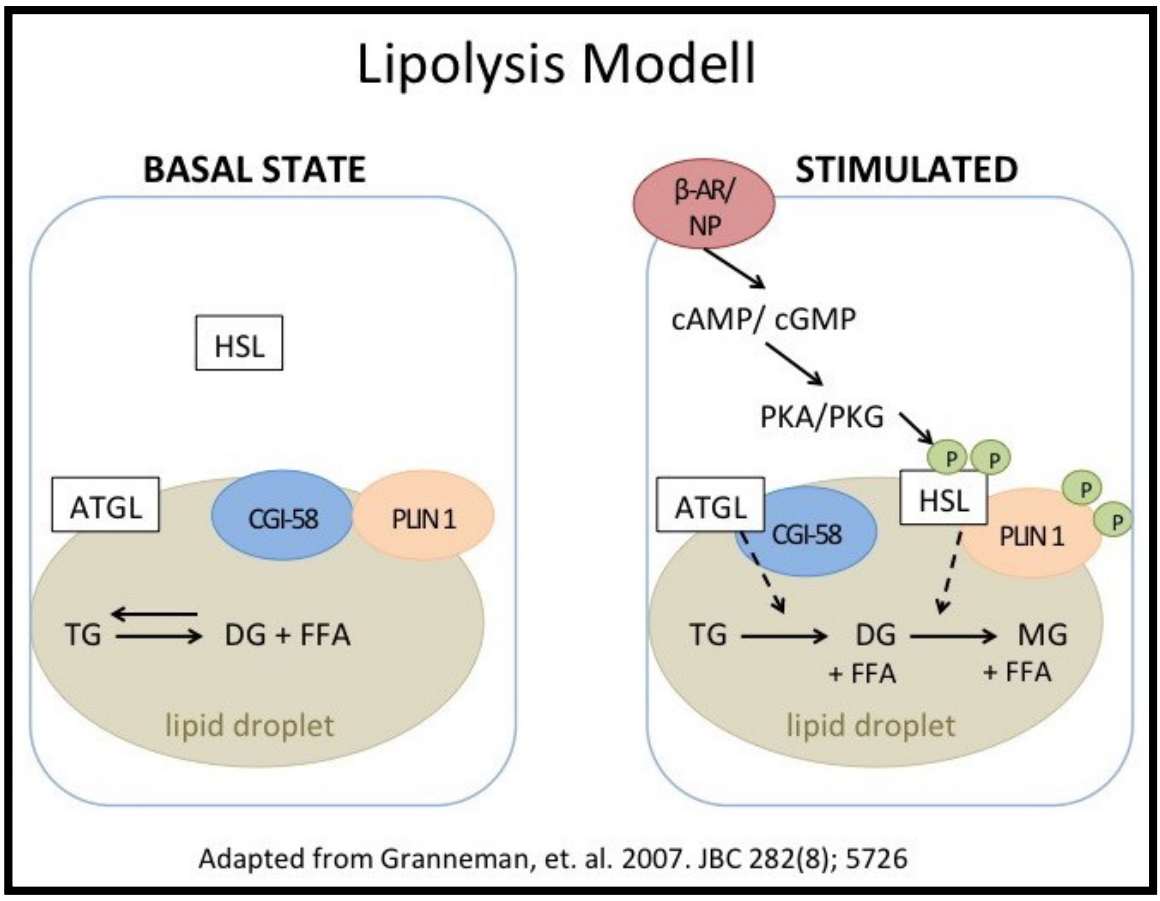
The catabolic process of lipolysis is carried out in essentially all tissues and cell types, but only adipocytes are able to secret FFAs for systemic needs to the own body (42). In pregnancy FFAs released of the placenta are used as a source of energy and also for supply of the fetus (24). This FFAs origin either from maternal circulation directly or are obtained by lipolysis from stored LD in the placenta (43).

So far the regulation of lipolysis in the human placenta is not studied so far in detail, since most of the studies cited below are focused on lipolysis in adipose

tissue. But it gives an overview of most important accepted mechanisms of lipolysis.

3.4.1 Sequence of lipolysis

Lipolysis of TGs is conducted in three steps by three different enzymes, so called lipases. In every step one FFA is released from the glycerol skeleton (Figure 2). Adipose triglyceride lipase (ATGL) converts TG to diglyceride (DG) and is the rate-limiting enzyme of lipolysis (44). For the hydrolysis of DG to monoglyceride (MG) the hormone-sensitive lipase (HSL) is the most important and rate-limiting enzyme, although HSL influences also hydrolysis of other acylesters including TG (45). In the final step of lipolysis monoglyceride lipase (MGL) separates MG into



glycerol and an FFA (46).

Figure 2: Modell of lipolysis in basal state and lipolysis stimulated state (47). TG = triglyceride; DG = diglyceride; MG = monoglyceride; FFA = free fatty acid; ATGL = adipose triglyceride lipase; HSL = hormone sensitive lipase; CGI-58; PLIN 1 = perilipin 1; PKA = protein kinase A; PKG = protein kinase G

3.4.2 Regulation of lipolysis

Four major pathways for the regulation of lipolysis are described in the literature, catecholamine and natriuretic induced promotion of lipolysis activity and reduction of lipolysis activity via catecholamine and insulin (41).

Catecholamines can either up or down regulate lipolysis depending to their relative affinity to either β- or α_2-adrenergic receptor (AR). Activation via β-AR shows pro-lipolytic effects, while α2-AR activation leads to anti-lipolytic effects (48).

Upregulation of lipolysis is mediated by phosphorylation of the LD-associated protein perilipin (PLIN1) and the cytoplasmatic HSL in adipocytes. This phosphorylation is performed by protein kinase A (PKA, cAMP-dependant kinase) activated by β-AR-stimulation or protein kinase G (PKG, cGMP-dependant kinase) activated by natriuretic peptides (41). (Figure 2)

Inhibitation of lipolysis is mainly carried out by insulin. Insulin is able to suppress PKA and thereby lipolysis with an Akt-(PKB)-dependant pathway that activates phosphodiesterase3B (PDE3B). And there is also an alternative Akt-independent pathway of inhibition via phosphoinositide-3 kinase (PI3K) that is conducted by insulin (49).

In early pregnancy insulin-resistance mediates a down-regulation of lipolysis while in late gestation heightened catecholamine levels during even short fasting periods are able to counter that effect (22).

Additionally to this main pathways how lipolysis may be regulated, there are some alternative pathways known that are able to modulate lipolytic activity. These pathways act mainly via cAMP signalling or modulation of β-adrenergic- and insulin-receptor sensitivity. Factors upregulating lipolysis are thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), angiopoietin-like protein 4 (ANGPTL4), tumour necrosis factor α (TNFα), growth hormone (GH) and

glucocorticoids. Anti-lipolytic effects are induced by neuropeptide Y (NPY) and peptide YY (PYY) (41).

3.4.3 Adipose triglyceride lipase (ATGL)

ATGL is the main enzyme for catalysing the first step of lipolysis. It is expressed on the surface of lipid droplets (LD) (44). Activation of ATGL depends on many factors and interactions. The most important co-activator of ATGL is comparative gene identification 58 (CGI-58) (3). Under basal conditions CGI-58 is bound to PLIN1, while upon PKA-mediated phosphorylation of PLIN1 leads to the release of CGI-58, hence CGI 58 is able to interact with ATGL and enhance lipolysis via protein-protein interactions (50).

Genetic defects of ATGL cause neutral lipid storage disease (NLSD) with fat deposition in various tissues. Also defects in CGI-58 lead to NLSD, but with different clinical outcome in comparison with ATGL deficiency (51). ATGL deficiency results in NLSD with myopathy especially cardiomyopathy. CGI-58 mutations on the other hand lead to NLSD with ichthyosis (Chanarin-Dorfman Syndrome) and is often corroborated by hepatic steatosis or neurological disorders, but neither of those gene defects causes obesity in human patients (52).

Next to its function as ATGL-co-activator for lipolysis enhancement, CGI-58 has additional functions. CGI-58 is needed to establish a functional skin barrier and seems to be important for hepatic fat and energy metabolism. Furthermore CGI-58 has lysophosphatidic acid acyl transferase (LPAAT) activity and is also involved in inflammatory signalling and insulin action (50).

A very important inhibitor of ATGL is the G0/G1 switch gene 2 (G0S2). G0S2 is interacting directly with ATGL to reduce its lipolytic activity thereby protecting LDs from depletion (53).

Studies on ATGL and its co-factors CGI-58 and G0S2 in the human placenta were carried out in pregnancies complicated by GDM. The results revealed an increase

of ATGL mRNA but no quantitative changes in ATGL protein expression could be found. Lipase activity was not measured in this study. The ATGL co-factors CGI-58 and G0S2 seamed not to be influenced by GDM (54).

The first time ATGL mRNA and protein in the human placenta was described by Barrett et al. in 2014 (54) in a GDM pregnancy cohort study. There is no literature of ATGL in normal, uncomplicated pregnancy available so far.

3.4.4 PAT family proteins (PLIN)

The PAT family includes 5 lipid droplet (LD) associated proteins, perilipin (PLIN) 1 to 5. The expression of these proteins is dependent on the type of tissue (42). PLIN1 is the most important protein family member in adipose tissue (AT) (42). In the liver PLIN2 is most dominant interestingly, the liver has the second biggest lipid store capacity in the body (55). On the other hand PLIN5 is higher expressed in organs of lipid hydrolysis like heart and other oxidative muscle (55).

PLIN3 is expressed in all cell types and is like all PLIN proteins protecting the LD from deprivation by ATGL (42). PLIN 3 is most dominant in the small intestine, there are lipids just transitionally stored. PLIN3 might has an more important role in transport of dietary lipids (55). As the placenta is responsible for balancing the lipid transition from the mother to the fetus (43) PLIN3 might has a leading role in placental LD too.

Although there is not a lot is known about PLIN alterations in placental tissue, recent studies showed PLIN alterations in muscle tissue in offspring rats of high fat diet mothers that lasted in PLIN3 overexpression even until young adulthood (56). There is no representative study showing the effect of human maternal obesity on PLIN alternations in the skeletal muscle of their offspring, but there is some evidence that PLIN3 expression in skeletal muscle is also influenced by diabetes and inactivity (57) which might be a further connecting factor of intrauterine environment and later live disease.

4 Hypothesis and Aims

The main objective of this study is to verify the hypothesis that high maternal pre-pregnancy BMI effects uptake and metabolism of fatty acids in the human term placenta. Therefore key genes involved in placental lipolysis after Nanostring® analysis (conducted by Birgit Hirschmugl, MSc.) was used to identify the key genes involved in placental FA metabolism.

From this gene data set 7 genes involved in lipolysis and cholesterol esterification were selected due to promising results in Nanostring® analysis. These selected genes namely ATGL, CGI-58, G0S2, PLIN3, ANGPTL4, SOAT1 and SOAT2 – were confirmed by qRT-PCR and on protein level.

The accumulated data from this testing was statistically analysed to find an association between genes involved in placental lipolysis and maternal characteristics like pre-pregnancy BMI, triglyceride, total cholesterol and phospholipid plasma levels or neonatal characteristics like ponderal index or placental weight. This was done in order to find out if modulation of placental lipolysis might be associated with maternal obesity and offspring health.

5 Methods and Materials

5.1 Subjects

For this from the Institutional Review Board of Metrohealth Medical Center (Case Western Reserve University) approved study 93 women were recruited with uncomplicated singleton pregnancies. Excluding factors were multiple gestation, fetal abnormalities, intrauterine growth restriction and diabetes, pre-gestational as well as gestational diabetes.

Placental tissue and blood samples were collected from term pregnancies (n =73) after elective caesarean section. All propends had to sign the written consent prior to this. The study cohort (n=55) was defined as obese by having a pre-pregnancy Body Mass Index (BMI) higher than 30 kg/m^2. The control group (n=18) was defined as normal weight with a BMI between 18.5 and 24.9 kg/m^2.

Further information about maternal ethnicity, gynaecological state (number of gravities and parities, PCOS or problems in former pregnancies) and medication was acquired from the medical report additionally to information about GDM, preeclampsia, and maternal infections in the respective pregnancy.

Information of the offspring as sex, birth weight, length of the neonate, head and chest circumference and skinfold of the flank was identified in the first neonatal check. In order to determine the neonatal metabolic state the Rohrer Ponderal Index (PI) in kg/m^3 was conducted. For a offspring, it is calculated with mass in kilograms (kg) and height in meters (m), giving a measure with the same dimensions as density.(58,59).

All obtained data and the placental biopsies were collected at Metro health Medical Centre, Case Western Reserve University Cleveland and kindly distributed for this study from Prof. Sylvie Hauguel de Mouzon. The ethical approval for the analysis of the placental tissue and data at the Medical University of Graz was assented by the ethical committee under the study number 25/401 ex 12/13.

5.2 Lipid analysis

TG, Chol and PL were measured using enzymatic reagents from Diasys (Holzheim, Germany) and were calibrated using secondary standards from Roche Diagnostics (Mannheim, Germany). All measurements were performed on an Olympus AU640 analyser in cooperation with Assoz. Prof. Priv.-Doz. Dr. Hubert Scharnagl (Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz). The coefficients of variation (between day) were < 5% (60).

5.3 RNA isolation and quantitative real-time PCR

For isolation of total RNA from the placental tissue samples RNeasy® mini kit (Qiagen®, Venlo, Netherlands) was applied according to manufacture's instructions. 2 μg of total RNA was than reverse transcripted using SuperScript® II Reverse Transcriptase (Invitrogen™, Life Technologies Ltd., Carlsbad, California) and Random Hexamer Primer (Fermentas, Thermo Scientific, Rockford, USA) according to manufacture's instructions.

Quantitative Real Time PCR assays were performed with TaqMan® gene expression assays (Life Technologies Ltd., Carlsbad, California) and TaqMan® Universal Master Mix (Life Technologies Ltd., Carlsbad, California) according to manufacture's instructions. The utilised primers (Table 1) were TaqMan® CGI 58, TaqMan® perilipin3 (PLIN3), TaqMan® angiopoetin-like 4 (ANGPTL4), TaqMan® G0/G1 switch 2 (G0S2), TaqMan® sterol O-acetyltransferase 2 (SOAT2), TaqMan® sterol O-acetyltransferase 1 (SOAT1) and TaqMan® adipose triglyceride lipase (ATGL). TaqMan® human TATA-Box Binding Protein (TBP) was used as reference gene (61).

Table 1: Primers used for qRT-PCR

gene (alternative name)	gene ID	assay ID	company
CGI-58 (ABHD5)	51099	Hs01104373_m1	Life Technologies Ltd., Carlsbad, California
perilipin3 (PLIN3)	10226	Hs00998416_m1	Life Technologies Ltd., Carlsbad, California
angiopoetin-like 4 (ANGPTL4)	51129	Hs01101127_m1	Life Technologies Ltd., Carlsbad, California
G0/G1 switch 2 (G0S2)	50486	Hs00274783_s1	Life Technologies Ltd., Carlsbad, California
sterol O-acetyltransferase 2 (SOAT2/ACAT2)	8435	Hs01573878_m1	Life Technologies Ltd., Carlsbad, California
sterol O-acetyltransferase 1 (SOAT1/ACAT1)	6646	Hs00162077_m1	Life Technologies Ltd., Carlsbad, California
adipose triglyceride lipase (ATGL/PNPLA2)	57104	Hs00386101_m1	Life Technologies Ltd., Carlsbad, California
TATA-Box Binding Protein (TBP)	6908	Hs00427620_m1	Life Technologies Ltd., Carlsbad, California

Cycling parameter for all genes were conducted at 50 °C for 3 minutes in stage 1, at 95 °C for 10 minutes for activation of the polymerase in stage 2 and than 40 cycles in stage 3 with 15 seconds at 95 °C and 1 minute at 60 °C in every cycle. Standard curves were carried out with 1 ng, 5 ng, 10 ng and 50 ng cDNA of an unknown sample to select the best sample size (50 ng) for the chosen primers. Each assay was performed with a negative control without template and each sample was analysed in 10 µl volume triplicates.

The geometric mean and standard deviation of the each triplet was calculated and every cycle threshold (ct), over 37 cycles was graded as negative result. For further statistical analysis of relative expression levels the 2^(-Δct) calculation was used (62).

5.4 Western Blot

80 – 150 mg placenta tissue was homogenized on ice in RIPA Puffer (Sigma-Aldrich, Vienna, Austria) containing protease inhibitor (Roche, Basel, Switzerland) to get protein extracts. The homogenized placenta tissue was centrifuged at

13.000 rpm and 4°C for 10 min and the supernatant was transferred to a tube. Protein concentration in the protein extract was determined with BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) according to manufacture's instructions and diluted to 2 μg protein/μl with RIPA Buffer (Thermo Scientific, Rockford, USA) containing 25mM Tris•HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS.

The protein extract (2 μg/μl dilution in RIPA Buffer) was mixed with Laemmli Sample Buffer (BioRad Laboratories, Vienna, Austria) containing 65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue in 1:1 ratio. The protein solution (1 μg/μl) was heated to 95 °C for 5 minutes.

Electrophoresis was made with 10% Mini-PROTEAN® TGX™ Gels (15 wells, BioRad Laboratories, Vienna, Austria). On every gel 2 wells were used for PageRuler™ Prestained Protein Ladder (4 μl), 12 wells for the investigated samples (10 μl) and for a reference sample (10 μl) chosen out of all investigated samples. Electrophoresis was run in Tris/Gycin/SDS-running buffer at 125 V and 400 mA for 65 minutes.

Proteincolumns were transferred from the gel to nitrocellulose membranes with Trans-Blot® Turbo™ Transfer System (BioRad Laboratories, Vienna, Austria) for 5 minutes with 2.5 A constant and up to 25 V. Membranes were blocked in 5 % skimmed milk in TBE (Tris/Boric Acid/EDTA Buffer with 0.1 % Tween) for 1 hour at room temperature, afterwards incubated overnight at 4 °C with the primary antibody (CGI 58) in 1:250 dilution. Therefor the ABHD5 monoclonal antibody (M01) clone 1F3 (Abnova® Corporation, Neihu District. Taipeh City, Taiwan) was used.

Afterwards membranes were washed 4 times for 10 minutes in TBE and incubated with the secondary antibody goat anti mouse (1:1000 dilution, BioRad Laboratories, Vienna, Austria) for 1 hour at room temperature. Thereafter the membranes were washed again 4 times for 10 minutes in TBE.
Protein lanes were detected with SuperSignal™ West Pico Chemiluminescent

Substrate (Thermo Scientific, Rockford, USA) or SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, USA) according to manufacture's instructions. Quantification was done with Alpha DigiDoc 1000 (Alpha Innotech, San Leandro, California).

For among one another normalization of the membranes, they were stripped with Restore™ Western Blot Stripping Buffer (Thermo Scientific, Rockford USA), blocked again for 1 hour in 5% skimmed milk in TBE and incubated with anti-beta actin antibody, AC-15, ab6276 (abcam® 1:10000 dilution) for 1 hour in room temperature. Afterwards washed again 4 times for 10 minutes in TBE and incubated with the secondary antibody goat anti mouse (1:15000 dilution, BioRad Laboratories, Vienna, Austria) for 1 hour in room temperature. Than washed 4 times for 10 minutes. Aktin protein were detected also with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, USA) and quantified with Alpha DigiDoc 1000 (Alpha Innotech, San Leandro, California).

The CGI 58 protein band (ABHD5 antibody) was detected between 55 and 40 kDa. Normalisation was done to reduce bias of differences in sample amount used for Western Blots and in order to compensate loading differences of each protein sample. Additionally, every blot was additionally normalised on single sample that was used for every Western blot to avoid differences in electrophoresis. The evaluation of the spot density (CGI-58 and actin) was measured and calculated with an Alpha DigiDoc 1000.

5.5 Statistical Analysis

Data was analysed with SigmaPlot® software (Systat Software Inc., Chicago, Illiois). Pearson correlation tests were performed between expression data of the genes, maternal and fetal parameters respectively. The results of these correlations are all described by R and p value. The P value expresses the significant differences between parameters and the R indicates the relation of the compared groups to each other. Additional calculations were carried out when total cohort was separated in male and female offspring groups. To compare the

obese study cohort with the lean control t-tests were performed. A calculated P value of < 0.05 was considered as significant difference.

6 Results

6.1 Maternal and neonatal characteristics

The participating women were first divided in a normal weight control group (n=18) with a BMI range of 18.5-24.9 kg/m^2 and in an obese study group (n=55) with a BMI >30 kg/m^2 according to WHO definition of obesity. Maternal characteristics like pre-pregnancy BMI and gestational weight gain were calculated from obtained clinical records. Neonatal parameters as gestational age (to exclude preterm deliveries) and birth weight were contemplated and ponderal index was computed (Table 2).

Table 2: Maternal and neonatal characteristics of the study cohort. Statistical comparison between the groups was done by t-test, significance $p < 0.05$.

maternal and neonatal characteristics					
	normal weight (BMI 18.5-24.9 kg/m^2)		**obese** (BMI > 30 kg/m^2)		
	mean	± SD	mean	± SD	p
prepregnancy BMI (kg/m^2)	22.33	1.50	37.85	6.83	**< 0.001**
weight gain (kg)	15.86	6.94	12.19	8.09	**0.044**
gestational age (weeks)	39.1	0.4	39.2	0.5	n.s.
birth weight (kg)	3.317	0.512	3.276	0.453	n.s.
ponderal index (kg/m^3)	27.12	5.33	28.25	5.07	n.s.
placental weight (g)	658.86	163.39	668.29	186.89	n.s.

In comparison to the lean the obese group had a significant higher ($p < 0.001$) pre-pregnancy weight, but the women of the lean group gained significant more weight while gestation ($p = 0.044$). The two groups showed no significant difference in gestational age, ponderal index and birth weight of their offspring at birth. There was also no significant difference in placental weight. (Table 2)

6.2 Maternal lipid levels

Maternal plasma samples were investigated for TG, Chol and PL levels. Because of poor sample quality after several plasma towing and freezing cycles of all samples (n = 73) only 21 (lean n = 3, obese n = 18) showed reliable lipid results of the plasma (Table 3).

Table 3: Maternal lipid levels. Statistical comparison between the groups was performed by t-test. Significance: $p < 0.05$.

maternal lipids					
	normal weight (BMI 18.5-24.9 kg/m^2)		obese (BMI > 30 kg/m^2)		
	mean	± SD	mean	± SD	p
Chol (mg/dl)	192.67	15.04	178.89	58.92	n.s.
TG (mg/dl)	122.00	19.47	149.00	63.72	n.s.
PL (mg/dl)	206.33	7.37	208.17	55.15	n.s.

In the plasma samples of the studied groups were compared with t-test and there were found no significant differences in Chol, TG and PL levels between these groups.

6.3 Expression of lipolytic genes in placental tissue

RNA (n=73) samples were investigated for mRNA expression of 7 selected genes with qRT-PCR. The tested genes were ATGL, CGI 58, G0S2, PLIN3, ANGPTL4, SOAT1 and SOAT2. From all samples just 10 (4 lean and 6 obese samples) reached a positive cycle threshold (ct > 37) for SOAT2 indicating a very low expression von SOAT2 in placental tissue. 2^(-Δct) calculation was performed and the result was taken as relative expression parameter (Table 4).

Table 4: qRT-PCR results of human placental tissue. Results are calculated from cycle threshold (ct) as described above. Results of normal weight and obese group were

compared with t-test, significance given by $p < 0.05$.

qRT-PCR results							
	all results		normal weight (BMI 18.5-24.9 kg/m^2)		obese (BMI > 30 kg/m^2)		
2^(-Δct)	mean	SD	mean	SD	mean	SD	p
ATGL	0.4629	0.2398	0.4249	0.1459	0.4756	0.2637	n.s.
CGI 58	2.6348	0.6855	2.3883	0.4947	2.7154	0.7230	**0.039**
G0S2	0.1134	0.0709	0.1258	0.0895	0.1094	0.0641	n.s.
PLIN3	6.3208	1.7303	6.0488	1.6952	6.4098	1.7477	n.s.
ANGPTL4	3.5598	1.7160	2.8501	1.3300	3.7921	1.7739	**0.021**
SOAT1	0.2551	0.0923	0.2518	0.0973	0.2562	0.0915	n.s.
SOAT2	0.0007	0.0010	0.0012	0.0016	0.0004	0.0001	n.s.

The higher the number of the relative expression result, the earlier the RNA in the qRT-PCR could reach the ct, what means that more of this RNA sequence was quantified in the tested placental tissue. In the comparison of these results with t-test could be found significant higher amount of CGI 58 RNA in the obese group ($p = 0.039$) and a significant higher amount of ANGPTL4 RNA in this group ($p = 0.021$). In the other tested RNA sequences ATGL, G0S2, PLIN3, SOAT1 and SOAT2 showed no significant differences when comparing the normal weight and the obese group (Table 4).

6.3.1 Association of gene expression in placental lipolysis and maternal BMI

Comparing maternal pre-pregnancy BMI (kg/m^2) with the relative expression levels via Pearson correlation coefficients (Table 4), revealed no significant relationships between maternal BMI, ATGL, G0S2, PLIN3, ANGPTL4, SOAT1 and SOAT2 respectively. Only CGI-58 showed a significant ($p=0.014$) positive ($R = 0.287$) correlation with maternal BMI (Figure 3). The higher the pre-pregnancy BMI was in the cohort the higher amount of CGI 58 RNA was quantified in the placenta.

This correlation is not significant anymore when the cohort is analysed separately in female and male offspring subgroups, although the correlation with the male subgroup can be described as borderline significant with a p-value of 0.057. (Table 5)

Table 5: Pearson correlation coefficients of maternal BMI related to mRNA expression of lypolytic genes in the placenta. $p < 0.05$ = significant

BMI [kg/m²]									
	both sex			male			female		
	n	R	p	n	R	p	n	R	p
ATGL	72	-0.103	0.391	40	-0.099	0.544	32	-0.135	0.462
CGI 58	73	**0.287**	**0.014**	40	0.304	0.057	33	0.235	0.188
G0S2	73	-0.779	0.512	40	0.145	0.372	33	-0.339	0.053
PLIN3	73	0.098	0.407	40	0.297	0.063	33	-0.137	0.446
ANGPTL4	73	0.182	0.124	40	0.284	0.076	33	0.079	0.664
SOAT1	73	0.076	0.521	40	0.178	0.271	33	-0.032	0.861
SOAT2	10	-0.259	0.470	5	0.242	0.694	5	-0.500	0.391

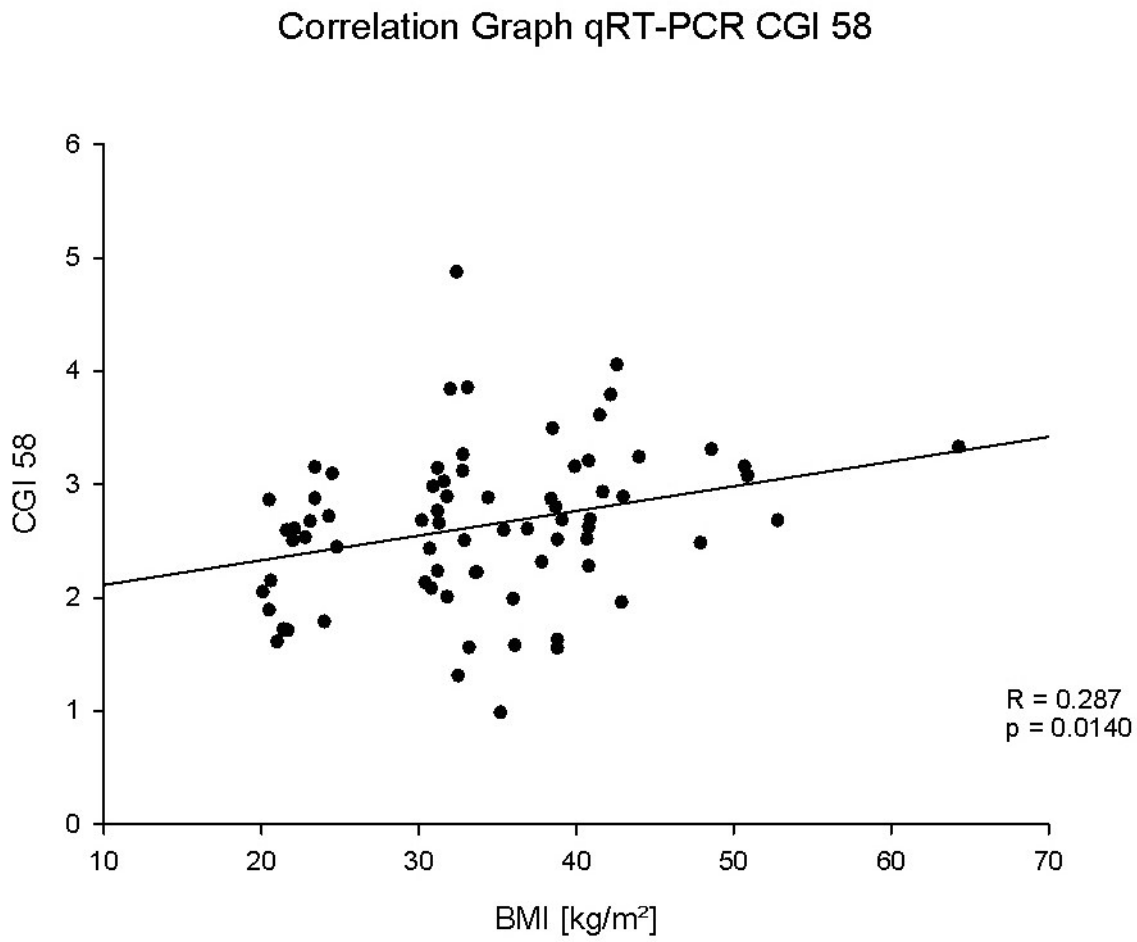


Figure 3: Correlation of maternal pre-pregnancy BMI and placental CGI 58 mRNA expression.

With a relation of R = 0.287 the correlation between pre-pregnancy BMI and CGI 58, the CGI 58 RNA amount in the placenta is just slightly accelerated with higher pre-pregnancy BMI.

6.3.2 Association of gene expression and neonatal ponderal index

Pearson correlation between the relative expression levels of the inspected genes and the neonatal ponderal index (m/kg^3) (Table 6) showed no significant connections in the group of both sexes. But in male subgroup a positive correlation (R = 0.383, p = 0.015) between PI and G0S2 was revealed (Figure 4).

Table 6: Pearson correlations for ponderal index of the neonates and relative mRNA

expression in the placenta is shown. Significance is given by $p < 0.05$.

Ponderal Index [kg/m³]									
	both sex			male			female		
	n	R	p	n	R	p	n	R	p
ATGL	72	-0.196	0.098	40	-0.287	0.072	32	-0.072	0.696
CGI 58	73	0.011	0.927	40	0.085	0.603	33	0.065	0.719
G0S2	73	0.172	0.147	40	**0.383**	**0.015**	33	-0.103	0.570
PLIN3	73	-0.143	0.227	40	-0.092	0.573	33	0.000	0.999
ANGPTL4	73	0.175	0.139	40	0.192	0.236	33	0.299	0.091
SOAT1	73	0.153	0.195	40	0.179	0.269	33	0.075	0.680
SOAT2	10	-0.189	0.600	5	0.399	0.506	5	-0.164	0.793

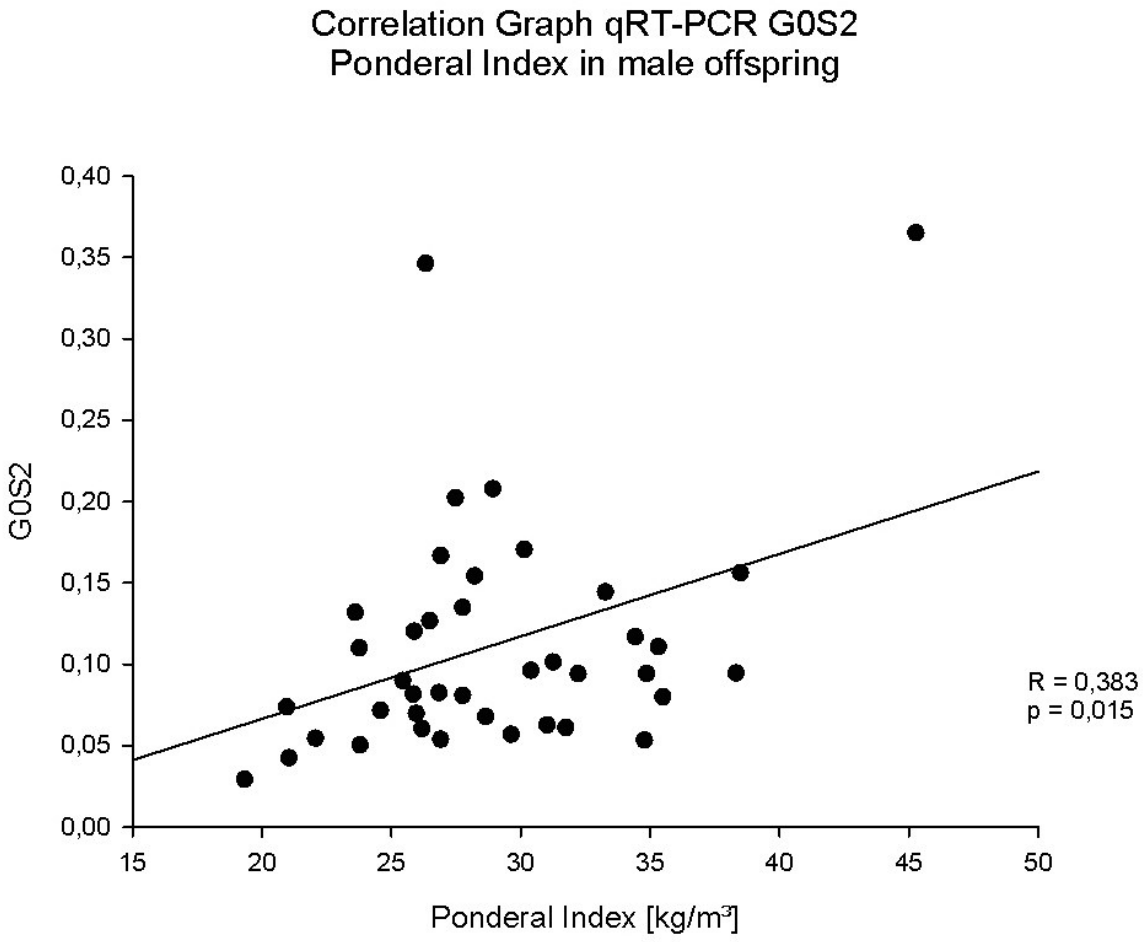


Figure 4: Correlation graph of relationship between neonatal ponderal index and placental G0S2 mRNA expression calculated from qRT-PCR results in male offspring.

6.3.3 Association of gene expression and placental weight

Pearson correlation test between placental weight and the relative mRNA expression discovered no significant correlations (Table 7). Neither in the whole group, nor in the sex divided groups, any correlations were found.

Table 7: Pearson correlations of placental weight and relative mRNA expression in the placenta are depicted. Significance is given by $p < 0.05$.

Placental Weight [g]									
	both sex			male			female		
	n	R	p	n	R	p	n	R	p
ATGL	72	-0.074	0.538	40	-0.278	0.082	32	0.237	0.192
CGI 58	73	-0.057	0.631	40	-0.013	0.939	33	0.062	0.732
G0S2	73	0.101	0.395	40	0.222	0.169	33	-0.075	0.679
PLIN3	73	-0.201	0.087	40	-0.228	0.157	33	0.067	0.712
ANGPTL4	73	0.029	0.807	40	0.041	0.802	33	0.173	0.334
SOAT1	73	0.121	0.308	40	0.078	0.633	33	0.153	0.394
SOAT2	10	0.060	0.869	5	-0.362	0.550	5	0.225	0.716

6.3.4 Association of gene expression and maternal lipids

Testing the Pearson correlation between relative mRNA-expression and maternal triglycerides (TG), total cholesterol (Chol) and phospholipids (PL) (Table 8) revealed just a single significant ($p = 0.041$) positive ($R = 0.448$) correlation between ATGL and PL (Figure 5), this correlation is only found in the both sex group and not in the sex divided groups.

Table 8: Pearson correlation of maternal lipid levels (triglycerides, cholesterol and phospholipids) and relative mRNA expression in the placenta is shown. Significance is

given by $p < 0.05$.

TG [mg/dl]									
	both sex			male			female		
	n	R	p	n	R	p	n	R	p
ATGL	21	0.300	0.187	8	0.169	0.690	13	0.292	0.333
CGI 58	21	0.024	0.918	8	-0.242	0.563	13	-0.009	0.978
G0S2	21	-0.350	0.119	8	-0.517	0.190	13	-0.166	0.587
PLIN3	21	0.007	0.978	8	-0.018	0.967	13	-0.223	0.464
ANGPTL4	21	-0.034	0.884	8	-0.252	0.548	13	-0.027	0.930
SOAT1	21	-0.058	0.802	8	0.016	0.970	13	0.023	0.940
SOAT2	5	-0.314	0.607	3	-0.651	0.549	2	-1.000	--
Chol [mg/dl]									
	both sex			male			female		
	n	R	p	n	R	p	n	R	p
ATGL	21	0.333	0.140	8	0.455	0.258	13	0.283	0.349
CGI 58	21	0.101	0.662	8	0.047	0.912	13	-0.005	0.987
G0S2	21	-0.179	0.439	8	-0.535	0.172	13	0.251	0.408
PLIN3	21	0.159	0.491	8	0.175	0.678	13	-0.008	0.980
ANGPTL4	21	0.060	0.797	8	-0.063	0.882	13	0.055	0.859
SOAT1	21	0.036	0.876	8	0.114	0.789	13	0.093	0.763
SOAT2	5	-0.596	0.288	3	-0.944	0.213	2	-1.000	--
PL [mg/dl]									
	both sex			male			female		
	n	R	p	n	R	p	n	R	p
ATGL	21	**0.448**	**0.042**	8	0.520	0.187	13	0.412	0.162
CGI 58	21	0.145	0.530	8	0.026	0.952	13	0.053	0.864
G0S2	21	-0.248	0.277	8	-0.648	0.082	13	0.161	0.598
PLIN3	21	0.194	0.399	8	0.159	0.706	13	0.035	0.911
ANGPTL4	21	0.148	0.522	8	-0.046	0.914	13	0.163	0.594
SOAT1	21	0.031	0.895	8	0.040	0.925	13	0.178	0.560
SOAT2	5	-0.491	0.401	3	-0.933	0.235	2	-1.000	--

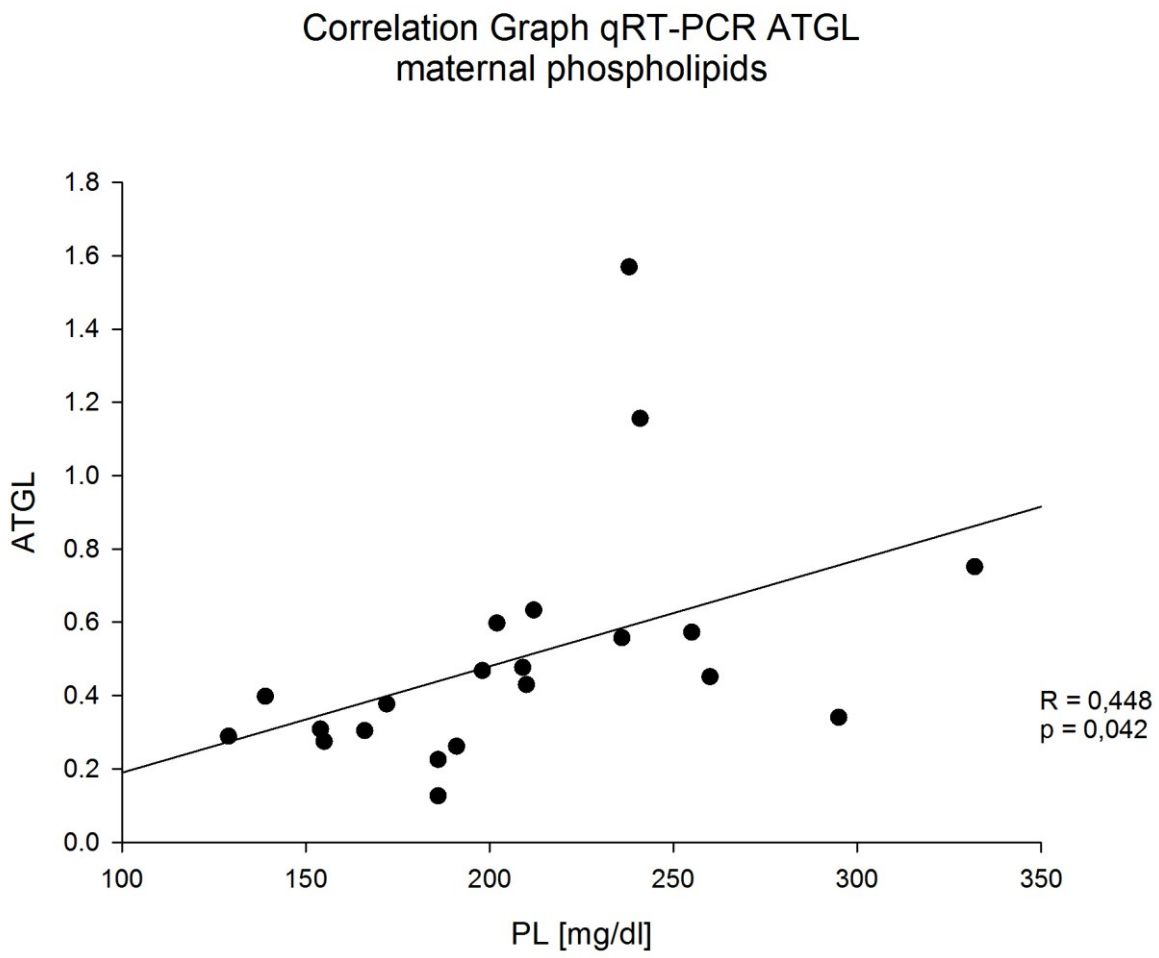


Figure 5: Correlation between maternal plasma PL and ATGL mRNA expression in male offspring.

6.4 Protein expression of CGI 58 in placental tissue

Placental CGI 58 protein expression was measured using Western Blot technique (as described in the methods) (Figure 6). The normalized results are shown in Table 9.

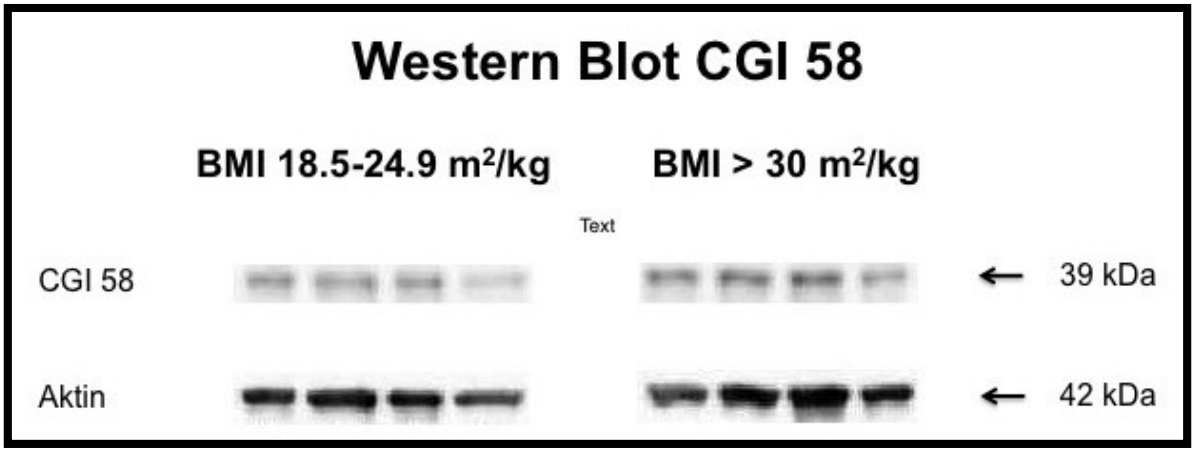


Figure 6: Western blot of one representative experiment with eight samples out of 71 samples.

Table 9: Western blot results of CGI 58 in mean with standard deviation (SD). Area results are normalized to a standard probe and actin protein area. Normal weight/obese and male/female offspring groups were compared with t-test. Significance is given by $p < 0.05$.

Western Blot results				
	CGI 58			
	n	mean	SD	p
all results	71	1.036	0.608	
normal weight (BMI 18.5-24.9 kg/m²)	17	0.581	0.169	< 0.001
obese (BMI > 30 kg/m²)	54	1.180	0.626	
male offspring	38	1.021	0.629	n.s.
female offspring	33	1.054	0.592	

The t-test between CGI-58 protein of the normal weight group and the obese group showed a highly significant ($p < 0.001$) difference between these groups. Less CGI-58 protein was found in the placentas of pre-pregnant normal weight women.

6.4.1 Association of CGI 58 protein expression and maternal/neonatal parameters

Western Blot results were used to substantiate the correlation between maternal BMI and CGI 58 mRNA expression. Protein expression results (n = 72) were correlated (Pearson correlation) with maternal BMI, fetal ponderal index (PI) and placental weight (Table 10).

Table 10: Pearson correlations of maternal BMI, offspring PI, placental weight and protein expression of CGI 58 in the placenta is described. Significance is given by $p < 0.05$.

CGI 58 protein (Western Blot)									
	both sex			male			female		
	n	R	p	n	R	p	n	R	p
BMI (kg/m2)	71	**0.497**	**< 0.001**	38	**0.469**	**0.003**	33	**0.531**	**0.001**
PI (kg/m3)	71	0.164	0.172	38	**0.367**	**0.023**	33	-0.097	0.593
placental weight (g)	71	0.105	0.383	38	0.262	0.112	33	-0.134	0.457

The positive correlation between CGI 58 protein and maternal BMI was highly significant ($p < 0.001$; $R = 0.498$) (Figure 7) and is in contrast to the mRNA results also detectable in the male ($p = 0.003$; $R = 0.469$) (Figure 8) and female ($p = 0.001$; $R = 0.531$) (Figure 9) subgroups. Importantly, CGI-58 protein correlates significantly with fetal PI in the male subgroup ($p = 0.023$; $R = 0.367$) (Figure 10).

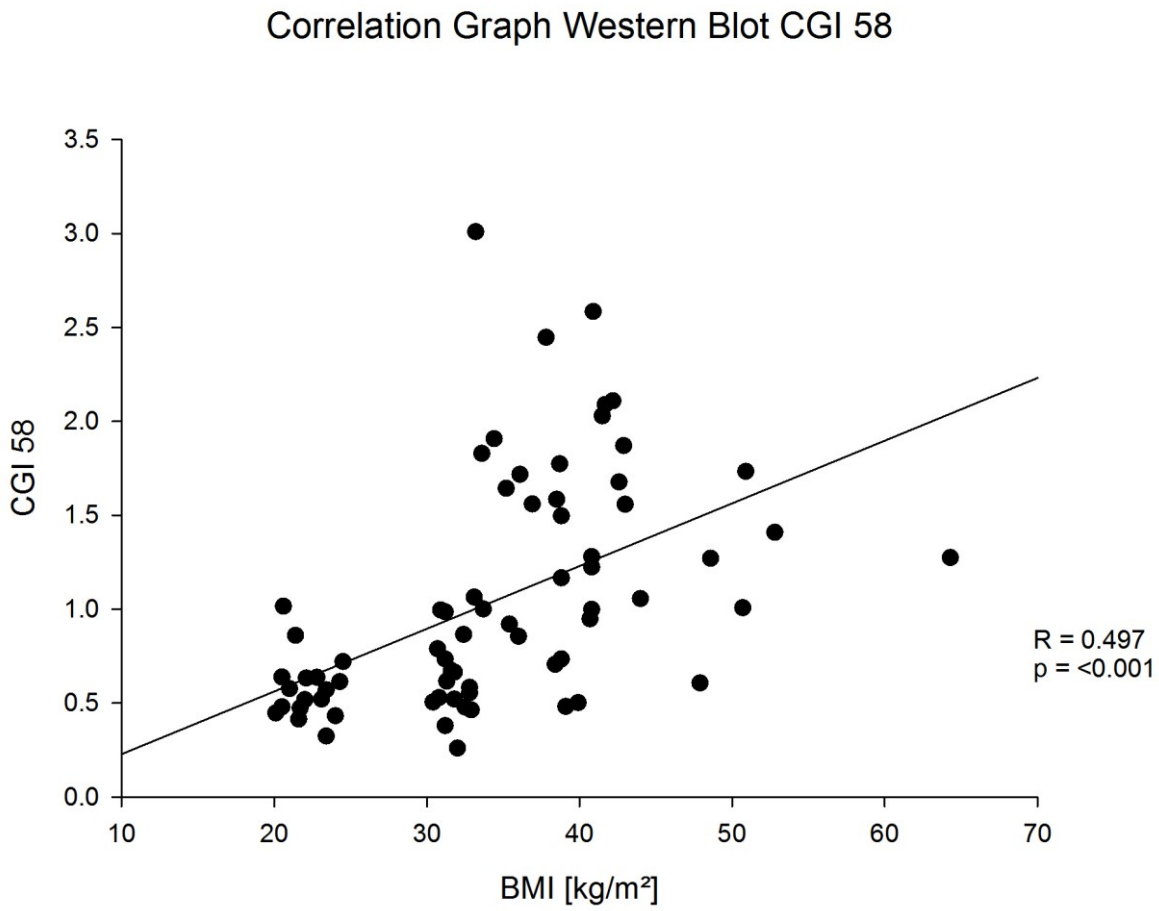


Figure 7: Correlation of pre-pregnancy BMI and CGI 58 protein expression.

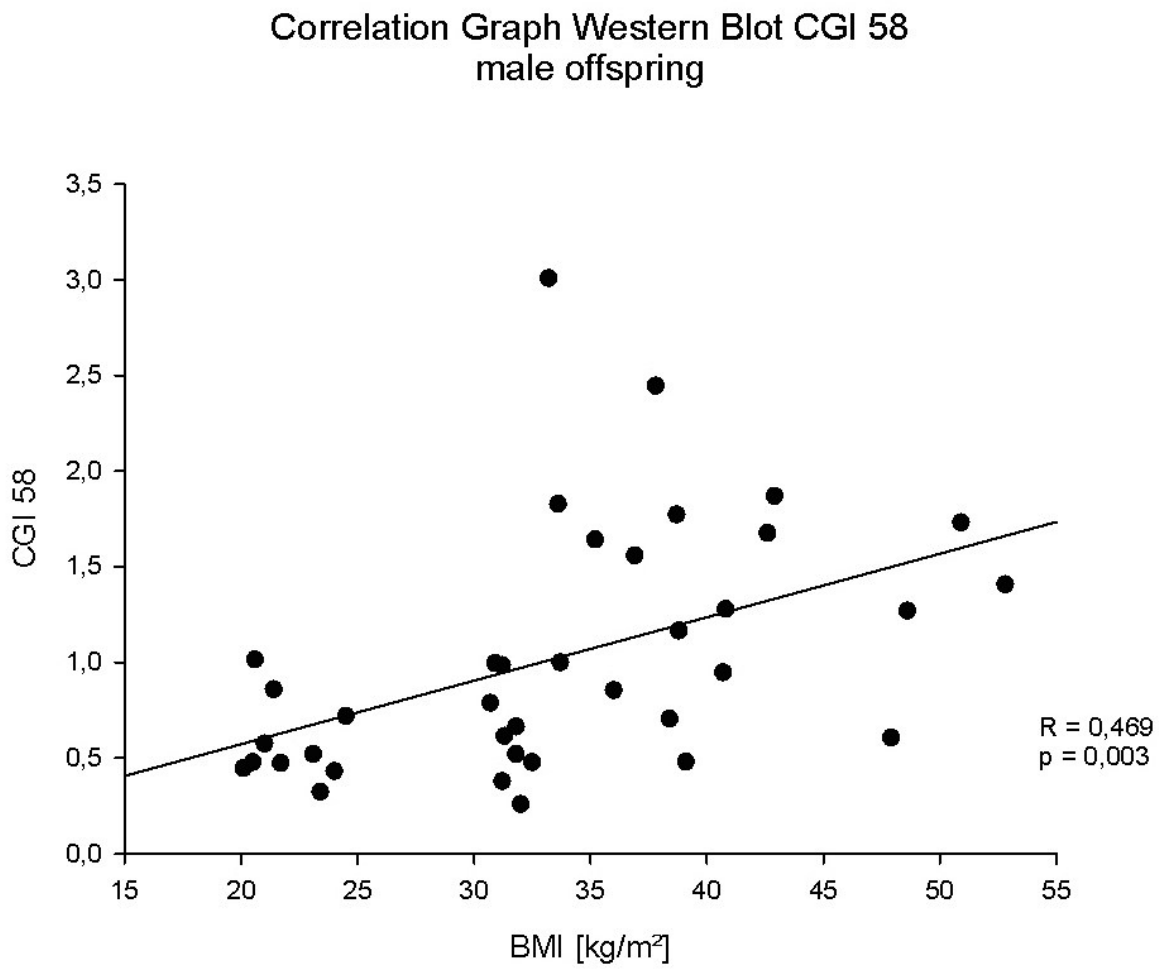


Figure 8: Correlation of maternal pre-pregnancy BMI and CGI-58 protein expression in placentas of male offspring.

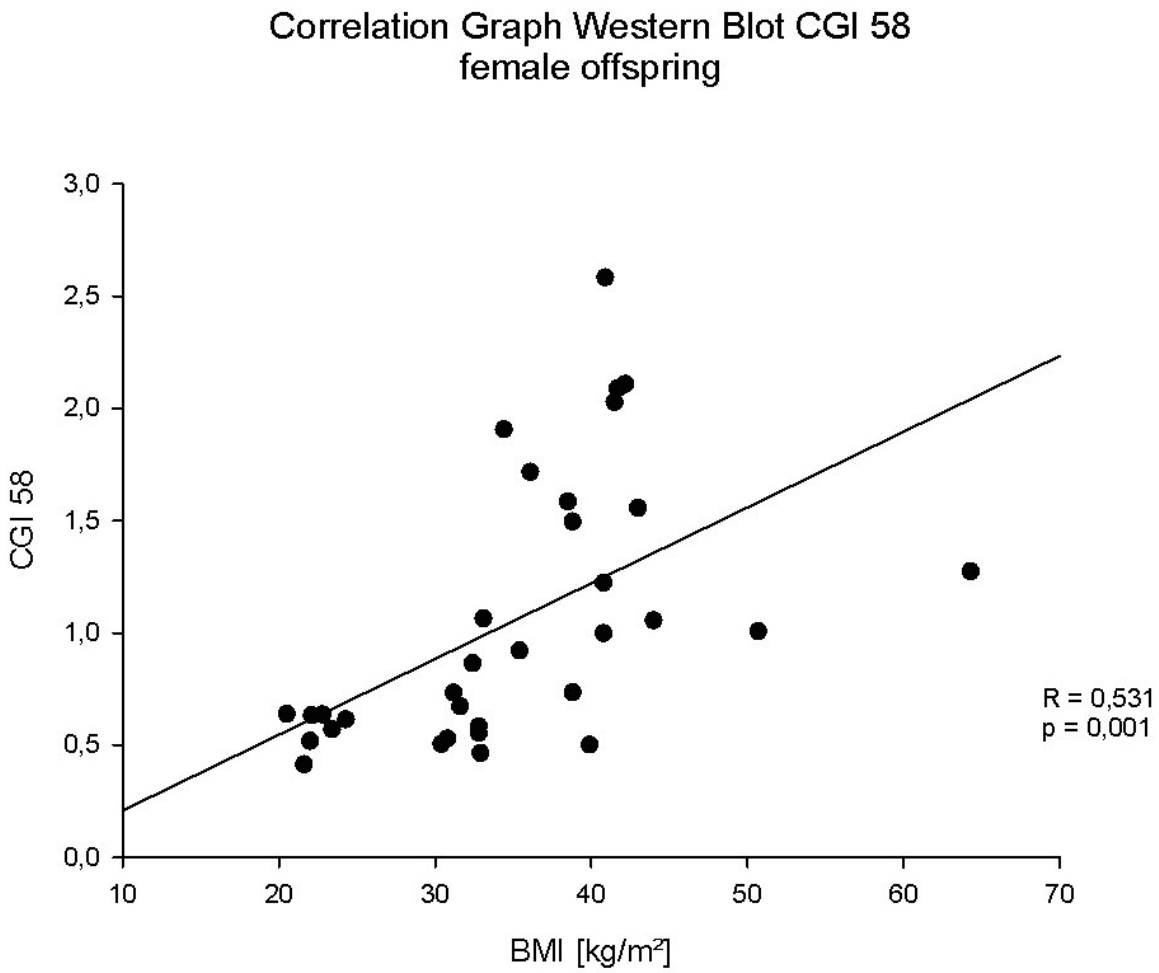


Figure 9: Correlation of maternal pre-pregnancy BMI and CGI 58 protein expression in placentas female offspring.

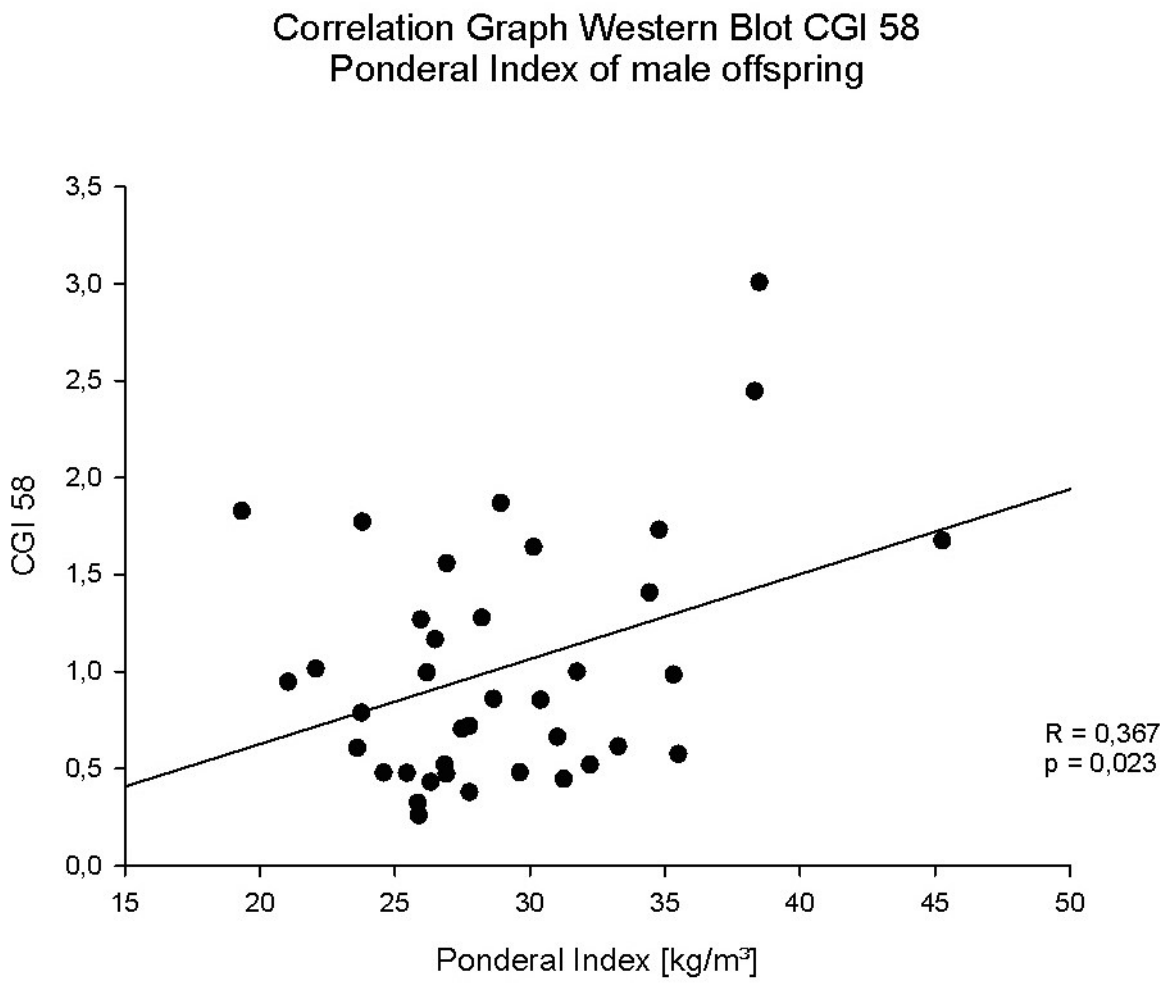


Figure 10: Correlation of neonatal ponderal index and CGI 58 protein expression in placentas of male offspring.

6.4.2 Association of CGI 58 protein expression an maternal plasma lipids

Further correlations were calculated (Pearson correlation coefficient) with results of the maternal plasma lipid analysis (Table 11). Negative correlations were found between CGI 58 and total cholesterol ($p = 0.034$; $R = -0.493$) (Figure 11) and phospholipid levels ($p = 0.030$; $R = -0.473$) (Figure 12) in the maternal plasma of the both sex groups. These correlations between maternal lipid levels and CGI protein levels were lost when analysing within the sex divided groups.

Table 11: Pearson correlations analysed between maternal lipids (TG, Chol and PL in plasma) and CGI 58 protein levels in the placenta. Significance = $p < 0.05$.

CGI 58 protein (Western Blot)									
	both sex			male			female		
	n	R	p	n	R	p	n	R	p
TG (mg/dl)	21	-0.396	0.075	8	-0.352	0.392	13	-0.354	0.236
Chol (mg/dl)	21	**-0.493**	**0.023**	8	-0.376	0.359	13	-0.517	0.071
PL (mg/dl)	21	**-0.473**	**0.030**	8	-0.433	0.284	13	-0.439	0.134

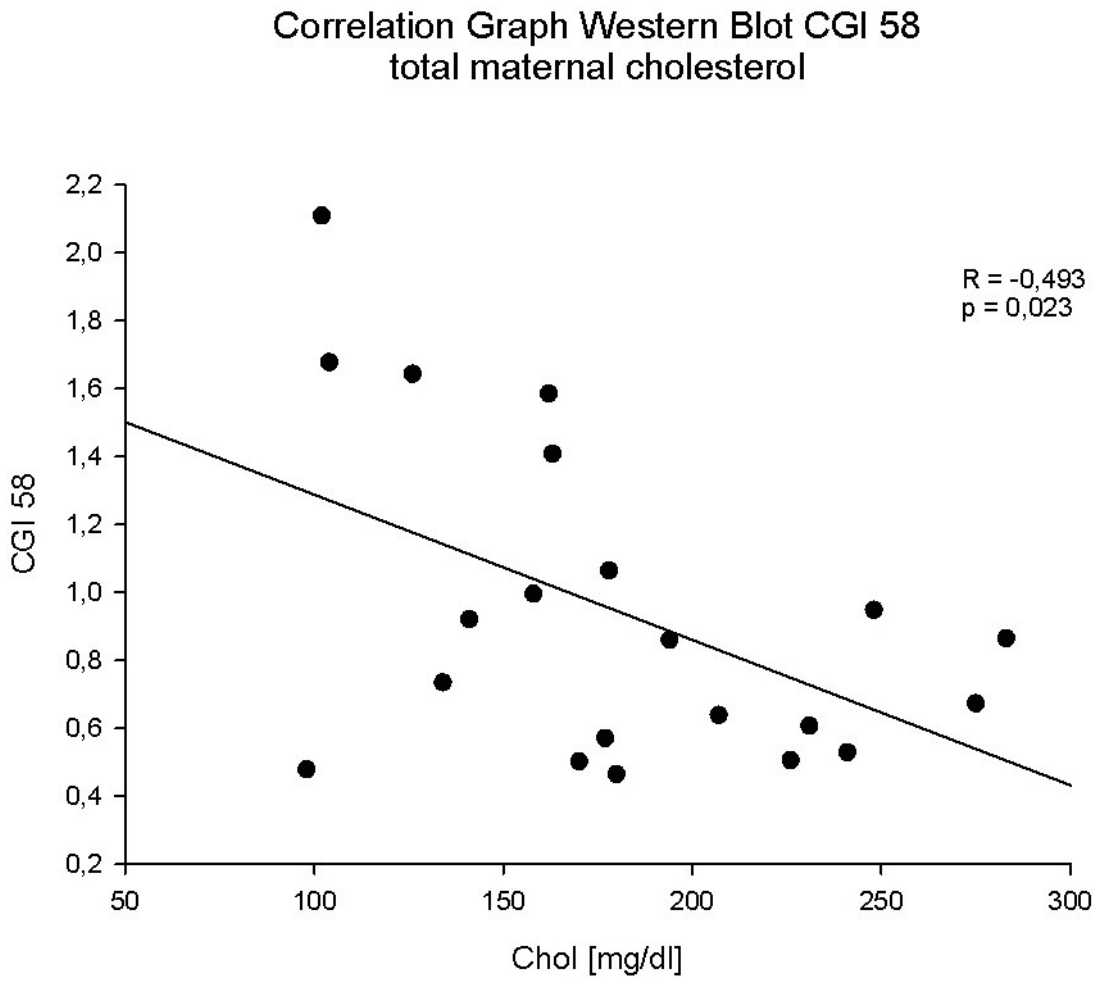


Figure 11: Correlation between maternal plasma total cholesterol and CGI 58 protein expression.

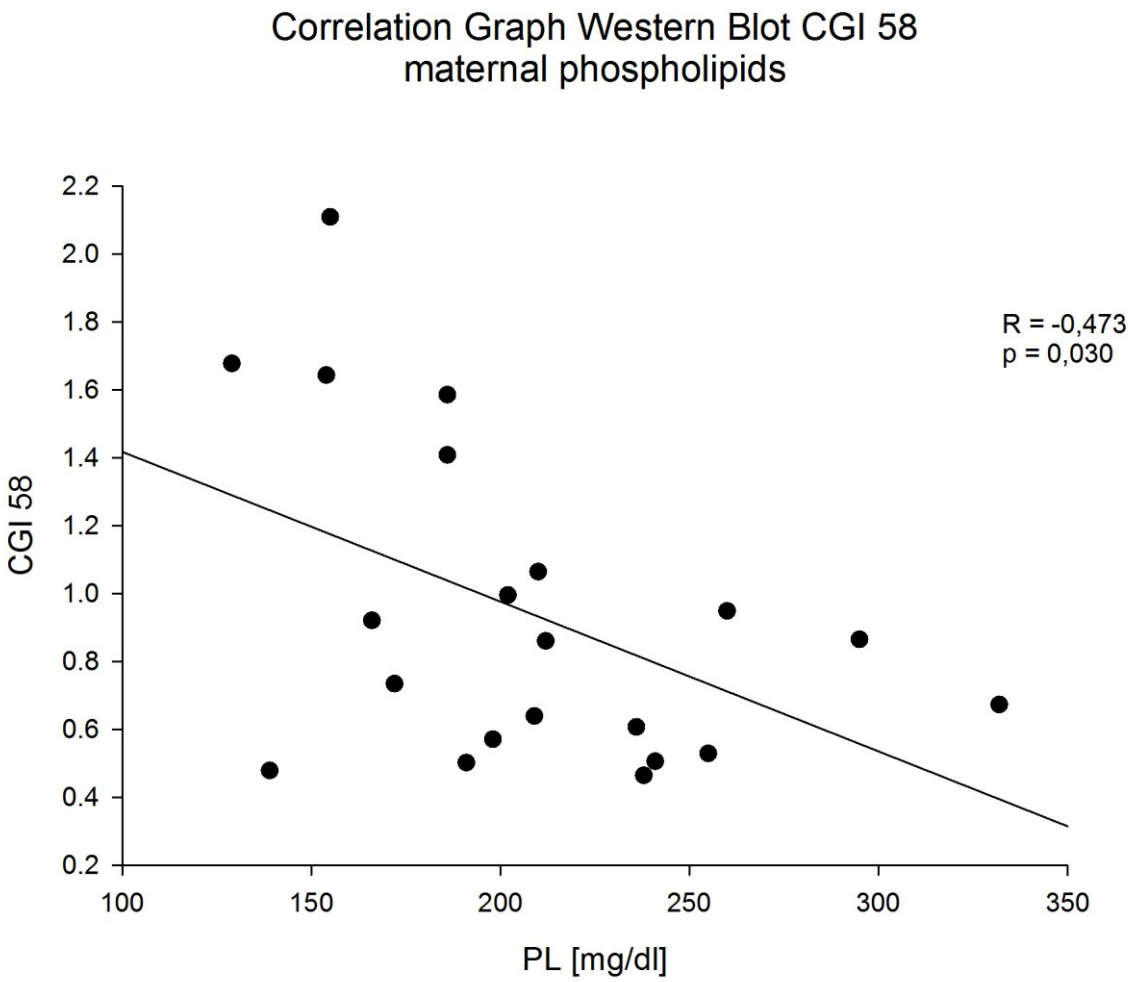


Figure 12: Correlation between maternal plasma phospholipids and CGI 58 protein expression.

7 Discussion

To prove the hypothesis that maternal pre-pregnancy BMI influences placental lipolysis the first approach was to determine the expression of the main lipolytic enzyme ATGL (44). ATGL mRNA expression in placentas of lean and obese women showed no significant correlations to maternal BMI or other clinical parameters. Changes of ATGL expression by obesity are still controversially discussed in the literature due to many oppositional findings of under- and overexpression of ATGL in adipose tissue of obese subjects (41).

Steinberg et al., found in 2007 an overexpression of ATGL mRNA in adipose tissue of obese individuals combined with lowered ATGL protein levels in subcutaneous fat, and normal ATGL protein in visceral fat (63). On the other hand in 2011 Yoa-Borengasser et al. described no differences in ATGL mRNA expression between normal and obese subjects in adipose tissue but decreased ATGL protein levels in obesity (64). Both studies showed substantial similarity in the results as mRNA and protein regulation of the ATGL expression in adipose tissue differs significantly.

ATGL mRNA and protein expression in human placenta was already studied in 2014 by Barrett et al., but focussed on mothers with gestational diabetes (GDM). They found an acceleration of ATGL mRNA in GDM placentas but no differences on ATGL protein levels and also no changes of the co-factors CGI 58 and G0S2 between the investigated groups (54).

In our study we did not find any correlations of maternal BMI and ATGL but to conclude about lipolysis activity we also concentrated on possible cofactors of ATGL activity: CGI 58, as an up-regulating factor of ATGL, G0S2, as an important long term in-activator of lipolysis, and PLIN3, as a PAT family protein member highly expressed in placental tissue (42,53).

The qRT-PCR results on CGI 58 mRNA revealed a significant positive correlation with maternal BMI ($p = 0.014$; $R = 0.287$). Western Blot testing pointed out an even stronger correlation between maternal BMI and CGI 58 protein ($p < 0.001$; R

= 0.498). The higher the BMI of the investigated women at start of pregnancy was the higher mRNA and protein CGI 58 levels were found in the placentas at term.

CGI 58 is important for lipid droplet binding and activation and the enzymatic activity of ATGL is strongly enhanced by this cofactor. Gene defects in CGI 58 are associated with the development of neutral lipid storage disease with ichthyosis, which is also known as Chanarin Dorfman syndrome. *In vitro* experiments demonstrated an up to 20-fold increase of ATGL activity in combination with CGI 58 (50,65). BMI dependent increase of CGI 58 mRNA and protein expression in the placenta is an indicator of an up-regulation of lipolysis in the placenta as a result of maternal overweight and obesity, suggesting metabolic changes as the main driver of these regulations.

Dysregulation of lipolysis leads to lipotoxicity and thereby to insulin resistance and other metabolic disorders (38). Women who enter pregnancy in an obese state are most likely to get a lipotoxic profile that is translated to the fetus *via* the placenta (37). As a consequence this pre-existing and maintained impaired metabolic profile could be one of the reasons for future health problems of these children (17). To find signs for up-regulation of lipolysis in the placenta might be an important step to a better knowledge of obesity inheritance from the mother to the child.

G0S2 was in general not correlated to maternal BMI in our different lean and obese groups. In order to get deeper insights about how maternal factors may regulate placental lipolysis and whether sex matters, we divided our study cohort in groups of female and male offspring. Due to evolutional differences placentas of boys work different to placentas of female offspring. Therefore male offspring's placentas are more vulnerable to intrauterine environment changes (66). Only few of our results showed sex specific effects. Just in the correlation between G0S2 mRNA and neonatal parameters showed a significant positive correlation ($R = 0.383$, $p = 0.015$) between ponderal index (PI) of male offspring and G0S2 mRNA count in the placenta.

PI is an index similar to the BMI for adults, but used for offspring. For calculation 100-times the birth weight (in g) is divided to the cubed birth length (in centimetres), which is an indicator for the volume of the offspring. This index is briefly used to estimate the nutritional state of new-borns (59). The positive correlation of PI from male offspring and G0S2 indicates that in male sex placental G0S2 expression might have influence on neonatal body composition.

The mRNA quantity of PLIN3, although relatively highly expressed in all placental samples, showed no correlations neither to maternal BMI or any of the other mentioned maternal and neonatal parameters. This indicates that there is no relation of PLIN3 expression in maternal obesity compared to normal weight mothers. Furthermore since PLIN3 regulates lipid storage and thereby lipolysis ANGPTL4 was investigated as one of the possible conductors of lipolysis up-regulation (41).

This analysis of ANGPTL4 mRNA in correlation to maternal BMI and other maternal and fetal parameters revealed no significant correlations. No evidence of change in ANGPTL4 due to maternal obesity was found in the placenta. In contrast to our results on the human placenta, a study by Robciuc et al. showed that decreased ANGPTL4 levels were correlated with increased bodyweight and ANGPTL4 concentrations were also positively correlated with circulating free fatty acids and waist-to-hip ratio, thereby suggesting a role of ANGPTL4 in fat storage (67).

In addition to genes primary associated in lipolysis we investigated two genes that are involved in cholesterol esterification: sterol O-acyltransferase 1 (SOAT1 or ACAT1) and sterol O-acyltransferase 2 (SOAT2 or ACAT2). These two isoenzymes contribute to cholesterol homeostasis in humans and are also discussed as possibly modifiable factor genesis of cardiovascular diseases (68). Alternations in cholesterol homeostasis in obese pregnant women might have negative effects to the fetus, at least in early pregnancy where the offspring is most dependent on maternal cholesterol (69).

In our study no significant correlation between maternal BMI and the placental mRNA expression of SOAT1 or SOAT2 could be found. In case of SOAT2 only 10 out of 73 samples could be evaluated due to the low detection of mRNA signal in the human placenta of. In total we concluded that SOAT2 is very poorly expressed in placental tissue and no relation to maternal weight could be observed.

Information on plasma lipid levels in the maternal circulation is limited since the low number of available samples. The calculated correlations between the remaining 21 samples and the mRNA expression revealed one significant correlation; ATGL mRNA expression is positive correlated to phospholipid quantity in the maternal plasma ($p = 0,041$; $R = 0,448$), indicating a synergistic metabolic regulation of PL- and TG-synthesis

Furthermore, our maternal lipid analysis revealed some negative correlations to CGI 58 protein levels in the placenta. Total cholesterol ($p = 0.034$; $R = -0.493$) and phospholipid ($p = 0.030$; $R = -0.473$) decrease with a higher quantity of CGI 58 protein. These findings might be interesting for further investigations in a bigger cohort and with better sample quality.

Along with these findings a paper has been published by Hirschmugl et.al., which shows this dysregulation in lipolysis by CGI 58. Of note, dysregulation of placental lipolysis also correlates with increased triglyceride levels in the placenta but irrespective of maternal plasma TG levels (70).

8 Prospect and limitation

To get better insights to the relation between maternal BMI and lipolysis activity in the placenta there are some more factors that should be considered and discussed in later studies. One of these factors is discriminate the cohort between maternal overweight in comparison to maternal obesity. In order to get effective information about lipolysis a protein activity assay of important key proteins like ATGL would provide some more detailed information.

Beside the strengths of this study there are a few limitations to mention. One limitation is the inconsistency of the study cohort in gestational weight gain, which is known as an influence factor of fetal environment next to obesity itself (71). Another limitation is that the size of the evaluable lipid plasma samples got very low after dismissing all the defect samples due to repetitive freezing and thawing cycles.

9 Conclusion

In conclusion this study revealed an association between maternal pre-pregnancy BMI and higher CGI 58 expression in the human placenta. This result indicates an up-regulation of placental lipolysis via ATGL and major contribution of CGI 58 in obese pregnancy. Although ATGL and the other studied genes (G0S2, PLIN3, ANGPTL4, SOAT1, SOAT2) were neither on transcriptional nor on protein levels changed in relation to the BMI, a potential contribution to this process on activity level can not be excluded.

10 Abbreviations

ABHD5	α/β hydrolase domain 5 or CGI 58
ACAT1	acetyl-CoA acetyltransferase 1 or SOAT1
ACAT2	acetyl-CoA acetyltransferase 2 or SOAT2
ACTH	adrenocorticotrophic hormone
ANGPTL4	angiopoetin-like protein 4
AR	adrenergic receptor
AT	adipose tissue
ATGL	adipose triglyceride lipase
BMI	body mass index
CGI 58	comparative gene identification 58 or ABHD5
Chol	total cholesterol
ct	cycle threshold
DG	diglyceride
e.h.	eigenhändig
FA	fatty acid
FABP	fatty acid binding protein
FATP	fatty acid transport protein
FFA	free fatty acid
G0S2	G0/G1 switch gene 2
GDM	gestational diabetes mellitus
GH	growth hormone
HDL	high-density lipoprotein
HSL	hormone sensitive lipase
LD	lipid droplet
LDL	low-density lipoprotein
LGA	large for gestational age
LPAAT	lysophosphatidic acid acyl transferase
MG	monoglyceride
MGL	monoglyceride lipase
m.p.	manu propria
NLSD	neural lipid storage disease
NPY	neuropeptide Y

n.s.	not significant
qRT-PCR	quantitative real time-PCR
PCOS	polycystic ovary syndrome
PDE3B	phosphodiesterase3B
PI	ponderal index
PI3K	phophoinositide-3 kinase
PKA	protein kinase A or cAMP dependant kinase
PKG	protein kinase G or cGMP-dependant kinase
PL	phospholipids
PLIN	perilipin
PPAR-γ	peroxisome proliferator-activated receptor-γs
PYY	peptide YY
SOAT1	sterol O-acetyltransferase 1 or ACAT1
SOAT2	sterol O-acetyltransferase 2 or ACAT2
TBP	TATA-box binding protein
TG	triglyceride
TNFα	tumour necrosis factor α
TSH	thyroid stimulating hormone
VLDL	very low density lipoprotein
WAT	white adipose tissue

11 References

1. WHO | Obesity and overweight [Internet]. World Health Organization; [cited 2015 May 6]. Available from: http://www.who.int/mediacentre/factsheets/fs311/en/
2. Data and statistics [Internet]. World Health Organization; [cited 2015 May 6]. Available from: http://www.euro.who.int/en/health-topics/noncommunicable-diseases/obesity/data-and-statistics
3. Schweiger M, Schreiber R, Haemmerle G, Lass A, Fledelius C, Jacobsen P, et al. Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. J Biol Chem. 2006 Dec 29;281(52):40236–41.
4. Virtue S, Vidal-Puig A. Adipose tissue expandability, lipotoxicity and the Metabolic Syndrome — An allostatic perspective. Biochim Biophys Acta - Mol Cell Biol Lipids. 2010 Mar;1801(3):338–49.
5. Jo J, Gavrilova O, Pack S, Jou W, Mullen S, Sumner AE, et al. Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth. PLoS Comput Biol. 2009 Mar;5(3):e1000324.
6. Cooke AA, Connaughton RM, Lyons CL, McMorrow AM, Roche HM. Fatty acids and chronic low grade inflammation associated with obesity and the metabolic syndrome. Eur J Pharmacol. 2016 Apr 12;
7. Han TS, Lean ME. A clinical perspective of obesity, metabolic syndrome and cardiovascular disease. JRSM Cardiovasc Dis. SAGE Publications; 2016 Jan;5(0):2048004016633371.
8. Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, et al. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss. Arterioscler Thromb Vasc Biol. 2006 May 1;26(5):968–76.
9. Rani V, Deep G, Singh RK, Palle K, Yadav UCS. Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. Life Sci. 2016 Feb 3;
10. McGuire W, Dyson L, Renfrew M. Maternal obesity: consequences for children, challenges for clinicians and carers. Semin Fetal Neonatal Med. 2010 Apr;15(2):108–12.
11. Bautista-Castaño I, Henriquez-Sanchez P, Alemán-Perez N, Garcia-

Salvador JJ, Gonzalez-Quesada A, García-Hernández JA, et al. Maternal obesity in early pregnancy and risk of adverse outcomes. PLoS One. Public Library of Science; 2013 Jan;8(11):e80410.

12. Poorolajal J, Jenabi E. The association between body mass index and preeclampsia: a meta-analysis. J Matern Fetal Neonatal Med. 2016 Jan 13;1–20.
13. Spradley FT, Palei AC, Granger JP. Immune Mechanisms Linking Obesity and Preeclampsia. Biomolecules. Multidisciplinary Digital Publishing Institute (MDPI); 2015 Jan 1;5(4):3142–76.
14. Voormolen DN, Abell SK, James R, Hague WM, Mol BW. Diagnostic Criteria and Treatment for Gestational Diabetes Mellitus. Semin Reprod Med. 2016 Mar;34(2):102–9.
15. Catalano PM. Trying to understand gestational diabetes. Diabetic Medicine. 2014.
16. Huda SS, Brodie LE, Sattar N. Obesity in pregnancy: prevalence and metabolic consequences. Semin Fetal Neonatal Med. 2010 Apr;15(2):70–6.
17. Alfaradhi MZ, Ozanne SE. Developmental programming in response to maternal overnutrition. Front Genet. Frontiers; 2011 Jan 3;2:27.
18. Samuelsson A-M, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EHJM, et al. Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. Hypertension. 2008 Feb;51(2):383–92.
19. Smith J, Cianflone K, Biron S, Hould FS, Lebel S, Marceau S, et al. Effects of maternal surgical weight loss in mothers on intergenerational transmission of obesity. J Clin Endocrinol Metab. Endocrine Society; 2009 Nov 2;94(11):4275–83.
20. Nelson SM, Matthews P, Poston L. Maternal metabolism and obesity:modifiable determinants of pregnancyoutcome. Human Reproduction Update, Vol.16, No.3. 2010. p. 255–75.
21. Tarantal AF, Berglund L. Obesity and lifespan health--importance of the fetal environment. Nutrients. Multidisciplinary Digital Publishing Institute (MDPI); 2014 Apr 1;6(4):1725–36.
22. Herrera E. Lipid metabolism in pregnancy and its consequences in the fetus

and newborn. Endocrine. 2002 Oct;19(1):43–55.
23. Duttaroy AK. Transport of fatty acids across the human placenta: a review. Prog Lipid Res. 2009 Jan;48(1):52–61.
24. Myatt L, Maloyan A. Obesity and Placental Function. Semin Reprod Med. 2016 Jan;34(1):42–9.
25. Oey NA, den Boer MEJ, Ruiter JPN, Wanders RJA, Duran M, Waterham HR, et al. High activity of fatty acid oxidation enzymes in human placenta: implications for fetal-maternal disease. J Inherit Metab Dis. 2003;26(4):385–92.
26. Ghio A, Bertolotto A, Resi V, Volpe L, Di Cianni G. Triglyceride metabolism in pregnancy. Adv Clin Chem. 2011 Jan;55:133–53.
27. Desoye G, Schweditsch MO, Pfeiffer KP, Zechner R, Kostner GM. Correlation of hormones with lipid and lipoprotein levels during normal pregnancy and postpartum. J Clin Endocrinol Metab. 1987 Apr;64(4):704–12.
28. Scifres CM, Catov JM, Simhan HN. The impact of maternal obesity and gestational weight gain on early and mid-pregnancy lipid profiles. Obesity. 2014 Mar;22(3):932–8.
29. Bozkurt L, Göbl CS, Hörmayer A-T, Luger A, Pacini G, Kautzky-Willer A, et al. The impact of preconceptional obesity on trajectories of maternal lipids during gestation. Sci Reports, Publ online 20 July 2016; | doi101038/srep29971. Nature Publishing Group; 2016;6:465–71.
30. Vahratian A, Misra VK, Trudeau S, Misra DP. Prepregnancy body mass index and gestational age-dependent changes in lipid levels during pregnancy. Obstet Gynecol. 2010 Jul;116(1):107–13.
31. Farias D, Franco- Sena A, Vilela A, Lepsch J, Mendes R, Kac G. Lipid changes throughout pregnancy according to pre- pregnancy BMI: results from a prospective cohort. BJOG An Int J Obstet & Gynaecol. 2016;123(4):570–8.
32. Woollett LA. Fetal lipid metabolism. Front Biosci. 2001 Mar 1;6:D536-45.
33. Herrera E, Desoye G. Maternal and fetal lipid metabolism under normal and gestational diabetic conditions. Horm Mol Biol Clin Investig. 2016 Jan 1;26(2).
34. Cunningham FG, Leveno KJ, Bloom SL, Hauth JC, Rouse DJ, Spong CY.

Williams Obstetrics. 23rd editi. United States of America: The McGraw-Hill Companies, Inc.; 2010.

35. Lewis RM, Demmelmair H, Gaillard R, Godfrey KM, Hauguel-de Mouzon S, Huppertz B, et al. The placental exposome: placental determinants of fetal adiposity and postnatal body composition. Ann Nutr Metab. Karger Publishers; 2013 Jan;63(3):208–15.
36. Higgins L, Greenwood SL, Wareing M, Sibley CP, Mills TA. Obesity and the placenta: A consideration of nutrient exchange mechanisms in relation to aberrant fetal growth. Placenta. 2011 Jan;32(1):1–7.
37. Jarvie E, Hauguel-de-Mouzon S, Nelson SM, Sattar N, Catalano PM, Freeman DJ. Lipotoxicity in obese pregnancy and its potential role in adverse pregnancy outcome and obesity in the offspring. Clin Sci (Lond). Portland Press Ltd; 2010 Aug;119(3):123–9.
38. Saponaro C, Gaggini M, Carli F, Gastaldelli A. The Subtle Balance between Lipolysis and Lipogenesis: A Critical Point in Metabolic Homeostasis. Nutrients. 2015 Jan;7(11):9453–74.
39. Saben J, Lindsey F, Zhong Y, Thakali K, Badger TM, Andres A, et al. Maternal obesity is associated with a lipotoxic placental environment. Placenta. 2014;
40. Jarvie E, Hauguel-de-Mouzon S, Nelson SM, Sattar N, Catalano PM, Freeman DJ. Lipotoxicity in obese pregnancy and its potential role in adverse pregnancy outcome and obesity in the offspring. Clin Sci (Lond). 2010;
41. Nielsen TS, Jessen N, Jørgensen JOL, Møller N, Lund S. Dissecting adipose tissue lipolysis: Molecular regulation and implications for metabolic disease. Journal of Molecular Endocrinology. 2014.
42. Lass A, Zimmermann R, Oberer M, Zechner R. Lipolysis – A highly regulated multi-enzyme complex mediates the catabolismof cellular fat stores. Progress in Lipid Research, Elsevier. 2010.
43. Gil-Sánchez A, Demmelmair H, Parrilla JJ, Koletzko B, Larqué E. Mechanisms involved in the selective transfer of long chain polyunsaturated Fatty acids to the fetus. Front Genet. 2011 Jan;2:57.
44. Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, et al. Fat mobilization in adipose tissue is

promoted by adipose triglyceride lipase. Science. 2004 Nov 19;306(5700):1383–6.

45. Haemmerle G, Zimmermann R, Hayn M, Theussl C, Waeg G, Wagner E, et al. Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. J Biol Chem. 2002 Feb 15;277(7):4806–15.
46. Karlsson M, Contreras JA, Hellman U, Tornqvist H, Holm C. cDNA Cloning, Tissue Distribution, and Identification of the Catalytic Triad of Monoglyceride Lipase: EVOLUTIONARY RELATIONSHIP TO ESTERASES, LYSOPHOSPHOLIPASES, AND HALOPEROXIDASES. J Biol Chem. 1997 Oct 24;272(43):27218–23.
47. Granneman JG, Moore H-PH, Granneman RL, Greenberg AS, Obin MS, Zhu Z. Analysis of lipolytic protein trafficking and interactions in adipocytes. J Biol Chem. 2007 Feb 23;282(8):5726–35.
48. Robidoux J, Martin TL, Collins S. Beta-adrenergic receptors and regulation of energy expenditure: a family affair. Annu Rev Pharmacol Toxicol. Annual Reviews; 2004 Jan 16;44:297–323.
49. Choi SM, Tucker DF, Gross DN, Easton RM, DiPilato LM, Dean AS, et al. Insulin regulates adipocyte lipolysis via an Akt-independent signaling pathway. Mol Cell Biol. 2010 Nov 1;30(21):5009–20.
50. Zierler K a, Zechner R, Haemmerle G. Comparative gene identification-58/α/β hydrolase domain 5: more than just an adipose triglyceride lipase activator? Curr Opin Lipidol. 2014;
51. Schweiger M, Lass A, Zimmermann R, Eichmann TO, Zechner R. Neutral lipid storage disease: genetic disorders caused by mutations in adipose triglyceride lipase/PNPLA2 or CGI-58/ABHD5. Am J Physiol Endocrinol Metab. 2009 Aug;297(2):E289-96.
52. Lord CC, Brown JM. Distinct roles for α-β hydrolase domain 5 (ABHD5/CGI-58) and adipose triglyceride lipase (ATGL/PNPLA2) in lipid metabolism and signaling. Adipocyte. Landes Bioscience; 2014 Oct 20;1(3):123–31.
53. Yang X, Lu X, Lombès M, Rha GB, Chi Y-I, Guerin TM, et al. The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. Cell Metab. 2010 Mar 3;11(3):194–205.
54. Barrett HL, Kubala MH, Scholz Romero K, Denny KJ, Woodruff TM,

McIntyre HD, et al. Placental lipases in pregnancies complicated by gestational diabetes mellitus (GDM). PLoS One. 2014 Jan 12;9(8):e104826.

55. Sztalryd C, Kimmel AR. Perilipins: lipid droplet coat proteins adapted for tissue-specific energy storage and utilization, and lipid cytoprotection. Biochimie. 2014 Jan;96:96–101.
56. Macpherson REK, Castelli LM, Miotto PM, Frendo‐cumbo S, Milburn A, Roy BD, et al. A Maternal High Fat Diet Has Long‐Lasting Effects on Skeletal Muscle Lipid and PLIN Protein Content in Rat Offspring at Young Adulthood. Lipids. 2015;50:205–17.
57. Covington JD, Noland RC, Hebert RC, Masinter BS, Smith SR, Rustan AC, et al. Perilipin 3 Differentially Regulates Skeletal Muscle Lipid Oxidation in Active, Sedentary, and Type 2 Diabetic Males. J Clin Endocrinol Metab. 2015 Oct;100(10):3683–92.
58. Prasad SR, Mohan M, Kumar A, Kapani V. Ponderal index as a marker of intrauterine growth. Indian J Med Res. 1989 Dec;90:442–7.
59. Roje D, Banovic I, Ivo B, Tadin I, Ivica T, Vucinović M, et al. Gestational age--the most important factor of neonatal ponderal index. Yonsei Med J. 2004 Apr 30;45(2):273–80.
60. Sreckovic I, Birner-Gruenberger R, Obrist B, Stojakovic T, Scharnagl H, Holzer M, et al. Distinct composition of human fetal HDL attenuates its anti-oxidative capacity. Biochim Biophys Acta. 2013 Apr;1831(4):737–46.
61. Lanoix D, Lacasse A-A, St-Pierre J, Taylor SC, Ethier-Chiasson M, Lafond J, et al. Quantitative PCR Pitfalls: The Case of the Human Placenta. Mol Biotechnol. Humana Press Inc; 2012 Apr 17;52(3):234–43.
62. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. Oxford University Press; 2001 May 1;29(9):e45.
63. Steinberg GR, Kemp BE, Watt MJ. Adipocyte triglyceride lipase expression in human obesity. Am J Physiol Endocrinol Metab. 2007;
64. Yao-Borengasser A, Varma V, Coker RH, Ranganathan G, Phanavanh B, Rasouli N, et al. Adipose triglyceride lipase expression in human adipose tissue and muscle. Role in insulin resistance and response to training and pioglitazone. Metabolism. 2011 Jul;60(7):1012–20.
65. Lass A, Zimmermann R, Haemmerle G, Riederer M, Schoiswohl G,

Schweiger M, et al. Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. Cell Metab. 2006 May;3(5):309–19.

66. Eriksson JG, Kajantie E, Osmond C, Thornburg K, Barker DJP. Boys live dangerously in the womb. Am J Hum Biol. Jan;22(3):330–5.
67. Robciuc MR, Tahvanainen E, Jauhiainen M, Ehnholm C. Quantitation of serum angiopoietin-like proteins 3 and 4 in a Finnish population sample. J Lipid Res. 2010 Apr;51(4):824–31.
68. Chang T-Y, Li B-L, Chang CCY, Urano Y. Acyl-coenzyme A:cholesterol acyltransferases. Am J Physiol Endocrinol Metab. 2009 Jul 1;297(1):E1-9.
69. Smilde- Baardman ME, Kerstjens- Frederikse WS, Berger RMF, Bakker MK, Hofstra RMW, Plösch T. The Role of Maternal-Fetal Cholesterol Transport in Early Fetal Life: Current Insights. BOR Papers in Press. 2012.
70. Hirschmugl B, Desoye G, Catalano P, Klymiuk I, Scharnagl H, Payr S, et al. Maternal obesity modulates intracellular lipid turn - over in the human term placenta. Int J Obes. (in press).
71. Lau EY, Liu J, Archer E, McDonald SM, Liu J. Maternal weight gain in pregnancy and risk of obesity among offspring: a systematic review. J Obes. 2014 Jan;2014:524939.