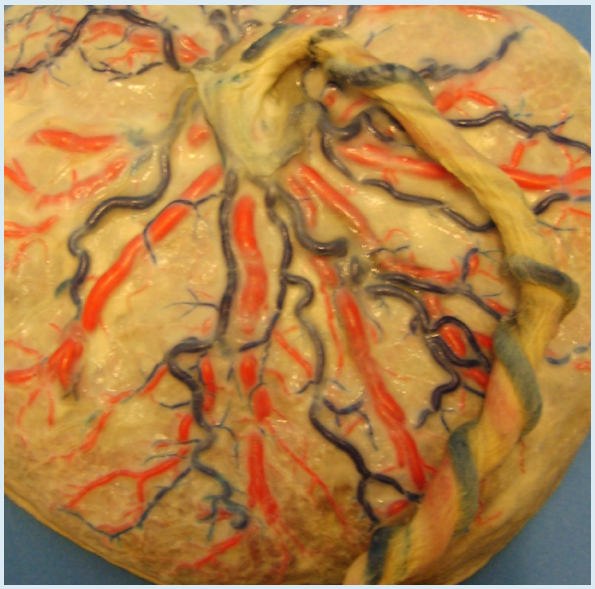
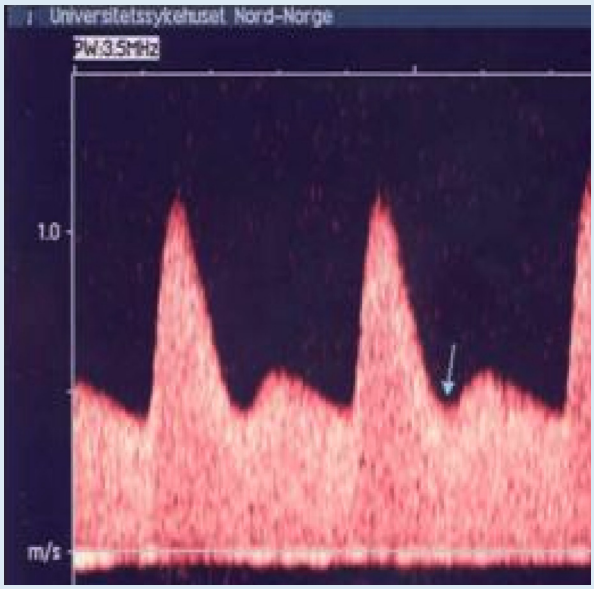
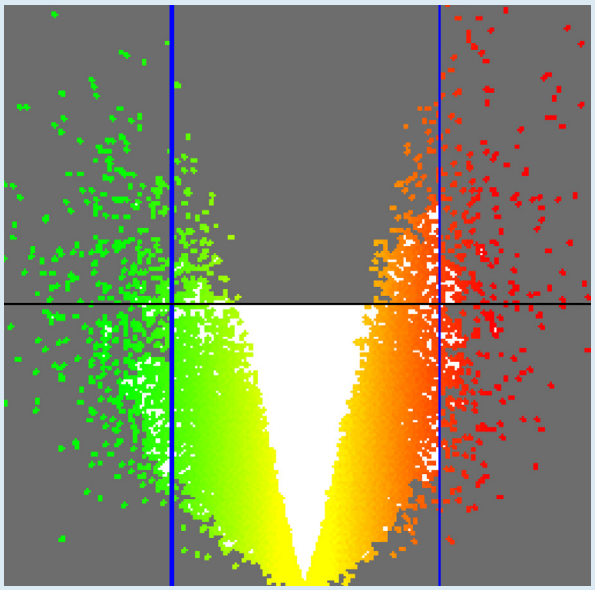
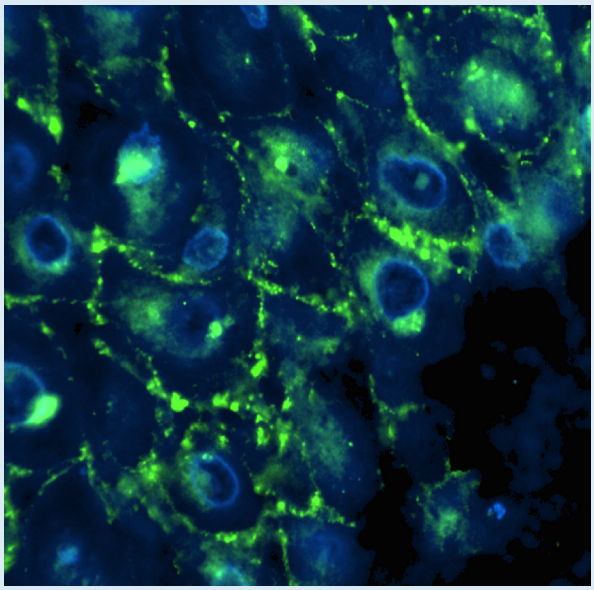
Gene Expression Profile of Normal and Compromised Placentas









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A dissertation for the degree of Philosophiae Doctor

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Cover pictures

Upper-left: Human term placenta plastinated by Vasilis Sitras, exhibited at the Department of Human Anatomy, Faculty of Medicine, University of Tromsø.

Upper-right: Doppler blood flow velocity waveforms of the uterine artery with protodiastolic "notching" (arrow) indicating increased utero-placental vascular impedance.

Lower-left: Volcano-plot showing differentially expressed genes from a microarray experiment. The color indicates up-(red) or down- regulation (green).

Lower-right: Microscopic view of chorionic villus demonstrating laeverin immunofluorescence (green) in the cell-membrane of the syncytiotrophoblasts.

To my sons

Vangelis and Alexandros

"The difficult is a bagatelle, the impossible is a challenge"

Solan Gundersen
Flåklypa Grand Prix (1975)

CONTENTS

ACKNOWLEDGEMENTS

This thesis is a result of consistency, inspiration and enthusiasm; values imbibed to me by my father and my supervisor. They both share two common characteristics: integrity and a warm heart. I am privileged to have Prof. Ganesh Acharya as my "guru"; he is a role model as a clinician, a researcher and a human being, but most of all he is "a friend for life".

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My parents, Voula and Vangelis, and my sister Dina have always been "here" for me even if we live in the two extremes of Europe. My optimistic attitude in life is a result of a wonderful childhood they gave me. Kjellaug and Ernst Sneve, my parents in law, accepted me as "a son" and I am deeply grateful for their love and support.

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to accomplish it and, most importantly, enjoy every minute of it. I miss his friendship and guidance.

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Tromsø, June 2009

Vasilis Sitras

ABBREVIATIONS

C_T	threshold cycle
DNA	deoxyribonucleic acid
ENG	endoglin
FOS	v-fos murine osteosarcoma viral oncogene homolog
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
GNGT1	guanidine nucleotide binding protein (G protein), gamma transducing activity polypeptide 1
hCG	human chorionic gonadotropin
HELLP	hemolysis, elevated liver enzymes, low platelets
hPL	human placental lactogen
HSD17B4	hydroxysteroid (17-beta) dehydrogenase 4
IUGR	intrauterine growth restriction
LEP	leptin
LRAP	leucocyte-derived arginine aminopeptidase
mRNA	messenger ribonucleic acid
PI	pulsatility index
PlGF	placental growth factor
RT-PCR	real-time reverse-transcriptase polymerase chain reaction
sFlt-1	soluble fms-like tyrosine kinase 1
VEGF	vascular endothelial growth factor

ABSTRACT

Objective

Placenta has an important function of sustaining life *in utero*. Role of placental gene expression in the physiology of parturition and pathogenesis of pregnancy disorders are poorly understood.

The aims of this thesis were to:

1. Investigate the effect of labor on global gene expression profile of normal placentas
2. Compare placental gene expression profile of uncomplicated pregnancies with that of pregnancies complicated by severe preeclampsia and intrauterine growth restriction (IUGR)

Materials and Methods

Hemodynamic assessment of the maternal uterine and feto-placental circulations was performed ≤72 hours before delivery using Doppler ultrasonography. Global gene expression profile was investigated using microarrays in placental samples collected after delivery from women with normal pregnancies (n=34), and pregnancies complicated by preeclampsia (n=16) and IUGR (n=8). The effect of parturition on gene expression was studied comparing placentas obtained from healthy women after normal delivery (n=17) with placentas obtained from women delivered by elective cesarean section (n=17). Placental gene expression profiles of women with severe preeclampsia (i.e. BP≥160/110 mmHg and proteinuria ≥2+ in dipstick) and IUGR due to placental insufficiency (i.e. estimated fetal weight <5th percentile for the gestational age with hemodynamic signs of redistribution of blood flow to the fetal brain) were compared with healthy controls. Microarray results were validated at the transcript and protein level by real-time reverse-transcriptase polymerase chain reaction (RT-PCR), placental immunofluorescence and urinary electrochemiluminescence immunoassay.

Results

Gene expression profile was similar in healthy placentas obtained following normal delivery and elective cesarean section. Placental genes were differentially expressed in preeclampsia and IUGR compared to normal controls indicating a central role of the placenta in the pathogenesis of these pregnancy-specific disorders. In particular, 16 genes were able to differentiate preeclamptic from normal placentas in our samples after supervised clustering. Several known (leptin, Flt-1, endoglin) and some novel genes (laeverin) and pathways (angiogenesis, hypoxia, Alzheimer, Notch) were found to be involved in the pathogenesis of preeclampsia. Subgroup analysis comparing early- (i.e. ≤34 weeks) with late-onset

preeclamptic placentas showed different genetic signatures with oxidative stress, inflammation and endothelin signaling pathways mainly involved in early-onset disease.
In IUGR, genes involved in glucocorticoid-metabolism and inflammation mediated by chemokine and cytokine signaling pathway were differentially expressed compared with controls. None of the known imprinted placental genes were differentially expressed.

Conclusion

Labor does not significantly alter the global gene expression profile in near term placenta. Placental gene expression profile is altered in severe preeclampsia and IUGR. Early-onset preeclampsia has a different genetic signature compared with late-onset preeclampsia supporting the possibility of different pathogeneses. Genes involved in inflammatory pathways are up-regulated both in early-onset preeclampsia as well as in IUGR, indicating that they might share common pathogenetic features.

LIST OF PAPERS

This thesis is based on the following papers, which are referred in the text by Roman numerals:

I. Sitras V, Paulssen RH, Grønaas H, Vårtun Å, Acharya G. **Gene Expression in Laboring and Non-laboring Human Placenta Near Term.** *Mol Hum Reprod.* 2008; 14(1):61-65.

II. Sitras V, Paulssen RH, Grønaas H, Leirvik J, Hanssen TA, Vårtun Å, Acharya G. **Differential Placental Gene Expression in Severe Preeclampsia.** *Placenta* 2009; 30 (5) : 424-433.

III. Sitras V, Paulssen RH, Leirvik J, Vårtun Å, Acharya G. **Placental Gene Expression in Intrauterine Growth Restriction Due to Placental Insufficiency.** *Reproductive Sciences* 2009; doi:10.1177/1933719109334256.

1. INTRODUCTION

Evolutionary changes, starting 120 million years ago, led to the creation of placenta and the appearance of new species, the eutherian animals. Although the placenta has similar function in all species, different species developed placentas of unique structural complexity. Indeed, from a simple membrane in the inner surface of the egg in reptiles and birds, the placenta has evolved through several developmental steps to a complex organ in primates. These phylogenetic changes necessitated drastic reorganization of the morphological and molecular structure of the placenta, driven by mitochondrial and nuclear genetic rearrangement (Carter and Mess, 2007). Thus far, however, the molecular mechanisms behind this process are unknown. Even the developmental processes that occur in the placenta, from conception to birth, in a single species are still not completely clear. Recent longitudinal genomic studies in the murine placenta suggest that a transition occurs during mid-gestation, with usage of different genes by the same cellular populations, in the absence of any major morphological change (Knox and Baker, 2008). In the human placenta, developmental changes are thought to be controlled by different factors such as oxygen tension, placental hormones (hPL, hCG), growth factors (VEGF, PlGF) etc. Unbalanced regulation of these factors could lead to impaired placental development causing pregnancy complications such as miscarriage, preeclampsia and IUGR (Lyall and Kaufmann, 2000).

1.1 Placenta: The organ of life

Placenta is a temporary but essential organ in human development. It is highly vascular and mediates gas, nutrient and waste exchange between the fetal and maternal circulations. It is also an important endocrine organ that produces and metabolizes hormones in autocrine, paracrine and endocrine fashion, influencing its own function as well as that of the fetus and the mother. Additionally, the placenta seems to protect the fetus-allograft from the maternal immune system. One could argue that the placenta is a living organism itself as it undertakes some of the functions performed after birth by the lungs, gut, kidneys and endocrine organs. Indeed, mutations in genes involved in placental development and morphogenesis result in early fetal loss (Rossant and Cross, 2001).

Recent, evolutionary morphologic analysis presents evidence that the human placenta has evolved from a common eutherian ancestor (Wildman et al., 2006). From a morphologic point of view, placentas of different species can be divided in several types depending on the: (i) *Feto-maternal interface*, which is the number of maternal tissue-layers penetrated by fetal

tissue (iii) *Feto-maternal interdigitation*, which is the form of contact between maternal and fetal tissue/blood and (iii) *Shape*. The human placenta has: (i) *Hemochorial interface*, because trophoblastic cells are highly invasive and penetrate in the maternal spiral arteries. (ii) *Villous interdigitation and* (iii) *Discoid shape*.

The human placenta undergoes a continuous remodeling throughout the pregnancy in response to changing functional needs. Formation of the placenta starts after the union between a sperm and an egg (*fertilization*) forming the embryo, which undergoes mitotic divisions to form the *blastocyst*. This has two different cell populations: the *inner cell mass* which develops in the embryo proper and the *trophectoderm* from which the placenta and the extraembryonic membranes are formed. Already at this early stage, during implantation of the *blastocyst* in the *decidua* (i.e. the uterine epithelium during pregnancy), the *trophectoderm* is responsible for the interconnection of the developing embryo with the mother and thus plays a crucial role in the successful outcome of pregnancy (Dey et al., 2004). Development of the *placental villi*, the functional units of the placenta, starts when cytotrophoblast proliferation gives rise to branches that protrude into the lacunae containing maternal blood to form the villous tree (*primary villi*). At 5 weeks gestation these villi are invaded centrally by extra-embryonic mesenchyma, transforming into *secondary villi*. During the second trimester, feto-placental capillaries are formed *de novo* (*vasculogenesis*) in the central stroma of the villi, transforming them into *tertiary villi* which have several branches and sub-branches with blunt-ended extremities that form the "villous tree".

At term, the feto-maternal *hemochorial* interface provides a surface of 15 m^2 for exchange and consists of five distinct layers: (i) Villous syncytiotrophoblast lining directly the intervillous space which bathes in maternal blood (ii) Basal membrane (iii) Villous core containing extracellular matrix and pericytes (iv) Basement membrane attached to capillary endothelial cells and (v) Fetal capillary endothelial cells where fetal blood circulates (Georgiades et al., 2002).

1.2 Feto-placental-maternal circulation

In the 16^{th} and 17^{th} century, Aranzio and William Harvey described that the uterine and umbilical vessels are not directly connected in the placenta and that fetal blood is circulated within fetal tissues and to some extent into the placenta (Fishman and Richards, 1964). The driving force directing blood in the placenta is probably the pressure gradient between the two umbilical arteries and vein on the fetal side, and between the uterine arteries and veins on the maternal side.

During embryogenesis, *hematopoiesis* occurs within the yolk-sac (Pereda and Niimi, 2008) and within the villous capillaries from hematopoietic stem cells (Demir et al., 1989). The feto-placental circulation is established by seven weeks of gestation. Morphological studies have demonstrated continuous evolution of the different villous types during gestation (Kaufmann et al., 1979). In the first and second trimester, trophoblastic sprouts generate new *mesenchymal villi* that transform into poorly vascularised *immature intermediate villi* causing high placental vascular impedance (Fisk et al., 1988). In the third trimester, *mesenchymal villi* transform to *mature intermediate villi,* which don't self-replicate but elongate and produce *terminal villi.* The decrease in trophoblast proliferation along with a relative increase in endothelial proliferation from 26 weeks of gestation until term causes a switch from *branching* to *non-branching angiogenesis* (i.e. formation and differentiation of new blood vessels). This results in the formation of long and slender villous trees containing one or two poorly branched capillary loops, which in turn, cause a decrease in feto-placental vascular impedance (Jauniaux et al., 1992). These morphological changes in villous tree development are reflected in the hemodynamic changes observed in the feto-placental circulation throughout pregnancy. Indeed, ultrasound assessment of the feto-placental circulation shows a gradual increase in the fraction of the fetal cardiac output distributed to the placenta in the second trimester (Vimpeli et al., 2008) with slight decrease towards term (Kiserud et al., 2006).

The maternal side of the placenta is perfused ~ 83% by the two uterine arteries and ~17% by the ovarian arteries (Wehrenberg et al., 1977), and about 75% of the uterine flow circulates in the intervillous space (Bartels and Moll, 1962). There is a steady increase in uterine blood flow with advancing gestation (Thaler et al., 1990), although the uterine blood flow normalized by fetal weight decreases (Konje et al., 2001). There is an increase in blood flow in the common iliac artery, but the blood flow in the external iliac artery decreases, indicating redistribution of lower extremity blood flow to the pregnant uterus and the placenta (Palmer et al., 1992). Clearly, a carefully orchestrated and balanced maturation of the feto-placental and utero-placental circulations is necessary for the physiologic function of the placenta, which is reflected in fetal and maternal wellbeing throughout gestation (Kingdom and Kaufmann, 1997). However, maternal uterine artery blood flow and oxygen delivery to the placenta, are not the sole determinants of fetal wellbeing. Indeed, a recent study on maternal oxygen transport in relation to fetal growth in high altitude pregnancies of two genetically different populations, concluded that the largest independent determinant of fetal weight was placental weight (Zamudio et al., 2007). Thus, genetic programming of the

placenta might play an essential role not only in determining pregnancy outcome, but also in the development of disease in adult life (Giussani, 2007).

1.3 Gene expression

The expression of deoxyribonucleic acid (DNA) follows the rules of a central dogma which occurs in two stages: (i) *Transcription*, during which DNA is transcribed into *messenger RNA (mRNA)* and (ii) *Translation*, during which *mRNA* serves as a template for protein synthesis.

The term *gene expression* refers to the process of decoding the *DNA* information, from gene to protein. The expression level of different genes among cells or tissues varies depending on the function, developmental stage or disease state, resulting in different *phenotypes* (i.e. the observable properties of an organism that are produced by the interaction of the genotype and the environment). Regulation of gene expression is complex, but schematically it can be divided in four different levels as shown in Figure 1: The first and most important level is transcription initiation. The next is maturation of pre-mRNA to mRNA by alternative splicing [removal of non-protein coding DNA sequences (introns) and putting together coding sequences (exons)]. Further regulation of gene expression occurs at the translation level, through mRNA editing, and finally, at the protein level through post-translational modifications that can determine protein activity. Recently, approximately 700 human small-RNAs (consisting of 20-30 nucleotides) have been shown to play an important role in gene expression (Kim et al., 2009) by interfering with chromatin formation, mRNA stability and translation control.

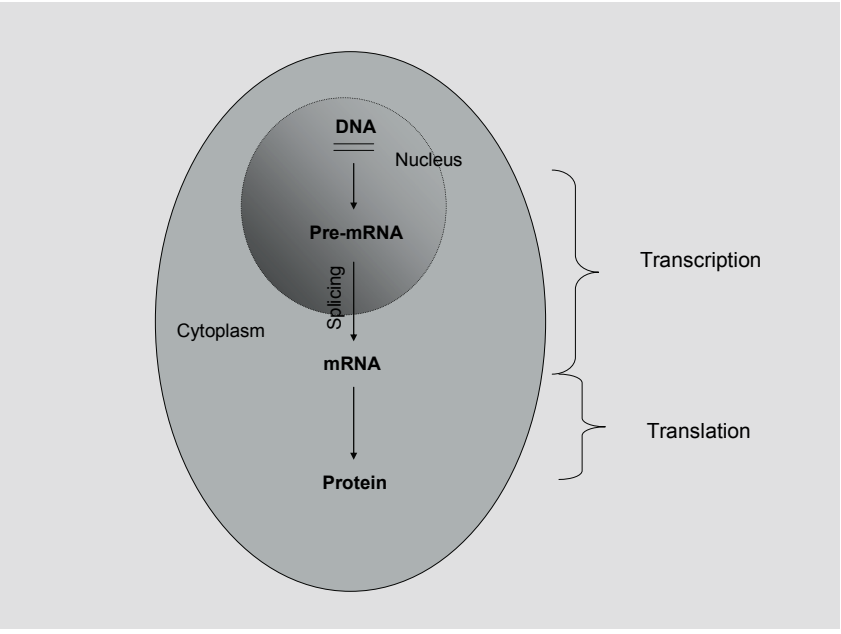

Figure 1. Schematic description of gene expression in a living cell

2. AIMS OF THE THESIS

The main aim of this thesis was to investigate the global gene expression profile in healthy and compromised human placentas. More specific objectives were to study the effect of:

a) normal labor, b) severe preeclampsia, and c) IUGR due to placental insufficiency on global placental gene expression profile.

Additional aims were to investigate the differences in placental gene expression profiles between early- and late-onset preeclampsia, and between IUGR with and without preeclampsia.

3. MATERIALS & METHODS

3.1 Study population

Pregnant women were recruited from the Department of Obstetrics and Gynecology, University Hospital of Northern Norway, during the period January 2005 - November 2006. The study protocol was approved by the Regional Committee for Medical Research Ethics (REK-Nord 94/2004). A total of 79 pregnant Caucasian women were recruited to the study. Informed written consent was obtained from all the participants. The flow of participants is shown in Figure 2.

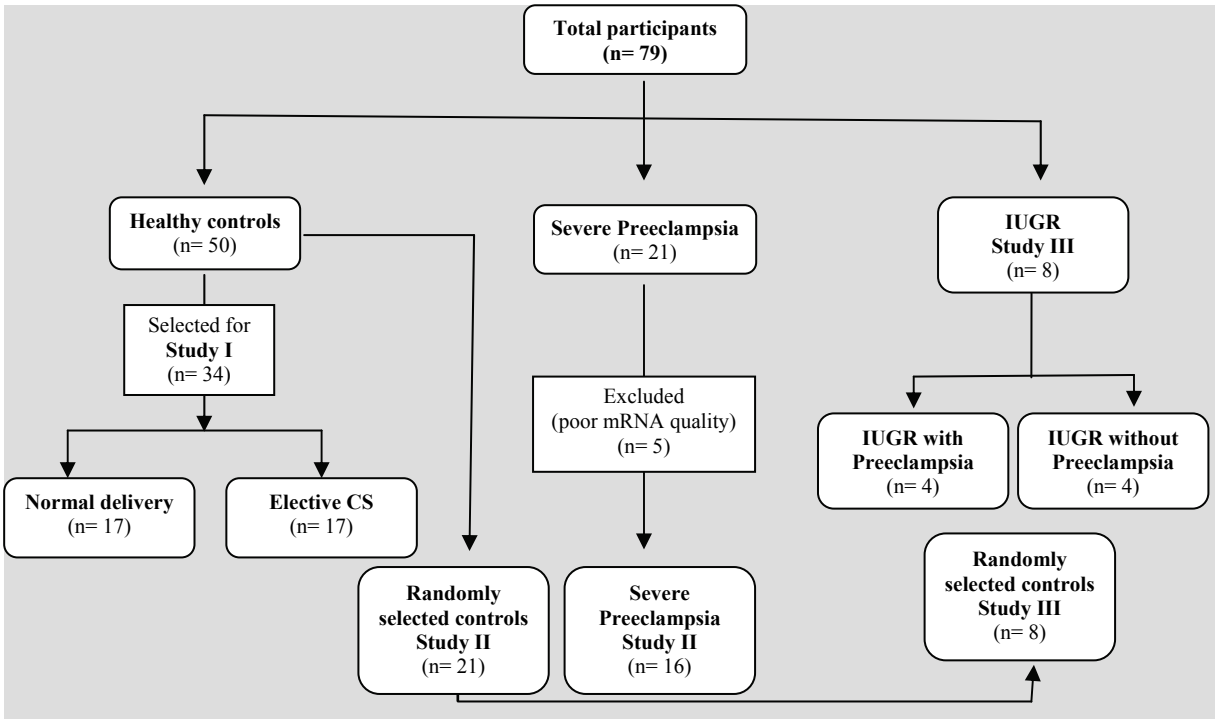

Figure 2. Flow diagram showing the phenotypes of the study participants

Gestational age was assigned by ultrasound at 18-20 weeks gestation. Severe preeclampsia was defined as a blood pressure (BP) of at least 160 mmHg systolic and/or 110 mmHg diastolic, with proteinuria ≥2+ on dipstick, measured on at least two occasions 6 hours apart while the patient was on bed rest, or hemolysis, elevated liver enzymes and low platelet (HELLP) syndrome, after the 20th week of gestation (Sibai et al., 2005). Women with pre-existing chronic hypertension, renal disease, lupus erythematosus, diabetes, and gestational hypertension without proteinuria were excluded. IUGR was defined as: (i) birth weight below the 5th percentile for the gestational age according to Norwegian nomograms (Skjaerven et al., 2000) (ii) oligohydramnios (i.e. amniotic fluid index ≤5th percentile for the gestational age) in the absence of fetal congenital urinary tract abnormality (Phelan et al., 1987) and (iii) abnormal fetal Doppler parameters (absent or reversed end-diastolic flow in the umbilical artery or signs of blood flow redistribution to the fetal brain, i.e. middle cerebral artery pulsatility index (PI) <5th percentile and umbilical artery PI >95th percentile). Women with small for gestational age fetuses with normal Doppler, known fetal chromosomal or structural congenital abnormality or infection were excluded.

Physical examination and ultrasonography were performed and blood and urinary samples were collected ≤72 hours before delivery. Placental and cord blood samples were collected immediately after delivery, following a standardized technique by two designated persons. Blood samples from the umbilical artery and vein were separately analyzed for acid-base status using a spectrophotometer ABL 725 Blood Gas Analyzer (Radiometer Medical AS, Copenhagen, Denmark) within 15 minutes of collection. Samples that had a pCO_2 difference of <1 kPa (7.5 mmHg) and pH difference of <0.03 units between umbilical arterial and venous blood were considered to have mixed blood and were discarded. The neonatal and placental weights were determined using electronic scales (Seca Model 727, Vogel & Halke, Hamburg, Germany). Outcome of pregnancy and information on the neonates were prospectively recorded. All collected data were stored in an electronic database (Figure 3).

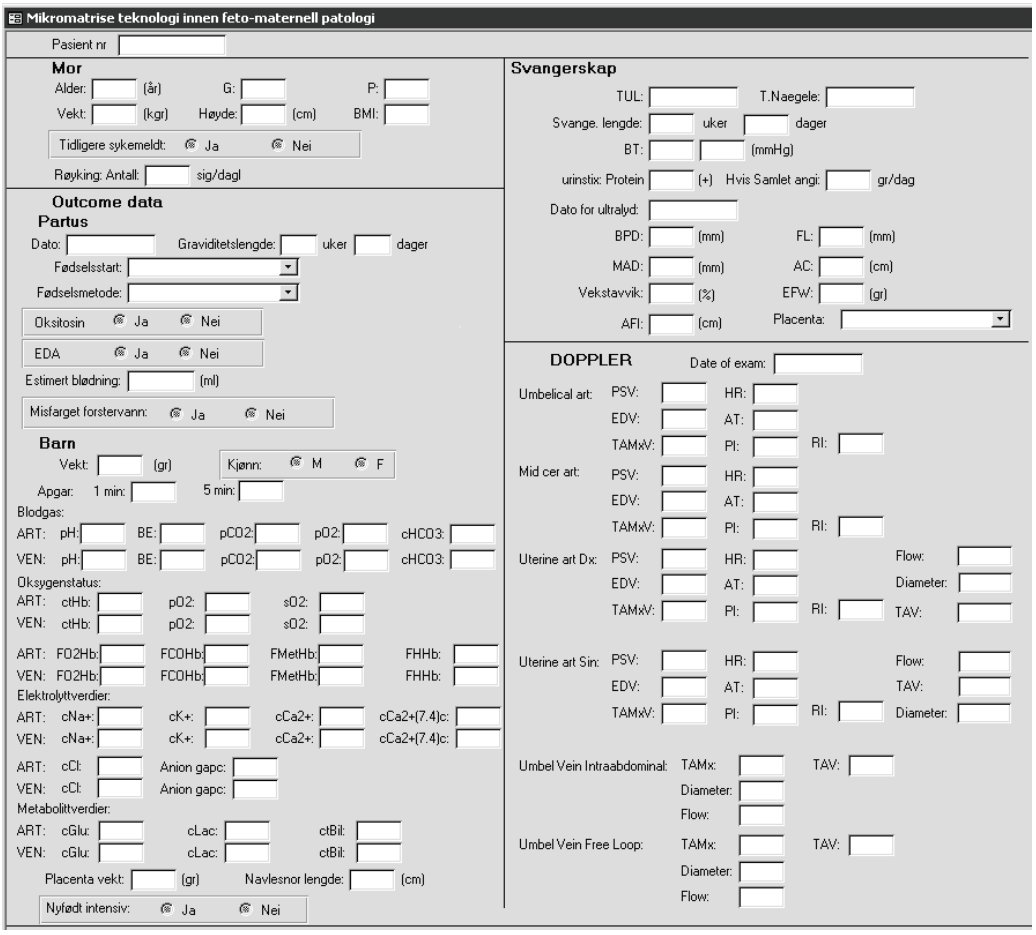

Figure 3. Microsoft Access data file showing the list of variables recorded for each participant of the study

3.2 Hemodynamics

Maternal uterine and feto-placental hemodynamics was studied using Acuson Sequoia 512 ultrasound system (Mountain view, CA, USA) with a 2.5 - 6 MHz curvilinear transducer. After a survey of fetal anatomy, biometry was performed to estimate the fetal weight. Blood flow velocity waveforms were obtained from the maternal uterine arteries, fetal middle cerebral artery, and umbilical artery and vein at a free-loop of the cord using color directed pulsed-wave Doppler. A large sample volume (5-10mm) and lowest possible insonation angle (kept below 20 degrees) were used. Velocity measurements were performed online and angle correction was used as required. Values used for statistics were an average of at least three cardiac cycles. The PI was calculated from the arterial blood velocity waveforms as:

PI = (peak systolic velocity − end-diastolic velocity)/time-averaged maximum velocity

Umbilical vein inner diameter was measured at a free-loop of the cord using B-mode ultrasound. Uterine artery diameter was measured using power Doppler angiography (Acharya et al., 2007; Konje et al., 2001). The values used for analysis were an average of three separate measurements. The volume blood flows (Q) of the umbilical vein and uterine

arteries were calculated as the product of time-averaged intensity weighted mean velocity (Vmean) and the cross-sectional area (CSA) of the blood vessel:

$Q\ (ml/min) = Vmean\ (cm/s) * CSA\ (cm^2) * 60$

Assuming that the blood vessel has a circular lumen, CSA was calculated as:

$CSA = \pi * Radius^2 = 0.785 * Diameter^2$

3.3 Microarrays

The founding report on microarray technique was based on the genome of the flowering plant Arabidopsis thaliana containing the smallest genome of any higher eukaryote examined to date (Schena et al., 1995). It was a pioneering study demonstrating a novel high-capacity system that could monitor the expression of 45 genes in parallel. Although recent advances in sequencing and microarray technology have produced high-density microarrays, the basic methodology of DNA microarray remains similar: RNA samples or targets are hybridized to known cDNAs/oligo probes on the arrays. One such sequencing effort is the Human Genome Project. It was established as an international project with the aim to identify the chromosomal position and genomic organization of all human genes. In 2004, the International Human Genome Sequencing Consortium completed 99% of the human DNA sequencing and announced that "the human genome could be regarded as effectively known" (IHGSC, 2004).

A microarray is a small (approximately 1.3x1.3 cm) glass, silicon slide or chip upon the surface of which a large number of known human gene sequences (usually from 500 to 35 000) are fixed in defined positions in a matrix-fashion. There are two types of microarrays: (i) cDNA microarrays where individual DNA sequences are spotted on the array (usually up to 1500 base pairs) (ii) Oligonucleotide-arrays where oligonucleotides (usually 70-mers) are either printed or robotically synthesized directly on the array. Microarray technology offers the potential to analyze the expression state of each individual gene represented in the genome in a single experiment. Yet, with this method it is impossible to quantify the absolute expression level of a gene. It is thus necessary to perform comparative studies, for example normal versus diseased tissue samples, in order to obtain the relative gene expression profile of a given sample. A typical microarray experiment is executed following a number of necessary steps (Figure 4).

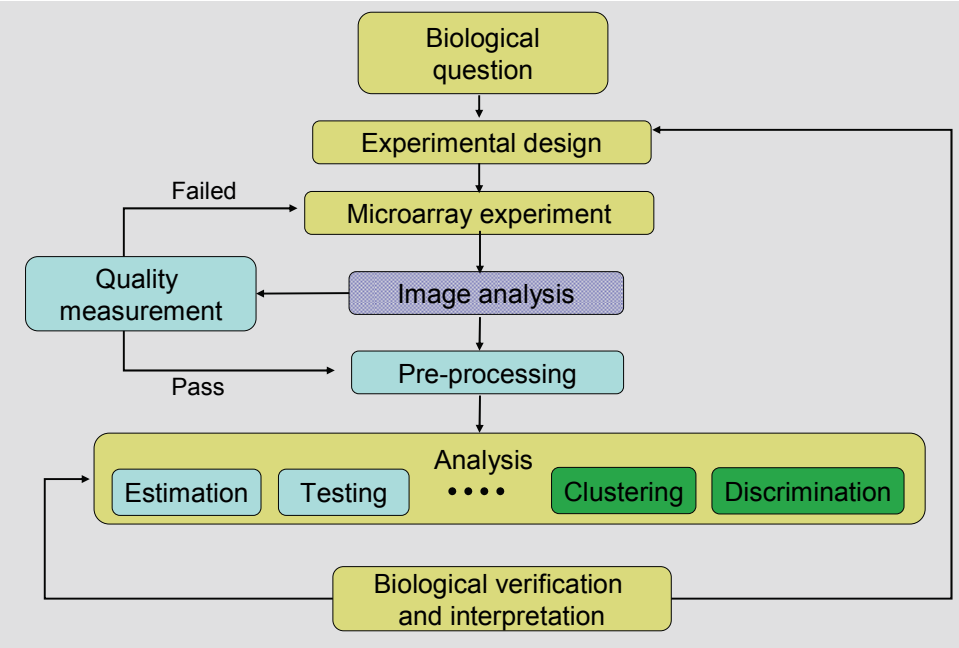

Figure 4. Outline of a typical microarray experiment

Experimental design

The first and most crucial step of microarray experiments is the design (Yang and Speed, 2002) which is dependent on the aim of the study. Microarray studies are comparative studies and the overall results depend on the phenotypic characteristics of the samples that are compared. Gene expression profiling of a diseased sample, is based on the relative up- or down-regulation of each gene, compared to an "assumed" healthy sample which is used as control. Possible confounding factors that would add "noise" to the data should also be taken into account. Given that y represents a measurement of gene expression in the placenta, the following linear model may be calculated:

$$y = parity + maternal\ age + delivery\ method + \ \ldots + healthy\ or\ diseased + \varepsilon$$

where ε is an "error-term" representing the inherent biological variation of the sample and the measurement error of the system.

There are three main types of experimental design: (i) Direct, where each diseased sample is matched to one healthy control (pair-wise comparison), (ii) Indirect, where each diseased sample is matched to a pool of several healthy samples (common reference) and (iii) Loop design, where each sample is compared with the others in consecutive pairs (Figure 5).

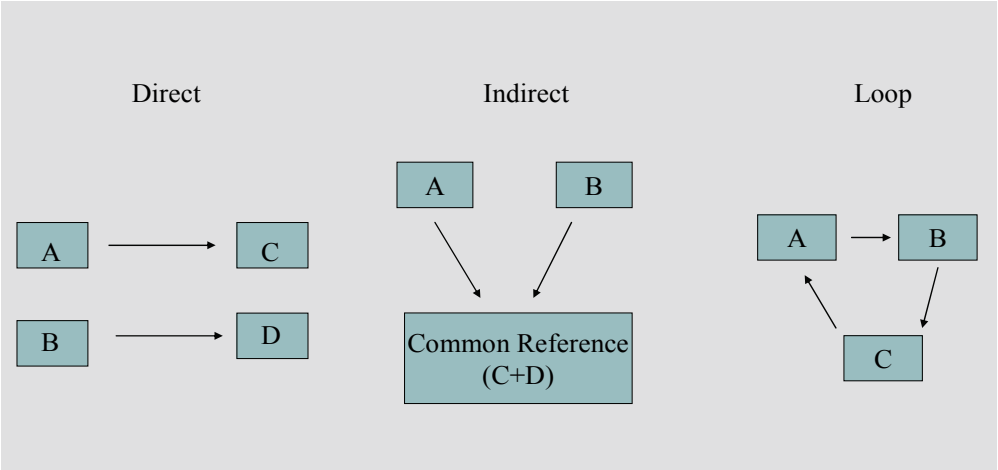

Figure 5. Graphical representation of microarray experimental designs. The arrows indicate hybridization between two mRNA samples (A, B, C etc)

Microarray laboratory procedures

There are five laboratory steps during a microarray experiment:

1. RNA extraction and quantity/quality control
2. Labeling
3. Hybridization
4. Washing
5. Image acquisition

RNA extraction and quantity/quality control

Disruption and homogenization of placental samples were performed in lysis buffer using the MagNa Lyser Instrument (Roche Applied Science, Germany), according to the manufacturer's instructions. Isolation of total RNA was performed using the MagNa Pure Compact RNA isolation kit and the MagNa Pure Compact Instrument (Roche Applied Science, Germany) (Paulssen et al., 2006). RNA was quantified by measuring absorbance at 260nm, and RNA purity was determined by the ratios OD260nm/280nm and OD230nm/280nm using the NanoDrop instrument (NanoDrop® ND-1000, Wilmington, USA). The RNA integrity was determined by electrophoresis using the Agilent 2100 Bioanalyser (Matriks, Norway). Only RNA samples with a ribosomal RNA 28S/18S ratio of >1 and/or RNA Integrity Number >7.2 were used for microarray experiments (Schroeder et al., 2006).

Two different microarray platforms were used for the studies of this thesis: (i) A two-channel platform, needing two fluorescent dyes *(direct labeling)* in study I and a one-channel platform, based on chemiluminescence *(indirect labeling)*, in studies II and III.

Microarray procedures for study I

For the two-channel platform, total RNA was reverse transcribed and labeled directly with Cy3- and Cy5- attached dendrimer (Figure 6), using the Genisphere 3DNA 350HS kit (Genisphere, Montvale, NJ) as described in the manufacturer's protocol. Hybridizations were carried out in a TECAN HS4800 instrument (TECAN, Austria) using the formamide-based hybridization buffer from Genisphere containing 5% dextrane sulphate and 5.5 ng/ml human COT1-DNA (GIBCO-BRL Life technologies) at 37º for 23 hours. 3DNA dendrimer hybridizations were carried out in formamide-based hybridization buffer alone. Post-hybridization washes were carried out at room temperature with 2x saline sodiumcitrate (SSC) for 1 min, 0.2% sodium dodecylsulphate (SDS) /2xSSC for 1 min and finally with 0.2xSSC for 30 sec. For global gene expression analysis 35K oligo microarrays were used that were obtained from the Norwegian Microarray Consortium (http://www.microarray.no). Briefly, the arrays contained spotted 70-mer oligonucleotides obtained from the Human Array-Ready Oligo Sets (AROS™) v3.0, OPERON, Germany (http://www.operon.com). The set contained 34,580 probes representing 24,650 human genes and 37,123 gene transcripts. The probe design is fully based on the Ensembl Human 13.31 Database (http://www.ensembl.org) and Human Genome Sequencing Project. As external control system, the Spot Report oligo validation system (Cat# 252170-7) from Stratagene was used. The arrays were scanned with the GenePix 4000B scanner (Axon Instruments Inc).

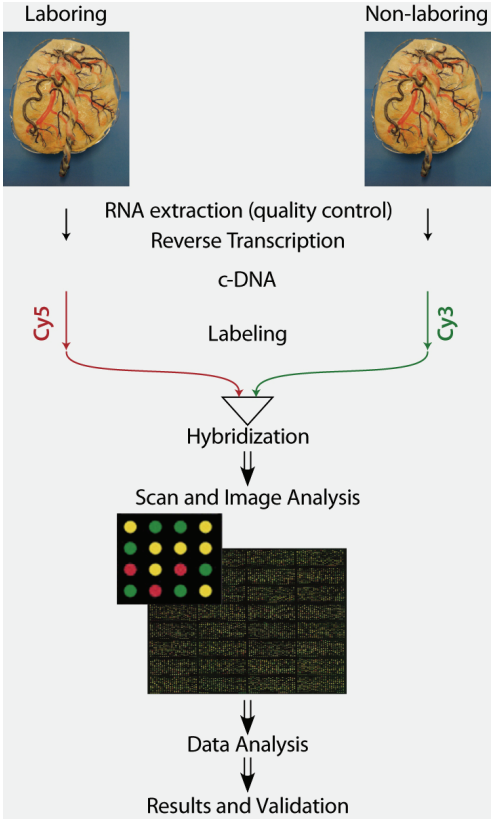

Figure 6. Flowchart describing the microarray experiment for study I

Microarray procedures for studies II and III

For the one-channel platform, total RNA samples were processed into digoxigenin (DIG)-labeled cRNA (Figure 7) using the Applied Biosystems Chemiluminescent NanoAmp™ RT-IVT Labeling Kit. The labeled DIG-cRNA (10 µg per microarray) was then injected into each microarray hybridization chamber. Following hybridization at 55°C for 16 hours, the unbound material was washed from the microarrays. Features that retained bound DIG- labeled cRNA were visualized using the Applied Biosystems Chemiluniescence Detection Kit. Anti-DIG alkaline phosphatase was used to hydrolyse a chemiluminescence substrate to generate light at 458nm which was than detected by the Applied Biosystems 1700 Chemiluninescent Microarray Analyzer. The Human Genome Survey Microarray v.2.0 (Applied Biosystems) with 32878 probes for the interrogation of 29098 genes was used for microarray analysis.

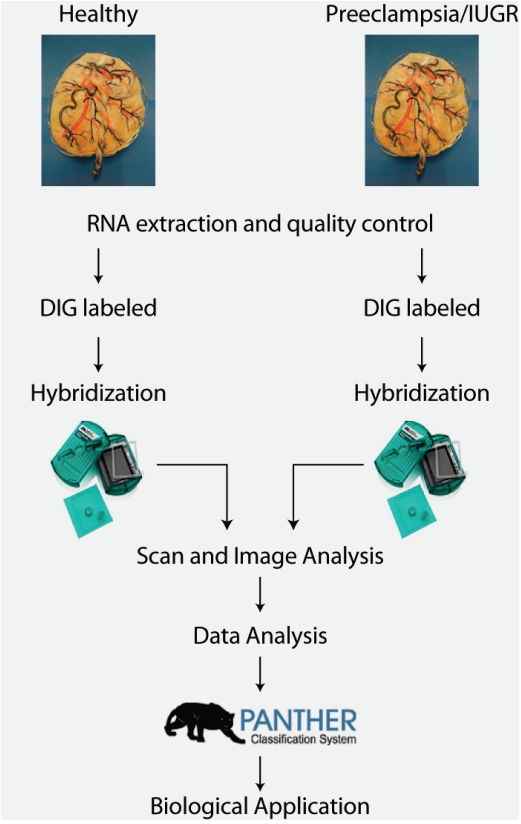

Figure 7. Flowchart describing the microarray experiments for studies II and III

Image analysis

The scanned images are then turned into numbers, called *raw data*. Each spot on the microarray is given a number depending on its fluorescence intensity. The main steps during image analysis are *normalization,* aiming to remove effects from the technical process from the data (for example different labeling efficiencies, different scanning parameters etc), and *filtering* aiming to eliminate spots that will bias or add noise to the results.

In study I, the features were extracted from the arrays using Genepix 6.0 software (Axon instruments Inc., 2004). The background estimates were calculated using the morphological opening method (Soille, 2006). Spots that displayed a signal-to-noise ratio of less than three or that were significantly saturated (more than 20% saturation among foreground pixels) were filtered out. The median was used as the averaging measure of the foreground pixels. After quality control, genes that were present in less than 50% of the arrays were filtered out. Normalization was carried out using Lowess normalization (Cleveland, 1979).

In study II, the features were extracted from the arrays using the ABI1700 software. Normalization was carried out using quantile normalization which forces all the slides to have the same intensity distribution. Spots on the arrays with less than excellent signal and with

expression values missing for ≥25% of the samples in the preeclampsia group or ≥35% in the control group were filtered out.

In study III, the features were extracted from the arrays using the ABI1700 software. The gene expression data were analyzed using J-Express pro 2.7 (Molmine, Bergen, Norway) (Dysvik and Jonassen, 2001). The pre-normalized intensity values were extracted per spot from the data files. Weak spots with signal to noise ratio <3.0 and control spots were filtered out. All arrays were normalized by the Lowess method (Cleveland, 1979). Missing expression levels were inferred using the LSimpute method (Bo et al., 2004).

Data analysis

Microarray experiments generate large amount of data and represent a challenge for conventional statistical methods. Novel computational methods that are able to handle large datasets are developed. A major problem in microarray experiments is the abundance of false positive findings and often adjustment with multiple testing and permutation (i.e. reordering) of the data is necessary in order to reduce type I errors (false positives). Microarray data analysis aims to find genes with different fluorescent intensity between arrays, i.e. differentially expressed genes between distinct phenotypic groups. A gene is differentially expressed between two groups when it is systematically up- or down-regulated in one group compared to the other. Differentially expressed genes are usually presented at two levels: (i) *statistical* level shown by p-values indicating the probability that the difference in gene expression of a particular gene between two samples is true and (ii) *biological* level which is the mean fold-change in relative over- or under-expression of a particular gene between the samples. The level of p-value and fold-change above which the genes are "significantly" differentially expressed is arbitrary. In addition, clustering methods and principal component analysis (PCA) (Raychaunhuri et al., 2000) that organize genes or samples into groups or hierarchy based on expression similarity are used in order to make microarray results easier to understand.

More specifically, for the purpose of finding differentially expressed genes in study I, we applied an empirical Bayes analysis (Smyth, 2004) using the LIMMA package (Smyth, 2000). The data were analyzed using a two-component linear model. The prior guess of the number of differentially expressed genes was set to 0.01. Multiple testing was accounted for by estimating the false discovery rate (set at <15-20%) applying the Benjamini-Hochberg (Benjamini, 1995) and Storeys less conservative Q-value (Storey, 2002) procedure. A 2.5-fold change in expression of any gene that was present on ≥ 8 arrays was considered significant, if

the difference in fluorescence intensity between two matched samples reached a p-value of < 0.01.

For the purpose of finding differentially expressed genes in study II, we applied an empirical Bayes analysis using the LIMMA package. The data were analyzed using an ANOVA two-component linear model accounting for preeclampsia versus normal and nulliparity versus multiparity. We further used the same method in order to identify potential differentially expressed genes between early- and late-onset preeclampsia. The cut-off time-point of early- versus late-onset preeclampsia was 34 weeks gestation. The prior guess of the proportion of differentially expressed genes was set to 0.01. A two-fold change in expression of any gene was considered significant, if the difference in fluorescence intensity between the samples reached a p-value < 0.01. A hierarchical cluster analysis was conducted on the 300 most differentially expressed genes. A Predictive Analysis of Microarrays (PAM) (Tibshirani et al., 2002) was conducted in order to investigate whether any set of genes provides predictive power between the groups (preeclampsia versus normal).

In study III, statistical significance was assigned to the genes by using the Significance Analysis of Microarrays (SAM) methodology (Tusher et al., 2001). PCA was performed on the 20 genes with best t-scores in the main analysis, in order to obtain discrimination between IUGR and normal placentas.

Database submission of microarray data

The Microarray Gene Expression Data Society (MGED; http://www.mged.org) has recommended that all publications using microarray technology should be compliant with the MIAME (Minimal Information About Microarray Experiments) requirements (Brazma et al., 2001) and raw data should be available in public repositories such as ArrayExpress (Brazma et al., 2003) or Gene Expression Omnibus (GEO)(Edgar et al., 2002). All our studies are MIAME compliant and raw data can be retrieved in GEO (http://www.ncbi.nlm.nih.gov/projects/geo/) with accession numbers GSE 8375, GSE 10588 and GSE 12216 for study I, II and III respectively.

Gene Annotations

The last step of microarray studies is the interpretation of the results. Lists of differentially expressed genes between groups are examined in order to find possible associations/interactions regarding their biological function (cellular events to which the gene product contributes), cellular component (location of a gene/protein), molecular function (at a

biochemical level) or pathways (molecular signaling networks of communication between genes/proteins). This process is performed via internet-databases with updated, evidence-based information/knowledge on gene/protein function by using software able to handle large datasets. In other words, the ultimate scope of every microarray experiment is to find answers on biological questions such as: What is the role of the differentially expressed genes in determining the disease phenotype? Which cellular/molecular pathways might be involved? How does the disease process affect cellular/tissue function and phenotype? Or vice versa, how does cell/tissue remodeling affect pathogenesis of disease? Are there single genes or sets of genes that could predict disease severity and outcome; and if so, through which pathways? Ultimately, could the findings be applied in clinical practice?

For the annotation of the genes, PubGene 2.6™ Database and Analysis Software (Jenssen et al., 2001) and DAVID Bioinformatics Resources 2007 (Dennis et al., 2003) were used in study I and Protein ANalysis THrough Evolutionary Relationships (PANTHER) (Mi et al., 2007) in studies II and III.

3.4 Real-time reverse-transcriptase polymerase chain reaction

As a large amount of genes might be differentially expressed in microarray experiments, the level of expression of selected genes must be confirmed by other independent methods.

RT-PCR is the method of choice to validate microarray data. It was invented by Kary Mullis (Mullis, 1990) and is a method for amplifying a specific region of a DNA strand, by mimicking the DNA duplication process occurring in nature. The process requires a DNA-template, one or more specific primers, two enzymes (reverse transcriptase and DNA polymerase) and several deoxynucleotide triphosphates that serve as “bricks” to build a new, complementary DNA strand (cDNA). The process consists of three steps: (i) Reverse transcription and denaturation from single stranded DNA-template to cDNA (ii) Primer annealing and (iii) Primer extension (elongation) with formation of cDNA. This procedure is repeated several times, in general 20-35 cycles, so that the amount of cDNA increases exponentially (Freeman et al., 1999).

In RT-PCR there is an additional fourth step during which a fluorogenic probe is annealed to the cDNA sequence, between the forward and reverse PCR primers. During extension the fluorescent molecule is cleaved, emitting fluorescence that is measured continuously (real-time) and is proportional to the amount of PCR product (Figure 8).

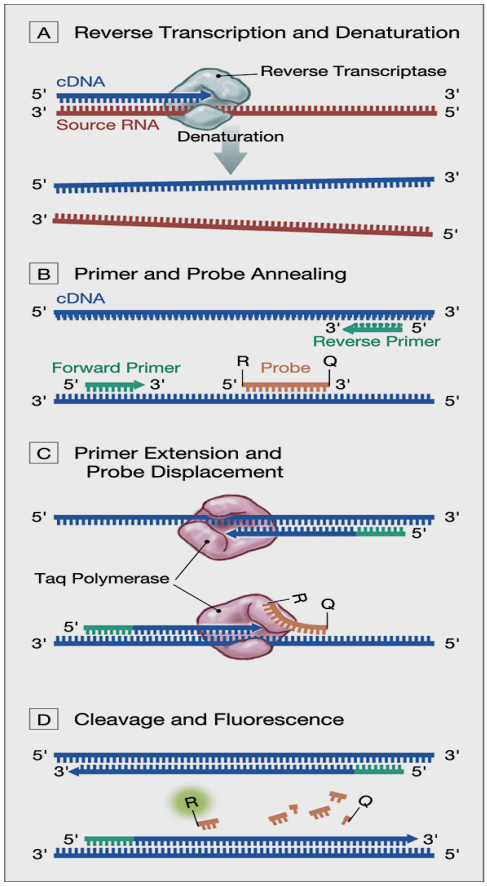

Figure 8. Outline of RT-PCT technique, reproduced with permission from King and Sinha, JAMA, 2001

An example of an RT-PCR amplification curve of a gene (MML3) is shown in Figure 9. The curves indicate the fluorescent signal of the PCR product (ΔRn) at each time point, reaching an exponential phase where there is a doubling of the product with each cycle. The higher the starting copy number of the gene, the earlier the exponential phase starts. A fixed fluorescence threshold is set by the operator, significantly above the baseline (red line). A parameter called C_T (*threshold cycle*) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. The lower the C_T, the higher the initial amount of target gene.

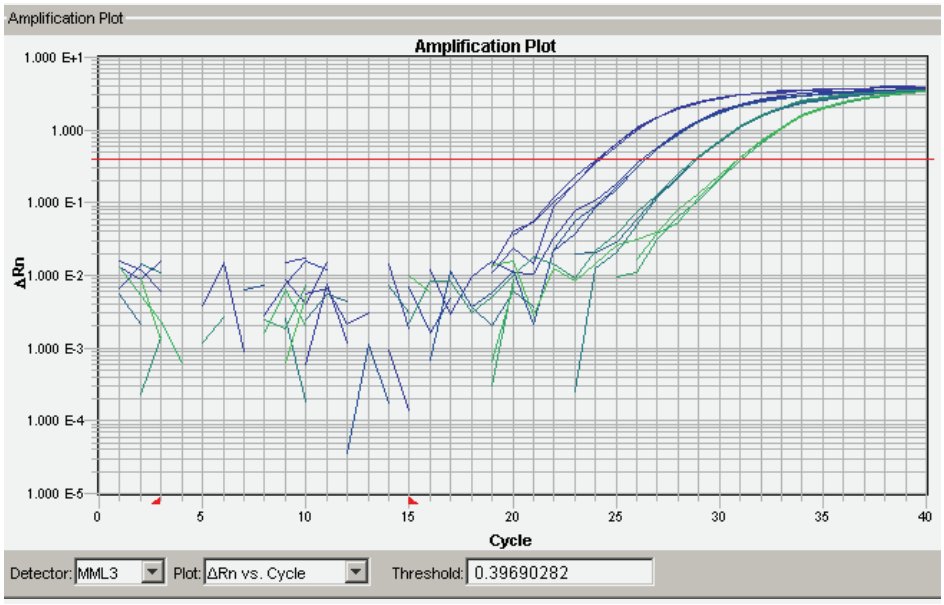

Figure 9. Example of RT-PCR amplification curve of a gene (MML3)

In RT-PCR the relative expression of a gene is normalized to genes that are constitutively active, called housekeeping genes. These genes have a constant expression regardless of the state of the cell. There are several housekeeping genes in the human placenta (Vandesompele et al., 2002; Meller et al., 2005). By comparing the C_T values of the genes in question with the C_T values of housekeeping genes, one can calculate the expression level of the genes in different states of the samples, by using different formulas (Livak and Schmittgen, 2001; Pfaffl, 2001).

3.5 Placental morphology

All placentas were macroscopically examined in the labor ward after delivery for completeness, number of umbilical cord vessels, color of the membranes and gross morphology of the maternal surface looking for signs of retroplacental clots, calcifications, infarctions etc. After taking biopsies, whole placenta was immersed in 4% formalin and sent for histopathologic examination. All placentas were examined according to our hospital's standard laboratory procedure. Five sections were examined that were sampled from the umbilical cord, membranes, central portion of placenta, basal plate and transmural central part, respectively. One pathologist with special training in placental microscopy, evaluated all sections of the preeclamptic placentas, without prior knowledge of clinical status, looking for signs of inflammation as described previously (Redman et al., 1999) in standard hematoxylin and eosin stained slides.

3.6 Urinary human chorionic gonadotropin measurement

Urinary samples were collected from the mother before delivery and stored at –70°C until further analysis. Urinary hCG concentration was measured in 41 normal pregnancies and 18 pregnancies affected by severe preeclampsia by an automated electrochemiluminescence immunoassay method using intact hCG + β-subunit kit (Modular E170 Analyser, Roche Diagnostics) which has previously been validated (Ajubi et al., 2005).

3.7 Leptin immunofluorescence

Tissue sections were fixed in formalin and embedded in paraffin blocks according to standard procedures. Glass slides were cleaned with 95% ethanol, treated with subbing solution and air dried. 4–6 micron thick tissue sections were cut and applied to slides. Slides were deparaffinized in xylene using three changes 5 minutes each. Sections were hydrated gradually through graded alcohols (100% ethanol twice for 10 minutes each, then 95% ethanol twice for 10 minutes each). Slides were then washed in de-ionized water for 1 minute with stirring and excess liquid aspirated from slides.

Antigen unmasking was performed using heat treatment. Slides were placed in a prewarmed solution of 10 mM sodium citrate buffer, pH 6.0 for 5 min. This step was repeated once. Slides were let to cool down at room temperature in the buffer for 20 minutes before they were washed in de-ionized water three times for 2 minutes each and excess liquid was aspirated from slides.

Slides were incubated with 500 µl 10% normal goat blocking serum (Santa Cruz Biotechnology, Inc, USA) in 1x phosphate buffered saline (PBS) for 20 minutes at room temperature. Suction was used to remove reagents (drying of the specimens was avoided between steps) before slides were washed in 1xPBS two times for 2 minutes each. Primary antibody for leptin 2.5 µg/ml (Ob (A-20) Santa Cruz Biotechnology, Inc, USA) in PBS with 1.5% normal blocking serum was incubated for 60 minutes. PBS only was used to test the specificity of the primary antibody (negative control). Slides were washed with three changes of PBS for 5 minutes each and then incubated for 45 minutes at room temperature with secondary goat anti-rabbit IgG-FITC 2.5 µg /ml (Santa Cruz Biotechnology, Inc, USA) in PBS with 1.5% normal blocking serum. Slides were washed with three changes of PBS for 2 minutes each before they were air dried and counterstained with DAPI (4',6-diamidino-2-phenylindole) II (Vysis, Abbott Diagnostics, USA).Images were obtained using a CytoVision (Applied Imaging, San Jose, CA, USA) digital system equipped with a charged-coupled device (CCD) camera (Cohu Inc., San Diego, USA).

4. MAIN RESULTS

4.1 Paper I

Gene expression profiling was carried out on mRNA extracted from placental samples that were obtained from 17 women after vaginal delivery (laboring) and 17 women after elective cesarean section before the onset of labor (non-laboring). All women were healthy and had uncomplicated pregnancies. There were no significant differences regarding maternal phenotype, hemodynamic parameters and pregnancy outcome between the groups. In the group of women delivering vaginally, the mean duration of labor was 7.6 (range, 2-15) hours. Out of approximately 25 000 genes that were represented on the array, one third (n=8000) passed stringent quality criteria. An empirical Bayes analysis was performed in order to find differentially expressed genes. Only three genes, namely FOS, FOSB and GNGT1 were differentially expressed more than 2-fold. Several other genes captured by microarrays were related to oxytocin degradation (LRAP) and placental hormone metabolism (HSD17B4), but did not seem to be significantly affected by the mode of delivery. Furthermore, validation using RT-PCR (Paper I, Table 2) showed only FOS to be actually down-regulated by 2.2 mean fold-change in laboring compared to non-laboring placentas (Figure 9), suggesting that the global placental gene expression profile is not significantly altered by labor.

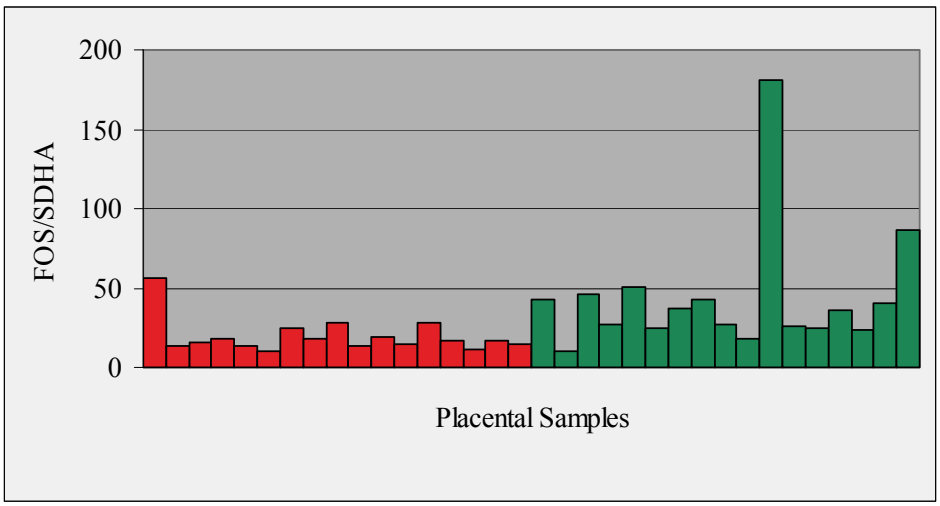

Figure 10. Relative expression of FOS normalized for SDHA (housekeeping gene) in for each of the placental samples estimated by RT-PCR. Red indicates laboring, green indicates non-laboring placentas; mean FOS/SDHA expression of 19.56 and 43.73 respectively

4.2 Paper II

Gene expression profiling was carried out on mRNA extracted from placental samples that were obtained from 21 women with severe preeclampsia and from 21 controls that were randomly selected from a group of 50 women with uncomplicated pregnancies. Preeclamptic women had an average mean arterial pressure of 132mmHg and proteinuria of 5g in a 24-hour urine specimen. They were not significantly different in terms of age, parity and body mass index (BMI) from their controls. However, they had significantly higher mean uterine artery PI and reduced uterine artery volume blood flow, although the fetal circulation was not compromised as indicated by normal cerebro-placental ratio (middle cerebral PI/umbilical artery PI) and normalized umbilical vein volume blood flow. Eleven out of sixteen preeclamptic women were delivered prematurely (at <37 weeks) by cesarean section before the onset of labor and among them 3 had HELLP syndrome. The method of delivery (vaginal versus cesarean) was balanced between the groups. The number of small for gestational age babies was significantly higher and placental weight lower among preeclamptic women compared with controls. Two preeclamptic women had growth restricted fetuses. However, the neonatal outcome was not different between the groups.

After RNA isolation, five preeclamptic placentas were excluded due to poor RNA quality. Thirty-seven hybridizations in a one-channel detection system of chemiluminescence emitted by the microarrays were performed. A total of 18811 genes on the arrays passed our quality criteria after image analysis. In preeclamptic placentas 213 genes were significantly (fold-change≥2 and p≤0.01) up-regulated and 82 were down-regulated, compared with normal placentas (Paper II, Table 1S). Supervised clustering analysis showed 16 of these genes able to differentiate preeclamptic from healthy placentas (Paper II, Figure 1). Microarray results were validated by RT-PCR for 16 selected genes and there was a good correlation for most of them (Paper II, Table 3S). Different subtypes of hCG were up-regulated in preeclamptic placentas from 3.5 to 6.4 fold. We validated this result by measuring hCG concentration in maternal urine. Mean concentration of urinary hCG in the control group was 20034 (95% CI: 13121-27687) IU/L and in preeclamptic patients 68271 (95% CI: 18917-117624) IU/L (p=0.04). There was no significant correlation between urinary hCG values and placental weight (r=0.03; p=0.823). Leptin RNA was highly (40 fold) up-regulated in the preeclamptic placentas both in microarray and RT-PCR. In order to correlate this result with leptin protein expression levels, immunofluorescence was performed in preeclamptic and normal placentas. Leptin was expressed in the villous trophoblast and was more abundant in the preeclamptic placenta (PaperII, Figure 4).

Pathway analysis using all differentially expressed genes between 16 preeclamptic and 21 normal placentas showed several differentially expressed genes to be associated with Alzheimer disease, angiogenesis, Notch-, TGFβ- and VEGF-signaling pathways. Preliminary biological link analysis showed that hypoxia, apoptosis, angiogenesis and folate metabolism were altered in the preeclamptic placentas (data not shown).

The preeclamptic placentas were further sub-grouped depending on (i) disease onset and (ii) level of proteinuria.

Five placentas were delivered before 34 weeks of gestation (early-onset preeclampsia) and 11 after 34 weeks (late-onset preeclampsia). Microarray data analysis showed 402 differentially expressed genes between these groups (Paper II, table 2S and Figure 2). Comparison of pathways showed that oxidative stress, inflammation mediated by chemokine and cytokine and endothelin signaling pathways were mainly involved in early-onset preeclampsia (Paper II, Figure3). This result was validated for selected genes by RT-PCR (Paper II, Table 3S).

Preeclamptic placentas were stratified in three different phenotypes, according to the level of proteinuria: Group A: four patients with proteinuria 1-2 g/l, Group B: five patients with proteinuria 3-4 g/l and Group C: five patients with proteinuria ≥ 5 g/l. A three-component ANOVA was performed that showed significant differences in gene expression profile between groups A and C (Figure 11).

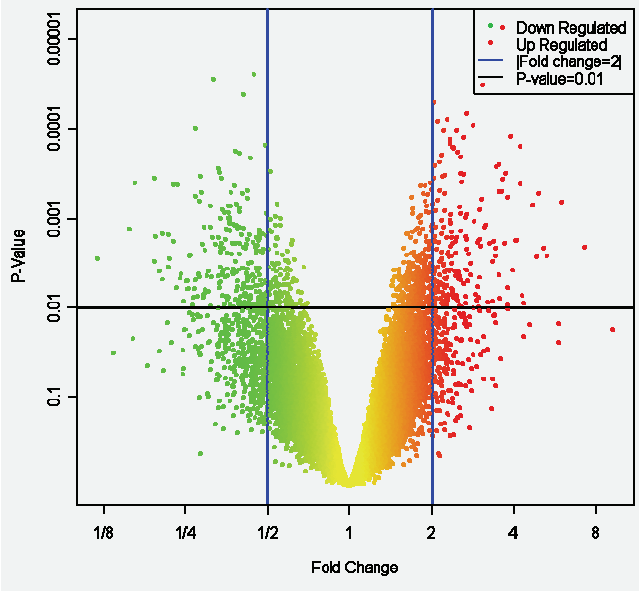

Figure 11. Volcano plot showing differentially expressed placental genes between preeclamptic women with proteinuria 1-2 g/l compared to those with proteinuria >5 g/l. Each spot represents a gene and color intensity represents the expression level. All the genes located on the upper-lateral quadrants are significantly differentially expressed at a statistical and biological level)

4.3 Paper III

Gene expression profiling was carried out on mRNA extracted from placental samples that were obtained from eight patients with IUGR due to placental insufficiency, and from eight women with uncomplicated pregnancies that served as controls. Four women with IUGR also had preeclampsia. There were no significant differences regarding maternal age, parity and weight gain during pregnancy between the groups. Gestational age, birth weight and placental weight were significantly lower in IUGR pregnancies. All patients with IUGR had oligohydramnios and hemodynamic signs of redistribution of blood flow to the fetal brain, as implicated by the inclusion criteria of the study. Two women in the IUGR group received a single course of antenatal corticosteroid. The mode of delivery (vaginal versus cesarean) was balanced between the study groups. Neonatal outcome was favorable in both groups as indicated by Apgar scores and cord blood acid-base status (Paper III, Table 1).

Microarray data analysis showed that in IUGR placentas 47 genes were up-regulated and 70 genes were down-regulated at a significant level (fold-change $\geq$1.5 and p-value$\leq$.05) compared with normal placentas (Paper III, Tables 2 and 3). PCA for the top 20 differentially expressed genes showed good separation in terms of gene expression patterns between the groups (Paper III, Figure 1). Pathway analysis of differentially expressed genes between IUGR and normal placentas, after Bonferroni correction for multiple testing, showed significant up-regulation of the inflammation mediated by chemokine and cytokine signaling pathway (pathway accession number P00031 in http://www.pantherdb.org). Subgroup analysis comparing IUGR with and without preeclampsia showed few (n=27) differentially expressed placental genes (Paper III, Table 4). Validation of the microarray results by RT-PCR showed good correlation between the two methods (Paper III, Table 5).

5. DISCUSSION

This thesis recapitulates three studies on global gene expression profile of healthy (laboring and non-laboring) and compromised (preeclamptic and IUGR) placentas using genome wide microarrays. Placental gene expression was altered by disease states but not by normal labor. Results have been validated at the transcript and protein levels for selected genes. These results indicate that gene expression profiling of the human placenta is a useful tool in the effort to understand the role of the placenta in parturition and in the patho-physiology of pregnancy-specific disorders, such as preeclampsia and IUGR.

5. 1 Gene expression profile of the normal placenta

The human placenta is a complex tissue composed by a broad variety of placental/fetal and maternal cells. Structural heterogeneity of placental villi depends mainly on the degree of maturity and on the sampling site of the placenta. Accordingly, global gene expression profile varies within the normal placenta (Sood et al., 2006). In particular, it appears that hypoxia-related genes are up-regulated in placental sites distant to the umbilical cord insertion and towards the basal and chorionic plates (Wyatt et al., 2005), possibly reflecting reduced blood perfusion at these sites (Matijevic et al., 1995). In fact, these changes in gene expression, although modest (up to three-fold), correlate well with villous histology, i.e. increased fibrin deposition and syncitial knots and reduced villous size, mostly observed at the periphery of the placental disc. Moreover, gene expression profiling of the feto-maternal interface (basal plate) between midgestation and term, showed dramatic changes regarding angiogenesis, cell motility, extracellular matrix modulation, gene transcription, signal transduction, immune response, protein biosynthesis, and lipid metabolism (Winn et al., 2007). Therefore, for the purpose of this thesis, placental samples were taken from a standardized location, approximately 2cm beside the umbilical cord insertion (a landmark that is easily recognizable), and the sample included tissue from the middle layer of placenta midway between maternal and fetal surfaces. This is a representative site to study placental gene expression as it includes central villous parenchyma that is well perfused. Additionally, all samples were collected by two designated persons to ensure technical reproducibility and washed thoroughly with isotonic saline to avoid contamination with maternal blood.

5.2 Placental gene expression in parturition

The molecular mechanisms regulating the duration of pregnancy and initiation of labor are still incompletely understood. Clearly, interactions of maternal, placental, fetal and environmental factors cooperate in order to activate the uterus in order to expel the fetus (Challis et al., 2000b). Deregulation of these factors might cause premature delivery (Goldenberg et al., 2008). Moreover, there is epidemiological evidence that genetic factors might play a role in preterm birth (Varner and Esplin, 2005), indicating that regulation of gene expression in feto-maternal tissues may be involved in the process of parturition. Indeed, several studies using high-throughput methods (i.e. genomics, trancriptomics, proteomics) identified genes involved in prostaglandin synthesis and inflammatory response being differentially expressed in the myometrium, cervix and chorio-amniotic membranes in laboring compared to non-laboring women (Romero et al., 2006). However, few genomic studies have focused on the role of the human placenta in labor. A recent microarray study on placental tissue collected from five laboring and five non-laboring women, using only one array and indirect design, showed that most placental genes were not differentially expressed in labor and that a slightly (mean ratio=2.71) increased expression of matrix metalloproteinase-1 (MMP-1) was seen in laboring women (Vu et al., 2008). Another study using two 15K, custom-made human cDNA arrays on placental samples from seven women undergoing labor longer than 15 hours and 10 samples from women undergoing elective cesarean section, showed 90 transcripts to be significantly altered in their expression levels by >1.35-fold. Many of the placental genes up-regulated in women with prolonged labor were involved in oxidative stress (Cindrova-Davies et al., 2007). In our study, Placental FOS was the only gene that was down-regulated (2.2-fold) in women who delivered vaginally following normal (not prolonged) labor. Interestingly, in the study by Cindrova-Davies et al. (2007) placental FOS was 4.96-fold up-regulated in women with prolonged labor but the confidence interval of the C_T values in RT-PCR were wide [6.72 (5.03-7.42) in non-laboring and 4.70 (2.96-6.93) in women after long labor]. However, ingenuity pathway analysis showed that FOS might play a role in the regulatory relationships among differentially expressed transcripts during labor.

Results of our study indicate that placental gene expression profile is not significantly altered by normal labor. The discrepancies between our study and other studies (Cindrova-Davies et al., 2007; Vu et al., 2008) might be due to different sample size, experimental design and image and data analysis criteria. We have used a larger sample size, direct experimental design and more stringent image and data analysis criteria compared with

previous studies. Uterine contractions cause intermittent reduction of blood flow to the uterus that might result in oxidative stress during prolonged labor. In our study, however, there were no significant hemodynamic differences in placental circulation. Moreover, the fetal oxygen uptake is not altered by normal labor (Acharya and Sitras, 2009). Our results support the assumption that the fetal genome and the maternal tissues play a key role in parturition, rather than the placenta (Challis et al., 2000a; Young, 2007). Nevertheless, it would be wise to balance between laboring and non-laboring women while matching subjects in comparative placental genomic studies.

5.3 Placental gene expression in pregnancy disorders

There is clinical, epidemiological, genetic and histopathologic evidence that abnormal placentation plays an important role in the pathogenesis of various pregnancy disorders, such as preeclampsia, IUGR, preterm birth and miscarriage (Roberts and Hubel, 2008). The results of this thesis provide evidence, at the transcript and protein level, that placental gene expression profile is altered in pregnancies complicated by severe preeclampsia and IUGR due to placental insufficiency. Our results are in concordance with previous microarray studies that consistently showed differential placental gene expression in preeclampsia (Founds et al., 2008; Enquobahrie et al., 2008) and IUGR (Roh et al., 2005; McMinn et al., 2006), and we have made a comprehensive list of differentially expressed genes available (Paper II, Table 1S).

A set of 16 genes was found to predict preeclampsia phenotype in the study samples and a set of 20 genes to separate IUGR from normal placentas. Some of these genes in combination with other biochemical and/or ultrasound markers (e.g. uterine artery Doppler, flow-mediated vasodilatation of brachial artery) might help in early identification of pregnancies at risk. In fact, some studies have already shown that the combined use of high maternal serum markers (e.g. hCG, sFlt1) and presence of notching in the uterine artery wave form in mid trimester could predict preeclampsia and/or IUGR better (Hershkovitz et al., 2005; Barkehall-Thomas et al., 2005; Stepan et al., 2007).

Pathway analysis of differentially expressed genes showed angiogenesis, oxidative stress, Notch and Alzheimer disease signaling pathways to be involved in severe preeclampsia. Moreover, severe preeclampsia appeared to affect placental gene expression in different ways depending on the time of disease onset, with inflammation mediated by cytokines and chemokines pathway involved in early-onset disease. Interestingly, the same inflammatory pathway seems to be up-regulated in IUGR as well, suggesting common

pathogenesis. Disturbance of placental development might cause preeclampsia phenotype in the mother and alter fetal programming (Myatt, 2006; Ness and Sibai, 2006).

Investigation on how differentially expressed placental genes link together in the biological context, highlighted possible synergistic effects among hypoxia, oxidative stress, inflammation, apoptosis and folate metabolism in our study population. 6-pyruvoyltetrahydropterin synthase (PTS), a key enzyme in the synthesis of tetrahydrobiopterin (H4B) and an essential cofactor of NO synthase, was significantly down-regulated in preeclamptic placentas. Placental H4B regulates NO synthase activity in late pregnancy (Kukor et al., 2000) possibly through stabilization by vitamin C (Toth et al., 2002). Furthermore, H4B, folic acid, vitamin C and reactive oxygen species are known to interact in order to maintain adequate endothelial function (Figure 12) and thus unbalanced regulation might cause vascular diseases (Das, 2003).

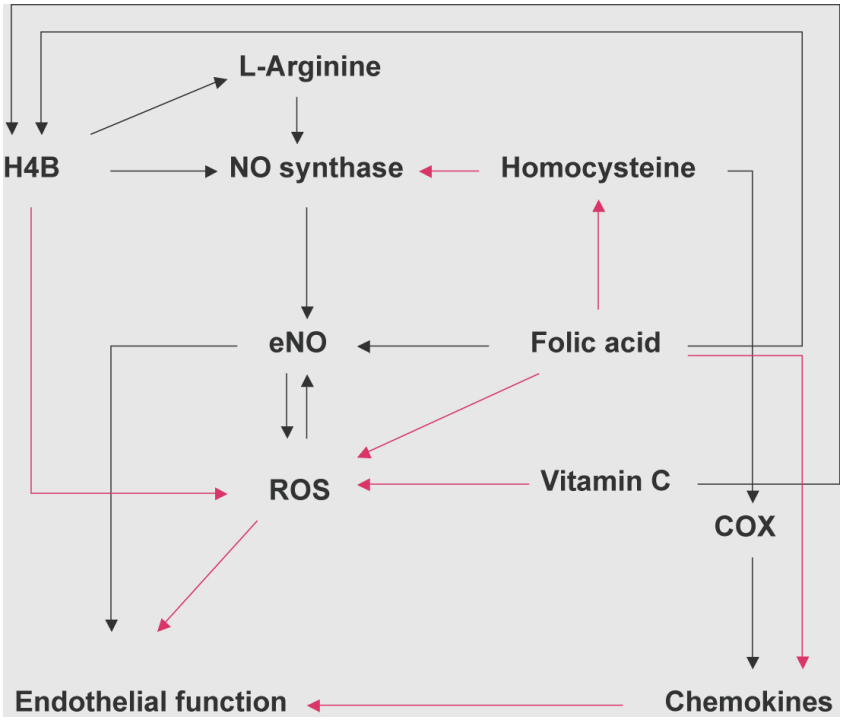

Figure 12. Schematic representation of interaction between chemokines, vitamins, reactive oxygen species (ROS), nitric oxide (NO) and cyclooxygenases (COX) affecting endothelial function. Red arrows indicate negative and black arrows indicate positive effect

Taken together these findings suggest that vitamins such as H4B, folic acid and vitamin C might have a role in the pathogenesis of preeclampsia. Indeed, there are ongoing clinical trials investigating the effect of vitamin and oxygen supplementation in the prevention of pregnancy disorders (Holmes et al., 2004; Rumbold et al., 2006). Although, several clinical

trials have so far failed to prove a clear prophylactic and/or therapeutic effect of antioxidant therapy (Kontic-Vucinic et al., 2008), it must be underlined that these trials had different designs regarding participants, intervention schemes and outcomes.

5.4 Placental angiogenesis in healthy and compromised pregnancies

In the course of pregnancy there is a continuous regulation of placental villous vascular development. This process is not completely understood but it is thought to be controlled by the expression of several angiogenic growth factors, which is influenced by genetic, endocrine, metabolic and environmental factors (Charnock-Jones et al., 2004). Oxygen tension in the intervillous space is a major factor affecting the expression of these angiogenic factors, and hence, placental vasculo- and angio-genesis (Kaufmann et al., 2004). In fact, conditions causing fetal hypoxia might be regulated by placental angiogenic factors, including their receptors and antagonists (Mayhew et al., 2004). In our study population, the utero-placental blood flow was reduced in preeclamptic pregnancies. This was reflected in placental gene expression profile with up-regulation of genes involved in angiogenesis, TGF-beta and VEGF signaling pathways in preeclamptic placentas. Recent studies suggest that an excess of placentally-derived anti-angiogenic factors (i.e. sFlt1 and ENG) in the maternal circulation might cause preeclampsia (Tjoa et al., 2007). Levels of VEGF and its receptors (VEGFR1 and 2) and PlGF are reported to be higher, lower or unaltered in late-onset preeclampsia and IUGR with positive end-diastolic flow in the umbilical arteries (Helske et al., 2001; Tse et al., 2001). However, less evidence exists for the involvement of angiogenic factors in early-onset preeclampsia and IUGR with absent end-diastolic flow in the umbilical arteries (Khaliq et al., 1999; Mayhew et al., 2004). Results of our studies suggest that the interpretation of the "angiogenic state" of the human placenta during preeclampsia and /or IUGR should be closely correlated to the phenotype of the disease, i.e. severity, time-onset and degree of hemodynamic compromise.

5.5 Inflammatory pathways in early-onset preeclampsia and IUGR

The placental genes involved in the inflammation mediated by cytokines and chemokines pathway were up-regulated in early-onset preeclampsia and IUGR in our study population. Cytokines include the interleukins, interferons and tumour necrosis factors (TNF) and have both pro- and anti-inflammatory actions (Jason et al., 2001). They play pivotal role in intercellular communication, especially between B- and T-cells, and affect neuronal, haematopoietic and embryonal development (Townsend and McKenzie, 2000). Chemokines,

called also chemotactic cytokines, comprise a large family of small (6-14 kDa) proteins. They mediate their effects through binding with trans-membrane receptors on different leukocyte lineages with activation of G-protein signaling pathways (Mellado et al., 2001). The final effect is regulation of chemotaxis of immune cells and regulation of gene expression and apoptosis (Vlahakis et al., 2002).

Maternal endothelial dysfunction during preeclampsia is linked to a generalized maternal inflammatory response (Redman et al., 1999). A pathophysiological model of preeclampsia where placental factors cause the clinical syndrome has been suggested (Redman and Sargent, 2005). Interactions among T-cells and placental cytokines and chemokines may alter maternal immune response and cause preeclampsia/IUGR (Visser et al., 2007). Results of our study show that the inflammation mediated by cytokines and chemokines pathway is altered in early-onset preeclampsia and IUGR, although it was not possible to match the gestational age of our patients with their controls. As normal pregnancy requires immune adaptation in the feto-placental interface, cytokine levels might change continuously throughout gestation, the validity of these results may be questioned. Nonetheless, cytokine-receptor microarray on placental samples (Pang and Xing, 2003) and serum cytokine (LEP, IL-10, TNF, IL-6 and IL-8) assay (Sharma et al., 2007) have also shown over-expression of inflammatory markers in preeclamptic women compared with gestational age matched healthy controls. Moreover, a microarray study of placental tissue obtained from two pregnancies complicated by early-onset preeclamptia with IUGR compared to a gestational age matched control placenta, showed up-regulation of inflammatory genes, many of which decode cytokines, in the absence of histopathologic inflammatory findings (Heikkila et al., 2005).

5.6 Limitations and strengths of the studies

Several approaches were used in order to optimize our experiments. We used direct comparison design which is more precise than the indirect design because the replicates available for comparison are doubled in number. Furthermore, it avoids the technical variation between the microarray slides caused by the instability of the common reference.

Relatively large number (n=50) of “healthy” placentas (biological replicates) from women with uncomplicated pregnancies was collected in order to match them randomly with compromised placentas. Randomization is a crucial step in experimental design, performance and interpretation as it reduces both technical and biological variation in microarray experiments. Indeed, in an ideal experiment the association of measured variables is

true/causative, when randomization ensures that exposed and unexposed experimental units are interchangeable (Hernan and Robins, 2006). By randomizing, we were able to balance the possible confounding effects of maternal age, parity, fetal sex and delivery method between the study groups.

The quality of mRNA was checked by electrophoresis and in study II 5 placentas that yield mRNA of poor quality even after repeated isolation were discarded. Poor mRNA quality may be due to apoptotic process that is shown to occur in the preeclamptic placenta (Allaire et al., 2000).

Microarrays are able to investigate gene expression only at a transcriptional level, i.e. measure the mRNA levels of a given gene at a given time. This has several drawbacks: (i) a single gene can give rise to multiple gene products (ii) RNA can be alternatively spliced or edited to form mature mRNA (iii) mRNA levels do not necessarily correlate with protein levels, which are the functional endpoints of coding genes (iv) proteins are regulated by additional mechanisms such as posttranslational modifications, compartmentalization and proteolysis (v) protein to protein interactions (folding) or cellular environmental conditions, such as pH and temperature, might affect protein function. Finally, biological function is determined by the complexity of these processes. All these factors are related to the functional, dynamic complexity of biological systems (such as cells, organs and organisms) which cannot be captured by microarrays.

On the other hand, microarrays are a powerful tool of investigating genome-wide expression profile, providing information not otherwise attainable. Traditionally, biomedical research has been based on "reductionism" in the sense that complex biological systems were divided in simple units that were easier to explore and understand. However, as disease probably affects several molecular mechanisms, one can hypothesize that in certain diseases sets of genes are affected rather than a single gene. With the help of bioinformatic tools that analyze the relations among genes forming multiple molecular pathways, microarray technology provides insights into the mechanistic molecular processes occurring in the diseased cell/tissue as a whole. Another important related issue is the intercellular signaling, i.e. the interactions between the different cells in a tissue. These behavioral interactions between cells are difficult to explore in *in vitro* studies, making microarray comparison among diseased and healthy tissues a method that can point out the biological pathways involved in the development of the disease state. Indeed, whole genome expression profiles have been successfully applied in cancer research aiming to classify different cancer subtypes on the basis of their differential genetic signature. Pathologically indistinguishable cancer

types with different global gene expression profiles, may share common “genomic-related” response to therapy, individual metastasizing capabilities and different prognoses (Segal et al., 2005). Moreover, signatures of differentially expressed sets of genes have been reproduced by several microarray platforms and laboratories, and validated in large patient populations using RT-PCR, indicating that results from microarray experiments can provide clinicians with prognostic and therapeutic tools.

6. CONCLUSIONS

Genome-wide expression profiling of the placenta is a powerful tool to detect differences in gene expression between healthy and compromised pregnancies. From the results of this thesis, the following conclusions can be drawn:

a. Placental gene expression is not significantly altered by normal labor, indicating a secondary role of this organ in the initiation and progression of normal parturition.
b. Placental gene expression is profoundly altered in severe preeclampsia, suggesting that the placenta plays a central role in the pathogenesis of this disease.
c. Placental angiogenic factors – including their receptors and antagonists – are involved in the pathogenesis of preeclampsia. However, the expression levels of placental angiogenic factors alone cannot clearly differentiate early- from late-onset preeclampsia.
d. There is no epigenetic modification of imprinted placental genes in placental insufficiency leading to IUGR.
e. Inflammation mediated by cytokines and chemokines signaling pathway in the placenta might be involved in the development of early-onset preeclampsia and IUGR.

7. FUTURE PERSPECTIVES

Early-onset preeclampsia and IUGR due to placental insufficiency might share common placental pathogenesis. Preeclampsia and IUGR frequently lead to preterm delivery and are associated with poor perinatal outcome. Therefore, early identification of women at high risk of developing these complications might improve pregnancy outcome. A set of placental genes/proteins detected in the maternal blood and/or urine together with the assessment of maternal, fetal and placental hemodynamics might help clinicians in the prediction and early diagnosis of these pregnancy-specific disorders. In this respect, laeverin, a placenta specific protein that was found to be over-expressed in severe preeclampsia in our study population, deserves further investigation. In particular, it would be interesting to further explore the downstream effects of this gene on placental development and function.

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