Placental transfer of proteogenic amino acids and taurine in healthy term pregnancies: a human *in vivo* study

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Contents

Acknowledgements...5

List of papers...7

Abbreviations...8

Summary...9

1. Introduction...11

1.1. The first nine months –the most consequential time of our lives...11

1.1.1. Fetal growth: short and long term consequences...11

1.1.2. The developmental origin of health and disease...11

1.1.3. The fetal nutritional environment...12

1.2. Placental anatomy and development...13

1.2.1. Gross anatomy of the placenta...13

1.2.2. Placental development...14

1.2.3. The placental membrane...18

1.2.4. The uteroplacental circulation...18

1.2.5. The umbilical cord and the umbilical circulation...20

1.3. A general overview of placental physiology...20

1.3.1. Endocrine function...20

1.3.2. Protective barrier...21

1.3.3. Transfer of nutrients, water and gases...21

1.3.4. Placental metabolism and nutrient sensing...25

1.4. Placental transport and metabolism of amino acids...26

1.4.1. Amino acid transporter systems in the placenta...28

1.4.2. Regulation of placental amino acid transporters...29

1.4.3. Placental amino acid metabolism...30

1.4.4. Placental transport of taurine...32

1.5. The placenta and fetal growth...36

1.6. Placental research...38

1.6.1. Human *in vitro* and *ex vivo* models...38

1.6.2. *In vivo* animal models...40

1.6.3. Human *in vivo* studies...43

1.7. The placenta –still "the least understood organ in the human body"...44

2. Aims...45

3. The placenta 4-vessel study –methods at a glance...46

3.1. Design and study population...46

3.2. Sampling method...46

3.3. Analyses 47
3.3.1. Glucose 47
3.3.2. Progesterone 47
3.3.3. Amino acids 47
3.3.4. Hemoglobin 47
3.3.5. CSAD expression 47
3.4. Definitions and calculations 48
3.4.1. Uteroplacental arteriovenous differences and umbilical venoarterial differences 48
3.4.2. Mass uptake and release in the fetal-placental unit 49
3.4.3. Adjustment for up-concentration of hemoglobin across the placenta 50
3.5. Ethics approval 51
4. Summary of the results **52**
5. Discussion **55**
5.1. Methodological considerations 55
5.1.1. Study design 55
5.1.2. Reliability 55
5.1.3 Internal validity 56
5.1.4 External validity 58
5.1.5. Statistical considerations 59
5.2. Interpretation of the results 59
5.2.1 The 4-vessel approach to study the human placenta in vivo 59
5.2.2. Large individual variations in amino acid concentrations and flux 60
5.2.3. Proteogenic amino acids: The relationship between supply and uptake in the fetal-placental unit 61
5.2.4. Transfer of taurine between the maternal, placental and fetal compartments 62
5.2.5. The unresolved question of taurine synthesis in the human placenta 63
5.2.6. Amino acid transfer and placental properties 65
5.2.7. Placental metabolism -comparisons between findings in animals and the present study on healthy pregnant women 68
5.2.8. Fetal uptake of amino acids and glucose 69
5.2.9. Amino acids and birthweight in humans 70
6. Conclusion, clinical application and future research **71**
6.1. Conclusions 71
6.2. Clinical applications 72
6.3. Future research 73
Errata paper I **74**
References **75**
Appendix
Papers I, II and III

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Oslo, April 2018

Maia Blomhoff Holm

List of papers

Paper I: Holme AM, Holm MB, Roland MC, Horne H, Michelsen TM, Haugen G, Henriksen, T: The 4-vessel Sampling Approach to Integrative Studies of Human Placental Physiology In Vivo. J. Vis. Exp. (126), e55847, doi:10.3791/55847 (2017).

Video: https://www.jove.com/video/55847

Paper II: Holm MB, Bastani NE, Holme AM, Zucknick M, Jansson T, Refsum H, Mørkrid L, Blomhoff R, Henriksen T, Michelsen TM: Uptake and release of amino acids in the fetal-placental unit in human pregnancies. PLoS ONE 12(10): e0185760, https://doi.org/10.1371/journal.pone.0185760 (2017)

Paper III: Holm MB, Kristiansen O, Holme AM, Bastani NE, Horne HE, Blomhoff R, Haugen H, Henriksen T, Michelsen TM: Placental release of taurine to both the maternal and fetal circulations in human term pregnancies. *In revision.*

Abbreviations

ATP: Adenosine triphosphate
A-V difference: Arterio-venous difference
BM: Basal membrane
BMI: Body mass index
CDO: cysteine dioxygenase
CSAD: Cysteine sulfinic acid decarboxylase
DOHaD: Developmental origin of health and disease
FDR: False discovery rate
GADL1: Glutamate decarboxylase-like protein 1
GLUT: Glucose transporter
Hb: Hemoglobin
Il: Interleukin
IUGR: Intrauterine growth restriction
LAT: L-type amino acid transporter
LDL: Low-density lipoprotein
mTOR: Mechanistic target of rapamycin
mRNA: messenger ribonucleic acid
MVM: Microvillous membrane
n: number
NADH: Nicotinamide adenine dinucleotide
Q1, Q3: quartile 1, quartile 3
qRT-PCR: Quantitative Real-time polymerase chain reaction
SD: Standard deviation
SNAT: Sodium-coupled neutral amino acid transporter
TAT: T-type amino acid transporter
TAMX: Time average maximum velocity
TAUT: Taurine transporter
TNF α: Tumor necrosis factor α
v-a difference: Venoarterial difference
VLDL: very low-density lipoprotein

Summary

Normal fetal growth and development depends on a continuous supply of amino acids. Furthermore, the nutritional environment in which the fetus develops has a major impact on both the immediate and the future health of the newborn child. The placenta constitutes the interface between the maternal and fetal circulations, and virtually all compounds that are exchanged between the mother and the fetus must pass through the placental tissue. Thus, to a large extent the placenta governs the fetal environment *in utero*, and a thorough comprehension of the functions of this complex organ is a key to understand fetal nutrition and its impact on fetal and adult health.

Our knowledge of the human placenta is mainly derived from animals and *in vitro* and *ex vivo* models, since it is difficult to access and study this organ *in vivo* in humans without imposing ethically unacceptable risks on the ongoing pregnancy. We therefore aimed to establish a sampling method to study the human placenta *in vivo* ("the 4-vessel method"), and to employ this method to assess placental transfer of amino acids in healthy human pregnancies.

Paper I

In paper I we aimed to make our established 4-vessel method accessible for the international research community, by in depth description and live visualization of the sampling techniques. We demonstrated that the sampling method is feasible in a busy clinical setting, and showed that our method can be used to investigate different aspects of the functions of the human placenta.

Paper II

In paper II we aimed to explore the interplay between uptake and release of amino acids in the fetal-placental unit *in vivo*. We determined and assessed the paired relationships between concentrations and arteriovenous differences of 19 proteogenic amino acids on the maternal and fetal sides of the placenta in our 4-vessel plasma samples from non-complicated human term pregnancies. We showed that in a fasting state, there are large individual differences in the flux of amino acids across the placental membrane in both the maternal and fetal circulations, indicating that placental amino acid transfer in the human at term is a highly dynamic process. We observed a net uptake of most amino acids in the fetus, but only a net uteroplacental uptake of a few amino acids from the maternal circulation. There was no correlation between the fetal uptake from the umbilical circulation and the uteroplacental

uptake from the maternal circulation in our mother-fetus pairs. Our findings illustrate the complex relationship between uteroplacental and fetal amino acid uptake, and highlight the role of placental metabolism and properties in the immediate government of amino acid transfer to the healthy human term fetus.

Paper III

Taurine is a vital non-proteogenic amino acid in fetal life. In paper III, we aimed to study the transfer of taurine between the maternal, placental and fetal compartments *in vivo* in healthy term pregnancies. Unexpectedly, we observed that the human placenta has the capacity for a concomitant bilateral release of taurine to both the maternal and fetal circulations, indicating that taurine may play a fundamental role in the placental homeostasis beyond the supply to the fetus. The pattern of placental taurine transfer led us to re-evaluate the prevailing view that human placental tissue lacks the ability to synthesize taurine. To this end, we studied the placental expression of both CSAD mRNA and protein. We found that the term placenta expresses CSAD mRNA, but our data regarding expression of CSAD protein were not consistent. Taken together, however, our results may suggest a potential for taurine synthesis in the human placenta.

In conclusion, we have established the 4-vessel sampling approach to promote *in vivo* studies of the human placenta and demonstrated that this method can be used to study and integrate a variety of placental properties. We have explored the impact of the placenta in determining amino acid uptake by the fetus in non-complicated term pregnancies, and, finally, we have shown that some current experimentally based concepts of placental functions are challenged by the results obtained by our human *in vivo* model.

1. Introduction

1.1 The first nine months –the most consequential time of our lives?

The first nine months of our life, from conception to birth, is spent inside our mother's womb. During this time, we develop from a single celled zygote to a fully-grown fetus, ready to enter the world outside the uterus. These nine months are perhaps the most consequential period in our lives, as the nutritional and environmental conditions we encounter *in utero* lay the foundation for our health, not only as a baby and child, but for decades to come.

1.1.1. Fetal growth: short and long-term consequences

Fetal growth is an important indicator of fetal health and developmental conditions. Under normal circumstances, the fetus' congenital growth potential yields a newborn of appropriate size within a broad range of normal birthweights. However, in addition to inherent determinants like genetics and fetal gender, fetal growth and development is fundamentally dependent on adequate nutrient supply. Several other factors, like the endocrine milieu, exposure to toxins, and infectious agents may also influence a fetus' growth trajectory (1). Intrauterine growth restriction (IUGR) occurs when the fetus fails to reach its potential for growth and development *in utero* and complicates, depending on the definition, 5-10 % of all pregnancies (2, 3). Growth restricted fetuses have a substantially higher risk of perinatal morbidity and mortality, including intrauterine death, preterm delivery, and asphyxia, as well as other adverse neonatal outcomes like respiratory distress syndrome, necrotizing enterocolitis, retinopathy of prematurity and metabolic disturbances (3, 4). Fetal overgrowth, i.e. growth *exceeding* the fetus' inherent growth potential, is also associated with adverse pregnancy outcomes. Perinatal complications include intrauterine death, birth asphyxia, shoulder dystocia, birth trauma both to the mother and the baby, polycythemia, hyperbilirubinemia and hypoglycemia (4, 5). In addition to the short-term complications, however, deviating fetal growth is associated with higher risk of substantial morbidity in adult life through the concept of "the developmental origin of health and disease" (DOHaD) (6, 7).

1.1.2. The developmental origin of health and disease

The idea that the conditions under which a fetus develops has a major impact on adult health has been evolving for several decades, but became widely accepted through the work of David Barker some twenty years ago (8). "The Barker hypothesis" was based on epidemiological studies from England and Wales showing a link between fetal growth and

weight in infancy, and later ischemic heart disease (9, 10). Fetal nutritional and environmental conditions are thought to alter gene expression during the development of tissues and organs permanently, with lasting consequences for the structure, physiology and metabolism of not only the individual exposed, but also the following generations (11). There is now evidence of a link, not only between intrauterine conditions and cardiovascular disease, but also an association with adult obesity, diabetes, certain cancers, asthma and mental illness (6, 7, 12-14). This makes the womb a promising target for prevention of several major disease burdens in our world today through interventions before birth. To understand the intrauterine environment and fetal nutrition is thus of great importance for public health around the globe.

1.1.3. The fetal nutritional environment

The nutritional environment of the developing fetus is dependent on several factors (15). The mother's diet during pregnancy provides the ultimate source of nutrients to the fetus, while her metabolism determines the composition of nutrients in the maternal plasma. Hormones, cytokines, adipokines and metabolic intermediates are all involved in the government of the maternal metabolism and thus the nutrients available for transfer to the fetus. Such factors are altered in maternal metabolic disturbances like obesity and diabetes (6, 16). Physical activity, stress, smoking and age are other maternal factors that may influence the intrauterine nutritional conditions, through alterations in maternal metabolism, hormonal balance and/or transfer of nutritionally active compounds to the fetus(17). The fetus' genetic growth potential and thus its demand of nutrients, as well as its own metabolism and hormonal status will also affect the intrauterine nutritional environment.

However, the maternal and fetal metabolic and endocrine systems are separated by the placenta; the organ situated between the maternal and fetal circulations. Virtually all compounds transferred between the maternal and fetal circulations must pass the placental parenchymal cells, and the placenta secretes a number of hormones and signaling molecules that may modify the pregnancy, as well as the maternal, fetal and placental metabolism. Consequently, the placenta governs to a large extent the nutritional and metabolic environment in which the fetus develops and grows. In accordance, our group has previously shown that placental weight, as a crude measurement of placental function, is an important determinant of both fetal growth and birthweight. The placental weight significantly modified the effects of maternal determinants like body mass index (BMI), gestational weight gain and plasma glucose on fetal growth and birthweight (18). A thorough comprehension of the

placenta and the functions of this complex organ is therefore a key to understand fetal nutrition and its impact on fetal and adult health.

1.2. Placental anatomy and development

1.2.1. Gross anatomy of the placenta

The human term placenta has a discoid shape, with an average diameter of 22 cm and a thickness of 2,5 cm (19, 20). Mean (standard deviation, SD) placental weight was in a recent population-based study of Norwegian pregnancies reported to be 660 (185) g (21). The placenta is of both maternal (uterine) and fetal origin and made up by two main components; the decidua basalis and the villous chorion (i.e., the chorion frondosum) (Fig 1). The placenta is a hemochorial villous organ, which means that maternal blood is in direct contact with the villous chorionic tissue (22). The fetal surface of the placenta is the chorionic plate which is covered by the amnion and faces the amniotic cavity. Chorionic vessels protrude on the surface, converging towards the umbilical cord which normally is inserted near the center of the placenta. The junctional zone between the placenta and uterine wall is a mixture of trophoblastic (fetal) and decidual (uterine) cells (20). The maternal surface of the placenta, the basal plate, is created when the placenta detaches from the uterus in the process of labor. With the placenta in situ, the basal plate cannot be separated from the underlying placental bed, which remains *in utero* when the placenta is delivered. Upon inspection, the basal plate is divided into slightly elevated areas called cotyledons by grooves formed by decidual septa.

Fig 1 Cross section of the placenta and membranes *in utero*

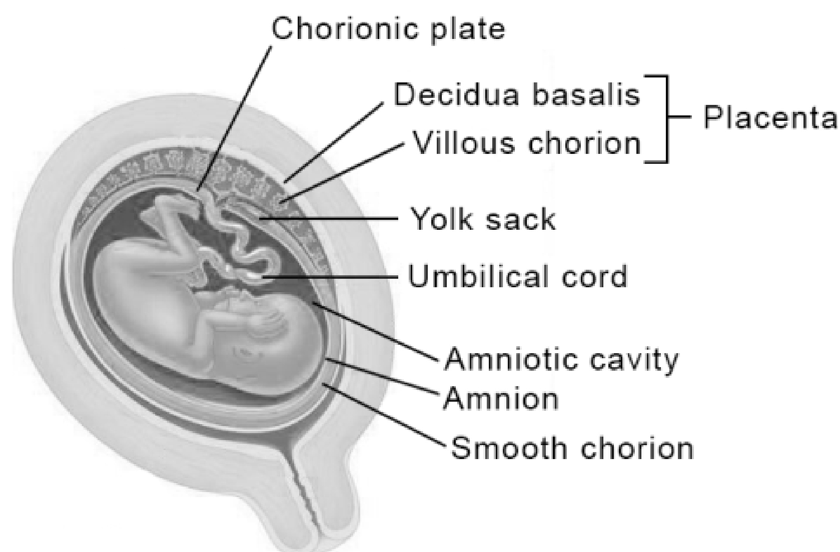


Illustration by Øystein Horgmo, University of Oslo

1.2.2. Placental development

The development of the placenta begins as soon as the zygote establishes contact with the endometrium in the uterine wall (19). Approximately three days after the oocyte is fertilized in the fallopian tube, the zygote reaches the uterine cavity as a morula (20). Fluid begins to enter the center of the cell mass, expanding the morula to form a blastocyst. The inner cells of the blastocyst, the embryoblast, gives rise to the embryo, while the outer cell mass forms the trophoblasts, which later contributes to the placenta (19). Around day 4-5 post conception, the blastocyst hatches from the surrounding zona pellucida and is ready to be implanted into the uterine wall.

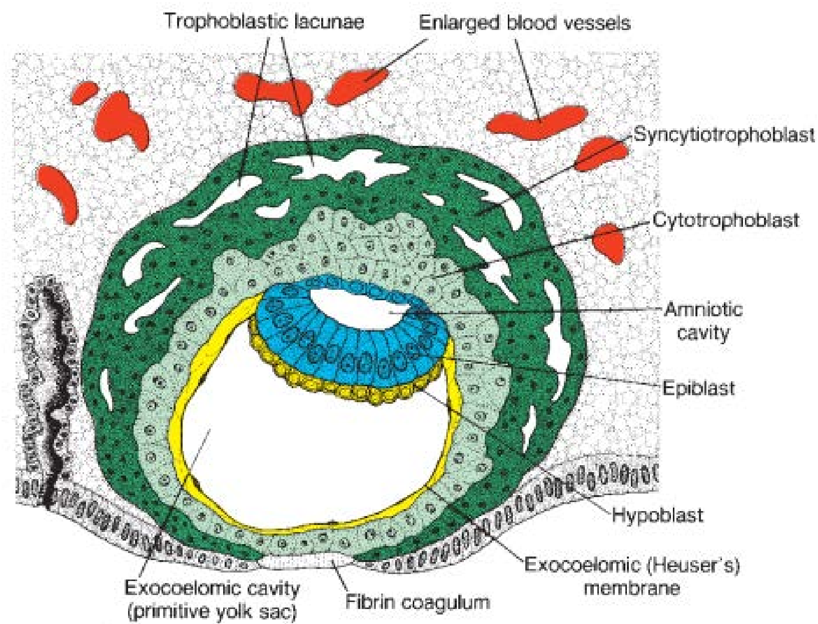
Around day six, the trophoblastic cells at the embryoblast pole start to penetrate into the endometrial stroma after having formed cell-cell contacts with uterine epithelial cells. The trophoblasts migrate between the endometrial cells, displacing them without causing apoptosis or necrosis. During this process, the trophoblasts differentiate into two layers; an outer continuous, multinucleated mass called the syncytiotrophoblast, and an inner layer of mononucleated cells called cytotrophoblasts (20). The cytotrophoblasts proliferate and fuse into the syncytiotrophoblast to maintain and expand the syncytial layer. The cells of the surrounding endometrial stroma differentiate to metabolically active decidual cells with high intracellular amounts of glycogen and lipids (19, 20, 22).

At day 8-9 vacuoles start to appear in the syncytium, fusing into a system of large communicating lacunae (Fig 2A). Within the following week, the lacunae establish open connections with glandulae in the highly active endometrial stroma. Glandular secrets can be seen entering the syncytial lacunae, and this phenomenon, called histotrophic nutrition, is considered to be essential in the nutrition of the fetal-placental unit during the first two-thirds of the first trimester (19, 23).

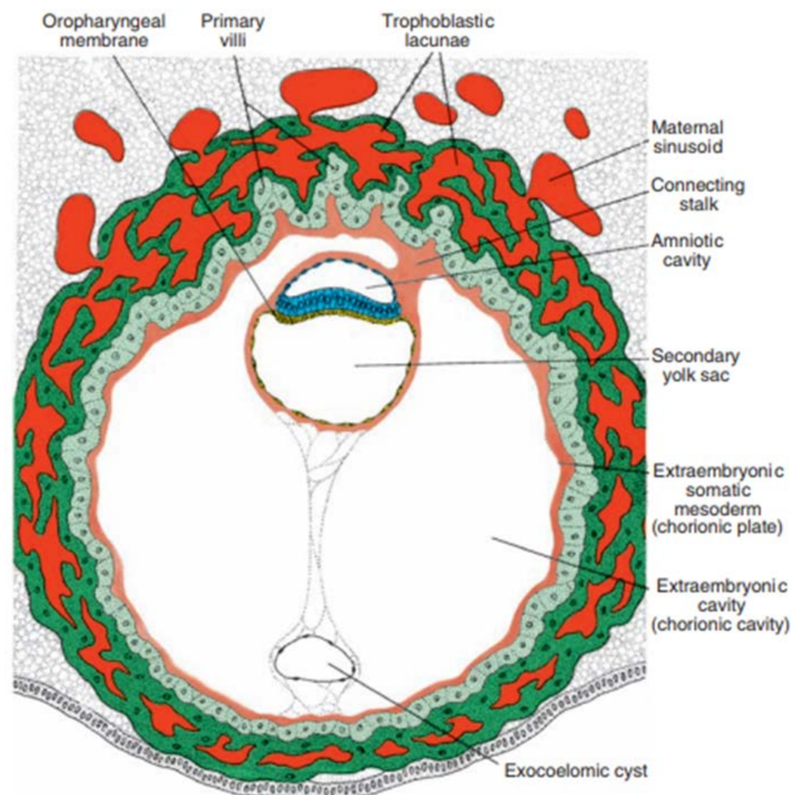
The syncytiotrophoblast expands deeper into the endometrium and erodes the endothelium of maternal sinusoid capillaries. The sinusoids come in contact with the syncytial lacunae, and as the syncytiotrophoblast continue to erode more and more sinusoids, maternal blood starts to flow through the lacunar system (Fig 2B). Thus, the uteroplacental circulation is established (20).

Fig 2 Human blastocyst

A)



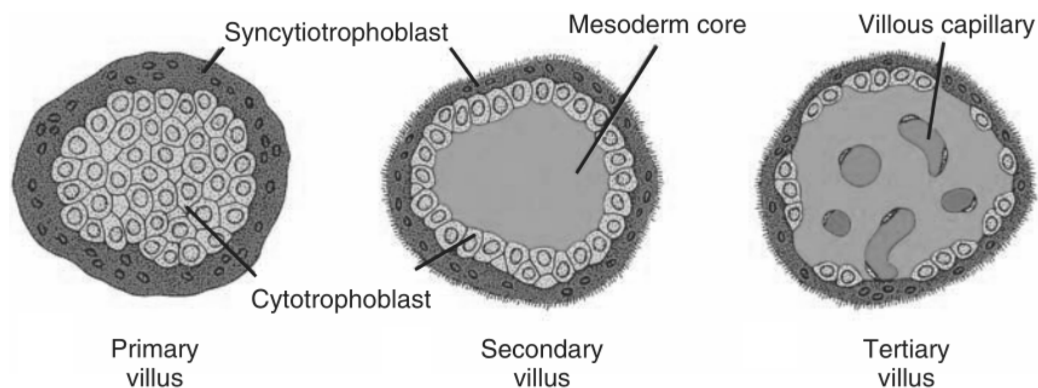
B)



A) day 9 (lacunar stage), and **B)** day 13 (beginning of uteroplacental circulation and formation of primary villi) *Reprinted with permission from Lippincott Williams & Wilkins Langman's Medical Embryology, 10th Edition by TW Sadler, Copyright 2006*

The cytotrophoblasts proliferate locally and begin to penetrate into the syncytiotrophoblast layer by the end of the second week. This results in the formation of primary villi, which are columns of cytotrophoblasts covered by syncytium (Fig 2B, Fig 3) (20). At the same time, cells derived from the embryonic yolk sac line the inner surface of the cytotrophoblasts and form a loose connective tissue called extraembryonic mesoderm. This becomes the chorionic plate. Mesodermal cells from the embryo also penetrate the core of the primary villi, giving rise to secondary villi. The mesodermal cells subsequently start to differentiate into blood cells and small blood vessels, forming the villous capillary system by the end of the third week. The villi are now called tertiary villi (Fig 3). The villous capillary system makes contact with capillaries developing in mesoderm in the chorionic plate and the connecting stalk, which later develops into the umbilical cord. These vessels, in turn, establish contact with the circulatory system in the embryo, thus connecting the embryo with the placenta (20).

Fig 3 Placental villi



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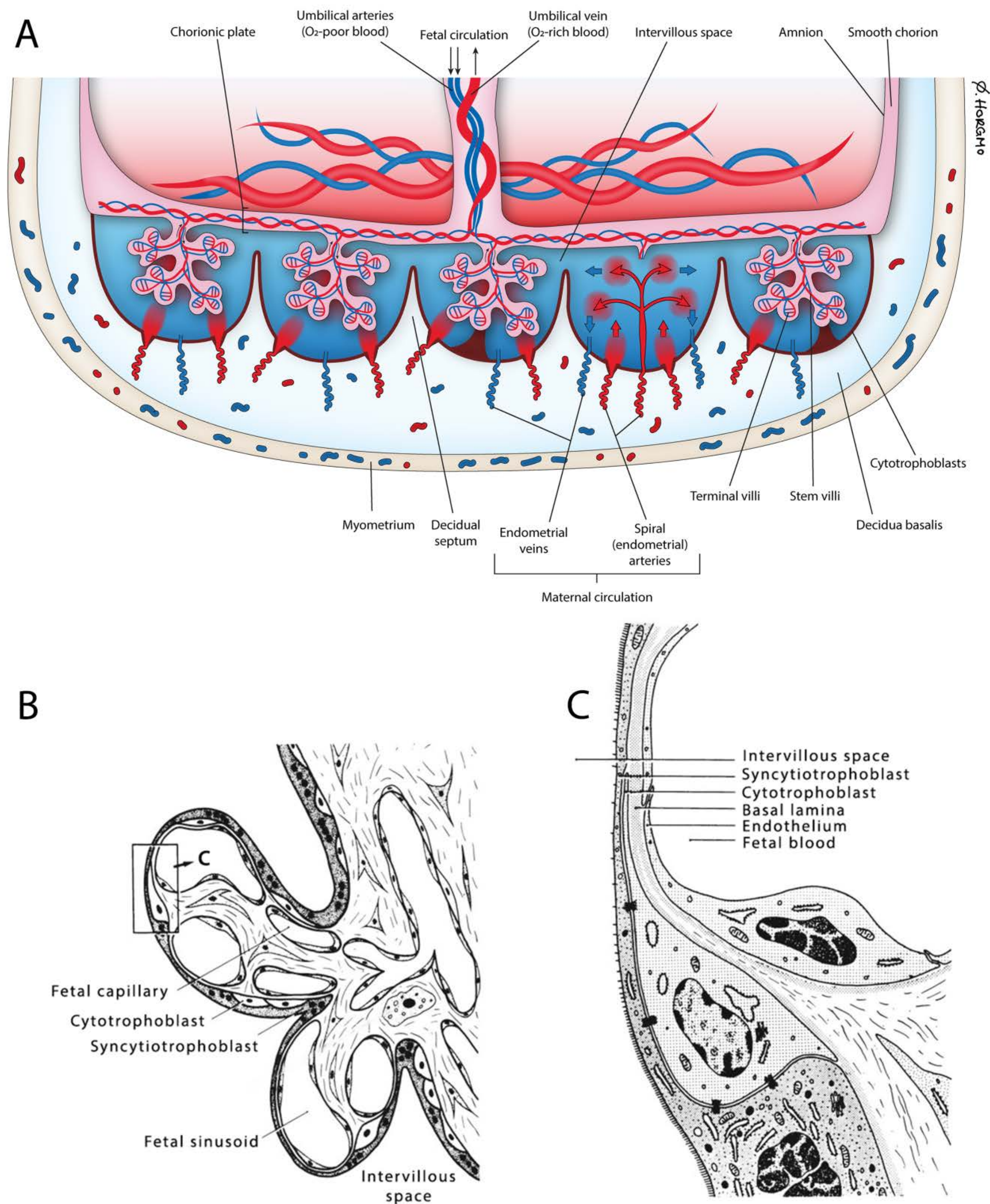
The cytotrophoblasts in the villi continue to proliferate. In distinct localities they penetrate the syncytial layer and enter the decidua, eventually forming stem (or anchoring) villi (Fig 4A). The villi that branch from the sides of stem villi are called terminal villi (19). It is in the terminal villi that the maternal-fetal exchange of nutrients and other substances will occur. As the pregnancy advances, numerous terminal villi extend from the stem villi into the surrounding blood filled intervillous (lacunar) spaces.

Fig 4 Detailed structure of the human placenta



A) Cross section of the mature placenta *Illustration by Øystein Horgmo, University of Oslo* **B)** Simplified light microscope section of two terminal villi, branching off a stem villus **C)** Schematic electron microscopic section demonstrating the layers in the placental membrane *Adapted with permission from Springer Nature: Springer-Verlag Berlin Heidelberg, Pathology of the Human Placenta, 6th edition by K Benirschke, G.J. Burton and R.N. Baergen, Copyright 2012*

The villi on the embryoblast pole continue to grow and expand, giving rise to the villous chorion (or the chorion frondosum) (Fig 1). The decidua over the villous chorion is called the decidua basalis (20). During the fourth and fifth months, the decidua forms septa which project into the intervillous spaces and divides the placenta into approximately 15-25 interconnected compartments called cotyledons (20). Each cotyledon consists of a main stem of a chorionic villous tree with its branches and sub branches of terminal villi. The volume of terminal villi increases exponentially throughout the pregnancy. This process depends mainly on angiogenesis, with longitudinal growth and coiling of the villous capillaries which bulges out on the trophoblast surface (19). The result is a continuous expansion of the total chorionic villi surface area, reaching 12-14 m^2 in the mature placenta (20). The growth trajectory of the human placenta follows an s-curve regression, flattening at the end of pregnancy (24).

1.2.3. The placental membrane

Exchange between the maternal and fetal circulations occurs across the placental membrane, i.e., the interface which separates the maternal blood in the intervillous spaces and fetal blood in the villous capillaries (Fig 4 B and C). Notably, exchange only happens in the terminal villi where the interface is extremely thin and the maternal and fetal blood come into very close proximity (19, 22). Initially, the placental membrane consists of four layers; the syncytiotrophoblast facing the maternal circulation, a layer of cytotrophoblasts, villous connective tissue and the fetal capillary endothelium (20). By week 20, however, the cytotrophoblasts and connective tissue in many of the villi disappear (22). Longitudinal capillary growth in the terminal villi stretches the covering syncytial layer, and the syncytiotrophoblast becomes thinner and free of nuclei and most organelles. This maturation or the maternal-fetal interface results in a close contact between, and sometimes fusion of, the syncytiotrophoblast and the fetal endothelium (19).

The syncytiotrophoblast has two polarized plasma membranes; the microvillous membrane (MVM) towards the maternal circulation, and the basal membrane (BM) facing the fetal capillary endothelium (19). The MVM surface has a brush border of numerous microvilli which increases the area towards the maternal blood five to seven times (25).

1.2.4. The uteroplacental circulation

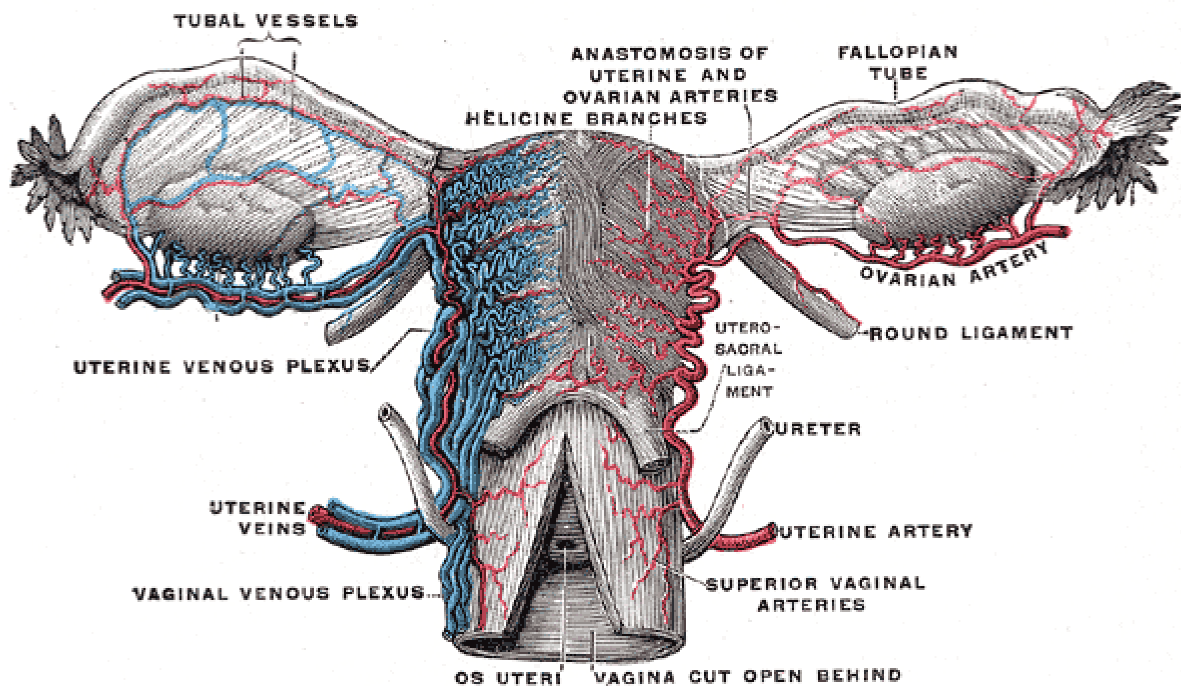
The maternal uteroplacental circulation is venous in the first weeks of the placental development. The intervillous spaces establish contact with maternal arterial blood through

erosion of uterine spiral arteries at the end of the first trimester (19). The cytotrophoblasts play a fundamental role in the remodeling of the spiral arteries. They invade the terminal ends of the vessels and replace the maternal endothelial cells in the vessel walls. This results in hybrid vessels containing both fetal and maternal cells (20). The endovascular invasion transforms the spiral arteries from small, high-resistance vessels to larger vessels with low resistance, resulting in increased maternal blood flow to the intervillous spaces.

The arterial blood supply to the uterus is mainly derived from the uterine arteries (26). In the non-pregnant state, the mean (SD) uterine blood flow has been estimated to be 21.14 (9.1) mL/min (27). Estimates of the uterine blood flow in pregnancy near term vary considerably, between approximately 0.5 and 1 L/min (28, 29). In early pregnancy, the uterine blood flow constitutes approximately 3.5% of the maternal cardiac output, while near term the fraction of the cardiac output distributed to the uterine circulation may reach 12% (30). The uterine arteries arise from the hypogastric artery, which is the anterior division of the internal iliac artery, and reach the uterus at approximately the level of the internal os of the uterine cervix, where they divide (Fig 5). The descending limb of the uterine artery travels downward along the cervix and the lateral vaginal wall, while the ascending limb goes upward alongside the uterus and continues below the fallopian tube. Notably, there are frequent anterior and posterior branches going off to the vagina, the fallopian tube and the ovary (26). The ovarian arteries, which arise from the aorta, anastomose with the uterine arteries and thus also contribute to the uterine blood supply.

The uterine arteries branch into thinner vessels, ultimately giving rise to the spiral arteries which penetrate the endometrium and during pregnancy enter the intervillous spaces in the placenta (Fig 4A). At term the intervillous spaces contain approximately 150 mL of blood, which is replenished 3-4 times per minute (20). The maternal blood is forced deep into the intervillous spaces as a funnel-shaped stream and bathes the numerous small villi in oxygenated blood (20, 31). As the pressure decreases, the blood flows back from the chorionic plate towards the decidua where it enters the endometrial veins. The endometrial veins converge to the uterine venous plexuses which lie alongside the uterine wall (Fig 5). The plexuses, in turn, are drained by a pair of uterine veins on either side which open into the corresponding hypogastric vein (26)

Fig 5 Blood supply to the uterus and adnexa



From Henry Vandyke Carter - Henry Gray (1918) Anatomy of the Human Body Bartleby.com: Gray's Anatomy, Plate 589, Public Domain, retrieved from Wikimedia commons

1.2.5 The umbilical cord and the umbilical circulation

During the third to sixth week the umbilical cord forms at the site of the connecting stalk, uniting the extraembryonic mesoderm in the chorionic plate with the embryo (31). The umbilical cord initially contains two arteries and two veins, but one of the veins regresses, leaving two umbilical arteries and one umbilical vein in the mature cord. The vessels are surrounded by stroma composed of Wharton’s jelly, covered by amniotic epithelium (20). Oxygenated blood from the capillaries in the villous trees in the placenta is transported to the fetus in the umbilical vein, while deoxygenated blood from the fetus is transported back to the placenta through the two umbilical arteries (Fig 4A).

1.3. A general overview of placental physiology

1.3.1. Endocrine function

The placenta secretes a variety of endocrine, paracrine and autocrine compounds to maintain and modulate pregnancy and parturition, as well as fetal growth, and maternal and fetal metabolism (22, 31). These compounds include steroid hormones like estrogens and

progesterone, and peptide hormones like human chorionic gonadotrophin, human placental lactogen and placental growth hormone. Furthermore, the placenta secretes various growth factors, angiogenetic factors, cytokines, chemokines and eicosanoids (22).

1.3.2. Protective barrier

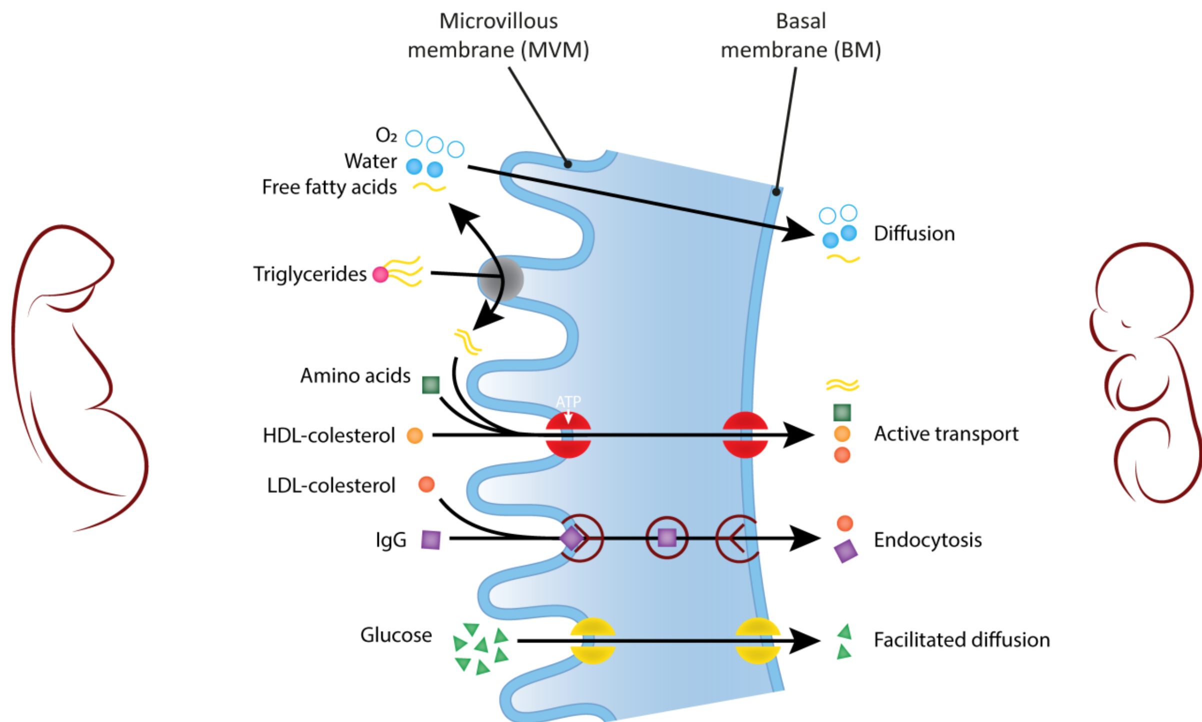
The placenta serves as a protective barrier against fetal exposure to toxic and infectious agents in the maternal circulation. Export pumps in the MVM and placental metabolizing enzymes like cytochrome P450 will, for instance, reduce the transfer of xenobiotics from the mother to the fetus (22). Further, maternal cortisol is oxidized to the less biological active cortisone, a system believed to protect the fetus against stress-mediated rise of cortisol in maternal blood (32). Several compounds and microorganisms, however, are fully able to cross the placental barrier, with potential detrimental effects for the fetus. These include alcohol, drugs, bacteria, protozoa and viruses.

1.3.3. Transfer of nutrients, water and gases

The placenta is responsible for the transfer of virtually all substances between the maternal and fetal compartments, including gasses, water and waste compounds, as well as the micro- and macro nutrients required by the fetus. Placental transfer is dependent on multiple factors like uteroplacental and fetal blood flow, placental area and syncytial layer thickness, concentration gradients, transport proteins and placental metabolism. The uterine blood flow relies on the successful remodeling of the spiral arteries and further a range of endocrine, paracrine and autocrine factors which are known to alter the resistance in the placental circulation (33). Umbilical blood flow relies on normal development of the placental villous tree and of the vessels at the chorionic plate.

The MVM and BM in the syncytial layer constitute the main barriers in maternal-fetal transfer (Fig 6). The MVM has a coat of carbohydrate-protein complexes called glycocalyx, which is the immediate interphase between maternal blood and placenta (19). Further, in addition to crossing the two syncytial plasma membranes, substances must pass through a layer of villous stroma (the basal lamina) and cross the fetal capillary endothelium.

Fig 6 Transfer across the placental syncytiotrophoblast



Adapted from AM Holme, Studies of the human placenta in vivo -The role of the placenta in glucose transfer and secretion of anti-angiogenic factors, 2017. Illustration by Øystein Horgmo, University of Oslo

Substances are transferred across the placental syncytiotrophoblast via three main mechanisms; simple diffusion, facilitated diffusion and active transport (33) (Fig 6). Diffusion refers to the net movement of molecules from higher to lower concentrations. A maternal-fetal concentration difference for any molecule, or electrochemical gradient for charged molecules, will lead to diffusion of that molecule across the placental barrier down its concentration gradient (33). Simple diffusion occurs when a substance passes through the plasma membrane without the aid of an intermediary such as an integral membrane protein. For instance, lipophilic substances like oxygen, carbon dioxide and ethanol dissolve freely through the entire syncytiotrophoblast plasma membrane. Simple diffusion is considered to be flow limited because of the large surface area available to this process. Net transfer will depend on the maternofetal concentration difference of the substance, which itself depends on the flow rates of the uterine and umbilical circulation. Facilitated diffusion, on the other hand, is spontaneous passive transport of molecules or ions across the plasma membrane via specific transmembrane proteins. An example of such diffusion is placental transfer of glucose via the glucose carrier GLUT-1. Facilitated diffusion is, in addition to concentration

gradients and flow, more dependent on the surface area of the barrier available for diffusion and the thickness of this barrier.

In contrast to diffusion, active transport uses cellular energy to move molecules across a plasma membrane against a gradient, polar repulsion, or other resistances. Active transport is more limited by the properties of the exchange barrier than by blood flow (33). The differences in the type, number and activity of transporters in MVM and BM thus provides the basis for active transport between the mother and the fetus. Amino acids are transported across the placental membrane through active transport. In addition to these main transport mechanisms, the syncytiotrophoblast may also take up compounds via endocytosis, i.e., invagination of the plasma membrane. Low density lipoproteins (LDL) and immunoglobulin G are transferred across the placenta via receptor-mediated endocytosis (34).

Transfer across the fetal endothelium can occur via transcellular or paracellular mechanisms (34). Small water-soluble nutrients, such as glucose and amino acids, diffuse freely through capillary endothelial junctions (35). The rate of this diffusion is determined by the permeability of the stromal interstitium to the different substances, the junctions between the endothelial cells and the endothelial glycocalyx (35). Although recent reports have suggested that the endothelial cell layer is an underestimated component in the maternal-fetal interface, the role of the fetal endothelium in the government of fetal-placental transfer remains unresolved (34).

Glucose and insulin

Glucose is the primary energy source for the fetus. At term, most of the fetal plasma glucose is derived from maternal plasma, since there is little evidence of gluconeogenesis in the healthy human fetus or in the placenta (36, 37). Glucose is transferred across the placental membrane by facilitated diffusion, mainly mediated by the insulin independent glucose carrier GLUT-1 in the last part of pregnancy (36). The maternal plasma glucose concentrations are higher compared to fetal plasma, and the main driving force for the placental glucose transfer is the maternal-fetal concentration gradient (38). GLUT-1 is more abundant in the MVM compared to the BM, and this asymmetrical distribution has been proposed to constitute a rate-limiting step in the transplacental glucose transfer from the mother to the fetus (39). The high density of GLUTs combined with the large surface area in the MVM promote efficient uptake of glucose by the syncytiotrophoblast. This extensive capacity for glucose transport in the MVM provides sufficient glucose for the placental metabolism, while maintaining the

gradient between the interior of the syncytiotrophoblast and the fetal circulation which is essential for net efflux to the fetus. The placental metabolism of glucose is known to be high (37), and in “the placenta 4-vessel study” we have shown that on average 31% of the glucose taken up from the maternal circulation is consumed by the placental and uterine tissue (unpublished data). GLUT-3, another insulin-independent glucose carrier, is also present in the placental membrane at earlier stages of gestation, but the expression decreases towards the end of pregnancy. Several other members of the GLUT family have also been identified in the placenta, but their role in glucose transfer remains uncertain (36).

Maternal insulin is not transferred across the placenta (36), and the prevailing opinion has further been that the placenta does not express insulin-sensitive glucose carriers. The influence of insulin on glucose uptake in the syncytiotrophoblast per se is therefore uncertain. Insulin may, nevertheless, affect placental glucose transfer by modulating glucose levels in the maternal circulation. Furthermore, stimulation of insulin receptors in the MVM may result in activation of upregulate various signaling pathways in the placenta, resulting in enhanced placental transport of amino acids.

Lipids

Lipids are important nutrients both for the fetus and the placenta. The fetus requires essential fatty acids and long-chained polyunsaturated fatty acids for normal growth and development, in particular of the nervous system. Furthermore, cholesterol is required for the construction of cell membranes and for synthesis of steroid hormones and as precursors for bioactive compounds (36, 40). The placenta is also in a continuous need of cholesterol, both to maintain its huge microvillous surface and to synthesize steroids, particularly progesterone (36). Fatty acids are highly integrated in the metabolism of glucose, and as such involved in the overall energy metabolism in the placenta.

The fetus itself is able to synthesize a portion of its required cholesterol and fatty acids, but it also relies on placental transfer from the maternal circulation (36). Cholesterol and fatty acids in LDL and very low density lipoprotein (VLDL) are mainly taken up by the syncytiotrophoblast through a lipoprotein-receptor-mediated mechanism based on endocytosis of the lipoprotein particles. Placental uptake of cholesterol is also mediated by other receptors without internalization of the receptor (36). Further, maternal triglycerides and phospholipids may be hydrolyzed by lipases at the microvillous surface, and the free fatty acids transferred across the syncytiotrophoblast by simple diffusion. Free fatty acids may also be taken up via the action of membrane-bound and cytosolic fatty acid binding proteins (22, 36). However,

the precise mechanism by which the different components involved in placental lipid transfer contribute to facilitate the transport is not fully understood.

Water

Homeostasis of water during pregnancy is also vital for normal fetal development. The fetal requirement of water increases markedly during pregnancy along with the exponential growth of the fetus, and is primarily met by placental transfer from the mother (41). The net transfer of water molecules to the fetus is considerably larger than the transfer of any other compound (42). Water transfer from the maternal to the fetal circulation is mainly determined by the colloid osmotic and hydrostatic forces at the placental interface (42). The flux of water across the human placenta occurs passively through both paracellular and transcellular pathways (41, 43). Although the molecular and cellular mechanisms of the maternal-fetal fluid balance in the human remain to be resolved, integral membrane water channel proteins known as aquaporins can affect the water permeability of the placental membrane. These aquaporins appear to be particularly important for facilitating transfer of water between the maternal and fetal circulations (43, 44). Furthermore, it has been demonstrated that fetal-placental venous constriction may influence the transplacental fluid balance and promote fetal-maternal fluid loss (45).

1.3.4 Placental metabolism and nutrient sensing

The placenta is a highly active metabolic organ, and consumes a substantial amount of nutrients, energy and oxygen. The maternal circulation is the main source of these elements, but they may also be derived from the fetus (37). The amount and composition of the various maternal substances taken up by the placenta are altered by the extensive placental metabolism. Consequently, the nutrients delivered to the fetus may significantly differ from those originally taken up from the maternal circulation. Changes in the placental metabolism may therefore have a significant impact on the transfer between the maternal and fetal compartments. This is particularly true for substances that are both transferred and consumed by the placenta in relatively large quantities, like glucose, oxygen and amino acids.

Emerging evidence suggests that the placenta plays a dynamic role in the regulation of fetal-maternal transfer by sensing concentrations of available nutrients, hormones and other signaling molecules, and adapting the placental metabolism accordingly to optimize fetal growth (37, 46). There have been identified a number of nutrient sensing signaling molecules and pathways in the human syncytiotrophoblast, which may participate in the integration of

maternal and fetal signals to regulate fetal nutrient availability (46). Mechanistic target of rapamycin (mTOR) is a major regulator of cell growth, and has in particular been suggested as an important integrator of maternal, placental and fetal signals to govern placental transfer (47). Growth factors, hormones, nutrients and oxygen tension are major upstream regulators of mTOR, and the activation of mTOR affects transcription of genes participating in the metabolism of nucleotides, lipids and amino acids (47). mTOR may also govern nutrient transporter activity in the syncytiotrophoblast by posttranslational modifications altering trafficking of the transporters to the plasma membrane (48).

1.4. Placental transport and metabolism of amino acids

Amino acids are essential for normal fetal growth and development, not only to build fetal proteins, but also as sources of energy, as neurotransmitters, and as precursors in metabolic pathways (49, 50). Amino acids are organic compounds containing an amine and a carboxyl group, in addition to side chains specific for each amino acid (51). Numerous amino acids are described, but only 20 are incorporated into proteins during translation and thus characterized as proteogenic amino acids (51). Nine of the proteogenic amino acids are termed essential, as they must be obtained through the diet because the body is unable to synthesize them from other compounds (Table 1). A few non-essential amino acids are considered as conditionally essential *in utero* and in early childhood, since the metabolic pathways that synthesize these amino acids are not fully developed.

Table 1 Essential and non-essential amino acids

Essential amino acids	Non-essential amino acids
Histidine	Alanine
Isoleucine	Arginine
Leucine	Asparginine
Lysine	Aspartate
Methionine	Cysteine
Phenylalanine	Glutamate
Threonine	Glutamine
Tryptophan	Glycine
Valine	Proline
	Serine
	Tyrosine

Placental amino acid transfer is a complex process, which involves many aspects of maternal, fetal and placental anatomy and physiology (Table 2). It is well established that amino acid concentrations in the fetal circulation are higher than the concentrations within the maternal circulation, whereas amino acid concentrations in the syncytiotrophoblast exceed those of the fetal circulation (52, 53). These observations implicate that amino acids are transported into the syncytiotrophoblast through active transport.

Table 2 Factors which affect amino acid concentrations and transfer in the maternal, placental and fetal compartments

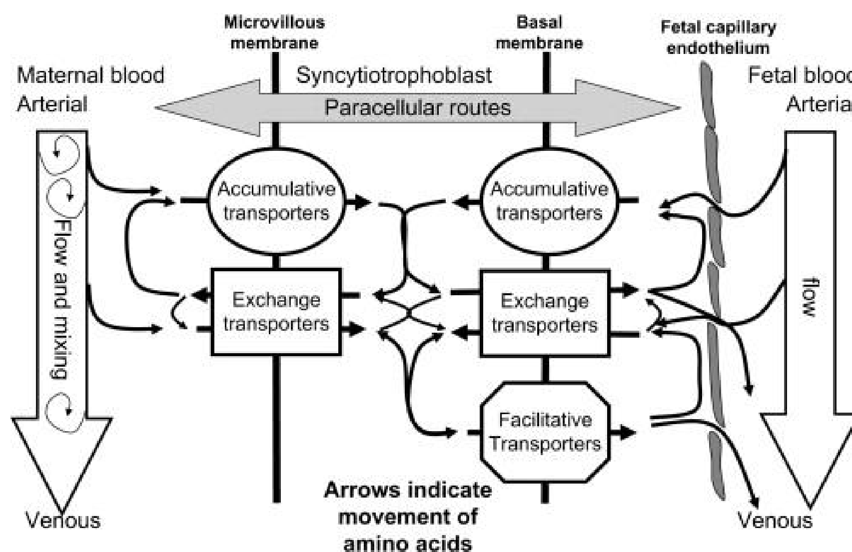
Factor	Effect on amino acid concentrations and placental transfer
Maternal metabolism	Determines amino acid concentrations in the uterine artery which affects concentrations in the intervillous space
Maternal spiral artery flow	Affects the rate at which arterial blood is delivered to the placenta and the rate at which amino acid depleted blood is removed
Volume and structure of the intervillous space	Determines how effectively amino acids from arterial blood will mix and reach the sites of transport on the MVM
MVM transporters	Change amino acids concentrations in the intervillous space and the syncytiotrophoblast
Syncytiotrophoblast volume and surface area	Volume will determine the concentration change due to the influx or efflux of a given amount of amino acids. Surface area will constrain the number of transporters which can be expressed
Placental metabolism	Amino acid concentrations will be affected by catabolism, anabolism, inter-conversion and flux into and out of the placental protein pool
BM transporters	Change amino acid concentrations between the syncytiotrophoblast and the fetal compartment
Volume of stroma	Determines the concentration change due to the delivery or removal of a given amount of amino acids
Diffusion through endothelial junctions	Affects the amino acid concentrations at the BM and flux into the fetal capillary
Fetal capillary volume	Will affect the rate blood flow, vascular resistance as well as the concentration of delivered amino acids
Fetal umbilical blood flow	Determines the rate of delivery of umbilical arterial blood and the rate at which transferred amino acids across the placenta are removed from the site of exchange
Fetal metabolism	Determines umbilical arterial amino acid concentrations which affect the concentrations at the BM

Adapted from R.M. Lewis, Placenta 2013 (Open access) (35)

1.4.1. Amino acid transport systems in the placenta

Amino acid transport across the placental membrane in humans is mediated by over 20 distinct amino acid transport systems with overlapping specificity (49, 54). The transport of different amino acids cannot be considered separately, as the transport of one may affect the transport of others, both due to competitive inhibition and the effect of the amino acid profile on both sides of the membrane on the transporters. There are three main classes of amino acid transporters in the syncytiotrophoblast; accumulative transporters, exchange transporters and facilitated transporters (49) (Fig 8).

Fig 8 Transfer of amino acid across the placental membrane via transport systems in the syncytiotrophoblast



From R.M. Lewis, Placenta 2013 (Open access) (35)

Accumulative transporters are driven by electrochemical gradients across the plasma membrane, and mediate amino acid uptake into the syncytiotrophoblast through cotransport with Na^+ against the amino acid concentration gradient (33). The inwardly directed Na^+ gradient is maintained by Na^+/K^+/ATPases in both the MVM and BM, which actively pump Na^+ out of the syncytiotrophoblast (33). The most important accumulative transporter system is the system A family (sodium-coupled neutral amino acid transporter, SNAT 1, 2 and 4), which primarily generates uptake of small, non-essential neutral amino acids like alanine, glycine, glutamine, serine and proline (33, 49, 55). Accumulative transporters are important in the MVM because they can establish amino acid gradients that drive the activity of exchangers and facilitated amino acid transporters, and thereby the overall placental uptake of

amino acids from the maternal circulation. The only accumulative transport system with an evident function in the BM is X^{-}_{AG}, which ensures uptake of fetal glutamic acid into the placenta for further metabolism (35). Otherwise, the role of accumulative transporters in the BM is uncertain, since they generate placental uptake of fetal amino acids from the umbilical circulation, but no efflux to the fetus.

Exchange transporters transfer one amino acid across the plasma membrane in exchange for another. Such transport systems alter the composition of amino acids in the syncytiotrophoblast, but not the overall quantity of amino acids transported (35). System L (L-type amino acid transporter, LAT1 and 2) are major amino acid exchangers in the placenta, mediating Na^+ independent transport of tryptophan, branched chain and aromatic neutral amino acids, many of which are essential (55, 56). Exchange transporters have an important function in both MVM and BM. Their activities are determined by amino acid concentrations on both sides of the plasma membrane, and thus by blood flow and the activity of other amino acid transport systems (35).

Facilitated transporters mediate diffusion of amino acids in both directions across the plasma membrane, with net transport in the direction of the concentration gradient. Facilitated transporters like system T (T-type amino acid transporter, TAT1), LAT3 and LAT4 are thought to be primarily located in the BM of the syncytiotrophoblast. These transporters generate net transfer of amino acids to the fetus down the concentration gradient established and maintained by transporters in the MVM (57). It is, however, important to notice that the facilitated amino acid transporters not necessarily operate like other facilitated transporters, such as for instance GLUT. Instead, they are reported to have complex kinetics with several possible affinities for different amino acids (58).

In addition to the active integral membrane transporters, there are studies suggesting that placental amino acid transfer may occur via simple diffusion through paracellular routes, for instance through areas of syncytial damage or via trans-syncytial channels (35). The fetal-maternal concentration and pressure gradients would in such cases promote net transfer from the fetus to the mother (35).

1.4.2. Regulation of placental amino acid transporters

The syncytiotrophoblast must integrate a magnitude of possibly divergent maternal, placental and fetal stimuli and modify its function accordingly. Placental amino acid transporters are

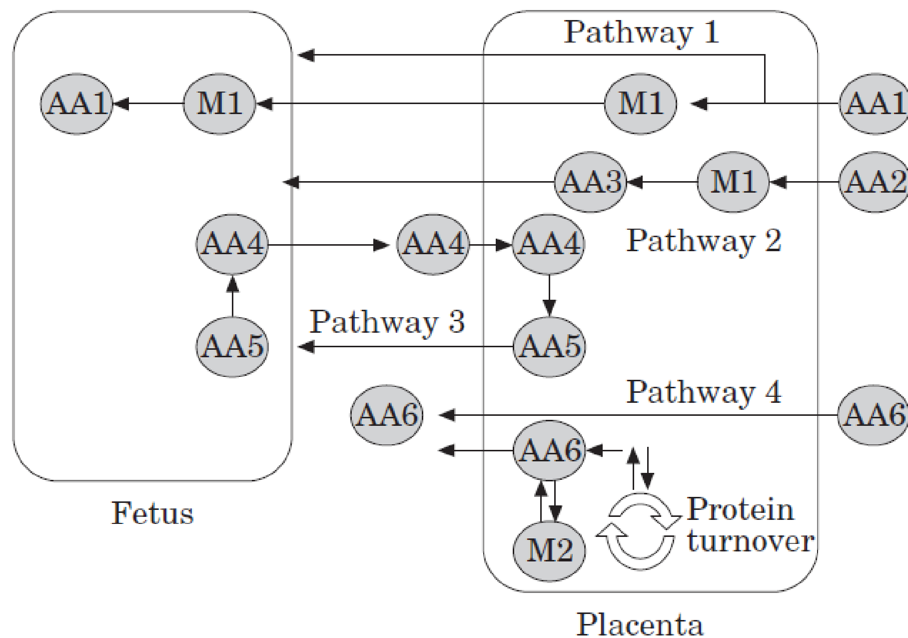
regulated by hormones, nutrients and cytokines (39). *In vitro* studies on cultured trophoblasts and villous fragments suggest that insulin, leptin, insulin like growth factor 1, amino acid deprivation (i.e. adaptive regulation), oleic acid, 1,25-Dihydroxy vitamin D_3, and cytokines such as interleukin (Il) 6 and tumor necrosis factor (TNF) α stimulates system A activity, resulting in increased amino acid uptake by the syncytiotrophoblast (33, 50, 59, 60). Activation of mTOR signaling stimulates system A by altering transporter translocation to the plasma membrane (39, 48). Hypoxia, Il 1β, adiponectin, nitric oxide, docosahexaenoic acid, and corticotropin-releasing hormone reduce system A activity (39, 50). Angiotensin II also reduces system A activity in placental villous fragments, primarily through a negative effect on a Na/K/ATPase activity and altered intracellular concentration of sodium (33).

There are more conflicting data regarding the regulation of the system L amino acid transporter. Increased intracellular calcium concentrations, protein kinase C and low extracellular pH stimulate system L activity (39). Studies of cultured trophoblasts and villous explants from term placentas have shown that mTOR is a positive regulator of system L, and that glucose increased system L activity in an mTOR-dependent mechanism (39, 61). Long term infusion of full length adiponectin reduced the activity of system L in studies of pregnant mice, but this has not been reproduced in *in vitro* studies. One study of cultured primary trophoblasts showed an increase in system L activity in response to insulin exposure, while another similar study observed no such effect (39).

1.4.3. Placental amino acid metabolism

Tracer studies in both animals and humans indicate that transfer of amino acids between the maternal and fetal compartments is closely linked to placental amino acid metabolism (62, 63). Interconversions and consumption of amino acids alter the concentration of amino acids available for transfer. Transport through exchangers and facilitated transporters are particularly influenced by the placental metabolism since their activity is governed by the concentration gradients. Metabolic processes involving amino acids in the placenta include deamination and oxidation to generate energy, interconversion and biosynthesis of other compounds, fetal-placental shuttling, and protein turnover (37) (Fig 9).

Fig 9 Potential metabolic pathways involved in amino acid transfer in the ovine placenta



AA: amino acid, M: metabolite. *Reprinted from Placenta,22, FC Battaglia and TRH Regnault, Placental Transport and Metabolism of Amino Acids, 145-161, Copyright 2001, with permission from Elsevier.*

A substantial part of the amino acids taken up by the ovine placenta is used for oxidative metabolism (63). Glutamic acid is likely the most important amino acid fuel for the human placenta (54). Glutamic acid can be transformed to α-ketoglutarate via the enzyme glutamate dehydrogenase, a process that generates NADH which in turn may produce ATP through the electron transport chain. When α-ketoglutarate is transformed back to glutamic acid, placental branched chain amino acids (valine, leucine and isoleucine) are transaminated to their corresponding α-keto acid. The α-keto acids are in turn decarboxylated to produce acetyl coenzyme A derivates which may enter the tricarboxylic acid cycle (37, 64). However, in the ovine placenta it has been shown that only a small amount of the α-keto acids are utilized for oxidation due to very low activity of α-keto acid decarboxylases (54). This is also suggested to be the case in human pregnancies (37). Instead, the transamination of branched chained amino acids could be important for nitrogen shuttling, for instance to the purine synthesis (63). Tracer studies in ovine pregnancies have further shown a net transfer of α-keto acids from the placenta to the fetus, while similar human studies in contrast have demonstrated a net output from the fetus towards the placenta (65). Oxidation of other amino acids like alanine, glycine, aspartate, phenylalanine, and proline to urea and carbon dioxide which occur

in other tissues presumably also occur in the placenta, but such placental mechanisms has not been explored in the human (37).

Animal studies have shown that glutamic acid is part of an inter-organ cycle between the placenta and the fetal liver (62, 64). Glutamic acid can be converted to glutamine in the placenta, and studies in pregnant sheep have shown that large amounts of glutamine are released to the fetal circulation (66). The fetal liver clears glutamine from the fetal circulation and produces glutamic acid, which in turn is released back in the fetal circulation for placental uptake and usage. This is an example of how the fetal liver controls the supply of an important oxidative fuel for the placenta. Data from perfusion studies of the human cotyledon agrees with the findings in animals demonstrating a glutamine-glutamic acid interconversion (67), but whether such a cycle takes place in the human fetal-placental unit has not been established *in vivo*.

Studies in pregnant sheep have further demonstrated an exchange of serine and glycine between the placenta and the fetal liver (62). Serine is taken up from the maternal and fetal circulations, and is converted to glycine in the placenta via the enzyme serine hydroxymethyltransferase. Placental glycine is released into the fetal circulation and taken up by the fetal liver where it is converted to glycine. In turn, glycine is released back into the fetal circulation. However, the activity of serine hydroxymethyltransferase in the human placenta is reported to be low, and the significance of the serine-glycine cycling in the human is uncertain (68).

Amino acids are an essential part of biosynthetic pathways also in the placenta. Protein synthesis and degradation has been demonstrated in the syncytiotrophoblast, but the knowledge about these processes is limited (37). Other biosynthetic pathways in the placenta include synthesis of polyamines from proline, and generation of nitric oxide from arginine (37). Tracer studies in sheep have shown a significant placental uptake of alanine from the maternal circulation, but only a small fraction is directly transported to the fetus (69). Most of the alanine transferred to the fetus may thus be a result of protein degradation, as well as alanine production from pyruvate through transamination reactions (63).

1.4.4. Placental transport of taurine

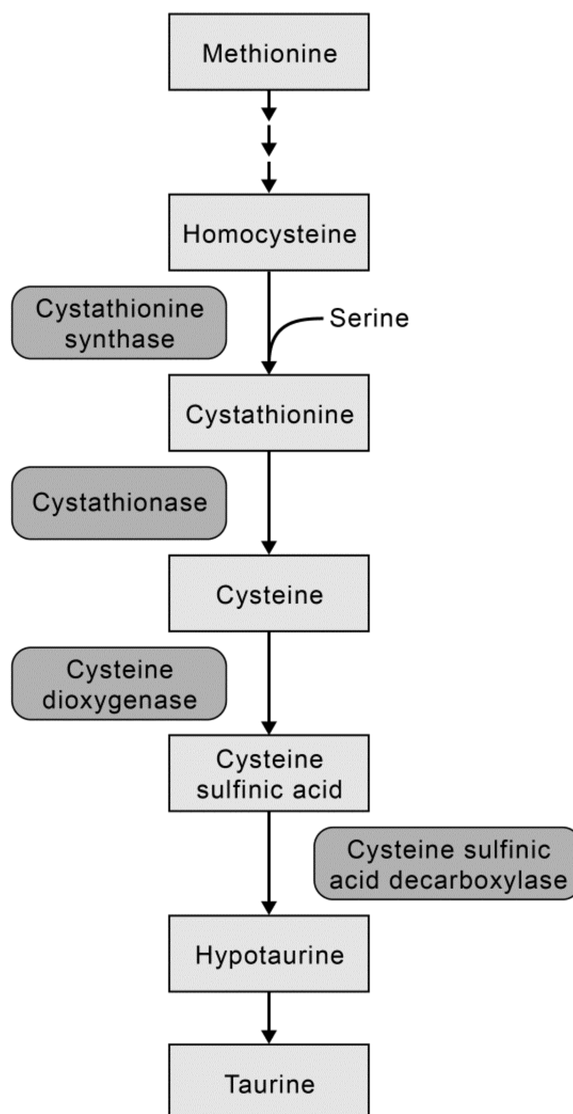
The amino acid taurine (2-aminoethane-1-sulfonic acid) is involved in vital cellular processes like regulation of cell volume, proliferation, apoptosis, and cytoprotection. It has numerous

biological properties in the human body, including conjugation of bile acids to aid fat absorption in the small intestine, blood pressure regulation, neuroinhibition and protection, and in regulation of retinal function (70, 71).

Taurine is not considered to be a usual amino acid in the common biochemical meaning of the term, because it carries its amine group on the β-carbon, not the α-carbon which is typical for most amino acids. Furthermore, taurine has a sulfonic acid group in place of the normal carboxyl group (72). Taurine is not incorporated into proteins, and thus remains free in the intracellular compartment. It is present in extremely high concentrations in many human tissues like liver, brain, retina, kidney, heart and skeletal muscle (70, 72). The taurine body pool is determined by taurine and sulfur amino acid intake from the diet, primarily meat and fish, as well as by taurine synthesis, and reabsorption of taurine by the kidneys (73, 74). The cellular taurine content is a result of biosynthesis, active uptake by the taurine transporter, and release via a volume sensitive leak pathway (75).

In adult humans, taurine is primarily synthesized from methionine and cysteine via the cysteine sulfinic acid pathway (Fig 10) (72, 76). Taurine may also be synthesized from cysteine via coenzyme A and cysteamine, but the relative contribution of this pathway to the net taurine production is not fully established (76).

Fig 10 Biosynthesis of taurine



In the cysteine sulfinic acid pathway, the enzyme cysteine dioxygenase (CDO) converts cysteine to cysteine sulfinic acid. This metabolite can either be transaminated by aspartate amino transferase and further converted to sulfur and pyruvate, or it can be decarboxylized to hypotaurine for taurine synthesis by the enzyme cysteine sulfinic acid decarboxylase (CSAD). CSAD activity may thus regulate the partitioning of cysteine sulfinic acid between decarboxylation and transamination, and has been considered to be the rate limiting step in the taurine synthesis (77, 78). The ability to synthesize taurine varies between different species, developmental stages and types of tissues (70). Livers from cats, monkeys and humans have, for instance, lower CSAD activity compared to livers from other species. Furthermore, livers and brains from young mammals are considered to have lower synthetic capacities than the same organs in adults (70, 72). In contrast, taurine levels in the human fetal liver and brain are

more than twice the levels in adults, and brain levels remain high several months after birth (79). This suggests that the fetus and infant are highly dependent on an exogenous source of taurine.

The high fetal taurine levels are in accordance with its imperative role in fetal development. Reduced placental taurine transporter activity and lower concentrations of taurine in fetal plasma are associated with intrauterine growth restriction, retinal degradation, disrupted skeletal and myocardial muscle development, and dysfunction of the nervous system and the pancreatic islets (53, 75, 80, 81). Like for the proteogenic amino acids, the fetal plasma concentration of taurine is higher than in the maternal circulation, whereas the tissue concentration in the placenta exceeds the fetal circulation in manifold (52). Despite the extremely high taurine concentration in the syncytiotrophoblast, it is widely reported in the literature that the human placenta, like human fetal organs, have limited or no ability to synthesize taurine (73, 74), implying that the human fetus relies solely on transfer of taurine from maternal plasma. This notion has been based on reports of lacking synthesizing enzymes in placental and fetal tissues (82, 83). Notably, these reports have only studied the enzymes converting the essential amino acid methionine to cysteine, several steps earlier in the synthesis pathway for taurine (Fig 10). Recently, however, a study by Korneeva and colleagues has shown expression of both CDO mRNA and protein, as well as CSAD mRNA, in the human placenta (77). Placental protein expression of CSAD, on the other hand, has not, to our knowledge, been studied in humans, despite this enzyme's vital role in the taurine biosynthesis.

Taurine is taken up in the syncytiotrophoblast against its concentration gradient in cotransport with Na^{+} and Cl^{-} by the taurine transport system (TAUT, system β) (39). *In vitro* studies on human placental tissues have shown that system β is almost exclusively polarized to the MVM, and this transport system is thought to generate the steep placental-fetal gradient which drives sodium independent transport across the BM towards the fetus (74, 81). The mechanism for the fetal taurine transfer is, however, largely unknown. Studies of isolated membrane vesicles and human placental explants have demonstrated that taurine also may be released across the MVM to the maternal circulation via chloride channels as a response to increased cellular volume and changes in osmolarity (84, 85).

Activation of protein kinase C, as well as the nitric oxide donor SIN-1 involved in oxidative stress in IUGR, both limits taurine transport in isolated MVM vesicles and villous tissue

explants (74). It has also been reported that high glucose levels diminish uptake of taurine in cultured trophoblast cells (39). The uptake of taurine in human villous tissue fragments is not affected by the cytokines and hormones that stimulate amino acid transporter system A, such as insulin-like growth factor I and II, Il Iβ, TNF α, growth hormone and leptin. Furthermore, the role of mTOR in regulating taurine transport is still uncertain (39).

In addition to its vital role in fetal development, recent evidence suggests that taurine is crucial for the development and function of the human placenta through facilitating syncytiotrophoblast renewal and survival. Renewal of the syncytiotrophoblast involves proliferation and fusion of underlying trophoblasts, and this renewal is required to uphold the transporting and endocrine functions of the syncytiotrophoblast and thus to provide nutrients to the fetus and to generate hormones that sustain pregnancy (86). In cultures of human cytotrophoblasts, inhibition of system β reduced intracellular taurine concentrations, impaired the differentiation and fusion of cells to form a multinucleated syncytium, and increased the susceptibility to TNF α-mediated apoptosis (87). In a villous explant model, intracellular taurine depletion lead to weakened regeneration of the trophoblasts and to increased sensibility to oxidative stress, resulting in nuclear and mitochondrial DNA damage (86). The observed results of taurine deficiency *in vitro* are consistent with features of placental dysfunction seen in pregnancy complications like preeclampsia and intrauterine growth restriction (86, 87). Interestingly, the placental tissue concentration of taurine in preeclamptic women is lower compared to normal pregnancies (88). The high intracellular taurine levels in the syncytiotrophoblast could thus reflect the cytoprotective functions of taurine. Notably, as in other cell types, taurine is an important osmolyte in the syncytiotrophoblast (85, 87). As all cells have to regulate their volume to survive and maintain their functions, this is another mechanism by which taurine may influence placental health and function. Inadequate placental taurine levels could consequently be a contributing factor to the placental insufficiency associated with pregnancy complications like preeclampsia and intrauterine growth restriction.

1.5. The placenta and fetal growth

There is a close relationship between birthweight and placental weight (24). The growth trajectories of the human fetus and the placenta, however, follow two different patterns; the placenta follows an S-shaped curve, while fetal growth follows an exponential curve (89). Towards the end of pregnancy, the fetus consequently grows at a faster rate than the placenta

and the fetal/placental weight ratio, often termed "placental efficiency", increases (24). To uphold this accelerated fetal growth, the placenta must increase its nutrient transfer capacity. This most likely occurs through changes in placental perfusion and maturation of the placental membrane, with stretching and thinning of the maternal-fetal interface and enhancement of transporter density in the syncytial plasma membranes (33, 89). Human and animal studies have demonstrated that amino acid transport activity is inversely correlated with fetal and placental size in normal, uncomplicated pregnancies (90-92). This is in line with the observations that lower placental weights are not associated with a substantial decrease in fetal weights in such pregnancies, indicating that the placenta has a considerable ability to compensate and maintain fetal growth (89).

In IUGR pregnancies, on the other hand, lower placental weights are associated with a marked reduction in fetal weights, indicating failure of the placental compensatory mechanisms (89). In addition to reduced blood flow limiting the diffusion rate of gasses and certain nutrients to the fetus, the activity of a range of nutrient transporters and carrier proteins in the MVM and the BM are shown to be decreased in human and animal IUGR placentas, including transporters for amino acids, lipids and ions (33, 50, 93). Glucose transporters are not affected in IUGR, and the activity of some transporters, such as the plasma membrane Ca^{2+} ATPase, are in fact increased. These observations from *in vitro* studies suggest that alterations in activity of some transporters may be a causal factor in IUGR, while others represent a fetal-placental compensatory activation stimulated by reduced fetal growth (33, 93).

Amino acid transporter activity decreases prior to the onset of IUGR in animal models, indicating a causal role in the pathogenesis of this pregnancy complication (94, 95). Accordingly, both *in vitro* and *in vivo* studies in humans have demonstrated a decrease in the expression of systems A, L and β, reduced placental transfer of labelled leucine and phenylalanine, and lower fetal amino acid concentrations in IUGR pregnancies (53, 80, 96-98). Findings from a recent study in human IUGR pregnancies suggest that decreased placental mTOR activity leads to down-regulation of placental system A activity by promoting proteasomal degradation of SNAT 2, thereby contributing to impaired fetal amino acid availability and restricted growth in IUGR (99). It is further possible that placental system A activity may be decreased as a result of reduced oxygen supply, which in turn may be due to the impaired blood flow frequently observed in this pregnancy complication (33).

Finally, microRNAs have been suggested to play a role in impaired placental nutrient transfer leading to IUGR (100).

Fetal overgrowth is associated with increased maternal nutrient availability. This has been shown as a result of high calorie diet in animal models, as well as in women with obesity and in women with poorly controlled gestational diabetes (50). An abundance of substrates during the intrauterine development is believed to stimulate fetal insulin secretion and subsequently fetal growth (101). Fetal overgrowth is further associated with increased activity of system A, L and β in isolated placental MVMs from obese and diabetic women (50). However, placental uptake of amino acids via system A has also been reported to be reduced in pregnancies of obese women and nonhuman primates. These seemingly contradictory results suggest that this process may be affected by levels of specific nutrients or hormones in maternal plasma, rather than maternal adiposity *per se* (50). Raised maternal insulin concentrations, for instance, may be a part of the explanation for the increased placental amino acid transport observed in diabetic and obese pregnant women (50). In a study of pregnant mice, obesity induced by a high fat/high sugar diet increased placental glucose and amino acid transfer to the fetus and was associated with the activation of placental insulin and mTOR signaling in fetal overgrowth (102). Placental insulin and mTOR pathways were also activated in human pregnancies complicated by maternal obesity (103). Other studies in human pregnancies suggest that placental function may be modulated by maternal hyperglycemia and obesity-related inflammation and oxidative stress, and that elevated leptin, IL 6 and TNF α, and low adiponectin may stimulate placental nutrient transport (104-108). Circulating maternal lipids may also contribute to placental inflammation and increased transport of nutrients across the placenta through toll-like receptor 4 signaling and thus influence the growth of the developing fetus (109).

1.6. Placental research

1.6.1. Human *in vitro* and *ex vivo* models

A wide variety of *in vitro* and *ex vivo* models have been used to study transfer across the human placenta (110-113) (Table 3).

Table 3 *In vitro* models for studying placental transfer

Model type	Material	Application	Advantages	Disadvantages
***Ex vivo* perfusion model**	Placental cotyledon	• Placental transfer of nutrients, xenobiotics etc. • Placental tissue metabolism • Role of placental transporters	• Maintain whole organ complexity • Easily available • Non-invasive	• Only mature placenta • Maternal side damaged • Technically challenging • Limited lifetime • Different protocols –no standard criteria
Tissue preparations	Placental explants	• Uptake of substances in the syncytiotrophoblast • Transport regulating factors	• Intact micro-structure • Cell-cell interaction • Any stage of gestation	• Syncytiotrophoblast may be damaged (despite intact microstructure, the exposures of the apical and endothelial surfaces to the medium may be distorted) • Limited lifetime
	MVM and BM vesicles	• Transporter expression and function • Uptake and efflux	• Allow comparison between MVM and BM	• Absence of regulatory factors and cell-cell interactions • May not mimic *in vivo* conditions
Cell cultures	Primary placental cells	• Uptake of substances in the syncytiotrophoblast	• Represents normal cells • Any stage of gestation	• Absence of tissue structure and interactions between different cells • High contamination level
	Cell lines (transfected, chorio-carcinoma)	• Transport and metabolism	• Replicate rapidly in culture • Easily cloned	• May not mimic normal syncytiotrophoblast

Adapted from R Levkovitz, Placenta 2013(113)

Human *ex vivo* placental perfusion and *in vitro* monolayer cell cultures on a permeable membrane are the only methods that reflect the structure of the placental membrane and thus can be used to study transfer across the placental interface between maternal and fetal blood (113-115). The placenta perfusion model has been employed for many years, and it is an effective and non-invasive method that may provide valuable information on a wide range of functions of the human placenta. It is, however, a highly experimental method which not necessarily reflects the *in vivo* milieu (pH, oxygen tension, binding proteins, flow patterns,

etc.). The generated results must therefore be interpreted within the limitations of the model (110, 112).

Other *in vitro* placenta models, like placental explants and cell cultures, do not constitute a continuous membrane. The uptake in such models thus represents the transfer of substances into the syncytiotrophoblast from the maternal circulation, but does not shed light on the transfer from the placenta to the fetus (110). Nevertheless, these methods may provide important data on metabolism and transporter functions which may modify transplacental transfer (111, 114). Preparation of membrane vesicles from placental homogenates is another useful method to study the placenta, in particular the transporter systems in the two syncytial membranes. Importantly, these studies occur in absence of the extensive regulatory mechanisms present in the human placenta *in vivo*.

In addition to the different *in vitro* and *ex vivo* models employed to study the human placenta, morphological studies using techniques like electron microscopy, immunohistochemistry and stereology have been decisive for the current understanding of placental functions and properties (116, 117).

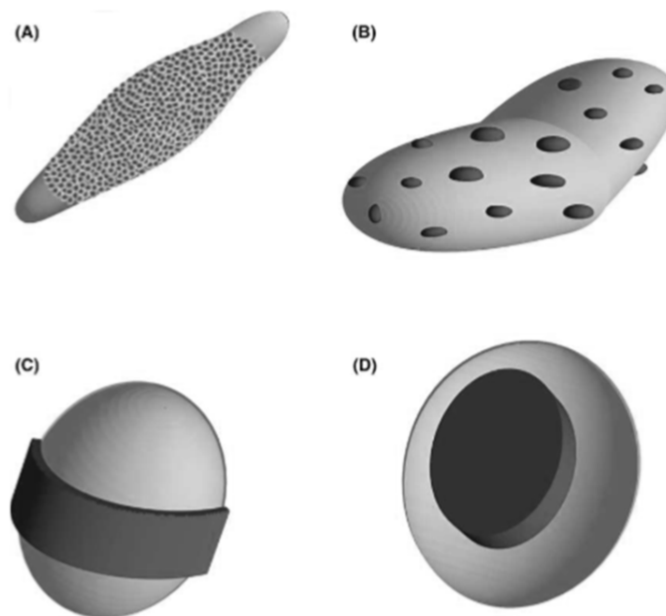
None of the *in vitro* or *ex vivo* placenta models, however, are able to explore or consider all aspects involved in placental transfer in pregnant women. This limits our ability to learn how the different factors contribute to the flux across the placenta as a whole integrated system, and to reveal which of the factors at play are most important in determining the fetal nutritional conditions. A newer approach is to use the pieces of knowledge generated from experimental studies in computational modeling to explore the complex functions in placental transfer and metabolism (35, 118, 119). By mathematically integrating the different factors influencing placental transfer, such systems biology studies aim to understand and determine how each factor contributes to the process as a whole, and thus to be able to predict how any changes in specific placental parameters will affect placental transfer.

1.6.2. *In vivo* animal models

The knowledge of placental physiology generated from *in vitro*, *ex vivo* and mathematical modeling must evidently be evaluated *in vivo*. The majority of *in vivo* studies of the placenta have been performed in animals. However, extrapolation of data obtained in animal models to human pregnancies is difficult since the placenta is one of the organs with the highest diversity among the species (110, 120, 121). Based on gross morphology, .i.e., the shape and

the area of contact between fetal and maternal tissue, placentas from different animal species can be grouped into 4 types (fig 11 A-D): diffuse (horses, pigs), multicotyledonary (ruminants), zonary (carnivores), and discoid/bidiscoid (primates, rodents, rabbits) (122).

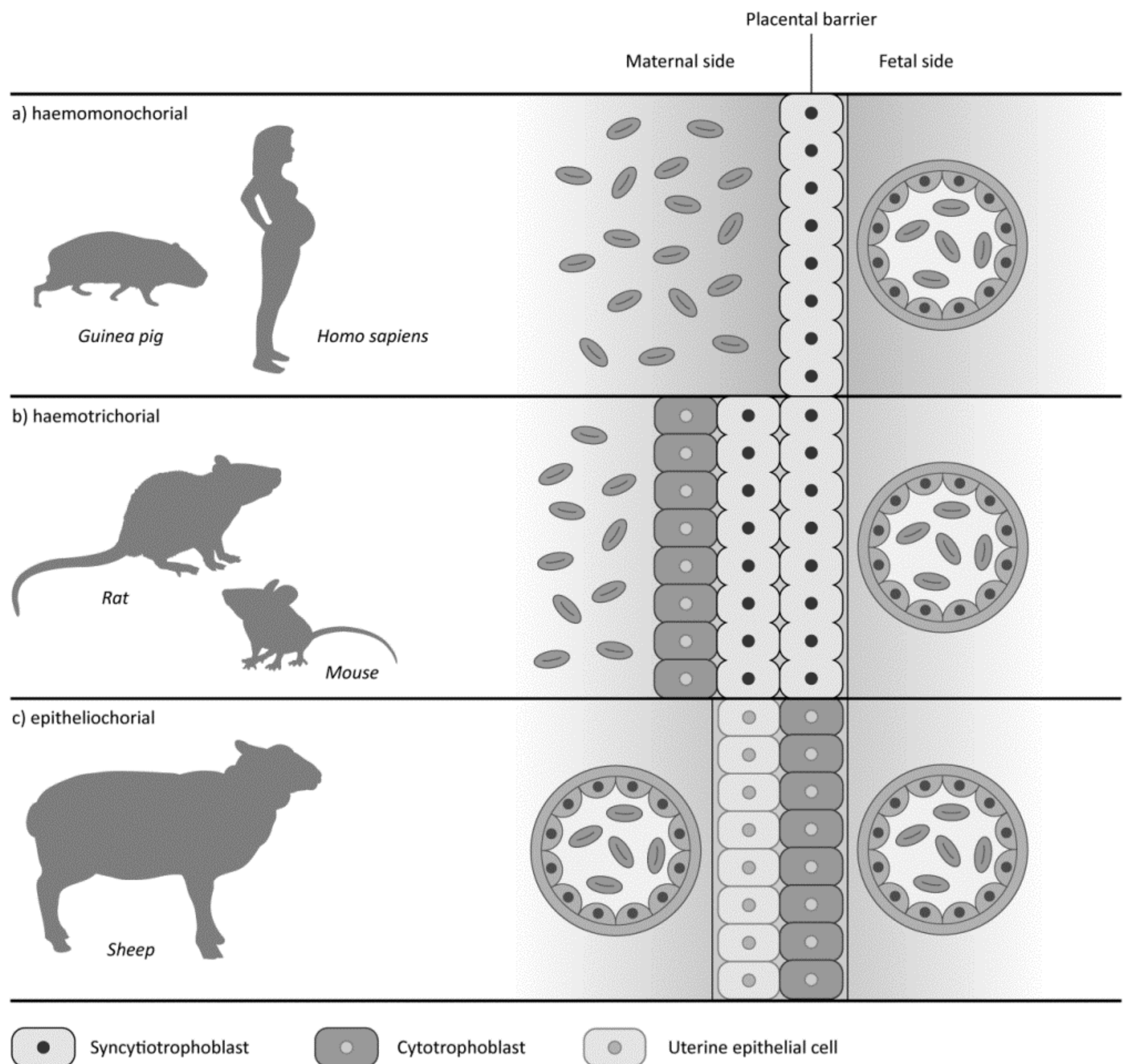
Fig 11 Placental types recognized by gross morphology A) diffuse, B) multicotyledonary, C) zonary, D) discoid



From K. Imakawa, Genes to Cells 2015 (Open access)(123)

Further, the placenta is subdivided according to the cell layers between fetal and maternal blood: epitheliochorial (horses, pigs, and ruminants), endotheliochorial (carnivores), and hemochorial (rodents, rabbit, and primates) (122) (Fig 12). In addition to anatomical features, there are extensive differences between the species related to placental immunology, endocrinology, protein expression etc. (121).

Fig 12 Placental types subdivided according to the cell layers between fetal and maternal blood



A) Hemomonochorial placental membrane (human, guinea pigs and chinchillas): only one syncytiotrophoblast layer separates the maternal intervillous space from the fetal capillaries B) Hemotrichorial placental membrane (mice and rats): three layers of trophoblast cells separate the maternal intervillous space from the fetal capillaries C) Epitheliochorial placenta membrane (sheep): one layer of uterine epithelium cells and one layer of trophoblast cells separate maternal and fetal capillaries *Adapted from E. Mikkelsen, R Soc Open Sci 2017 (Unrestricted use) (124) by Øystein Horgmo, University of Oslo*

Many different animals have been used in studies of placental physiology, including rodents (mouse, rat, guinea pig), rabbits, domestic animals (sheep, cow, horse, pig) and non-human primates. All of these species have their advantages and limitations as models of human

placental physiology (120, 122), and there is thus no single ideal animal model for studying the human placenta. In recent years, there has been an extensive debate as to whether animal pregnancy models can be used to draw firm conclusion about human pregnancies (121, 125, 126). Some argue that due to the increasing number of known human-specific factors in human placentation, "many aspects of human placentation can only be understood on the basis of experiments on human cells and tissues in combination with data collections from human subject studies" (121). Others claim that "although the molecules involved may differ to some degree between [animals] and humans, the homology is sufficient to guide investigation of the human condition in scientifically productive directions" (126). Nevertheless, while animal models may provide valuable clues as to what to look for in humans, it is imperative to validate the data in pregnant women.

1.6.3. Human *in vivo* studies

In vivo studies of placental transfer in the human are essential, but may be ethically, logistically and technically challenging. A simple approach has been to draw blood from maternal peripheral vessels and from one or both umbilical vessels during vaginal delivery or cesarean section (96, 127-129). Umbilical blood samples have also been obtained percutanously under ultrasonic guidance at different gestational ages (53). The comparison between fetal and maternal plasma concentrations of any given compound can be viewed as an index of placental transport and metabolism (62, 130).

The optimal approach to study the placenta *in vivo*, however, is to obtain blood samples from both incoming and outgoing vessels on the maternal and fetal sides of the placenta ("4-vessel sampling"), combined with corresponding measurements of blood flow. Ideally, this would be done at several time points during gestation, but methods like amniocentesis, chordiocentesis or chorionic villous sampling contains a risk of harming the fetus (0.5–1% risk of miscarriage/preterm delivery), and longitudinal blood sampling is consequently ethically unacceptable without a medical indication (115). Further, the maternal uterine artery is only accessible through invasive procedures like abdominal incision and arterial catheterization. Several national and institutional ethical committees have approved cross-sectional studies using 4-vessel sampling during cesarean section (131). Only a few studies employing the 4-vessel sampling technique have been published, however, possibly due to the logistically demanding procedure which requires a close coordination between the patient, her doctors,

the delivery department, the operative and anesthesiology units and the laboratory services (131).

It is possible to expand the *in vivo* blood sampling methods with maternal infusions of unlabeled or labeled compounds (i.e., "tracer studies"), for instance glucose or various amino acids (54, 62) A comparison between the enrichment of various compounds in fetal and maternal plasma may provide more detailed information on placental transfer, as well as on placental and fetal metabolism (97, 132-136). However, this approach introduces an intervention and does not necessarily explore placental functions under normal physiological conditions in human pregnancies.

1.7. The placenta – still "the least understood organ in the human body"

Accumulating evidence highlight the placenta as a major determinant of both perinatal and adult health. In despite of this, the placenta is claimed to be the least understood organ in the human body (137). Our knowledge of the human placenta is mainly derived from animals and *in vitro* and *ex vivo* models, since it is difficult to access and study this organ *in vivo* in humans without imposing ethically unacceptable risks on the ongoing pregnancy. The experimental studies have generated invaluable conceptual models of how nutritional, metabolic, and hormonal factors works together to determine the environment under which the fetus develops. Due to the limited *in vivo* studies of the human placenta, however, we largely do not know to which extent these models are applicable in the human. The placenta 4-vessel study aims to expand the current understanding of human placental functions and how the placenta regulates the intrauterine nutritional environment under normal physiological conditions.

2. Aims

The overall aims of the present thesis were to establish a method to study the human placenta *in vivo*, to collect an extensive biobank of maternal and fetal plasma, as well as placental tissue for future research, and to employ this biobank to assess placental transfer of amino acids *in vivo* in healthy human pregnancies. We aimed to explore, under the given conditions, the impact of the placenta in determining the amino acid supply to and uptake by the fetus and to evaluate some of the experimentally derived current concepts of fetal-placental exchange and metabolism of amino acids in a human *in vivo* model.

Specific aims:

I. Make our established 4-vessel sampling method accessible for the international research community, by in depth description and live visualization of the techniques. We also aimed to demonstrate that the 4-vessel sampling method can be employed to investigate and integrate different aspects of placental functions.

II. In order to explore the interplay between uptake and release of amino acids in the fetal-placental unit *in vivo*, we aimed to determine and assess the paired relationships between plasma concentrations and arteriovenous differences of 19 proteogenic amino acids on the maternal and fetal sides of the placenta in our large sample from human mother-fetus pairs.

III. Taurine is a vital non-proteogenic amino acid in fetal life. We aimed to study the transfer of taurine between the maternal, placental and fetal compartments in our 4-vessel plasma samples from non-complicated human term pregnancies. Based on current concepts, we hypothesized that increased plasma concentrations and uptake of taurine in the fetus is associated with increased maternal plasma concentrations, uteroplacental uptake from the maternal circulation and concentrations of taurine in placental tissue. The high placental tissue concentrations of taurine and the pattern of placental taurine transfer led us to re-evaluate the potential for a synthesis of taurine in the human term placenta by assessing the placental expression of cysteine sulfinic acid decarboxylase (CSAD).

3. The placenta 4-vessel study –methods at a glance

3.1. Design and study population

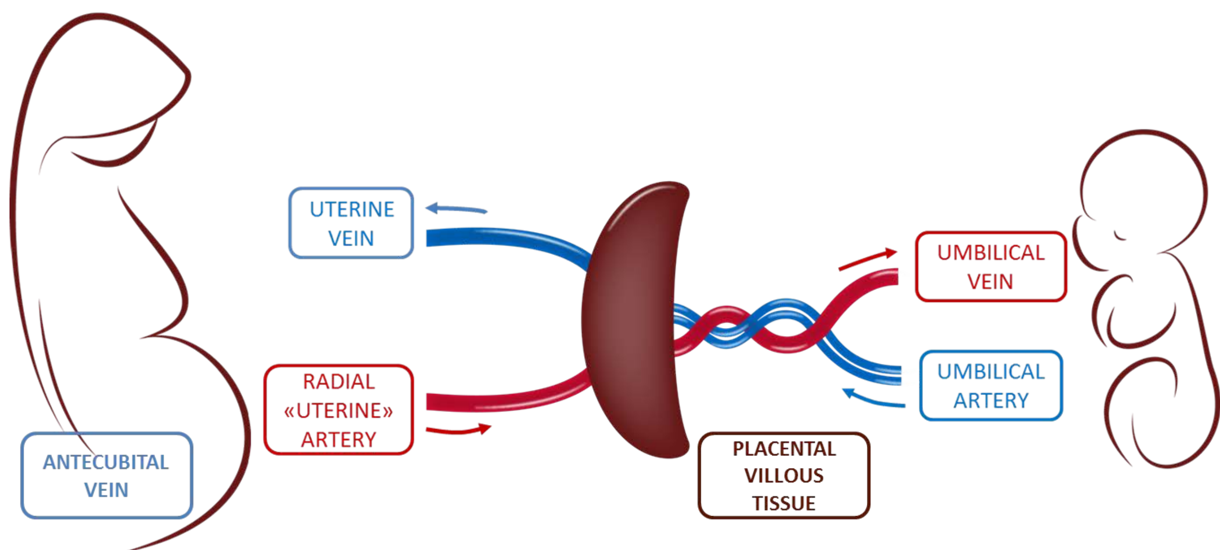
The present thesis is based on a cross-sectional *in vivo* study of 179 women who were scheduled for planned cesarean section at Oslo University Hospital, Rikshospitalet between October 2012 and June 2015. This study also included their infants. We invited healthy women with uncomplicated singleton pregnancies to participate. Exclusion criteria were significant pre-existing comorbidity, medication (other than levothyroxine and occasional use of antiallergics, antiemetics, antibiotics, and antacids), smoking, pregnancy complications and onset of labor prior to scheduled cesarean section.

3.2. Sampling method

Ultrasound measurements of blood flow in the uterine and umbilical circulations were performed in the morning before the cesarean section.

The cesarean section was performed in a fasting state, in spinal anesthesia (bupivacaine 10 mg, fentanyl 20 µg). Before the uterine incision, we simultaneously obtained maternal blood samples from the radial artery, the antecubital vein and the uterine venous plexus on the anterolateral surface of the uterus. Immediately after delivery of the infant, but before the delivery of the placenta, we obtained fetal blood samples from the umbilical artery and vein (Fig 13).

Fig 13 Sampling sites, the 4-vessel sampling method



We collected the placenta immediately after delivery. We removed the decidua and collected pieces of chorionic villous tissue (1-2 cm^3) from 4-5 sampling sites, randomly located in the center and each quadrant of the placenta.

3.3. Analyses

3.3.1. Glucose

Glucose concentrations were measured using the hexokinase/glucose-6-phosphate dehydrogenase method (Roche) at the Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet.

3.3.2. Progesterone

Progesterone concentrations were measured using an electrochemiluminescence immunoassay (Cobas) at the Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet.

3.3.3. Amino acids

Amino acid concentrations were analyzed by liquid chromatography–tandem mass spectrometry at the Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo. We identified each amino acid by MS, corresponding to each particular internal standard, and we determined the concentration from the ratio of analyte peak area/internal standard peak area against a linear multiple point calibration curve as previously described (138).

3.3.4. Hemoglobin

Hemoglobin (Hb) concentrations were analyzed in whole blood directly after sampling using photometric techniques. Hb in maternal blood was measured in a blood gas analyzer (Radiometer ABL825 Flex) at the Department of Intensive Care Medicine, Oslo University Hospital, Rikshospitalet. Hb in fetal blood was measured in a Sysmex hematology analyzer at the Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet.

3.3.5. CSAD expression

The placental expression of CSAD mRNA was analyzed by Quantitative Real-time polymerase chain reaction (qRT-PCR), at the Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo. We used beta-actin as an endogenous control.

The placental expression of CSAD protein was analyzed using western blot analyses at the Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo. We used both a commercial mouse anti-human CSAD antibody and a custom made sheep anti-mouse CSAD antibody, which has been shown to react against human CSAD protein (139). We used mouse liver homogenates and recombinant CSAD protein as positive controls, and peptide competition as a negative control.

3.4. Definitions and calculations

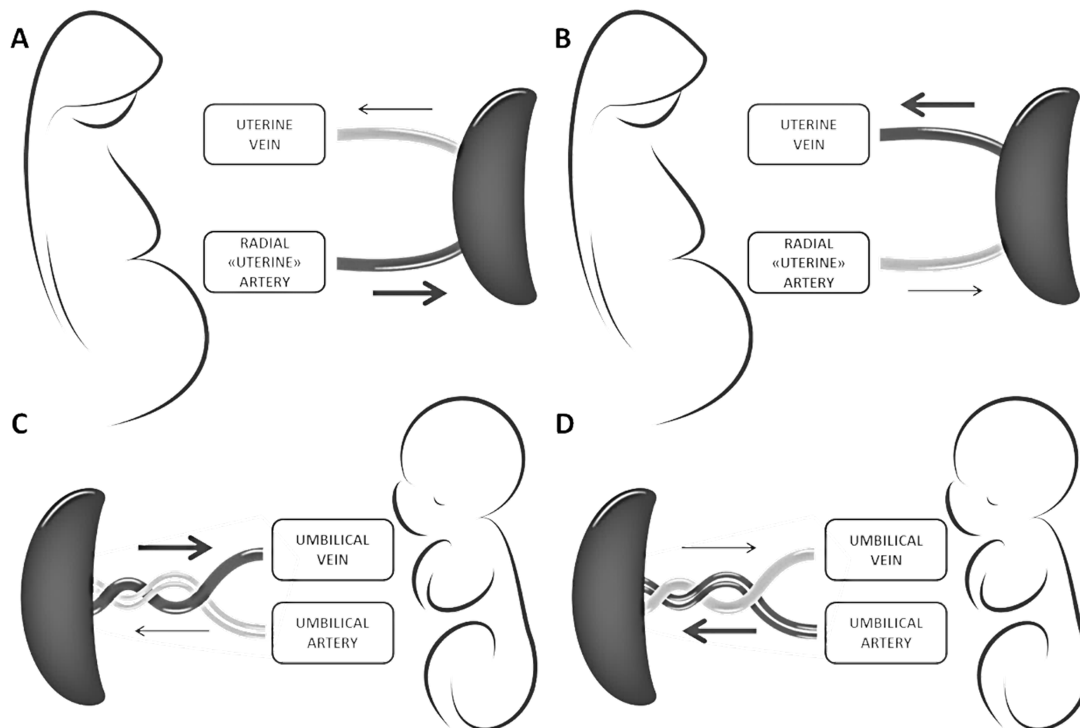
3.4.1 Uteroplacental arteriovenous differences and umbilical venoarterial differences

The concentration of a given substance in the radial artery was defined as the maternal supply (µmol/L) to the placenta. The concentration of a given substance in the umbilical vein was defined as the umbilical supply (µmol/L) to the fetus.

The uteroplacental arteriovenous (A-V) concentration difference (µmol/L) for any given substance was calculated as the difference between the concentration in the radial artery and the uterine vein. A higher concentration in the radial artery compared to the uterine vein was interpreted as a uteroplacental uptake (µmol/L) from the blood passing in the maternal circulation (fig 14 A). A higher concentration in the uterine vein than in the radial artery was interpreted as a uteroplacental release (µmol/L) to the maternal circulation (Fig 14 B).

The umbilical venoarterial (v-a) difference (µmol/L) for any given substance was calculated as the difference between the concentration in the umbilical vein and the umbilical artery. A higher concentration in the umbilical vein than in the umbilical artery was interpreted as a fetal uptake (µmol/L) from the blood passing in the umbilical circulation (Fig 14 C). A higher concentration in the umbilical artery compared to the umbilical vein was interpreted as a fetal release (µmol/L) to the umbilical circulation (Fig 14 D). By definition, a fetal uptake corresponds to a placental release to the umbilical circulation, while a fetal release corresponds to a placental uptake from the umbilical circulation.

Fig 14 A) Uteroplacental uptake from the maternal circulation B) Uteroplacental release to the maternal circulation C) Fetal uptake from (placental release to) the umbilical circulation D) Fetal release to (placental uptake from) the umbilical circulation



3.4.2. Mass uptake and release in the fetal-placental unit

We calculated the blood flow (Q) in the uterine arteries and the umbilical vein as

$$Q = h \times \left[\frac{D}{2}\right]^2 \times \pi \times TAMX$$

where D is the vessel diameter, TAMX is the time averaged maximum velocity and h is the coefficient for the spatial blood velocity profile (0.5 for the umbilical vein and 0.6 for the uterine artery (29, 140, 141).

Based on Fick's principle we calculated the uteroplacental mass uptake or release in µmol/min by multiplying the uterine blood flow with the uteroplacental A-V difference. We calculated the fetal mass uptake or release in µmol/min by multiplying the umbilical blood flow with the umbilical v-a concentration difference. We calculated the positive or negative placental consumption of taurine as the difference between the uteroplacental and the fetal uptake or release.

3.4.3. Adjustment for up-concentration of hemoglobin across the placenta

In representative subsamples in our study (maternal circulation n=40, umbilical circulation n=30) we studied the Hb concentrations in the incoming and outgoing vessels on both the maternal and fetal sides of the placenta during maternal fasting. The median [Q1, Q3] Hb concentration was slightly higher in the uterine vein (11.1 [19.2, 11.9] g/dL) compared to the radial artery (11.1 [9.8, 11.8] g/dL), with a median [Q1, Q3] uterine vein vs. radial artery Hb ratio of 1.017 [1.002, 1.029] (range 0.98-1.06). The median [Q1, Q3] Hb concentration was higher in the umbilical artery (15.8 [15.1, 16.8] g/dL) compared to the umbilical vein (15.2 [14.1, 16.1] g/dL), with a median [Q1, Q3] umbilical artery vs vein Hb ratio of 1.048 [1.01, 1.077] (range 0.94-1.16).

It is highly reasonable to assume that Hb is not taken up or released by the syncytiotrophoblast or the fetal endothelium when the blood passes the placenta. A possible explanation for the observed up-concentration of Hb across the placenta in both the maternal and umbilical circulation is therefore a net transfer of water between the maternal, placental and fetal compartments. The observed Hb ratio between the uterine vein and the radial artery is consistent with a uteroplacental uptake of water from the maternal circulation. The Hb ratio between the umbilical artery and vein is consistent with a fetal uptake from the umbilical circulation. We observed similar results for albumin concentrations across the placenta. Albumin is another compound which is considered not to be taken up or released by the placenta in significant amounts. Regardless of the cause of the up-concentration of Hb and albumin, we argue that this phenomenon also will influence the A-V and v-a concentration differences of other substances across the placenta. In order to take this into account, we divided the different amino acid concentrations ([X] for amino acid X) in the uterine vein and umbilical artery in all the mother-fetus pairs in the study, by the median maternal and fetal Hb ratio, respectively, to adjust the A-V and v-a concentration differences:

$$Uteroplacental\ arteriovenous\ difference\ [X] = [X]_{radial\ artery} - \left(\frac{[X]_{uterine\ vein}}{1.017}\right)$$

and

$$Umbilical\ venoarterial\ difference\ [X] = [X]_{umbilical\ vein} - \left(\frac{[X]_{umbilical\ artery}}{1.048}\right)$$

3.5. Ethics approval

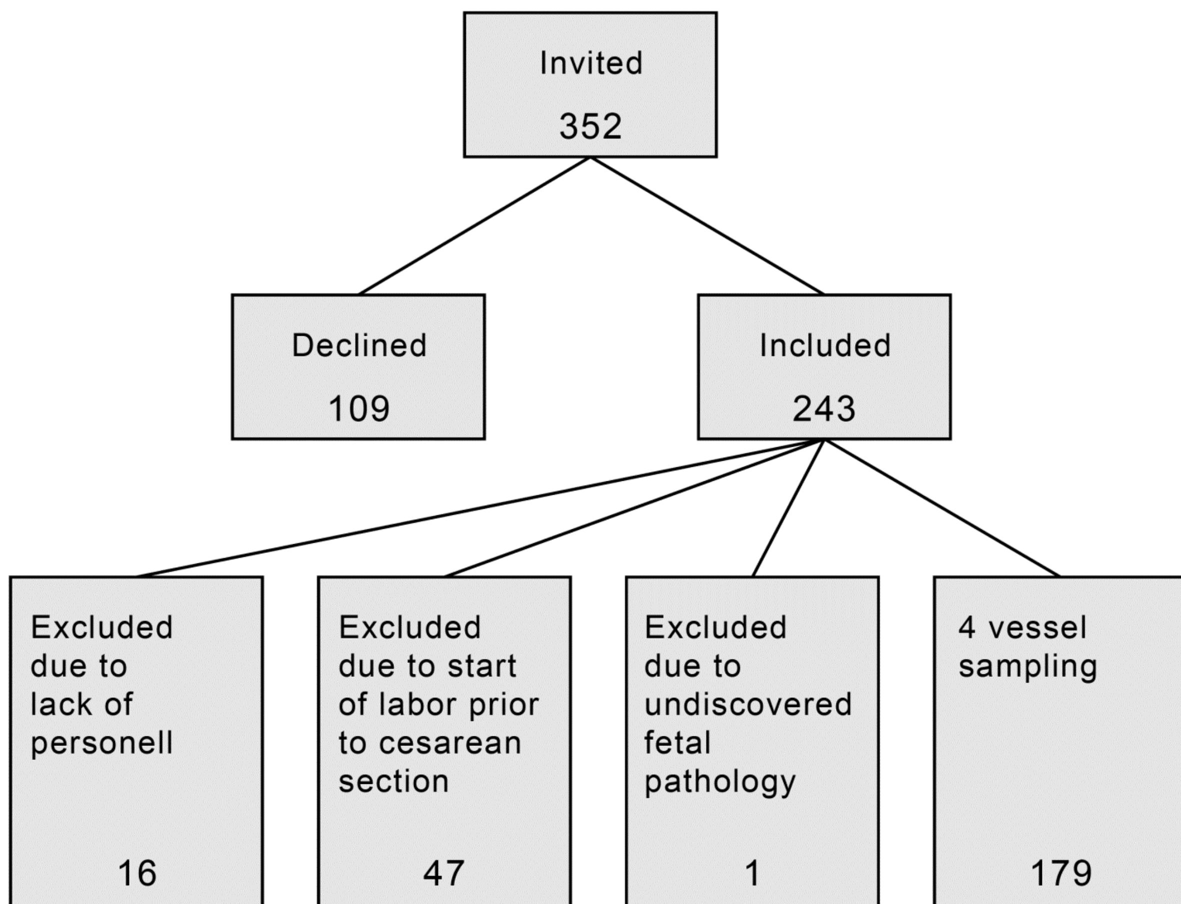
All participants signed a written informed consent. The study was approved by the data protection officials at Oslo University Hospital and the Regional Committee for Medical and Health Research Ethics, Southern Norway (#2419/2011).

4. Summary of the results

In vivo studies of the human placenta are important to test current experimentally based concepts and to generate new hypotheses regarding the functions of this unique organ. However, *in vivo* investigation of the human placenta is highly challenging. In **paper I** we described and visualized the placenta 4-vessel sampling method. A video of the method is published at https://www.jove.com/video/55847.

We here demonstrated that the sampling method is feasible in a busy clinical setting, but that it is time consuming and technically challenging. Of the 352 women invited to participate in our study, blood samples were obtained from 179 healthy mother-fetus pairs (Fig 15). We obtained complete 4-vessel sampling and good quality blood flow measurements of both maternal and fetal vessels in 70 mother-fetus pairs.

Fig 15 Flow chart of inclusion



Paper I further exemplified how our method may be employed to investigate different aspects of placental functions. We demonstrated a placental *transfer* of glucose from mother to fetus,

with a uteroplacental uptake from the maternal circulation of 0.29 [0.13, 0.41] mmol/L and a fetal uptake from the umbilical circulation of 0.54 [0.29, 0.75] mmol/L. The fetal mass glucose uptake from the umbilical circulation was 0.10 [0.05, 0.15] mmol/minute.

We demonstrated a placental *uptake* of glutamic acid from both the maternal and fetal circulations; a uteroplacental uptake of 10.4 [1.6, 21.2] µmol/L from the maternal circulation, and a placental uptake of 8.7 [-0.2, 16.0] µmol/L from the umbilical circulation.

We also demonstrated an expected placental *release* of progesterone of 1187 [404, 1855] nmol/L to the maternal circulation.

In **paper II** we determined and assessed the relationship between the concentrations and arteriovenous differences of 19 circulating proteogenic amino acids on the maternal and fetal side of the placenta in the same healthy mother-fetus pairs at term.

We observed a net fetal uptake of 14 amino acids from the blood passing in the umbilical circulation (µmol/L), adjusted for the up-concentration of Hb across the placenta. Most of the essential amino acids showed a fetal uptake. Glutamic acid was taken up by the placenta from the umbilical circulation. On the maternal side of the placenta, we observed a net uteroplacental uptake of glutamic acid, arginine, and the essential amino acids leucine and isoleucine from the blood passing in the maternal circulation, adjusted for the up-concentration of Hb across the placenta. We further observed a uteroplacental release of glutamine, glycine and tyrosine to the maternal circulation.

For each of the amino acids, except tryptophan, we observed an expected correlation between the concentration in the umbilical vein, representing the umbilical supply to the fetus, and the concentration in the radial artery, representing the maternal arterial supply to the placenta (r_s= 0.24-0.79, $p<0.001$-0.002; glutamic acid: r_s=-0.29, p=0.01). However, neither the concentration in the umbilical vein nor the fetal uptake of the different amino acids from the umbilical circulation was correlated with the uteroplacental uptake from the maternal circulation. Further, only the fetal uptake of leucine (r_s=0.31, $p<0.001$) and alanine (r_s=0.27, p=0.001) was significantly correlated with the maternal concentrations in the radial artery. The limited relation between umbilical supply and uptake of amino acid in the fetus on one hand, and uteroplacental uptake from the maternal circulation on the other hand, illustrates the complex relationship between amino acid uptake in the placenta and transfer to and uptake in

the fetus. Our findings highlight the role of placental metabolism and properties in the immediate government of amino acid transfer to the healthy human term fetus.

In **paper III** we studied transfer of the non-proteogenic amino acid taurine between the maternal, placental and fetal compartments in healthy term pregnancies. It is currently assumed that the primary role of the placenta in this regard, is to secure fetal demand through transfer of taurine from maternal plasma. However, human studies of placental taurine transfer *in vivo* are scarce.

We observed a median [Q1, Q3] uteroplacental release of 0.7 [-2.1, 6.2] µmol/min to the maternal circulation, and a median [Q1, Q3] fetal uptake of 4.0 [-1.1, 8.9] µmol/min from (i.e., a placental release to) the umbilical circulation. We further observed a median negative placental consumption of taurine (-8.9 [-12.8, 3.6] µmol/min). These results demonstrated an unexpected bilateral placental release of taurine to both the maternal and fetal circulations. The median [Q1, Q3] taurine concentration in placental tissue was 67.8 [57.3, 76.0] µmol/mg placental protein. Contrary to our hypothesis, increased umbilical plasma concentrations and uptake of taurine by the fetus were neither associated with increased maternal arterial plasma concentrations, with uteroplacental uptake from the maternal circulation, nor taurine concentrations in placental tissue. Conversely, increased fetal uptake and concentrations in the umbilical vein was associated with the uteroplacental release of taurine to the maternal circulation ($r=-0.19$, $p=0.02$ and $r=-0.24$, $p=0.003$, respectively). The concomitant bilateral placental release of taurine observed in fasting women, indicates that the placenta has the capacity to be a source of taurine in healthy term pregnancies.

The unexpected pattern of placental taurine transfer led us to re-evaluate the prevailing view that human placental tissue lacks the ability to synthesize taurine. To this end, we studied the placental expression of both CSAD mRNA and protein. Whereas the qRT-PCR analyses clearly demonstrated expression of CSAD-mRNA in the human term placenta, the western blot analyses using two different antibodies gave us somewhat conflicting results. Taken together, however, our results may suggest a potential for taurine synthesis in the human placenta.

5. Discussion

5.1. Methodological considerations

5.1.1. Study design

The placenta 4-vessel study is an observational cross-sectional study. Due to the need to access maternal and fetal vessels we could only obtain blood samples at term in normal pregnancies. Thus, our study does not provide any opportunity to study earlier stages or longitudinal changes during pregnancy. The observational nature of the design, due to both ethical and technical aspects, limits the possibility to perform controlled mechanistic studies or determine causality. However, our 4-vessel sampling method gives a unique opportunity to study human placental physiology *in vivo*, where the measured variables are resultants of all the interacting factors at play in a way that is not possible to study *in vitro* or *ex vivo*.

5.1.2. Reliability

Random errors in a study reduce the reliability of the data, i.e., the degree to which the results can be reproduced (142). Precise measurements and a sufficient sample size are two main ways to limit random error.

The coefficient of variation (%) indicates the precision of a laboratory analysis. The coefficient of variation for the amino acid analyses in the present study was below 10%, except for lysine and serine. Intra observer studies assess the precision of clinical measurements. In the present study, the intra observer variation measured as the intra class correlation coefficient for the diameter in the uterine artery was 0.97 (n= 16). The intra class correlation coefficient for the diameter in the umbilical vein has previously been reported to be 0.97 (n=381) for the same observer (143). The clinical data was plotted by two people to reduce the risk of random typing errors.

It was difficult to assess the statistical power and calculate the required sample size for the studies in the present thesis, both due to limited previous data to base such calculations on, and because of the large number of different substances that we aimed to explore. Further, when assessing concentration differences between two vessels, it is not apparent how large the concentration differences should be to be classified as “physiologically relevant”, and thus of interest to detect. We performed power calculations with standardized differences and Altman’s nomogram (144) with the aim of detecting a 10% difference between the incoming

and outgoing vessels on the maternal side of the placenta (where the concentration differences were reported to be smallest). We estimated a required sample size of 29-240 for the different proteogenic amino acids (α= 0.05, β= 0.9). For taurine, we calculated that we needed less than 20 to detect relevant concentration differences across the placenta. In addition to these estimations, we took into account the practical aspects of the sampling and the available time frame. Taken all these factors into consideration, we aimed to collect a study sample of 150-200 mother-fetus pairs.

5.1.3. Internal validity

Internal validity refers to the presence of systematic errors (bias) due to collection, analysis or interpretation of the data (145).

Selection bias

Selection bias is systematic difference between the characteristics of the people selected for a study and the population from which the samples are drawn (145). This may be caused by inappropriate sampling, missing data or loss of study objects.

Due to the necessity to access the uterine vein, we could only include women scheduled for planned cesarean section. The main indications for cesarean section among the healthy women eligible for inclusion in our study were two previous cesarean sections or the mother's own request due to earlier traumatic delivery. Few healthy first time mothers are delivered by cesarean section, except in the case of breech presentation. Consequently, the women in the present study are older, with higher parity than average. Both age and parity have an effect on fetal growth (146), and this could therefore cause bias in our study. Provided that they were healthy (i.e., absence of diagnosed disease), we included women across the range of BMIs and metabolic status. However, vaginal delivery is often recommended in cases of obesity and gestational diabetes, which may have limited and biased the recruitment of the outer ranges of these physiological spectra.

The missing data in the current study was mainly caused by lack of qualified personnel. The ultrasound flow measurements, in particular, were carried out by one observer and were consequently entirely dependent on this person's availability. Such selection of data must be considered as random. However, the ultrasound measurement of the diameter in the uterine artery is technically highly challenging, and we did observe a slightly higher BMI in the group of women with unsuccessful flow measurements compared to the women with complete flow measurements (median 28.9 vs. 27.5 kg/m^2, respectively, p=0.04). This may

cause bias in our flow sub sample. Another source of missing data was unsuccessful blood sampling, most often from the umbilical or radial artery. We observed higher maternal BMI (median 28.6 vs 26.2 kg/m^2, p=0.03), and lower birthweights (mean 3326 vs 3571 g, p=0.03) and placental weights (523 vs 626 g, p<0.001) in the maternal-fetus pairs lacking blood from the umbilical artery, thus this could also be a source of selection bias.

Loss of study objects was not an issue in the current study, due to the cross-sectional design.

<u>Information bias</u>

Information bias, or alternatively, detection bias, occurs in the case of measurement error or if the data has not been collected in the same way for all study subjects (142).

In order to limit measurement errors and inter observer variability, we limited the number of personnel conducting the sampling and analyses. As mentioned previously, only one highly skilled person performed the ultrasound flow measurements, and one highly qualified person performed all the amino acid analyses. To adjust for any day-to-day variation in the analyses, we normalized all of the amino acid data to the mean value of two quality control samples.

The flow measurements were performed as close in time to the surgery as possible. However, the uteroplacental (and possibly the fetal-placental) blood flow may vary over time or be affected by different aspects of the surgery. Preliminary data from the intra observed study of the blood flow measurements showed no significant change in the blood flow in the uterine artery or in the umbilical vein measured with a two hours interval. Furthermore, preliminary data from a sub group in our study showed no significant alteration in cardiac output before and after spinal anesthesia.

Our estimates of uteroplacental amino acid uptake and release in the maternal circulation are based on concentration differences between the uterine vein and the radial artery, as a proxy for the uterine artery. The radial artery is easier to access, and we assumed that the composition of blood is similar throughout the entire arterial part of the circulation. The uterine vein, on the other hand, drains the uterine tissue in addition to the placenta, and we can therefore not exclude a uterine contribution to our observations.

Another possible source of bias/error in our measurements is the observed up-concentration of Hb across the placenta. It is likely that this phenomenon also applies to the placental A-V and v-a differences of the amino acids. Consequently, when we calculated the differences in amino acid concentration, we also considered how an adjustment for the Hb up-concentration

would affect the results. Unfortunately, we did not obtain Hb measurements in all of our mother-fetus pairs. We were therefore not able to adjust the amino acid concentration differences based on individual Hb-measurements in the studies included in the present thesis. Rather, we used the median Hb ratios measured in the subsample. Since there is no evidence of Hb production in the placenta, the cause of the up-concentration of Hb across the placenta is most likely due to net water exchange between the maternal, placental and fetal compartments at the time of sampling.

5.1.4 External validity

External validity, or representativity, describes to which degree the findings in a study can be generalized to populations outside the study sample (145). We wished to study placental physiology in healthy pregnancies across the whole range of metabolic conditions, including BMIs, birthweights, glucose levels, etc. The fact that we did not exclude women based on such criteria increases the study's external validity, as the pregnancies of the women included in our study reflect the whole spectrum of what we consider as normal gestational physiology. However, the current study was carried out at Oslo University Hospital, Rikshospitalet. The women giving birth at this hospital (with the exception of those referred due to maternal or fetal pathology) are generally older than the average pregnant Norwegian woman, with higher education and lower BMI (147). Further, some aspects of placental and fetal physiology may vary between different geographical regions and ethnic groups. For instance, the placental activity of system A and L amino acid transporters in pregnancies complicated by diabetes and fetal overgrowth has been reported to be different in British and Swedish women, and Jansson and colleagues have consequently suggested that "there may be population differences in the placental response to maternal metabolic disease" (103). This must be kept in mind when comparing our findings with other studies.

The present thesis aimed to study placental physiology under normal, physiological conditions without any manipulation or intervention. Although placental physiology presumably is less affected by cesarean section than by the stress of vaginal delivery, the effects of surgery and anesthesia, as well as the uterine incision and delivery of the fetus, may still alter the arteriovenous and venoarterial concentration differences compared to the normal condition *in utero*. All of the women in the present study had been fasting for minimum 6, and median 9 hours at the time of the blood sampling. Despite the gradual decline in maternal and fetal umbilical vein glucose levels during fasting, we considered the women to be in a basal

euglycemic state (mean glucose (SD): 4.41 (0.44) mmol/L, median insulin [Q1, Q3]: 51.7 [34.2, 77.2] pmol/L). The duration of fasting did not influence the measured amino acid concentrations or concentration differences in the present thesis. Milsom and colleagues reported that about half of the proteogenic amino acids were significantly lower in fasting compared to fed individuals in a study of non-pregnant women (148). In pregnant sheep, fetal uptake from the umbilical circulation did not appear to change qualitatively or quantitatively during a prolonged fast (149). Nevertheless, it is conceivable that we could have observed a different picture if the samples had been obtained in the postprandial state. For instance, we observed a net release of non-essential, but not of essential amino acids across the forearm of the women in our study (determined as the concentration difference between the radial artery and the antecubital vein, data not shown), which may reflect a moderate catabolic state at the time of sampling. Alanine and glutamine, in particular, showed a considerable release in the forearm, while glutamic acid showed a large uptake. In sum, although euglycemia prevails for a large proportion of the day, a state of prolonged fasting does usually not occur in the healthy pregnant woman, and this could influence the transmissibility of our results to the "normal, physiological conditions" that we aimed to study.

5.1.5. Statistical considerations

The distributions of most of the amino acid concentrations are skewed, and we have therefore employed non-parametric statistics, i.e., paired comparisons with Wilcoxon sign rank tests and correlation analyses with Spearman's rank correlation coefficient. In general, a two-sided p-value <0.05 was considered significant. In paper II, however, we performed a large number of Spearman correlations. Consequently, we adjusted for multiple testing by controlling the false discovery rate (FDR) based on the method of Benjamini-Hochberg (150). Due to the exploring nature of this particular study, we chose the FDR-adjustment in order to avoid "over correcting" and thus lose possibly important biological information. For the same reason, we used a FDR significance level of 0.1, which is an accepted approach when adjusting for multiple testing in big data analyses.

5.2. Interpretation of the results

5.2.1. The 4-vessel approach to study the human placenta *in vivo*

Paper I demonstrates how an integrative *in vivo* approach to study the human placenta at term can be performed. A large part of the work with the present thesis has been to establish the 4-

vessel sampling method at our facilities and to collect an extensive biobank. Of the 352 eligible women approached for inclusion between October 2012 and June 2015, we obtained blood samples of 51%. The main reasons for loss of participants after inclusion, was start of labor prior to caesarean section and lack of sufficient personnel to conduct the study (figure 15). A minimum of seven different researchers and members of the hospital staff were involved in different tasks in each sampling procedure, in addition to the operating team performing the surgery. Of the 179 women subjected to blood sampling during their cesarean section, 43 % was considered as "complete", i.e., that we obtained ultrasound blood flow measurements and blood samples from all four vessels. This illustrates the challenging logistics and time-consuming nature of this type of study, and the amount of resources needed to establish a biobank of material obtained from human mother-fetus pairs in vivo.

Paper I also shows how the 4-vessel sampling method can be used to study different aspects of placental functions in both healthy and pathological pregnancies. We wished to describe and visualize the logistically challenging method to endorse more *in vivo* studies of the human placenta. It is particularly time consuming to collect human *in vivo* data from pathological pregnancies, and a standardization of the sampling method makes it easier to pool and compare data from different studies. The amino acid data from our large sample of healthy pregnant women presented in paper II and III may thus form a reference material which in the future can be compared with samples from other studies of both healthy and pathological pregnancies. Hence, our collected samples might make it possible to better understand the complex relationships between maternal amino acids supplied to the placenta, provision of amino acids to the fetus and the fetal and placental utilization of amino acids.

5.2.2. Large individual variations in amino acid concentrations and flux

In the current study of healthy human term pregnancies, there were considerable individual variations in the amino acid concentrations in all four vessels and in the A-V and v-a differences. This is in agreement with previous human and animal studies of amino acid concentrations and concentration differences across the placenta (66, 96, 127, 151-157). There have also been reported large variabilities in plasma amino acid concentrations in non-pregnant women, both in a fasting and post prandial state (148). We have employed the current gold standard method for amino acid analyses (158), and the coefficient of variation for the amino acid analyses in the current study was generally acceptable. It is therefore most likely that the individual variability we, and others, observe in the human reflects the

biological variations in normal, healthy pregnancies, notably after a prolonged fast. For all the amino acids, including the proteogenic as well as taurine, we observed both a uteroplacental uptake and release, and a fetal uptake and release in different mother-fetus pairs. The large individual differences in the flux of amino acids across the two layers of the placental membrane in human term pregnancies, suggest that placental amino acid transfer is a highly dynamic process, altering between placental and fetal uptake and release. The fact that this large diversity in placental amino acids fluxes occurs even in a basal (fasting) state may indicate that there are considerable inherent variabilities in the placental properties regarding metabolism and transfer of amino acids in different individuals.

5.2.3. Proteogenic amino acids: The relationship between supply and uptake of amino acids in the fetal-placental unit

Both ovine and human studies, including the present thesis, have demonstrated a positive correlation between the concentrations of proteogenic amino acids in the maternal and fetal circulations, indicating that the supply of amino acids to the placenta in the maternal circulation is an important determinant of the supply to the fetus in the umbilical circulation (66, 96, 159). This relationship between maternal and fetal amino acid concentrations has also been interpreted to indicate a direct link between the placental uptake of amino acids from the maternal circulation and the fetal supply and uptake from the umbilical circulation (62). In paper II, we therefore expected to find an association between the umbilical amino acid concentrations and fetal uptake (µmol/L) from the blood passing in the umbilical circulation, and the uteroplacental uptake of amino acids from the maternal circulation. Interestingly, there was no relationship between the fetal supply or fetal uptake respectively, and the uteroplacental uptake in our healthy term mother-fetus pairs. Despite an extensive fetal uptake of amino acids from the umbilical circulation we only observed a net uteroplacental uptake of a few amino acids from the mother. This finding is in contrast to observations in both fasting and non-fasting pregnant sheep, where studies of arteriovenous differences across the placenta have shown that, for many amino acids, the uterine uptake nearly matches the simultaneously determined umbilical uptake (66, 160).

The strong correlation between maternal and fetal concentrations of proteogenic amino acids in the present and previous studies implies that the maternal nutritional state is related to the fetal nutritional environment. However, the data presented in paper II suggest that in the human this is a result of net uptake or “influence” over time, rather than a moment-to-moment

management of amino acid transfer. At least in a fasting state, the net trans-placental amino acid transport from the mother to the fetus seems to be limited, indicating that the placenta plays an important role in the immediate provision of amino acids for the fetus. Interconversions of amino acids, amination of keto acids, and breakdown of proteins may explain how the placenta can be an immediate source of both essential and non-essential amino acids for the fetus. For instance, tracer studies in ovine pregnancies have shown that approximately half of the leucine flux from the placenta to the fetus is produced within the placenta through breakdown of placental proteins (161). Similar studies in humans found that 10% of fetal plasma leucine is derived from protein breakdown within the placenta (62). Our *in vivo* observations support a concept where the immediate placental transfer of proteogenic amino acids to the human fetus to a large degree is determined by placental metabolism and properties, and not primarily by the uptake from the mother. This is in line with the variable results from studies in humans and animals on the effect of maternal infusions of amino acids on fetal delivery and uptake (134, 162-164).

It is interesting to note that we made similar observations regarding the placental transfer of glucose in the present human *in vivo* study. Although maternal and fetal glucose concentrations are shown to be closely linked (128), we did not observe any correlation between the fetal uptake from the umbilical circulation (µmol/L) and maternal plasma concentrations or the uteroplacental uptake of glucose from the maternal circulation (38). These lacking correlations may be caused by the considerable portion of glucose that is metabolized by the placenta. It is often assumed that controlling maternal plasma glucose levels will secure control of fetal glucose uptake during gestation. However, this assumption does not take into account the consumption and conversion of nutrients that occurs in the placenta. Individual variation in placental consumption of glucose may at least partly explain why diabetic mothers with apparently well controlled blood glucose still deliver large for gestational age infants. Together, the *in vivo* findings in the placenta 4 vessel study highlight the placental metabolism as key in the government of maternal-fetal transfer of nutrients.

5.2.4. Transfer of taurine between the maternal, placental and fetal compartments

In similarity with the proteogenic amino acids, maternal and fetal concentrations of taurine have been reported to be correlated (159). Based on this relation, as well as results from animal studies, maternal taurine supply has been suggested as an important determinant of fetal taurine levels and uptake (165-168). In paper III, however, we observed no relationship

between maternal and fetal taurine concentrations. Furthermore, our findings did not confirm our initial hypothesis of an association between increased umbilical plasma concentrations and uptake of taurine by the fetus, and increased maternal arterial plasma concentrations, uteroplacental uptake from the maternal circulation and placental concentrations of taurine. Despite the large individual variations in the flux across both the MVM and BM of the syncytiotrophoblast, our data from healthy term pregnancies, notably after a prolonged fast, rather showed a median concomitant bilateral placental release of taurine to both the maternal and fetal circulations. The fact that we observed a highly significant correlation between the taurine flux across the MVM and across the BM in the same placentas suggests that properties of the placenta itself are governing the bilateral placental release. Interestingly though, the placental release was independent of the taurine concentration in the placental tissue. This observation may seem counterintuitive, since the high taurine concentration in placental tissue is regarded as the main driving force for the flux of taurine across the BM towards the fetus. However, the taurine concentration was on average 27 times higher in placental tissue compared to the umbilical vein, and it is plausible that this concentration difference is much higher than what is required to facilitate taurine transfer to the fetus. Thus, the level of taurine in the umbilical circulation may not necessarily be directly related to the amount of taurine in the placental tissue. The data presented in paper III indicates that other factors than placental taurine concentrations determine the flux of taurine across the MVM and BM of the syncytiotrophoblast. The paper discusses several possible explanations for the concomitant bilateral placental taurine release, including taurine's role in maintaining placental function and homeostasis.

5.2.5. The unresolved question of taurine synthesis in the human placenta

The bilateral placental release of taurine described in paper III indicates that the placenta has the capacity to be an immediate source of taurine in healthy term pregnancies. We and others have shown that human placental tissue contains very high levels of taurine (52), which may be a result of placental uptake and accumulation across gestation or in the postprandial state. The high placental taurine concentrations could also be due to biosynthesis of taurine in the placenta itself. Decarboxylation of cysteine sulfinic acids via the enzyme CSAD is considered to be a major pathway for taurine synthesis in humans, although other pathways also may be involved (72, 76, 169). In paper III we were able to convincingly demonstrate the expression of CSAD mRNA in human placental tissue. Our findings agree with the recent observations made by Korneeva and colleagues (77), and challenge the prevailing view that the placenta

lacks taurine synthesizing enzymes. Together, our data suggest a potential for biosynthesis of taurine in the human placenta. However, we were not able to firmly conclude that the placental CSAD mRNA was translated into protein. The western blot analyses of CSAD protein expression were performed with both commercial and custom made antibodies, but the results varied between different western blots. This is in accordance with data presented in the Human Protein Atlas, showing "highly variable immunohistochemical staining in trophoblastic cells in sections of human placenta" (https://www.proteinatlas.org/). There might be several reasons for these inconclusive results. Winge and colleagues described unspecific staining of the CSAD protein with commercial antibodies (139), and we cannot exclude this possibility when we stained our western blot with the commercial anti-CSAD antibody from Abcam. The custom made antibody that we used was purified against glutamate decarboxylase-like protein 1 (GADL1), which are known to show cross reaction with CSAD (139). Contaminating anti-GADL1 could thus be a possible source of false positive CSAD expression both in the western blots using the commercial antibodies in the present study, and in the analyses of data presented in the Human Protein Atlas. Interestingly though, the enzyme GADL1 has been shown to demonstrate cysteine sulfinic decarboxylase activity, potentially being involved in taurine biosynthesis (169). Therefore, any detection of GADL1 instead of CSAD in the present study, might still suggest the possibility of placental taurine synthesis.

The potential discrepancy between CSAD mRNA and protein expression could of course also be due to lacking or variable translation of CSAD mRNA in the syncytiotrophoblast. It is, for instance, possible that taurine synthesis in the placenta is only conditionally induced, like in cases of low taurine supply in the maternal circulation or high fetal or placental taurine needs.

It may seem paradoxical that the placenta should spend energy to actively transport taurine across the MVM, if the placenta itself is able to synthesize this amino acid. However, the contribution of uptake versus a possible synthesis to the placental taurine pool, and thus the taurine available for transfer to the fetus could for instance vary across gestation and even according to the prandial status. The ability to synthesize taurine gradually increases after birth in most mammalian species (72). It is conceivable that healthy placentas at term with an abundance of substrate available may be able to synthesize taurine, while they depend on taurine uptake from the maternal circulation at earlier gestational stages. All the same, although the data regarding CSAD protein expression in the human placenta is not conclusive

as yet, the prevailing view in the literature claiming that there is no taurine synthesis in the human placenta is not necessarily valid. More studies are clearly needed.

5.2.6. Amino acid transfer and placental properties

The discrepancy between fetal uptake of amino acids from the umbilical circulation and the uteroplacental uptake from the maternal circulation was a striking finding in both paper II and III. Our findings highlight the role of placental metabolism and properties, and possibly also demand, in the immediate government of amino acid transfer to the healthy human term fetus. In evolutionary terms, development of effective placental adaptions to periods of variable maternal nutrition may have been an advantageous trait. Given this, several questions arise, including which cues govern these adapting properties, as well as the placental functions in general.

There are different theoretical models presented to explain how the placenta responds to changes in the maternal and fetal nutritional environment (93). In the placental nutrient sensing model, the placenta responds to maternal nutritional signals, representing a mechanism by which fetal growth is matched to the maternal resources. The fetal demand model, on the other hand "represents a classic homeostatic mechanism by which the fetus compensates for changes in nutrient availability by regulating nutrient supply (i.e. placental transport) in the opposite direction" (93). Emerging evidence, however, indicates that the placenta actively responds to both maternal and fetal nutritional and metabolic cues, and that it integrates this information with information from intrinsic nutrient sensing signaling pathways to match fetal demand with maternal supply (46).

Signals from the placenta itself may also regulate placental functions. There is a wide range of hormones, cytokines and other autocrine/paracrine signaling molecules produced and secreted by the placenta that may both alter the properties of the placenta itself, and also affect the maternal and/or fetal milieu. These responses may in turn influence the placental functions (50). Further, placenta-specific genomic imprinting, i.e., epigenetic suppression of alleles inherited from one parent that causes genes to be expressed in a parent-of-origin-dependent manner, is thought to be involved in the regulation of fetal growth and development (15, 170). It should be emphasized that the placenta carries out the function of several adult organs during pregnancy, like the lungs, the liver, the kidneys, the endocrine system, and the immune system. It is therefore likely that the placenta's own requirements of energy and substrates may be of vital importance to maintain its functions.

Placental efficiency and transfer of proteogenic amino acids

Placental efficiency is often defined by the fetal-placental weight ratio (birthweight/placental weight), i.e. g fetus produced per g placenta (171). When we studied the sum of all 19 proteogenic amino acids in our material, we found a negative correlation between placental efficiency and the maternal uteroplacental A-V difference, and a positive correlation between placental efficiency and the fetal umbilical v-a difference (unpublished data, see Table 1 in appendix). These results suggest that more efficient placentas are associated with lower uteroplacental amino acid uptake or a net release (µmol/L) to the blood passing in the maternal circulation, and increased fetal uptake/placental release to the blood passing in the umbilical circulation. Less efficient placentas, on the other hand, are associated with increased uteroplacental uptake or a lower release to the blood passing in the maternal circulation, and increased fetal release/placental uptake from the umbilical circulation.

Our observations indicate differential placental properties at term in healthy pregnancies. The functional state of some placentas is such that amino acids are effectively delivered to the fetus, whereas other placentas possibly have adapted to a state of low release or net uptake from both the maternal and fetal circulations. It has been suggested that also in normal pregnancies the placenta may reach its physical limits towards term, when placental growth through expansion of terminal villi occurs at the expense of the volume of the intervillous space (172). This could compromise the intervillous perfusion and contribute to reduced oxygen tension, syncytial stress and injuries and diminished placental capacity. It is conceivable that such placentas may require more amino acids to maintain normal structure, metabolism and functions, like transfer of glucose and oxygen and hormonal production. This is an example of how placental properties and demand may adjust placental transfer, also, at least temporarily, at a certain expense of the fetus.

Placental efficiency and transfer of taurine

In accordance, we observed that placental efficiency was positively correlated with taurine concentrations in the umbilical vein and the fetal umbilical v-a difference, i.e., the fetal uptake/placental release (µmol/L) to the blood passing in the umbilical circulation (unpublished results, see Table 2 in appendix). We also found a positive correlation between placental efficiency and placental taurine concentrations. Thus, it seems that efficient placentas both have higher taurine concentrations and deliver more taurine to the fetus. However, these two functions are apparently independent of each other in healthy term pregnancies, since the results presented in paper III showed no correlation between placental

taurine and transfer to the fetus. Our observations regarding taurine and placental efficiency may suggest that efficient placentas are in a state of surplus; they function optimally, have sufficient amount of taurine to maintain its own health and are thus able to effectively export taurine to the fetus. Less efficient placentas, however, have lower taurine levels to uphold its health and normal functions, and thus prioritize itself in terms of retaining or accumulating taurine from both circulations.
Placental weight shows a tight negative correlation with placental efficiency expressed as fetal-placental weight ratio and is also negatively correlated with umbilical vein taurine concentrations, the fetal umbilical v-a difference and placental taurine concentrations in our material (unpublished results, see Table 2 in appendix). The concept that larger placentas are less efficient towards term, and show less delivery or even uptake of taurine and have lower placental taurine concentrations, is in line with a notion that the placenta towards term reaches its physical limits, with possible weakened placental function. Larger placentas are reported to reach the flattening of the sigmoid shaped growth curve earlier than smaller placentas (173), and are also associated with increased risk to develop late onset preeclampsia (174). Taurine is an effective antioxidant and has been shown to be vital for the health of the syncytiotrophoblast (86, 87). It is conceivable that larger placentas have higher taurine demands, and thus compensate with less release or net uptake, while the smaller placentas, notably within the "normal" ranges, are far from reaching their limits, and uphold their optimal function with both taurine delivery to the fetus and accumulation of high intracellular taurine levels.

Our observations regarding placental efficiency and amino acid transfer in human term pregnancies agree with the observations in paper II and III, arguing for the role of placental properties and demand in the immediate transfer of amino acids to the fetus in healthy term pregnancies. Further, our observations support a notion of placental adaptive mechanisms that ensure maintenance of placental structural and functional integrity. One reasonable interpretation of our findings is that placental metabolism and demand, under certain circumstances may overrule its primary task of providing amino acids to the fetus. This is biologically and evolutionary plausible because it is vital for the developing fetus that an appropriate placental function is secured, irrespective of maternal nutritional status. It is important to keep in mind, however, that this may not apply to earlier stages of gestation. Further, in cases of small placentas with overt pathology, for example compromised

uteroplacental circulation due to thrombosis and infarction, the functional adjustments discussed on the basis of the present work may not apply.

5.2.7. Placental metabolism -comparisons between animal studies and the present study on healthy pregnant women

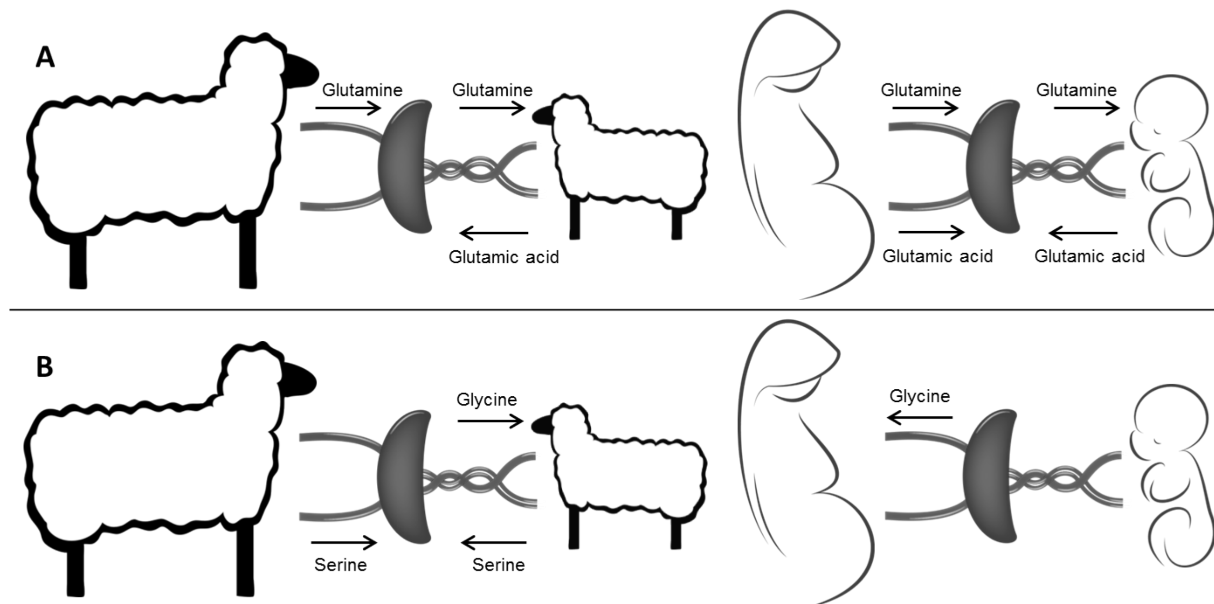
The observations in the present thesis highlight the importance of the placental metabolism in the immediate government of placental amino acid transfer. The placental metabolism alters the concentration of amino acids available for transfer to the fetus, and several interconversions and inter organ-cycles between the placenta and the fetal liver have been described in animals. It is, however, less clear to what degree these metabolic interactions are present in the human. The 4-vessel-method gives an opportunity to test to which extent the current, and mainly experimentally based, models of placental nutrient transfer can predict observations made in humans *in vivo*.

The glutamic acid-glutamine cycle is one of the inter-organ cycles between the placenta and the fetal liver described in animals (62, 64). In humans, we observed a placental release and fetal uptake of glutamine from the umbilical circulation and a fetal release and placental uptake of glutamic acid from the umbilical circulation, consistent with this inter-organ cycle. However, on the maternal side of the placenta, our human *in vivo* study differed from that of animal studies. In paper II, we showed a significant uteroplacental uptake of glutamic acid and a release of glutamine into the maternal circulation, whereas animal studies have reported no significant uteroplacental uptake of glutamic acid and/or a considerable uteroplacental uptake of glutamine (66, 175) (Fig 15A).

The serine-glycine cycle between the placenta and the fetal liver is also described in animals (62, 63). Although the transfer of glycine from the mother to the fetus has been shown to be limited in both animal models and in humans (66, 98, 132, 176), our *in vivo* data do not support a similar cycling in humans. In paper II, we observed no significant placental delivery of glycine to the umbilical circulation and no significant placental uptake of serine from the maternal or umbilical circulations (Fig 15B). This observation is consistent with results from Lewis et al., who demonstrated a much lower activity of serine hydroxymethyl transferase in the human placenta compared with the ovine placenta (68). In contrast to previous findings in sheep, we also observed a significant uteroplacental release of glycine to the maternal circulation. The correlation between this release and the glycine concentration in the umbilical

artery suggested that the uteroplacental release of glycine in humans may be a result of excess glycine in the fetal- placental unit, possibly produced by the fetus itself.

Fig 15 Findings in ovine and human pregnancies



A) Glutamine and glutamic acid B) Serine and glycine

In our study of human term pregnancies, alanine was the amino acid that showed the largest fetal uptake from the umbilical circulation, but we observed no significant net uptake from the maternal circulation. This is in contrast to a similar study in sheep, demonstrating a significant placental uptake of alanine from the maternal circulation (66). Interestingly, both human and ovine tracer studies have shown that only a smaller fraction of the maternally derived alanine is directly transported to the fetus (69, 177). This indicates that most of the alanine transferred to the fetus may be a result of placental metabolism, such as protein degradation and alanine production from pyruvate through transamination reactions. The suggested role of the placental metabolism in alanine delivery to the fetus agrees with our observation of a lacking relation between uteroplacental and fetal uptake of alanine in fasting pregnant women at term (paper II).

5.2.8. Fetal uptake of amino acids and glucose

The 4-vessel sampling method also provides the opportunity to study the relations between a large variety of nutritional and regulatory factors operating in the maternal, placental and fetal compartments. The observed relations are the resultants of a number of pathways and signal systems that are continually integrated in the *in vivo* situation. Paper I describes the fetal

uptake of glucose from the umbilical circulation. Glucose is the primary energy source for the fetus, and most of the glucose in fetal plasma at term is derived from the maternal circulation. It has been hypothesized that when energy sources other than glucose, such as amino acids, are abundantly available to the fetus, umbilical glucose uptake is decreased. This may result in additional glucose being available for placental consumption to energize placental amino acid transport. Based on this hypothesis, increased fetal amino acid concentrations and uptake should be associated with decreased glucose uptake by the fetus and increased placental glucose consumption. However, our *in vivo* human model did not support these results. Instead we observed that the fetal uptake of proteogenic amino acids (µmol/min) was positively correlated with the fetal uptake of glucose (µmol/min) and negatively correlated with the placental glucose consumption (µmol/min) (unpublished data, see Table 3 in appendix). This indicates that there is limited interchange between amino acid and glucose utilization in healthy human term fetuses. Rather, our data suggest that "more takes more", meaning that the fetuses who take up and consume the majority of one nutrient also consume more of the others. This could for instance be due to larger blood flow in the umbilical circulation and consequently a higher mass of substrates delivered to these fetuses. However, we observed the same positive correlation between amino acid and glucose v-a concentration differences (µmol/L). The positive correlation between mass uptake of amino acids and glucose by the fetus may also be a result of genetics and/or due to a programming of the fetal metabolism to high consummation of nutrients earlier in pregnancy

5.2.9. Amino acids and birthweight in humans

An adequate supply of amino acids to the fetus is fundamental for intrauterine growth and development (50). Small for gestational age fetuses are reported to have significantly lower total amino acid concentration in the umbilical circulation, as well as reduced abundance and activity of placental amino acid transporters, compared with optimal sized fetuses (50, 96). In our large sample of healthy mother-fetus pairs, the sum of proteogenic amino acids in the umbilical vein was positively correlated with birthweight (unpublished data, see Table 4 in appendix). There was no significant correlation between fetal uptake of amino acids and birthweight. This discrepancy indicates that the amino acid concentration in the umbilical vein, reflecting the nutritional environment of the fetus, has a continued impact on fetal growth and consequently is associated with birth weight. The fetal uptake of proteogenic amino acids, on the other hand, is highly dynamic according to the results from paper II, and the fetal uptake (or release) of amino acids at any given moment is thus not decisive for

birthweight. The sum of amino acids taken up or released over a period of time may indeed be correlated with birthweight, but we were unable to show this in our cross-sectional study. In addition, it is possible that the concentration of amino acids entering the fetus may have effects on fetal growth that are independent of the actual fetal uptake/consumption, for instance related to fetal insulin secretion or to other hormones/growth factors.

6. Conclusions, clinical application and further research

6.1. Conclusions

1. We have established and described the 4-vessel sampling approach to promote *in vivo* studies of the human placenta. In paper I we showed that although logistically challenging, the 4-vessel sampling method is feasible in a clinical setting. We have demonstrated that the method can be used to study and integrate different aspects of the functions of the human placenta. Furthermore, we have shown that some current experimentally based concepts regarding placental functions are challenged by the results obtained by our human *in vivo* model, underscoring the need for such studies.

2. We have shown in fasting women with healthy term pregnancies, that there are large individual differences in the flux of amino acids across the two layers of the syncytial membrane. These observations suggest that placental amino acid transfer in the human at term is a highly dynamic process, altering between uptake and release. Furthermore, the observed net uptake of most amino acids (both proteogenic and taurine) in the fetus, without a corresponding uptake from the maternal circulation, suggest that the placenta may serve as an immediate source of amino acids for the healthy term fetus. Finally, our results imply that placental properties, metabolism, and possibly demand, are more important for the instant government of amino acid transfer to the fetus than what at any time is supplied or taken up from the maternal circulation.

3. We have shown that the human placenta has the capacity for a concomitant bilateral release of taurine to both the maternal and fetal circulations, indicating that taurine may play a fundamental role in the human placental homeostasis beyond the supply to the fetus. We clearly observed expression of CSAD mRNA in human term placentas, while our data regarding placental expression of CSAD protein not were consistent. Our findings suggest a potential for taurine synthesis in the human placenta, although further studies are needed.

6.2. Clinical implications

Emerging evidence suggests a link between intrauterine developmental conditions and later health. This makes the fetal period a promising target for prevention of both perinatal and adult morbidity, and thus to reduce the burden of non-communicable diseases around the globe. The results presented in the present thesis represent a small piece in the large puzzle of trying to deduce the complexity of the intrauterine environment and fetal nutrition, with the hope to develop potent and safe interventions before birth. Observational studies in healthy populations have shown a strong impact of placental weight on the relationship between maternal metabolic state (e.g., BMI, plasma glucose and gestational weight gain) and newborn anthropometric outcomes (e.g., birthweight and percentage of body fat) (18, 178, 179). These findings, as well as morphological studies and gene expression analyses both on normal and complicated pregnancies, underscore the potential of the placenta as a target of clinical intervention.

The current thesis has focused on the physiology of the placenta in healthy term pregnancies. Insight into the range of normal placental functions is fundamental to understand, prevent and treat pregnancy complications associated with placental dysfunctions. Still, studies of placental functions have been performed in pathological settings, and conclusions related to both normal and abnormal properties have often been drawn on the basis of such studies. However, knowledge derived from pathological pregnancies is not necessarily transferable to non-complicated pregnancies. For example, the activity of the system A amino acid transporter has been shown to be positively correlated with neonatal anthropometric measures in small for gestational age-pregnancies, but inversely correlated with birth weight in pregnancies resulting in appropriate sized babies (90, 91). Guidelines for optimized fetal nutrition in normal, healthy pregnancies should therefore preferably be based on research related to normal conditions.

Taken together, we have shown that the 4-vessel sampling method has the potential to provide new knowledge that is essential to advance our understanding of the human intrauterine nutrition. It is our hope, that this acquired knowledge may ultimately form the basis for promotion of the immediate and future health of the newborn child.

6.3 Future research

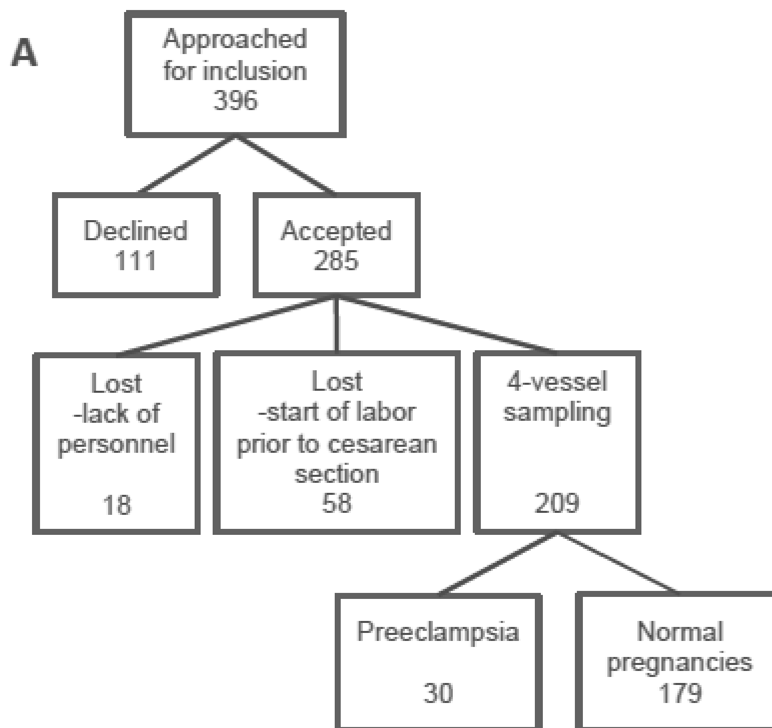
The placenta 4-vessel study provides exciting opportunities for future studies of the human placenta. *In vivo* data generated by the 4-vessel sampling method may both be employed to test current experimentally derived models, and to generate new hypotheses, which in turn may be further explored experimentally. The present thesis underscores the role of placental properties and metabolism in amino acid transfer in healthy human term pregnancies. Consequently, crucial questions for the future is to determine what governs the placental metabolic properties, transfer capacity and other placental functions, and how the maternal and fetal conditions interact with the placenta. By taking advantage of the placenta 4-vessel sampling method, we may compare uptake and release of nutrients across the placental membrane with hormones, cytokines and other signaling molecules (insulin, adiponectin, etc.) in the maternal and fetal circulations. Further, in order to understand the complex interplay between the different nutrients supplied to the fetus and their transfer across the placenta, it is of interest to integrate data on uptake and release across the placental membrane from different nutrient classes. High scale omics-methods may be applied on our study material for a more in-depth classification of the metabolites taken up and released across the placenta. More sophisticated statistical methods, like principal component analyses and other cluster studies, may be useful to discern patterns in a large and complex data material. In the 4-vessel biobank, maternal subcutaneous and omental tissues are available, making studies on interaction between maternal adipose tissue and placental nutrient handling possible. It is also of interest to pair our *in vivo* human data with *in vitro* studies of placental tissue, for instance vesicle studies of activity and localization of relevant transporter proteins and studies of nutrients and energy sensing systems in the placenta. Further investigations on the role of CSAD in placental taurine synthesis should also be performed. Lastly, our findings from healthy human term pregnancies may be compared with studies on pathological pregnancies, like IUGR and preeclampsia.

Errata paper I

Table 1 and Table 2: Umbilical vein – umbilical artery

The reported glutamaic acid concentrations are not adjusted for day-to-day variation and thus differ slightly from the concentrations presented in paper II.

Figure 3: Flow chart (Fig 3 A) is missing



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Appendix

Unpublished data

Table 1 Spearman correlations between placental efficiency and maternal and fetal concentration differences for the sum of proteogenic amino acids

	Maternal uteroplacental A-V difference (µmol/L)	Fetal umbilical v-a difference (µmol/L)
Placental efficiency	r_s= -0.17, p=0.03*	r_s= 0.17, p=0.04*

*p<0.05

Table 2 Spearman correlations between placental efficiency, placental weight, and taurine concentrations

	Umbilical vein (umol/L)	Fetal va-difference (umol/L)	Placental tissue (umol/mg protein)
Placental efficiency	r_s=0.28, p<0.001*	r_s=0.29, p<0.001*	r_s=0.25, p=0.001*
Placental weight	r_s=-0.17, p=0.02*	r_s=-0.19, p=0.02*	r_s=-0.20, p=0.008*

*p<0.05

Table 3 Spearman correlations between the sum of proteogenic amino acid in the umbilical vein, fetal proteogenic amino acid uptake, fetal glucose uptake, and placental glucose consumption

	Fetal glucose uptake (mmol/min)	Placental glucose consumption (mmol/min)
Proteogenic amino acids in the umbilical vein (µmol/L)	r_s=0.16, p=0.08[NS]	r_s=-0.13, p=0.27[NS]
Fetal uptake proteogenic amino acids (µmol/L)	r_s=0.27, p=0.003*	r_s= -0.26, p= 0.03*

*p<0.05, NS= non-significant

Table 4 Spearman correlations between birthweight, the sum of proteogenic amino acid in the umbilical vein, and the fetal concentration difference for the sum of proteogenic amino acids

	Umbilical vein (umol/L)	Fetal umbilical v-a difference
Birthweight	r_s= 0.17, p=0.02*	r_s= 0.11, p=0.16[NS]

*p<0.05, NS= non-significant

Invitation letter

Forespørsel om deltakelse i forskningsprosjektet

"Preeklampsi, placentafunksjon, kardiovaskulær risiko og endoteldysfunksjon"

Bakgrunn og hensikt

Vi spør deg om å bidra til et forskningsprosjekt der hensikten er å studere svangerskapsforgiftning (preeklampsi). Preeklampsi rammer 1800-2000 gravide hvert år i Norge. Nyere forskning tyder på at preeklampsi oppstår fordi morkaken frigjør stoffer til mors blod der de blant annet fører til høyt blodtrykk. Kvinneklinikken, Rikshospitalet behandler mange kvinner med preeklampsi årlig. Kvinneklinikken gjennomfører derfor et forskningsprosjekt der formålet er å påvise hvilke stoffer det er som frigjøres fra morkaken og fettvev og som kan bidra til utvikling av preeklampsi.

Hva innebærer studien?

Studien gjelder gravide som forløses med keisersnitt og som har preeklampsi og på kvinner som forløses med keisersnitt og ikke har preeklampsi.

Mor:

Ultralyd: Før keisersnittet ønsker vi å gjøre en ultralyd undersøkelse hvor vi vil undersøke blodgjennomstrømningen fra deg til morkaken og fra morkaken til fosteret.

Blodprøver: Vi tar en blodprøve fra livmorens vene under keisersnittet og en "vanlig" prøve fra armen (både fra vene og arteriekanyle). Etter forløsning tar vi også blodprøver fra navlestrengen.

Vevsprøver: Vi tar en liten prøve (ca 1 gram) fra fettvev i bukveggen og inne i bukhulen. I tillegg tas det en vevsprøve fra morkaken. Vi tar også vare på slimhinnen fra innsiden av livmoren som ellers tas ut og kastes.

Hva skal prøvene brukes til? Prøvene vil bli testet med hensyn på næringsstoffer og stoffer som kan skade celler i åreveggen (endotelceller) og som derfor kan føre til bl.a. høyt blodtrykk og proteiner i urinen.

Barn:

For å få inntrykk av barnets ernæringsstatus registrer vi lengde, vekt og omkrets av mage, lår og overarm. Deretter måler vi tykkelsen av hudfolder, dette innebærer ikke ubehag for barnet.

Mulige fordeler og ulemper

Det tar 2-3 minutter å ta prøvene og det medfører ingen smerter eller ubehag utover det som er vanlig ved keisersnitt. I denne undersøkelsen legges det inn arteriekanyle på hånden for å kunne ta blodprøver. Dette innbærer ett ekstra stikk, et stikk du kan unngå ved ikke å delta i studien. Etter at kanylen er anlagt innebærer det lite plager og ingen risiko for deg eller babyen. Du har ingen direkte fordeler av å være med på denne studien. Vårt mål er at resultater av prosjektet kan bidra til å forbygge og behandle preeklampsi.

Hva skjer med prøvene og informasjonen om deg?

Blodprøvene lagres nedfrosset inntil testingen starter. Ansvarlig for denne biobanken er prosjektleder i denne studien. Analysene vil bli utført i Norge og ved samarbeidende institusjon i USA.

Prøvene og informasjonen som registreres om deg skal kun brukes slik som beskrevet i hensikten med studien. Alle opplysningene og prøvene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger og prøver gjennom en navneliste. Det er kun autorisert personell knyttet til prosjektet som har adgang til navnelisten og som kan finne tilbake til deg. Hele databasen fra denne studien vil være anonymisert etter at siste pasient er inkludert i studien, dvs. senest innen 2015. Det vil ikke være mulig å identifisere deg i resultatene av studien når disse publiseres. Oslo universitetssykehus ved administrerende direktør er databehandlingsansvarlig for studien.

Frivillig deltakelse
Det er frivillig å delta i studien. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke til å delta i studien. Dette vil ikke få konsekvenser for din videre behandling. Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Om du nå sier ja til å delta, kan du senere trekke tilbake ditt samtykke uten at det påvirker din øvrige behandling. Dersom du senere ønsker å trekke deg eller har spørsmål til studien, kan du kontakte Ane Moe Holme, lege/stipendiat tlf 23070245/ 02770.

Du er forsikret på vanlig måte gjennom Pasientskadeforsikringen.
Prosjektet er vurdert og tilrådd av Regional Etisk Komité for medisinsk forsknings-etikk (REK Sør-Øst) og meldt til personvernombudet ved Oslo Universitetssykehus.
Hvis du sier ja til å delta i studien, har du rett til å få innsyn i hvilke opplysninger som er registrert om deg. Du har videre rett til å få korrigert eventuelle feil i de opplysningene vi har registrert. Dersom du trekker deg fra studien, kan du kreve å få slettet innsamlede opplysninger. Opplysningene blir senest slettet 2015.

Vennlig hilsen

Prosjektleder:	Tore Henriksen, professor, Kvinneklinikken, Rikshospitalet.
Medarbeidere:	Lege Ane Moe Holme
	Lege Hildegunn Horne
	Lege Maia Blomhoff Holm
	Leiv Arne Rosseland, professor, Akuttklinikken, Rikshospitalet
	Guttorm Haugen, professor, Kvinneklinikken, Rikshospitalet

Samtykke til deltakelse i studien
Jeg er villig til å delta i studien / Jeg bekrefter å ha gitt informasjon om studien

-- / ---
(Signert av prosjektdeltaker, dato) (Signert av prosjektlege, dato)

Errata

Maia Blomhoff Holm

Placental transfer of proteogenic amino acids and taurine in healthy term pregnancies: a human in vivo study

Page	Fig	Original text	Corrected text
69	15 A	[Arrow showing glutamine on the maternal side of the placenta points right]	[Arrow showing glutamine on the maternal side of the placenta points left]