Studies of the human placenta in vivo
-The role of the placenta in glucose transfer and secretion of anti-angiogenic factors

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November 2017
Alt er komplisert og henger sammen
(All is complicated and intertwined)

-Bjørg Lorentzen
# Table of Contents

**ACKNOWLEDGEMENTS** .......................................................................................................................... 5
**ABBREVIATIONS** ...................................................................................................................................... 7
**TERMINOLOGY AND CALCULATIONS** ............................................................................................................. 8
**SUMMARY OF THE THESIS** ......................................................................................................................... 9
**LIST OF PUBLICATIONS** ............................................................................................................................ 13

**INTRODUCTION** ........................................................................................................................................... 14

- The placenta's implication for maternal and fetal health ............................................................................ 14
- Placental development and morphology ........................................................................................................ 16
- Placental functions ........................................................................................................................................ 20
- Placental adaptations ................................................................................................................................... 33
- Understanding placental nutrient transfer - why bother? .............................................................................. 39
- Preeclampsia ................................................................................................................................................ 44
- Methods to study placental transfer and metabolic function ......................................................................... 48
- Knowledge gaps .......................................................................................................................................... 52

**OBJECTIVES OF THESIS** ............................................................................................................................ 54

- Overall aim ................................................................................................................................................ 54
- Specific aims ................................................................................................................................................. 54

**MATERIALS AND METHODS** .................................................................................................................... 56

- Ethical aspects ............................................................................................................................................. 56
- Study design ............................................................................................................................................... 56
- Study population ......................................................................................................................................... 56
- Data collection and measurements ............................................................................................................. 58
- Statistical analysis ...................................................................................................................................... 62

**SYNOPSIS OF RESULTS** .......................................................................................................................... 63

- Paper I ......................................................................................................................................................... 63
- Paper II ....................................................................................................................................................... 63
- Paper III ..................................................................................................................................................... 65
- Paper IV ..................................................................................................................................................... 66

**METHODOLOGICAL CONSIDERATIONS** .................................................................................................. 68

**GENERAL DISCUSSION** ............................................................................................................................... 76

- Placental glucose transfer in human ............................................................................................................. 76
- Factors affecting placental glucose transfer: ................................................................................................. 77
- The placental origin of PLGF and sFlt-1? ....................................................................................................... 86

**SUMMARY AND CONCLUSIONS** .................................................................................................................... 89

**CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES** ....................................................................... 91

**APPENDICES** ........................................................................................................................................... 93

**ERRATA** ................................................................................................................................................... 97

**BIBLIOGRAPHY/REFERENCES** .................................................................................................................. 98

**PAPERS** ..................................................................................................................................................... 111
Acknowledgements

The present work was carried out at the Division of Gynecology and Obstetrics at Rikshospitalet, Oslo University Hospital. It was done in collaboration with the Institute of Basic Medical Sciences, Department of Nutrition, University of Oslo and Jansson-Powell Lab at University of Colorado, School of Medicine, Department of Obstetrics and Gynecology, Division of Reproductive Sciences. If was funded by the South-Eastern Norway Regional Health Authority and helped by a “start-up” grant from Norwegian National Advisory Unit on Women’s Health, Oslo University Hospital.

This research project, with its comprehensive collection of samples, would not have been possible without the positive support from all the clinical colleagues, midwives, anesthesiologists, operation nurses and anesthetic nurses at Rikshospitalet. Thank you all for creating such an inspiring working environment based on cooperation between personnel and specialties, and for supporting research despite buzzy days at the clinic.

My three supervisors are all prime examples of how great enthusiasm, effort and interest make it possible to combine research with great clinical care. They all contributed, in different ways, to evoke my interest and introduce me to the project at the time it was being planned. I am so grateful that they included me in this project. Trond Melbye Michelsen deserves my gratitude for being my main supervisor and for being infinitely patient providing me guidance throughout this thesis. His working capacity is plainly impressive. He has tried hard to teach me how to “boil it down”, but it is evident from this thesis that I still may have to study the art of reduction. Professor Tore Henriksen is inspiration incarnated. He expresses, in both his clinical work and as a researcher, pure delight in face of a puzzle. He has conveyed his enthusiasm for understanding clinical situations by its underlying mechanisms to everyone who have been fortunate to work with him. I feel very lucky to have the opportunity to discuss both clinical and research questions with him. Marie Cecile Paasche Roland has been more than a “kinder egg”. When I met her, she was an inspiring PhD-student who provided me comfort in my clinical work during my transition from a small hospital up north to the more complex world of Rikshospitalet. She then encouraged me to start my PhD, and was the best fellow PhD-student I could imagine, offering generously of her time and giving me a flying start when we wrote the first paper together. I am happy that she accepted to become my supervisor when she started her postdoctoral period and ever grateful that she shares so generously of her laughter, support and wisdom.

I also want to thank Professor Guttorm Haugen, for his enormous contribution to this project, examining our women by ultrasound in the early hours before surgery. Furthermore, for sharing his knowledge, expanding the discussions in our group and for returning every manuscript I have ever sent him with major improvements. To my fellow PhD’s in the project: 50 points are awarded to Ms Maia Blomhoff Holm, for the cool use of intellect when others were in great peril, and to Ms Hildegunn Horne for always keeping the deluminator at hand. Happiness can be found in the darkest of times if one only remembers to turn on the light. Together we solve tasks
that are too big to handle alone. This project would not have been half the size or
quality without you.
I am happy to have shared this period with Gun Lisbeth, Camilla and Gry, superb
colleagues and solid accomplices in science who have become good friends, with
whom I have shared joys and frustrations of life. Thanks to Oddrun Kristiansen for her
snaps and snats, and for bringing back the “naive enthusiasm” when the rest of us had
lost it.

I will also use this opportunity to thank collaborators and coworkers: Professors
Thomas Jansson and Theresa Powell are both experts in the field who opened their
laboratory for our group and taught me vesicle prep., western blots and shared their
wisdom in their co-authorships. The anesthesiologists Leiv Arne Rosseland, Eldrid
Langesæter, Ivar Omenås and Lasse Gronningsæter provided excellent care for our
patients while assisting in our sampling. Professor Lars Mørkrid shared his time, wit
and tremendous knowledge. Manuela Zucknick’s statistical expertise has been most
valuable. Heidi Stand helped with laboratory guidance and fun during long lab hours.
Kathrine Frey Froslie has stubbornly convinced us all that we can enjoy and
understand statistics. Øystein Horgmo deserves lots credit for all the effort of
translating my crude illustrative ideas into beautiful and comprehensive illustrations.

I am grateful to the past and present Heads of Department, Prof. Thomas Åbyholm and
Kristi Hjelle, and to Britt Frisell and Marit Gullesen, all of whom have facilitated and
helped me through the administrative challenges of this work. I am also grateful to
Anne-Sofie Letting who kindly let me keep a foot in the clinical world.

Several PhD’s, roommates and good colleagues at “E3” have, by sharing ideas,
projects or just laughs, made my days brighter and more interesting. My deepest
gratitude and respect to Liv Ellingsen and Bjørg Lorentzen who, ever since I met them
at the ALSO course, have been my greatest inspirers and mentors in all aspects of my
clinical profession. Bjørg helped establishing the project and has been invaluable in
the collection of samples.

Finally, I want to thank my big, extended family and my friends. Mum and dad who by
 genetics, learning and living life have encouraged me to wonder and seek knowledge.
Thanks for showing me that every obstacle can be modified though peaceful
interaction with nature or climbing a mountain.
To my children, who I hope have thrived in utero despite my sweet tooth. Thank you
for reminding me to counterbalance the seriousness of work with the joy of being a
mother. Thank you for giving me the excuse for still reading fairytales and dive into
dreams. Thanks to Rowling, Andem, Toneff, Baker, Hamsun and the rest of the usual
suspects for always distracting me, and for laying the ground for intergenerational
interactions for generations to come.
Lastly, I am most fortunate to have found in my love, not only a tolerant man, a skilled
doctor, a caring father to our children, but also a fellow researcher -who undeniably
provoked me when he was “demonstrating that it is possible to complete a PhD without
ever being stressed”...
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>arteriovenous</td>
</tr>
<tr>
<td>BM</td>
<td>basal membrane of the syncytiotrophoblast (facing the fetus)</td>
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<tr>
<td>BMI</td>
<td>body mass index (weight kg/height m²)</td>
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<tr>
<td>FFA</td>
<td>free fatty acids</td>
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<td>GLUT</td>
<td>glucose transporter protein</td>
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<td>GDM</td>
<td>gestational diabetes mellitus</td>
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<td>GWG</td>
<td>gestational weight gain</td>
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<td>hCG</td>
<td>human chorionic gonadotrophin</td>
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<td>hPGH</td>
<td>human placental growth hormone</td>
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<td>hPL</td>
<td>human placental lactogen</td>
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<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
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<tr>
<td>IUGR</td>
<td>intrauterine growth restriction</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>MVM</td>
<td>microvillous membrane of the syncytiotrophoblast (facing the mother)</td>
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<tr>
<td>PAPP-A</td>
<td>pregnancy-associated plasma protein A</td>
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<tr>
<td>PIGF</td>
<td>placental growth hormone</td>
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<tr>
<td>pp-13</td>
<td>placental protein 13</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>sFlt-1</td>
<td>soluble Fms-like tyrosine kinase-1</td>
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<tr>
<td>TAMX</td>
<td>time averaged maximum velocity</td>
</tr>
<tr>
<td>v-a</td>
<td>venous-arterial</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>vascular endothelial growth factor receptor 1</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>Q</td>
<td>Volume blood flow</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Terminology and Calculations

**Placental transfer** is used as a general term covering all aspects of transfer over the placenta, not as a quantitative unit.

**Net placental transfer** is used for the net mass of a substrate that is transferred from the placenta to the umbilical circulation.

**Fetal glucose consumption** is, in this thesis, considered equivalent to the net placental glucose transfer to- or the fetal uptake from- the umbilical circulation as there is little gluconeogenesis in a healthy human fetus.

\[
\text{Volume blood flow } Q = h \times \left( \frac{D}{2} \right)^2 \times \pi \times \text{TAMX}
\]

D is the vessel diameter (cm), TAMX is time averaged maximum velocity and h is the coefficient for the spatial blood velocity profile, 0.5 was used as the coefficient for the umbilical vein and 0.6 for the uterine artery (1-4).

**Uteroplacental arteriovenous concentration difference** = \([X]_{MA} - [X]_{MV}\)

**Umbilical veno – arterial concentration difference** = \([X]_{fv} - [X]_{fa}\)

**Maternal – fetal gradient** = \([X]_{MA} - [X]_{fa}\)

**Maternal supply** = \([X]_{MA} \times Q_M\)

**Uteroplacental uptake** \(\Delta [X]_M Q_M = ([X]_{MA} - [X]_{MV}) \times Q_M\)

**Umbilical supply to the fetus** = \([X]_{fv} \times Q_f\)

**Fetal glucose consumption** \(\Delta [X]_f Q_f = ([X]_{fv} - [X]_{fa}) \times Q_f\)

**Uteroplacental consumption** = \(\Delta [X]_M Q_M - \Delta [X]_f Q_f\)

Similar blood composition of substance X in the radial and uterine artery was assumed. The calculations are done under the assumption of steady state, supported by the fasting state of the mothers. Subscripts: MA, the radial
artery; MV, the uterine vein; fv the umbilical vein and fa, the umbilical artery.

Figure 1 | Operational definitions relevant for the thesis. The mass of glucose transferred from the placenta to the umbilical circulation is considered equivalent to fetal glucose consumption as there is little gluconeogenesis in a healthy human fetus.

Summary of the thesis

The placenta is essential for the fetal nutritional and endocrine environment, and thus for immediate and future health of the child. Most of the current concepts of placental transfer, metabolism and function in the human are extrapolations from epidemiological studies, studies of pathological conditions, animal models and in vitro studies. These models have provided invaluable insight into placental function, yet many of the results are conflicting and have not been tested in vivo in human pregnancies. There are reasons to believe that some of the diverging results can be explained by the diversity of the currently used models.

The placental 4-vessel study was initiated with two main aims:

To study human placental physiology in vivo, with focus on nutrient transfer in relation to maternal metabolic factors and infant outcomes.
To investigate placental factors contributing to pathological pregnancies, especially preeclampsia.

Specifically, the study was designed to calculate the uteroplacental uptake, consumption and release of different substances by determining the volume blood flow and the arteriovenous (AV) concentration differences at the maternal and fetal sides of the placenta. The study of placental transfer was elaborated by the assessment of placental nutrient transporters, maternal metabolic status and newborn anthropometry. The study design was observational and cross-sectional, conducted during caesarean section near term of pregnancy. We aimed to describe physiological relationships in healthy pregnancies as they are fundamental for understanding pathological mechanisms. We sought to determine if previous findings and current concepts of placental function, based on animal and in vitro studies, could be applied to normal pregnancies in vivo. Moreover, the 4-vessel method was used to study pathological pregnancies. By comparing placental release into the maternal circulation in normal and preeclamptic pregnancies in a case-control design, we aimed to investigate the origin of factors that might contribute to the clinical features of preeclampsia.

The four papers of this thesis represent the first phase of a more comprehensive project. They cover the method (paper I), placental glucose transfer in normal pregnancies (paper II and III), and placental release of vasoactive compounds to the maternal circulation in preeclampsia (paper IV).

In paper I, we present a comprehensive protocol for studies of human placental physiology at term including a video demonstrating the procedures. We demonstrated net placental transfer of glucose to the fetal circulation, placental secretion of progesterone to the maternal circulation and placental uptake of glutamic acid from both fetal and maternal circulations as a proof of principle. The method is discussed with its limitations, strengths and prospects.

In paper II, we determined the AV-concentration differences of glucose on the maternal and fetal sides of the placenta in vivo and found, in contrast to
what was expected, that these were not correlated. Moreover, despite a strong correlation between maternal and fetal glucose levels, we found no correlation between maternal glucose levels and the fetal uptake of glucose (mmol/L) given by the umbilical venous-arterial (v-a) concentration difference. Ovine studies have demonstrated that the umbilical glucose v-a concentration difference can be separately regulated by the fetal concentrations of glucose, independent of maternal levels (5). Consistent with this notion we observed that the fetal umbilical v-a glucose difference of glucose was strongly correlated to the glucose concentration gradient between the maternal and fetal circulations, which is the main driving force for placental glucose transfer. Taken together, our findings may suggest that fetal v-a glucose difference is not just affected by incoming maternal glucose.

In paper III, we determined the mass of glucose taken up by the placenta from the maternal circulation and the mass consumed by the placenta and fetus. The fetal glucose consumption correlated with birthweight and fetal insulin levels. Fetal glucose consumption (mmol·min⁻¹·kg⁻¹ newborn) was not correlated with maternal body mass index (BMI) and gestational weight gain (GWG), but with maternal glucose concentration. We elaborated on our findings in paper II, by demonstrating that uteroplacental glucose uptake was correlated to placental, but not to fetal glucose consumption. Fetal and placental glucose consumption were inversely correlated, but neither correlated with the expression of glucose transporter 1 (GLUT1) in the maternal and fetal facing membranes of the syncytiotrophoblast. Placental glucose consumption was not related to glucose in the incoming arteries on either side of the placenta. This contrasts findings in ovine studies which have implied that placental glucose consumption is regulated by maternal and fetal glucose levels (6, 7). Our findings do not, however, exclude the notion from in vitro placental transfusion studies and experimental animal studies, that the mass of glucose available for fetal consumption is affected by placental metabolism (5, 8).

In paper IV, we used our in vivo 4-vessel sampling method to investigate one of the prevailing questions concerning preeclampsia. We found that the
placenta released soluble Fms-like tyrosine kinase 1 (sFlt-1) to the maternal circulation in early onset preeclampsia, but not in healthy pregnancies. Placenta released placental growth factor (PlGF) to the maternal circulation in both healthy and preeclamptic pregnancies, and we found no support for an impaired placental release of PlGF in preeclampsia.

To summarize the main conclusions; BMI or GWG correlated with birthweight, but not with fetal glucose consumption in healthy pregnancies at term. Even though maternal and fetal glucose levels are closely correlated, uteroplacental uptake of glucose does not directly relate to fetal levels or consumption of glucose. The mass of glucose consumed by the placenta seems to interfere with this relationship as it is inversely correlated to fetal glucose consumption. In support of existing concepts, and in contrast to normal pregnancies, we found a significant placental release of sFlt-1 in early-onset preeclampsia. Placental release of PlGF was not impaired in preeclampsia, thus low circulating levels of PlGF in preeclampsia are more likely to be caused by binding to excess sFlt-1.
List of publications


Introduction

The placenta’s implication for maternal and fetal health

Pregnancy is a coexistence of two individuals and cannot be understood outside this fundamental frame. It has been argued that the mother and her fetus have opposing interests, but maternal health and fetal development are mutually dependent on a healthy pregnancy. Situated at the interface between the mother and her offspring, the placenta represents the third entity of the pregnancy, and may orchestrate maternal and fetal adaptations to pregnancy and, as such, to each other.

Intrauterine conditions affect fetal growth, body composition and development and have immediate consequences for intrauterine health, perinatal morbidity and mortality. What’s more, the environment of the fetus has consequences for the life-long health of the offspring (9, 10). Growth deviating from its genetically determined growth potential, both as intrauterine growth restriction (IUGR) and as fetal overgrowth, can result in obstetrical complications. IUGR gives increased risk for stillbirth, preterm delivery, hypoxic ischemic encephalopathy and increased neonatal morbidity (11, 12). Fetal overgrowth is associated with complicated deliveries (protracted delivery, operative delivery, postpartum haemorrhage, uterus rupture, vaginal lacerations and shoulder dystocia), asphyxia and neonatal hypoglycaemia. Since Barker et al. demonstrated an association between birthweight and the risk for cardiovascular disease, insulin resistance and type 2 diabetes later in life (13-15), a large body of evidence has confirmed these associations and found link between intrauterine conditions and other non-communicable diseases (16). The concept of developmental programming provides a mechanistic understanding of these associations (17, 18). Intrauterine conditions in general, and nutrition in particular, affects gene expression through epigenetic modifications and thereby permanently sets the functional properties and structural features of major organs. These programming effects are sensitive to factors like maternal obesity, other states of malnutrition, diabetes, hypoxia and infection. Each organ system
will be susceptible at distinct times of gestation depending on the timing of differentiation and maturation. The duration and severity of the perturbations will also determine the effect of the epigenetic modifications.

Worldwide, maternal obesity-related metabolic disorders including gestational diabetes have become main risk factors for disturbed fetal development and pregnancy complications (19). The intrauterine nutritive conditions are influenced by maternal diet, nutrient stores and metabolic state. However, as most substances must pass the placenta to reach fetal circulation, current evidence clearly indicates that the exchange of nutrients, gases and other compounds between the maternal and fetal circulations are governed also by placental properties. Furthermore, the placenta has a high metabolic activity and secretes several hormones and growth factors that modifies maternal metabolism profoundly. Placental insufficiency and vascular dysfunction is a prominent feature of several pregnancy complications like preeclampsia and IUGR.

The placenta is particularly complex in the sense that it as a single organ performs tasks that require several organs in the born individual. Furthermore, its functions, structure and efficiency are under continuous development alongside fetal maturation. This makes placental physiology difficult to study. Moreover, the major differences between animals and humans in placental anatomy, pregnancy duration and complications hamper the extrapolation of experimental studies to humans (20-22). The human placenta lies rather inaccessible for studies during pregnancy and in vivo studies of normal placental physiology are few (23). These considerations have been acknowledged by the National Institute of Health (NIH), which in 2015 addressed the placenta as the least understood human organ despite its uttermost importance for the immediate and future health of the mother and her offspring (24).
Placental development and morphology

During a lifespan limited to 38 weeks, the placenta goes through a continuous development from a fertilized oocyte to a highly versatile and specialized organ.

At term the human placenta is a discoid organ of about 500g with a 12m² surface area (microvilli not included), which separates the maternal and fetal circulation by two main cell layers, the syncytiotrophoblast and the umbilical capillary endothelium. It is perfused on the fetal side, by blood of reduced content of oxygen and nutrients, through the two umbilical arteries. After passing through the capillaries of the placental vasculature blood returns to the fetus via the single umbilical vein providing oxygen and nutrients (Figure 2). The placental capillaries and the overlying trophoblast cells form an elaborately branched structure (the villous tree) which projects into the intervillous space perfused with maternal blood. The maternal side of the placenta is mainly supplied by the two uterine arteries, with varying contribution from the ovarian arteries (25-27). Maternal blood reaches the placenta though the spiral arteries, which open directly in to the intervillous space before it drains through openings in the uterine veins (Figure 2). Thus, maternal blood is in direct contact with the trophoblast lined villous tree of fetal origin and this constitutes the basic functional unit of the human
The human placenta evolves from cells derived from the conceptus and thus shares genetic composition with the fetus. At day 3 after fertilization, when the conceptus consists of 16 cells, it is organized in an inner and outer cell layer. At the time of implantation the conceptus has become polarized to form the blastocyst, consisting of the inner cells at one pole (which gives rise to the embryo) and an outer layer of trophoblast cells. At day 6 the trophoblastic cells of the outer layer begins to penetrate and invade the uterine mucosa. Together with extraembryonic mesoderm these are the forerunners of the chorion and the placenta (29).

The outer part of the invading trophoblast layer transform into a continuous multinucleated mass called the syncytiotrophoblast. The remaining trophoblast cells are cytotrophoblasts, which provides growth of the syncytiotrophoblast by continuous proliferation and subsequent fusion. Over the next days, vacuoles appear and fuse within the syncytiotrophoblast to form a system of continuous lacuna which will later become the intervillous space filled with maternal blood. Pillars of cytotrophoblasts protrude into the syncytiotrophoblast layer forming primary villi. Other cytotrophoblasts penetrate the entire syncytiot to create a cytotrophoblastic shell towards the maternal decidualized endometrium. Subsequently, mesenchymal cells of the extra embryonal mesoderm penetrate the primary villi (secondary villi), and from day 18 fetal capillaries differentiate from hemangioblastic stem cells in the mesenchyme (tertiary villi). Surrounded by the newly formed endothelial cells, the same progenitor cells are origin of hematopoietic stem cells. Thus, the fetal and placental circulations develop separately, and complete feto-placental circulation is achieved at the beginning of week 6 post conception.

Some of the cells in the cytotrophoblastic shell differentiate and migrate into the endometrium. These cells are called extravillous trophoblasts and serve two main functions. The interstitial extravillous trophoblasts serve to anchor the placenta, and through paracrine interaction with maternal endometrial cells they contribute to the formation of the decidua. The endovascular
extravillous trophoblasts invade the maternal spiral arteries and are of essential importance for the adaptation of the maternal vessels to the pregnancy. The endovascular villous trophoblasts replace the endothelium and smooth muscle layers of maternal spiral arteries securing a non-responsive, low resistance blood supply to the intervillous space. The definitive placenta is created by the establishment of the maternal-placental circulation, a process that starts by 8-9 weeks of human pregnancy and is completed by week 20.

Figure 3 | Basic morphology of human placental villi A) Longitudinal section across the uterus, placenta, and membranes in the human. The chorionic sac, consisting of placenta and membranes, is black. B) The mature villous tree protruding into the intervillous space which is bathed in maternal blood. A stem villus (1) continues in a bulbous immature intermediate villus (3). The slender side branches (2) are the mature intermediate villi, the surface of which is densely covered with grape-like terminal villi (4). C) Highly simplified section of two terminal villi. D) Schematic section of the vasculosyncytial exchange surface, demonstrating its layers. From left to right: intervillous space (perfused with maternal blood), microvillous membrane (MVM) of the syncytiotrophoblast, the basal membrane (BM) of the syncytiotrophoblast, the basal lamina, the fetal endothelial cell and the fetal capillary (perfused with fetal blood). Illustration and text: Reproduced with permission from Benirschke K et al.(29)
The placental villus is the basic functional unit of the human placenta where gas, nutrients and waste are exchanged between the maternal and fetal circulations. The villus consists of a continuous syncytiotrophoblast layer in direct contact with maternal blood, which overlays a discontinuous layer of cytotrophoblasts, stromal fibroblasts, macrophages and the placental capillaries of the umbilical circulation. The villous structure is in the shape of branching trees and the expansion of the placental surface area is exponential with continuous syncytial sprouts becoming new villi (Figure 3B). Throughout the pregnancy the placenta becomes more and more refined, a process called maturation (30). The villi become longer and their diameter less. The continuous syncytiotrophoblast layer becomes thinner, the cytotrophoblast layer becomes dispersed and ongoing angiogenesis of fetal capillaries in the terminal villi form a looped network with sinusoidal dilations bulging beneath the syncytium (Figure 3C). In sum, the distance between the maternal and fetal circulation is reduced significantly and in a mature tertiary villi at term it may be as little as 2-3 μm. It consists of the two cell membranes of the syncytiotrophoblast, the basal lamina, and the two cell membranes of the fetal endothelial cell (Figure 3D) (31). Together, these are the areas of the tertiary villi where maternal-fetal exchange is most efficient, and they are designated the vasculosyncytial exchange surface.

The syncytiotrophoblast is polarized with a microvillous plasma membrane (MVM) facing the maternal blood of the intervillous space and a basal plasma membrane (BM) facing the cytotrophoblasts and the villous capillaries (Figure 3D). The MVM is highly folded, with microvilli which makes a surface area 5-7 times the surface of the BM. It is the site of transport proteins and receptors to a vast numbers of proteins, hormones, growth factors, immunoglobulins and other ligands. The base of the microvilli is central in vesicle formation and the placental transfer of macromolecules. The polarity and the properties of the syncytiotrophoblast are of vital importance for placental transfer.

The capillary endothelium of the umbilical circulation is of the continuous type with paracellular clefts consisting of tight junctions without directional
preference and adherence junctions. These clefts allow passage of molecules below 1500g/mol which includes glucose and amino acids, but partly restrict transfer of larger molecules such as immunoglobulins and albumin (21, 32, 33).

Placental functions

The placenta exerts functions that are later taken over by several separate organs like the lungs, kidneys, liver, gut, endocrine and hematopoietic organs and is involved in all aspects of fetal wellbeing (34). It is essential for gas and nutrient transfer throughout the pregnancy. Furthermore, it is the site of hematopoiesis early in pregnancy, it governs pH and water balance and secures heat transfer (29). It protects the fetus against certain drugs, xenobiotic molecules and infectious agents, as well as against the maternal hormones and inflammatory response. A unique feature of the trophoblastic cells is that their combination of surface molecules does not stimulate a maternal reaction to the foreign antigens expressed by paternal genes. The placenta has numerous metabolic, endocrine, secretory and excretory functions essential for waste elimination, for fetal growth, for maternal metabolic adaption to pregnancy and for maternal-fetal cross talk. Thereby it governs to a large extent the intrauterine environment of fetal development.

Maternal-fetal exchange - Placental transfer

The transfer of most substances between the mother and the fetus is not unidirectional, but rather bidirectional exchange resulting in a net transfer from the mother to the fetus or vice versa. Considering that placenta is metabolic active and synthesizes hormones and other compounds, the exchange may be between the mother and the placenta, or between the fetus and the placenta. The polarized syncytiotrophoblast, with distinct differences between the maternal and fetal surfaces, contributes to directional transfer of certain substances.

In general substances pass either through cells (transcellular transport) or between cells (paracellular transfer). The maternal side of the placenta is
covered by the continuous syncytiotrophoblast in which there are no lateral intercellular spaces, thus transcellular transport is most important for placental transfer. There is evidence for the presence of water filled “trans trophoblastic channels” or temporal breaks in the syncytiotrophoblast, but the significance of such channels is debated (35).

The placental transfer can occur by diffusion down a gradient or energy requiring active transport. The mode of transport can be non-mediated or mediated by transporter protein or binding to a receptor (Figure 4). The size, polarity, solubility and degree of protein binding of a substrate will influence the mode and energy cost of placental transfer. Furthermore, the sum of the driving forces (differences in concentrations, membrane potentials, osmotic and hydrostatic pressures) between the maternal and fetal side of the placental exchange surface will determine the energy required to move the substrate or the rate of diffusion (Figure 5) (32, 34).

Figure 4 | Simplified overview over placental transfer mechanisms; exemplified by some of the essential substrates for the fetus. (In nature each substrate is transferred by highly specific transporters.) Illustration: Øystein H. Horgmo, University of Oslo

Small lipid soluble molecules, like respiratory gases, are transferred non-mediated by simple diffusion (Figure 4). Transfer of larger or charged molecules are mediated by specific transport mechanisms depending both on
the substrate and the driving forces. The mediated transport most commonly involves transporter proteins which mediate facilitated diffusion (glucose transporters) or energy requiring active transport (amino acids transporters) (Figure 4). The membrane proteins that catalyze transfer of molecules across the plasma membrane are relatively substrate specific and follow saturation kinetics. Furthermore, transfer can typically be inhibited by competition between several substrates for the same transporter protein (as with amino acid transporters) (32, 34).

Transfer can also occur by endocytosis and transcytosis (36). This involves invagination of the plasma membrane at one side of the syncytiotrophoblast and release of the content either to the intracellular or the opposite extracellular compartment. The endocytosis may be non-mediated or mediated by ligands binding to specific receptors in the plasma membrane (transfer of immunoglobulin G) (Figure 4).

Lastly, fragments of the MVM and vesicles are released to the maternal circulation. They may have different content depending on the size, and on the gestational age and state of pregnancy. Some of these vesicles, called exosomes, are of endosomal origin and contain signaling molecules including microRNAs which are important for intercellular communication and possibly vital for the maternal immune tolerance of the fetus (37). The exosomes and the circulating fragments of the MVM are believed to be important in the maternal-fetal interplay (37, 38). The shedding of MVM to the maternal circulation may also be part of pathological processes caused by stress to the syncytiotrophoblast like oxidative stress, hypoxia and turbulent blood flow (37, 39).

**Overview over factors influencing maternal fetal exchange**

In sum the transfer over the human placenta is unique for all substances. Transport through all the above described pathways depends on the qualities of the placental exchange surface and the sum of factors that alters the driving forces. These factors are listed in Figure 5 and Box 1. Some illustrative aspects of the integrative comprehension of placental transport function are addressed below.
### The Mother
- Volume, velocity and turbulence of blood flow to the placenta.
- Maternal supply of a substrate is affected by maternal hormones, nutritional intake, consumption and metabolism.

### The Substrate
- Physiochemical characteristics as solubility, charge and size in addition to the degree of binding to transporter proteins.

### The Driving forces
- The concentration differences, membrane potentials, hydrostatic and osmotic pressures between the maternal circulation, the intracellular compartment of the syncytiotrophoblast and the fetal circulation.

### The Energy
- ATP, -directly or indirectly.

### The Pathway
- Transcellular vs transcellular channels, mediated vs non-mediated, endocytosis/transcytosis, vesicles.

### The Placenta
- Exchange surface area and thickness (maturity), placental metabolism, catabolism and interconversion. Properties of MVM and BM (expression and activity of transporter proteins, enzymes, receptors, lipid composition, fluidity, channels), cytosolic transport, (binding proteins, cytoskeleton) and transtrophoblastic channels or tears. Diffusion across basement membrane, villous stroma and capillary endothelium.

### The Fetus
- Volume, velocity and turbulence of blood flow to the placenta. Flow on the fetal side will influence distention of the placental villi and thus the area of the vasculosyncytial exchange surface. Fetal expenditure and demand is influenced by fetal hormones, metabolism and genetics.

### The Interplay
- Maternal supply versus fetal demand. Placental versus fetal demand.

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**Figure 5 and Box 1 | Factors influencing maternal fetal exchange (32, 34).**

**Illustration: Øystein H. Horgmo, University of Oslo**

**Regarding the abundance of substrate, exchange surface area and flow:**

The maternal supply of a substrate may be determined both by maternal plasma levels, which is a result of intake, consumption and metabolism, as well as the blood supply to the placenta through the uterine arteries. The transfer of substances is not only dependent on the blood volume passing...
through the intervillous space, but on the velocity at the exchange surface. *In vitro* studies have demonstrated that the thickness of the vasculosyncytial exchange surface is dependent on the perfusion pressure difference between the intervillous space and fetal capillaries. In addition, the pressure dependent distention of the fetal capillaries is likely to affect the total area of the placental exchange surface (40, 41). Similarly to the maternal side, the placental transfer on the fetal side is likely to be dependent on both the volume of blood passing the placenta in the fetal placental capillaries per time unit as well as the velocity. Lastly, the fetal consumption of a substrate will affect the concentrations on the fetal side and may therefore affect the transfer directly by altering the transfer gradient between the maternal and fetal circulations.

**Regarding placental properties and metabolism, and changes throughout pregnancy:**

First, the area and the thickness of the vasculosyncytial exchange surface are believed to be proportional to the mass of nutrient transferred (Figure 6, #1). Though it is difficult to study this entity, it has been shown to be closely correlated to placental and birthweight (42). Second, the properties of the MVM and BM, especially the density and activity of transporter proteins, enzymes and receptors as well as the lipid composition and fluidity of the membranes, are possible rate limiting steps in the transfer (Figure 6, #2, #3). Thirdly, the transfer may also be affected by the diffusion through the basement membrane, villous core and capillary endothelium (Figure 6, #4). Finally, the transport through syncytial cytosol may be dependent on binding proteins or the cytoskeleton (Figure 6, #5) and on placental consumption for its own metabolism or interconversion of metabolites (Figure 6, #6 and Figure 6). Energy and substrates are needed to support placental development, the active transport of some substrates, as well as other essential placental functions like productions of hormones, growth factors etc.
The transfer of several substances is likely to change during pregnancy as a result of the continuous growth and development of the villous three, the thinning and expansion of the vasculosyncytial exchange surface, and changes in transporter expression and activities, as well as the fluctuations in substrate availability and demand (43-46). Total fetal nutrient and energy requirements will increase during pregnancy, whereas the demand for specific substrates (e.g. cholesterol) might decrease due to gradual initiation of the fetus’ own synthetic and catabolic ability (47).

**Transport of gases**

Oxygen and carbon dioxide are small, lipid soluble molecules that diffuse rapidly over the plasma membranes of the syncytiotrophoblast. Thus, the net placental transfer of these substances is believed to be limited only by the size and thickness of exchange surface and by blood flow to and from the site of transfer. The oxygen transfer is facilitated by the large difference between maternal and fetal pO₂. The fetal hemoglobin has a higher affinity for oxygen and lower affinity for carbon dioxide than adult hemoglobin, which favor the
transfer of oxygen to the fetus and carbon dioxide to the mother (48, 49). This mechanism protects the fetus from the consequences of suboptimal placental blood flow which may affect fetal-placental gas exchange.

**Transport of water**
Water is transported from the maternal circulation across the placenta against a higher hydrostatic pressure on the fetal side. The compounds accounting for the osmotic gradients that drive water towards the fetus have not been identified. However, the syncytiotrophoblast has a large surface and high water permeability, and it has been suggested that a very small osmotic gradient is needed to promote water movement. There is evidence that aquaporins are expressed in the placenta, but their role in overall water transport has not been established (32).

**Transport of glucose**
Glucose is the main substrate providing energy for the growing fetus and the placenta. There is no evidence of significant fetal gluconeogenesis in normal pregnancies and the fetus is believed to be dependent on glucose transfer from maternal circulation (50, 51). In pregnancy, maternal glucose metabolism is altered by hormone regulated adaptations which result in increased hepatic glucose production and peripheral insulin resistance. These metabolic changes ensure glucose supply to the fetus also in a fasting state. Glucose is transferred across the placenta by facilitated diffusion through insulin independent glucose transporters (GLUT), thus the main driving force is the maternal-fetal glucose concentration gradient (52).

There are several isoforms of the glucose transporters present in placental tissue, but their expression, activity and relative importance vary both between the different cells of the placenta and throughout pregnancy.

GLUT1 is expressed in the syncytiotrophoblast, cytotrophoblast cells, endothelium, and vascular smooth muscle cells as well as in stromal cells (45, 53). It is insulin independent, especially highly expressed in the two membranes of the syncytiotrophoblast and believed to be the primary glucose
transporter at term (54). The role of this transporter will be further discussed in the Discussion section of this thesis.

GLUT3 has high glucose affinity and is found in the cytotrophoblasts and the extravillous cytotrophoblasts in first trimester. The GLUT3 transporter may be of importance for glucose transfer early in gestation as it is expressed in mitotic trophoblasts, but not in terminally differentiated syncytiotrophoblast (45, 55). In the third trimester, it is expressed in the endothelium of the fetal placental capillaries (55). Transfer of glucose over the placental capillary endothelium is thought to occur by simple diffusion. As such, this high affinity transporter in the endothelial cells is hypothesized to ensure glucose uptake from fetal blood with low glucose levels following fetal consumption, for storage as glycogen in the placental endothelial cells (55). A further decrease in capillary glucose levels will also steepen the maternal-fetal glucose concentration gradient. Together this may secure glucose supply to the fetus even when the maternal glucose supply is sparse.

GLUT4 is an insulin responsive transporter which is found in the stromal cells, amnion and chorion fibroblasts and co-localize with insulin receptors. In certain tissues insulin promote the transfer of pre-synthesized GLUT4 to the membrane, which ensures a rapid upregulation in transport activity, followed by a slower phase of de novo synthesis. GLUT4 has not been confirmed to be expressed in the syncytiotrophoblast. This explains why a rapid effect of insulin on the placental glucose transport cannot be demonstrated, and GLUT4 is not considered to be important for the maternal-fetal transfer (56). However, insulin receptors are present in the syncytiotrophoblast and complete syncytial insensitivity to glucose cannot be assumed (57).

GLUT9 is the least studied transporter in the placenta, it is found in the syncytiotrophoblast, and vascular endothelium in two specific isoforms. It transfers fructose as well as glucose and might have role in diabetic pregnancies (58).
The maternal-fetal gradient can be affected both by maternal and fetal glucose concentrations as demonstrated both in humans and animal models (5, 44). Apart from the importance of the maternal-fetal gradient, there are still some controversies regarding which factors that are considered important for the maternal-fetal transfer of glucose in human pregnancies. The high capacity of the human placenta for glucose transfer has led some to conclude that the transfer is not limited by transporter availability, but rather limited by flow and/or the vasculosyncytiotial surface (59, 60). Nevertheless, GLUT1 density at the BM is, by others, considered a rate-liming step in glucose transfer, a notion supported by an asymmetrical glucose transfer over the two membranes in vitro (8, 45). In animal models, the proportion of uteroplacental glucose uptake that is metabolized by the placenta seems to influence net placental glucose transfer (5). On the other hand, some argue that the vasculosyncytiotial exchange surface in humans is deprived for energy requiring organelles and that placental consumption of glucose does not influence maternal-fetal transfer (61). Maternal, fetal and placental factors considered to affect placental glucose transfer are listed below and discussed more thoroughly in the Discussion section.
### Factors affecting placental glucose transfer

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<tr>
<th><strong>Maternal-fetal gradient</strong></th>
<th><strong>This thesis</strong></th>
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<tbody>
<tr>
<td>- Maternal glucose concentrations (Pedersen hypothesis)</td>
<td>Maternal and fetal glucose concentrations</td>
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<td>- Fetal glucose concentrations</td>
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<td>- Placental glucose concentrations??</td>
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<th><strong>Placental three-dimensional structure</strong></th>
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<td>- The total area of the vasculosyncytial exchange surface (correlated to placental weight)</td>
<td>Placental weight</td>
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<td>- Distance between maternal and fetal blood at the exchange surface</td>
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<th><strong>Placental volume blood flow</strong></th>
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<tr>
<td>- Maternal side</td>
<td>Volume blood flow in uterine artery and umbilical vein</td>
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<tr>
<td>- Fetal side</td>
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<th><strong>Placental glucose metabolism</strong></th>
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<td>- Glycolysis, aerobe and anaerobe</td>
<td>Placental glucose consumption</td>
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<td>- Glycogenesis and glycogenolysis?</td>
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<td>- Interconversion</td>
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<td>- Polyol pentose pathway</td>
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<th><strong>Transporter density</strong></th>
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<td>- GLUT1 in the MVM (sensitive to glucose concentrations? (62))</td>
<td>GLUT1 expression in MVM and BM</td>
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<td>- GLUT1 in the BM (rate-limiting step of glucose transfer?(45, 63))</td>
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<tr>
<td>- GLUT3 in the umbilical capillary endothelium (capillary glycogen storage, reduced blood glucose in the fetal plasma entering the placenta?(55))</td>
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<td>- Other GLUTs (GLUT9?(58))</td>
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<th><strong>Hormones and growth factors</strong></th>
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<td>- Fetal insulin (Affects fetal glucose consumption, lowers the fetal glucose levels and steepens the maternal fetal gradient (64, 65))</td>
<td>Maternal and fetal insulin concentrations</td>
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<tr>
<td>- Maternal hormone mediated increase maternal hepatic glucose production and peripheral insulin resistance (progesterone, HPGH, HPL, leptin, resistin)</td>
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<tr>
<td>- Resistin (stimulate GLUT1 expression?)</td>
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<tr>
<td>- Fetal IGF1 (stimulate GLUT1 expression?(66))</td>
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| **Hypoxia (67)** | |

Box 2 | The driving forces, the structural and the regulatory factors of placental glucose transfer. The factors studied in this thesis in the right column.

### Transport of amino acids

There are over 20 amino acids in the plasma, - some of which the fetus can synthesize from intermediates and some of which has to be transferred from the mother (essential amino acids). There are many families of amino acid transport proteins present in the human placenta. Each transports relative specific, but overlapping, groups of amino acids selected on the structural form of both amino acids and transporters (68). Most amino acids occur in
higher concentrations in the fetal plasma than the maternal plasma, necessitating active transport by transport proteins. The driving forces for these transporters are the electrochemical gradients. The energy is primarily generated by the \( \text{Na}^+\text{K}^-\)-ATPase, which extrudes sodium from the syncytiotrophoblast in exchange for potassium. Thus, the cell interior of the syncytiotrophoblast becomes negative in relation to the outside and there is a sodium gradient across the MVM membrane which is of particular importance. The majority of amino acids are co-transported with sodium through transporter systems like system A amino acid transporters (e.g. glycine and alanine). Cationic amino acids (e.g. lysine) benefit from their positive charge and are transported though \( \text{Na}^+ \) independent transporters like system \( \text{y}^+ \). The resulting high concentrations in the syncytiotrophoblast permit most amino acids to diffuse down their concentration gradient into the fetal circulation as well as back to the mother through system L transporters. Some amino acids, like leucine, is believed to be exchanged with other amino acids by transporter systems (e.g. system L), which takes advantage of this gradient between the placenta and the maternal and fetal circulations (68). The amino acids can be transported unchanged or be metabolized in the placenta into other amino acids or byproducts which are transported to the fetus. It is believed that the placenta is involved in placental-fetal amino acid cycles which are part of optimizing unique fetal supplies and organ specific delivery of certain amino acids (69, 70).

The active, transporter mediated nature of the amino acid transfer in the placenta enables a specific regulated transport of each amino acid. This regulation makes it possible to maintain appropriate levels of certain amino acids which may be critical in fetal development. Experimental studies have shown that regulations of amino acid transport can occur as a response to intracellular levels in the trophoblast as part of a feedback system or a response to maternal hypoglycemia, hypoxia or reduced uterine blood flow (32). Expression of transporters may be partly subjected to endocrine control (71), and there is some evidence suggesting that expression can be modulated under adverse conditions to compensate for maternal undernutrition, reduced vasculosyncytial exchange surface or perfusion (72).
Transport of lipids

Dietary fat, primarily fatty acids and sterols, are essential for the normal growth and development of the human fetus. Fatty acids are important for energy storage and as part of cell membrane phospholipids. Cholesterol and other sterols are obligatory components of all cell membranes, involved in intercellular signaling, important gene regulators and precursors of steroid hormones. The fetus starts to synthesize cholesterol at about mid-gestation, thus transfer of cholesterol from the maternal to the fetal compartment is crucial in early gestation (73). Thereafter, the fetus makes use of cholesterol derived from its own synthesis in addition to the maternal cholesterol transferred across the placenta (47). The levels of total cholesterol in the fetus are only 25% of the maternal levels, and the distribution of the lipoprotein classes is markedly different with larger proportions of high-density-lipoprotein (HDL) in the fetal circulation (74). Likewise, the total levels of fatty acids are lower in the fetal circulation, but the proportion of long polyunsaturated fatty acids larger. Cholesterol may be taken up by the placenta through internalization of low-density-lipoprotein (LDL) receptor or by the HDL binding scavenger receptors at the MVM (Figure 4). There is experimental evidence that cholesterol efflux to the fetal circulation is regulated by adenosine triphosphate (ATP)-binding cassettes in the placental endothelial cells in accordance with the general model of HDL mediated cellular efflux of cholesterol (75).

Triglycerides are hydrolyzed by lipases in the MVM and free fatty acids can then move across the membrane (Figure 4). The transport of un-esterified fatty acids involves membrane-bound fatty acid transport proteins, fatty acid translocase and cytosolic mobilization by fatty acid binding proteins, which may account for the preferentially transport of long polyunsaturated fatty acids. Further, an upregulation of fatty acid transporters in face of low fatty acid concentrations suggests a regulated transport and metabolism (76). In the placenta, lipids can be esterified, oxidized, interconverted, stored or transported to the fetal circulation (77). Lipids are stored in droplets in the trophoblast, both for later use and as a means to protect the cells from lipo-toxicity.
The transport, storage and metabolism of lipids in the trophoblast is affected by endocrine signals including insulin, glucocorticoids and adiponectin and thus suspected to be altered during hyperglycemia (32).

**Endocrine functions**

One of the major functions of the human placenta is the capacity to synthesize hormones and other mediators, as this endocrine function is crucial for gestational success. The placenta secretes a number of hormones into the maternal and/or fetal circulations; among them are the known pregnancy hormones like human chorionic gonadotrophin (hCG), human placental growth hormone (hPGH), human placental lactogen (hPL), estrogen and progesterone. Leptin, resistin, pregnancy-associated plasma protein A (PAPP-A) and placental protein 13 (PP-13) are also believed to be produced by the placenta and of importance in different stages of the pregnancy. Together these hormones affect placental establishment, development and function, fetal development, uterine quiescence and maternal adaptions to pregnancy through autocrine, paracrine and endocrine effects (29).

The major source of placental hormones is the syncytiotrophoblast, but almost all placental cells are involved in hormone production or regulation in some way. Both the synthesis and the response to hormones are tightly regulated in a cell specific manner, as exemplified by the differential effect of peroxisome proliferator receptor gamma (PPARγ) which stimulates hCG secretion in extracellular trophoblasts, but inhibits production in the syncytiotrophoblast. In extracellular trophoblasts, autocrine and paracrine hormones like hCG, hPGH, PAPP-A and resistin stimulates pathways that are vital for their migration and invasion of and contribute to vascular and uterine remodeling (78).

The placental hormone secretion and response are subjects to profound changes throughout gestation, e.g. hCG and leptin levels decrease towards term, whereas the steroid hormones continue to rise. These changes can be understood as a placental response to maintain the “plastic adaptation” that has to occur between the mother and her fetus in order to fulfill the fetal and
maternal demands as the pregnancy progresses. They may also be the cause of these altered demands, important for the regulation of distinct pregnancy stages like implantation, remodeling of the uterine vasculature, maternal metabolic adaptation, labor, breastfeeding etc. The effect of each placental hormone on the pregnancy is regulated by feedback systems and by several factors, including other placental hormones. Because of this intertwined regulation of each placental hormone, their implication and direct effects are still somewhat elusive. However, it is clear that altered levels of plasma hormones may have a negative effect on the gestational adaptation and the pregnancy outcome (78). A deficient or altered placental production of hormones is associated with several pregnancy complications like hyperemesis, preeclampsia, intrauterine growth restriction, gestational diabetes or chromosomal abnormalities (79). The changes overlap between the conditions, is blurred by the fluctuations of hormone levels in normal pregnancies, and for many conditions it remains unclear if these changes are causal or a placental adaptive response. However, in some pathologic conditions these changes occur prior to the diagnosis and thus their role as potential biomarkers together with clinical measurements and risk factors is subjected to research (80).

Placental adaptations

General considerations

The close relationship between placental weight and birthweight suggests that placental capacity adapts to meet fetal demand or vice versa. Heinonen et al. found that even though infants born small for gestational age have lower placental weight, the birthweight/placental weight ratio (by some called placental efficiency) is higher than for infants appropriate for gestational age (81). Placental adaptation can be defined as cellular mechanisms that serve to maintain overall homeostasis, including extension of the vasculosyncytial exchange surface and altered density and activity of transporters. The definition can be widened to include placental changes throughout pregnancy
which may serve to protect the growing fetus, including changes in placental oxygen and nutrient consumption, protein synthesis, enzyme activity, mitochondrial function and growth. Importantly, the fetus may also respond to the actual functional capacity of the placenta. Rapid growth of the placenta earlier in pregnancy may promote accelerated fetal growth, which may subsequently increase fetal demands (82).

**Placental interconversion of substrates**

Recent studies have revealed how specific levels of different nutrients may affect placental metabolism not only by possible effects on placental transporters, but also by driving the metabolic pathways directly. High glucose levels were found to reduce mitochondrial fatty acid oxidation and increase triglyceride accumulation in human placenta (83). The placenta has high nutrient and energy requirements in order to support the placental growth, the transfer of substances and the synthesis of proteins etc. It may use several metabolites interchangeably to meet these requirements, and it may adjust the relative contribution of each metabolite as a metabolic response to stress (e.g. hypoxia, hyperglycemia, or undernutrition) (Figure 7) (67, 84). In sheep, the maternal glucose supply and the amount of uteroplacental uptake have been found to directly regulate the oxidative metabolism of glucose in the uteroplacental unit, as the fraction of oxygen used to oxidize glucose was increased by increased uteroplacental glucose uptake. Furthermore, there was a reciprocal relationship between glucose oxidation and oxidation of other substrates (85). Thus, net placental transfer of a substrate is inevitably affected by the abundancy of other substrates and the interconversion of metabolites in the placenta.
Figure 7 | Placental metabolism and interconversion of substrates, simplified overview over possible pathways. Glucose that is not transferred to the fetus may be directed into glycolysis to produce pyruvate, fermentation to lactate or the pentose phosphate or polyol pathways both of which are important in the production of precursors of nucleotides for DNA and RNA synthesis. Glucose may also be stored as glycogen. Amino acids can be converted to and exchanged for other amino acids, used as building blocks for protein synthesis, or as fuel for the citric acid cycle. Free fatty acids can be used as fuel via beta-oxidation to produce NADH and acetyl-CoA, stored as triglycerides, or they may be converted to other free fatty acids before transported to the fetus. The relative importance of these pathways in human placenta depends on oxygen and substrate availability and is yet to be determined. AA: amino acids, FFA: free fatty acids, TG: triglycerides, NADH: nicotinamide adenine dinucleotide, ATP: adenosine triphosphate.

Oystein H. Horgmo, University of Oslo

Maternal supply and fetal demand

Although often presented as opposing, both maternal supply and fetal demands are likely to cause adaptations in placental transfer and metabolism (86). Several ovine models of maternal nutritional stress (undernutrition, hyper- and hypoglycemia) have demonstrated that the placental consumption of glucose varies with maternal glycaemia. However, when fetal glucose concentrations were manipulated independently of the maternal, these models also showed that placental consumption is dependent on fetal glucose.
concentrations (5, 7). It is plausible that placental adaptations to fetal signals occur in normal pregnancies supporting normal fetal growth and development. Failure to adapt or sustained adaptations may be part of the etiology in intrauterine fetal growth deviations (86).

The notion that the placenta adapts to maternal nutritional cues is supported by several studies. These cues may be oxygen levels, overall or specific nutrients levels or hormones known to be sensitive to maternal nutritional state and to affect placental transport and metabolism, e.g. insulin, cortisol, adiponectin and leptin (87, 88). The mechanistic target of rapamycin (mTOR) signaling pathways respond to changes in nutrient levels, hormones and growth factors to control cell growth and metabolism. In the placenta, mTOR likely regulates amino acid transporter activity and may be involved in regulating placental growth, and thus provides a possible pathway by which the placenta may respond to maternal signals (89). The relative importance of maternal versus fetal cues on regulation of placental transfer may vary though gestation. As an example, Desoye et al. have demonstrated that in early pregnancy the placental insulin receptors are located at the MVM of the syncytiotrophoblast, whereas at term, they are predominantly found at the placental endothelium. They suggest that this entails a shift in control of insulin-dependent processes from the mother at the beginning of pregnancy to the fetus at the end (90). Bozzetti at al. analyzed umbilical plasma samples obtained by cordocentesis. They demonstrated that at mid-gestation fetal glucose levels may supersede maternal if the maternal levels are low, a situation that is not described in late gestation (44). Both ovine and human studies have demonstrated that while maternal glucose levels are stable throughout pregnancy, the fetal levels in the umbilical artery decrease probably in response to increased insulin production (5, 44). This steepens the maternal-fetal gradient, and thereby increases the glucose supply, to match the increased needs of the growing fetus (91). This may indicate that the fetus’ own metabolism might be of increasing importance in the regulation of glucose transfer as pregnancy progresses. Finally, it is likely that responses to maternal and fetal cues are affected by each other (84, 87).
Acute and chronic (mal-) adaptation

Necessary and positive adaptations may become dysfunctional when longstanding (84, 92). Acute stress typically alters the placental handling of certain nutrients and hormones transiently. In the experimental ovine studies, acute maternal hypoglycemia reduced placental glucose consumption per kg placenta and maintained the glucose transfer to the fetus (5). Severe or chronic stress, or stress at specific periods of pregnancy, may alter placental nutrient transfer permanently and result in deviation of fetal and placental growth (84, 87, 92). In the ovine model chronic hypoglycemia early in pregnancy reduced placental growth, resulted in proportional larger utilization of glucose by the uteroplacental unit than by the fetus, and altered the placental transport capacity for glucose at any given maternal-fetal glucose gradient (84).

The placental and fetal responses to pregnancy perturbations may be understood as the product of timing, duration, treatment and severity of the insult. Importantly, the outcome of placental adaptions depends on the continuous environment. This is illustrated in general in the Barker hypothesis suggesting that adaptions to undernutrition increase the risk of diseases if the offspring is exposed to excess of nutrients (17). In parallel it has been hypothesized that adaptions early in pregnancy may persist after resolution of the perturbation and become maladaptive (64).

Adaptions versus dysfunction

Many current concepts of placental transfer arise from studies of pathological states or extreme experimental settings. Such settings may not demonstrate placental physiology, but dysfunction. The factors that are part of physiological placental adaption to maternal supply/fetal demand remain elusive and so does the distinction between placental adaptions and placental dysfunction.

Placental dysfunction in its widest sense is altered function of the placenta that results in an unhealthy outcome for the newborn. However, the term is often used synonymously with placental insufficiency, which usually refers to
placental transfer insufficient to meet the fetal nutrient or oxygen demands, resulting in deviating fetal growth. Several different pathological conditions, pregnancy perturbations and complications involve a dysfunctional placenta. Early-onset preeclampsia and IUGR are associated with the small insufficient placenta. However, also the large placentas present in many diabetic pregnancies may be considered dysfunctional. Whether the placental dysfunction is the cause or a consequence of a pregnancy complication is often unclear (Figure 8 and 9). It has been difficult to achieve international consensus on the definitions of pregnancy complications related to placental insufficiency, like IUGR and preeclampsia, because they are often based on overlapping sets of symptoms, sometimes without clear distinction to normal physiology, and with no consistent set of pathological features of the placenta (93, 94).

It is likely that the combined adaptations of the three entities of the pregnancy (mother, fetus and placenta) contribute to the inconsistency of both the symptoms and the findings, and as such hamper the research into placental complications (Figure 8 and 9).

Figure 8 | Placental adaptions may promote healthy growth despite an unhealthy environment or be part of a causal chain leading to deviating fetal growth. Failure to adapt may also cause fetal growth to deviate from its natural trajectory. Maternal perturbations may predispose failure of placental invasion and placental insufficiency.
In sum, placental dysfunction can be attributed to failure in the placentation process (invasion, vascular transformation and growth), perturbed villous development including premature or impaired maturation, altered placental blood flow, placental hypoxia or oxidative stress and unbalance between maternal supply and fetal needs.

Figure 9 | Failure of placental invasion may also occur in healthy mothers and cause placental insufficiency, growth restriction and preeclampsia.

Understanding placental nutrient transfer - why bother?

_Sibley, C.P._(95)

**Examples of how placental nutrient transfer is challenged in clinical conditions**

*Maternal overweight* is defined as BMI of 25 or above at the start of pregnancy. Both maternal overweight and excessive GWG increase the risk of gestational diabetes, preeclampsia, macrosomia, prolonged labor, operative deliveries, shoulder dystocia, miscarriages, and fetal death. The mechanisms linking maternal obesity to fetal macrosomia are largely unknown, but some of the effect seems to be mediated through increased placental weight (96). Overweight and excessive GWG are associated with increased nutrient availability for the placenta, increased inflammation and altered maternal hormones, cytokines and adipokines. It is believed that the combined modifying effects of these factors affect placental transport, possible by
influencing nutrient sensing pathways including insulin like growth factor-1 (IGF-1), mTOR and peroxisome proliferator-activated receptor-α (PPAR-α) (87, 97). A study of obese mothers giving birth to infants large for gestational age demonstrated a correlation between birthweight and GLUT1 receptors in the syncytiotrophoblast, but an independent correlation between maternal BMI and glucose transporters is yet to be demonstrated (98). Placenta derived cytokines related to overweight, like resistin and placenta derived hepatocyte growth factor, have been shown to stimulate glucose uptake and GLUT1 expression in trophoblast cells (99). Maternal obesity alters amino acid transporter expression in MVM, but the studies are conflicting as some showed upregulation, some unaltered levels and some downregulation (87, 100, 101). The discrepancies between these studies may be attributed to the type and size of the study population. In particular, some studies were restricted to cases of fetal overgrowth (100) whereas others studied unselected cases of maternal obesity (101). Animal models of maternal obesity have been studied extensively, some of the models resulting in restricted fetal growth and some in overgrowth. In sum they present deviating findings with regard to placental transport and transporter expressions (87, 102).

Maternal diabetes is divided into pre-gestational (type 1 or type 2) or gestational diabetes (GDM). Most of current knowledge about gestational diabetes is based on the old WHO definition (plasma glucose 7.8-11.1 mmol/L 2 hours after a 75g oral glucose load), which was based on the risk for future maternal diabetes rather than the direct effect on pregnancy outcome. Recent studies have demonstrated that the risk of pregnancy complications gradually increases with increasing maternal glucose levels, without obvious thresholds (103, 104).

The range and severity of pregnancy complications associated with maternal diabetes is somewhat different between pre-gestational and gestational diabetes. Although both represent increased risk for preeclampsia,

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1 The new WHO definition is plasma glucose 5.1-6.9 mmol/l in a fasting state, or ≥8.5≤11.0 mmol/L 2 hours after a 75g oral glucose load.
macrosomia, higher proportion of newborn body fat independent of birthweight, prolonged labor, shoulder dystocia and operative delivery, the risk is significantly greater for diabetes requiring insulin treatment in pregnancy. Pre-gestational diabetes is also associated with increased risk of spontaneous abortion, intra uterine fetal death, placental insufficiency, IUGR and congenital disorders.

Pre-gestational diabetes is associated with hyperglycemic effects early in pregnancy, fluctuating glucose levels, sustained stress and insulin treatment, all of which are associated with greater risk for macrosomia and in fact with alterations in GLUT1 in the syncytiotrophoblast (58, 99, 105, 106). GLUT1 in the BM have been shown to be consistently upregulated in type 1 and insulin dependent diabetes, but inconsistently in GDM (58, 63, 107, 108). GDM is often associated with higher BMI and altered metabolic and hormonal profile, but may also be considered a continuum of normal physiology where placental adaptations may arise later in pregnancy and are different from in pre-gestational diabetes.

More than 50 years ago, the Danish doctor Jørgen Pedersen in his thesis “Diabetes and Pregnancy: blood sugar of newborn infants” advanced a hypothesis that linked maternal diabetes to fetal overgrowth. He proposed that maternal hyperglycemia leads to excessive placental glucose transfer and fetal hyperglycemia, which then stimulates the fetal pancreas and results in fetal hyperinsulinemia accelerating fetal growth (109). (Interestingly, Pedersen also suggested that the average maternal glucose levels in pregnancy, through fetal insulin levels, affect the homeostatic set-point of the glucose handling in the fetal liver. Thus, even if he did not elaborate on long-term consequences, he touched upon matters later included in the Barker hypothesis.) While the Pedersen hypothesis has been implemented to suggest that there is an unopposed glucose transfer from the mother to the fetus, there is evidence that the mass of glucose transfer is influenced by several of the factors listed in Box 2. The HAPO study has demonstrated that the effect of maternal glucose levels on newborn weight is linear also within the normal range of maternal blood glucose (110). The STORK study showed that the
relations between maternal glucose and newborn weight, growth and adiposity are attenuated when the placenta weight is included in the statistical model (96, 111). In vitro studies have demonstrated unaltered glucose transfer in GDM, and hyperglycemia is associated with decreased placental glucose uptake and lower expression of transporters in experimental studies (112, 113).

The Pedersen hypothesis does not explain why macrosomia is common even in women with good glycemic control. This seeming paradox may have several, not mutually exclusive, explanations. One is the definition of “good glycemic control”. Another explanation is the association between GDM and high BMI, which comes with a range of other metabolic and hormonal changes that may alter placental function. In general, diabetes is associated with higher maternal circulating levels of glucose, amino acids, triglycerides, FFA and intermediate metabolites and thus altered maternal nutrient supply to the placenta (114, 115). There is evidence that diabetes alters placental and fetal uptake and metabolism of FFA, and thus promotes fat accretion in both (114, 116). Several studies have demonstrated that combined maternal adiposity and gestational diabetes are more consistently associated with increased fetal growth than obesity or GDM alone (reviewed in (117)). A third explanation for unexpected cases of macrosomia is given by lines of evidence demonstrating that maternal hyperglycemia and insulin affect placental function and set the growth trajectory early in pregnancy (64, 82). This conceptual framework has been named the fetal glucose steal. It proposes that pulsatile hyperglycemia and/or poor glycemic control in early and even pre-pregnancy might be of importance for fetal insulin levels throughout pregnancy (5, 61). The high fetal insulin levels are believed to result in higher fetal glucose consumption (and thus increase the maternal-fetal gradient and placental glucose transfer) and promote fetal growth despite later maternal glycemic control (64). The authors speculate that the increased fetal glucose consumption contributes to maternal euglycemia later in pregnancy (64). The direct effect of insulin treatment on the development of the conceptus, beyond reduction of malformations, is largely unknown (57, 58, 99, 106).
Altogether, the mechanisms linking maternal glucose levels and fetal growth are not fully understood and still a subject of interest for researchers.

**Maternal mal- or undernutrition** is associated with IUGR and altered placental size and function. In developed countries IUGR is believed to be predominately caused by primary placental insufficiency and/or reduced perfusion of the placenta in the absence of maternal malnutrition, both of which may cause reduced maternal supply of nutrient to the placenta.

Experimental fetal IUGR caused by undernutrition, hypothermia, reduced placental flow by uterine ligation and fetal hypoglycemia are perhaps the perturbations best studied in relation to placental transfer and metabolism in a variety of animal models (5, 7, 118). Studies of IUGR in humans are largely restricted to retrospective designs due to the relative low incidence and obvious ethical reasons. Clinical studies have demonstrated lower fetal glucose and amino acid levels and lower maternal/fetal ratios of long-chain polyunsaturated fatty acids in IUGR compared to infants appropriate for gestational age. Elegant *in vivo* studies of radiolabeled amino acids administered to the maternal circulation prior to cesarean section demonstrated altered amino acid transfer in IUGR (119). Several animal models of IUGR and placental insufficiency (made by caloric or specific nutrient restrictions and high ambient temperature) showed reduced net placental transfer of both glucose and amino acids (120-122). In experiments using uterine arterial constriction, however, the placenta uses less glucose and the glucose transfer to the fetus is sustained. The mechanisms regulating placental glucose metabolism under these conditions may be different as they involve altered blood flow and oxygen supply to the uteroplacental unit (84).

Glucose transporter expression has not consistently been shown to be altered in rodent IUGR models, but interestingly Kavita et al. found reduced expression of GLUT1 in the MVM in a baboon model of maternal undernutrition (121). System A amino acid transporter (SNAT) expression and activity has been found to be reduced in the MVM of human pregnancies affected by IUGR and in the non-human primate model of maternal undernutrition (87, 121, 123). However, in a cohort of newborns appropriate
for gestational age, Godfrey et al. found that the SNAT activity in the MVM was highest in the smallest babies (124). This may be interpreted to imply that SNAT activity is upregulated to meet fetal demand and that failure of such upregulation may be part of the IUGR etiology (86). The actual contribution of these changes in transporter expression to the overall quantity of nutrient transfer remains to be settled.

**Hypoxia** is believed to be part of the causal cascade in many different pregnancy complications associated with placental insufficiency and IUGR, including preeclampsia. Many aspects of placental function are altered in response to hypoxia. In experimental studies, uteroplacental O\(_2\) consumption is unaffected by a range of acute and chronic nutritional stresses, and it is maintained faced with reduced oxygen delivery (anemia or uterine artery ligation) by increased extraction (84). In contrast, chronic stress, whether it is hypoxia or nutritional, may alter placental mitochondrial function and affect the ATP-production and thus the placental function in general. Altered placental consumption of oxidative substrates might affect the transfer of nutrients (84). There are experimental indications that the fetus grows in relation to its overall O\(_2\) availability (84). On the other hand, Zamudio et al. concluded that hypoxia related growth restriction in human may be due to lower fetal glucose consumption and insulin levels rather than O\(_2\) availability (125). By studying human pregnancies at high altitude, Zamudio et al. described the isolated effect of hypoxia on placental glucose transfer and O\(_2\) consumption. According to their interpretation, placental O\(_2\) consumption did not increase, but fetal glucose levels and consumption was decreased. Preferential anaerobe glucose metabolism in the placenta was believed to maintain fetal O\(_2\) delivery and consumption (125).

**Preeclampsia**

Preeclampsia is a hypertensive pregnancy disorder believed to be caused by the placenta, as it occurs only in the presence of a placenta and is related to placental mass. Preeclampsia represents a major cause of maternal and neonatal morbidity and mortality worldwide with an incidence of 3-6% (126).
Definition

Preeclampsia is a syndrome of rather inconsistent coexistence of certain clinical symptoms, and the definition has changed both in time and between regions. The current definition according to Norwegian guidelines is new onset of hypertension (blood pressure $\geq 140/90$) and proteinuria (ratio of protein/creatinine $\geq 0.3$) after 20th gestational week (127). Several guidelines including the latest from the American College of Obstetricians and Gynecologists, and the International Society for the Study of Hypertension in Pregnancy (ISSHP), have omitted proteinuria as a mandatory criterion in their revised definitions (128, 129). The new definition according to ISSHP is hypertension and the coexistence of one or more of the following new-onset conditions including proteinuria, signs of maternal organ dysfunction (renal, liver, hematological or neurological complications) or fetal growth restriction.

Clinical symptoms

The maternal clinical symptoms and signs are diverse (elevated blood pressure, severe headache, visual symptoms, eclampsia, severe abdominal pain in the upper quadrants, vomiting, liver dysfunction, thrombocytopenia, hemolysis, renal dysfunction, general and pulmonary edema) and reflect general endothelial dysfunction. When preeclampsia occurs early in pregnancy (<34 gestational week) it is associated with inadequate cytotrophoblast invasion and remodeling of the spiral arteries, placental pathology like infarctions and acute atherosis, and fetal complications like IUGR and oligohydramnios. Late onset preeclampsia, on the other hand, is associated with both low and high birthweights. There is still no cure for preeclampsia except delivery of the placenta. Hence, the clinical decisions must balance the health of the mother and her fetus, and prematurity is an important and severe consequence.

Pathophysiology

Even though there are some distinct differences between early and late onset preeclampsia, no clear support for a dichotomized etiology has been found
It is likely that several different factors act together and at different stages of pregnancy, predisposing and causing the development of the clinical syndrome we recognize as preeclampsia. Thus, the understanding of the pathophysiology, the search for diagnostic tools and development of preventive strategies has been challenging (131).

The placental origin of preeclampsia is challenged by the fact that no placental pathology or biochemical markers are consistently associated with preeclampsia. This might support the idea that the syndrome is a consequence of a troubled interplay between the mother and the conceptus (131, 132). There are common risk factors (chronic hypertension, diabetes, obesity, metabolic syndrome, renal disease and certain autoimmune diseases) for preeclampsia and cardiovascular disease, which support the hypothesis that pregnancy is a stress test that exposes underlying sub-clinical vascular disease (133). It is likely that the risk factors may contribute to the development of preeclampsia both by affecting the placentation process with transformation of the spiral arteries and villous development, but also by affecting the baseline sensitivity of the maternal systemic endothelium to placental substances released from a dysfunctional placenta.

Recent theories suggest that diverse types of cellular stressors (e.g. hypoxia, oxidative stress, inflammation) may affect the syncytiotrophoblast at any stage of the pregnancy and induce a cellular stress response (134). The outcome will depend on the type, suddenness, persistence and impact of the stress and on the balance of the response. In addition, the clinical picture will depend on when in pregnancy the syncytiotrophoblast is affected, contributing to the diverging debut and consequences for the mother and neonate (134). The syncytiotrophoblast stress, regardless of timing and type, is thought to result in an alteration of syncytial compounds secreted into the maternal circulation. Protein containing compounds have been most extensively studied, especially proteins with vasoactive properties. The altered secretion is believed to cause an imbalance of circulating angiogenic and antiangiogenic factors engaged in the endothelial maintenance, and thus contributing to the maternal endothelial dysfunction (134). Since the
production of angiogenic factors is considered central for the regulation of placental vascular development and trophoblast invasion, this imbalance can be both a cause and consequence of inadequate invasion of the cytotrophoblasts and shallow placentation (135, 136). This highlights the possibility that intrinsic factors in the mother as well as placental dysfunction may cause this syndrome and its diversity.

The vascular endothelial growth factor (VEGF) family including Placental Growth Factor (PlGF) are involved in angiogenesis and have been shown to promote proliferation, migration and survival in endothelial cells by binding to their transmembrane receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR) (Figure 10A). VEGFR-2 exclusively binds VEGF, whereas VEGFR-1 binds both PlGF and VEGF. The two ligands for VEGFR-1 display distinctive intracellular effects (137). Soluble Fms-like tyrosine kinase-1 (sFlt-1) is a soluble form of VEGFR-1 lacking the transmembrane and intracellular domain and thus acts as an anti-angiogenic factor by binding a fraction of VEGF and PlGF in the circulation (Figure 10B). PlGF potentiates VEGF signaling, by promoting intermolecular cross talk between the VEGF receptors and perhaps by displacing VEGF from VEGFR-1 and sFlt-1, promoting binding to the more potent VEGFR-2 (Figure 10A). Furthermore, heterodimers of both the ligands and the receptors display a more potent angiogenic effect in *in vitro* studies (137).

In healthy adults, sFlt-1 is found to be expressed in endothelial cells and monocytes and PlGF is expressed in endothelial and smooth muscle cells (138, 139). In normal pregnancies, VEGF ligands and receptors are shown to be highly expressed in the placental tissue and invasive cytotrophoblasts express VEGF, PlGF and sFlt-1 in early first trimester (140, 141). The plasma concentrations of these factors throughout pregnancy have been studied in several longitudinal studies in nested case-control design. sFlt-1 increase with gestational age in normal pregnancies, but is significantly higher in preeclamptic pregnancies (142). PlGF concentrations follows a bell-shaped curve with an initial rise that plateaus around weeks 32–33 in normal pregnancies, then falls toward term (143-145). In preterm preeclamptic
women, the levels are lower, the rise is slower, the peak plateau come earlier (week 24–27), and the decline is less marked. The altered levels of sFlt-1 and PIGF have been shown to occur several weeks prior to the clinical manifestations of early-, but not late-onset preeclampsia, and to correlate with the severity of the disease (143, 146, 147).

Altogether, current concepts suggest that an altered placental release of these factors is part of the pathogenesis of preeclampsia. On the other hand, there are some indications of a general endothelial contribution to the elevated sFlt-1, which might be in line with concept that maternal inherent endothelial dysfunction contributes to the disease (138, 148-151). The placental and endothelial contribution to the circulating pool of these factors in vivo remains to be settled.

Figure 10  
A: In normal pregnancies PIGF and VEGF binds to the transmembrane receptors promoting migration, proliferation and cell survival in endothelial cells. By displasing VEGF from VEGFR-1 PIGF is thought to promote VEGF binding to the more potent receptor VEGFR-2.  
B: Preeclamptic pregnancies are characterized by more circulating sFlt-1 which binds PIGF and VEGF in the circulation, and thereby prevent binding to the bioactive transmembrane receptors.  
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Methods to study placental transfer and metabolic function

It is difficult to access and study the human placenta in vivo without exposing the mother and fetus to unethical risks. Studies of placental function in the
human are therefore largely based on *in vitro* and *ex vivo* models. The majority of previous *in vivo* studies of placental transport and metabolism have been performed in animals (5, 47, 152).

**Animal vs human**

Placental structure and functions vary considerably between species. The thickness of the placental exchange surface is a function of the invasiveness of the placenta and will have important impact on the nutrient transfer. Based on the histological structure of the placental exchange surface, placentas are classified (from thinnest to thickest exchange surface) as hemomonochorial (primates and guinea pig), hemodichorial (rabbits), hemotrichorial (rodents), endotheliochorial (carnivores), and epitheliochorial (horses, pigs and ruminants) (Figure 11) (153). In addition to the structural differences the placentas differ in their immune expression and nutrient transporters. Whereas humans predominantly express GLUT1 in the syncytiotrophoblast of term placentas, the most frequent used animal models (mouse, rat and sheep) also express GLUT3. What’s more, for some of these animals the proportion of GLUT1 decreases as the proportion of GLUT3 increases towards term (154, 155). Furthermore, the duration of pregnancy and frequency of multiple pregnancies vary between species. Most human pregnancy complications do not naturally occur in animals, from this follows that experimental animal studies of placental pathology are performed in engineered models. This raises question about their relevance, especially considering the absence of clear pathophysiological understanding of most pregnancy complications. Of uttermost relevance when studying nutrient transport are the highly different diets / energy sources between human and some animals. As an example, the adult sheep and her term fetus use lactate as an important an energy source, whereas humans do not (156). Lastly, the amount of subcutaneous fat is 12-15% in human term newborn, but only 3-5% in the rhesus monkey and even less in other mammals used as models. The placental weight is an independent determinant of infant body fat mass indicating an important role of the placenta in human fetal fat deposition (157). However, animal models have important advantages over humans in their reproductive rate and in the possibilities that lie in altering factors that
may influence the pregnancies, including genes, hormones and nutrients. Furthermore they represent a unique possibility to study placental transfer in vivo at different stages of the pregnancy. Important current concepts of placental transfer originate from ovine and other animal studies (5, 158, 159). Moreover, the mouse model has been very important for studying the effect of maternal obesity, diabetes, undernutrition and hypoxia as well as for modeling preeclampsia.

In sum, extrapolation from animal models to humans must be done with caution, as they can never be more than just models (20-22). Importantly, these models may be used to test causal hypothesis and to understand mechanistically observations made in humans.

Figure 11 | Schematic of different placental exchange surfaces. (a) Haemomonochorial placenta. Only one layer of syncytiotrophoblast separates the maternal blood space from the fetal capillaries. (b) Haemotranchorial placental exchange surface (e.g. mice and rats). Three layers of trophoblast cells separate the maternal blood space from the fetal capillaries. (c) Epitheliochorial placental exchange surface (e.g. sheep). Maternal capillaries are intact. One layer of uterine epithelium cells and one layer of trophoblast cells separate maternal and fetal capillaries. Figure reprinted with permission, Mikkelsen E et al. Royal Society open science. 2017;4(4):161098.(160).
In vitro vs in vivo

*Ex vivo* studies of human placental explants, trophoblast cells and perfused placentas allow for experimental testing of hypotheses, by manipulating the study conditions. However, even if these methods can test the effect of single factors on trophoblast cells or overall placental function, they cannot directly extrapolate to the complex *in vivo* setting where manipulation of one factor is likely to cause alteration in several others. Clinical cohorts and epidemiological studies have been used to study the relationship between maternal and placental factors and outcomes in the offspring. They have unraveled how placental weight modifies the effect of maternal metabolic factors on fetal outcome of pregnancy outcomes, but give limited information of placental physiology *in vivo* (96, 157). Studies of human placentas will, with some exceptions, be restricted to after the delivery, which hampers the possibility to differentiate causal findings from adaptive in pathological pregnancies (20).

Research questions regarding placental transport and endocrine functions *in vivo* require blood samples and thus poses both ethical and methodological difficulties. Apart from a few small studies conducted by cordocentesis, (44, 91, 161) which hardly can be replicated for ethical reasons, studies are restrained to observe the normal or diseased placenta at term or at the point of delivery. Ideally, studies conducted *in vivo* can measure an integrative effect of the variables studied, but the observational and cross-sectional design hampers the possibility to study causal relationships. Studies of traceable markers/stable isotopes have been conducted in humans and represent a powerful approach that would provide more dynamic and mechanistic information (162).

Ultrasound studies are safe, feasible and are used to study human placental and fetal development *in vivo* in healthy and pathological pregnancies throughout gestation e.g. in longitudinal designs.

Physiology vs pathophysiology

Comparing healthy with pathological human pregnancies is useful to gain knowledge about pathology, and might tell us about placental function and
adaptions. Nevertheless, extrapolating placental features of pathological pregnancies to the physiological setting might not necessarily be correct. The forces that balance human physiology might be different from the ones that are changed in pathology.

Knowledge gaps

In general, studies of human placental functions and dysfunctions meet major challenges for several reasons. First because of the inaccessibility of the human placenta in vivo, second because of the comprehensive interplay that occurs both at the cellular, organ and individual (mother and fetus) level and last because the definitions of several pathological conditions are inconsistent, as discussed above.

The paper-specific knowledge gaps in the present thesis are as follows:

- Current concepts of basic placental transfer and secretory functions are largely based on in vivo animal studies and experimental in vitro studies. Methods to study human placental transfer and secretion in vivo are scarce (paper I).
- The Pedersen hypothesis of the relationship between maternal glucose levels and fetal overgrowth implies that fetal access and consumption of glucose is an unmodified consequence of maternal glucose levels. However, animal models demonstrate a more fine-tuned interplay between maternal supply of glucose and fetal glucose consumption. Less is known about the in vivo transfer of glucose in human term pregnancies (paper II and III).
- The STORK study demonstrated, in a human clinical cohort, that placental weight modifies the strength of the statistical relation between maternal glucose levels and fetal outcome (birthweight, fetal growth and neonatal adiposity). Animal studies also indicate that maternal-fetal transfer of glucose cannot be understood without considering the effect of the placenta, because placental consumption of glucose influence the fetal glucose levels and consumption. The
placental glucose consumption has not been quantified and studied in human (paper III).

- The GLUT1 density of the BM is considered rate-limiting for placental glucose transfer, but the relation between GLUT1 expression in the syncytial membranes and the net placental glucose uptake and release have not been explored in vivo (paper III).

- In pathological pregnancies like preeclampsia and placental dysfunction related to IUGR, the circulating levels of PlGF and sFlt-1 are consistently found to be altered. In vitro studies of trophoblasts and human placental explants suggest a placental origin of these alterations. The general endothelial expression of these factors and the large systemic endothelial compared to placental surface may indicate a possible endothelial contribution of these factors in pregnancies. The fact that preeclamptic mothers display the combined risk factors for preeclampsia and cardiovascular disease prior to their pregnancies supports inherent subclinical endothelial dysfunction. The in vivo placental release of PlGF and sFlt-1 has not been determined and compared to the release from an endothelial surface in normal and preeclamptic pregnancies (paper IV).
Objectives of thesis

Overall aim

The overall aim of the thesis was to investigate to which extent current models and hypotheses of placental physiology and pathophysiology are applicable to a human in vivo setting.

Specific aims

PAPER I

The aim was to establish an in vivo method for studying human placental physiology and pathophysiology.

PAPER II

Using the 4-vessel sampling method described in paper I on a healthy pregnant population, we aimed to study placental glucose transfer in vivo by determining the AV- concentration differences on the maternal and fetal sides of the placenta together with the maternal-fetal glucose gradient. Next, we aimed to investigate how current concepts of placental glucose transfer fit the human in vivo observations. We tested the hypothesis that the fetal umbilical v-a glucose difference is mainly determined by maternal glucose concentration and the uteroplacental AV- glucose difference.

PAPER III

Given a correlation between fetal consumption of glucose and birthweight, the overall was aim to identify factors related to fetal glucose consumption.

Specifically, we aimed to quantify uteroplacental glucose uptake in healthy pregnancies, and to determine the allocation of glucose between the placenta and fetus. We hypothesized that maternal factors known to be independently associated with birthweight, i.e. maternal BMI, glucose levels and GWG, positively correlate with uteroplacental uptake and fetal consumption of glucose. Next, we hypothesized that fetal glucose consumption also is related
to fetal and placental factors, i.e. placental weight, GLUT1 expression and glucose consumption, and fetal insulin and glucose levels.

PAPER IV

By using the unique 4-vessel sampling method we aimed to answer some of the prevailing questions about placental dysfunction in preeclampsia. Specifically, we aimed to investigate whether the human placenta releases s-Flt1 and PlGF to the maternal circulation in normal pregnancies and whether this release is altered in preeclamptic pregnancies. Further, we tested whether we could demonstrate an extra-placental release by measuring the AV-difference in the forearm.
Materials and methods

Ethics

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 S-07174a and 2419/2011. All patients signed a written informed consent.

Study design

The thesis is based on a clinical cross-sectional study and a case-control study conducted at Oslo University Hospital, Oslo, Norway.

Study population

The study consists of two populations, study arm 1 (fall 2012- spring 2015) including healthy women, with uncomplicated pregnancies and study arm 2 (2008-ongoing) including women with preeclampsia (Figure 12). Study arm 1 was designed to study nutrient transfer and placental metabolic properties in vivo, and also to serve as a control group for study arm 2 in case-control study design.

Study arm 1:
Healthy, non-smoking women with uncomplicated singleton pregnancies who were scheduled for cesarean section were approached for inclusion in the study. Indications for cesarean section were more than one prior cesarean section, breech presentation with disadvantageous pelvic measurements, mechanical disproportion, previous rupture of the anal sphincter with sequela, previous traumatic delivery, mother’s request/anxiety and previous myomectomy. Exclusion criteria were pre-existing co-morbidity (except mild asthma, well controlled hypothyreosis), medication (except thyroxine and the occasional use of antibiotics, paracetamol and antihistamines), start of labor prior to the planned caesarean section, and pregnancy complications.
Figure 12  Study population study arm 1 healthy pregnancies. Among the 179 included women with normal pregnancies complete 4-vessel blood samples were obtained in 162 (91%). Fifty-one (28%) participants were not included for ultrasound measurements due to logistical limitations. Of the 128 participants (72%) subjected to ultrasound, blood flow measurements at the fetal side of the placenta were obtained in all participants, whereas complete blood flow measurements at both the maternal and fetal side were obtained in 77 (60%). The microvillous and basal membranes were isolated in a sub-cohort of 33 maternal-fetal pairs. (Maternal A indicates the radial artery and V the uterine vein; Fetal a the umbilical artery and v the umbilical. M+F 4-vessel indicates complete arteriovenous samples on maternal and fetal side of the placenta).
Study arm 2: Women, with singleton or duplex pregnancy, diagnosed with preeclampsia before gestational week 34 and delivered by cesarean section were included in study arm 2. Preeclampsia was defined as hypertension (systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg) and proteinuria (repeated positive protein urine dipstick (≥+1) or urine protein/creatinine ratio >30 mg/mmol). All women approached for inclusion accepted the invitation. Exclusion criteria were smoking, start of labor prior to caesarean section, and considerable preexisting morbidity apart from preexisting hypertension.

Paper I is a method article and include the whole healthy cohort (Figure 12). Paper II is a sub cohort of the 40 first women included in our study. Paper III includes the whole healthy cohort (Figure 12), and results from a sub-cohort of 33 women from which placenta samples were processed to separate the two membranes of the syncytiotrophoblast. Paper IV is a case-control study and includes 23 patients from study arm 2 and 20 healthy controls, randomly chosen from the first 40 women of study arm 1.

Data collection and measurements

The method has been described in paper I. A brief summary is presented in the following text and in Figure 13.

Clinical data

Medical and obstetric history was noted at inclusion. Clinical data was collected at delivery. Mothers were weighed on a calibrated scale at the morning of delivery (Tanita Body composition Analyser, Tokyo, Japan). Newborns and placenta were weighed and measured by the attending midwife using a calibrated scale within two hours of delivery.
Overview of the 4-vessel sampling method

The 4-vessel sampling method refers to the sampling of blood from the incoming and outgoing vessels at the maternal and fetal side of the placenta (Figure 13). A blood sample from the antecubital vein was sampled in addition to serve as a reference. These samples were used to calculate paired arteriovenous concentration differences on both sides of the placenta and maternal-fetal gradients of different compounds (Calculations on page 8). Combined with volume blood flow in the maternal uterine artery and the umbilical vein measured by ultrasound, placental and fetal uptake, consumption and release were quantified according to the calculations listed on page 8 and in Figure 1.

Figure 13 | Overview over the 4-vessel sampling method. Abbreviations: Arad, Radial artery used as proxy for uterine artery; Vut, Uterine vein; Aumb, Umbilical artery; Vumb, Umbilical vein. Reproduced from Michelsen TM, Holme AM, Henriksen T. Transplacental nutrient transfer in the human in vivo determined by 4-vessel sampling, Placenta. 2017 (23), Øystein H. Horgmo, University of Oslo
**Ultrasound measurements**

Uterine and umbilical volume blood flow was determined by ultrasound at the day of delivery by a single experienced fetal-medicine specialist (Guttorm Haugen) using the same equipment (Acuson Sequoia 512, Siemens Healthcare GmbH, Erlangen, Germany). Doppler ultrasound was used to identify the vessels, to measure time-averaged maximum velocity (TAMX) and to identify branches between the site of diameter and TAMX measurements. The velocity was obtained as the mean velocity of three heart cycles (uterine artery) or over 3-5 s in vessels with non-pulsatile flow (umbilical vein) with an insonation angle below 30°. The uterine artery was visualized as it crossed over the external iliac artery, and TAMX was measured close to this site. The internal vessel diameter was measured in a perpendicular angle as close to the site of velocity measurements as possible and calculated as the mean value measured in 5-10 optimal frames. The internal vessel diameter and TAMX of the umbilical vein were measured in the straight portion of the intra-abdominal umbilical vein, before any visible branches. Intra observer variation measured as intra class correlation coefficient for umbilical vein diameter by the present observer has previously been reported to be 0.97 (163). The equivalent coefficient for the uterine artery diameter was 0.97 (n=16).

**Blood samples**

During planned cesarean section, when the uterine vein was exposed, blood samples were collected simultaneously from the radial (as a proxy for the uterine) artery, the uterine vein and the antecubital vein (Figure 13). Fetal blood samples from the umbilical artery and vein were obtained immediately after delivery of the infant, before delivery of the placenta. All blood samples were immediately transferred to ethylenediaminetetraacetic acid (EDTA) vacutainers (Vaccuette, Greiner bio-one, Kremsmunster, Austria), put on ice and centrifuged within 30 min at 6 °C, 2500 x g for 20 min. Plasma were stored at –80° C.

Plasma glucose was measured by the hexokinase/glucose-6-phosphate dehydrogenase enzymatic *in vitro* test on a Modular P800 (CV 1.9%)
Plasma insulin was analysed by immunoassay with monoclonal specific antibodies using a sandwich principle and detection by electro-chemiluminescence on a Modular E170 (CV 4.9%) (Roche, Mannheim, Germany). Plasma progesterone was analysed by immunoassay with monoclonal specific antibodies using a competition principle and detection by electro-chemiluminescence on a Modular E170 (CV 2.0%) (Roche, Mannheim, Germany). All the above analyses were performed at the Department of Medical Biochemistry, OUS, Rikshospitalet. Plasma glutamic acid was analyzed by an Applied Biosystems 4000 Q TRAP linear MS/MS spectrometer (Foster City, CA, USA), liquid chromatography/tandem mass spectrometry-LC/MSMS, at the Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Norway, using modifications of a previously described method (164). Briefly, plasma samples were thawed and underwent protein precipitation without derivatization. The chromatographic separation was performed on a C-18 high-performance liquid chromatography column. Then glutamic acid was identified by mass spectrometry, corresponding to a particular internal standard. The concentration of glutamic acid was determined from the ratio of analyte peak area/ internal standard peak area against a linear multiple point calibration curve (CV< 10%).

PlGF and sFlt-1 plasma concentrations were measured in one run and in duplicates by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) by the PhD-candidate with assistance of a bioengineer with experience. The reported intra- and inter assay variation coefficients were 7.0% and 11.8%, respectively, for PlGF; corresponding numbers for sFlt-1 were <10%

**Placental samples**

Placentas were weighed, and placed on ice immediately after delivery. They were trimmed and placental homogenates were prepared from 50g of placental tissue and frozen at -80°. The further preparation of the placental tissue and transporter analysis was performed in collaboration with the Powell/Jansson lab in Denver, Colorado. Syncytiotrophoblast MVM and BM were isolated using differential centrifugation and Mg²⁺ precipitation and stored at -80°C in
buffer D containing protease and phosphatase inhibitors (Sigma-Aldrich Co. LLC) according to a well-established protocol (45, 165). Membrane protein content was determined using Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

GLUT1 protein expression in the MVM and BM was determined by Western Blotting. Five μg of protein was separated on an any kD Mini-Protean TGX gel (Bio-Rad, Hercules, CA, USA) and transferred to an Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA, USA) overnight. The membranes were blocked with 5% Blotting-Grade Blocker (nonfat dry milk) in 0.1% Polysorbate 20 in tris-buffered saline (TBST), and incubated with 1:1000 Anti-GLUT1 antibody (Millipore, Billerica, MA, USA) in 5% Bovine Serum Albumin (BSA) in TBST. Antigens were detected using 1:3000 Anti-rabbit IgG, HRP-linked antibody (Cell Signaling, Danvers, MA, USA) in 5% BSA/TBST and enhanced chemiluminescence detection with Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA). Amido Black total protein staining was used to control for protein loading or transfer.

Statistical analysis

Descriptive data was presented as mean with standard deviation (SD), median with interquartile range [IQR] or [Q1, Q3] or as numbers (%). Groups were compared using independent t-test, Mann–Whitney U-test, chi square or Fisher’s exact test as appropriate.

Comparisons between concentrations in the artery and vein in maternal and fetal circulations were performed by paired t-test when variables were normally distributed, or Wilcoxon signed rank test when they were skewed. We used Pearson’s correlation coefficient to describe relationships between two normally distributed variables, and Spearman’s rank correlation coefficient in the case of correlation between skewed variables or when a nonlinear relationship was expected.
Synopsis of results

Paper I
In the first paper our method for studying human placental physiology was presented. The combined 4-vessel sampling technique and ultrasound volume blood flow measurements were described both in text and in a video. We demonstrated that by determining the volume blood flow, collecting blood samples from the incoming and outgoing vessels on both sides of the placenta, placenta tissue samples, as well clinical data of the mother and infant many aspects of placental function can be studied \textit{in vivo}.

Placental function, and the interaction between the mother and the fetus, relies on specific transfer, release and uptake of substances from the two circulations. We illustrated how the 4-vessel method can be used to study these elements of placental function \textit{in vivo} by demonstrating a placental net-transfer of glucose from the maternal to the fetal circulation, a placental net-uptake of glutamic acid from both circulations and a placental release of progesterone to the maternal circulation.

Paper II
Plasma was collected from the uterine vein, radial artery, antecubital vein, umbilical artery and vein of the 40 first maternal-fetal pairs included in study arm 1. The samples were analysed for glucose and insulin, and AV-differences were calculated (Figure 14).

The placenta extracted 6% of the glucose available in maternal blood, and the fetus consumed approximately 10% of the glucose in the fetal-umbilical circulation.

In these 40 maternal-fetal pairs, the glucose concentration in the umbilical vein was not related to birthweight or placental weight, whereas the correlation between the fetal umbilical v-a glucose difference and birthweight reached significance (r 0.32, \(p=0.049\)).
Figure 14 | Glucose concentrations and gradients (mmol/L) in the arteries and veins on the maternal and fetal side of the placenta. There were significant AV-differences on both sides of the placenta. Maternal arterial glucose concentration was higher than the glucose concentration in the umbilical artery in all subjects. *p<0.001, paired t-test.

The glucose and insulin concentrations were found to correlate on the maternal (r 0.52, p=0.001), but not on the fetal side, (r 0.12, p=0.48). Fetal insulin was however correlated with the fetal v-a glucose difference (r 0.43, p=0.007), placental weight (r 0.46, p=0.003) and birthweight (r 0.58, p<0.001) in line with the notion that insulin is an important growth factor for the fetus.

Maternal arterial and fetal venous concentrations of glucose were highly correlated (r 0.86, p<0.001). In contrast to what was expected maternal arterial glucose concentration was not significantly correlated to the fetal umbilical v-a glucose difference (r 0.3, p=0.07). Furthermore, the uteroplacental AV-glucose difference was neither correlated to the level of glucose in the umbilical vein, nor the fetal umbilical v-a glucose difference (r 0.16, p=0.33 and r 0.18, p=0.28).

The main driving force for placental glucose transfer, the maternal-fetal gradient, was highly correlated to the fetal umbilical v-a difference (r 0.8, p<0.001) and negatively correlated to glucose concentration in the umbilical artery (r -0.38, p=0.017), whereas no correlation was found with the glucose concentration in the umbilical vein. Similarly, the fetal umbilical v-a glucose difference was negatively correlated to the glucose concentration in the
umbilical artery (r = -0.45, \( p = 0.004 \)), but not to the glucose concentration in the umbilical vein.

In sum, the maternal glucose concentrations and uteroplacental AV–difference did not correlate with fetal uptake of glucose (given by the fetal umbilical v-a difference), whereas the maternal-fetal gradient was negatively correlated with the fetal glucose concentrations in the artery and strongly positively correlated to the fetal glucose uptake.

**Paper III**

Volume blood flows and glucose concentrations were measured in the participants of study arm 1. The uteroplacental glucose uptake (\( n = 70 \)), fetal glucose consumption (\( n = 125 \)) and the placental glucose consumption (\( n = 70 \)) were calculated in \( \mu \text{mol/min} \) (calculations are listed on page 8).

The median \([Q_1, Q_3]\) volume blood flow was 488.4 [358.5, 604.4] mL·min\(^{-1}\) in the uterine artery and 196.2 [158.3, 232.2] mL·min\(^{-1}\) in the umbilical vein.

The median uteroplacental glucose uptake was 117.1 [59.1, 224.9] \( \mu \text{mol·min}^{-1} \).

The fetal glucose consumption was 96.8 [52.7, 144.5] \( \mu \text{mol·min}^{-1} \) and 28.9 [15.4, 41.8] \( \mu \text{mol·min}^{-1}\cdot\text{kg}^{-1} \) when corrected for fetal weight. The placental glucose consumption was 31.9 [-40.6, 92.6] \( \mu \text{mol·min}^{-1} \) and 51.4 [-65.8, 185.4] \( \mu \text{mol·min}^{-1}\cdot\text{kg}^{-1} \) when corrected for placental weight.

36% had higher fetal glucose consumption than uteroplacental uptake. Expressed as median percentage with quartiles, the fetal and placenta glucose consumption constituted 31% [-17, 66%] and 69% [33, 117%] respectively of the uteroplacental uptake.

In line with the results in paper II, the fetal glucose consumption was correlated with birthweight (Spearman’s rho 0.34, \( p < 0.0001 \)) and ponderal index (rho 0.42, \( p = 0.002 \)). Fetal insulin is considered growth factor in utero and was correlated with the fetal glucose consumption as well birthweight in our study.
The fetal glucose consumption was not correlated with early pregnancy BMI, GWG or parity, indicating that the effect of these factors on fetal growth goes through other mechanisms than fetal glucose consumption. In contrast to what was expected fetal glucose consumption was rather weakly correlated with maternal glucose levels (rho 0.20, p=0.03), and not with uteroplacental glucose uptake (rho 0.09, p=0.45).

Several findings indicated that these relationships were attenuated by placental and fetal factors. Fetal glucose consumption was positively correlated with placental weight (rho 0.2, p=0.002), and inversely correlated with placental glucose consumption (rho -0.47, p<0.001). In the sub cohort consisting of 33 women, there were no correlations between GLUT1 density in BM and maternal glucose, uteroplacental uptake, placental or fetal consumption of glucose. However, GLUT1 MVM and BM expression were positively correlated with glucose levels in the umbilical artery (rho 0.38, p=0.03 and rho 0.36, p=0.045 respectively). Furthermore, and contrary to what was expected, the uteroplacental glucose uptake was inversely correlated with the MVM (rho=-0.44, p=0.01), also after adjustment for placental and fetal weight. Altogether, the latter results did not support a clear-cut relationship between density of GLUT1 transporters and fetal glucose consumption.

**Paper IV**

The maternal samples of the 4-vessel sampling method were used to study pathological pregnancies. In a case-control study we investigated the *in vivo* placental release of Placental growth factor (PIGF) and soluble Fms-like tyrosine kinase 1 (sFlt-1) by determining the uteroplacental AV- differences in human. Further we investigated whether this release was altered in early onset preeclampsia compared healthy pregnancies (controls), and whether there was a release of PIGF and sFlt-1 from systemic endothelium.

Women with preeclampsia had, as expected, lower plasma concentrations of PIGF and higher concentrations of sFlt-1 (p<0.001) compared with women with a healthy pregnancy. There were significant uteroplacental AV-
differences of sFlt-1 in preeclampsia (p<0.001), but not in controls (Figure 15). The median uteroplacental AV-difference constituted 29.2 % (12.6, 61.0) of the circulating level of sFlt-1 at a given time. The uteroplacental AV-differences of PlGF were significant in both groups (p<0.001), and constituted 24.5 % (2.2, 72.3) and 73.2% (21.4, 97.7) of the circulating PlGF in controls and preeclampsia respectively. Thus, despite the lower concentrations of plasma PlGF in preeclamptic women, we did not find a reduced placental release, not even when adjusted for placental weight. We found no PlGF or sFlt-1 concentration differences between the radial artery and the antecubital vein.

In sum we found a net release of sFlt-1 from the placenta in early onset preeclampsia. Placenta released PlGF to the systemic circulation, but we found no support for an impaired placental release of PlGF in preeclampsia.

Figure 15| Summary of results from paper IV, showing the uteroplacental (left) and antebrachial (right) AV-differences of PlGF (green) and s-Flt-1 (yellow).
Methodological considerations

Ethical aspects

All patients were approached for inclusion after the decision about delivery mode was made, to ensure that indications for cesarean delivery were not influenced by participation in the research project. Apart from one incidence of a local hematoma in relation to the arterial line resulting in temporary paresthesia of the hand, we have not experienced adverse effects during sampling from any of the 4 sites.

Study design

The observational cross-sectional design limits the opportunity to draw conclusions on causality. The method applied does not allow for longitudinal sampling, or sampling earlier in gestation and thus our findings only apply to late pregnancy. We are restrained to compare our data to models and hypothesis generated from experimental studies and in vitro studies. Ideally, factors shown to affect glucose transfer in vitro or animal studies should have been manipulated also in the human in vivo setting to gain a more mechanistic understanding of the glucose transfer.

Validity

Selection bias and representativeness

Selection bias can be caused by non-representative or selective sampling, loss of study objects and by missing data. Due to the cross-sectional design, the loss of study objects is not relevant, but so are the two others. The fact that this study had to be conducted during cesarean section created a selection bias (papers I-IV). Study arm 1 carefully aimed to exclude preexisting pathological conditions. Given that planned cesarean section is less common in nulliparous women, the participants were older and of higher parity than the average population. Parity is a determinant of fetal growth (111) and this selection bias might affect the validity of our results. The glucose consumption was significantly lower in fetuses of nulliparous compared to multiparous women (mean difference (CI %) 0.04 mmol/min (0.08, 0.002),
p=0.04, unpublished data). Even though this is an interesting finding, it might indicate that our findings concerning fetal glucose consumption may not be entirely valid for nulliparous pregnancies.

The participants were lean compared to the average age matched population which could bias our findings. We did not exclude dietary treated GDM, maternal BMI outside normal range or infants large or small for gestational age, based on the fact that these labels are founded on thresholds and the physiology is likely to be more continuous. We wanted to study human placental physiology within a wide spectre of maternal metabolic states and by not selecting on these variables we enhanced the representativeness of our data and preserved the potential for later sub-group analyses. Furthermore the list is endless if every maternal or fetal metabolic factor should be considered, and it would be extremely challenging to control them all. Excluding the four and 48 maternal-fetal pairs with GDM or BMI> 25 respectively, did not alter our main conclusions regarding fetal glucose consumption.

**Missing data**

The demanding logistic of the procedure limits the study population and resulted in some missing values. In particular, the fetal-medicine specialist was not available for ultrasound measurements all days. These missing data are considered random and do not pose a bias. However, high maternal BMI at delivery may have contributed to sub-optimal conditions for ultrasound examination and thus a possible bias as the median [Q1, Q3] BMI at delivery (27.5 [25.6, 30.1]) was slightly lower in the ones with complete ultrasound measurements of the uterine artery than in the ones without (28.8 [26.5, 31.6]). On the other hand, we did not find that maternal pre-pregnancy BMI, fat percentage, fetal weight or fetal glucose consumption differed between these groups.

In paper IV we did not have samples from the antecubital vein in some of the case subjects. This was because the collection of preeclamptic patients started before the healthy cohort, and the idea to compare the uteroplacental AV-
difference to the difference between the radial artery and antecubital vein came later. However, the data is very consistent and we do not think this represents a bias other than reducing the general strength of the statistical analysis.

External validity

Our findings are only valid for term pregnancies, and the placental transfer and metabolism of glucose might be different in earlier gestation (91). Furthermore, the women were fasting, which might be viewed as a strength, but may also hamper the validity of our data in the fed state.

For study arm 2 (paper IV), the design restricted the study to women with early-onset preeclampsia, which is not necessary a bias as there are distinct differences between late and early onset preeclampsia in risk factors, clinical manifestations and perhaps different underlying etiology. However, the results are only representative for early onset pre-eclampsia. The controls were not matched with the cases on gestational age in paper IV, which is an obvious bias. It can easily be argued that to compare early onset preeclampsia with gestational aged match controls in itself poses a bias, because no preterm delivery can be considered to be without underlying pathology. In addition, our method requires delivery by cesarean section which makes matching impossible. However, this will evidently affect the validity of the results as discussed in the paper.

Measurements errors

All data collection were performed by medical doctors and kept on few hands to lessen variability in the data. A single experienced fetal-medicine specialist (Professor Guttorm Haugen) performed all volume blood flow measurements by ultrasound using the same equipment. The high intra class coefficient of variation indicates good precision.

Because biochemistry is a vital part of the present work, we ensured high-quality measurements by analyzing the plasma samples (paper I-III) at the laboratory at the Department of Medical Biochemistry, OUS, Rikshospitalet.
However, due to the number samples that were analysed, the analyses had to be performed over several days and we cannot completely rule out that variation in analysis have affected our work. For all analyses the coefficients of variation were well below 10%. The intra assay variation of 11.8% during the ELISA to determine PLGF was the exception. PLGF forms complex with sFlt-1. This complex interferes with the measurements of PLGF levels in late pregnancy and in preeclampsia, because the assay presumably measure only free PLGF at high sFlt-1 levels. This is discussed in paper IV and the results interpreted thereafter. The separation of MVM and BM and GLUT1 protein expression was done according to a well-characterized protocol in collaboration with the experienced Powell/Jansson lab in Denver, Colorado (45, 166).

The 3 maternal blood samples were collected simultaneously, followed immediately by the umbilical samples. The whole sampling procedure took 2-4 minutes. We sampled both umbilical vessels after cord clamping, but before delivery of the placenta in the first part of study arm 1 (all papers). During the study period we adapted the procedure to implement late clamping of the umbilical cord. As a result we sampled the umbilical artery before clamping of the cord and the vein immediately after, for the second part of study arm 1 (papers I and III).

We used EDTA vacutainers to collect the samples, which do not contain inhibitors of glycolysis. However, inhibitors of glycolysis have been shown to be ineffective the first two hours, whereas cooling and prompt separation by centrifugation, in line with our protocol, is effective (167).

The measurements of the volume blood flow in the uterine artery were hampered by technical difficulties as they were performed late in pregnancy when it is unlikely to get a perpendicular angle for diameter measurements at the same site as TAMX is measured. Care was taken to exclude cases where vessels branched of between the two sites of measurements. The contribution of the ovarian arteries to the placental circulation cannot be accounted for. A number of studies have calculated volume blood flow in the uterine artery in late gestation, with estimates varying from ~450 to 1200 ml/min (at week 36
to term) (3, 168-170). The volume blood flow in the umbilical vein has been reported to be about ~200 to 310 ml/min (1, 2, 171, 172). Our measurements were in the lower ranges, especially the vessel diameters. In general measurements of vessel diameters are sensitive to variation in methodology. The use of color Doppler ultrasound may give rise to larger diameter measurements than B-mode. Furthermore, the resolution of the vessel wall as well as the placement of the marker for measurements will have an impact on the estimation. Notably, several of the studies, including ours, demonstrate great variation at the individual level. This may illustrate the technical difficulties, but also the physiological variation. Regardless of the cause, it may have great impact on the result in studies with low number of study subjects. We minimized the variation caused by these factors in our study by using the same equipment and having one examiner only. It is possible that the estimates differ by the method of calculation. We used TAMX where others have used a calculation based on time averaged mean velocity (168, 170). However, for the umbilical vein, Acharya et al. have demonstrated that the difference between the two calculations is minimal (2).

The blood samples and ultrasound measurements were obtained within hours of each other (Mean (SD) 2.4 h (1.5), unpublished data). Although the ultrasound measurements are conducted as close in time to the surgery as possible, they are inherently conducted prior to the spinal anesthesia and the blood sampling. From this follows that maternal cardiac output (CO) may change and possibly affect uteroplacental (and even feto-placental) blood flow. Thus an information bias cannot be excluded. The possible change in CO caused by spinal anesthesia can be compensated by phenylephrine which was used in the current study. Preliminary data from a subset of our participants (n=23) show no significant alteration in maternal CO before spinal anesthesia and at the time of sampling (paired t-test, p= 0.84, mean difference (CI %) -0.09 L/min (-0.96, 0.79), unpublished data).

In a sub-cohort, we performed analyses that revealed an up-concentration of hemoglobin in the uterine vein and the umbilical artery (Median [Q1, Q3] Hb ratio between uterine vein and radial artery in n= 40 was 1.017 [1.002, 1.029] and between umbilical artery and vein in n=30 1.048 [1.010, 1.077]) (70).
This may be related to the movement of water in the uteroplacental unit and could impose a measurement bias because it may affect the size of the AV-differences.

**Statistical considerations**

The human physiology is not linear in nature, but largely based on feedback systems and complex dynamic interactions. In the case of placent al glucose transfer, the feedback systems are evident and hamper the possibility to draw true direct acyclic graphs (DAGs) and do pathway analysis exploring causality. Exploration of such feedback systems would benefit from longitudinal datasets, which our study design cannot provide. The physiological interdependence of many of our observations makes several of our correlations related to each other. We were interested in investigating the difference (a-b) between two variables a and b and the relationship of (a-b) with other variables, but also with a and b themselves. Since the difference (a-b) is a function of a and b, the correlation coefficient between (a-b) and a (or b) will be affected by the character of the relationship between a and b. As an example the maternal-fetal gradient is calculated as the difference between the radial and umbilical arteries, which are correlated. This will affect the relationship between the maternal-fetal gradient and the maternal and fetal glucose concentrations. Furthermore the correlation could be affected by the scale on which these individual variables are measured, e.g. if b is substantially smaller than a, the relationship between a and a-b would approach the relationship between a and a. As such, in some of the correlations studied, a mathematical relationship may influence our findings and we must therefore be cautious in our interpretations. Despite the lack of longitudinal data to surmount these methodological issues our findings might still be of relevance when we compare what we observe to more mechanistically derived data.

The “missing values” lead to low power in certain of the correlations studied, and the results must be interpreted thereafter. For example in paper III, GLUT1 was analyzed in a sub-cohort of 33 maternal-fetal pairs. We found that GLUT1 in the MVM was inversely correlated to uteroplacental uptake,
whereas we did not find an effect of GLUT1 in BM on fetal glucose consumption. The latter might be due to lack of statistical power, and our conclusions were adjusted thereafter to avoid type 2 error (the error of not rejecting a null hypothesis when the alternative hypothesis is the true state of nature). Likewise, the case-control study of preeclampsia was relatively small and would have been strengthened by a doubling of the control group.

Many of the variables studied are products of two or more measured variables, for example glucose consumption and uteroplacental uptake and the absolute measurements of GLUT1. The variance in each of these measurements has impact on the total variance of the variable and may contribute to blur biological relations. In this perspective we have to be cautious when interpreting negative results as we risk type 2 errors.

A major strength of our analysis is that we could do paired analysis within each mother-fetal pair. We considered the need for adjusting for multiple testing in paper III, but as the variables were selected based on the biological relevance according to preexisting models, we decided to present the data without adjustment. The correlations between fetal glucose consumption and maternal glucose, and between uteroplacental uptake and GLUT1 in the MVM would become borderline significant (p=0.06 and 0.07 respectively) by Benjamini-Hochberg correction with an overall false discovery rate of 5%. In paper II there are several correlations presented in a supplementary table. The correlation between maternal glucose levels and umbilical v-a glucose difference and the correlation between the umbilical v-a glucose difference and birthweight would become insignificant after a Benjamini-Hochberg correction. The correlation between the maternal-fetal glucose gradient and the uteroplacental AV-difference (p=0.06) and the glucose levels in umbilical artery (p=0.05) would become borderline significant in n=40.

**Methodological contributions to the field of placental research**

Current concepts of basic placental transfer and secretory functions are based on observational cohorts, *in vivo* animal studies and experimental *in vitro* studies and the validity of these concepts need to be tested in humans in vivo.
The 4-vessel sampling method provides a unique possibility to study human placental physiology and pathophysiology \textit{in vivo}, with all the interacting factors at play, a situation that can never be reproduced \textit{in vitro}. The method allows studies of the maternal, placental and fetal unit together rather than as separate entities, which could prove valuable in understanding pathologic pregnancies and maternal-fetal interaction. It may prove most beneficial used in concert with experimental studies. Such a dual approach would allow to test of the validity of existing experimental models in humans, and to test mechanistically the hypotheses generated \textit{in vivo}. The 4-vessel sampling method may be applied to a wide range of research questions in both normal and pathological pregnancies depending on the further analyses of the samples.
General discussion

Placental glucose transfer in human

Most previous studies of glucose and insulin concentrations in the human uteroplacental unit have been conducted during vaginal delivery, making comparison to our study difficult because of the non-fasting state and the physiological stress. Based on a systematic literature search, we found two small studies (n=6 and n=11) which reported arterial and venous glucose concentrations at both the maternal and the fetal side of the placenta (23, 173, 174). All studies, including ours, demonstrated positive maternal uteroplacental AV- and fetal umbilical v-a- differences for glucose. The glucose concentrations were consistently lower in the umbilical circulation compared to the maternal circulation, supporting that glucose is transported along a concentration gradient across the placenta. However, compared to animal studies, the maternal-fetal glucose gradient is low, indicating that the human placenta has a high glucose transport capacity (7, 8). In our first paper, we found that the placenta extracted 6 % of the glucose available in the maternal circulation and that the fetus extracted approximately 10 % of glucose per liter blood passing in the umbilical circulation. When we included the whole cohort, the corresponding percentages were 6 and 14. This was somewhat lower than reported by Metzger et al. (10% and 17%), but comparable to what was calculated from the data presented by the Schaefer et al. (8% and 14%) (173, 174). This could not be explained by differences in insulin concentrations between the studies. Interestingly, Metzger et al reported larger placentas and lower placental efficiency measured as birth/placental weight ratio.

Zamudio et al. studied plasma glucose concentrations in maternal arterIALIZED and umbilical samples at term at high and low altitudes (125). They measured flow in the uterine arteries and the umbilical vein, and calculated the fetal glucose consumption (mean ±standard error; 68.7 ±5.8 μmol min⁻¹ kg⁻¹ newborn) and maternal glucose delivery (0.73±0.04 mmol min⁻¹ kg⁻¹ placenta and newborn), which was higher than what we found (median [Q1, Q3]; 28.9
\[15.4, 41.8\, \mu\text{mol min}^{-1} \text{kg}^{-1} \text{ and } 0.56 [0.39, 0.68] \, \mu\text{mol min}^{-1} \text{ kg}^{-1} \), respectively). The differences could be attributed mainly to differences in volume blood flow. Supposedly, we used comparable techniques to measure the vessel diameter (ultrasound, regular B-mode, information gained by personal notification), but Zamudio et al. used time mean, rather than maximum, averaged velocity for calculations. They reported the calculated rather than the measured values, which makes it difficult to further penetrate the cause of the divergence. Furthermore, as they did not sample from a maternal artery and the uterine vein, they did not calculate uteroplacental uptake or placental consumption of glucose. The fetal glucose consumption was lower at high altitudes and correlated to the mass fetal glucose in the umbilical vein, which was interpreted to be due to increased placental consumption under conditions of mild chronic hypoxia. Zamudio et al. proposed that placental glucose consumption may vary, and affect glucose available for fetal consumption, a concept that may be in line with the wide range of placental glucose consumption and the negative relationship with fetal glucose consumption that we observed in paper III.

Factors affecting placental glucose transfer: 
Existing concepts and our findings

**Maternal BMI and GWG**
Maternal overweight and excessive GWG are associated with increased maternal insulin resistance and increased nutrient availability for the placenta. Altered glucose transfer has been suggested to be part of the mechanism linking BMI to birthweight. It is, however, well established that maternal BMI is a determinant of birthweight independent of maternal glucose levels (111). In accordance with this, maternal BMI and gestational weight gain (GWG) were not associated with placental or fetal consumption of glucose, but consistently related to fetal insulin, placental weight and birthweight (paper III). Altogether, our findings indicate that the effect of maternal BMI on birthweight does not involve increased net placental glucose transfer/fetal consumption. This highlights the role of maternal metabolic
components besides glucose (like triglycerides, free fatty acids, amino acids or intermediate metabolites) known to be correlated to fetal insulin, neonatal adiposity and/or birthweight (157, 175).

**Maternal-fetal glucose gradient**

Placental ex vivo perfusion studies and ovine *in vivo* studies have demonstrated that placental glucose transfer is mainly driven by the maternal-fetal glucose gradient (52, 176). Importantly, studies have demonstrated that this gradient is not merely dependent on maternal glucose concentrations but affected independently by fetal glucose levels (5, 8). Furthermore, ovine studies have demonstrated that fetal glucose consumption may separately regulate fetal glucose concentrations, and determine the maternal-fetal gradient independent of maternal glucose concentrations (5, 77). In line with these experimental data we observed that, in normal human pregnancies, the maternal-fetal gradient was inversely correlated to glucose concentrations in the umbilical artery, and strongly positively correlated to the fetal glucose v-a difference and to fetal glucose consumption (papers II and III). However, the mathematical relations between these parameters must be kept in mind.

**Insulin**

Maternal and fetal hormones, growth factors and cytokines might have an indirect impact on the maternal-fetal glucose gradient by influencing maternal and fetal glucose concentrations. The effects of fetal insulin on the maternal-fetal gradient and placental glucose transfer have been studied extensively in sheep (5). These studies show that fetal insulin and glucose levels additively affect the total fetal glucose consumption. We found that the fetal consumption of glucose (mmol·min⁻¹·kg⁻¹) and the maternal-fetal gradient correlated with the fetal insulin levels (paper II and III). This could be in line with the concept that fetal hyperinsulinemia might lead to exaggerated placental glucose transfer even at normal maternal glucose levels (64). Insulin is believed to be an important growth factor in the fetus and correlated with birthweight and placental weight also in our study (paper II). Given this role, secretion of insulin might be subjected to different regulation in the fetus than in the born individual. This was noticed in several older
studies describing a different insulin response to glucose from what is seen in adults (177, 178). While fetal insulin levels were correlated with fetal glucose consumption (papers II and III), we did not find that they were correlated with fetal glucose levels (rho 0.12, p=0.5) in paper II. It must, however, be kept in mind that our mothers were fasting for more than eight hours. This relation became significant in the total cohort rho 0.26, p<0.001 (unpublished data), still it seems like insulin is less correlated to glucose levels in the fetus than in the mother (rho 0.5, p<0.001, paper II and total cohort) (178). In their ovine studies, Hay and colleagues concluded that basal insulin secretion has little effect on fetal glucose metabolism, but that glucose stimulated insulin secretion does occur, increases over gestation and is enhanced in fetuses subjected to pulsatile hyperglycemia (179, 180).

The effects of maternal insulin on placental glucose or nutrient transfer have not been addressed to any extent in the papers of this thesis. At term there are, according to current concepts, no insulin sensitive glucose transporters in the MVM. In accordance with this there were no correlation between uteroplacental glucose AV-difference or uptake and maternal insulin levels. However, the effect of insulin via the insulin signaling pathway might be important for placental growth, the placental nutrient sensing system and the regulation and abundance of nutrient transporters in the syncytiotrophoblast (57). We found no correlation between maternal insulin and placental weight, in contrast to results earlier published by this group (157). Given that our samples were collected at term, we are not able to evaluate the impact of maternal insulin on placental growth in early pregnancy. O’Thierny et al. found that maternal insulin secretory response early, but not late, in pregnancy correlated with placental weight and placental growth as demonstrated by ultrasound (181).

**Maternal glucose levels and uteroplacental uptake**

Perhaps our most striking observation was that there was no correlation between the uteroplacental AV-concentration difference and the fetal umbilical v-a difference of glucose in paper I, which was underscored by the lack of correlation between the uteroplacental uptake and fetal consumption
of glucose in paper III. Furthermore, the maternal glucose concentrations were relatively weakly correlated to the fetal glucose consumption and v-a difference, just reaching significance with a Spearman’s rho of about 0.2 in the total cohort. These findings are in contrast to the close correlation between maternal and fetal glucose levels. We cannot fully explain these findings as we have only observational data. It has been hypothesized that fetal consumption at term is primarily regulated by fetal factors and less dependent of maternal glucose concentrations or uptake (at least within normal ranges) (90). Thus is tempting to speculate that this notion may partly explain our finding, especially taken together with the above mentioned relationship between fetal insulin and glucose consumption. The lack of correlations between uteroplacental uptake and fetal glucose consumption (paper III) might also be due to placental glucose consumption.

**Placental consumption**

By measuring the volume blood flow immediately before delivery, we were able to estimate the mass of glucose consumed by the placenta and the uterus (paper III); in the thesis referred to as placental consumption because the relative consumption by the uterus is assumed small. We found that the placental consumption of glucose varied greatly among individuals both in terms of mass (mmol/min) and in terms of proportion (%) of the uteroplacental uptake. At median the placental consumption constituted 30% of the total uteroplacental glucose uptake, a low proportion compared to experimental and ex vivo perfusion studies (30-80%) (8, 32). An in vivo setting includes the uterine in addition to the placental glucose consumption as opposed to in experimental placenta perfusion experiments, which underscore the discrepancy. On the other hand, placenta perfusion experiments are conducted in hypoxic conditions compared to in vivo and with glucose as the only energy source, thus placental glucose consumption may not be comparable in these settings (182). Summarizing findings in domestic animals, Vaughan and Fowden found that the placental glucose consumption (200-400 μmol⋅min⁻¹⋅kg placenta⁻¹) was consistently five to ten-fold higher per kg tissue than the fetal consumption (30-40 μmol⋅min⁻¹⋅kg fetus⁻¹) (84). In our human material, median placental glucose consumption
per kg tissue was lower (51.4 [-65.8, 185.4] μmol·min⁻¹·kg placenta⁻¹), while fetal glucose consumption was comparable to the domestic animals (28.9 [15.4, 41.8] μmol·min⁻¹·kg fetus⁻¹). Accordingly, the fetal use of glucose relative to the glucose available in the uteroplacental unit was higher in humans compared to domestic animals. Comparing placental glucose consumption across species is difficult because of diversities in methodology and the above discussed anatomical variances between species. Fetal lambs have lower arterial glucose concentrations (~ 1.1 mmol/L) compared to human fetuses (3.3 mmol/L, paper III). A larger maternal-fetal glucose gradient (3-5 mmol/L in lamb compared to 1.2-1.5 mmol/L in humans) (5, 183, 184) is needed to sustain an effective maternal-fetal glucose gradient because of the longer diffusion distance between maternal and fetal blood. A placental exchange surface consisting of several cell layers may consume more of the glucose in transit. The partition of glucose between the placenta and fetus may be different in ovine than human pregnancies because the human fetus has the ability to store excess glucose as fat, while the leaner fetal lamb predominantly oxidizes transferred glucose (184). Moreover, the fetal lamb uses lactate as an energy source.

Interestingly, we observed negative placental glucose consumption in about 1/3 of the participants. We did not measure the ovarian volume blood flow which may contribute to the uteroplacental blood flow in about 10% of individuals, as has been demonstrated in baboons and rhesus monkeys. However, their quantitative importance is debated (25, 27). The variation of contribution from the ovarian arteries between individuals may theoretically contribute to the variance in the calculations of net placental transfer. While it may also contribute to a slight underestimation of uteroplacental flow, it is very unlikely that the ovarian flow is of such volume that it may explain our observation. To evaluate the possibility that the negative placental glucose consumption was a consequence of an underestimation of uterine volume blood flow we hypothetically doubled the measured flow, but still found negative placental consumption in ¼ of our observations (paper III). This observation is surprising, and needs to be interpreted with caution. Theoretically, it may indicate that the placenta apparently produce glucose to
meet fetal glucose demands when uteroplacental uptake is low. There are few indications of placental glucose production in experimental studies, but an in vivo tracer study in humans by Prendergast et al. also calculated a negative placental consumption. A drop in glucose tracer enrichment between maternal arterialized and umbilical venous blood at term was interpreted as support for placental production of glucose (185). Importantly, in our study women were fasting for more than 8 hours before sampling and our finding may not apply to the fed state.

It has been recognized in several experimental settings that placental consumption may influence the relative placental transfer of glucose, but it has been uncertain whether this applies to humans, and whether placental consumption is dependent on the maternal-fetal gradient or not. Ex vivo perfusion studies have demonstrated the placental consumption to be relatively independent of the maternal-fetal gradient (8), whereas ovine studies have found the placental consumption to be positively correlated to fetal glucose levels in the umbilical artery (5). We found no correlation between the placental glucose consumption and the maternal-fetal gradient or the arterial glucose concentrations at the maternal or fetal side of the placenta (paper III). However, the placental glucose consumption was negatively correlated to the glucose levels in the umbilical vein. The former observations are in line with the in vitro perfusion experiments describing that higher maternal-fetal glucose gradient favors transplacental glucose transfer rather than placental glucose consumption (8). Reciprocally it can be speculated that the placental glucose consumption affects the amount of glucose transferred to the fetus separately of the maternal-fetal gradient. This last notion can be supported by the correlation between the placental glucose consumption and the uteroplacental uptake, and between the placental and the fetal glucose consumption.

In sum, our findings are in line with ovine studies demonstrating that net placental glucose transfer is regulated separately by alterations in maternal supply, placental consumption and fetal concentrations demonstrating that placental transfer of nutrients cannot be understood without considering a
three-compartment model (176). However, our studies do not support that placental consumption is regulated by maternal or fetal glucose levels or the maternal-fetal gradient.

**Flow**

The maternal-fetal glucose gradient is suggested to be affected not only by maternal and fetal arterial concentrations, but also by blood flow, as movement to and from the transfer sites will affect the local concentration gradient (56).

Our observational data can add only limited information regarding this issue. The amount of glucose taken up by the uteroplacental unit and consumed by the fetus and placenta was dependent on volume blood flow by calculation, but we did not find a correlation between the uterine or the umbilical flow and the maternal-fetal gradient or the AV-concentration differences on either side of the placenta (rho from -0.08 to 0.05, p-values from 0.43 to 0.72, unpublished data). In line with previous studies, umbilical volume blood flow was strongly correlated to birthweight (rho 0.59, p < 0.001) (186, 187). The correlation between uterine volume blood flow at term and birthweight (rho 0.2, p = 0.09) did not reach significance in our 70 healthy maternal-fetal pairs. The uterine volume blood flow may still be a determinant for fetal growth when it is pathologically low, besides the uterine flow may be more important earlier in pregnancy by having an effect on placental growth (188, 189).

**Placental size and surface**

The placental exchange surface is believed to be important for the transfer of most nutrients and, although it is difficult to study this entity, has been shown to be closely correlated to placental weight and birthweight (42). Interestingly, we did not find a relation between placental weight and the placental glucose consumption or the uteroplacental uptake (paper III). We did, however, find that placental weight was correlated to the fetal glucose consumption, but not to the fetal glucose consumption mmol·min⁻¹·kg⁻¹ newborn. As such, the first correlation seems to depend on the close relation between birthweight and placental weight. There were no correlations between placental weight and the AV glucose difference on either side of the
placenta (paper II and III). In sum, we could not explain the observed variation in placental glucose consumption at term by the variation in placental weight between healthy pregnancies.

**Glucose transporter density in the maternal and fetal facing syncytiotrophoblast membranes**

The glucose transporter densities in the syncytiotrophoblast are considered regulators of placental glucose transfer, at least in humans (5, 45, 56, 107). There are several lines of evidence that support this, still the results are somewhat conflicting both concerning regulation of the expression and activity of these transporters as well as their importance for glucose transfer in quantitative terms (5, 58, 107). Factors altering the GLUT1 expression may be of maternal, placental and fetal origin, and might be different in normal and pathological pregnancies. Several endogenous and exogenous hormones, like glucocorticoids, chorionic-releasing hormone, insulin like growth factor 1 (IGF1), insulin and resistin have been shown to affect GLUT1 expression in vitro (32, 58, 66).

It has been demonstrated that the distribution of GLUT1 in the syncytiotrophoblast is asymmetric, with higher protein expression in the maternal facing membrane (MVM) than in the fetal facing membrane (BM) (45). Taking the larger surface area of the MVM into account, it has been estimated that the expression and activity of the GLUT1 transporter is several folds higher in the MVM than in the BM. This has led to the hypothesis that the density of GLUT1 in the BM is a rate limiting step in placental glucose transfer independent of placental exchange surface area. Accordingly, it has been proposed that GLUT1 density in MVM is irrelevant for regulation of placental glucose transfer due to the high glucose transfer capacity (45). These notions are supported by the coordinated increase in GLUT1 BM expression and glucose transfer throughout pregnancy, and could be of importance to increase glucose concentrations in the cytosol and thus the gradient between the syncytiotrophoblast and umbilical circulation (45). A polarity of transplacental glucose transfer have been demonstrated in in vitro perfusion studies, and interpreted to be a combined result of asymmetrical
transport capacity between the membranes and placental glucose consumption (8). Activity and expression of GLUT 1 in the BM was found to be increased in type1 diabetes and associated with higher glucose transfer in vitro, even when the mother was euglycemic for weeks prior to term (105).

We found higher density of GLUT1 receptors in the MVM compared to BM as previously described (paper III). In our healthy population, the GLUT1 density in BM was not correlated to maternal glucose, uteroplacental uptake, placental or fetal consumption of glucose, but borderline correlated to the glucose levels in the umbilical vein. It must be taken into account that these associations might be lost due to low statistical power. Our results do not, however, clearly support that high GLUT1 transporter expression increases net placental glucose transfer/fetal glucose consumption. In contrast, and unexpectedly, we found that the density of GLUT1 in MVM was inversely correlated with the umbilical v-a glucose difference (rho -0.35, p=0.45, unpublished data) and the uteroplacental glucose uptake, also when adjusted for placental weight (paper III). We found that there was a positive correlation between GLUT1 (both in BM and MVM) and the glucose levels in the umbilical artery (paper III).

Interestingly, while pre-gestational diabetes is related to an upregulation of GLUT1 in the BM, there are conflicting results regarding diet-regulated GDM and GLUT1 expression (58, 108). Furthermore, and in contrast to findings in diabetic pregnancies, studies exposing human trophoblasts and placental explants from normal term pregnancies to hyperglycemia demonstrated decreased glucose uptake and lower GLUT1mRNA and protein expression, (112, 190). What’s more glucose had auto-regulative effect on the placental trophoblast by altering GLUT1 partitioning between the MVM and the intracellular space (62). A correspondingly regulation of GLUT1 expression and activity by glucose have been found in other tissues, and GLUT1 seems to be regulated by glucose demand in cancer cells (191). Depriving human cytotrophoblasts cells of glucose in an experimental setting increased glucose uptake and expression of GLUT1 (190). While the effect on glucose transporters occurred at extreme glucose levels (25mmol/L and 0 mmol/L
respectively), the effect on glucose uptake occurred within normal physiological glucose levels. The partitioning of GLUT1 between MVM and the intracellular space is in contrast to the prevailing notion that the density GLUT1 in MVM is too large for a regulative function. Still, this may hold true within physiological glucose levels. Gaither et al. found in a group of predominantly diabetic pregnancies (n= 21) that the GLUT1 in the MVM, but not the BM, was inversely correlated with birthweight (63). Summing up, an auto-regulative role of GLUT1 in normal term pregnancies have been proposed (58, 62). Ovine studies have demonstrated that the relative importance of the maternal-fetal gradient for the net placental glucose transfer is decreased at term and that the transfer becomes more dependent on total placental glucose transport capacity (including the exchange surface area and transporters) (5, 192). Our findings are not incompatible with such a concept, but as they are not conclusive we cannot claim that they provide support it. The inverse relationships between the GLUT1 density in the MVM and the uteroplacental uptake and umbilical v-a difference were unexpected, and based on few observations. More observations are needed to validate our findings. Further analyses of GLUT1 activity and glucose transport in our placenta samples in an experimental setting need to be done.

The placental origin of PlGF and sFlt-1?

Placental origin of PlGF and sFlt-1 in normal pregnancies?
The placental origin of the ligands and receptors of the VEGF family is supported by high expression in normal placental tissue (140, 141, 193). In vivo placental release of PlGF could not be demonstrated in a small study (9 cases and 9 controls) by Bujold et al. However, in support of a placental origin of PlGF, we found a significant uteroplacental release of PlGF in both early onset preeclamptic and healthy pregnancies (paper IV). We doubled the number of subjects studied compared to two earlier studies, but in line with their results we could not demonstrate an in vivo release of sFlt-1 to account for that sFLT-1 levels increase until delivery also in normal pregnancies.
(194, 195). This might be due to lack of statistical power, but may also be indicative of extra-placental sources of sFlt-1 in normal pregnancies.

**Placental release of PlGF and sFlt-1 in preeclampsia?**

Early onset preeclampsia is associated with lower circulating PlGF and higher sFlt-1 concentrations compared to healthy pregnancies, which was confirmed in our study. The placental origin of these alterations is supported by several studies. sFlt-1 has been found to be upregulated in cultured placental trophoblast from preeclamptic women (193). Furthermore, sFlt-1 is abundant in syncytial knots in preeclamptic placentas (196). Hypoxic conditions have been shown to stimulate production and activity of antiangiogenic proteins, e.g. sFlt-1, in cultured trophoblasts. Regulation of PlGF transcription in response to hypoxia is tissue specific, but there is evidence of decreased expression of PlGF mRNA in hypoxic trophoblasts (197, 198). Studies of preeclamptic placental explants showed increased sFlt-1 production, but did not confirm decreased production of PlGF (142, 199). Our study could not demonstrate that the lower levels of PlGF observed in preeclampsia was caused by an impaired uteroplacental release. In fact, the uteroplacental PlGF release relative to the actual levels of PlGF in the circulation tended to be higher in the preeclamptic group than in the controls. The finding is consistent with the hypothesis that lower maternal PlGF levels in preeclampsia is caused by increased removal of free PlGF from the circulation and/or binding to sFlt-1 (141, 142). However, we cannot exclude that our study was underpowered to demonstrate a difference in placental PlGF release (type II error) or that our interpretation was hampered by the difference in gestational age between cases and controls. The results from our human *in vivo* study support the concept that the uteroplacental unit contributes to the increased levels of sFlt-1 in preeclampsia, in contrast to in healthy pregnancies (194, 195) (paper IV).

**Support for a general endothelial production?**

There are several lines of evidence supporting that a pre-existing susceptibility of the maternal vasculature might be contributing to the development of preeclampsia including the shared risk factors for
cardiovascular diseases (131, 133, 200, 201). Moreover, preeclampsia has been associated with an altered expression of PIGF and sFlt-1 also in endothelial cells and monocytes (138, 202). Studies have indicated extra-placental, most likely vascular, origins of sFlt-1 in pregnant women (138, 148, 151). Furthermore, in non-pregnant women, a releasable vascular store of sFlt-1 and PIGF did not differ between nulliparous and women with previous preeclamptic pregnancies. Interestingly, the amount of releasable sFlt-1 in non-pregnant women reached concentrations found in third-trimester pregnancies; however, they were far below the levels found in preeclampsia (148). We did not find an AV-difference between the radial artery and the antecubital vein to support a systemic endothelial contribution of sFlt-1 and PIGF in healthy nor preeclamptic pregnancies. However, our study was not designed to exclude such a contribution. The properties of endothelial cells vary between organs and the size of the vascular bed in the forearm is just a fraction of the total endothelial surface of the human body. The possible pre-existing susceptibility of the maternal endothelium likely involves other mechanisms than altered release of anti-angiogenic factors.

**Clinical implications**

Although we cannot exclude general endothelial or other extra-placental sources of sFlt-1 and PIGF, our study supports the concept that the placenta is the main source of PIGF in pregnancies. This is important to establish because they are both implemented as predictive biomarkers of preeclampsia in clinical practice at several medical centers around the globe. A marker that is derived from the placenta solely may display a direct correlation with placental function, whereas markers that originate from several sources (i.e. maternal, placental and even fetal) might be more difficult to interpret. However, consistent with earlier findings, we did not find a uteroplacental release of sFlt-1 in normal term pregnancies although the serum levels are known to increase until delivery. This may be indicative of extraplacental sources of sFlt-1, which may contribute to the difficult interpretation of sFlt-1 as a biomarker. It is important to know that the use of these factors as biomarkers is hampered by several factors that our study does not address. They are not specific for preeclampsia, but also predict IUGR (132, 145).
addition, they do not effectively predict late onset preeclampsia, which accounts for about 80% of preeclampsia. The incidence of early onset preeclampsia is less than 1% in nulliparous women, so the positive predictive values of these markers are inevitable low despite fairly good sensitivity and specificity.

Summary and conclusions

- This thesis has demonstrated that it is possible to study human placental physiology in vivo, although the model is challenging both in terms of practical implementation and in terms of interpretation of the results. Such studies leave few options for manipulation and thus remain largely observational. The in vivo method presented here is useful to test concepts obtained otherwise (e.g. the relationship between placental glucose consumption and incoming glucose on both sides) and the method can be used to generate new hypothesis relevant in human pregnancies (e.g. based on the paradox that uteroplacental glucose uptake is not related to fetal glucose consumption even though maternal and fetal glucose are closely correlated).

- BMI or GWG correlated with birthweight, but not with fetal glucose consumption in healthy pregnancies at term.

- The Pedersen hypothesis of maternal glucose excess leading to increased fetal glucose and insulin levels and fetal growth applies to our findings in vivo. Importantly, our results also demonstrate that there are forces counteracting this cascade:
  - Even though maternal and fetal glucose levels are closely related, uteroplacental uptake of glucose does not correlate with fetal levels or consumption of glucose.
  - Fetal glucose consumption correlates with birthweight and fetal insulin levels. Our findings may be taken to support the concept obtained in ovine studies that the fetal demand may be an important regulator of transplacental glucose transfer at term.
  - Placental glucose consumption correlates strongly with uteroplacental uptake in normal pregnancies. Furthermore, the
placental glucose consumption varies greatly among individuals possibly demonstrating a flexibility of the placental glucose transfer system.

- Although we were not able to test this specifically in our model,- a tentative interpretation would be that the above suggested regulative mechanisms provide the fetus with means to keep the glucose consumption stable.

- Contrary to what was hypothesized, we did not find clear evidence for a regulative function of GLUT1 in the BM on fetal and placenta glucose consumption. The negative correlation between GLUT1 in the MVM and uteroplacental glucose uptake was unexpected, not easy to interpret and needs further investigation.

- The 4-vessel sampling method has proven highly useful to study the maternal-placental interplay in pathological pregnancies by determining specific factors released by the placenta to the maternal circulation in vivo. We demonstrated that the uteroplacental unit releases sFlt-1 to the maternal circulation in early-onset preeclamptic, but not in normal pregnancies. Placental release of PlGF was not impaired in preeclampsia compared to normal normal pregnancies, which is consistent with the concept that the low levels of PlGF in preeclampsia are related to excess binding of sFlt-1.

- We did not observe any endothelial release of sFlt-1 or PlGF by the vascular bed of the forearm in either preeclamptic patients or controls.

- Finally, to quote dr. Pedersen,- this thesis includes a number of speculations, and I shall therefor abstain from drawing further conclusions (109).
Clinical implications and future perspectives

To clinicians this heading immediately refers us to look for how new knowledge can be applied in clinical practice, - alter the way we deal with disease. This is jumping too far too fast. No pathology may be fully understood without profound knowledge about the normal physiology.

Furthermore, in our clinics we examine and advise many perfectly normal women with healthy pregnancies. The advice we give them should be based on knowledge about normal physiology and not on knowledge applying to unhealthy pregnancies. Even though the Pedersen hypothesis still holds true, our findings support the notion that fetal demand may be an additional regulator of placental glucose transfer. There is emerging evidence that this demand may be set at an early stage of pregnancy and that this might lead to permanent changes in placental growth and glucose transfer (64). As seen from the discussion of this thesis there are likely to be placental mechanisms and adaptions in healthy pregnancies, as well as in GDM, that may serve to keep glucose delivery to the fetus fairly stable. The known linear relationship between maternal glucose levels and birthweight and pregnancy outcome has highlighted the somewhat artificial lines between healthy and GDM pregnancies (104). Traditionally, we act when a pregnancy has past the defined line of pathology. The adaptions may already be well established, and may be less modifiable, at the point where we set the diagnosis of GDM. The intrauterine conditions and fetal growth have consequences for the immediate maternal and fetal health, but also for the future health of the offspring. This underscores the need for advising women, diabetic or not, to optimize their metabolic conditions BEFORE the onset of pregnancy. In practice, it means that we need to care for the health of the adolescents and find a way to reach this group who is not regularly in contact with our healthcare systems.

Taken together I shall not claim that this thesis is of direct value to the practicing clinician, however as our understanding of normal physiology increases, so will our insights of the mechanisms that fail in pathological pregnancies.
Our comprehensive and unique data bank has just been opened, the first samples analysed and the first results are presented in this thesis. The potential use of our collected material is described in paper 1, and if these considerations were to be compared to what I have done in my thesis, it becomes very evident that a lot of research lies ahead. There is an enormous potential to dig deeper in the factors affecting fetal glucose metabolism and growth. We can look into the signalling molecules or hormones that may affect glucose transfer. Notwithstanding the potential to do integrative analyses of several factors and metabolites that together affects fetal growth and metabolism.

The unique cohort of preeclamptic pregnancies can be used to explore placental secretion into maternal and fetal circulations and help sort out placental causes versus maternal and fetal responses to preeclampsia.

Summing up, there lays a potential for several masters, PhDs and post-docs in our freezers.
Appendices
Forespørsel om deltakelse i forskningsprosjektet

"Preeklampsii, placentafunksjon, kardiovaskulær risiko og endoteldysfunksjon"

Bakgrunn og hensikt

Hva innebærer studien?
Studien gjelder gravide som forløses med keisersnitt og som har preeklampsii, og kvinner som forløses med keisersnitt og ikke har preeklampsii.
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 navlestrgenen.
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.
I forbindelse med keisersnittet ønsker vi å måle blodtrykk og hjerteminuttvolum med to metoder som sammenlignes. Den ene metoden benytter måling av trykk inne i en blodåre (arteriekanylen, se ovenfor), mens den andre måler dette via en mansjett utenpå en finger (Finometer).
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. Målinger av blodtrykk og hjerteminuttvolum vil kreve ca 10 minutter ekstra tidsbruk, men selve operasjonen tar ikke lenger tid.
**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.


**Frivillig deltakelse**


**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)
Table 1 corrected:

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<th>p-value*</th>
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Paired differences

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Table 2 corrected:

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Paired differences

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<td>-8.7 [-16.0, 0.2]</td>
<td>&lt;0.001</td>
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Errata
Bibliography/References


201. Ghosh A, Freestone NS, Anim-Nyame N, Arrigoni FIF. Microvascular function in pre-eclampsia is influenced by insulin resistance and an imbalance of angiogenic mediators. Physiological reports. 2017;5(8).