Dissertation

Obesity Effects on the Maternal-placental Dialogue in the First Trimester of Pregnancy

submitted by

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Department of Obstetrics and Gynaecology

under the supervision of

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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. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice”.

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Disclosures

This dissertation is based on the following original publications:

Bandres-Meriz J, Majali-Martinez A, Hoch D, Morante M, Glasner A, van Poppel MNM, Desoye G, Herrera E. Maternal C-Peptide and Insulin Sensitivity, but Not BMI, Associate with Fatty Acids in the First Trimester of Pregnancy. Int J Mol Sci. 2021;22(19).

Bandres-Meriz J, Kunz C, Havelund JF, Faergeman NJ, Majali-Martinez A, Ensenauer R, Desoye, G. Distinct maternal metabolites are associated with obesity and glucose-insulin axis in the first trimester of pregnancy. Int J Obes (Lond). 2023;47(7).

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I further contributed to the following publications during my doctoral studies:

Bandres-Meriz J, Dieberger AM, Hoch D, Pochlauer C, Bachbauer M, Glasner A, et al. Maternal Obesity Affects the Glucose-Insulin Axis During the First Trimester of Human Pregnancy. Front Endocrinol (Lausanne). 2020;11:566673.

Majali-Martinez A, Weiss-Fuchs U, Miedl H, Forstner D, **Bandres-Meriz J**, Hoch D, et al. Type 1 Diabetes Mellitus and the First Trimester Placenta: Hyperglycaemia-Induced Effects on Trophoblast Proliferation, Cell Cycle Regulators, and Invasion. Int J Mol Sci. 2021;22(20).

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   2019 - Group Prof. Martin Gauster: lab rotation, staining of first trimester placenta sections of different gestational ages

   2022 – Group of Prof. Berthold Huppertz: imaging of stress granules in scan microscope

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I dedicate this thesis to my **grandma**, who taught me to read.

Table of contents

Abbreviations

A

AGEs: Advanced glycation products

AV: Anchoring villous

B

BCAA: Branched-chain amino acids

BMI: Body mass index

BV: Blood vessel

C

CC: Cell column

CRL: Crown-rump length

CT: Cytotrophoblast

D

DHA: Docosahexaenoic acid

DNA: Deoxyribonucleic Acid

E

ELISA: Enzyme-linked immunosorbent assays

ER: Endoplasmic reticulum

F

FFPE: Formalin-fixed paraffin embedded

FV: Floating villous

H

hCG: Human choriogonadotropin

HGF: Hepatocyte growth factor

4-HNE: 4-hydroxy-2-nonenal

hPL: Human placental lactogen

I

IS_{HOMA}: Homeostatic model of insulin sensitivity

ISR: Integrated stress response

IVS: Intervillous space

L

LMP: Last menstrual period

LC-PUFA: Long chain polyunsaturated fatty acids

M

MCM: Minichromosome maintenance complex

MDA: Malondialdehyde

MVS: Microvillous membrane

O

O_2: Oxygen

P

PUFA: Polyunsaturated fatty acid

R

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT-qPCR: Reverse transcription quantitative polymerase chain reaction

S

SCT: Syncytiotrophoblast

ST: Stroma

U

UPR: Unfolded protein response

Zusammenfassung

Mütterliche Adipositas ist ein Risikofaktor für Schwangerschaftskomplikationen und die künftige Gesundheit des Nachwuchses. Im ersten Trimester der Schwangerschaft wächst die Plazenta schnell und reagiert daher empfindlich auf Veränderungen der intrauterinen Umgebung, wie z. B. auf mütterliches Übergewicht. Wir stellten die Hypothese auf, dass sich der Stoffwechsel zwischen normalgewichtigen und übergewichtigen/adipösen Schwangeren im ersten Trimester der Schwangerschaft unterscheidet. Außerdem stellten wir die Hypothese auf, dass diese Veränderungen im mütterlichen Umfeld die Entwicklung der Plazenta bereits im ersten Trimester beeinflussen. Ziel der vorliegenden Studie war es, Veränderungen in den zirkulierenden Metaboliten bei Frauen mit Übergewicht/Adipositas und im Proteom der Plazenta zu identifizieren, die einer Adipositas-assoziierten Umgebung ausgesetzt sind.

Zu diesem Zweck wurden mütterliches Serum und entsprechendes Plazentagewebe bei einem freiwilligen Schwangerschaftsabbruch zwischen der 4. und 11. Schwangerschaftswoche gewonnen. Gestationsalter, mütterliches Alter und Body-Mass-Index (BMI) wurden erfasst. Darüber hinaus wurden Leptin, Glukose, C-Peptid und die Insulinsensitivität (IS_{HOMA}-Index) im mütterlichen Serum gemessen bzw berechnet. Um Veränderungen der zirkulierenden Fettsäuren zu untersuchen, wurden die wichtigsten Fettsäurespezies (n = 18) bei 123 Frauen gemessen (Gaschromatographie) und zwischen normalgewichtigen und übergewichtigen/adipösen Frauen verglichen. Zusätzlich wurden andere klinische Merkmale wie Leptin, Glukose, C-Peptid und IS_{HOMA} in die Analyse einbezogen. Wenn möglich, wurde das fetale Geschlecht anhand von Plazentagewebe bestimmt (RT-qPCR) und in der statischen Analyse berücksichtig. Zur tiefergehenden und umfassenden Charakterisierung des mütterlichen Stoffwechsels wurde eine hypothese-freie Metabolomik-Methode mit anschließender robuster statistischer Analyse durchgeführt (n = 111). Um Veränderungen in Plazenten zu untersuchen, die der mit mütterlicher Adipositas verbundenen Umgebung ausgesetzt sind, wurde bei 19 Plazenten aus dem ersten Trimester (Woche 5^{+0} - 6^{+6} nach der letzten Menstruation) das Proteom analysiert. Um die Ergebnisse der Proteomik zu bestätigen und weiter zu untersuchen, wurden die wichtigsten identifizierten Proteine mit Western blot quantifiziert und *in situ* immunolokalisiert. Darüber hinaus wurde die Zelllinie ACH-3P als Model des Trophoblasten im ersten Trimester für funktionelle Tests in vitro verwendet.

Die wichtigsten Ergebnisse waren:

1) Der mütterliche BMI und Leptin standen in keinem Zusammenhang mit den zirkulierenden Fettsäurekonzentrationen. Die Gesamtmenge an n-3-Fettsäuren war bei Frauen mit hohem C-Peptid oder niedrigem IS_{HOMA} verringert. Darüber hinaus wiesen Frauen mit hohem C-Peptid, die einen weiblichen Fötus zur Welt brachten, eine geringere Konzentration an zirkulierender Docosahexaensäure auf, was bei männlichen Föten nicht festgestellt wurde.

2) Proxies der Glukose-Insulin-Achse (Glukose, C-Peptid und IS_{HOMA}) waren mit mehr zirkulierenden Metaboliten assoziiert als Proxies für Adipositas (BMI und Leptin). Die stärksten Assoziationen bestanden zwischen C-Peptid und Palmitoleoylethanolamid sowie zwischen C-Peptid und N-Acetyl-L-Alanin.

3) Die Exposition gegenüber einer Adipositas-assoziierten Umgebung *in utero* veränderte das Plazenta-Proteom im ersten Trimester. Mütterliche Insulinsensitivität (IS_{HOMA}) war das klinische Merkmal, das signifikant mit diesen Veränderungen verbunden war. Die Plazenta von Frauen mit niedriger IS_{HOMA} wies erhöhte Konzentrationen von MCM-Proteinen auf, die an der DNA-Replikation und der Reparatur von DNA-Schäden beteiligt sind.

4) MCM-Proteine korrelierten außerdem mit C-Peptid und befanden sich hauptsächlich in den Kernen des Zytotrophoblasten. Darüber hinaus wurde MCM6 mit γH2AX (Marker für DNA-Schäden) ko-lokalisiert. Die *In vitro* Behandlung von ACH-3P-Zellen mit einer patho-physiologischen Insulinkonzentration oder Sauerstoffspannung führte weder zu einem Anstieg der DNA-Schäden (γH2AX) noch der DNA-Schadensreparatur (MCMs).

Diese Ergebnisse zeigen metabolische Unterschiede zwischen Frauen mit Normalgewicht und Übergewicht/Adipositas im ersten Trimester der Schwangerschaft und ein verändertes Plazenta-Proteom in Plazenten, die *in vivo* einer mit Adipositas assoziierten Umgebung ausgesetzt sind.

Abstract

Maternal obesity is a risk factor for pregnancy complications and future health of the offspring. In the first trimester of pregnancy, the placenta grows rapidly, making it sensitive to changes in the intrauterine environment such as maternal obesity. We hypothesized that metabolism differs between pregnant women with normal weight and overweight/obesity in the first trimester of pregnancy. In addition, we hypothesized that these changes in the maternal environment affect placental development already in the first trimester. The present study aimed to identify disarrangements in circulating metabolites in women with overweight/obesity and in the proteome of placentas exposed to an obesity-associated environment.

For this purpose, maternal serum and matched placental tissue were obtained during voluntary pregnancy termination between 4 and 11 weeks of pregnancy. Gestational age, maternal age and body mass index (BMI) were recorded. In addition, leptin, glucose, C-peptide and insulin sensitivity (IS_{HOMA} index) were measured in maternal serum. To investigate changes in circulating fatty acids, main fatty acid species (n = 18) were measured (gas chromatography) in 123 women and compared between normal weight and women with overweight/obesity. Additionally, other clinical traits such as leptin, glucose, C-peptide and IS_{HOMA} were considered in the analysis. When possible, fetal sex was determined (RT-qPCR) using placenta tissue and included in the analysis. For a deeper characterization of maternal metabolism, untargeted metabolomics followed by stringent statistical analysis was employed (n = 111). To investigate changes in placentas exposed to the obesity-associated environment, untargeted proteomics was used in 19 first trimester placentas (week 5^{+0} - 6^{+6} based on last menstrual period). To confirm and further explore the proteomics findings, the key identified proteins were immunolocalized *in situ*. In addition, the first trimester trophoblast cell line ACH-3P was used for functional assays *in vitro*.

The key findings were:

1) Maternal BMI and leptin did not associate with circulating fatty acid concentrations. Total n-3 fatty acids were decreased in women with high C-peptide or low IS_{HOMA}. In addition, women with high C-peptide bearing a female fetus had decreased circulating docosahexaenoic acid concentration, which was not found in male fetuses.

2) Proxies of the glucose-insulin axis (glucose, C-peptide and IS_{HOMA}) associated with more circulating metabolites than proxies of obesity-adiposity (BMI and leptin). The most robust associations were those between C-peptide and palmitoleoyl ethanolamide and between C-peptide and N-acetyl-L-alanine.

3) Exposure to an obesity-associated environment *in utero* altered first trimester placental proteome. Maternal IS_{HOMA} was the clinical trait significantly associated with these changes. Placentas of women with low IS_{HOMA} had increased levels of MCM proteins, which are involved in DNA replication and DNA damage repair.

4) MCM proteins further correlated with C-peptide and mainly located in cytotrophoblast nuclei. In addition, MCM6 was co-localized with γH2AX (DNA damage marker). *In vitro*, treatment of ACH-3P cells with a patho-physiological insulin concentration or oxygen tension did not increase DNA damage (γH2AX) nor DNA damage repair (MCMs).

These findings show metabolic differences between women with normal weight and overweight/obesity in the first trimester of pregnancy and altered placental proteome in placentas exposed to an obesity-associated environment *in vivo*.

1. Introduction

1.1. The human placenta

The placenta is a temporary organ of fetal origin that forms during gestation. The role of the placenta is to provide the fetus with oxygen and nutrients to support its growth. It also protects the fetus against xenobiotics and infections and by removing carbon dioxide and waste products that otherwise would be toxic for the fetus (1).

The development of the feto-placental unit is highly conserved among mammals. However, each species has developed its own strategy, e.g., morphology and function, to maximize oxygen and nutrient transport. In humans, as well as in other primates and rodents, the placenta is haemochorial, meaning that maternal blood is in direct contact with the placenta through its epithelium, the syncytiotrophoblast (cf. 1.1.1.) (2). To stablish an optimal blood flow, the extravillous trophoblast, i.e., trophoblast cells outside the chorionic villi, invade the decidua and reach the uterine spiral arteries (3). Failures in placentation, e.g., invasion and remodelling of the spiral arteries, can lead to spontaneous abortion (4, 5) and obstetrical syndromes such as pre-eclampsia and intrauterine growth restriction (6). The first trimester is a critical period for placentation and most severe pregnancy complications have their origin in aberrant early placentation (6, 7).

1.1.1. Cell types of the Placenta

The branches of the villous tree are the main functional unit of the placenta (**Figure 1**). They are formed by syncytiotrophoblast, cytotrophoblast, mesenchymal cells, Hofbauer cells, i.e., tissue-resident macrophages, and endothelial cells. Cytotrophoblast cells are trophoblast stem cells and differentiate into villous or extravillous cytotrophoblasts (1). **Extravillous cytotrophoblast cells** migrate, invade the decidua and participate in the remodelling of uterine spiral arteries. **Villous cytotrophoblast cells** proliferate and divide asymmetrically providing two daughter cells, of which one will differentiate into a new progenitor villous cytotrophoblast cell and another will fuse with the syncytiotrophoblast. DNA replication in the trophoblast compartment takes place exclusively in villous cytotrophoblast cells and by fusion, the villous cytotrophoblast provides new nuclei, organelles and mRNA transcripts to the syncytiotrophoblast (8). The **syncytiotrophoblast** is a multinucleated layer at the feto-maternal barrier in direct contact with maternal blood. This layer is involved in the transport of oxygen and nutrients from the mother to the fetal circulation and synthesizes hormones, e.g., human choriogonadotropin (hCG), human placental lactogen (hPL), progesterone, prolactin,

leptin and others (1, 9). The **stroma** is made up by different cell types, e.g., **connective tissue cells**, **endothelial cells** and **Hofbauer cells** (fetal macrophages).

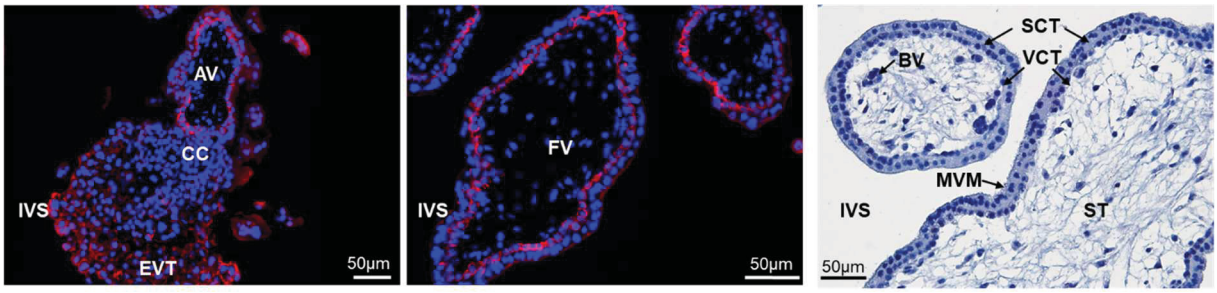

Figure 1. Structure of first trimester placenta villi. A, B) Immunofluorescence staining of a first trimester placenta section (gestational age 6^{+2} weeks LMP) showing cell nuclei in blue (DAPI) and villous cytotrophoblast and extravillous cytotrophoblast in red (E-cadherin staining). A) Anchoring villous (AV). B) Floating villous (FV). C) Hematoxylin-eosin staining of two placental villi (gestational age 5^{+0} weeks LMP). IVS: Intervillous space; EVT: Extravillous cytotrophoblast; CC: Cell column; MVM: Microvillous membrane; SCT: Syncytiotrophoblast; VCT: Villous cytotrophoblast; ST: Stroma; BV: Blood vessel (fetal capillary). Source: J. Bandres-Meriz.

1.2. Implantation and development of the first trimester placenta

The first step of implantation of the conceptus into the uterine wall (apposition) is thought to occur a day 5.5 to 6 post conception. At 7.5 days post conception, the conceptus is partially implanted into the superficial endometrium (10). About day 13 post conception, the primary villi begin to form. These villi consist of highly proliferative cytotrophoblast surrounded by syncytiotrophoblast. Approximately on day 15 post conception, mesenchymal cells derived from the extraembryonic mesoderm, start invading the primary villi, which will be now called secondary villi. At days 18 – 20 post conception, the first fetal capillaries are formed within the mesenchyme of the now called tertiary villi (2) (**Figure 2**).

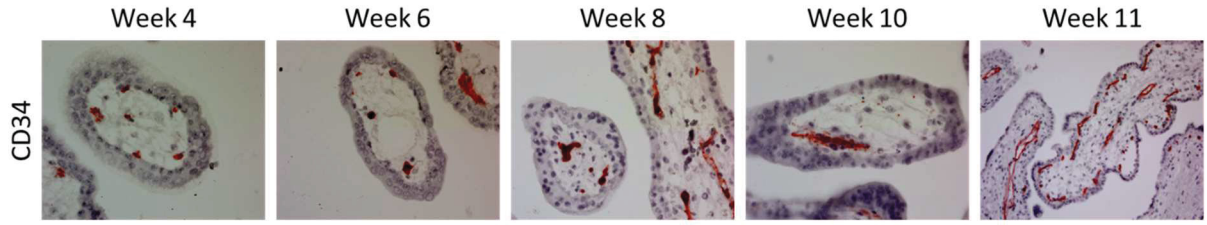

Figure 2. Different developmental stages of first trimester placenta (gestational age week 4^{+0}-11^{+6} LMP) showing CD34 positive cells indicative of fetal capillaries (red) and hematoxilin-eosin staining showing cell nuclei (blue). Source: J. Bandres-Meriz.

The placental and fetal circulations develop independent of each other. A complete feto-placental circulation is established only at week 6 post conception (11). However, effective blood flow from the mother to the feto-placental unit does not occur until the end of the first trimester. Therefore, early in pregnancy, the placenta develops at low physiological oxygen tension (cf. 1.2.3.), which is a stimulator of cytotrophoblast proliferation (12, 13).

The highest rate of placental growth takes place on the first trimester of pregnancy (14). Placental growth is mostly driven by villous cytotrophoblast proliferation, and is sensitive to environmental changes (cf.1.4. and 1.6.) (15). In contrast to tumour cells, in which proliferation is triggered by endogenous mutations that increase growth rate, in the early pregnancy period trophoblast proliferation is triggered by external factors derived from the endometrial glands and by imprinted genes such as IGF2 (16-18). For instance, epidermal growth factor (EGF) and insulin-like growth factors promote cytotrophoblast proliferation in placental explants (19-21).

1.2.1. Histiotrophic nutrition

During the first two months of pregnancy, endometrial glands can be found in the depth of the basal plate. These structures consist of a lumen surrounded by cubical epithelium and decidualized endometrial stromal cells (2) (**Figure 3**). Until the end of the first trimester, the endometrial glands supply the conceptus with nutrients in a process known as histiotrophic nutrition. During this period, the uterine arteries are plugged by extravillous cytotrophoblast cells (cf. 1.2.2.), which prevents the flow of maternal blood into the intervillous space (22). Therefore, histiotrophic nutrition is the main source of nutrients until the end of the first trimester.

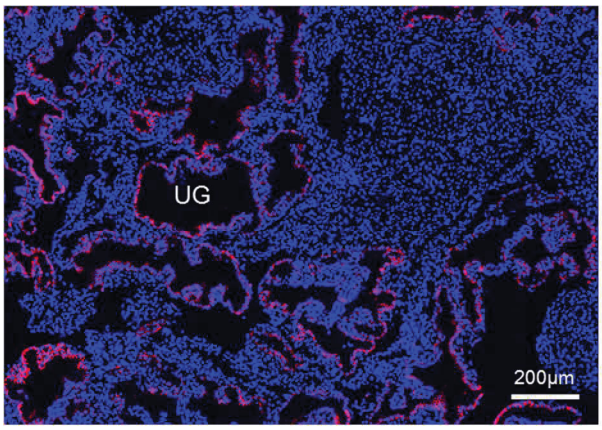

Figure 3. Staining of a decidua section (gestational age 6^{+3} weeks LMP) showing E-cadherin positive cells indicative of endoglandular extravillous trophoblast (red) and DAPI positive nuclei (blue). UG: Uterine gland. Source: J. Bandres-Meriz.

The composition of glandular secretions is similar to the composition of maternal plasma (**Table 1**) and has two main sources: 1) the transudate from capillaries surrounding the glands, and b) specific proteins, carbohydrates and cytokines synthesized by the uterine glands (16). The secretions of the uterine glands are rich in amino acids. Interestingly, proline and cysteine are the only two amino acids that have not been found in these secretions (23). Both are non-essential amino acids. Phagocytosis of maternal proteins by the syncytiotrophoblast serves as a source of amino acids for the fetus. However, how amino acids are transported from the poorly vascularized villi at this stage of early pregnancy to the fetus is not known. A possibility is that amino acids are transported through stromal channels to the coelomic cavity (24). The yolk sac could absorb these nutrients and pass them to the fetus through the vitelline circulation (24). Interestingly, the first stimuli for fetal insulin production are amino acids (arginine and leucine) and not glucose (25-27). This suggests that components of the uterine glands' secretions might be the first inducers of fetal insulin secretion. Interestingly, the amino acid composition of uterine glands' secretions is affected by maternal diet. Women with an unhealthy diet have increased concentrations of branched-chain amino acids (BCAA: valine, leucine, isoleucine), asparagine, histidine, serine, glutamine and phenylalanine (23).

Histiotrophic nutrition has the advantage to occur under low oxygen tension, which prevents the formation of reactive oxygen species (ROS). Avoiding ROS formation is especially important early in pregnancy, to avoid teratogenicity when organogenesis takes place.

In addition to the secretions of the uterine glands, the first trimester placenta is also exposed to signalling molecules produced by stroma cells of the decidua. These molecules are prolactin, insulin-like growth factor binding protein 1 (IGFBP-1), vascular endothelial growth factor (VEGF), transforming growth factor β1 (TGF-β1), and interleukin 15 (IL-15) and they promote trophoblast invasion, angiogenesis and immune regulatory processes (28).

Table 1. Composition of uterine glands' secretions.

Molecule	Chemical entity	Function	References
Glycodelin A	Glycoprotein	• Carrier for small hydrophobic molecules such as retinol or retinoic acid • Maternal tolerance of the placenta	(22) (29) (21)
Mucin 1 (MUC-1)	Glycoprotein	• Regulation of uterine receptivity and implantation. • Supply of amino acids	(22) (24)
Glycogen	Carbohydrate	• Energy supply	(22)
Tumor necrosis facor α (TNF-α)	Cytokine	• Spiral artery remodelling	(30, 31)
Uteroglobin	Cytokine	• Immunomodulation	(32)
Epidermal growth factor (EGF)	Hormone	• Trophoblast proliferation and migration	(19, 33)
Insulin-like growth factor 1 (IGF-1)	Hormone	• Cytotrophoblast proliferation, differentiation, apoptosis	(20)
Vascular endothelial cell factor (VEGF)	Hormone	• Trophoblast proliferation and migration	(33)
Aspartate (Asp)	Non-essential AA	• Source of energy • Synthesis of proteins • Synthesis of nucleotides • pH regulators • Antioxidants • Cell signalling molecules • Cell growth and differentiation	(23)
Glutamate (Glu)	Non-essential AA		
Asparagine (Asn)	Non-essential AA		
Histidine (His)	Essential AA		
Serine (Ser)	Non-essential AA		
Glutamine (Gln)	Non-essential AA		
Arginine (Arg)	Non-essential AA		
Glycine (Gly)	Non-essential AA		
Threonine (Thr)	Essential AA		
Alanine (Ala)	Non-essential AA		
Tyrosine (Tyr)	Non-essential AA		
Methionine (Met)	Essential AA		
Valine (Val)	BCAA		
Tryptophan (Trp)	Essential AA		
Phenylalanine (Phe)	Essential AA		
Isoleucine (Iso)	BCAA		
Leucine (Leu)	BCAA		
Lysine (Lys)	Essential AA		

AA: Amino acid; BCAA: Branched-chain amino acid

1.2.2. Spiral artery remodelling and blood flow establishment

Spiral arteries are uteroplacental arteries that connect the maternal uterine arteries with the intervillous space. The endothelium of the spiral arteries is invaded by extravillous trophoblast that form plugs thereby blocking the arterial lumen. The plugs prevent the flow of maternal blood and erythrocytes into the intervillous space (34). Nonetheless, the intervillous space is filled with an exudate, derived from plasma filtering and secretions of the endometrial glands. (cf. 1.2.1.) (22). The plugs disappear progressively from week 8 onward, which is accompanied by flow of fully-oxygenated blood into the intervillous space (2). Although some maternal blood flow in the intervillous space can already be detected at week 6, continuous blood flow is not

stablished until week 13 (35). The establishment of continuous blood flow comes along with shear stress, which promotes the development of microvilli at the syncytiotrophoblast membrane (36, 37). Impaired spiral artery remodelling and, therefore, reduced placental perfusion, is associated with pregnancy complications (7, 28).

1.2.3. Oxygen tension, glycolysis and oxidative stress

As discussed above, the utero-placental circulation is not stablished until the end of the first trimester. Therefore, the physiological oxygen tension in the intervillous space in this period is very low (2.5% O_2). The gaps between the endovascular trophoblast cells at the plugs of the spiral arteries are not wide enough to allow the passing of maternal erythrocytes; therefore, in this period the only source of oxygen for the feto-placental unit is the oxygen dissolved in the plasma (17).

Early placental development under low oxygen tension is necessary for villous cytotrophoblast proliferation (12). Under this low oxygen tension, glycolysis is the preferred pathway for energy (ATP and NADH) production in the early first trimester placenta. Glycolysis is intimately connected with the pentose-phosphate and polyol pathways. Although the energy obtained through glycolysis (2 ATP molecules) is inferior to the energy obtained by mitochondrial oxidative phosphorylation (36 ATP molecules), glycolysis presents several advantages for the placenta and fetus: 1) It provides carbon skeletons that can be used to build nucleotides, amino acids and lipids via the pentose-phosphate pathway, and 2) provides NADPH, which is required to produce reduced glutathione (antioxidant), via the polyol pathway (17). In a highly proliferative tissue, such as the first trimester placenta, the availability of sufficient building blocks (carbon skeletons) is essential to support cell growth as well as effective antioxidant defences, i.e., glutathione, that prevent DNA damage.

Opening of the spiral arteries and, hence, the beginning of maternal blood flow into the intervillous space, starts at the periphery of the placenta and progresses towards the central villi (38). This creates micro-niches of oxygen. Even after full establishment of the circulation, oxygen tension varies between placenta regions as can be inferred by more oxidative stress and apoptosis occurring in villi located in the periphery rather than those villi located rather centrally (38).

1.3. Maternal adaptations and metabolism in pregnancy

In pregnancy, maternal physiology and metabolism undergo major physiological adaptations to ensure enough supply of nutrients to the fetus and to prepare the pregnant woman for lactation. Maternal blood volume and cardiac output increase and systemic vascular resistance

decreases to facilitate blood flow into the placenta (39). As a consequence of increased maternal blood flow, glomerular filtration rate in the kidney also rises, leading to decreased serum concentrations of creatinine and urea (40). The increased metabolic rate in the mother requires extra supply of oxygen, which is achieved by hyperventilation (40). In the pancreas, insulin production is enhanced by β-cells hyperplasia (40).

Early in gestation maternal metabolism favours storage of nutrients, whereas in late gestation, it switches to a catabolic state to increase nutrient availability to the fetus (41). These changes in maternal metabolism are partially mediated by cytokines such as TNF-α and adiponectin secreted by the adipose tissue, and hormones and extracellular vesicles secreted by the placenta (cf. 1.5.) (41-43).

The glucose-insulin axis is a key mediator in the transition from an anabolic to a catabolic state. From week 6 based on the last menstrual period (LMP) to week 11^{+6} LMP, hepatic insulin sensitivity (IS_{HOMA} index) increases (44). Increased insulin sensitivity favours glycogen synthesis and *de novo* lipogenesis in the liver and triglyceride synthesis in the adipose tissue (anabolism) (45). With advancing gestational age, hepatic insulin sensitivity decreases and circulating insulin concentrations increase (46). Hepatic insulin resistance promotes glycolysis in the liver and breakdown of triglycerides into free fatty acids and glycerol in the adipose tissue (catabolism). The transition from an anabolic state early in pregnancy to a catabolic state in the second and third trimester ensures sufficient supply of glucose, amino acids, essential fatty acids and ketones to the fetus when its growth rate is the highest (1, 47).

Maternal lipid metabolism also changes with gestational age. Early in pregnancy, long-chain polyunsaturated acids (LC-PUFA) obtained from the diet and through endogenous metabolism are stored in maternal adipose tissue (48). In the third trimester, lipolysis increases and there is a peak in triglycerides, free fatty acids, cholesterol, phospholipids and lipoprotein concentrations in maternal blood. This increase in lipids is mediated by increased placental production of estrogens, placental lactogen, progesterone and 17β-estradiol, by hepatic production of triglycerides and by increased maternal insulin resistance (48-50).

Amino acids are also used by the feto-placental unit. Therefore, their concentrations in maternal circulation also change with gestational age. In addition, non-essential amino acids can be synthesized (anabolic state) from intermediates formed during glycolysis and citric acid cycle or are used for producing glucose (gluconeogenesis) in the catabolic state (1). Thereafter, amino acid concentration is tightly associated with the glucose-insulin axis and its concentration will change with the progressive increase in maternal insulin resistance. As already mentioned above, amino acids secreted by the uterine glands are the first stimuli for

insulin secretion in the fetal pancreas. This highlights the importance of amino acid metabolism early in pregnancy in the regulation of fetal metabolism.

1.4. Maternal obesity in the first trimester of pregnancy

Obesity is a chronic condition and has become a global epidemic. Obesity is defined as an individual having a body mass index equal or above 30 (BMI ≥ 30 kg/m^2) (51). Obesity associates with increased incidence of multiple diseases, e.g., diabetes mellitus, cardiovascular and respiratory diseases, mental health disorders, and has a major impact on life quality, morbidity and mortality (52). Furthermore, obesity is a major economic burden for healthcare systems (53).

In pregnancy, obesity is a complex health issue that has significant implications for the health of the mother and the developing baby. Newborns of mothers with obesity have increased adiposity and insulin resistance, suggesting that they develop insulin resistance *in utero* (54). In addition, women with obesity who become pregnant "transmit" the cycle of developmental overnutrition to their offspring, creating a vicious cycle of obesity across generations called intrauterine programming (55, 56). The distinct mechanisms are unclear but are likely a combination of environmental influences such as unhealthy diet, lack of physical activity as well as genetics and epigenetics (49, 57). Epigenetic modifications in DNA methylation occur in response to environmental changes and are transmitted to the daughter cells during mitosis (49, 58-60). Chronic hypoxia and derangements in one-carbon metabolism affect developmental plasticity, i.e., the ability to change gene expression in response to environmental stimuli, via epigenetic modifications (39, 61).

In non-pregnant individuals, obesity is often accompanied by impaired glucose and lipid metabolism as well as chronic inflammation and oxidative stress (62). Recently, we found that pregnant women with overweight (BMI 25 - 29.9 kg/m^2) and obesity (BMI ≥ 30 kg/m^2) have increased C-peptide (proxy for insulin) and decreased insulin sensitivity (IS_{HOMA}) compared to lean women (BMI < 25 kg/m^2*)* in the first trimester of pregnancy (weeks 4^{+0} - 11^{+6} LMP) (44). Of interest, glucose levels were not significantly different between groups. These results suggest an impairment in the glucose-insulin axis in pregnant women with overweight/obesity very early in pregnancy. The glucose-insulin axis plays a pivotal role regulating metabolic and endocrine adaptations in pregnancy. Fasting hyperglycaemia in the first trimester of pregnancy associates with increased risk of gestational diabetes mellitus, rate of caesarean sections and large for gestational age babies (63).

Lipid metabolism varies between lean and women with obesity despite normal glucose levels (41). In lean women, the first trimester is an anabolic period and lipogenesis is

enhanced. In contrast, in women with obesity, lipolysis prevails (41). This is probably due to reduced insulin sensitivity and, therefore, insulin's inability to supress lipolysis. High circulating free fatty acids further impair insulin secretion and are associated with the development of Type 2 diabetes (64).

The contribution of maternal hyperglycaemia and hyperlipidaemia on fetal growth and birthweight are well recognized (65-67). However, these factors alone do not accurately predict the association between high maternal BMI and birthweight. In order to identify other parameters that better predict adverse pregnancy outcomes, several studies have used targeted or untargeted metabolomics (68-72). Interestingly, maternal metabolism has a stronger association with pre-pregnancy BMI than with events occurring during gestation such as weight gain or diet (73).

Maternal obesity is acknowledged as a significant factor for fetal overgrowth. In pregnancies complicated by obesity there is a decrease in growth during early pregnancy, which is subsequently followed by a period of catch-up growth later on (74, 75). This catch-up growth *in utero* not only often leads to neonatal overgrowth, but may also increase the risk for developing obesity in the offspring (75). In addition, the chronic-low grade inflammation in pregnancies complicated by obesity and the potential delay in the opening of the spiral arteries (28) are contributing factors to fetal growth regulation and may further compromise fetal health.

1.5. Placental control of maternal metabolism

The placenta is an organ lacking innervation. Therefore, the dialogue between mother, placenta and fetus occurs through molecules transported in the blood. The placenta produces and secretes a variety of signalling molecules to "inform" the mother on the pregnancy status and enhance the transfer of nutrients to the placenta.

The changes in maternal insulin sensitivity are, at least partially, regulated by hormones secreted by the placenta, e.g., human choriogonadotropin (hCG), human placental lactogen (hPL), hepatocyte growth factor (HGF), progesterone, prolactin, leptin, kisspeptin (1, 9). These hormones stimulate β-cell expansion in the maternal pancreas (hPL, HFG) and stimulate (hCG, kisspeptin) or reduce (leptin) maternal insulin secretion. From week 9 onwards, the placenta is also the major producer of estrogens. Estrogens participate in the remodelling of the uterine vasculature (76), and induce epigenetic changes by DNA methylation and changes in histone proteins (77).

The placenta also releases exosomes. Exosomes are extracellular vesicles surrounded by a membrane. They transport proteins, bioactive lipids and RNAs and participate in feto-maternal communication. Placental exosomes are released into the maternal circulation

already at week 6 of gestation and are thought to reflect the physiological status of the tissue of origin, i.e., placenta (43). The abundance of exosomes in the maternal circulation increases in pathologies such as gestational diabetes mellitus and maternal obesity (78).

In addition, during pregnancy there is bidirectional exchange of cells between the mother and the fetus. This exchange is known as feto-maternal chimerism and implies the presence of cells of fetal origin in maternal tissues (fetal microchimerism) as well as of maternal cells in the placenta and fetal tissues (maternal microchimerism) (79). Although both forms of microchimerism co-exist, there is higher trafficking and implantation of fetal cells into maternal tissues (80). Feto-maternal cell trafficking has been described to start between week 4 and 6 of gestation in humans (81) and to increase with advancing gestational age. Some fetal cells can be found in maternal blood up to three decades after delivery (82). Fetal cells have also been found in numerous maternal tissues e.g., skin, spleen, liver, brain, lung, heart, kidney, breast, thyroid. These fetal cells are stem-like, highly plastic and can differentiate into tissue specific cell types e.g., cardiomyocytes, endothelial cells, neurons, hepatocytes (79). The role of feto-placental michrochimerism is controversial. A possibility is that fetal cells contribute to the allocation of resources. For example, at late stages of pregnancy the migration of fetal cells into the maternal breast could increase milk production, migration into the brain could increase maternal bonding and migration to the thyroid increase heat production (83).

1.6. Placental response to metabolic stress

Embryonic and fetal growth are delayed in pregnancies complicated by obesity (84). Since placental growth precedes fetal growth, it is plausible that placental growth is also impaired in obese pregnancies, similarly to type 1 diabetes (85). Indeed, at the end of the first trimester, placental volume and vascularization are reduced in placentas exposed to maternal obesity (86), suggesting reduced placental growth. Although the adverse effects of maternal obesity on placental development and function at the end of pregnancy are well recognised, obesity-associated changes in early pregnancy and the underlying specific mechanisms are poorly understood.

Placenta and fetus develop in a protected intrauterine environment (50). The low oxygen tension until the end of the first trimester in the intervillous space protects placenta and fetus from oxidative stress. However, highly proliferative tissues, such as the villous cytotrophoblast, are especially prone to lesions in the DNA (87, 88). One of the most common hazards causing DNA damage is oxidative stress. Oxidative stress is a consequence of increased production of reactive oxygen species (ROS) such as O_2^-, H_2O_2 and $\bullet O_2$. ROS are formed endogenously during cellular metabolism, i.e., mitochondrial respiration, peroxisome

activity and protein folding in the endoplasmic reticulum (89). Paradoxically, mitochondria and endoplasmic reticulum themselves are also vulnerable to oxidative damage. ROS can damage nuclear DNA by oxidizing guanine nucleosides to 8-oxo guanine (8-OHdG), which induces G-A transitions or G-T transversions and results in DNA double strand breaks (89). ROS can also degrade mitochondrial DNA (90). Therefore, the low oxygen tension in which the placenta develops in the first trimester (period of maximum villous cytotrophoblast proliferation) can be regarded as a protective mechanism to minimize oxidative stress and DNA damage. Towards the end of the first trimester, when maternal blood flow into the intervillous space is established paralleled by oxygen rise, the activity of antioxidant enzymes in the placenta increases (50). These enzymes, e.g., superoxide dismutases, peroxidases, catalases, vitamins, transform ROS into inactive molecules, thus, reducing cellular oxidative stress that may occur with the rise of oxygen tension.

However, outside pregnancy metabolic disturbances such as obesity enhance ROS production and oxidative stress in multiple tissues, which results in increased oxidative DNA damage and higher serum concentrations of 8-OHdG (91). In addition, obesity also impairs DNA-damage repair pathways (91). Therefore, *in utero* exposure to maternal obesity is likely to increase oxidative DNA damage and to impair DNA damage repair in the placenta. Term placentas of women with obesity have increased triglyceride content likely due to increased esterification and decreased mitochondrial fatty acid β-oxidation (92). Decreased mitochondrial β-oxidation can be compensated by β-peroxisomal oxidation, which increases oxidative stress by generation of H_2O_2 (93). In addition, oxidative stress leads to peroxidation of fatty acids at the cellular membranes forming malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) among others, which can further induce DNA damage (94).

Another important stress pathway in the placenta is endoplasmic reticulum (ER) stress and its unfolded protein response (UPR). The function of the UPR is to restore ER homeostasis, but under chronic ER dysfunction, it can also activate pro-apoptotic signals, e.g., upregulate CHOP, JNK and caspase 12 (95). ER stress has been observed in highly active metabolic tissues, e.g., liver, pancreas, muscle and adipose tissue, of people with obesity (96). In animal models, maternal obesity was associated with increased transcription of ER stress proteins in the placenta (97). ER stress has also been identified in placentas of pregnancies complicated by preeclampsia and intrauterine growth restriction, suggesting that ER stress can impair placental development and function (98). Indeed, protein synthesis accounts for up to 30% of the oxygen consumption in the term placenta (99), highlighting the importance of protein synthesis for placental growth and function. Some known inducers of ER stress are oxidative stress, free fatty acids and inflammation (98). In a first trimester trophoblast cell line

(ACH-3P) we have shown that insulin treatment (10nM) at 2.5%O_2 increased eIF2α phosphorylation (marker of ER stress) (100). These results suggest that in the first trimester placenta ER-stress results from the interplay of metabolic factors and patho-physiological oxygen tension.

Very early in pregnancy, placental metabolic pathways leading to energy generation (ATP and NADPH) show a preference for the pentose phosphate pathway and polyol metabolism over the TCA cycle (oxidative phosphorylation), which is beneficial to reduce oxidative stress (17). However, in a situation of long term nutrient overload such as in obesity, it can have also adverse effects by increasing the formation of advanced glycation products (AGEs). AGEs form as a result of polyol metabolism and accumulation of sorbitol. In addition, AGEs can activate the NLRP3 inflammasome, which results in secretion of pro-inflammatory molecules, e.g. IL-1β, in term placenta explants (101). Outside of pregnancy, activation of NLRP3 inflammasome is enhanced in individuals with obesity and induces chronic-low grade inflammation, cell death by pyroptosis and worsens metabolic control, e.g., insulin resistance (102). Placentas exposed to a maternal obesogenic environment *in utero* also exhibit increased markers of inflammation e.g., IL-1β, IL-8, MCP-1 and CXCR2 at term of pregnancy (103).

Cell cycle regulators like Cyclin D1, Chk1, Chk2, Rad52 and BRCA1 among others are upregulated in first trimester placentas exposed to maternal obesity (104). The bivalent role of these proteins as cell cycle regulators as well as DNA-damage repair proteins suggest altered proliferation in placentas exposed to maternal obesity. Indeed, DNA damage (increased γH2AX) and apoptosis (increased Caspase 3) are increased in first trimester placentas exposed to maternal obesity *in utero* (105). In principle, all the mechanisms described above, i.e., oxidative stress, ER stress and inflammasome activation, can directly or indirectly induce DNA damage. However, which molecule(s) of the obesity-associated environment can activate these pathways and the precise mechanisms involved remain largely unknown.

1.7. *In vitro* models to investigate the first trimester placenta

Due to ethical and technical reasons, sampling maternal blood and placenta tissue in the first trimester of pregnancy is challenging. This poses a major limitation to investigate early metabolic changes and placental development in humans. Collecting placental tissue from healthy donors is a major limiting factor for studies into early placental development. The placental tissue needs to be obtained during voluntary pregnancy termination and requires an obstetrical technique that preserves tissue structure, e.g., curettage. Alternatively, placental tissue can be obtained from chorionic villous sampling during the screening for genetic

abnormalities, but this procedure is only indicated for high risk pregnancies and not performed routinely (106).

In vitro culture of first trimester chorionic villus explants resemble the *in vivo* situation the best. Explants preserve the spatial conformation and relation between cell types, the cytotrophoblast remains viable for several days and the syncytiotrophoblast undergoes regeneration (20). Since access to first trimester placenta tissue is the main limiting factor, other *in vitro* models are widely used. For instance, trophoblast organoids keep the proliferative capacity for up to one year and preserve the 3D structure (107, 108). The limitations of the organoid model are again the need of first trimester placenta tissue as starting material (107, 108)

The most widely used first trimester placenta models are first trimester trophoblast cell lines. Since primary trophoblasts do not proliferate *in vitro* (109), first trimester placental cell lines had to be stablished. The most commonly used first trimester trophoblast cell lines are HTR-8/SVneo, Swan71 and ACH-3P cells (110). Each cell line resembles different trophoblast cell types and the decision which cell line to use depends on the specific study question (110, 111).

As a further consideration, to mimic the physiological oxygen tension in the first trimester of pregnancy, all these models should be cultured at 2.5% O_2 (concentration before opening of the spiral arteries) or at 6.5% O_2 (concentration at the end of the first trimester after opening of the spiral arteries) in a hypoxia bench. Exposing the cells to 21% oxygen (atmospheric concentration) or hypoxia-reoxygenation cycles (lowering and increasing oxygen concentration) should be avoided to prevent cellular stress and syncytiotrophoblast apoptosis (112).

For my research question, explants and primary trophoblasts would be the best *in vitro* model. However, scarce availability of fresh first trimester tissue precluded using explants, organoids and primary trophoblasts. Therefore, the first trimester trophoblast cell line ACH-3P was used for in *vitro* experiments and the cells were cultured in a hypoxia bench mimicking the physiological oxygen tension in the first trimester of pregnancy.

2. Hypothesis and Objectives

In a first study developed within my PhD project, C-peptide (proxy of insulin) was increased and insulin sensitivity (IS_{HOMA} index) decreased in women with overweight/obesity compared to normal weight in the first trimester of pregnancy (44). Since the glucose-insulin axis is a key regulator of metabolic pathways, we **hypothesized** that:

There is a distinctive metabolic fingerprint in pregnant women with overweight/obesity compared to normal weight in the first trimester of pregnancy and these dysregulated metabolites affect early placental development.

The first objective was to identify changes in the metabolism of women with overweight/obesity compared to women with normal weight. Specifically, the aims were to:

1) Compare the concentration of individual saturated, unsaturated and polyunsaturated fatty acids in maternal serum of women with overweight/obesity and normal weight women. (Paper 1)

2) Analyse all serum metabolites present in women with overweight/obesity and normal weight and to identify distinctive clusters of metabolites and metabolic pathways. (Paper 2).

The second objective was to explore processes impaired in first trimester placentas exposed to an obesity environment *in vivo*. Specifically, the aims were to:

3) Compare the proteomes of first trimester placentas of women with overweight/obesity and normal weight, and to select key identified proteins for further analyses.

4) Identify the most relevant traits related to maternal metabolism that associate with changes in the placental proteome.

3. Materials and Methods

Part of this section was published in adapted form in:

***Bandres-Meriz J**, *Dieberger AM*, Hoch D, Pochlauer C, Bachbauer M, Glasner A, Niedrist T, MNM van Poppel, Desoye G. Maternal Obesity Affects the Glucose-Insulin Axis During the First Trimester of Human Pregnancy. Front Endocrinol (Lausanne). 2020;11:566673.

Bandres-Meriz J, Majali-Martinez A, Hoch D, Morante M, Glasner A, van Poppel MNM, Desoye G, Herrera E. Maternal C-Peptide and Insulin Sensitivity, but Not BMI, Associate with Fatty Acids in the First Trimester of Pregnancy. Int J Mol Sci. 2021;22(19).

Bandres-Meriz J, Kunz C, Havelund JF, Faergeman NJ, Majali-Martinez A, Ensenauer R, Desoye, G. Distinct maternal metabolites are associated with obesity and glucose-insulin axis in the first trimester of pregnancy. Int J Obes (Lond). 2023;47(7).

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3.1. Study participants

The study was approved by the ethical committee of the Medical University of Graz (no. 29-095 ex 16/17, December 23, 2016, and 31-094 ex 18/19, March 1, 2019). Women undergoing legal elective pregnancy termination were recruited after providing written informed consent. Gestational age of the participants was determined based on their last menstrual period (LMP) and verified by measuring the fetal crown-rump length (CRL). The inclusion criteria were: maternal age of 18 years or older, gestational age less than 12 weeks and a singleton pregnancy. Exclusion criteria included known co-morbidities such as pre-existing diabetes mellitus, hypertension, or autoimmune diseases. The participants provided self-reported information regarding their age and smoking status (cf. 3.3). Characteristics of the participants included in each study are shown (**Appendix Table 1-4)**.

3.2. Human blood and placenta tissue collection

Maternal venous blood (8 ml) was drawn after overnight fast and prior to elective pregnancy termination. Blood was collected (S-Monovette® Sarstedt, Nümbrecht, Germany REF.: 02.1063, clot activator), centrifuged and the serum fraction immediately frozen at -80°C until further use.

First trimester placental tissue (gestational age week 4^{+0} - 11^{+6}) was obtained after legal pregnancy termination. The tissue was washed with phosphate buffered saline (PBS, Sigma Aldrich, St. Louis, MO, USA) and either snap-frozen in liquid nitrogen or fixed with formalin and paraffin embedded (FFPE). Fetal sex was assessed by gene expression analysis (RT-qPCR) (cf. 3.8.).

Processing time, defined as the time (minutes) between sample (blood and tissue) collection and freezing at the laboratory, was carefully recorded.

3.3. Serum measurement of clinical traits

Maternal body mass index (BMI) and circulating leptin were used as proxies of obesity and adiposity, respectively. Participant's height (centimetres) and weight (kilograms) were objectively measured by the practitioner prior to pregnancy termination and utilized to calculate the BMI index (kg/m^2). Leptin (ng/ml) was measured by an enzyme-linked immunoassay (ELISA) (DRG, Marburg, Germany, Cat# EIA2395).

Circulating glucose, C-peptide and an insulin sensitivity index were used as proxies of the glucose-insulin axis. Glucose (mg/dl) was measured in an automated analyser (cobas R8000 c701, Roche Diagnostics, Mannheim, Germany) by the hexokinase method (Glucose HK Gen.3, Roche Diagnostics). C-peptide (pmol/l) was measured as a surrogate of insulin by

ELISA (R&D Systems Minneapolis, USA, Cat# DICP00; respectively). Glucose and C-peptide measures were used to calculate the homeostatic model of insulin sensitivity (IS_{HOMA}) (113):

$$IS_{HOMA} = 22.5 / (\text{C-peptide(pmol/l)} * \text{glucose (mg/dl)})$$

The assessment of smoking status involved a two-step procedure. Firstly, participants were asked to provide self-reported information regarding their smoking habits (yes/no) through a questionnaire. Secondly, among the participants who self-reported as non-smokers, the concentration of serum cotinine was measured by ELISA (Abnova, Taipei, Taiwan Cat# KA0930) (44). Only participants who reported themselves as non-smokers and whose serum cotinine concentration was below the cut-off 4.47 ng/ml were considered non-smokers (114).

3.4. Serum measurement of fatty acids

Serum fatty acid concentrations were determined as previously described (115). In brief, serum lipids were extracted in a mixture of chloroform and methanol (2:1, v/v), which included 0.005% (w/v) butylated hydroxytoluene (BHT) as an antioxidant and nonadecenoic acid (19:1) as internal standard. The resulting lipid extracts were then evaporated under vacuum until dryness. The dried extracts were subsequently reconstituted in toluene and subjected to methanolysis for 2.5 hours at 80°C in a solution of methanol and toluene (4:1, v/v) containing acetyl chloride and methyl-heptadecanoate (C 17:0) as a reference standard. The fatty acid methyl esters were separated and quantified using a Perkin Elmer gas chromatograph (Autosystem; Norwalk, CT, USA) equipped with a flame ionization detector and a 30m × 0.25 mm Omegawax capillary column. Nitrogen was employed as the carrier gas, and the fatty acid methylesters were compared against purified standards (Sigma Chemicals Co., St. Louis, MO, USA). The concentrations of fatty acids (mg/l) were determined by calculating the peak areas relative to that of the internal standard.

3.5. Serum metabolomics

Metabolites were extracted from 100 µl of serum using a procedure similar to Folch's extraction (116). In summary, the serum samples were mixed with 600µl of a chloroform/methanol solution (2:1, v/v). The mixture was incubated in a thermoshaker at 1000 rpm, 4°C for 1 hour. Following this, 200 µl of water were added to each sample and incubated at 1000 rpm, 4°C for 15 minutes. The samples then underwent two consecutive centrifugation steps at 13000 rpm, 4°C for 15 minutes. The resulting aqueous phase was further diluted in 200µl of a chloroform/methanol/water solution (86/14/1, v/v/v). This mixture was again incubated at 1000 rpm, 4°C for 15 minutes. Subsequently, the aqueous phase was lyophilized and then resuspended in 30 µl of a 1% formic acid solution. For metabolomic analysis, 2 µl of the

resuspended sample were injected using a Vanquish Horizon HPLC system (Thermo Fisher Scientific, Germering, Germany) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) for mass spectrometric analysis.

To investigate whether GLP-1 mediates the association between palmitoleoyl ethanolamide and C-peptide, GLP-1 concentrations were measured in maternal serum by ELISA (Abcam, Cambridge, UK, Cat# ab184857).

3.6. Untargeted proteomics in first trimester placenta

Untargeted proteomics was performed to analyse the proteome of 19 first trimester placentas with a gestational age ranging from week 5^{+0} to 6^{+6} LMP. In brief, 10 µg of placenta tissue per sample were thawed and resuspended in Dulbecco's Phosphate Buffered Saline (PBS) and incubated for 1 hour at 37°C. The tissue was then lysed using sonication, and the protein content was measured using the PierceTM BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA, #23250). A total of 50 µg of protein were treated with tris (2-carboxyethyl)-phosphine hydrochloride (TCEP) (Sigma Aldrich, Burlington, MA, USA) and 2-chloracetamide (Sigma Aldrich) for reduction and alkylation, followed by heating to 95°C for 10 minutes. Acetone precipitation was performed overnight (-20°C) and the resulting protein pellet was dissolved in trifluoroethanol (TFE) and ammonium bicarbonate. Proteins were pre-digested for 2h with LysC (Promega, Madison, WI, USA) and further digested overnight with trypsin (Promega). The resulting peptides were desalted and dried before being dissolved in a solution containing 0.1% formic acid and 2% acetonitrile. The samples were then analysed using nano-HPLC coupled with a Q-TOF mass spectrometer. For label-free quantitation, MaxQuant software with the Andromeda peptide search engine was used (117). The software's match-between-runs feature was enabled with a retention window of 0.7 min and an alignment window of 20 minutes.

3.7. Immunostaining

Formalin-fixed paraffin embedded (FFPE) placental tissues were sectioned into 3.5 µm slices and placed on Superfrost Plus slides (Menzel. Braunschweig, DE). To retrieve antigens, heat-induced antigen-retrieval was performed in citrate buffer (pH 6.5) using a decloaking chamber (Biocare Medical, Concord, CA, USA) at 120°C for 15 minutes. Prior to antibody incubation, peroxidase and protein blocking solutions (Thermo Scientific, Kalamazoo, MI, USA) were added.

For immunohistochemistry, placental sections were incubated with primary antibodies (MCM6 1:500) for 1 hour at room temperature in a humidified chamber. Primary antibody

enhancer (Dako, Glostrup, Denmark) was applied for 10 minutes, followed by incubation with HRP polymer (Dako) for 15 minutes and incubation with AEC single solution (Thermo Scientific) for 5 minutes. Nuclei were counterstained with Mayer's haematoxylin (Gratt Koller, Abcam, Austria) and mounted with Aquatex (Merck). Images were captured using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) equipped with a digital camera (Olympus, Tokyo, Japan) and AxioVision software (Zeiss, Oberkochen, Germany).

For immunofluorescence, first trimester placenta sections were incubated with primary antibodies (MCM6 1:500, Abcam; γH2AX, 1:100, Merck) overnight at 4°C. After washing with 1% Tween-TBE buffer, the sections were incubated with secondary antibodies (1:1000 anti-rabbit Alexa Fluor 550; 1:200 anti-mouse Alexa Fluor 488; Thermo Scientific) for 1.5 hours. Nuclei were stained with DAPI. To reduce autofluorescence, the tissue was treated with 0.3% Sudan Black B (Sigma Aldrich) in 70% (v/v) ethanol for 5 minutes.

To examine the co-localization of γH2AX and MCM6 in response to various DNA damage inducers, ACH-3P cells were incubated with primary antibodies (MCM6 1:100, Abcam; γH2AX, 1:100, Merck) for 1 hour at room temperature. Subsequently, secondary antibodies (1:200 anti-rabbit Alexa Fluor 550; 1:200 anti-mouse Alexa Fluor 633; Thermo Scientific) were applied and incubated for 1.5 hours. For an overall assessment of MCM6 and γH2AX localization, images were captured using a 40x objective on a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) equipped with a digital camera (Olympus, Tokyo, Japan). AxioVision software (Zeiss, Oberkochen, Germany) was used for image acquisition. For visualization purposes, adjustments to brightness and contrast were made afterward using the OlyVia software (Olympus).

Co-localization analysis involved acquiring images using an A1R confocal microscope (Nikon CEE GmbH, Vienna, Austria) with a 100x objective and consistent settings for all images. Three images from different regions were taken for each sample. Image J Fiji software was utilized to assess co-localization by drawing a scan line in the region of interest and quantifying the intensity (a.u.) per pixel (µm). The obtained results were plotted using GraphPad Prism (Version 8.4.0, San Diego, CA, USA). Z-stacks (0.4 µm) were utilized to construct 3D images using NIS-Elements software (Nikon). For visualization purposes, adjustments to brightness and contrast were made subsequently in ImageJ Fiji (118).

3.8. RNA isolation and RT-qPCR

In a subgroup of pregnancies (n = 83), placental tissue was available, allowing for sex determination using a previously established method (119). Placental tissue was homogenized in RLT Plus Buffer (Qiagen, Venlo, Netherlands) containing 1% β-Mercaptoethanol (v/v, Merck,

Darmstat, Germany) using a tissue lyser (MagNa Lyser, Roche, Basel, Switzerland). RNA isolation was performed with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer's instructions. Reverse transcription was carried out using the LunaScriptTM RT SuperMix Kit (New England BioLabs, Frankfurt, Germany). For RT-qPCR analysis, TaqMan Universal PCR Master Mix (Life Technologies, Carlsbad, CA, USA) and the primers DDX3Y (FAM labeled) and XIST (VIC labeled) (Life Technologies, DDX3Y: Hs00965254_gH, XIST: Hs01079824_m1) were utilized. The RT-qPCR was performed on a CFX96 Thermocycler (BioRad Laboratories, Hercules, CA, USA). Cycle threshold (Ct) values were obtained using the BioRad CFX Manager 3.1 software, and fetal sex was determined based on the ΔCt (XIST Ct - DDX3Y Ct) calculation.

For cell culture samples, the miRNeasy Tissue/Cells Advanced Micro Kit (Qiagen, Hilden, Germany) was used to isolate total RNA. The RNA was then reverse-transcribed using the Luna Script RT SuperMix Kit (New England BioLabs, Ipswich, MA, USA). To assess mRNA expression TaqMan universal PCR master mix (Life Technologies, Carlsbad, CA, USA) and TagMan gene expression assay for MCM6 (Hs00172459_m1, Life Technologies) was employed in RT-qPCR using a CFX384 RT-qPCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The Bio-Rad CFX Manager 3.1 software automatically generated Ct values. ΔCt values were calculated using the mean value of housekeeping genes TATA-binding protein (TBP; Hs03929085_g1, Life Technologies) and Peptidylprolyl isomerase A (PPIA; Hs04194521_s1, Life Technologies). For data visualization, the results were normalized to the reference category using the $2^{-\Delta\Delta Ct}$ method.

3.9. Protein isolation and western blotting

Placental tissue and ACH-3P cells were suspended in RIPA buffer (Sigma-Aldrich) containing protease inhibitors (Roche) and phosphoprotease inhibitors (MedChemExpress, Princeton, NJ, USA). Placental tissue was homogenized using a MagNa Lyser device (Roche). Protein concentration was determined using the bicinchoninic acid assay (BCA, Thermo Fisher Scientific). Protein lysates were mixed with Laemmli buffer 2x (Sigma Aldrich) and denatured at 95°C for 5 minutes. Equal amounts of total protein (10µg/well for tissue lysates and 5µg/well for cell lysates) were loaded onto 4-20% SDS-PAGE gels (BioRad Technologies) and resolved at 120 V for 75 minutes. The proteins were then transferred to a nitrocellulose membrane (BioRad Technologies) and stained with Ponceau S (Thermo Scientific). Non-specific binding sites were blocked with 5% non-fat dry milk (BioRad Laboratories) in tris-borate-EDTA (TBE) + 0.1% Tween 20 (Sigma Aldrich) for 1 hour. Following blocking, the membranes were incubated overnight at 4°C with primary antibodies against MCM6 (1:500 Abcam), γH2AX (1:1000, Merck), α-Tubulin (1:1000, Merck) or β-actin (1:20000). After washing the membranes

three times with TBE, they were incubated with the appropriate secondary antibodies (anti-mouse or anti-rabbit) conjugated with horseradish peroxidase. Immunodetection was performed using the SuperSignal West Pico kit (Thermo Scientific) and a ChemiDoc Touch imaging system (Bio-Rad Laboratories). Density of protein bands was quantified using image Lab 6.0.1 software (Bio-Rad Laboratories). When applicable, data was further normalized to an internal standard run on each membrane to account for variation between membranes.

3.10. Cell culture and hypoxia workbench

ACH-3P cells were seeded in 6-well plates ($2.5x10^4$ cells/well) for RNA and protein isolation or in 4-well chamber slides ($1x10^4$ cells/well) for immunohistochemistry. Three technical replicates were included per experimental condition. ACH-3P cells were cultured in Ham's F12 medium supplemented with glutamine (Thermo Fisher Scientific, Waltham, MA, USA; Biowest, Nuaillé, FR), containing 10% (v/v) defined fetal bovine serum (FBS, Cytiva, Marlborough, MA, USA) and 1% (v/v) penicillin/streptomycin (Thermo Fisher Scientific). The cells were then transferred to a hypoxic workstation (Xvivo System Model X3, BioSpherix, Redfield, NZ, USA) and allowed to adjust to the preset oxygen levels for 48 hours. All experiments were conducted in the hypoxia workstation at 6.5% O_2, except for the oxygen challenge experiment, for which cells were cultured at 2.5%, 6.5%, or 21% O_2 (cf. 3.10.3.).

3.10.1. DNA damage induction and recovery

To titrate the induction of DNA damage with etoposide, ACH-3P cells were exposed to 1µM, 5µM or 10µM etoposide (Cayman Chemicals, Ann Arbor, MI, USA) or DMSO (vehicle) (Sigma Aldrich, Burlington, MA, USA) for 1 hour at 6.5% O_2 (n = 1, in triplicates). Subsequently, cells were collected for gene expression and protein analysis.

To investigate DNA-damage repair, ACH-3P cells were maintained in culture for 72 hours in a hypoxia bench (6.5% O_2) and then treated with 10µM etoposide or DMSO (vehicle) for 1 hour (n = 3, in triplicates). The medium was removed, and the cells were allowed to recover for 3 hours or 24 hours in fresh medium (Ham's F12, 10% FBS, 1% penicillin/streptomycin), followed by cell collection for subsequent gene expression and protein analysis.

To investigate the co-localization of γH2AX and MCM6 in response to different DNA damage inducers, ACH-3P cells were cultured in 4-well chamber slides for 48 hours at 6.5% O_2. Thereafter, cells were treated with 10µM etoposide or 10µM bleomycin (Sigma Aldrich) (DMSO or F12 medium as respective vehicle controls) for 1 hour and then allowed to recover in fresh medium (Ham's F12, 10% FBS, 1% penicillin/streptomycin) for 1 hour at 6.5% O_2.

3.10.2. Insulin treatment

After 72 hours in culture in a hypoxia bench at 6.5% oxygen, the medium was replaced with low serum medium (Ham's F12, 2% FCS, 1% penicillin/streptomycin) for 24 hours to reduce insulin levels in the serum (final insulin concentration: $1.4x10^{-4}$nM) (n = 3, in triplicates). Subsequently, cells were cultured in the absence or presence of 10nM insulin (Calbiochem, San Diego, CA, USA) for an additional 24 or 48 hours at 6.5% O_2 and then collected for gene expression and protein analysis. This insulin concentration (10nM) was chosen to mimic physiological hyperinsulinaemia while preventing IGF-1 receptor activation (120).

3.10.3. Oxygen challenge

To closely mimic *in vivo* physiology, cells were cultured for 48 hours at 2.5% O_2 (physiological oxygen tension before the opening of the spiral arteries) (n = 4, in triplicates). After 72 hours, cells were either maintained at 2.5% O_2 or transferred to 6.5% O_2 or 21% O_2 for 48 hours, respectively. Then, cells were collected for gene expression and protein analysis.

3.10.4. ShRNA silencing

To investigate whether MCM proteins are required to initiate DNA damage repair in the first trimester placenta, MCM6 and MCM3 were silenced in ACH-3P cells using sort hairpin RNA (shRNA). Initial experiments were conducted during my stay abroad at the group of Prof. Louise T. Dalgaard, Department of Science and Environment, University of Roskilde and further continued at the Medical University of Graz. All the plasmids were kindly provided by Prof. Louise T. Dalgaard. pGIPZ plasmids containing a reporter gene (TurboGFP), a puromycin resistance cassette and the shRNA sequences of interest (shMCM6 and shMCM3) or negative controls (scramble shRNA and empty plasmid) were isolated from E. Coli following the manufacturer instructions (Midiprep plasmid isolation kit, Qiagen, Hilden, Germany). The lentiviral plasmids psPAX (packaging plasmid) and VSV-G or PMD2.G (envelope plasmids) were also isolated form E- Coli. For quality control, the isolated plasmids were digested enzymatically with KpnI (New England Biolabs) and 10xFast Digest Buffer (New England Biolabs) and the fragments separated in 1% agarose gel. To produce lentiviral particles, HEK293T cells were seeded in 6 cm^2 dishes ($1x10^6$ cells/dish) and incubated for 24h at 37°C. The day of transfection, HEK293T cells were co-infected with the shRNA plasmid of interest (2.5µg), psPAX (2.5µg) and VSV-G or PMD2.G (1µg) and lipofectamine 2000 (15µl; ThermoFisher) in 500µl DMEM high glucose, glutamax medium (ThermoFisher) without serum or antibiotics and incubated for 24h at 37°C. Next day, the media was replaced by DMEM supplemented with 10% FCS and 1% P/S. The supernatant containing the lentiviral particles was collected 48h and 72h after transfection and used to infect ACH-3P cells (80%

confluence). To facilitate infection, spinfection (1800rpm, 45min) in presence of sequa-brene (5µg/ml; Merck) was used. After 3h of incubation at 37°C, 1ml fresh media (Ham´s F12, 10% FBS, 1% P/S) was added and the cells further incubated for 48h.

To generate a stable cell line expressing the transgene of interest, positive cells were selected with puromycin (1µg/ml, Merck) for 7 days. Transduction of ACH-3P cells did not work in the laboratory. Therefore, the plasmids of interest (shMCM6, shMCM3) and negative control (shScramble, shEmpty) were sent to a company for packaging into lentiviral particles (VectorBuilder, Chicago, IL, USA). ACH-3P cells were transduced (multiplicity of infection = 7) with the commercially-packed lentiviral particles and positive selected with puromycin (1 µg/ml) for 7 days. FACS sorting was used to purify successfully transduced ACH-3P cells using the GFP+ marker (FACSARia IIIu, BD Biosciences, Franklin Lakes, NJ, US; performed at Imaging Core facility, ZMF, Graz) and classified into low, medium and high GFP positive cells. FACS sorted ACH-3P cell were kept in culture for 7 additional days. For quality control, protein extracts were used for western blotting and incubated with anti-MCM3 (1:500, Cell Signalling, #4012S, Danvers, MA, USA) or anti-MCM6 antibodies (1:500).

3.11. Bioinformatics and statistical analysis

3.11.1. Fatty acid analysis

Normal distribution of the variables was assessed by Shapiro-Wilk test and visually with histograms and Q-Q plots. Not normally distributed variables were ln-transformed. Associations between categorized exposures (BMI, leptin, glucose, C-peptide, IS_{HOMA}) and outcomes (fatty acids) were analysed in multivariable linear regression models. The models were adjusted for a priori defined confounders: maternal age (years), gestational age (days), processing time (minutes). The cut-off for the categorization of the leptin, glucose, C-peptide and IS_{HOMA} was based on tertiles (calculated from the cohort). The category representing the most favourable metabolic group, i.e., normal weight women, lowest leptin, lowest glucose, lowest C-peptide, highest IS_{HOMA}, were used as the reference category.

Gestational age changes in fatty acid concentration were examined using linear regression models for fatty acid classes as well as individual fatty acids. Linearity of gestational age changes was assess comparing the β-estimate between gestational age categories (week 4^{+0} - 6^{+6}, 7^{+0} - 9^{+6}, 10^{+0} - 11^{+6}). In addition, fatty acid measurements were standardized (z-score) and the median of each fatty acid at each gestational age category (week 4^{+0} - 6^{+6}, 7^{+0} - 9^{+6}, 10^{+0} - 11^{+6}) calculated. The median value was used to create a heat map based on length of the carbon chain (X-axis) and the degree of saturation (Y-axis).

For fetal sex analysis, an interaction term between fetal sex and the exposure variable was included in the model. If the interaction term was significant, the data was split into male and female groups and the association with the exposure analysed in a multivariable model. To correct for multiple testing, Benjamini-Hochberg procedure was applied. The threshold for significance was set at $p < 0.05$ for all analysis and $p < 0.1$ for interaction terms. Data analysis was done in IBM SPSS statistics (version 25, IBM Corp, Armonk, NY, USA) and GraphPad Prism (version 8.4.2, GraphPad Software, San Diego, CA, USA).

3.11.2. Metabolomics data analysis

Extreme outliers, defined as those above mean ± 4 * SD were considered measurement errors or contamination and excluded from analysis, i.e., counted as missing data (121). Missings denoting extreme outliers were imputed by k-nearest neighbours approach (121). All metabolites were log2 transformed for further analysis.

Associations between exposure variables (BMI, leptin, glucose, C-peptide, IS_{HOMA}; continuous and categorized) and outcomes (metabolites) were analysed using univariable analyses. P-values were adjusted for multiple testing using the Benjamini-Hochberg method to control for the false discovery rate (FDR). Machine learning (partial least squares discriminant analysis, random forest, supported vector machine algorithms) was applied to the set of annotated metabolites using the R-package Biosigner. The objective was to identify sets of metabolites that better predict a phenotype, e.g., obesity, hyperinsulinaemia. All the metabolites that were significantly associated with at least one exposure at these initial steps were further used for multivariable analysis. The multivariable models included three *a priori* defined confounders: maternal age (years), gestational age (days) and processing time (min). Cubic splines were used for those models where assumption of linearity was not fulfilled.

In addition, network analysis was used to identify sets of correlating metabolites that are simultaneously associated with an exposure. These analysis were performed with the "MoDentify" R-package. To determine the robustness of the results, univariable analyses including all the measured metabolites (n = 2449, annotated and non-annotated) were performed.

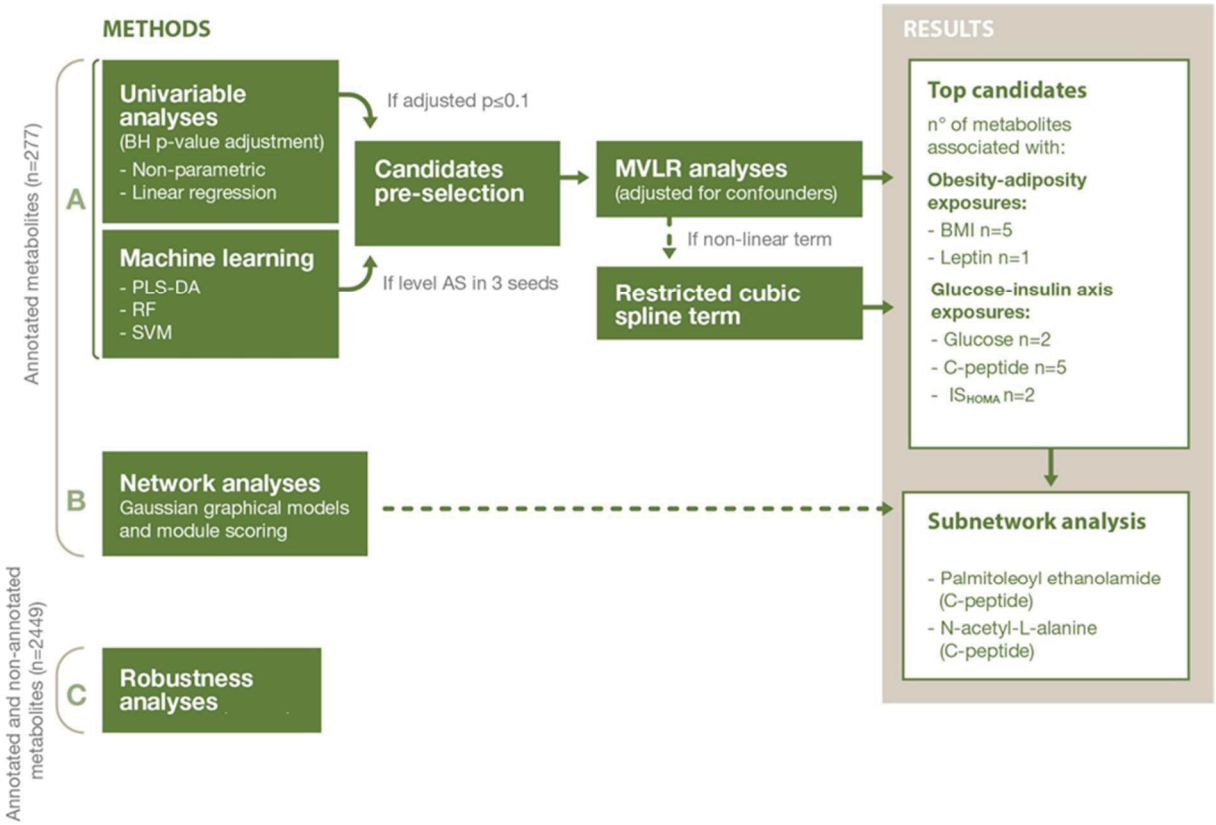


Figure 4. Flow chart of data analyses. A) Univariable analysis and machine learning methods were used to select metabolites significantly associated with at least one exposure (BMI, leptin, glucose, C-peptide or IS_{HOMA}). Next, to select the most significant candidates, multivariable linear regression (adjusted for confounders) or restricted cubic spline terms were used. B) Network analysis was used to identify sets of correlating metabolites that are simultaneously associated with an exposure (BMI, leptin, glucose, C-peptide or IS_{HOMA}). C) To test the robustness of the results, univariable analyses were performed in the total set of metabolites (annotated and non-annotated). BH: Benjamini-Hochberg; PLS-DA: partial least squares discriminant analysis; RF: Random forest; SVM: Support vector machine; MVLR: Multivariable linear regression analysis; BMI: Body mass index, IS_{HOMA}: Homeostatic model assessment of insulin sensitivity. *This figure was adapted from (116) with publisher's permission.*

To investigate whether GLP-1 mediates the association between palmitoleoyl ethanolamide (exposure) and C-peptide (outcome), a mediation analysis adjusted for the confounders gestational age (days), maternal age (years) and processing time (min) was conducted in a sub-cohort (n = 95) (**Figure 5**). To estimate the average effects, nonparametric bootstrap with 1000 bootstrap samples was used. The nonparametric bootstrap-based confidence intervals of the estimates were calculated via the bias-corrected and accelerated method. In addition, concentrations of palmitoleoyl ethanolamide and GLP-1 (n = 95), GLP-1 and C-peptide (n = 95) and C-peptide and palmitoleoyl ethanolamide (n = 111) were correlated using Spearman correlation.

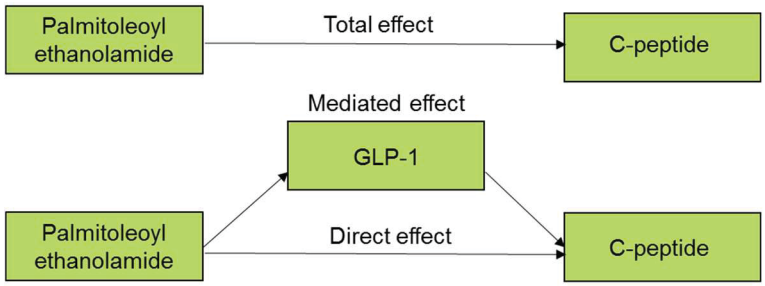


Figure 5. Mediation analysis. The total effect is the effect of the exposure (palmitoleoyl ethanolamide) on the outcome (C-peptide) without accounting for the mediator (GLP-1). The direct effect is the effect of the exposure on the outcome after taking into account a mediation effect of the mediator. The mediation effect is the total effect minus the direct effect. If the effect of the mediated effect is statistically significant ($p < 0.05$), then it can be concluded that the association between palmitoleoyl ethanolamide and C-peptide is mediated by GLP-1.

3.11.3. Proteomics

Downstream bioinformatic analysis was performed using Perseus software (version 1.6.10.43) (122). The filtering criteria set in the analysis required proteins to have a minimum of 9 valid values (greater than 0) in at least one biological group (low or high IS_{HOMA} group). Missing values were then imputed by random numbers using a normal distribution based on the overall data matrix (122). Following this, unsupervised principal component analysis was conducted. Additionally, a two-sided unpaired t-test with permutation-based false discovery rate (FDR) control at a significance level of 5% was performed. Protein-protein interaction, functional enrichment analysis and gene ontology analysis were conducted in STRING software (version 11.5) (123). The analysis included all proteins that were found to be significant ($p < 0.05$) in the low or high IS_{HOMA} group. The Homo sapiens library was used as background for the analysis. From each type of analysis (pathway, biological process, molecular function, cellular component), the top enriched terms were selected based on Benjamini and Hochberg false discovery rate p-values.

3.11.4. Search and use of public database repositories

Data repositories containing transcriptomic data were used to compare the first trimester placenta proteomics results with data available online (**Table 2**). Corresponding authors of the respective research articles gave permission to include the data in this doctoral thesis.

Table 2. Transcriptomic datasets that were used to compare self-generated results with published data.

Content	Database	Accession code	Source
Gene expression dependent on fetal sex in placenta tissue	NCBI Gene Expression Omnibus	GSE160076	(124)
	Supp. data; Additional File 4: Table S3		(125)
Gene expression in first trimester placenta cell types	Supp. data. Data File S1		(126)

All the datasets are available online.

3.11.5. Online tools and free use software

String database (123) was used to predict protein-protein interactions (networks) between the proteins upregulated or downregulated in placentas of women with low insulin sensitivity. String database shows interactions derived from genomic context predictions, high-throughput lab experiments, conserved co-expression, automated text mining and previous knowledge in databases. In addition, String database was used to find functional enrichments in the set of input genes (KEGG pathways).

Protein-protein interactions were exported from String database and imported into Cytoscape for visualization (127).

Enrichr (128) was used to identify transcription factors associated with genes codifying the proteins that were significantly upregulated and downregulated in placentas of women with low insulin sensitivity. Enrichr is linked to the manually curated database Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRUST) (129).

4. Results

Some of the results presented in this dissertation have been published in the following scientific papers:

Bandres-Meriz J, Majali-Martinez A, Hoch D, Morante M, Glasner A, van Poppel MNM, Desoye G, Herrera E. Maternal C-Peptide and Insulin Sensitivity, but Not BMI, Associate with Fatty Acids in the First Trimester of Pregnancy. Int J Mol Sci. 2021;22(19).

***Bandres-Meriz J**, Kunz C, Havelund JF, Faergeman NJ, Majali-Martinez A, Ensenauer R, Desoye, G. Distinct maternal metabolites are associated with obesity and glucose-insulin axis in the first trimester of pregnancy. Int J Obes (Lond). 2023;47(7).

*Metabolomics experiments were performed in the laboratory of Prof. Faergeman (Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense M, Denmark) during my research stay abroad in 2019. Metabolomics data was analysed at the department of Prof. Ensenauer (Institute of Child Nutrition, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany) during my research stay abroad in 2020.

In addition, silencing experiments (shRNA) in ACH-3P cells were performed at the laboratory of Prof. Dalgaard (Department of Science and Environment, Roskilde University, Roskilde, Denmark) during my stay abroad in 2021. These results are unpublished.

4.1. Metabolism of women with obesity in the first trimester of pregnancy

Maternal metabolism undergoes significant changes throughout pregnancy, and disruptions in metabolism, such as in obesity, can have negative effects on placental and fetal development. Moreover, maternal obesity can be “transmitted” contributing to a vicious cycle of obesity across generations. During the early stages of pregnancy, maternal metabolism plays a crucial role in regulating the growth and development of the placenta and fetus. Therefore, metabolic disturbances in the first trimester can potentially influence placental and fetal growth. However, there has been limited research on how maternal metabolism differs between lean and women with overweight/obesity during the first trimester. In this study, circulating fatty acids and metabolites in maternal serum were analysed and compared among women with varying BMI and clinical characteristics (leptin, glucose, C-peptide, and IS_{HOMA}). Additionally, when the sample size permitted, potential fetal sex influence on maternal metabolism was investigated.

4.1.1. Gestational age changes in fatty acid concentration

The study included 123 pregnant women (gestational age week 4^{+0} - 11^{+6}), all non-smokers. There was a general increase in total fatty acid concentrations (CI: 2.0 - 12.1, p = 0.007) with increasing gestational age. Individual fatty acids which increased with gestational age were palmitic acid (C 16:0), oleic acid (C 18:1), α-linolenic acid (C 18:3 n-3), dihomo-γ-linolenic acid (C 20:3 n-6), arachidonic acid (C 20:4 n-6), adrenic acid (C 22:4 n-6), osbond acid (C 22:5 n-6) and docosahexaenoic acid (C 22:6 n-3). Despite overall increase in fatty acid concentrations with advancing gestation, no distinctive pattern based on fatty acid composition (length of the carbon chain and degree of saturation) was observed (**Figure 6**). The small sample size (n = 17) at week 10^{+0} - 11^{+6} precluded investigating whether fatty acids changes throughout the first trimester are different in women with overweight/obesity compared to normal weight women. However, there was no significant interaction between gestational age and BMI, leptin, glucose, C-peptide or IS_{HOMA}, suggesting that fatty acid changes in concentrations during the first trimester are comparable between women with overweight/obesity and normal weight women. To account for gestational age influences, all analyses were adjusted for gestational age.

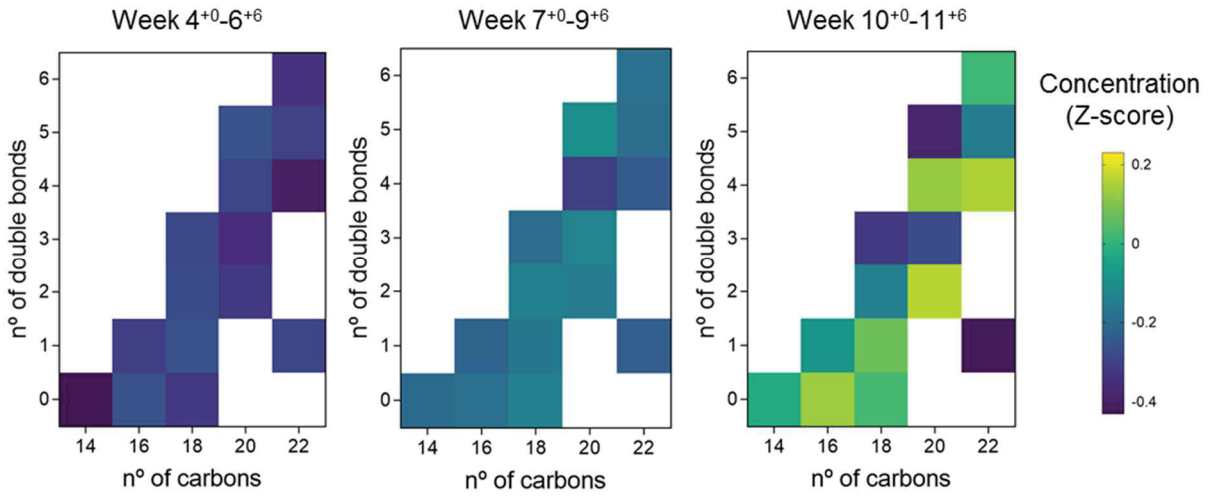


Figure 6. Fatty acid concentrations increase with advancing gestational age. Heat map showing the concentration (z-standardized) of each fatty acid classified according to the length of the carbon chain (X-axis) and the degree of saturation (Y-axis) at different gestational age categories. A general trend towards increased fatty acid concentration with advancing gestational age is observed, but not distinctive pattern based on carbon chain length and saturation. Colour code reflecting concentration is on the gradient scale on the right. *This unpublished figure is based on published data (44) with publisher's permission.*

4.1.2. Fatty acid metabolism in obesity

No significant associations between BMI, leptin or glucose and fatty acid concentrations were found, nor in fatty acid classes (saturated, unsaturated, n-3 PUFA, n-6 PUFA) nor in individual fatty acid species. In contrast, high C-peptide levels and low IS_{HOMA} were associated with decreased n-3 PUFA concentrations (**Figure 7**). The category with the most adverse, e.g., unhealthy, C-peptide (highest tertile, C-peptide ≥ 437.8 pmol/l) levels and lowest insulin sensitivity (lowest tertile, IS_{HOMA} ≤ 0.59) had decreased n-3 PUFA concentration (CI: –35.82– –6.28, p = 0.006; CI: –36.48 – –5.61, p = 0.008; respectively), compared to the reference category, e.g., the metabolically most favourable (C-peptide < 315.4 pmol/l; IS_{HOMA} > 0.85). Among the individual fatty acids, eicosatrienoic acid (C 20:3 n-3), eicosapentaenoic acid (C 20:5, n-3) and docosapentaenoic acid (C 22:5 n-3) were decreased in women with the highest C-peptide levels. Docosapentaenoic acid was also decreased in women with very low insulin sensitivity (IS_{HOMA} ≤ 0.58).

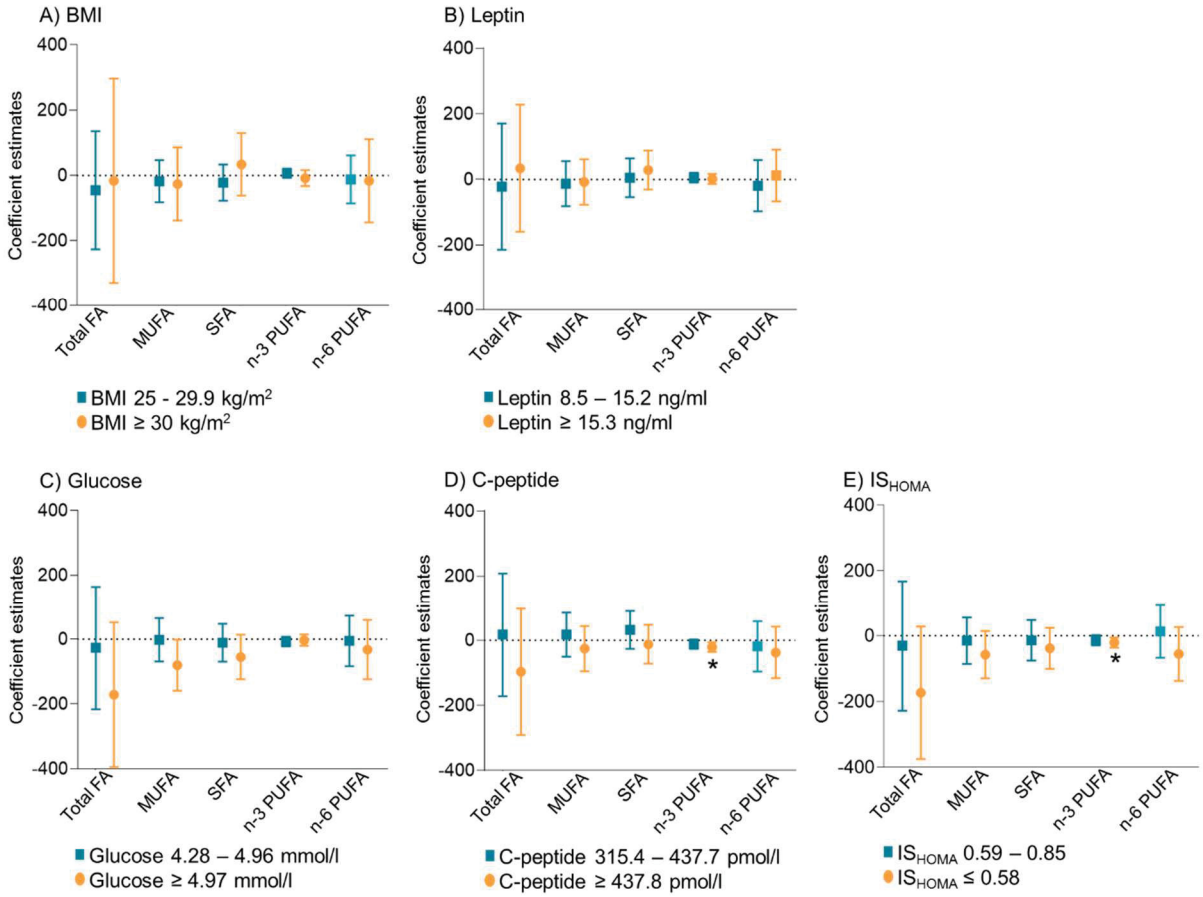


Figure 7. High C-peptide and low IS_{HOMA} associate with decreased n-3 PUFA in maternal serum. Exposure variables were categorized into normal weight, overweight and obese (BMI) or tertiles (leptin, glucose, C-peptide and IS_{HOMA}). Associations between categorized exposures and outcomes (total fatty acids, saturated, monounsaturated, n-3 polyunsaturated and n-6 polyunsaturated fatty acids) were analysed by linear regression adjusting for confounders (gestational age, maternal age and processing time). The most metabolically favourable category was selected as reference category: BMI < 25 kg/m^2, leptin < 8.5 ng/mL, glucose < 4.28 mmol/l, C-peptide < 315.4 pmol/L, IS_{HOMA} > 0.85. SFA: Sum of 14:0, 16:0, and 18:0; MUFA: Sum of 16:1, 18:1, and 22:1; n-3 PUFA: Sum of 18:3, 20:3, 20:5, 22:5, and 22:6; n-6 PUFA: Sum of 18:2, 18:3, 20:3, 20:4, 22:2, 22:4, and 22:5. * p < 0.05 vs reference category. SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids, n-3 PUFA: n-3 Polyunsaturated fatty acids; n-6 PUFA: n-6 Polyunsaturated fatty acids. *This figure was adapted from (44) with publisher's permission.*

4.1.3. Fetal sex effects on maternal fatty acid metabolism

When available, placental tissue was used (n = 83) to determine fetal sex. A significant interaction was observed between fetal sex and C-peptide (p = 0.072), and fetal sex and IS_{HOMA} (p < 0.070) in the model for total n-3 PUFA. These interactions remained significant after correcting for multiple testing. Consequently, separate analyses were conducted for women carrying female or male fetuses. Nevertheless, no significant associations were found between C-peptide or IS_{HOMA} with total n-3 PUFA concentration in these subgroups. Regarding individual n-3 PUFA, a significant interaction was observed between fetal sex and C-peptide

in the model for docosahexaenoic acid (C 22:6 n-3), even after adjusting for multiple testing. Subsequent analyses showed that the association between C-peptide and docosahexaenoic acid is specific for mothers carrying female fetuses (**Figure 8**). Adjusting for BMI or leptin did not significantly alter the effect size.

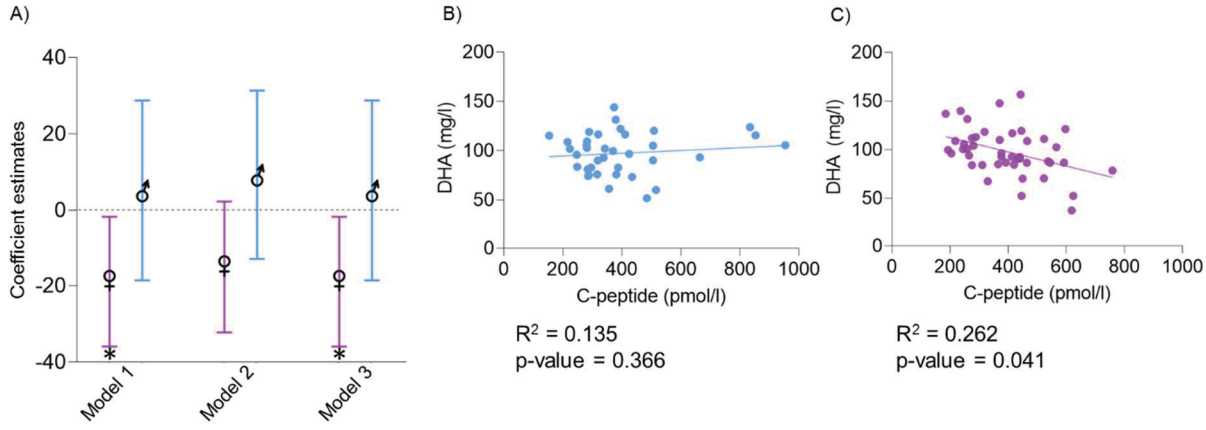


Figure 8. C-peptide associates with docosahexaenoic acid only in female-bearing mothers. A) Association between C-peptide and docosahexaenoic acid (DHA) in the female (pink; n = 46) and male (blue; n = 37) subgroups. Model 1: Adjusted for gestational age, maternal age and processing time; Model 2: Model 1 + adjustment for BMI; Model 3: Model 1 + adjustment for leptin). B) Correlation between docosahexaenoic acid and C-peptide in the male subgroup. C) Correlation between docosahexaenoic acid and C-peptide in the female subgroup. * $p < 0.05$. *This figure was adapted from (44) with publisher's permission.*

4.1.4. Untargeted metabolomics in maternal serum

After stringent statistical analysis, 15 metabolites were significantly associated with BMI (n = 5), leptin (n = 1), glucose (n = 2), C-peptide (n = 5) or IS_{HOMA} (n = 2) (**Table 3**). To evaluate the reliability of the results, an additional analysis that included all identified metabolites, including annotated and not-annotated metabolites (n = 2449) was conducted (**Figure 9, Table 4**). Interestingly, palmitoleoyl ethanolamide and N-acetyl-L-alanine remained significantly associated with C-peptide. Subnetwork analysis was performed to identify other metabolites simultaneously associated with C-peptide and correlated with palmitoleoyl ethanolamide and N-acetyl-L-alanine. Among the metabolites meeting these criteria (n = 26), most were amino acids or proteins (n = 9; 35%) or lipids (n = 7; 27%). There was a positive correlation between palmitoleoyl ethanolamide and the concentrations of L-proline, sphingosine-1-phosphate and tetradecanedioic acid. Similarly, N-acetyl-L-alanine showed a positive correlation with (2E,4E)-2,4-hexadienoic acid and aniline concentrations (**Figure 10**).

Table 3. Metabolites significantly associated with BMI, leptin, glucose, C-peptide and IS_{HOMA}.

	Confidence interval 95%	Effect size (%)	*p*-value
Obesity/Adiposity			
BMI			
Serine-Tyrosine	0.12 – 0.31*	0.30	<0.001
Phenylalanine-Threonine	0.14 – 0.31*	0.27	<0.001
Stachydrine	-0.22 – -0.06	0.18	<0.001
Proline-Hydroxyproline	-0.07 – -0.02	0.23	0,007
S-methyl-L-cysteine	-0.09 – -0.02	0.12	0,002
Leptin			
Phenylalanine-valine	0.04 – 0.29	0,17	0,021
Glucose-insulin axis			
Glucose			
Hexamethylphosphoramide	-0.02 – 0.41	0.17	0,034
15S-hydroperoxy-11Z,13E-eicosadienoic acid	-0.21 – -0.30	0.17	0,019
C-peptide			
Palmitoleoyl ethanolamide	0.10 – 0.34	0,21	<0.001
Androsterone glucuronide	0.12 – 0.60*	0,21	0,05
N-acetyl-L-alanine	0.04 – 0.10	0,18	<0.001
7α,17α-dimethyl-5β-androstane-3α,17β-diol glucuronide	-0.20 – 0.15*	0.17	0,003
Uridine	-0.14 – 0.04*	0.13	0,004
IS_{HOMA}			
S-Methyl-L-cysteine	-0.02 – 0.04	0.11	0,004
1-Myristoyl-sn-glycero-3-phosphocholine	-0.11 – -0.02	0.21	0,001

Linear regression terms or restricted cubic spline terms, as appropriate, were used to model the influence of exposures on metabolites. The models were adjusted for gestational age (days), maternal age (years), and processing time (minutes), assuming linear relationships. An interaction term between the exposure and gestational age was included in the model. The effect size (%) represents the coefficient of determination (R^2), indicating the proportion of variance in the outcome variable that can be predicted from the influence variables (R^2 ranges from 0 to 100%). * The association was not linear and, therefore, modelled with restricted cubic spline terms. *This table was adapted from (116) with publisher's permission.*

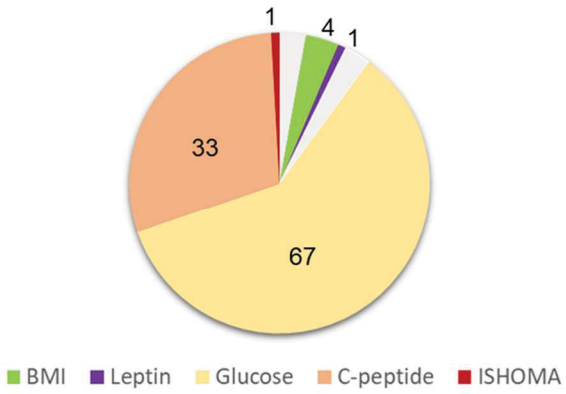


Figure 9. Robustness analysis. Number of metabolites (annotated and non-annotated) significantly associated with one of the exposures. One hundred and six different metabolites (4.3% of the total number of metabolites) were associated with at least one of the exposures (number of metabolites: BMI = 4, leptin = 1, Glucose = 67, C-peptide = 33, IS_{HOMA} = 1). *This figure was adapted from (106) with publisher's permission.*

Table 4. N-acetyl alanine and palmitoleoyl ethanolamide are robustly associated with C-peptide.

Exposure	Metabolite
BMI	NA
Leptin	NA
Glucose	Serine-Tyrosine (S)-Malate 3,5-Dihydroxyphenyl dodecyl benzene-1,3-diol S-Methyl-L-cysteine
C-peptide	N-acetyl-L-alanine Palmitoleoyl ethanolamide 3-Hydroxybutyrylcarnitine γ-Glutamylleucine
IS_{HOMA}	NA

Univariable analysis adjusted for multiple testing (Benjamini-Hochberg procedure) was used to find associations between the exposure variables and all the measured metabolites (annotated and non-annotated, n = 2449). Only significant associations are shown (adjusted p-value ≤ 0.1). NA: Not applicable (no metabolites significantly associated with the exposure). Bold: Metabolites significantly associated with a variable (cf. Table 3) that were again significantly associated in the robustness analysis. *This figure was adapted from (116) with publisher's permission.*

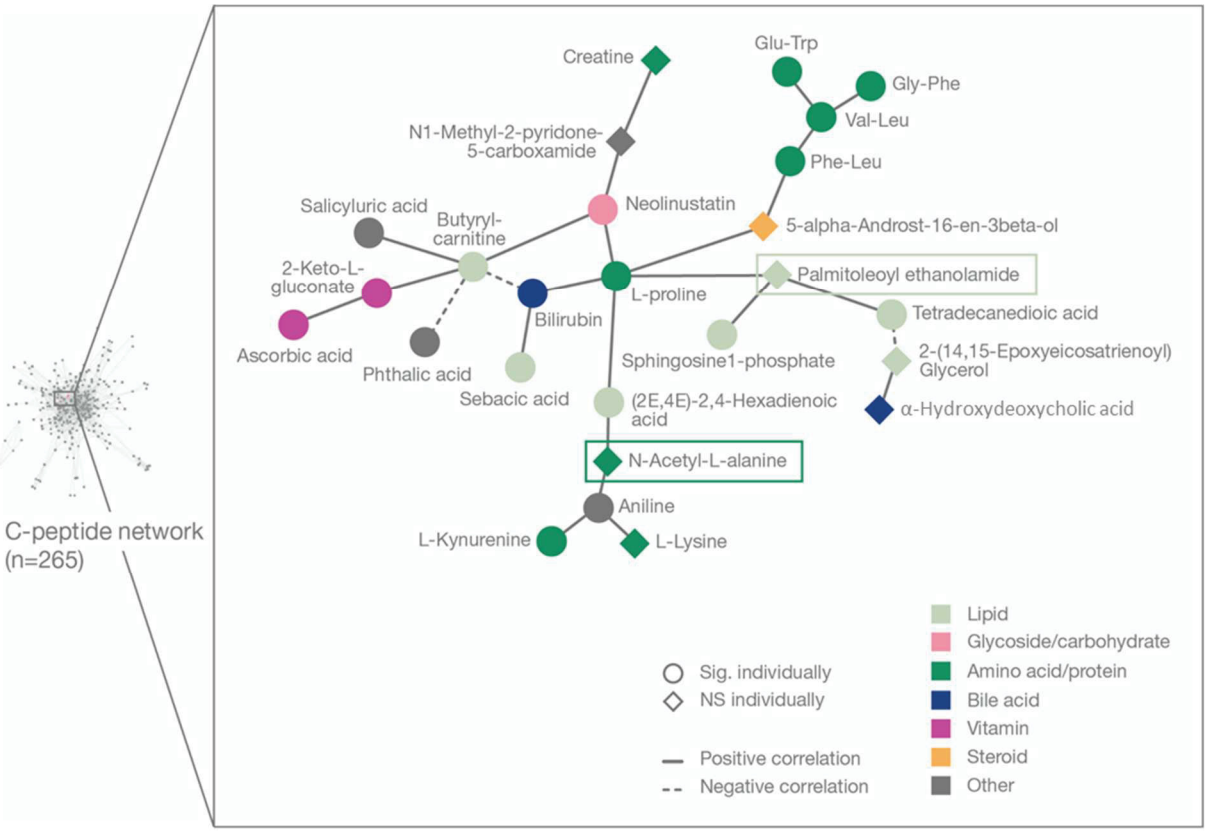


Figure 10. Subnetwork of metabolites (n = 26) that correlate with palmitoleoyl ethanolamide and are simulatenously associated with C-peptide. The nodes represent metabolites and the edges significant partial correlations between metabolites after multiple testing correction (Benjamini-Hochberg, $p \leq 0.1$). Round nodes represent metabolites significantly associated with C-peptide in the network but not when considered alone. Diamond nodes represent metabolites significantly associated with C-peptide also when analysed alone. Adjusted for confounders (gestational age, maternal age and processing time). *This figure was adapted from (116) with publisher's permission.*

4.1.5. Mediation analysis

To investigate if GLP-1 mediates the association between palmitoleoyl ethanolamide and C-peptide, a mediation analysis was conducted. GLP-1 did not mediate the association between palmitoleoyl ethanolamide (exposure) and C-peptide (outcome) (**Figure 11**). However, there was a significant correlation between C-peptide and palmitoleoyl ethanolamide (R^2 = 0.235, p = 0.014) and GLP-1 and palmitoleoyl ethanolamide (R^2 = 0.217, p = 0.035) but not between C-peptide and GLP-1 (R^2 = 0.188, p = 0.068).

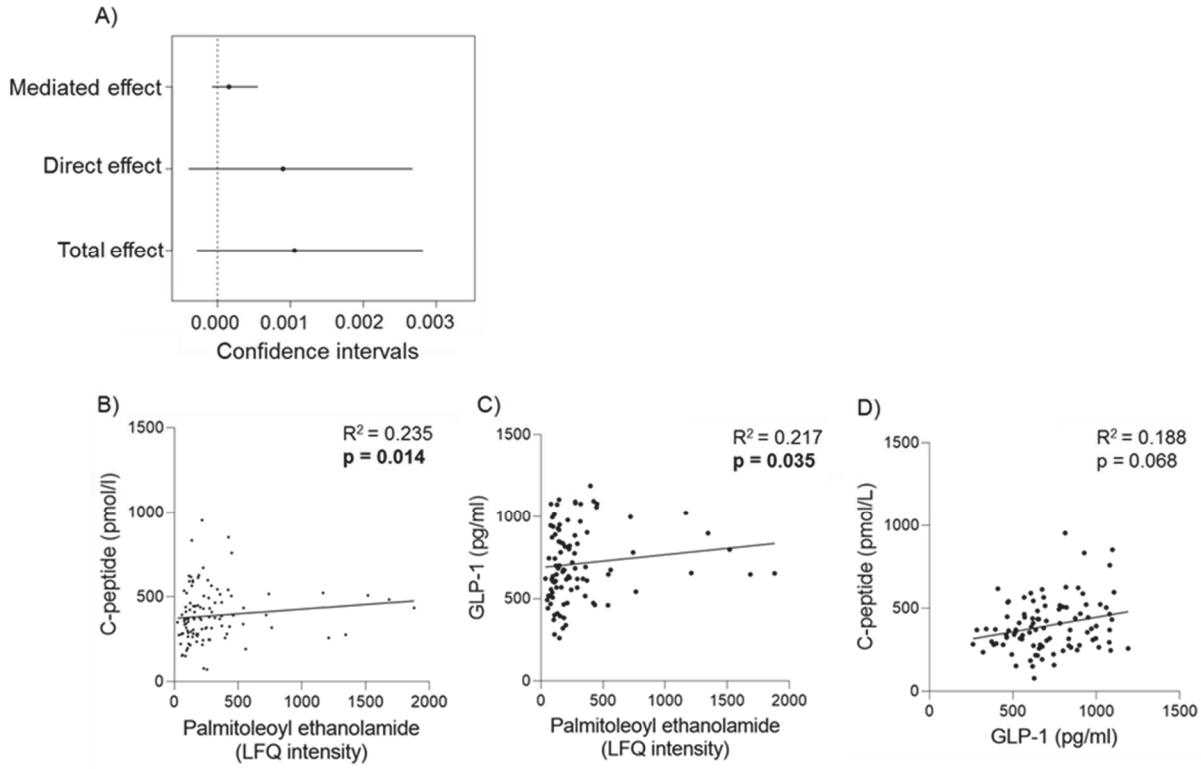


Figure 11. Mediation effect of GLP-1 in the association between palmitoleoyl ethanolamide and C-peptide. A) Confidence intervals of the mediated effect (total effect minus direct effect), direct effect (effect of palmitoleoyl ethanolamide on C-peptide accounting for GLP-1 effect) and total effect (effect of palmitoleoyl ethanolamide on C-peptide without accounting for GLP-1). Because the mediated effect is not statistically significant, it cannot be confirmed that the association between palmitoleoyl ethanolamide and C-peptide is mediated by GLP-1. B) Spearman correlation between C-peptide and palmitoleoyl ethanolamide. C) Spearman correlation between GLP-1 and palmitoleoyl ethanolamide. D) Spearman correlation between C-peptide and GLP-1.

4.1.6. Metabolic differences in women with overweight/obesity and low insulin sensitivity

Obesity is a heterogeneous metabolic condition and not all obese individuals are unhealthy, e.g., have a dysregulated metabolism (130). To acknowledge this heterogeneity, women with overweight/obesity in our cohort were classified as healthy (n = 23) if their insulin sensitivity was within the range of the normal weight population values ($IS_{HOMA} \geq 0.5$) or unhealthy (n = 9) if it was lower ($IS_{HOMA} < 0.5$). The metabolites tryptamine and 2,3,4,9-tetrahydro-1H-carboline-3-carboxylic acid were increased in the ´unhealthy obese´ group (**Table 5**).

Table 5. Metabolites significantly different between healthy and unhealthy women with overweight/obesity.

Metabolite	Confidence interval (95%)	Effect size (95%)	*p*-value
Tryptamine	-1.62 – -0.05	0.37	0.038
2,3,4,9-Tetrahydro-1H-carboline-3-carboxylic acid	-1.59 – - 0.06	0.38	0.036

Linear regression analysis adjusted for gestational age (days), maternal age (years) and processing time (min) was used to test for associations between IS_{HOMA} and a subset of metabolites (n = 19). Significantly different metabolites between women with normal insulin sensitivity and low insulin sensitivity who have overweight/obesity are shown in the table. Effect size (%) refers to the coefficient of determination (R2) and is the proportion of the variance in the outcome variable that is predictable from the influence variables (R2 lies between 0 – 100%). *This figure was adapted from (116) with publisher's permission.*

4.2. Changes in placentas exposed to maternal obesity

Rapid growth of the placenta during the first trimester makes it particularly vulnerable to disturbances in the intrauterine environment. Exposure to adverse metabolic conditions such as maternal obesity are associated with reduced placental growth in the first trimester. However, the specific metabolic triggers and cellular mechanisms leading to impaired placental growth in maternal obesity are still unclear. Whether exposure to an obesogenic environment affects early placental development has been investigated in this thesis by examining changes in the placental proteome. Subsequently, a well-established first trimester trophoblast cell line was used to investigate the potential effect of dysregulated traits in the obesogenic intrauterine environment on the identified proteins.

4.2.1. Proteome in placentas exposed to an obesogenic environment *in utero*

Untargeted proteomics was used to analyse the proteome of 19 placentas of women spanning a wide BMI range (19 - 34 kg/m^2). Using principal component analysis (PCA), two groups (group 1: n = 15; group 2: n = 4) of placenta samples that separated in component 1 (PC1) were identified (**Figure 12 A**). PC1 explained 15.4% of the variance and insulin sensitivity (IS_{HOMA}) was the clinical trait that best correlated (r = 0.713, p < 0.001) with PC1 (**Figure 12 B)**. In a group comparison test between samples clustered in group 1 and group 2, IS_{HOMA} was the only clinical trait significantly different (p < 0.05) (**Figure 12 C**).

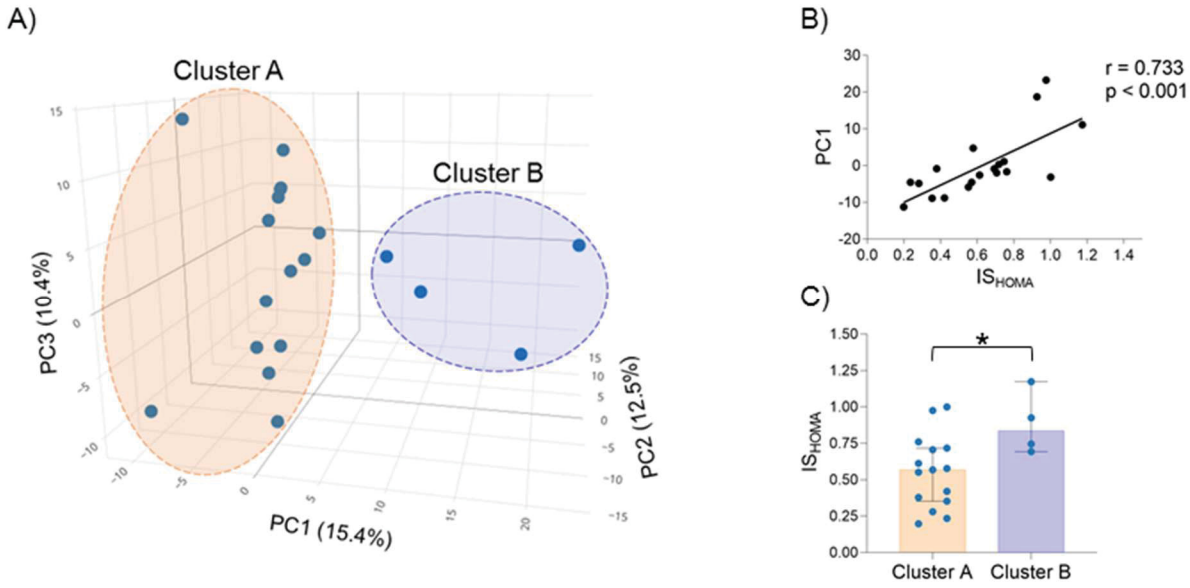


Figure 12. Changes in the placental proteome are associated with maternal insulin sensitivity. The proteome of 19 first trimester placental tissues was analysed by untargeted proteomics (MS/MS). A) Placenta samples separated into two clusters in principal component analysis. B) IS_{HOMA} was the maternal clinical trait that best correlated (highest Spearman correlation coefficient) with PC1. C) In group comparison analysis (Mann-Whitney U test), maternal IS_{HOMA} was the only clinical trait significantly (p < 0.05) different between group 1 and 2. Individual data as well as median and 95% confidence intervals are presented. IS_{HOMA}: Homeostatic model assessment of insulin; * p < 0.05

Since insulin sensitivity was the most relevant clinical trait explaining variation in the placenta proteome, we aimed to identify proteins differing between the placentas of women with high and low insulin sensitivity (**Figure 13 A**). The median IS_{HOMA} of the cohort (median IS_{HOMA} = 0.61) was used as cut-off to classify placental samples of women with high (healthy; IS_{HOMA} > 0.61) or low (unhealthy; IS_{HOMA} ≤ 0.61) insulin sensitivity. Eighty-six proteins were upregulated and 84 downregulated in the low insulin sensitive group. Upregulated pathways based on KEGG database were DNA replication, cell cycle, and ribosome processes (**Figure 13 B**). The most downregulated pathways (KEGG) were pentose phosphate pathway, glycolysis, fructose and mannose metabolism, galactose metabolism and biosynthesis of amino acids (**Appendix Figure 1; cf. Figure 34).** Because we hypothesized that exposure to maternal obesity affects placenta growth early in pregnancy, we decided to further investigate the proteins involved in DNA replication and cell cycle. These proteins were MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7 (**Figure 13 C**). They form the MCM-complex and play a crucial role as components of the pre-replicative complex at the origins of replication during the G1 phase of the cell cycle. MCM proteins are also involved in DNA damage repair mechanisms and, hence, were of particular interest since DNA damage is increased in first trimester placentas of women with obesity (105).

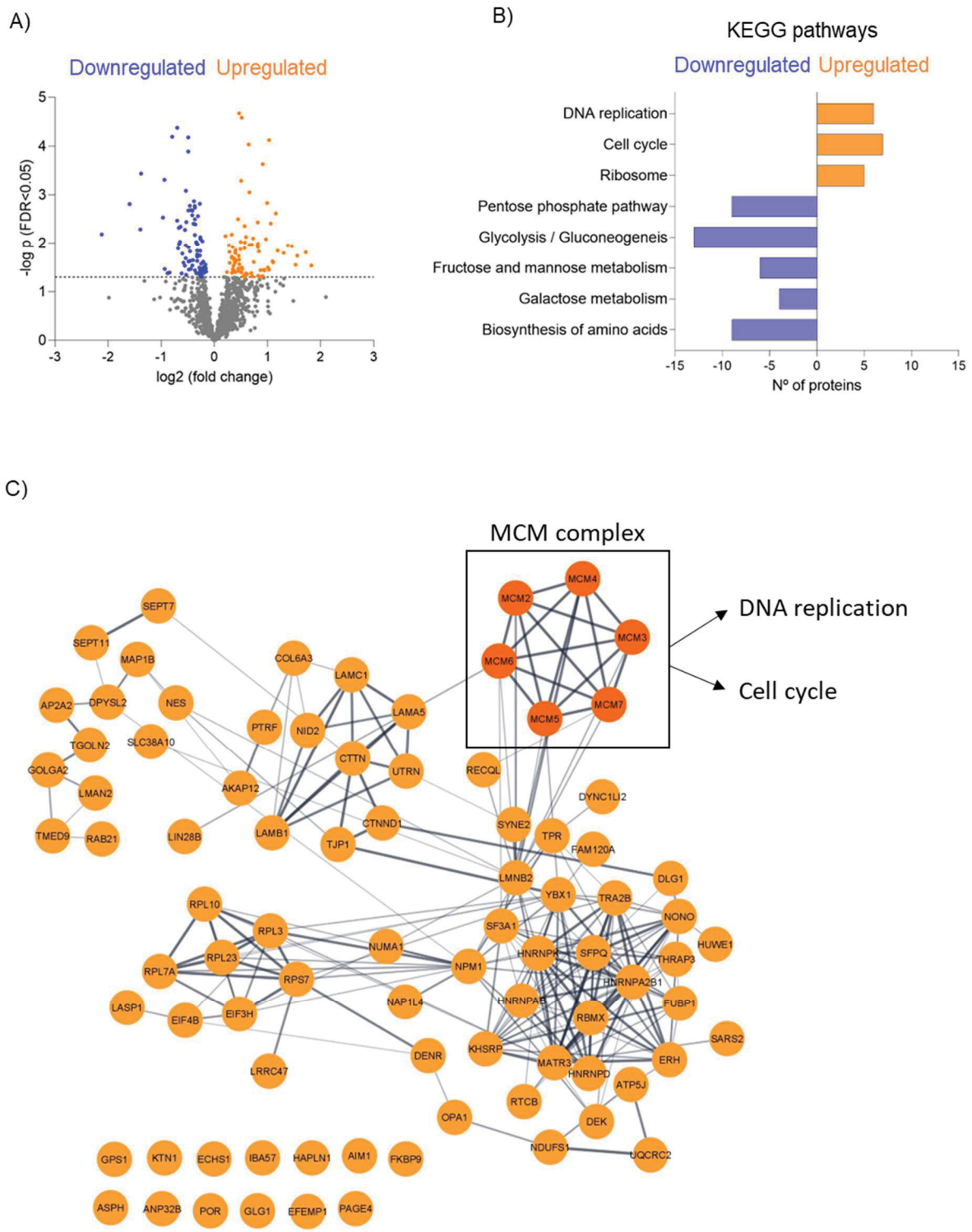
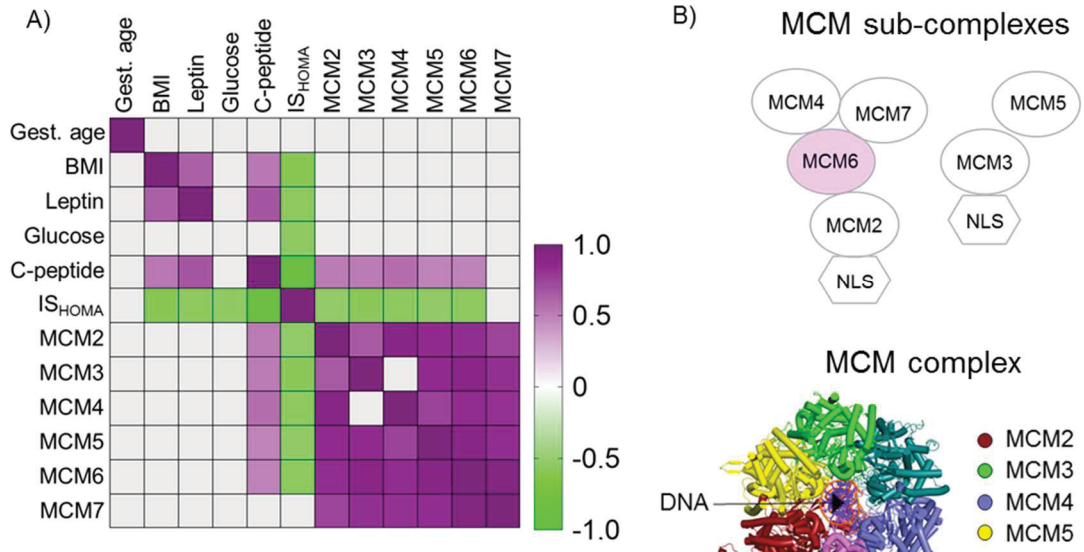


Figure 13. DNA replication and cell cycle pathways are upregulated in the placental proteome of women with low insulin sensitivity and the MCM-complex drives this enrichment. A) The proteome of placentas of women with high (IS_{HOMA} > 0.61) and low (IS_{HOMA} ≤ 0.61) insulin sensitivity was compared in a volcano plot. B) Proteins significantly upregulated (n = 86) and downregulated (n = 84) in the low IS_{HOMA} group were selected for pathway analysis (KEGG pathway; String V.11.5). DNA replication, cell cycle and ribosome pathways were the top upregulated KEGG pathways in the low IS_{HOMA} group. C) Six proteins of the MCM-complex (MCM2-7) were the drivers for enrichment of DNA replication and cell cycle pathways. KEGG: Kyoto Encyclopedia of Genes and Genomes; ECM: Extracellular matrix.

Subsequently, a potential relationship between maternal clinical traits (BMI, leptin, glucose, C-peptide, IS_{HOMA}) and levels of MCM2-7 proteins (raw LFQ values, prior imputation or normalization) (**Figure 14 A**) was examined. Both, IS_{HOMA} and C-peptide correlated with MCM proteins, suggesting that women with low IS_{HOMA} and high C-peptide have increased levels of MCM proteins. In addition, MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7 protein levels correlated with each other, indicating that MCM2-7 proteins function synergistically as components of the MCM-complex (**Figure 14 B**). MCM6 was the most enriched MCM in the low IS_{HOMA} group (**Figure 14 C**). For this reason, MCM6 was selected as a representative protein of the MCM-complex in subsequent experiments.



C)

Protein	- Log P value
MCM6	2.13
MCM7	2.02
MCM4	1.91
MCM3	1.83
MCM5	1.65
MCM2	1.64

Figure 14. MCMs correlate with maternal C-peptide and insulin sensitivity. A) Maternal clinical traits were correlated with LFQ values (Label Free Quantification; semi-quantitative measure of concentration) of MCM2-7 proteins. All proteins of the MCM-complex, except MCM7, were positively correlated with C-peptide and inversely correlated with IS_{HOMA}. Most individual MCM proteins were positively correlated with each other. B) The MCM complex is formed by two sub-complexes that are imported separately into the nucleus, where they assemble to form the MCM complex. Each complex has a nuclear localization signal (NLS). The structure of the MCM-complex was created in PyDock using the protein data base (PDB) identifier 7PLO. The image was created with PyDock. C) In proteomic analysis, MCM6 was the most significantly upregulated proteins in placentas of women with low insulin sensitivity. Therefore, MCM6 was selected as representative of the whole MCM complex.

MCM6 protein was predominantly localized to the nuclei of villous cytotrophoblast cells, which are the proliferative cell type in the placenta (**Figure 15**). Extravillous cytotrophoblast cell nuclei and some stromal cell nuclei also showed MCM6 signal.

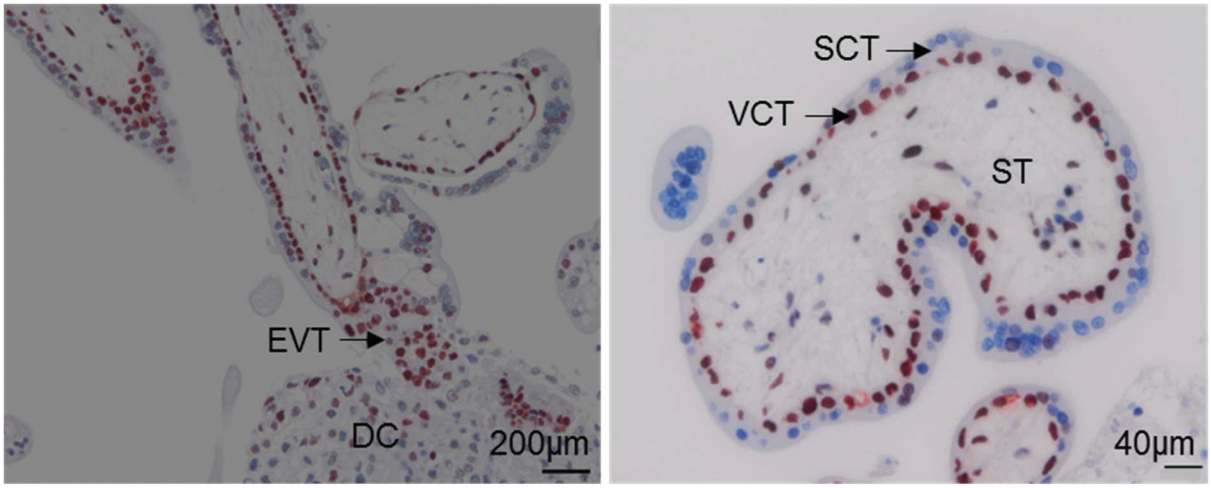


Figure 15. MCM6 is present in nuclei of villous cytotrophoblasts and extravillous trophoblast cells in the first trimester placenta. Placenta sections (gestational age: 5^{+0} weeks LMP) were stained with anti-MCM6 antibodies and nuclei counterstained with Mayer´s hematoxylin.

Increased levels of DNA damage repair proteins in placentas exposed to an obesity environment *in utero* suggest increased DNA damage in these placentas. To explore this possibility, the cellular and subcellular distribution of MCM6 and γH2AX (marker for DNA damage) in four placenta samples was examined (**Figure 16 A**). Similar to MCM6 location, γH2AX foci were primarily present in the nuclei of villous cytotrophoblast cells, with some nuclei in the syncytiotrophoblast and stroma cells also showing immunostaining for γH2AX. Co-localization analyses were performed to investigate whether MCM6 and γH2AX are in close subcellular proximity (**Figure 16 B**). MCM6 and γH2AX fluorescence intensities overlapped in the nuclei of villous cytotrophoblast cells, suggesting co-localization of these proteins in this cell type. This co-localization was observed in placentas exposed to both low and high maternal insulin sensitivity levels. Interestingly, MCM6 foci were also present in certain regions of the cytoplasm of villous cytotrophoblasts and syncytiotrophoblast (**Figure 17**).

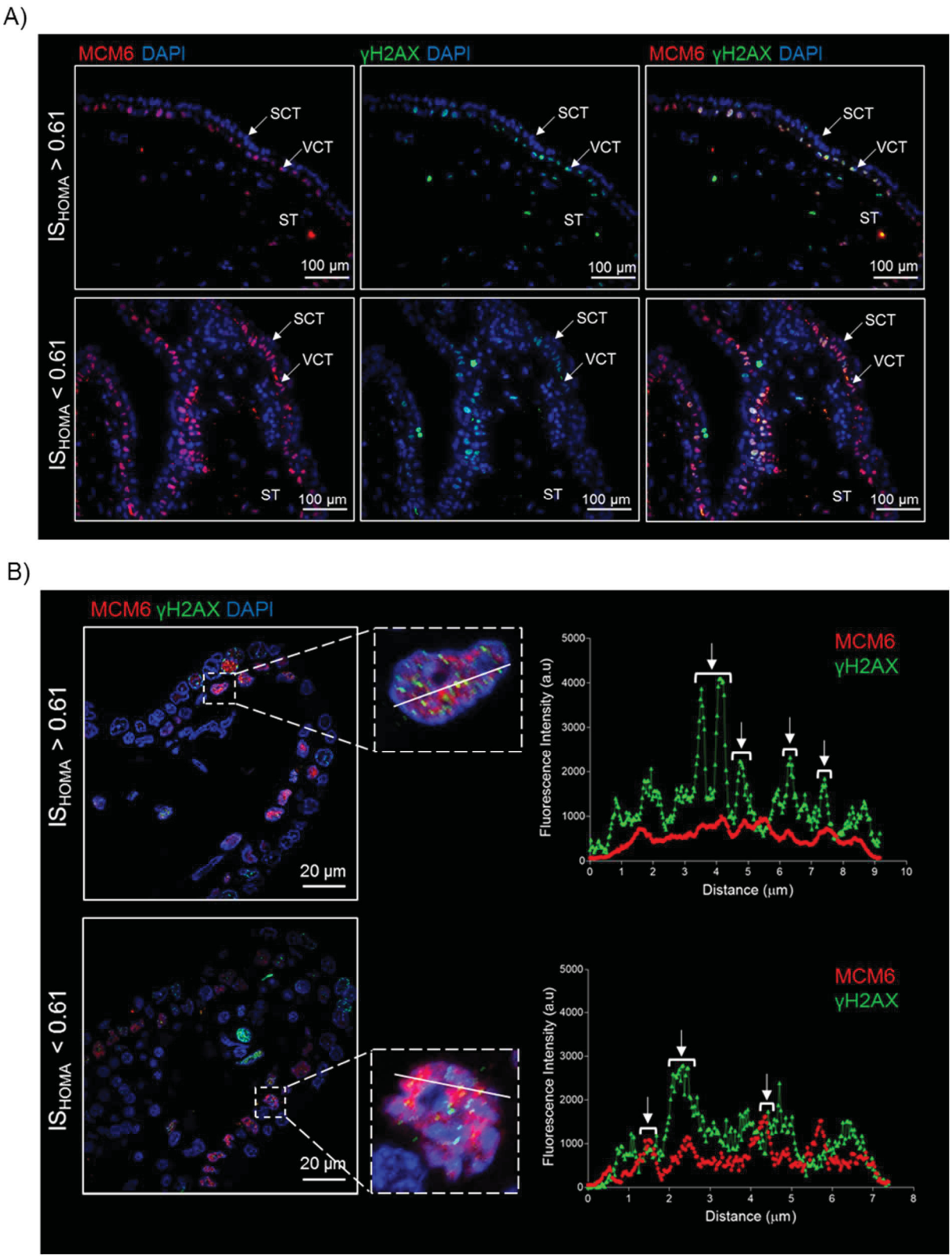


Figure 16. MCM6 and γH2AX co-localize to the nuclei of villous cytotrophoblast cells in the first trimester placenta. Placenta tissue of high (n = 2; IS_{HOMA} > 0.61) and low (n = 2; IS_{HOMA} ≤ 0.61) insulin sensitive women was stained with anti-MCM6 and anti-γH2AX antibodies as well as with DAPI (nucleus). A) Overview of MCM6 and γH2AX location. Images acquired in a light microscope (40x). MCM6 and γH2AX were found predominantly in the nuclei of cytotrophoblast cells. B) For co-localization assessment, z-stacks were acquired in a confocal microscope (100x). The line scan tool (ImageJ, FIJI) was used to measure fluorescence intensity (a.u.) per pixel (μm) and the data plotted using GraphPad prism. Brightness and contrast adjusted for better visualization.

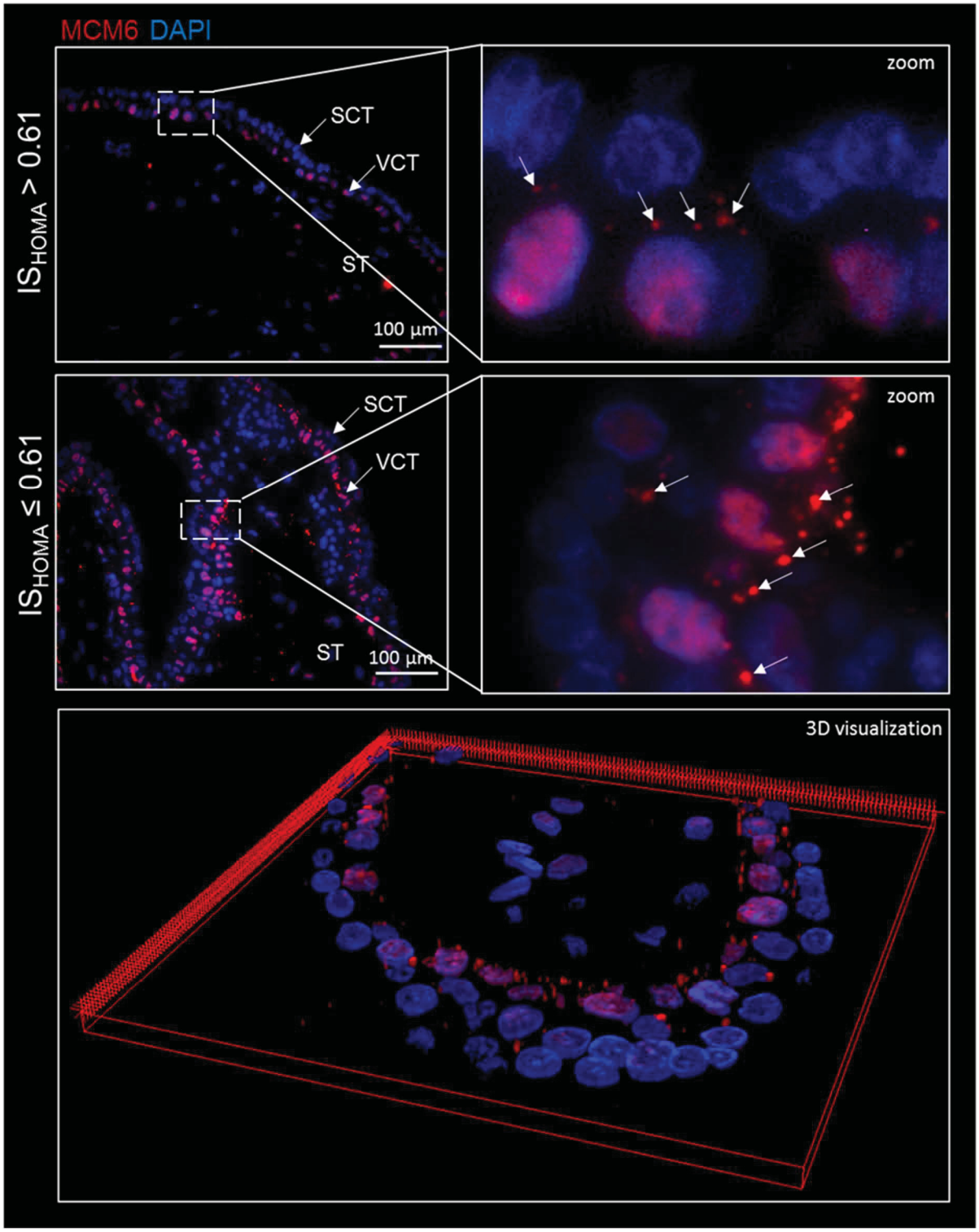


Figure 17. MCM6 foci form in the cytoplasm of villous cytotrophoblast cells and the syncytiotrophoblast in the human first trimester placenta. Placenta tissue of women with high (n = 2; $IS_{HOMA} > 0.61$) and low (n = 2; $IS_{HOMA} \leq 0.61$) insulin sensitivity was stained with anti-MCM6 antibody and DAPI (nucleus). Tissue overview of MCM6 staining acquired in a light microscope (40x) (top) and in a confocal microscope (100x) (bottom). Z-stacks were acquired in a confocal microscope to create a 3D-image. MCM6 is located predominantly in the nuclei of cytotrophoblast cells, but MCM6 foci are also present in the cytoplasm of villous cytotrophoblast cells and syncytiotrophoblast. Brightness and contrast adjusted for better visualization.

4.2.2. DNA damage induction in ACH-3P cells

To study whether DNA damage can be induced in ACH-3P cells, they were exposed to different etoposide concentrations for 1h. Etoposide is a topoisomerase II inhibitor that causes DNA double strand breaks (131). Exposure to 10nM etoposide for 1h was sufficient to induce DNA damage (γH2AX increase) (**Figure 18**). To investigate ACH-3P potential for DNA damage repair, the cells were allowed to recover for 3h and 24h and γH2AX measured. DNA damage was partially repaired (γH2AX decrease) after 3 hours of culture in absence of etoposide. However, reduction in DNA damage was not accompanied by changes in MCM6 levels.

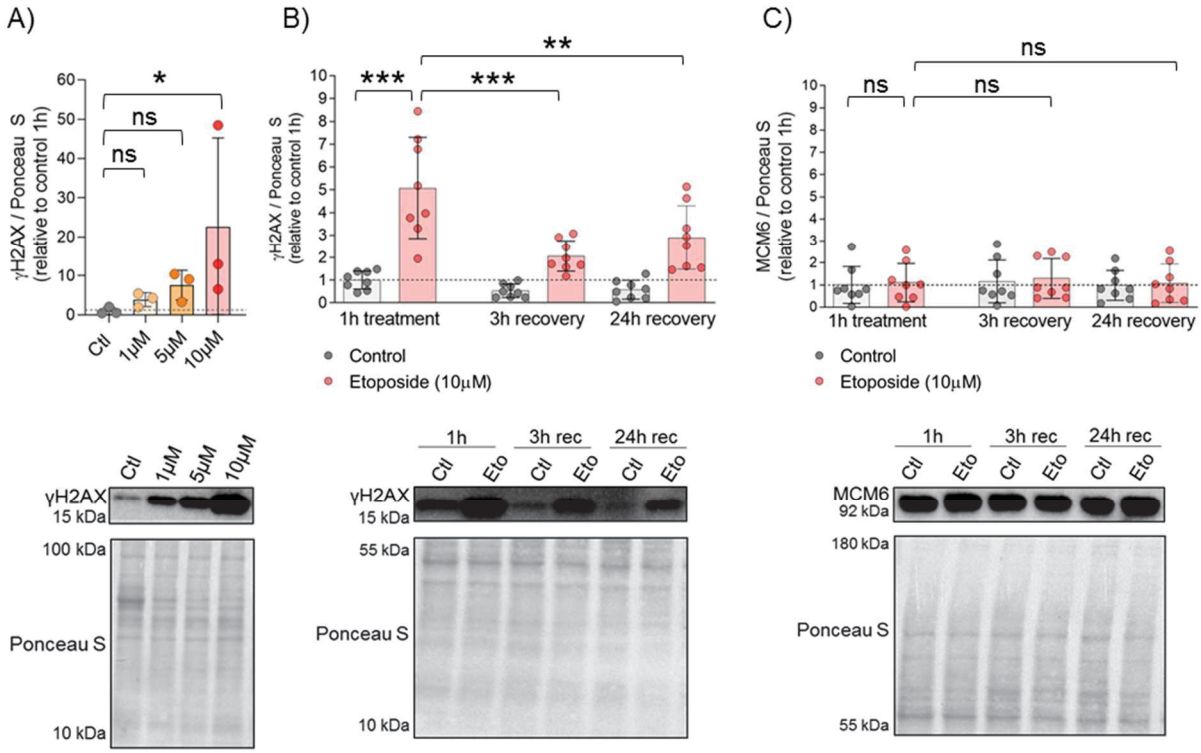


Figure 18. Etoposide causes DNA damage in ACH-3P cells and the damage can be repaired within 3h. A) ACH-3P cells were cultured at 6.5% O_2 in absence (control) or presence of etoposide (1μM, 5μM, 10μM) for 1h. Cells were harvested and lysates used for Western blotting. DNA damage (measured as γH2AX) was quantified and protein levels normalized to Ponceau S. Data are presented as mean ± SD. B-C) To investigate DNA damage repair, ACH-3P cells were allowed to recover in fresh media for 3h or 24h. Then, cells were harvested and lysates used for Western blotting using anti-γH2AX and anti-MCM6 antibodies. Results are shown relative to control at 1h. Data are presented as mean ± SD. One-way ANOVA with post hoc test and Bonferroni correction was used to compare protein levels between conditions. Brightness and contrast of Ponceau S staining adjusted for better visualization. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. ns: Not significant. Ctl: Vehicle control (DMSO); Eto: Etoposide; rec: Recovery.

To investigate the potential recruitment of MCM6 to DNA damage sites, ACH-3P cells were exposed to etoposide, which induces DNA damage at replication origin sites, or bleomycin, which induces damage at various locations across the chromosomes. Both treatments resulted in the formation of γH2AX foci, and in both experimental conditions, γH2AX and MCM6 co-localized. The co-localization of γH2AX and MCM6 following bleomycin treatment suggests that MCM6 can indeed be recruited to DNA damage sites (**Figure 19**).

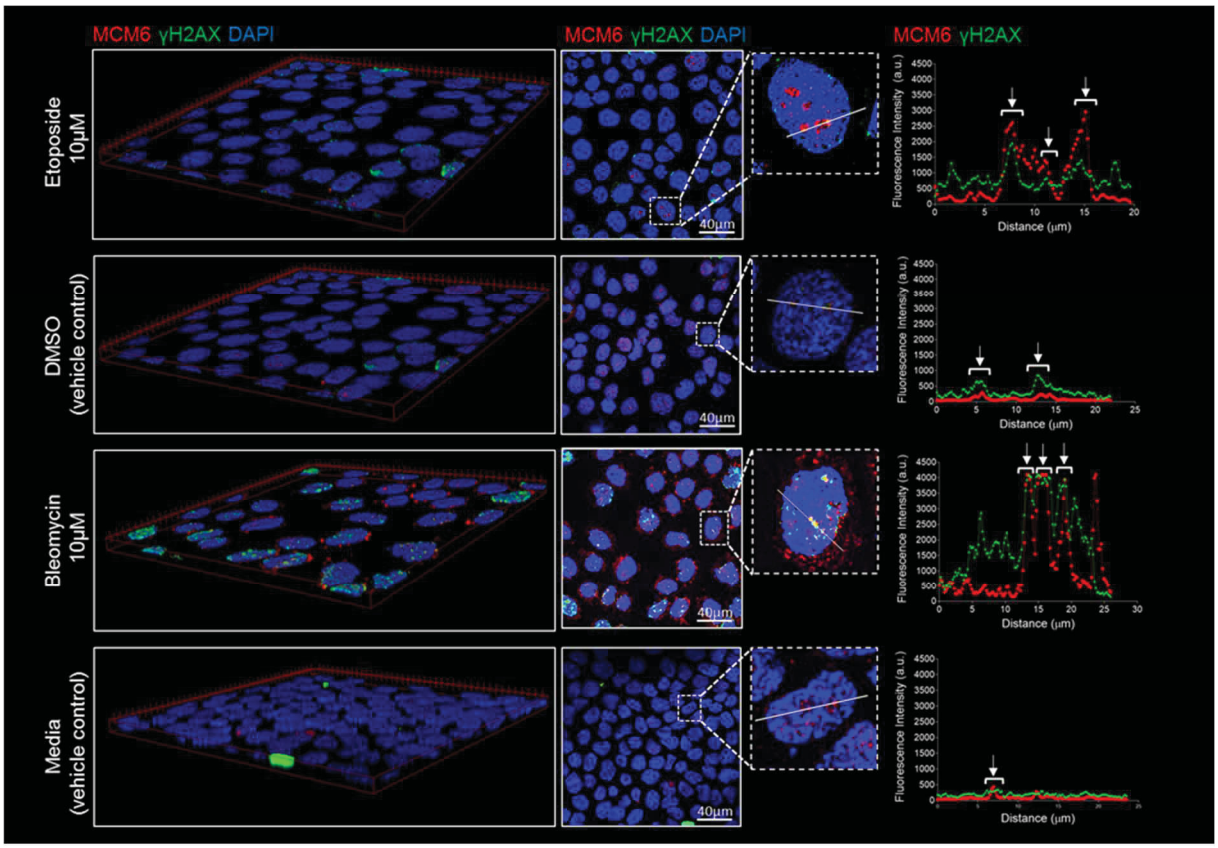
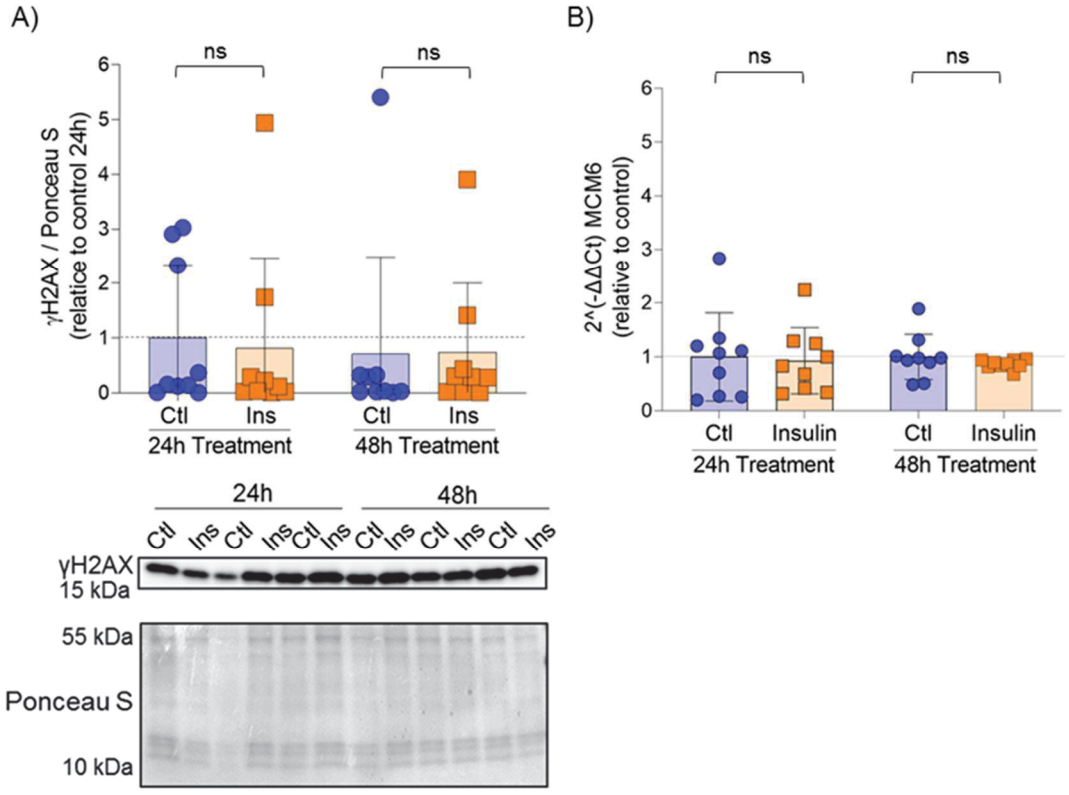


Figure 19. MCM6 is recruited to sites of DNA damage. ACH-3P cells were cultured at 6.5% O_2 in presence of etoposide (10μM), bleomycin (10μM) or vehicle control (DMSO and F12 media, respectively) for 1h. Thereafter, cells were allowed to recover in fresh media (F12 media) for 1h. Subsequently, cells were incubated overnight with anti-MCM6 and anti-γH2AX. DAPI was used to visualize nuclei. Images were acquired with a confocal microscope. 3D images and co-localization analysis were performed in Image J - Fiji. Arrows indicate regions, i.e. pixels, where γH2AX and MCM6 signals co-localize. Brightness and contrast of immunofluorescence staining adjusted for better visualization.

4.2.3. Mimicking the obesity environment *in vitro*

Because in first trimester placenta tissue C-peptide and MCM protein levels significantly correlated, we speculated that insulin may induce DNA damage and that this will lead to increased transcription of DNA damage repair proteins, e.g., MCMs. The first trimester trophoblast cell line ACH-3P was used as an *in vitro* model. To mimic the oxygen tension *in utero* early in pregnancy, the cells were cultured for 72h in a hypoxia workbench at 6.5% O_2. Then, the cells were exposed to 10nM insulin for 24 or 48h. No increase in DNA damage (γH2AX) nor changes in expression or protein levels of MCM6 (**Figure 20 A-C)** were observed. Interestingly, there was a significant correlation between protein levels of γH2AX and MCM6 ($r = 0.703$, $p < 0.0001$) (**Figure 20 D**). However the lack of significant interaction between γH2AX and treatment (control or insulin) suggest that the association between γH2AX and MCM6 is independent of insulin. Therefore, insulin could not be validated as the clinical trait altered in maternal obesity, which is associated with DNA damage in villous cytotrophoblasts in the first trimester of human pregnancy.



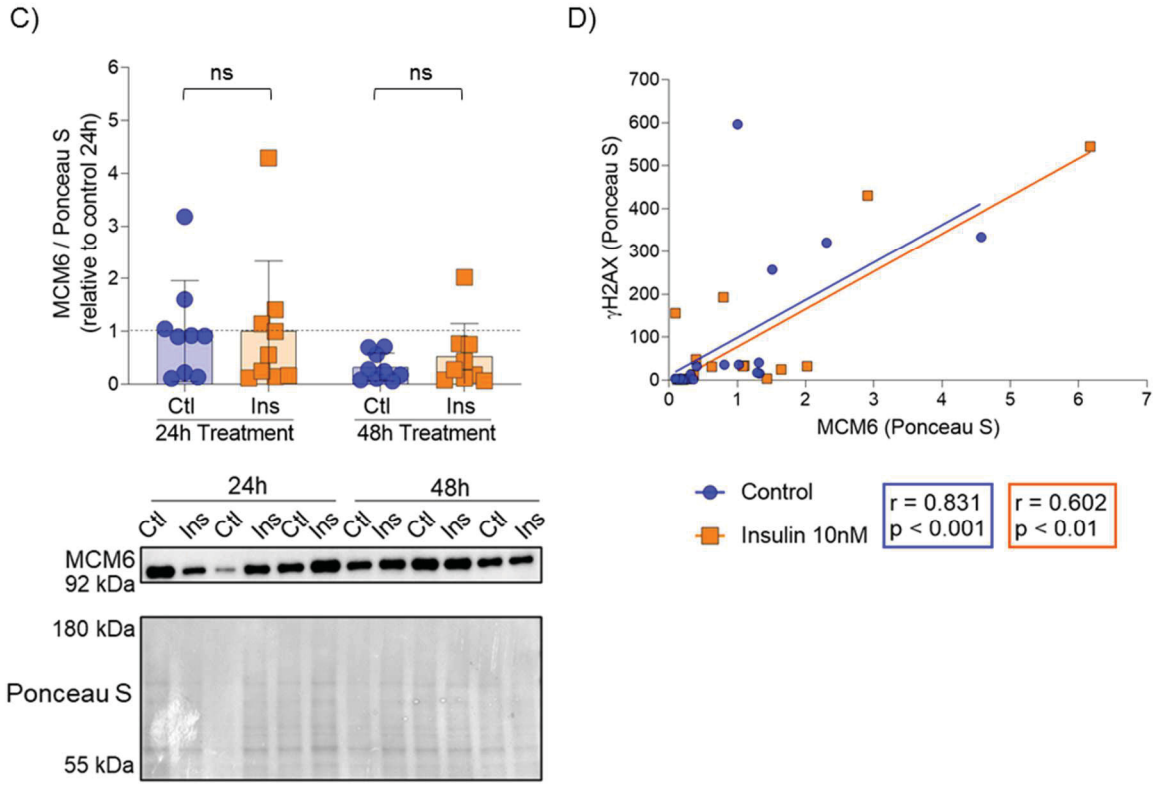
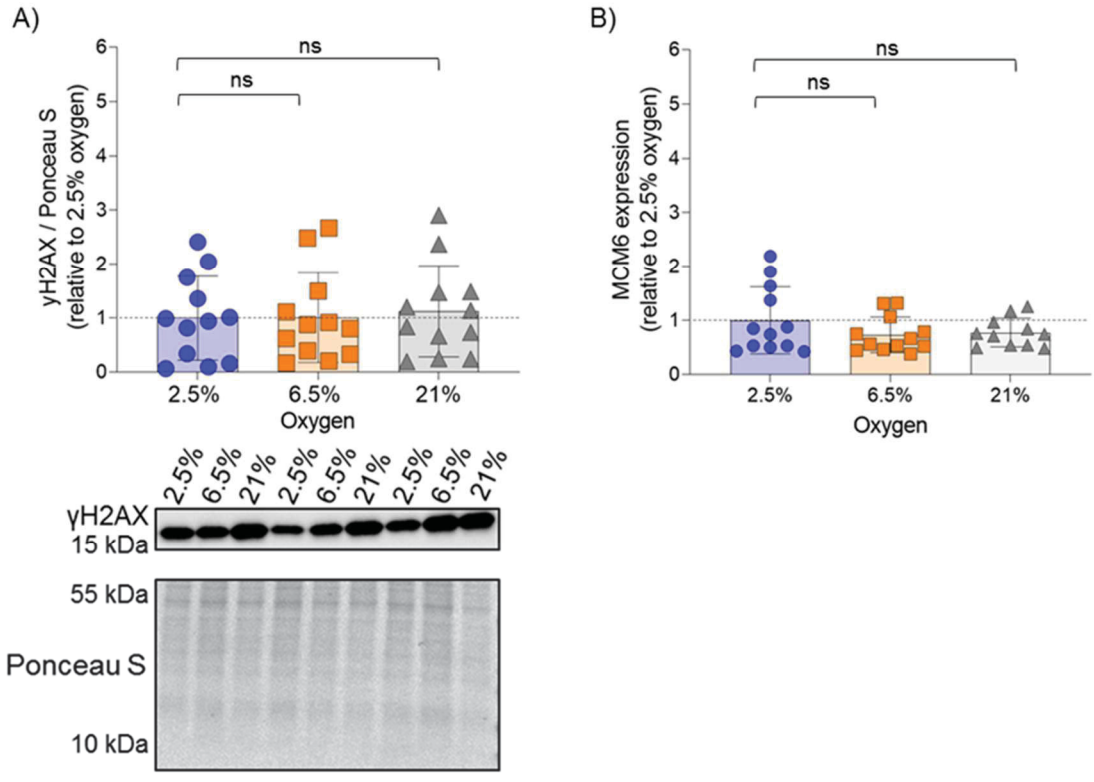


Figure 20. The correlation between γH2AX and MCM6 is independent of insulin. ACH-3P cells were cultured at 6.5% O_2 for 72h to accommodate to the oxygen tension (n = 3, in triplicates). Then, the cells were treated with vehicle control (F12 media) or 10nM insulin for 24h or 48h. Cells were harvested after 24h and 48h and cell lysates used for RT-qPCR and western blotting. A) DNA damage (measured as γH2AX) was quantified by western blotting and protein concentration normalized to Ponceau S. B) MCM6 expression was quantified by RT-qPCR and normalized to the mean of the housekeeping genes peptidyl-prolyl isomerase (PPIA) and TATA-binding protein (TBP). C) MCM6 was quantified by western blotting and protein concentration normalized to Ponceau S. D) γH2AX and MCM6 protein levels were correlated using Spearman correlation. Results (A-C) are shown relative to control 24h. Data are presented as mean ± SD. Kruskal-Wallis test was used to compare expression and protein levels between controls and insulin treated samples. Brightness and contrast of Ponceau S staining adjusted for better visualization. ns: Not significant.

Some studies suggest that there is a delay in spiral artery remodelling in pregnancies complicated by obesity (28), resulting in prolonged exposure of the growing placenta to low oxygen levels (28). This notion is further supported by absence of oxidative stress in first trimester placentas (week 5-10) of women with obesity as well as decreased gene expression of placental antioxidants (MGST3, SOD3, GSTP1, GLRX) in these placentas (105). We speculated that this extended exposure to low oxygen could lead to increased DNA damage and MCMs levels. To test this, ACH-3P cells were cultured at 2.5% oxygen concentration, which represents the physiological oxygen tension before spiral artery remodelling, for 72 hours. Then, a plate was kept at 2.5% O_2 and one plate transferred to 6.5% O_2 (oxygen tension after spiral artery remodelling by the end of the first trimester) for additional 48 hours. In addition, another plate was transferred to 21% O_2 as a control of hyperoxygenation. No differences in the protein levels of γH2AX nor MCM6 between cells kept at 2.5% O_2 and cells transferred to 6.5% O_2 or 21% O_2 were found (**Figure 21 A-C**). Similar to the previous finding, there was a significant correlation (r = 0.370, p = 0.026) between the protein levels of γH2AX and MCM6 but there was no significant interaction between γH2AX and oxygen tension, suggesting that the correlation between MCM6 and γH2AX is independent of oxygen tension. However, if MCM6 and γH2AX correlation is explored for each oxygen tension separately, there is a significant correlation only at 2.5% O_2, suggesting that oxygen may indeed have an effect (**Figure 20 D**).



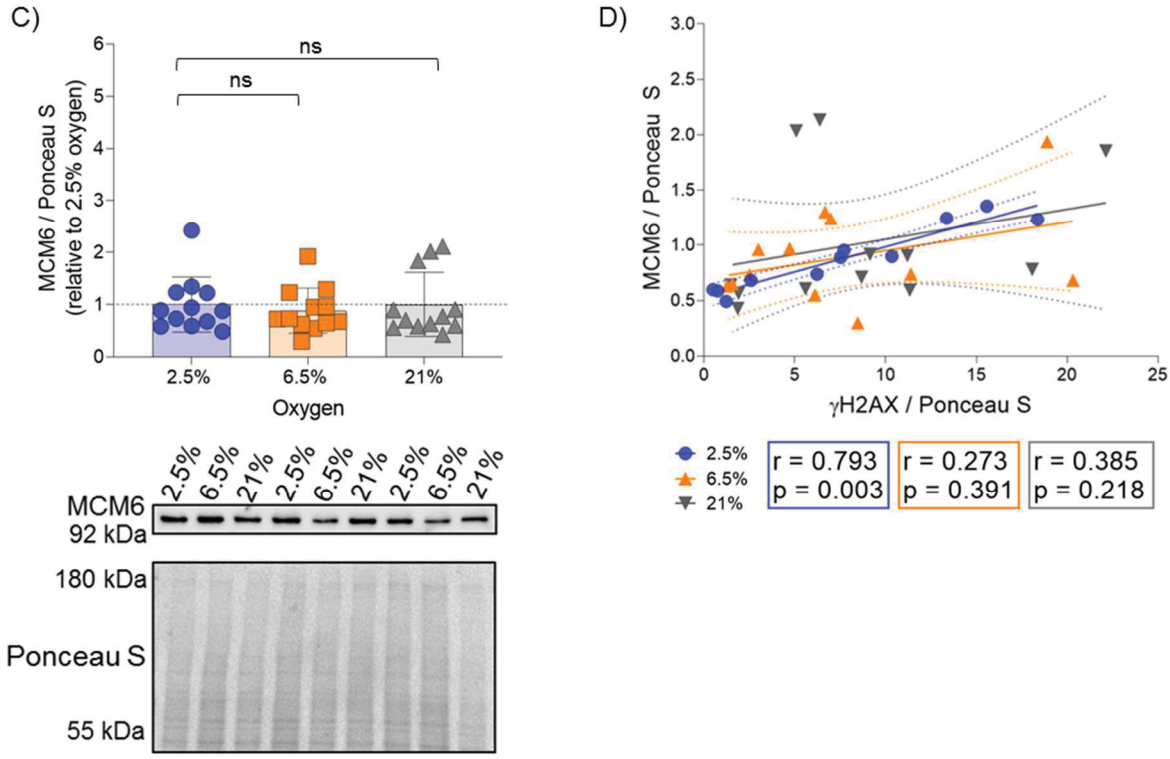


Figure 21. The correlation between γH2AX and MCM6 is independent of oxygen tension. ACH-3P cells were cultured at 2.5% O_2 for 72h (n = 4, in triplicates). Then, one plate was kept at 2.5% O_2, while the other plates were transferred to 6.5% O_2 or 21%O_2 for 48 additional hours. Thereafter, cells were harvested and lysates used for PCR or western blotting. A) DNA damage (measured as γH2AX) was quantified and protein level normalized to Ponceau S. B) MCM6 expression was quantified by RT-qPCR and normalized to the mean of the housekeeping genes PPIA and TBP. C) MCM6 was quantified and protein level normalized to Ponceau S. D) γH2AX and MCM6 protein levels were correlated using Spearman correlation. Results are shown relative to 2.5% O_2. Data are presented as mean ± SD. Kruskal-Wallis test was used to compare protein levels between samples at 2.5% O_2 (reference category) and samples exposed to 6.5% and 21% O_2. Brightness and contrast of Ponceau S staining adjusted for better visualization. ns: non-significant.

4.2.4. Silencing of MCM6 and MCM3 in ACH-3P cells

To further investigate the role of MCM proteins in the placenta, MCM6 and MCM3 genes were post-transcriptionally silenced in ACH-3P cells using shRNA. Despite good plasmid purity (**Figure 22)**, self-packed lentiviral particles failed to infect ACH-3P cells.

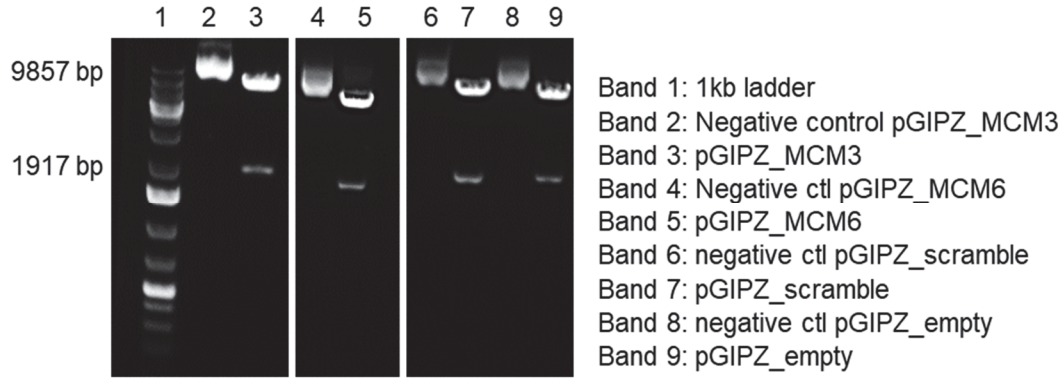


Figure 22. Quality control for isolated plasmids. pGIPZ plasmids containing the transgenes of interest (shMCM6, shMCM3) or negative controls (shScramble, shEmpty) were enzymatically digested with KpnI and the fragments visualized in an agarose gel. A negative control (no enzyme added in the digestion) was included for each plasmid. All the plasmids were digested resulting in two fragments of the expected base pair length (9857bp and 1917bp). bp: base pairs.

Using commercially packed lentiviral particles, ACH-3P were successfully infected (visually assessed as GFP fluorescence) and FACS sorting was used to obtain pure colonies of transduced cells (**Figure 23**; only ACH-3P shMCM6 cells shown). However, transduced cells progressively lost GFP expression after a few days in culture (assessed by fluorescence microscopy) and MCM6 and MCM3 protein levels were not decreased compared to the negative controls (assessed by western blotting) (**Figure 24**). The expression of the transgene in pGIPZ plasmids is under control of a cytomegalovirus promoter. It is possible that after some days in culture ACH-3P cells methylate the CMV promoter which silences it and prevents expression of the transgene (shMCM6 or shMCM3) (132, 133).

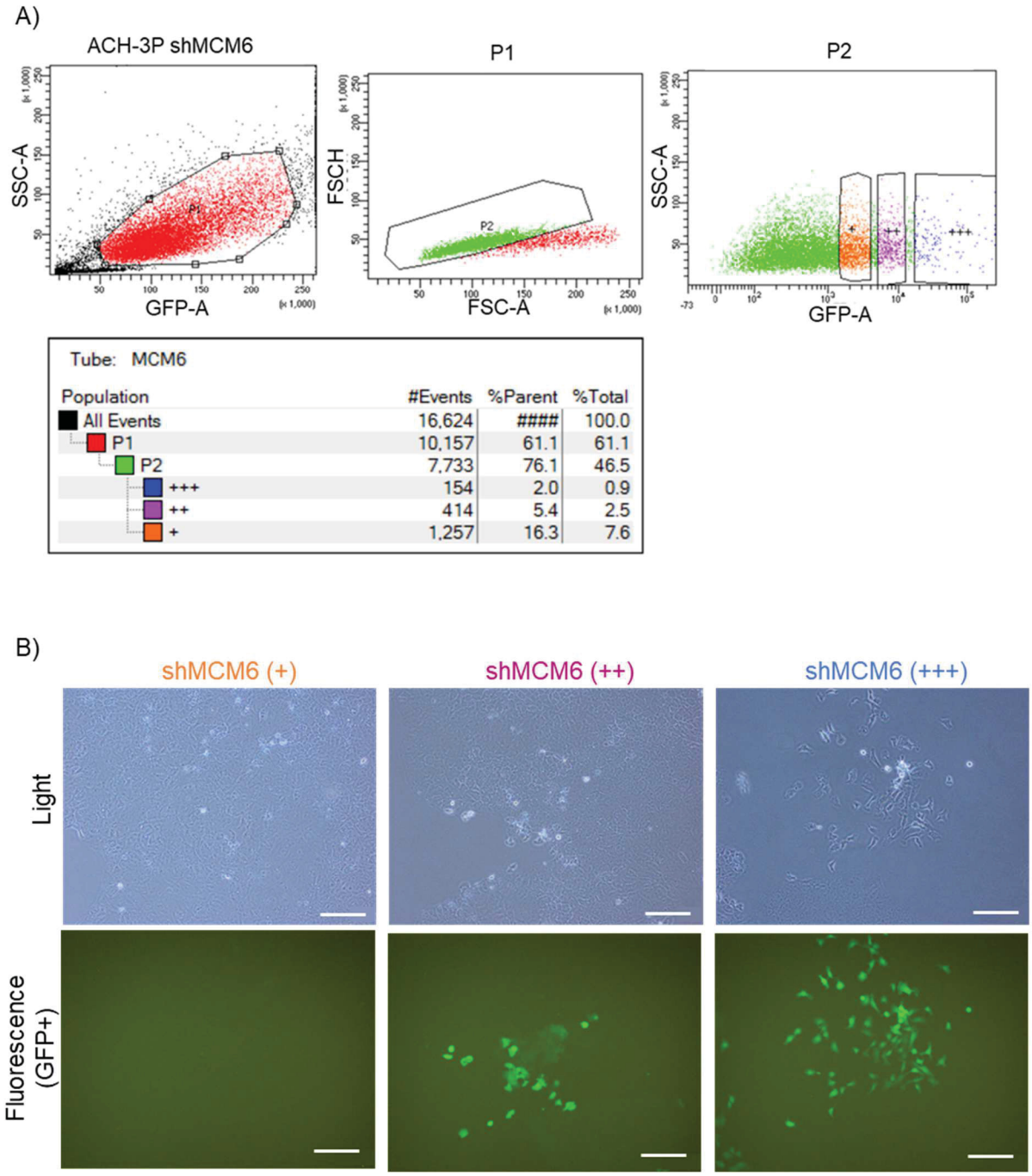


Figure 23. Lentivirus transduction of ACH-3P cells with shMCM6. A) ACH-3P cells transduced with shMCM6 were sorted based on GFP fluorescence intensity (marker of transduction) using FACS. The cells were separated into very GFP positive (+++), medium GFP positive (++) or low GFP positive (+). B) ACH-3P cells were further cultured at 37°C for 48h prior to microscope visualization. Scale bar: 200µm.

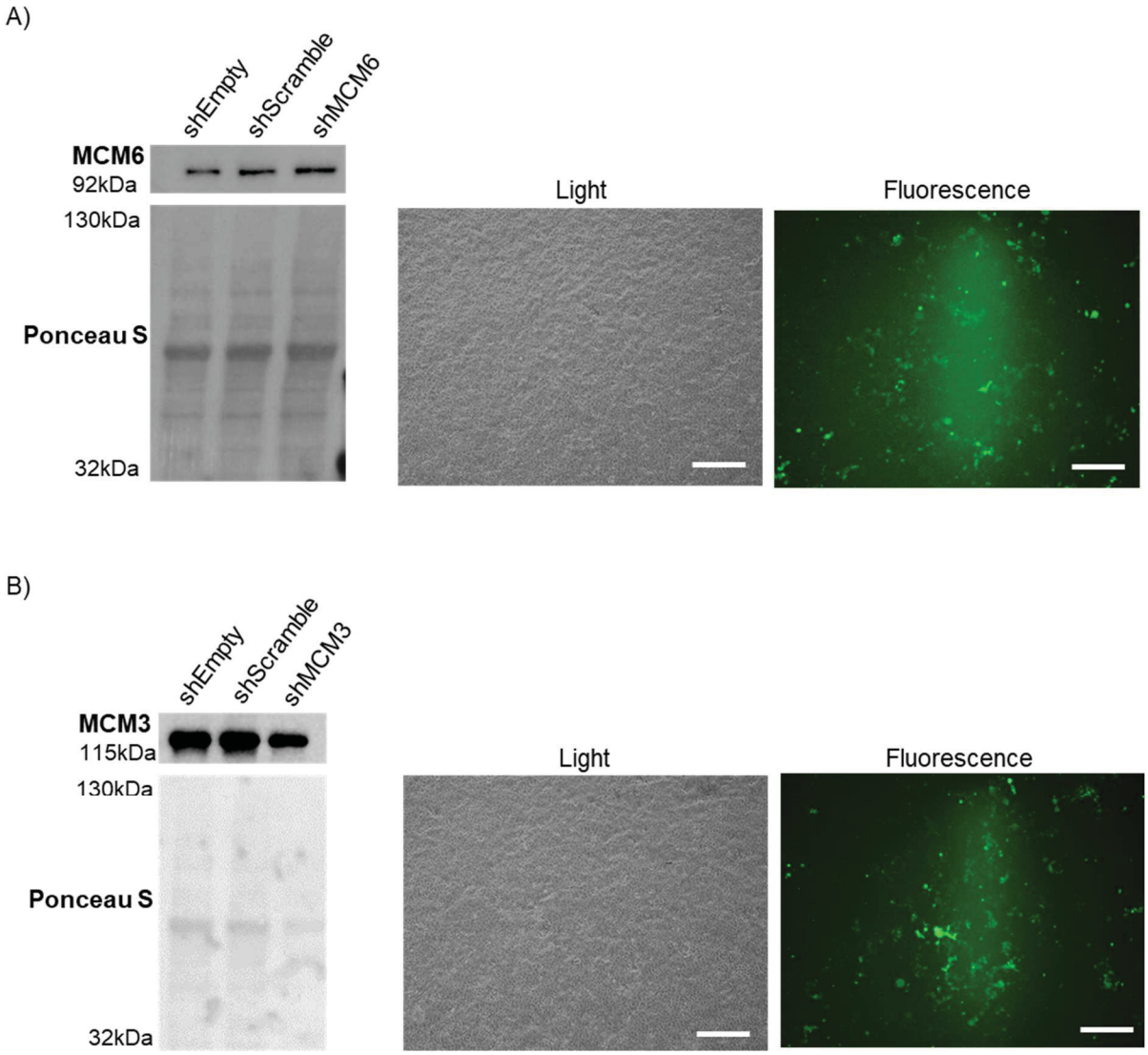


Figure 24. Transduced ACH-3P cells do not express the transgene after 5 days in culture. ACH-3P cells transduced with shMCM6, shMCM3, shScramble and shEmpty and sorted by FACS with very strong GFP signal intensity (+++) were cultured for 5 days at 37°C. Thereafter protein extracts were isolated for western blotting with anti-MCM6 and anti-MCM3 antibodies. A) MCM6 and B) MCM3 protein levels were not diminished in shMCM6 and shMCM3 ACH-3P cells compared to negative controls (shScramble and shEmpty). In addition, ACH-3P shMCM6 and ACH-3P shMCM3 cells lost GFP signal within some days. Scale bar: 200µm.

5. Discussion

The first trimester of human pregnancy is a critical period for placentation and may determine later development of the fetus. Throughout pregnancy, maternal metabolism needs to adapt dynamically to adequately cover fetal requirements. Metabolic disorders such as obesity impair maternal metabolic adaptability and affect fetal health in the short and long term. The adverse effects on fetal growth might be partially mediated by decreased placental growth in the first trimester. Despite increasing interest in how the peri-conceptional period and early-life events shape childhood and adulthood health, there is still little known about early events in pregnancy and how they are altered by maternal obesity. During this thesis, differences in the maternal metabolism of women with overweight/obesity compared to normal weight women during the first trimester of pregnancy were investigated. In addition, how *in vivo* and *in vitro* exposure to an obesity-associated environment affects the first trimester placenta proteome, and specifically MCM proteins, was explored.

Overall, this work contributes to the understanding of a relatively under-researched stage of pregnancy that is being increasingly acknowledged for its role in shaping pregnancy outcome and long-term health of the offspring. Thus, this thesis adds information on metabolic pathways dysregulated in women with overweight/obesity in early pregnancy and provides first clues on potential metabolic candidates that may be involved in compromised placental development in the first trimester of pregnancy.

5.1. The heterogeneity of maternal metabolism in the first trimester of pregnancy

Pregnant women with overweight/obesity have higher incidence of gestational diabetes mellitus, which is characterized by fasting hyperglycaemia (134). Despite the recognised link between obesity and altered glucose metabolism, studies in the first trimester considering other metabolic parameters such as insulin, insulin sensitivity, lipids or small metabolites are scarce. Including other clinical traits in addition to BMI is important because obesity is a heterogeneous metabolic condition (130). Therefore, not all women with overweight/obesity are unhealthy at the time of conception. To acknowledge the metabolic heterogeneity of obesity in pregnancy, in a first study we investigated fasting glycaemia, insulinaemia (measured as C-peptide) and insulin sensitivity (IS_{HOMA} index) in a cohort spanning a wide BMI and gestational age range (BMI: 16.6 to 41.4 kg/m^2; gestational age: week 4^{+0} - 11^{+6} LMP) (44). In addition to BMI, leptin was included as a proxy of maternal adiposity. We found a consistent increase in C-peptide and decrease in insulin sensitivity in women with overweight/obesity compared to lean women (44). The same trend was observed with leptin (44). Interestingly,

fasting glucose was not significantly different between BMI or leptin groups, suggesting that insulin secretion was sufficient to maintain normoglycaemia in this group (44). However, absence of hyperglycaemia does not imply that the women with overweight/obesity included in the study were metabolically healthy since they presented hyperinsulinaemia and low insulin sensitivity. In fact, these results further reflect the metabolic heterogeneity of obesity. In the general population many individuals with normal glucose levels are insulin resistant, which is the major risk for the development of type 2 diabetes (45). In addition, each clinical trait (hyperglycaemia, hyperinsulinaemia, insulin resistance) predisposes for a different trajectory of disease progression and risk of diabetic complications (135, 136). Therefore, investigating various clinical traits, alongside BMI, may provide a more comprehensive understanding of maternal metabolism. Based on this, in the next studies we included leptin, glucose, C-peptide and insulin sensitivity as predictors of changes in fatty acid levels and metabolites.

Maternal lipid metabolism during gestation has been extensively studied. The transition from anabolism to catabolism around mid pregnancy leads to profound changes in the concentration of circulating lipids in the mother and in consequence in the fetal circulation. Hyperlipidemia, a common feature in obesity, is a risk factor for fetal macrosomia in gestational diabetes (67, 137). Moreover, decreased n-3 PUFA in the maternal circulation correlates with cognitive impairment in the offspring (138, 139). To investigate changes in circulating fatty acids in the context of obesity, 18 fatty acids were compared individually and in classes based on their degrees of saturation (saturated, unsaturated, n-3 PUFA, n-6 PUFA). BMI and leptin did not significantly associate with individual fatty acids nor classes (140). In contrast, C-peptide and IS_{HOMA} were consistently associated with decreased total n-3 PUFA and some individual n-3 PUFA fatty acids (140). These results emphasize the importance of including other exposures in addition to BMI to capture the heterogeneity of obesity.

In non-pregnant individuals, obesity associates with decreased n-3 PUFA concentrations, especially docosahexaenoic acid (DHA, C 22:6 n-3) (141, 142). n-3 PUFA have insulin sensitizing properties, which may be mediated by increased mitochondrial biogenesis and by the secretion of anti-inflammatory molecules e.g., adiponectin, protectins, resolvins, and maresins (143, 144). Therefore, dietary supplementation with n-3 PUFA might appear as an attractive option to improve insulin sensitivity in individuals with overweight/obesity. Interestingly, in children, despite increased dietary intake of n-3 PUFA and similar α-linolenic acid (C 18:3 n-3; n-3 PUFA precursor) concentrations in circulation, DHA levels were lower in children with obesity compared to lean children (141). This observation suggests a dysregulation in the biosynthesis, e.g., desaturation and elongation steps, of n-3 PUFA in obesity rather than dietary influence. Different from other n-3 PUFA, DHA is

synthesized in the peroxisomes by β-oxidation of LC-PUFA C 24:6 n-3 (**Figure 25**). Therefore, it is possible that peroxisomal β-oxidation leading to DHA synthesis is reduced in obesity. Indeed, in our cohort low IS_{HOMA} was significantly associated with decreased DHA/α-linoleic ratio, suggesting impaired DHA synthesis. Genes involved in mitochondrial and peroxisomal oxidation are also downregulated in first trimester placentas exposed to maternal obesity (145). Interestingly, DHA concentration in the maternal serum as well as peroxisomal function in the placenta seem to depend on fetal sex (cf. 5.2.).

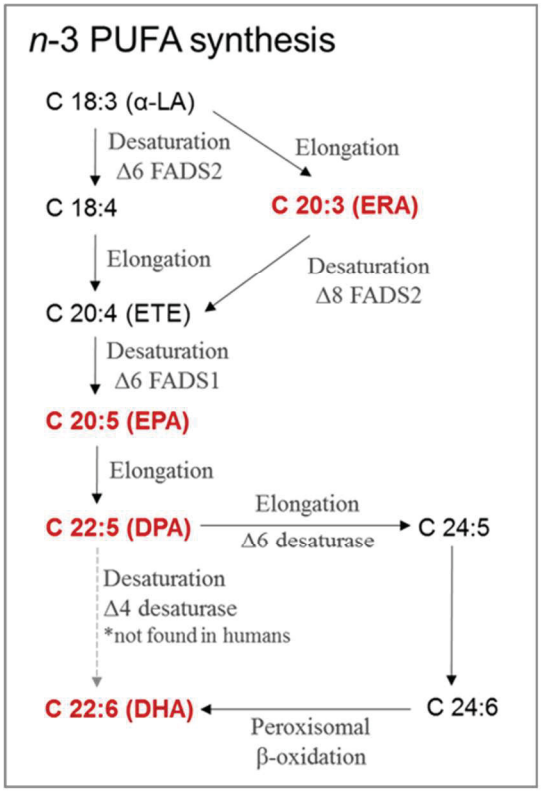


Figure 25. **Biosynthesis of n-3 PUFA**. n-3 PUFA are synthesized from their essential precursor α-linolenic acid (C 18:3) which is acquired with the diet. Subsequent PUFA synthesis involves a series of desaturation and elongation steps that take place at the endoplasmic reticulum or mitochondria, depending on the tissue. However, the conversion of n-3 C 24:6 to n-3 C 22:6 (DHA) takes place at peroxisomes.

For a comprehensive understanding of maternal metabolism during the first trimester of pregnancy, we further analysed the metabolome of 111 pregnant women using untargeted metabolomics. This is a hypothesis free method which allows the detection and semi-quantification of hydrophilic molecules present in the serum. We designed a stringent data analysis with the aim of identifying only few metabolic candidates that are robustly and significantly associated with clinical traits (BMI, leptin glucose, C-peptide or IS_{HOMA}). This analysis should enable the identification of only very relevant metabolites. After stringent data analysis, 15 metabolites significantly associated with BMI (n = 5), leptin (n = 2), glucose (n = 2), C-peptide (n = 5) and/or IS_{HOMA} (n = 2) were identified. These results again highlight the

importance of including other exposures alongside BMI to better capture the metabolic heterogeneity of obesity. The most robust metabolites, i.e., significantly associated with a clinical trait also after robustness analysis, were N-acetyl-L-alanine and palmitoleoyl ethanolamide, both positively associated with C-peptide. The limitation of untargeted metabolomics is that also identifies metabolites which have not yet been described, i.e., not annotated, yet. This makes biological interpretation of the results challenging. In addition, many identified metabolites are uncommon. To date, less than 20 publications in PubMed include the term n-acetyl-L-alanine, and only five include palmitoleoyl ethanolamide.

To obtain biological insight in the association between palmitoleoyl ethanolamide and C-peptide, I developed a theoretical model (**Figure 26**). Palmitoleoyl ethanolamide is an endocannabinoid-like lipid endogenously synthesized from palmitoleic acid (146). Although it shares the biosynthetic and degradation pathways of endocannabinoids, palmitoleoyl ethanolamide does not have affinity for the classical cannabinoid receptors CB1 and CB2 (147). Instead, palmitoleoyl ethanolamide has affinity for the GPR119 receptor which is mainly expressed in enterocytes and β-cells of the pancreas (148). I speculated that the mode of action of palmitoleoyl ethanolamide differs between the postprandial and fasting states. In the postprandial state, ingested palmitoleoyl ethanolamide can bind to G protein-coupled receptor 119 (GPR119) located at the apical membrane of enterocyte L-cells, facing the gut cavity. Binding of palmitoleoyl ethanolamide to the receptor induces intracellular production of cAMP, which stimulates the release of the incretin GLP-1 from enterocytes to the maternal circulation. Circulating GLP-1 reaches the pancreas, its main target organ, where it binds to the GLP-1 receptor in β-cells. In presence of high glucose levels, as occurs in the postprandial state, GLP-1 binding to GLP-1 receptor stimulates insulin release (149). This mechanism could explain the association between high palmitoleoyl ethanolamide and high C-peptide. To validate this model, GLP-1 concentrations were measured in a sub-cohort (n = 95) and correlated with palmitoleoyl ethanolamide and C-peptide levels. There was a significant correlation between palmitoleoyl ethanolamide and C-peptide as well as between palmitoleoyl ethanolamide and GLP-1. However, using statistical mediation analysis we could not confirm that GLP-1 mediates the association between palmitoleoyl ethanolamide and C-peptide levels. This is not surprising since the women included in our study were fasted overnight. In the fasted state, palmitoleoyl ethanolamide should not bind to GPR119 at the enterocytes and GLP-1 mediated insulin release in the pancreas would also not be possible because of low blood glucose levels. Therefore, there might be an alternative mode of action of palmitoleoyl ethanolamide in the fasting state. Since β-cells also express GPR119, it is possible that circulating palmitoleoyl ethanolamide stimulates insulin secretion by directly binding to pancreatic GPR119. We are currently testing the potential effect palmitoleoyl ethanolamide as

insulin secretagogue *in vitro* using an insulin-producing cell line (INS832/13 cells) in collaboration with Prof Chris Nolan and Dr. Viviane Delghingaro-Augusto, Australian National University, Canberra. As a consideration, both models e.g., postprandial and fasting, can only work if β-cell function and insulin secretion are preserved. Since in our study the glucose levels were not significantly increased in women lwith overweight/obesity, we can speculate that β-cell function is preserved for most women.

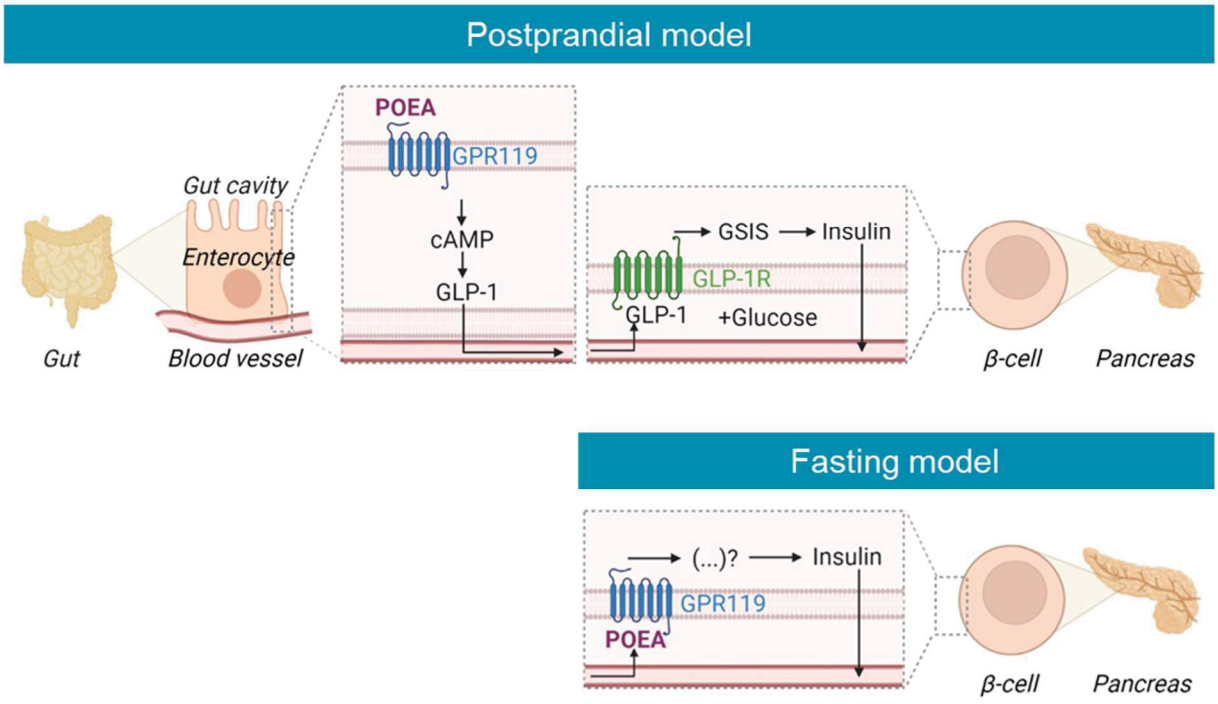


Figure 26. Hypothetical model of palmitoleoyl ethanolamide mode of action in the postprandial and fasting state. In the postprandial state palmitoleoyl ethanolamide binds to GPR119 receptor at enterocytes and stimulates GLP-1 secretion into the circulation. In presence of high glucose, GLP-1 binding to pancreatic GLP-1 receptor induces glucose stimulated insulin secretion (GSIS), resulting in insulin secretion. In the fasting state, palmitoleoyl ethanolamide binds to GPR119 in β-cells and, via an unknown mechanism, stimulates insulin secretion.

In fact, in a sub-analysis aiming to identify changes on the metabolome of healthy and unhealthy women with overweight/obesity, only 9 out of 34 women had impaired insulin sensitivity, suggesting that insulin secretion is indeed preserved in most women. We found that in this sub-cohort of women with overweight/obesity, insulin sensitivity (IS_{HOMA}) was inversely associated with tryptamine and 2,3,4,9-Tetrahydro-1H-carboline-3-carboxylic acid. Tryptamine is a derivative of tryptophan produced by gut microbiota, which contributes to maintaining the integrity of the intestinal barrier (150). Interestingly, hyperglycaemia drives intestinal barrier dysfunction, which is thought to be a determinant for healthy or unhealthy obesity (151). The reason is that increased permeability of the intestinal barrier allows entry of microorganism by-

products, like lipopolysaccharide (LPS), into the circulation which causes systemic inflammation. At the same time, systemic inflammation is a risk factor for developing obesity complications. In our sub-analysis, women with high BMI and low insulin sensitivity had increased tryptamine levels, which is counterintuitive. However, the robust finding of associations between metabolic traits and tryptophan by-products, points out the relevance of tryptophan metabolism in the regulation of the glucose-insulin axis and obesity. Unfortunately, there is no literature available in the context of obesity for the metabolite 2,3,4,9-Tetrahydro-1H-carboline-3-carboxylic acid. However, this compound is structurally similar to tryptamine, DL-tryptophan and other indoles, again supporting the notion of tryptophan metabolism as an interesting metabolic pathway in the context of healthy and unhealthy obesity.

5.2. Fetal sex

Fetal sex plays a crucial role in pregnancy, affecting placental function and influencing the health outcomes of both mother and fetus (152-155). The placenta is an organ of fetal origin and therefore has the same sex as the fetus. Despite increasing awareness of sexual dimorphism in the placenta, most studies early in pregnancy do not take it into account. The main reason is that fetal sex determination by ultrasound in the first trimester is not reliable (156). Placental tissue, if available, offers an opportunity to determine fetal sex by RT-qPCR based on XIST (X-linked) and DDX3Y (Y-linked) gene expression (119).

Sexual dimorphism in the placenta may be due to differential expression of X and Y chromosomes, downstream regulation of autosomal genes by X and Y chromosomes or signalling by sex hormones (124). Interestingly, female fetuses are more resistant to adverse environmental exposures *in utero* (157). This may be mediated by non-random X inactivation in female placentas in response to environmental stress (157, 158). In female placentas, one X chromosome is randomly inactivated meaning that some cells will express the maternal and others the paternal allele. Different from other tissues, the placenta is the only tissue capable of reactivating the inactivated X chromosome, at least *in vitro* (159). This suggests that female placentas might be capable of activating one or another allele depending on the environmental conditions (157). In addition, it has been proposed that under adverse intrauterine conditions male and female fetuses follow different growth strategies. Whereas males adapt their placental function to keep on growing, females reduce their growth which confers them partial resistance to further insults (157). Indeed, in the first trimester of pregnancies complicated by maternal obesity, fetal growth is reduced in females, but not in males (84). Since placental growth precedes fetal growth, such sex differences could also be present in the first trimester placenta.

Transcriptomic studies at the end of the first and beginning of the second trimester have found sexual dimorphism already in the early placenta. These differences are not only present on genes at X and Y chromosomes, but also in autosomal genes. For instance, extracellular matrix components, transcriptional regulation, DNA damage response and vesicle mediated transport were upregulated in female placentas, whereas genes involved in mitochondrial metabolism, immune regulation and response to nutrient deficit were upregulated in male placentas (124, 125). Of note, the genes and pathways identified as significantly different between female and male placentas differed considerably between studies. To obtain a more reliable signature of sex-dependent genes, I compared the identified as sexually-dimorphic expressed genes in both studies (**Figure 27 A**). Only 10 genes were commonly attributed to females in both studies and all were located on the X chromosome. Similarly, 10 genes were common for males in both studies and all were located on the Y chromosome. The few gene overlaps and the lack of overlap in autosome genes between studies might be due to differences in gestational age, since one study included placenta samples from week 10.5 to 13.5 (125) and the other from week 11 to 16 (124) of gestation. As a speculation, gestational age might have a stronger effect on autosomal gene expression than fetal sex and may have hidden subtle effects of fetal sex on autosomal gene expression.

One of the limitations of our proteomic analysis in first trimester placentas is the lack of fetal sex determination for most samples which precludes examining sexual dimorphism. The small amount of placental tissue available precluded isolating both protein (for proteomics) and RNA (for fetal sex determination) in most samples. Therefore, we cannot exclude a fetal sex contribution to the results. To overcome this limitation, we compared distinct genes for female and male placentas from published studies with proteins upregulated and downregulated in the low insulin sensitive in the proteomic analysis (**Figure 27 B**). None of the sexual dimorphic genes were among the upregulated/downregulated proteins in the low insulin sensitive group. Thus, the differences found are unlikely attributable to overrepresentation of males or females in the proteomics cohort. In addition, MCM expression was not significantly different in the transcriptomic analysis of male and female placentas in three published studies (124, 125, 160).

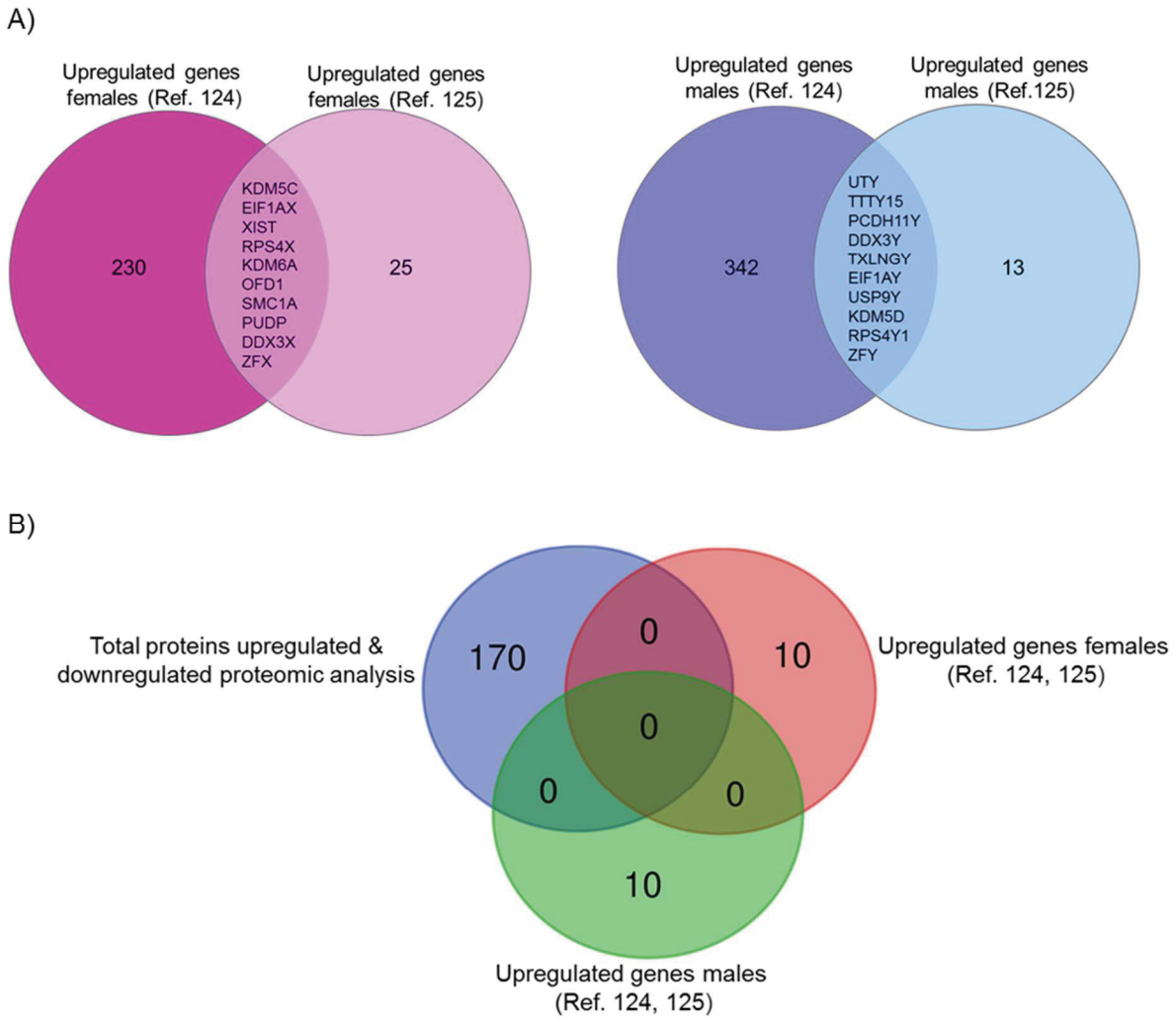


Figure 27. **Sexual dimorphism in placental gene expression.** A) Venn diagrams showing genes upregulated in female placentas or male placentas in two transcriptomic analysis. B) Venn diagram showing no presence of fetal dimorphic proteins among the significantly different (up- or downregulated) proteins in the low insulin sensitive group. *Constructed using data from (124, 125) with permission of the authors and publishing license.*

Because of the bi-directional dialogue between mother and feto-placental unit, fetal sex likely contributes to maternal metabolism regulation in the first trimester of pregnancy as reflected in a 4% higher GDM risk in pregnant women carrying a male fetus (154). In fact, the association between increased C-peptide and decreased DHA concentration in maternal serum in my cohort was dependent on female fetal sex (140). In the general population, diet supplementation with eicosapentaenoic acid (EPA) and DHA has been reported to improve insulin sensitivity in women, but not in men (161). These differences are probably the result of sex hormones, e.g., estrogens, which upregulate the activity of desaturases and synthesis of DHA (162). During pregnancy, the placenta becomes the primary organ of estrogen synthesis (163) and fetal sex may have an impact on estrogen synthesis.

Fetal sex is known to affect the regulation of the glucose-insulin axis in the mother. Carrying a male fetus is associated with impaired β-cell function and increased risk of developing GDM (154, 164, 165) and late onset pre-eclampsia (153). The mechanism is unclear but it has been suggested that reduced secretion of hPL and prolactin by male placentas may affect β-cell mass expansion in the maternal pancreas (154, 166). In addition, steroids can reverse the effect of placental lactogen in β-cell mass expansion (167). However, the effect of fetal sex on the glucose-insulin axis is controversial since other studies found that carrying a female fetus is rather a risk factor for developing insulin resistance (168, 169). Nonetheless, there is some agreement that carrying a male fetus associates with higher cardiovascular and metabolic load for the mother and increased risk of pregnancy complications (153). In preliminary analysis, we did not find significant differences in glucose, C-peptide or IS_{HOMA} between male and female-bearing mothers in our first trimester cohort (unpublished data). However, this might be due to small sample size and considerable metabolic heterogeneity in the women.

The small sub-cohort for which fetal sex determination was available and the inclusion of several *a priori* defined confounders in the statistical models used for analyses of metabolomic data precluded addition of fetal sex as a further confounder since it would have overadjusted the statistical models. In a metabolomics study in maternal serum in the second and third trimester (gestational age 12, 20, 28, 36 weeks), several metabolites were associated with fetal sex (160). None of these metabolites associated with fetal sex were among the enriched metabolites differing between women with overweight/obesity and normal weight or other clinical traits of obesity in our serum metabolomics analysis. This adds further evidence to the absence of overrepresentation of male or female fetuses in our cohort and/or that the fetal sex effect is weaker than the effect of the obesity environment.

5.3. Gestational age effect

The increasing requirement of nutrients and oxygen by the feto-placental unit with increasing gestational age requires adaptations of maternal metabolism. Therefore, maternal and placental metabolism change throughout pregnancy (41). In the first trimester of pregnancy, the major changes are the transition from histiotrophic to haemotrophic nutrition of the feto-placental unit as well as the opening of spiral arteries with subsequent rise in oxygen supply to the placenta (24, 170). In addition, maternal influence on placental development decreases over time and the fetus takes over most of the control (171, 172).

5.3.1. Gestational age effect in the maternal metabolism

The glucose-insulin axis is central to many of the metabolic adaptations in the mother. Therefore, gestational changes in glycaemia and insulinaemia regulate many other metabolic processes. Similar to other studies, we found a decrease in fasting glucose and C-peptide from week 6 LMP to the end of the first trimester (44, 173-175). In the follow up studies measuring fatty acids and metabolites in the maternal circulation, we consistently found associations with C-peptide and insulin sensitivity rather than BMI. To exclude a potential gestational age effect mediating these associations, we included gestational age as a confounder in the models as well as interaction terms when appropriate. A limitation of the study is the cross-sectional design which precludes investigating metabolic changes in the individuals over time but rather at the group level. Therefore, it is possible that the metabolic heterogeneity at the group level conceals relevant metabolic changes at the individual level for a given time point. For instance, we could not identify a distinct pattern of decreasing glucose levels between women with overweight/obesity and normal weight during weeks 6 to 10 post conception, despite previous reports suggesting such variations (44, 173). Another metabolic adaptation that can affect the concentration of circulating metabolites is the rise in plasma volume, which increases by 7-10% from week 6 to week 12 of gestation (41, 176, 177). In addition, placental secretion of hormones and exosomes changes over gestation, also contributing to regulating the maternal metabolic pool (42, 78, 178).

The only longitudinal study in healthy pregnant women analysing changes in metabolites over trimesters found a general decrease in amino acids, LC-PUFA, free carnitine, acetyl-carnitine, phosphatidylcholines and sphingomyelins with advancing gestational age (179). The authors proposed that the decreasing concentrations of most metabolites with increasing gestation indicates increased placental uptake. I speculate that amino acid concentrations in maternal serum are higher early in pregnancy to provide amino acids for uterine gland secretions and to support the histiotrophic nutrition of the placenta. Alternatively, the decreasing amino acid concentrations over gestation could reflect a dilution effect resulting

from above-mentioned rise in plasma volume. Increased plasma volume could also partially explain the decrease that we observed in glucose (-14%) and C-peptide (-18%) concentration between week 4-6 and 10-12 LMP (44) (**Figure 28**).

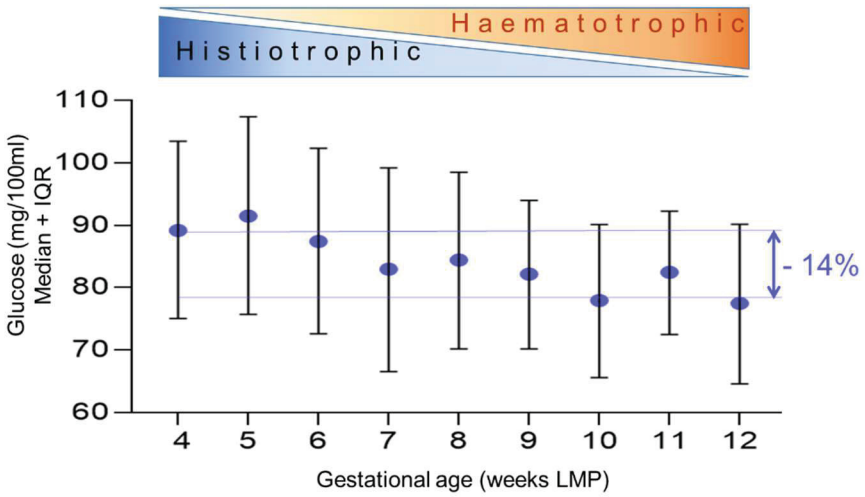


Figure 28. Decrease of maternal fasting glucose parallels the transition from histiotrophic to haemotrophic nutrition. Maternal fasting glucose was measured in a cohort of 323 pregnant women spanning a wide BMI and gestational age range in the first trimester of pregnancy. The median and interquartile range for glucose values on each week was calculated and plotted. The progressive decrease of maternal glucose parallels the opening of uterine spiral arteries and consequent transition from histiotrophic to haemotrophic nutrition. *This figure is based on published data (44) with publisher's permission.*

5.3.2. Gestational age effect on the placenta

The placenta also changes with gestational age to accommodate to changes in the maternal environment and to adapt to increasing fetal requirements. The first trimester placenta grows rapidly mostly by a high degree of villous cytotrophoblast proliferation. Similar to cancer tissue, the general hypomethylation of many placental genes in the first and second trimester may contribute to this rapid proliferation (180, 181). Placental gene expression differs between the first and third trimester (182). Early in pregnancy genes involved in cell proliferation, cell differentiation and angiogenesis are upregulated, whereas in the third trimester genes involved in surface receptor mediated signal transduction, G-protein mediated signalling and ion transport are upregulated (182). A transcriptomic analysis comparing chorionic villous samples collected at week 7-8 and 13-14 showed numerous differences likely reflecting the establishment of blood flow to the intervillous space and, hence, changes to haemotrophic nutrition and increased oxygen tension (183). Processes related to the endoplasmic reticulum were upregulated in the first trimester compared to the early second trimester, suggesting increased protein synthesis (183). Genes involved in transport of nutrients and oxygen were upregulated in the early second trimester (183). Similarly, in another

study cell cycle, DNA, amino acids, and carbohydrate metabolism were significantly upregulated and signal transduction downregulated in first trimester placentas (184). Indeed, it is not surprising that upregulation of genes involved in proliferation and protein synthesis are upregulated in the first trimester since first trimester cytotrophoblast proliferation is increased under low oxygen tension (12).

Because we aimed to identify changes in the proteome of placentas exposed to maternal obesity, we matched samples for gestational age to avoid confounding effect. Restricting the gestational age window was important to avoid differences caused by the transition to haemotrophic nutrition and by the oxygen rise and to rather capture effects associated with exposure to maternal obesity *in utero*. We selected samples of gestational age week 5^{+0} - 6^{+6} LMP because this early in pregnancy maternal influences are likely to prevail and oxygen tension in the intervillous space is not rising yet (170). In addition, differences in glucose, C-peptide and IS_{HOMA} between lean and women with overweight/obesity appeared more pronounced at week 4^{+0} - 6^{+6} LMP than later in the first trimester (**Figure 29**). These results should be taken with caution since the interaction term between BMI and gestational age in the glucose, C-peptide and IS_{HOMA} models was not statistically significant. Nonetheless, they suggest more pronounced metabolic differences in the glucose-insulin axis between women with overweight/obesity and normal weight very early in pregnancy.

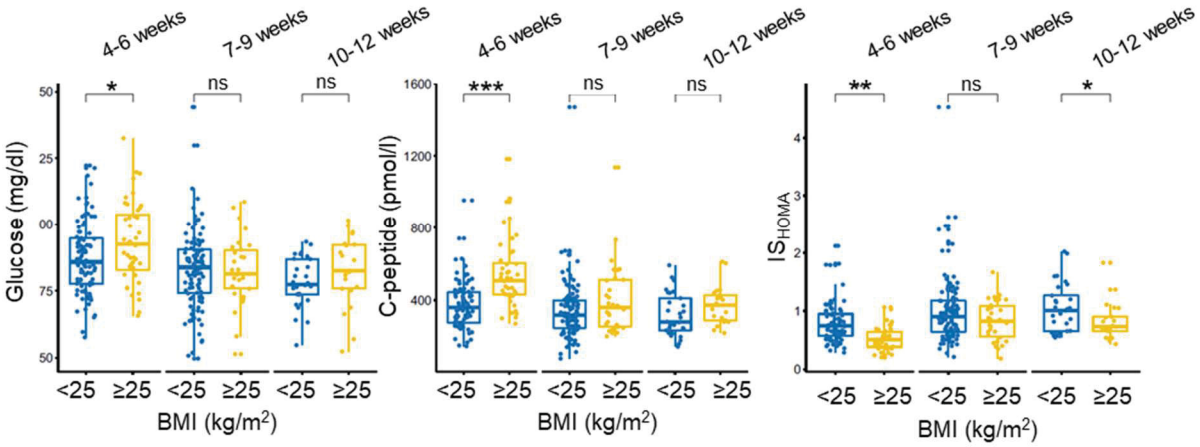


Figure 29. Metabolic differences between women with overweight/obesity and normal weight seem more pronounced very early in pregnancy. Maternal fasting glucose and C-peptide seem to be higher and insulin sensitivity lower in women with overweight/obesity compared to lean at week 4-6 lpm and not afterwards. However, the interaction term between BMI and gestational age was not statistically significant in the glucose, C-peptide and IS_{HOMA} models and therefore the results need to be taken with caution. *This unpublished figure is based in published data (44) with publisher's permission.*

Therefore, if the maternal environment induces changes on the placental proteome, then one would expect more pronounced placental changes in the very early period. We found that proteins related to DNA replication and cell cycle were upregulated in placentas exposed to maternal low insulin sensitivity. Since trophoblast proliferation is upregulated under low oxygen tension, these results add further support to the notion of delayed spiral artery opening and subsequent establishment of blood flow in pregnancies complicated by obesity/low insulin sensitivity (28).

5.4. *In utero* exposure to maternal obesity

Fetuses exposed to maternal obesity *in utero* follow a bi-phasic growth pattern compared to those in a metabolically normal pregnancy (75). During the early stages of pregnancy, fetal growth is reduced in obesity compared to normal pregnancies (84). This is followed by a period of accelerated growth during the later stages of pregnancy (75). Since placental growth precedes fetal growth, it is likely that placental growth also follows a bi-phasic pattern when exposed to an obesogenic environment *in utero*. Indeed, 3D ultrasound analysis showed decreased placental volume and vascularization in first trimester placentas of women with overweight (86).

Rapid villous cytotrophoblast proliferation in the first trimester placenta increases the likelihood of spontaneous DNA lesions and replicative stress (15, 87, 185). In our untargeted proteomic analysis in first trimester placentas, MCM proteins i.e., MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7, were increased in placentas of women with low insulin sensitivity. These proteins are involved in replication and DNA damage repair suggesting increased DNA damage in placentas of women with obesity, i.e., low insulin sensitivity. These results support previous findings showing increased DNA damage in the villous cytotrophoblast of first trimester placentas exposed to maternal obesity (105). Indeed, MCM6 co-localized with γH2AX, a DNA damage marker, in cytotrophoblast nuclei of first trimester placentas (cf. 4.2.1, Figure 16). At the single-cell level transcriptomics in published first trimester placenta data (126), MCM expression is highest in villous cytotrophoblast cells, corroborating our immunolocalization findings. This is not surprising, since MCMs have been shown to be expressed in replicating, but not in quiescent cells (186).

MCMs are highly conserved among eukaryotes. After synthesis, MCMs assemble into two sub-complexes (MCM2-4-6-7 and MCM3-5) which are imported separately into the nucleus owing to the nuclear localization sequence present in MCM2 and MCM3 (187). Once in the nucleus, both sub-complexes form a heterohexamer (MCM-complex) following a 1:1:1:1:1:1 stoichiometry (187). Knock-out of one MCM prevents MCM-complex assembly and

disrupts its function (187). The MCM-complex is a helicase and unwinds DNA during replication (188). During G1 phase, the MCM-complex is recruited to the origins of replication and in S-phase starts origin firing, i.e., start of DNA replication (189). For origin firing, the MCM-complex needs to be activated by CDKs and further recruitment of CDC45 and GINS (Sld5, GINS1, GINS2, GINS3) is necessary (189). Tight control of MCM-complex loading to origins of replication ensures that DNA is replicated only once per cell cycle (188).

Traditionally, MCMs function has been limited to cell cycle initiation. However, MCMs concentration is 40-100 fold higher than the number of origins of replication (MCMs paradox) suggesting additional roles for these proteins (186, 190). These additional roles are participating in DNA damage repair, interacting with checkpoint regulators and maintaining chromosome stability (191, 192). Interestingly, a novel role for MCMs may be the stabilization of cytoplasmic stress granules (193). Although we found that MCM6 localizes predominantly to cytotrophoblast nuclei in first trimester placenta, MCM6 foci were also present at the cytoplasm of the villous cytotrophoblast and syncytiotrophoblast. As a speculation, this may indicate the presence of stable cytoplasmic stress granules in placentas exposed to maternal obesity. This is interesting because it suggests a dual function of MCM6 depending on its subcellular location. In the cytoplasm MCM6 may regulate protein translation whereas in the nucleus it is involved in DNA related processes (**Figure 30**).

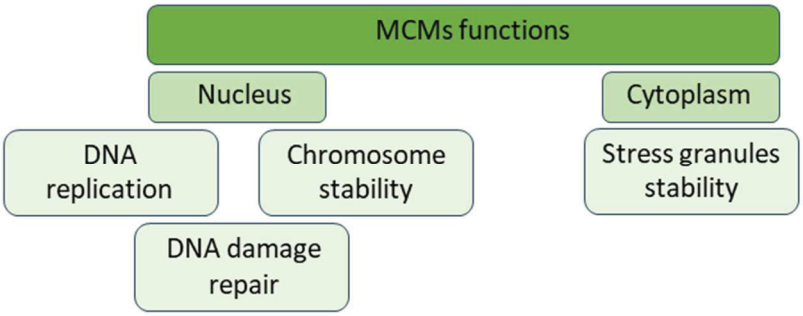


Figure 30. Functions of MCMs proteins at the nucleus and cytoplasm. In the nucleus, the MCM-complex acts as a helicase at origins of replication (ORC) and is necessary for DNA replication. In addition, MCMs participate in chromosome stability and DNA damage repair (191, 192). In the cytoplasm, MCMs participate in stress granules stability by delaying stress granule disassembly (193).

Previous data generated in our lab suggest increased DNA damage and apoptosis in placentas of women with obesity (104, 105). Therefore, investigating the role of MCMs in DNA damage repair and the metabolic conditions that may lead to increased MCM levels in the placenta was of particular interest. *In vitro*, silencing MCM proteins in HEK293 cells leads to decreased phosphorylation of proteins involved in DNA damage repair and chromatin

remodelling, e.g., H2AX, CHK2, ATM, BRCA1, 53BP1 after etoposide treatment, suggesting that MCMs are necessary to initiate DNA damage response pathways (194). Consequently, increased placental MCM levels in women with low insulin sensitivity may reflect upregulation of DNA damage repair pathways. Cytotrophoblast apoptosis is increased in placentas exposed to maternal obesity (105) suggesting that upregulation of DNA-damage repair pathways is insufficient to effectively repair DNA damage in placentas exposed to the obesity environment *in utero*.

Steroid hormones have been suggested to regulate MCM expression and protein levels. In ovariectomized mice, estradiol treatment increased the loading of MCM-complex into chromatin in uterine epithelial cell lysates (195). In addition, the combined action of estradiol and progesterone downregulated MCM protein levels (195). Interestingly, in the same study, treatment with progesterone alone or in combination with estradiol caused translocation of MCMs from nuclei to cytoplasmic regions (195). In MCF-7 cells, treatment with phytoestrogens increased MCMs expression and protein level (196). Estradiol is required for normal placenta development (197), but patho-physiologically high estradiol levels early in pregnancy associate with impaired placentation (198). In addition, in baboons, high estradiol in the first trimester of pregnancy impairs remodelling of the uterine spiral arteries, delaying the establishment of blood flow (199). *In vitro,* first trimester placenta explants treated with high estradiol show increased apoptosis (200). Therefore, it is possible that increased MCM protein levels in placentas of women with low insulin sensitivity are a consequence of exposure to high estradiol in the obesogenic environment. Unfortunately, untargeted metabolomics does not detect lipophilic estradiol and not enough serum was left in our cohort for estradiol quantification by ELISA. This precluded further studies on the possibility of MCM regulation by estradiol. However, body mass index is positively associated with estrogen synthesis (201) and in our metabolomics cohort, other steroid hormones, i.e., androsterone glucuronide and 7α,17α-dimethyl-5β-androstane-3α,17β-diol glucuronide were significantly associated with C-peptide. This suggests dysregulated steroid metabolism in the pregnancy environment associated with obesity (116).

MCMs have been extensively studied in cancer, but their role in metabolic conditions or the placenta is largely unknown. In cancer, high MCMs expression is a marker of worse prognosis and chemoresistance (202, 203). In publically available data repositories, MCM2 is overexpressed in the liver and MCM4 in the skeletal muscle of insulin resistant subjects (204). Similarly, MCM6 is overexpressed in pancreatic islets and adipose tissue in a mouse model of obesity-insulin resistance (BTBR ob/ob) (205). Based on this and the positive correlation between C-peptide (proxy of insulin) and MCM protein levels in our cohort, ACH-3P cells were treated with insulin to investigate potential insulin-induced changes in MCM6 expression and

protein. Unfortunately, a direct effect of insulin treatment on MCM6 expression *in vitro* could not be demonstrated. Interestingly, a study showed that insulin (1nM) induces DNA damage in primary first trimester trophoblasts, but not in a lung fibroblast cell line (IMR-90) (206). Therefore, it is plausible that some genotypic changes introduced during the generation of cell lines confers resistance to some insulin effects.

ACH-3P cells were generated by somatic hybridization of a choriocarcinoma cell line (AC1-1) with primary first trimester trophoblasts (week 12) of a male fetus and are tratraploid (94-98 chromosomes) (207). It is conceivable that doubling the amount of DNA together with the choriocarcinoma phenotype confers additional mechanisms to prevent or overcome DNA damage. Treatment with DNA damage inducers such as etoposide and bleomycin induced DNA damage in ACH-3P cells. However, it is possible that milder insults such as high insulin concentrations, are better tolerated. Alternatively, it has been proposed that insulin induces DNA damage by increasing ROS (208, 209). Since ACH-3P cells were cultured under low oxygen tension to mimic the *in vivo* situation, the low availability of oxygen might have prevented ROS generation. However, DNA damage was also not significantly different in ACH-3P cells exposed to different oxygen tensions (2.5%, 6.5%, 21% O_2). Perhaps only the interaction of high oxygen with insulin may have overwhelmed protective mechanisms in ACH-3P cells to ultimately lead to DNA damage, but this hypothesis was not tested. Additionally, the insulin concentration (10nM) used may not have been high enough to induce DNA damage in ACH-3P cells. However, 10nM insulin is considered a patho-physiological concentration. Higher insulin (supra-physiological) concentrations would not resemble the *in vivo* situation and could also activate IGF-1 receptors (120). Another factor that could explain the differences observed *in vivo* and *in vitro* is the time of exposure to the adverse stimuli. The first trimester placenta tissues used in untargeted proteomics were exposed to maternal obesity *in utero* for at least 21 days (5 weeks LMP), whereas ACH-3P cells were exposed to high insulin or high oxygen for a maximum of 2 days. Long term exposure *in vivo* enables the tissue to undergo allostatic adaptations that cannot be captured in the short term exposures of *in vivo* experiments. Moreover, the *in utero* situation is metabolically more complex and the placenta tissue is exposed to a much wider range of molecules. This is a common issue of the reductionist approach of *in vitro* experiments, in which replicating the complex molecular matrix of the *in vivo* metabolic milieu, shear stress and spatial-organization of the cells is not possible.

5.5. Insulin (C-peptide) versus insulin sensitivity (IS_{HOMA})

As discussed above, BMI is not a good measure of obesity and other clinical traits such as glucose, insulin, lipids and insulin sensitivity better reflect the metabolic status of the mother. Based on my results, C-peptide and IS_{HOMA} seem more relevant than glucose to explain first trimester changes in maternal metabolism and placental proteome in the context of obesity. For example, both C-peptide concentrations and $IS_{HOMA,}$ but not glucose concentrations in the maternal circulation were associated with maternal n-3 PUFA changes (140) and were correlated with MCMs levels at weeks 5^{+0} - 6^{+6} LMP. Absence of significant differences of fasting serum glucose between women with overweight/obesity and normal weight may account for this (44). Also in the general population, insulin resistance is not always accompanied by abnormal glucose levels (45, 136, 210). Hyperinsulinaemia and reduced insulin sensitivity go often hand in hand, which makes it difficult to distinguish the contribution of each to the ultimate phenotype, e.g., increased DNA damage in the first trimester placenta. For example, comparing MCM6 expression in the BTBR ob/ob mouse model with control mice does not enable distinguishing between effects of hyperinsulinaemia and insulin resistance, since BTBR ob/ob mice present both clinical traits. Despite the frequent co-occurrence of hyperinsulinaemia and insulin resistance, their metabolic effect on the placenta should be opposite. In other words, if the changes observed in the placental proteome are caused by hyperinsulinaemia in the maternal circulation, these changes should not be present when the placenta is insulin resistant. Therefore, is it hyperinsulinaemia or decreased insulin sensitivity inducing changes on the placental proteome?

To address this question, several considerations have to be made. First, insulin sensitivity was calculated using the homeostatic model assessment (IS_{HOMA}). This is a mathematical index which reflects maternal hepatic insulin sensitivity and does not provide information on peripheral insulin sensitivity (113, 211). However, offspring exposed to maternal obesity develop insulin resistance *in utero*; therefore it is likely that the placenta also becomes insulin resistant (54). In fact, the transcriptome of first trimester trophoblasts exposed to maternal obesity *in utero* are less responsive to insulin treatment *in vitro* than trophoblast of normal weight women, suggesting *in utero* development of placental insulin resistance (212). However, these results were generated at ambient oxygen levels (21%) and may not truly reflect the *in utero* situation.

The second consideration is that insulin was measured using C-peptide as a surrogate (213). Insulin and C-peptide are secreted in equimolar concentrations by the β-cells. Since C-peptide has a longer half-life than insulin and its concentrations are less sensitive to haemolysis (214), C-peptide is a better biochemical parameter to measure in a non-clinical set up as we had in our study. Although C-peptide levels are widely accepted as an inert surrogate

of insulin concentration, it can activate its own insulin-independent signalling cascades through protein kinase A, protein kinase C and activation of MAPK kinase (215). The specific mechanisms of activation have remained elusive. It has been proposed that C-peptide binds to a GPR, but the specific receptor has not been identified yet (216, 217). Therefore, it is possible that the associations that we found between C-peptide and other metabolic parameters with placental proteins reflect individual signalling characteristics of C-peptide.

One argument speaks for **insulin** as the top candidate inducing observed changes in the first trimester placenta: treatment of primary trophoblasts isolated from healthy first trimester placentas (gestational age week 6 - 8) with 10nM insulin resulted in increased DNA damage (γH2AX), apoptosis (caspase-3) and decreased cell survival (206). These results are very similar to our observations (105) and are also aligned with increased levels of DNA damage repair proteins, e.g., MCMs, in the first trimester placenta. Further support for insulin as key in inducing at least some of the observed changes in the placental proteome come from a mouse study. In that study, upon insulin binding, the insulin receptor translocated to the nucleus and interacted with MCM3 and MCM7 (218). This suggests that insulin binding to the insulin receptor and subsequent translocation of the receptor to the nucleus is capable of inducing transcription of target genes and obviates the need of insulin induction of intracellular signalling cascades that lead to the same effect. This concept is complicated by the spatial location of the insulin receptor at the microvillous membrane of the syncytiotrophoblast in the first trimester placenta. How could insulin, or the insulin-insulin receptor complex, translocate through the syncytiotrophoblast and induce DNA damage at the cytotrophoblast? This subject will be discussed in sections 5.6 and 5.7.

Systemic **insulin resistance** reflects impaired insulin action in the major insulin-responsive organs: liver, adipose tissue and muscle. In a study comparing gene expression in liver and muscle samples of insulin resistant and non-insulin resistant subjects, cell cycle, apoptosis, DNA double strand breaks and extracellular matrix organization processes were upregulated in the insulin resistant group (204). These results are very similar to the pathways upregulated in our proteome analysis of first trimester placentas of low insulin sensitive, i.e., high insulin resistant, women. In another study assessing gene expression in subcutaneous adipose tissue of insulin resistant and non-insulin resistant participants, cell cycle was also upregulated in the insulin resistant group (204). However, other upregulated pathways in adipose tissue, e.g., fatty acid and sterol metabolism, carbohydrate metabolism, angiogenesis and inflammation differed from the observations in the placenta. Overall, these results suggest a common response involving DNA damage and cell cycle in insulin resistant tissues together with specific responses in each organ. The specific responses in each organ might be explained by the concept of ‘selective insulin resistance’.

‘Selective insulin resistance’ is an interesting concept which implies that only some downstream pathways of insulin signalling are disturbed by insulin resistance. For example, ´selective insulin resistance´ in the liver can result in insulin resistance of the FoxO1 pathway and therefore lead to continuous gluconeogenesis, but not affect insulin sensitivity of the SREP-1c pathway resulting in extremely high fatty acid synthesis (219). This is possible if insulin resistance is regulated by distal components of the insulin signalling cascade rather than at the level of the insulin receptor (45). If the first trimester placenta develops insulin resistance *in utero*, it is likely ´selective´, with only specific signalling pathways altered. Although insulin resistance appears to regulate common processes across organs, e.g., upregulation of cell cycle, DNA damage and apoptosis, the main obstacle is the absence of a known mechanism. Insulin sensitivity per se cannot induce these changes. Therefore, the crucial question is whether IS_{HOMA} (index of hepatic insulin resistance) is reflecting maternal hyperinsulinaemia alone or a combination of metabolites highly correlated with insulin and/or insulin resistance.

5.6. A matter of location: which molecules could pass the syncytiotrophoblast and induce changes in MCMs in the villous cytotrophoblast?

As discussed above (cf. 5.5.), maternal **insulin** appears as the main candidate to mediate changes in the placenta proteome, e.g., affect cell cycle, replication and DNA damage repair pathways, and specifically in MCMs. First, maternal insulin levels (C-peptide) are increased in women with overweight/obesity (44). Second, C-peptide concentration correlates with MCM protein levels, which are involved in DNA-damage repair and cell cycle pathways at the villous cytotrophoblast of the first trimester placenta. Third, the insulin receptor is located at the microvillous membrane of the syncytiotrophoblast in the first trimester placenta (220). In addition, treating primary trophoblasts with insulin increases DNA damage and apoptosis (206), which resembles findings of my group in first trimester placentas exposed to maternal obesity (105).

However, the adverse effects of *in utero* exposure to maternal obesity may also be the consequence of a combination of factors related to insulin and/or insulin sensitivity rather than induced by exposure to a single molecular species. An additional consideration is that during the histiotrophic period of nutrition the molecular pool of the intervillous space may not fully resemble maternal serum composition since flow of maternal blood is not yet stablished (2). Nonetheless, the secretions of the uterine glands exhibit a composition similar to that of maternal serum (16).

Several molecules known to be altered in maternal obesity are present in the intervillous space and could, directly or indirectly, affect cytotrophoblast proliferation and/or survival through MCM regulation (**Table 6)**.

Table 6. Molecules altered in obesity in the maternal circulation, released from decidual cells and/or present in the intervillous space that could affect cytotrophoblast proliferation.

Metabolite	Function	Reference
Insulin	Cytotrophoblast proliferation	(221)
IGF-1*	VCT proliferation, differentiation, apoptosis	(20)
IGF-2*	VCT proliferation, EVT invasion and migration	(20, 222)
EGF*	VCT proliferation (week 4-5), SCT secretion of hCG and hPL (week 6)	(19)
Glucose	Energy supply	(223)
Lipids	Energy supply and storage, signaling, building blocks	(16)
Amino acids	Signaling, building blocks	(23)
Nucleosides	Building blocks, vasoconstriction	(224)
Leptin*	EVT invasion, SCT secretion of hCG, pro-inflammatory cytokines and prostaglandins	(225-227)
Estradiol*	Placentation, vascular remodeling by EVT, villous trophoblast viability	(200, 228)
Oxygen	Oxidative phosphorylation – energy, ROS production, signaling	(229)

* Molecule also secreted by the first trimester placenta. IGF-1: Insulin-like growth factor 1; IGF-2: Insulin-like growth factor 2 EGF: Endothelial growth factor; VCT: Villous cytotrophoblast; EVT: Extravillous trophoblast; SCT: Syncytiotrophoblast; hCG: Human choriogonadotropin; hPL: Human placental lactogen; ROS: Reactive oxygen species.

However, this raises a key question: how can insulin, or any other molecule(s) altered in maternal obesity, reach the intervillous space, signal through the syncytiotrophoblast and lead to MCM regulation in the villous cytotrophoblast (**Figure 31**).

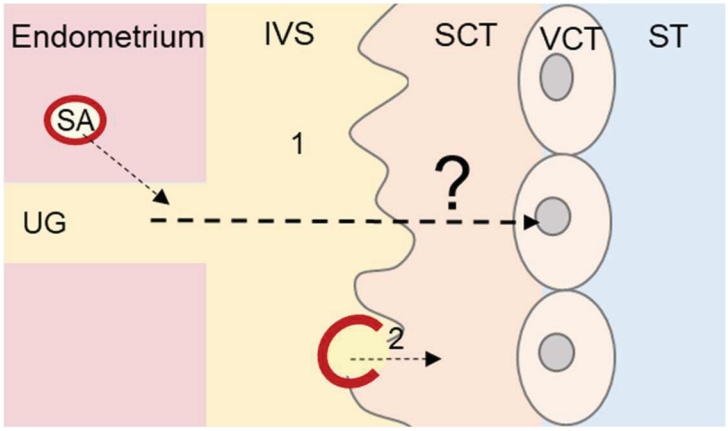


Figure 31. Schematic representation of the pathway for molecules from the mother to the villous cytotrophoblast. Early in pregnancy histiotrophic nutrition prevails (1) and secretions from the uterine glands together with transudate from the spiral arteries reach the intervillous space. After spiral artery remodelling, maternal blood flow opens to the intervillous space (2). Maternal secretions are taken up by the syncytiotrophoblast by pinocytosis or by receptors located at the microvillous surface. How maternal secretions and or molecular signals in the syncytiotrophoblast pass to the villous cytotrophoblast remains largely unknown. IVS: Intervillous space; SA: Spiral artery; SCT: Syncytiotrophoblast; ST: Stroma; VCT: Villous cytotrophoblast; UG: Uterine gland.

Several receptors and transporters for molecules in the intervillous space (cf. Table 6) are expressed in the trophoblast compartment in the first trimester (**Figure 32).** They are located on the microvillous membrane of the syncytiotrophoblast and/or on the plasma membrane of the villous cytotrophoblast. Thus, the molecular machinery is present to relay signals from the intervillous space to the villous cytotrophoblast.

Of note, not all molecules need a receptor to exert signalling functions and/or reach the villous cytotrophoblast. Some molecules such as oxygen can diffuse passively trough cell membranes and others are taken up by pinocytosis or phagocytosis, which is especially relevant for the period of histiotrophic nutrition. In addition, the syncytiotrophoblast and the villous cytotrophoblast are connected by gap junctions, which allow diffusion of ions, cAMP, cGMP, IP_3 and Ca^{2+} (230). These gap junctions are important for cytotrophoblast proliferation (20, 231).

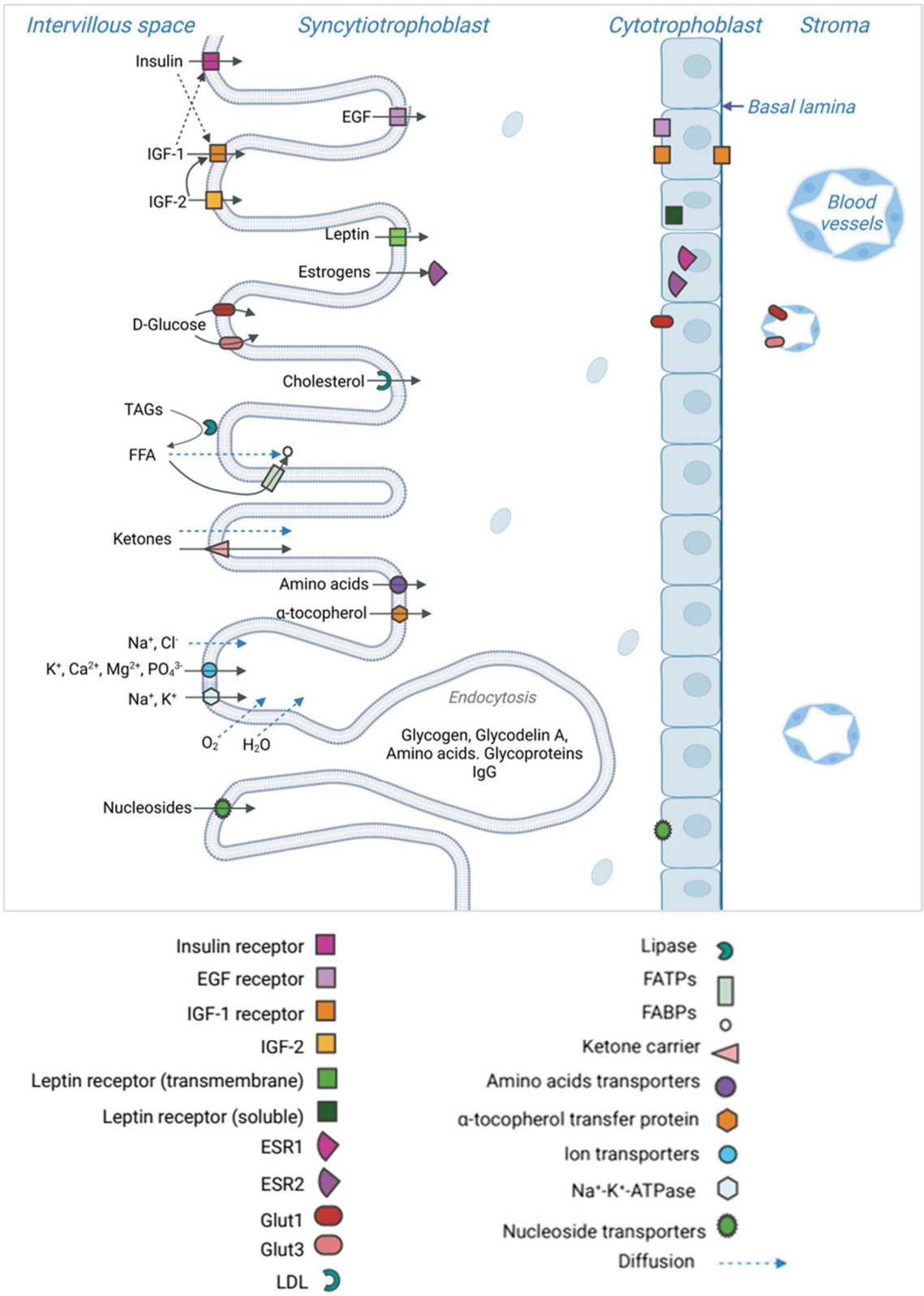


Figure 32. Location of several receptors and transporters on syncytiotrophoblast and villous cytotrophoblast in first trimester placenta. To exert signalling functions on the villous cytotrophoblast, molecules from maternal uterine secretions (histiotrophic period) or intervillous circulation (haemotrophic period) need to be taken up at the microvillous membrane of the syncytiotrophoblast. Gap junctions communicating syncytiotrophoblast and cytotrophoblast allow the pass trough of small molecules. How signalling between intervillous space, syncytiotrophoblast and cytotrophoblast occurs is poorly understood. Scheme created with BioRender.com.

An added challenge when addressing which molecules might cross the syncytiotrophoblast and exert its function at the cytotrophoblast is that signalling events change temporally and spatially with gestational age. For example, in the first trimester, the insulin receptor is expressed at the microvillous membrane of the syncytiotrophoblast but in the third trimester the insulin receptor locates predominantly to endothelial cells. This change in location implies that first trimester placenta is more responsive to maternal insulin whereas third trimester placenta predominantly responds to fetal insulinaemia (171). It also suggests that the placenta is especially sensitive to changes in the maternal metabolism early in pregnancy and that it becomes more independent of maternal influences with advancing gestational age (171, 172). Therefore, the relevance of a specific maternal molecule executing its function and/or mediating changes in MCMs in the villous cytotrophoblast is likely time-dependent. Another level of complication is the existence of redundant signalling cascades. Therefore, it is unlikely that any single metabolite mediates the full range of effects observed in the villous cytotrophoblast in obesity. Rather the chronic exposure to a combination of dysregulated molecules is likely to trigger the activation of obesity-related stress response pathways, e.g., DNA damage repair response, in the placenta. In addition, it should be considered that intermediate products generated during placental metabolism such as ROS or lactate might be the activators of stress responses in the villous cytotrophoblast.

ROS such as O_2-, H_2O_2 and $\bullet O_2$ are formed endogenously as a by-product of cellular metabolism. In pregnancy, oxidative stress plays a key role in the pathology of preeclampsia and intrauterine growth restriction (229). Functionally, ROS can induce DNA damage and affect cell proliferation, increase lipid peroxidation and induce endoplasmic reticulum stress (89). Although oxidative stress is increased in obesity, our group did not find oxidative stress in first trimester placentas of women with obesity (105).

Lactate can be transported from the mother to the fetus and vice versa (232). In addition, lactate is produced in the placenta as result of aerobic glycolysis, as occurs early in pregnancy before the opening of the spiral arteries. Interestingly, lactate has signalling functions by modifying histones (histone lactylation) (233). Epigenetic modifications in histones affect gene expression, DNA replication and DNA repair mechanisms. In macrophages, histone lactylation in lysine residues enhances transcription of genes involved in restoring homeostasis (233). Histone lactylation may also occur in the placenta. Furthermore, considering that early placental development shares characteristics with tumour development and its microenvironment (17), it could be expected that lactic acid participates in trophoblast invasion, angiogenesis and immune tolerance (234).

Most of the molecules discussed, directly stimulate placental growth (insulin, IGF1, IGF2, EGF) or are necessary building blocks for proliferating placental cells (amino acids, lipids). Whereas all are required for adequate placental development and function, excessive exposure as occurs in the intrauterine obesity milieu can activate obesity-related stress and stress response pathways. Chronic exposure will activate allostatic mechanisms that change the set points of intracellular and systemic responses, e.g., enhanced synthesis of DNA damage repair proteins, metabolic adaptations. If these allostatic mechanisms do not succeed restoring cellular balance, villous cytotrophoblast cells may undergo apoptosis, as is sometimes the case in first trimester placentas of women with obesity (105).

5.7. Replicative stress and/or endoplasmic reticulum stress in the placenta in obesity?

In the first trimester, placental and fetal growth are reduced in pregnancies complicated by obesity (74, 84, 86). These observations suggest that maternal obesity activates stress responses resulting in reduced placental growth very early in pregnancy. In contrast, in the late second trimester, estimated fetal weight, head conference, femur and humerus length are higher in fetuses exposed to maternal obesity, suggesting a catch up-growth (191). This may be the result of a change in maternal metabolism and/or an adaptive response of the placenta to the adverse intrauterine environment between the first and late second trimester. Placentas of women with low insulin sensitivity had increased MCM proteins levels, which are involved in replication and DNA damage repair. Therefore, upregulating MCMs synthesis may be a mechanism to increase villous cytotrophoblast proliferation and ultimately catch up with placental growth. I discuss here several pathways affected by obesity that could activate stress responses in the placenta ultimately leading to decreased placental growth early in pregnancy and/or adaptation.

Obesity associates with increased DNA damage and genomic instability in multiple tissues (235), including the first trimester placenta (104). Decreased growth of placentas exposed to maternal obesity could be explained by a) increased DNA damage and/or b) impaired DNA damage repair. One possibility is that factors altered in maternal obesity (inflammatory molecules, ROS) induce direct **DNA damage**, by for example oxidizing guanine nucleosides to 8-oxo guanine (8-OHdG) which results in DNA double strand breaks (cf. 1.6.). However, the proposed delay in spiral artery remodelling in placentas exposed to obesity and, therefore, development under lower oxygen tension, make oxygen a less likely candidate. In a caloric restriction experiment in mice, weight loss accompanied by decreased concentrations of circulating insulin, leptin and inflammatory markers resulted in decreased DNA damage, decreased oxidative damage of purines and increased DNA damage repair in multiple tissues (236). The authors proposed that activation of the insulin-signalling cascade leads to activation

of NADPH-oxidase and translocation of AKT into the mitochondria resulting in ROS production and subsequent DNA damage (94). However, the specific mechanism remains unclear and it is difficult to explain how insulin or other factors altered in maternal obesity could damage DNA at the cytotrophoblast nuclei in first trimester placentas of women with obesity.

Impaired **DNA damage repair** seems a more likely mechanism to account for increased DNA damage levels. DNA damage occurs in every replicating cell and organisms have developed strategies to repair it and continue with cell cycle progression. In the first trimester of human pregnancy, proteins involved in cell cycle regulation (Cyclin D1, CHK1, CHK2, Rad52) and repair (MCMs, BRCA1, SFPQ, NONO) are upregulated in placentas exposed to obesity (104). Despite increased repair protein levels, DNA damage (measured as γH2AX foci) and apoptosis (measured as Caspase 3) are higher in the villous cytotrophoblast of placentas exposed to maternal obesity (105). These results suggest that a) the amount of DNA lesions is too high to be adequately repaired by increasing the synthesis of DNA-damage repair proteins, or b) there is a shortage of nucleotides to repair the DNA double strand breaks.

Under low oxygen tension, anaerobic glycolysis enhances the pentose phosphate pathway, which provides precursors for nucleotide synthesis. I hypothesize that under physiological conditions, the nucleotide pool is sufficient to sustain DNA replication and repair the DNA lesions generated during replication. However, when nucleotide requirements for proliferation are higher than the availability of nucleotides that can be incorporated into nascent DNA strands, cells encounter replicative stress. Replicative stress results in cell cycle arrest and activation of repair pathways. Imbalance between proliferation factors and nucleotide reserves is observed in early stages of cancer development (237) and could also occur in first trimester placentas exposed to excessive growth under the influence of mitotic factors (insulin, IGF1, IGF2, EGF) as happens in obesity. In the likely scenario of delayed spiral artery opening in obesity (28, 105), which will then be associated with low oxygen tension and further stimulation of trophoblast proliferation (12), we can expect placentas exposed to maternal obesity to undergo considerable replicative stress. MCM genes are targets of N-myc (**Figure 33**). N-myc is a paralogue of C-myc, a transcription factor highly expressed in the villous cytotrophoblast and promotes cell proliferation (238). Interestingly, C-myc is also required for nucleotide biosynthesis (237, 239-241). It is also known that some DNA damage repair proteins, e.g., P53, supress glycolytic enzymes, e.g., PFKBP3, to enhance nucleotide synthesis via the pentose phosphate pathway and promote DNA damage repair, showcasing the interplay between DNA damage repair systems, glucose metabolism and nucleotide synthesis (242).

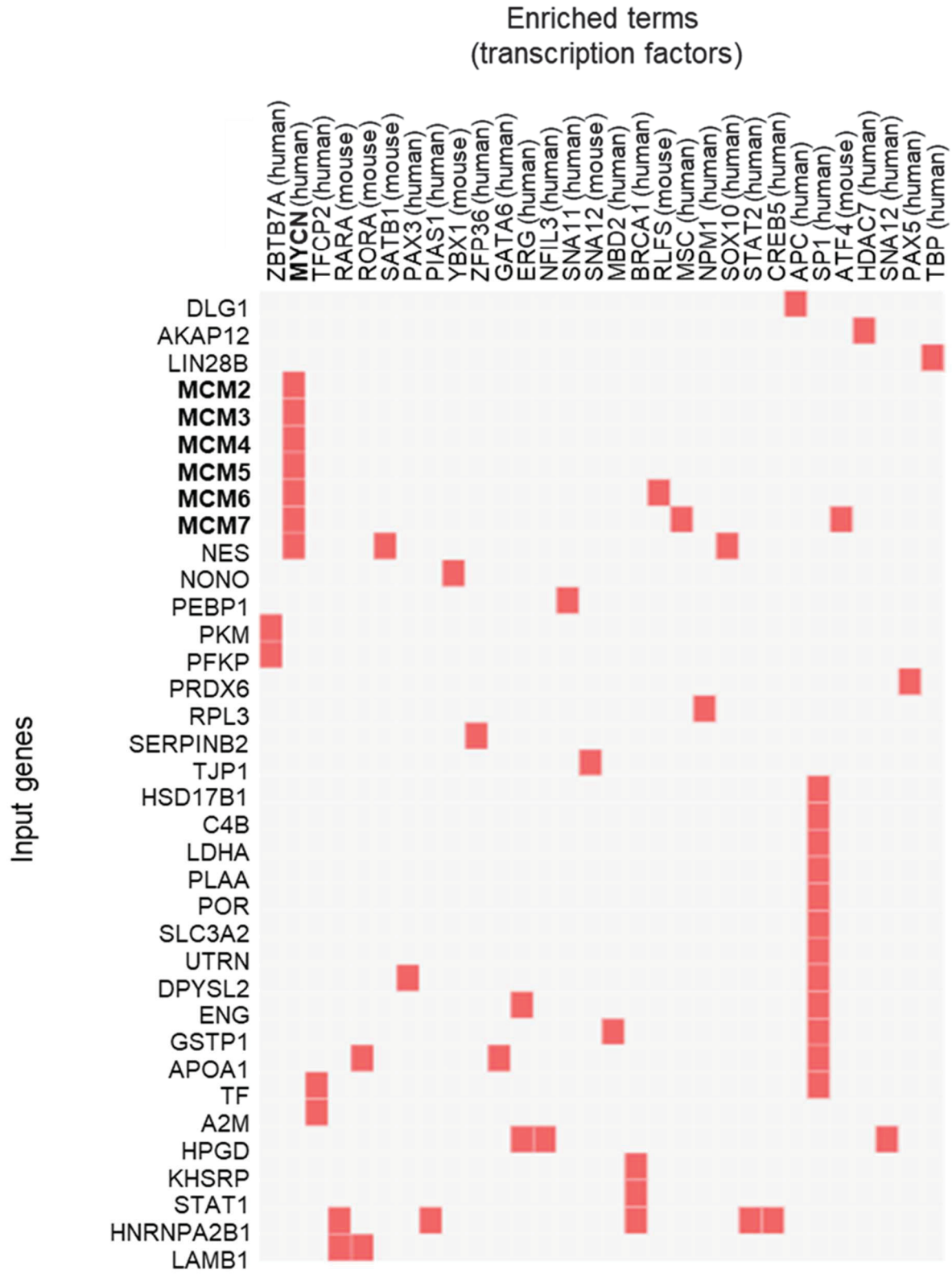


Figure 33. Transcription factors known to interact with the proteins upregulated and downregulated in the low IS_{HOMA} group. Rows show input genes (gene names of proteins significantly up- and downregulated in placentas of women with low IS_{HOMA} in the proteomic analysis), columns list enriched transcription factors, and cells in the matrix indicate whether a gene is associated with a transcription factor. Analysis performed using the online tool EnrichR (128), which performs searches at Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRUST) (129).

Importantly, many enzymes involved in glycolysis, i.e., PGI, PFKP, PFKL, ALDOC, PGM, ENO, were downregulated in the low IS_{HOMA} group in the proteomic analysis, suggesting a shift towards the pentose phosphate pathway (**Figure 34**) which may provide nucleotides for DNA synthesis and repair. However, since DNA damage (γH2AX) is increased in first trimester placentas of women with obesity (105), increasing nucleotide synthesis may not be enough to fully repair DNA damage.

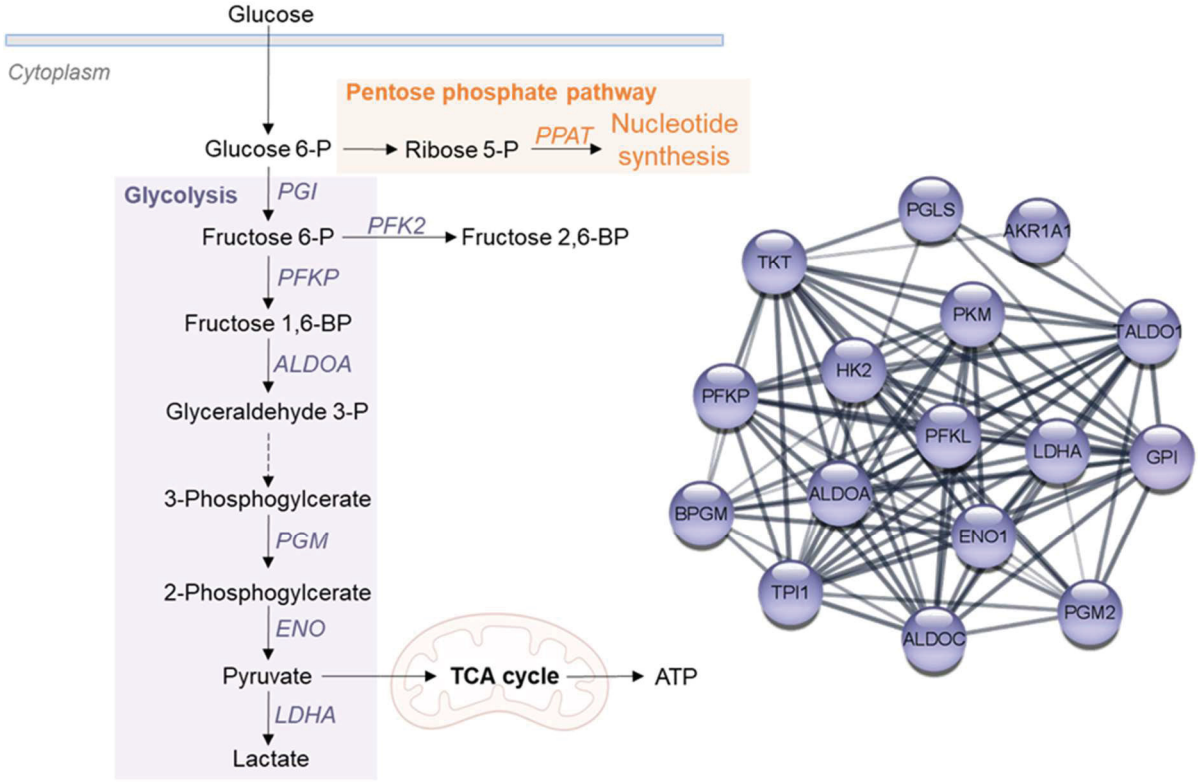


Figure 34. Proteins downregulated in the low IS_{HOMA} group that play a role in glycolysis and pentose phosphate pathway. Proteins downregulated in placentas of women with low insulin sensitivity are shown in blue. PPAT (in orange) was only detected in three placenta samples, all of which were from women with low insulin sensitivity. AKR1A1: Alcohol dehydrogenase; ALDOA: Fructose-bisphosphate aldolase A; ALDOC: Fructose-bisphosphate aldolase C; BPGM: Bisphosphoglycerate mutase; ENO1: Alpha-enolase; GPI: Glucose-6-phosphate isomerase; HK2: Hexokinase-2; LDHA: L-lactate dehydrogenase A chain; PFKL: ATP-dependent 6-phosphofructokinase; PGLS: 6-Phosphogluconolactonase; PGM2: Phosphoglucomutase-2; PFKP: ATP-dependent 6-phosphofructokinase; PKM: Pyruvate kinase; PPAT: Phosphoribosyl pyrophosphate amidotransferase; TALDO1: Transaldolase; TKT: Transketolase; TPI1: Triosephosphate isomerase.

The collective evidence above supports my hypothesis that increased DNA damage is a consequence of impaired DNA damage repair caused by nucleotide deficit. Nucleotide deficit may be a consequence of very high proliferative activity in the villous cytotrophoblast as a result of mitogenic input associated with obesity. This increased nucleotide demand would be accompanied by inadequate provision of nucleosides by the mother, despite expression of

nucleoside transporters (SLC29A1, SLC29A2, and SLC29A3) at the syncytiotrophoblast and villous cytotrophoblast of the first trimester placenta (126). In our metabolomics analysis in maternal serum, we found a positive association between C-peptide and uridine (nucleoside) (116). However, we did not find significant associations with other nucleosides (adenosine, guanosine, inosine and hypoxanthine), suggesting that the nucleoside pool is not different between women with overweight/obesity and normal weight.

Should these findings be confirmed in future studies, it would indicate that the nucleotide deficit in placentas exposed to (over)proliferative stimuli is not compensated by maternal influx of nucleosides in pregnancies complicated with obesity. Accepting the notion of increased DNA damage resulting from (over)proliferative stimuli and nucleotide deficit, then placental catch-up growth may be related to a reduction of (over)proliferative stimuli in the late second trimester. The decrease in maternal circulating insulin from week 6 LMP until the end of the first trimester along with the opening of the spiral arteries and subsequent increase in oxygen would reduce replicative pressure. As a result, the available nucleotide pool would be sufficient to support DNA replication, effectively repair the natural DNA damage that occurs during each cell cycle and allow villous cytotrophoblast proliferation to continue at higher pace. Under these new metabolic conditions, the placenta would grow “normally” and catch up despite exposure to an obese environment (**Figure 35**).

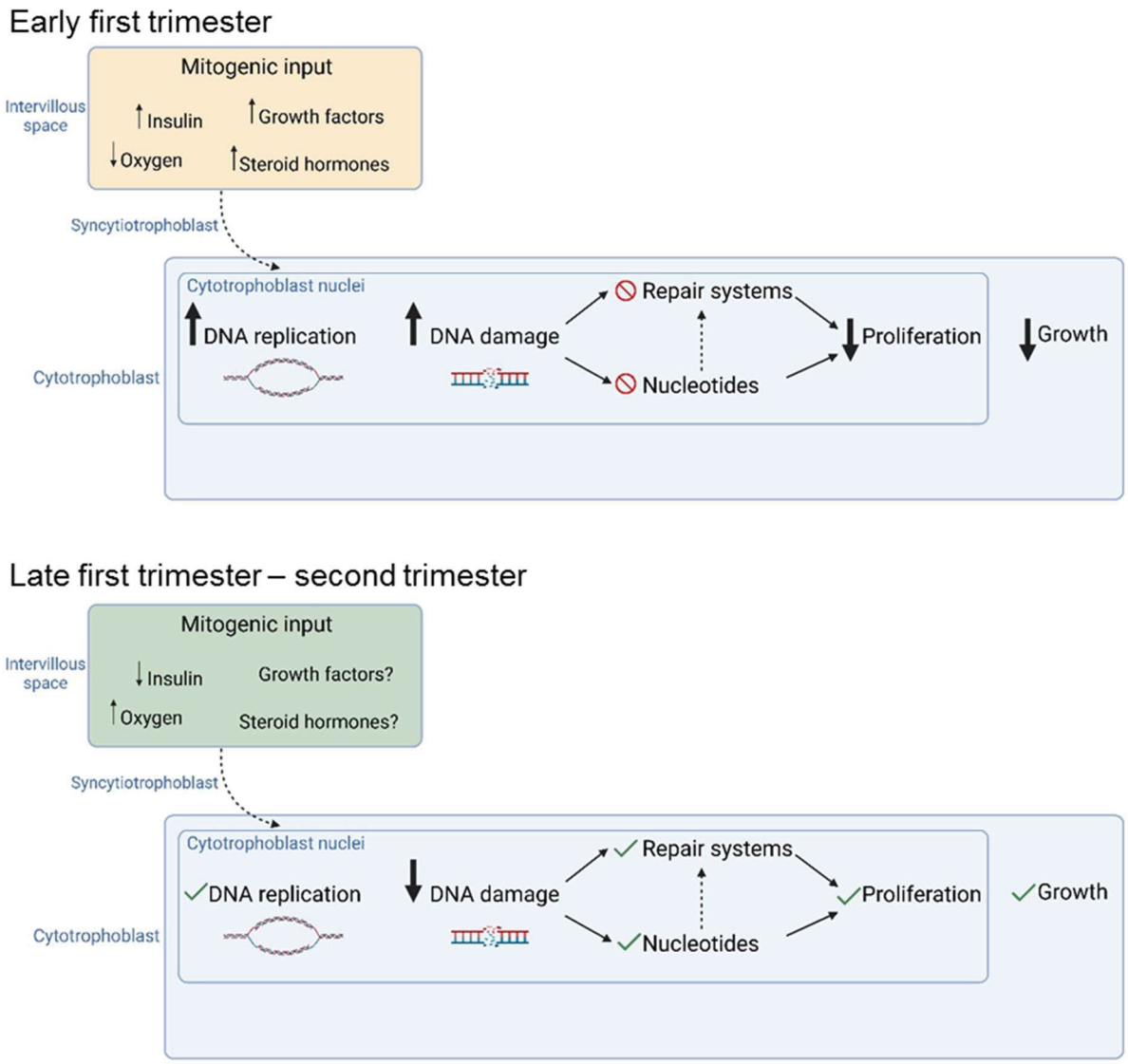


Figure 35. (Over)proliferative stimuli in the obesogenic environment saturate DNA-damage repair capacity of the villous cytotrophoblast early in pregnancy. High mitogenic input overstimulates DNA replication which results in increased DNA double strand breaks, i.e., DNA damage. Insufficient availability of nucleotides precludes DNA damage repair systems to successfully repair DNA damage, resulting in decreased villous cytotrophoblast proliferation. Ultimately, this decreased cytotrophoblast proliferation could result in reduced placental growth early in pregnancy. Towards the end of the first trimester, decreased (over)proliferative stimuli, e.g., decreased insulin and increased oxygen, alleviates replicative pressure. This together with allostatic mechanisms developed in survivor cells, e.g., increased nucleotide synthesis and DNA damage repair proteins, results in successful repair of spontaneous DNA lesions and normal cell proliferation and growth.

In addition to DNA damage and cell cycle pathways, processes related to protein translation are altered in placentas of women with low insulin sensitivity. In the network analysis of upregulated proteins, a sizable group of interacting proteins not recognised in pathway enrichment analysis, included multiple proteins involved in regulation of translation, e.g., SFPQ, NONO, YBX1, MATR3, several RPL and HNRNPD proteins (cf. Figure 13).

Interestingly, analysing intracellular location (gene ontology) of these proteins, paraspeckles and stress granules were the enriched organelles, suggesting that RNA translation and protein synthesis are halted in placentas of women with low insulin sensitivity (data not shown). One of the most studied stress response pathways resulting in translational arrest and protein synthesis is the unfolded protein response (UPR) that originates at the endoplasmic reticulum (ER). Protein synthesis, folding and maturation take place at the endoplasmic reticulum (ER). If the protein folding capacity of the ER is exceeded, also known as **ER stress**, Ca^{2+} is released to the cytosol and the unfolded protein response (UPR) is activated until ER homeostasis is restored (96). Under acute stress, the UPR stops RNA translation and protein synthesis and activates adaptive mechanisms to increase synthesis capacity and size of the ER to restore homeostasis. Under chronic stress, the UPR upregulates CHOP and Caspase-4 expression, which results in apoptosis (243). ER stress has been found in placentas of pregnancies complicated by intrauterine growth restriction and preeclampsia, probably caused by oxidative stress resulting from hypoxia-reoxygenation (15). ER stress is also observed in tissues exposed to obesity, and can be caused by a) oxidative stress-induced S-nitrosylation of IRE-1α, (15) (244), b) excessive nutrient availability that stimulates protein synthesis and overloads ER capacity (15, 96), c) prolonged exposure to saturated fatty acids which causes protein palmitoylation in the ER and impairs protein folding (161) and d) changes in lipid composition at the ER membrane which alter ER function (245). In the placenta, chronic ER stress could reduce placental growth by decreasing protein and lipid synthesis which are necessary building blocks for organelles and membranes as well as by induction of apoptosis. In the proteomic analysis of first trimester placentas, ER stress was not an enriched term. However, the large number of upregulated proteins related to control of protein translation in placentas of women with low insulin sensitivity suggests that protein synthesis may be decreased in these placentas. Temporally inhibiting translation of non-essential survival proteins could be a downstream effect of replicative stress, aiming at investing energy and resources into synthesis of DNA damage repair proteins, e.g., MCMs.

In addition to DNA damage and ER stress, other stress pathways are likely activated simultaneously in first trimester placentas exposed to maternal obesity. At term, mitochondrial β-oxidation is reduced in placentas exposed to obesity *in utero,* resulting in lower ATP generation (246). Also at term of pregnancy, the mitochondrial translocator protein TSPO is downregulated in placentas exposed to maternal obesity, resulting in decreased placental production of estradiol and progesterone (247). In the first trimester, genes involved in long-chain fatty acid oxidation, e.g., CPT2 and fatty acid esterification, e.g., DGAT1 and ACACA were downregulated in obesity, again suggesting **mitochondrial dysfunction** (145). However, in our proteomic analysis in first trimester placentas neither mitochondrial proteins nor proteins

in the steroid biosynthesis pathway were affected by exposure to the obesity environment. This may be due to the gestational age (week 5^{+0} - 6^{+6} LMP) of the placentas included in our study, since at this early gestational age mitochondrial oxidative phosphorylation may be less relevant due to the low oxygen tension at the intervillous space (2.5% – 6% O_2). Indeed, in placentas of women with low insulin sensitivity, proteins of the glycolysis pathway, which generates pyruvate for oxidative phosphorylation were downregulated.

Stress response pathways such as DNA damage repair, UPR and mitochondrial stress in placentas exposed to maternal obesity *in utero* are likely interconnected as part of the **integrated stress response**. The integrated stress response is an adaptive pathway conserved between eukaryotes that ensures homeostasis restoration to face acute stress but that activates cell death processes under chronic stress exposure (248). Therefore, the adverse effects of maternal obesity that lead to decreased placental growth in the first trimester are likely caused by a combination of factors and activation of the integrated stress response rather than single events. From an evolutionary perspective, it makes sense for an essential reproductive organ as the placenta, to develop redundant allostatic responses to maintain homeostasis. Nonetheless, the critical question on how signals from the intervillous space reach the cytotrophoblast and activate stress response pathways remains elusive and multiple further studies are required.

6. Conclusions and Perspectives

Overall, the results of this thesis lead to the following conclusions:

1) The first trimester of pregnancy in women with overweight/obesity is associated with a range of changes in circulating molecules and metabolites. These include increased leptin, C-peptide, palmitoleoyl ethanolamide, N-acetyl-L-alanine and decreased insulin sensitivity and total n-3 polyunsaturated fatty acids.

2) MCM proteins, which are involved in DNA replication and DNA damage repair, are upregulated in the proteome of placentas exposed to maternal obesity, i.e., low insulin sensitivity. However, none of the dysregulated molecules and metabolites identified in the maternal serum is directly and singly inducing DNA damage in first trimester placentas.

3) Other changes in the placental proteome of women with low insulin sensitivity include downregulation of proteins involved in glycolysis. This suggests a preference for the pentose phosphate pathway over mitochondrial oxidative phosphorylation.

4) It is unclear which metabolites or molecules in the intervillous space can activate signals in the syncytiotrophoblast that are transmitted to the villous cytotrophoblast and increase MCM levels ultimately causing DNA damage in this cell type. Rather than a single signal inducing these changes, it will likely be the combination of (over)proliferative stimuli. As a speculation, a key element of increased DNA damage may be nucleotide deficit in villous cytotrophoblast nuclei.

The present work adds a small piece of evidence to the complex processes governing regulation of villous cytotrophoblast proliferation and its dysregulation in the maternal environment associated with obesity. MCMs are only one member of the intricate molecular networks regulating DNA replication and DNA damage repair. Future studies are required to help understand placental growth under normal conditions and in pregnancies complicated by obesity. Because of the strong interrelationship between metabolic and signalling pathways, holistic approaches may be more suitable than reductionistic approaches dissecting complex processes into single molecule regulation. In addition, fetal sex is an important contributor not only to placental regulatory mechanisms but also of maternal metabolism and needs to be considered whenever possible.

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8. Appendix

Appendix Table 1. Characteristics of study participants included in the maternal serum fatty acids study (N = 123).

	n (%)	Mean ± SD	Median (IQR)
Age (years)	122	31.4 (± 7.2)	
Gestational age (days)	123	51.0 (± 15.4)	
4–6 weeks	58 (47.0%)		35.0 (35.0–42.0)
7–9 weeks	48 (39.0%)		56.0 (51.0–61.0)
10–12 weeks	17 (13.8%)		81.0 (78.0–82.0)
BMI (kg/m^2)	123		22.6 (20.6–25.7)
Under-/normal weight (< 25 kg/m^2)	85 (69.2%)		21.1 (19.8–22.8)
Overweight (25.0–29.9 kg/m^2)	30 (24.4%)		26.9 (25.9–27.4)
Obese (≥ 30.0 kg/m^2)	8 (6.5%)		32.6 (31.9–39.5)
Metabolic parameters			
Leptin (ng/ml)	123		11.8 (7.1–17.4)
1st tertile (< 8.5)	41		5.4 (3.5–7.1)
2nd tertile (8.5–15.3)	41		12.0 (10.2–13.9)
3rd tertile (≥ 15.3)	41		19.6 (17.4–28.6)
Glucose (mmol/l)	118	4.76 (± 0.85)	
1st tertile (< 4.28)	39		4.01 (3.65–4.14)
2nd tertile (4.28–4.97)	40		4.65 (4.49–4.82)
3rd tertile (≥ 4.97)	39		5.52 (5.16–5.98)
C-peptide (pmol/l)	123		371.0 (281.5–484.9)
1st tertile (< 315.4)	41		258.3 (218.5–281.7)
2nd tertile (315.4–437.8)	41		371.0 (346.6–402.8)
3rd tertile (≥ 437.8)	41		537.0 (476.4–621.3)
IS$_{HOMA}$	118		0.74 (0.53–1.00)
1st tertile (≤ 0.59)	40		0.46 (0.36–0.53)
2nd tertile (0.58–0.85)	39		0.73 (0.68–0.79)
3rd tertile (> 0.85)	39		1.13 (1.00–1.41)
Fatty acids (mg/L)			

Total fatty acids	123	2724.2 (± 415.4)	
SFA	123	918.9 (± 148.2)	
C 14:0	123		22.7 (17.3–32.8)
C 16:0	123		618.3 (539.4–694.0)
C 18:0	123	273.6 (± 45.5)	
MUFA	123	610.5 (± 132.8)	
C 16:1	123		54.8 (39.7–67.8)
C 18:1 (n-9)	123	546.1 (± 114.2)	
C 22:1 (n-9)	123	6.5 (± 2.8)	
n-3 PUFA	123	158.0 (± 28.5)	
C 18:3 (n-3)	123		10.7 (8.7–14.8)
C 20:3 (n-3)	123		4.9 (3.6–7.3)
C 20:5 (n-3)	123	17.2 (± 7.3)	
C 22:5 (n-3)	123	26.6 (± 9.6)	
C 22:6 (n-3)	123	97.5 (± 24.1)	
n-6 PUFA	123	1036.9 (± 161.8)	
C 18:2 (n-6)	123	751.4 (± 131.9)	
C 18:3 (n-6)	123		9.7 (7.8–12.9)
C 20:2 (n-6)	123		6.2 (5.0–7.3)
C 20:3 (n-6)	123	45.4 (± 14.0)	
C 20:4 (n-6)	123	216.4 (± 49.7)	
C 22:4 (n-6)	123	7.4 (± 2.4)	
C 22:5 (n-6)	123	5.8 (± 4.0)	

Normally distributed variables are presented as the mean ± SD and not normally distributed variables as median (IQR). BMI: Body mass index; gestational age: postmenstrual period; IS_{HOMA}: Homeostatic model assessment of insulin sensitivity; SD: standard deviation; IQR: Interquartile range. *This table was adapted from (40) with publisher's permission.*

Appendix Table 2. Characteristics of study participants included in the maternal serum metabolomics study (N = 111).

	BMI < 25 kg/m² (n = 77)			BMI ≥ 25 kg/m² (n = 34)			
	n	median	IQR	n	median	IQR	*p*-value
Gestational age (days)	77	50	42 – 63	34	42	35 – 50	0.03
Maternal age (years)	76	31	25 – 38	34	34	27 – 39	0.23
Leptin (ng/ml)	77	9.4	5.8 – 14.5	34	16.1	11.6 – 24.2	< 0.001
Glucose (mmol/l)	74	4.6	4.1 – 5.1	32	4.8	4.2 – 5.4	0.22
C-peptide (pmol/l)	77	355.1	269.8 – 435.5	34	437.3	332.2 – 523.3	0.009
IS_{HOMA} index	73	0.8	0.6 – 1.0	32	0.6	0.5 – 0.8	0.002

BMI: body mass index (BMI < 25 kg/m²: underweight-normal weight; BMI ≥ 25 kg/m²: overweight/obese); IS_{HOMA}: homeostatic model assessment of insulin sensitivity; IQR: Interquartile range. Given are the p-values of the Kruskal-Wallis test comparing BMI groups. *This table was adapted from (106) with publisher's permission.*

Appendix Table 3. Characteristics of study participants (BMI ≥ 25 kg/m²) included in the sub-analysis of ´healthy 'and ´unhealthy` obesity in the maternal serum metabolomics study (N = 32).

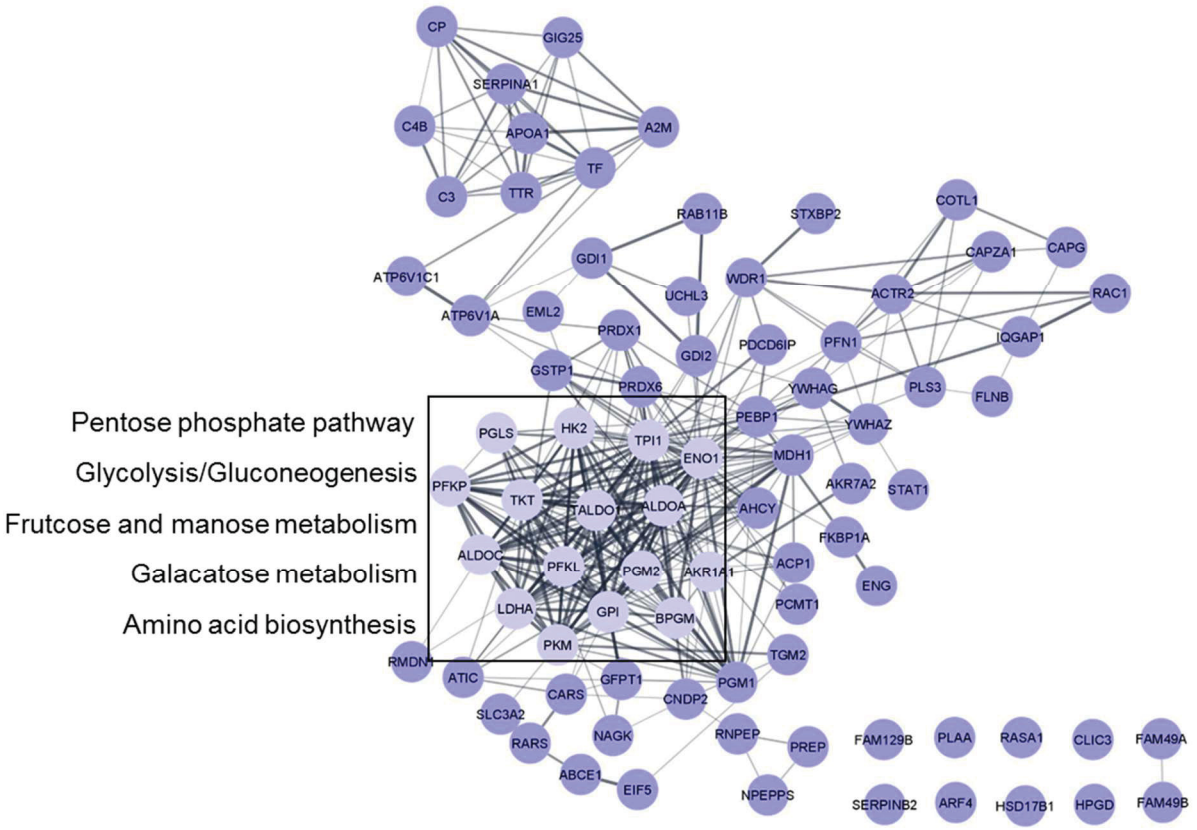
	IS_{HOMA} ≥ 0.5 (n = 23)			IS_{HOMA} < 0.5 (n = 9)			
	n	median	IQR	n	median	IQR	*p*-value
Gestational age (days)	23	48	35-64	9	35	35-42	0.12
Maternal age (years)	23	31	27-39	9	35	28-38	0.97
BMI (kg/m²)	23	27.1	25.8-28.9	9	27.4	26.6-29.3	0.59
Leptin (ng/ml)	23	17.4	12.2-23.1	9	16.3	11.5-24.7	0.66
Glucose (mmol/l)	23	4.6	4.2-5.0	9	5.5	5.2-6.0	0.006
C-peptide (pmol/l)	23	370.9	321.6-457.5	9	599.2	523.4-759.3	< 0.001

IS_{HOMA}: homeostatic model assessment of insulin sensitivity; BMI: body mass index; IQR: Interquartile range. Given are the p-values of the Kruskal-Wallis test comparing IS_{HOMA} groups. *This table was adapted from (106) with publisher's permission.*

Appendix Table 4. Characteristics of study participants whose placenta tissue was used for proteomics analysis (N = 19).

	Median; IQR
Gestational age (days)	36 (35-46)
Maternal age (years)	32 (28-39)
BMI (kg/m^2)	21.8 (19.7-29.0)
Leptin (ng/ml)	13.6 (6.1-20.6)
Glucose (mmol/l)	5.4 (4.7-6.0)
C-peptide (pmol/l)	421.2 (306.4-599.2)
IS_{HOMA} index	0.61 (0.37-0.76)

BMI: Body mass index; IS_{HOMA}: Homeostatic model assessment of insulin sensitivity; IQR: Interquartile range; p: p-value.



Appendix Figure 1. Pathways related to energy metabolism are downregulated in the placental proteome of women with low insulin sensitivity. Proteins significantly downregulated (n = 84) in the low IS_{HOMA} group were selected for pathway analysis (KEGG pathway; String V.11.5). Pentose phosphate pathway, glycolysis/gluconeogenesis, fructose and mannose metabolism, galactose metabolism and amino acid biosynthesis were downregulated in the low IS_{HOMA} group. KEGG: Kyoto Encyclopaedia of Genes and Genomes.