The placenta as modulator of fetal prosperity
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Discussion
Most obstetricians and paediatricians would agree that examination of the placenta can often explain abnormal pregnancy outcome. The placenta clearly is a pluripotent organ and a modulator of key processes supporting fetal prosperity. This thesis deals with the relation of placental characteristics to maternal and fetal health and disease. The spectrum of characteristics that is addressed ranges from simple weight to mRNA expression profiles.

**Placental Weight and Birth Weight**

Like birth weight, placental weight is also a proxy for health later in life. An association of placental weight to subsequent childhood growth and later health has indeed been reported. As discussed in chapter 1 of this thesis, Placental Ratio (PR) is the classical measure of placental function, defined as placental weight divided by birth weight. A low PR generally explains growth restriction, but it turns out not to be an accurate marker of fetal growth, since in multivariate analysis it is not an independent risk factor in combination with race, fetal sex, gestational age, maternal body mass index, socioeconomic status, anaemia, and smoking. Moreover, FGR is also found with normal and high PRs.

We used the cohort of pregnancies described in Chapter 5 to assess the relation of PR with gestational age. As these are cross sectional data one has to keep in mind that data are influenced by clinical decision-making in the timing of deliveries. In most cases interventions were done in the last stages of fetal compromise as a consequence of extreme placental insufficiency. Interestingly, there is no relation between gestational age and PR, and the PR values are rather uniformly lower than expected and in the range for placentas at term, with a median value of 0.15. This suggests that clinical decisions led to delivery of the fetuses at a comparable end stage of their pregnancy with an ill-functioning placenta.

![Figure 1: Placental Ratio (placental weight divided by fetal weight) as a function of gestational age at birth in the cohort described in Chapter 5. There is no significant linear trend.](image)
Since we argued in chapter 3 that BWR is superior to centiles in the classification of birth weight, it is an obvious choice to classify abnormal placental weight with a Placental Weight Ratio (PWR). PWR is then defined as observed placental weight divided by expected placental weight. Figure 2 shows the same placenta data as in Figure 1, expressed as PWR.

This example illustrates, given the significant linear trend, that placentas with a higher relative weight are more able to sustain these complicated pregnancies. This supports Kloosterman’s suggested positive relation between placental weight and duration of pregnancy. Further research is needed to demonstrate whether PWR is associated to perinatal outcome measures.7

Concluding, the PWR might, more than the PR, add to our understanding of placental function in compromised fetal growth. Normal PRs do not exclude FGR. We advise to use the PWR in future perinatal studies instead of centile thresholds.

In clinical obstetrics we need antenatal estimation of placental and fetal weight as this will probably help evaluating fetal prognosis and guide antenatal intervention. Estimated Fetal weight Ratio (EFWR) and Estimated Placental Weight Ratio (EPWR) then are relevant measures according to our former arguments. At present, however, there are no clinimetric methods to determine fetal weight and placental weight reliably enough to use as a parameter to predict neonatal outcome and guide timing of intervention. Placental weight can be approximated by 3D measurement of its volume, using ultrasound8-10 but the reproducibility is low.11 At term this heterogeneity is mainly due to a variable amount of circulating maternal blood.12 Probably, MRI might be a method to calculate the second trimester non-vascular
placental volume, the measurement of placental perfusion by echo planar imaging is also a way to evaluate early placental function. These technical innovations however are not generally available. There are other diagnostic tests to approximate placental function antenatally. Ultrasound Doppler Pulsatility index is used as a parameter of placental function and as an early marker to predict PE. Predictive performance of this single parameter in isolation is not enough to rigorously apply this diagnostic test in clinical practice. Therefore we agree with Romero that prediction on the basis of a single determinant will be inferior to a combination of markers. We advise to develop predictive models based on a combination of biochemical and biophysical determinants to predict the risk for developing PE and guide clinical research and practice.

Thyroid Hormones

In the second part of this thesis thyroid hormones are studied, firstly in relation to glucocorticoids in severely ill preterm neonates, secondly in relation to disease, in preeclamptic mothers and their neonates. Since optimal thyroid hormone levels are essential for brain development in fetal and early postnatal life, it is warranted to evaluate the effects of glucocorticoids on thyroid function in every developmental stage specifically. Results from studies on thyroid hormone effects of dexamethasone administered to preterm infants illustrate that hormonal interactions depend on developmental stage and on clinical condition. Antenatally administered glucocorticoids are associated with increased plasma T₃ and rT₃ in the fetal sheep. We found a different pattern: decreased plasma T₃ and increased rT₃ in severely ill preterm neonates.

Interactions between glucocorticoids and thyroid hormones are even more complex if studied in different tissues. In rat pups, intracellular T₃ may be increased in liver and decreased in brain as result of dexamethasone administration, but an opposite effect can be seen before birth. We made clear that gestational hypertensive disorders also have an effect on thyroid function, particularly in the neonate. The clinical relevance of these findings is not yet clear, since due to the design of our study we were not able to study longitudinal thyroid hormone effects in the neonates. If these low fT₄ values last extensively in fetal life or if they persist after birth, they can have a negative impact on brain maturation. Long term follow up of the children might elucidate the relevance of these findings. In our cohort we were not able to replicate findings of other researchers who suggested that thyroid dysfunction is one of the causes of PE. The incidence of maternal thyroid dysfunction in our cohort of pregnant women

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with PE was 2.5%, about equal to the 2% in the normal population. Our data are also of interest to the discussion on screening on thyroid dysfunction in pregnancy. The data show that maternal hypertensive disease is associated with higher TSH values, and underline that general health of the mother must be taken into account when interpreting screening results.

In conclusion, it appears that infants with FGR are prone to have low thyroid hormone levels, both antenatally and postnatally. It is possible that these low levels are an epiphenomenon of their severely compromised condition, but they may be an independent risk factor for abnormal neurodevelopmental outcome. The ultimate aim of future research should be enough knowledge to decide whether antenatal glucocorticoids should be accompanied by thyroid hormone to ensure proper neurodevelopment.

Gene Expression

The third main topic of this thesis concerns the molecular basis of placental physiology and pathophysiology. Sometimes, PE and HELLP syndrome run in families. Analyses of genome wide scans have revealed several chromosomal susceptibility loci for PE and resulted in the association of the STOX1 Y153H variation with familial PE. The role of STOX1 in PE is not clear since the initial results could not be confirmed by independent studies and the maternal imprinting and monoallelic expression of the gene that was essential for the disease mechanism in fact does not occur. Moreover, since most cases of PE are non-familial and do not follow classical Mendelian inheritance, the overall significance of these data is limited. Despite intensive research focusing on the pathways known to be involved in PE and HELLP, no mutations or polymorphisms have been identified in any factor known to be involved in these pathways except an extremely rare mutation of LCHAD affecting fetal mitochondrial fatty acid oxidation, as described in the Introduction. The pathways that in general explain the molecular basis of PE or HELLP syndrome include impaired trophoblast invasion leading to defective placentation, placental oxidative stress and systemic endothelial activation leading to a generalized inflammatory response. Figure 3 illustrates a diversity of markers assessed in relation to PE. At best, the analysis of markers only show a weak association with PE, in accordance with the weak predisposition of pre-existing vascular disease or thrombophilia disorders described in the Introduction. There are two main reasons to explain this failure to substantiate an association. Firstly, PE is a clinically heterogeneous disorder, making the characterization of the phenotype of extreme importance.
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Activated Protein C$^{41}$ Angiotensin Converting Enzyme$^{42}$ Angiotensinogen$^{43}$
Angiotensinogen receptor$^{44}$ Annexin$^{55}$ Apolipoproteine E$^{46}$
C-Reactive Protein$^{47}$ Calcitonin$^{48}$ Catalase$^{49}$ Cystathionine b-Synthase$^{50}$
Endoglin$^{51}$ Epoxide Hydrolase$^{52}$ Estrogen Receptor alpha$^{53}$ Factor$^{86}$ Factor$^{36}$
Factor V Leiden$^{57}$ Glycogen Phosphorylase$^{58}$ Glycoprotein IIIa$^{59}$ Glutathione peroxidase$^{49}$ Glutathione S transferase alpha$^{38}$
Human Leukocyte Antigen DR$^{60}$ Human Leukocyte Antigen G$^{61}$ $eta$-Hydroxysteroid Dehydrogenase type 2$^{64}$ Hydroxyprostaglandin hydrogenase$^{65}$
Hypoxia Inducible Factor$^{166}$ Insulin like Growth Factor$^{166}$ Insulin like Growth Factor Receptor$^{67}$ Integrin A$^{68}$ Intercellular Adhesion Molecule$^{1}$
Intercellular Adhesion Molecule$^{270}$ Interleukin$^{18}$ Interleukin-2$^{73}$
Interleukin-2 Receptor$^{73}$ Interleukin-4$^{74}$
Interleukin-6$^{62}$ Interleukin-10$^{75}$ Jun aminoterminal Kinase$^{76}$
Interleukin-1$^{77}$ Leptin$^{81}$
Lipid hydroperoxides$^{82}$ Lipoprotein(a)$^{83}$ Macrophage Colony Stimulating Factor$^{78,84}$ Malondialdehyde$^{82}$ Matrix Metalloproteinase$^{136}$
Matrix Metalloproteinase 2$^{266}$ Matrix Metalloproteinase 9$^{86,87}$ Matrix
Metalloproteinase 10$^{86}$ Matrix Metalloproteinase 12$^{89}$ Matrix Metalloproteinase 13$^{88}$
Matrix Metalloproteinase$^{158}$ Methylene Tetrahydrofolate Reductase$^{89}$ Neurokinin B$^{91}$ Neuropeptide Y$^{92}$ Nitric Oxide Synthase$^{84}$ P-selectin$^{78}$ Parathyroid Hormone Related Protein$^{90}$
Periostin$^{91}$ Placenta Growth Factor$^{91}$ Plasma membrane-associated pY397FAK$^{97}$ Plasminogen$^{91}$ Plasminogen Activator Inhibitor$^{95}$
Platelet Endothelial Cell Adhesion Molecule$^{49}$ Pregnancy associated protein A$^{96}$
Pro Atrial Natriuretic Factor$^{90}$ Prostacyclin Synthase$^{136}$ Prothrombin$^{101}$
Renin$^{102}$ sFLT, soluble version of VEGF receptor$^{103}$ Superoxide Dysmutase$^{49}$ Synctin$^{104}$ Thrombin activatable fibrinolysis inhibitor$^{105}$
Thrombomodulin$^{106}$ Thromboxane -A2 Synthase$^{100}$ Tissue Inhibitor of Metalloproteinase$^{98}$ Tissue Inhibitor of Metalloproteinase$^{23}$ Tissue
density lipoprotein$^{78}$ Tumor Necrosis Factor$^{106}$ Tumor Necrosis Factor$^{107}$
MMP-1$^{39}$ Transforming growth factor$^{107}$ Tumor Necrosis Factor$^{108}$
 Factor$^{71}$ Vascular Endothelial Growth Factor$^{109}$ Very Low von Willebrand factor$^{111}$

#### Figure 3: Markers investigated in relation to preeclampsia

Only a very clearly characterized phenotype will allow detailed analysis of contributing factors. Additionally, if the phenotype is not described in enough detail, comparison and generalization of studies will prove impossible.$^{112}$ Secondly, PE is a complex genetic disease in which the risk of disease is influenced by the contribution of multiple susceptibility genes together with environmental factors. For these complex diseases it is not likely that the pathophysiological mechanism will be elucidated by testing a single marker in a small cohort of patients. As an illustration, it took the analysis of 500,000 Single Nucleotide Polymorphisms in over 2,800 subjects to identify two additional susceptibility loci for SLE, also a complex genetic disease.$^{113}$ These reasons motivated us to take an alternative approach and use a non-selective high throughput technique in order to study multiple markers and disease mediators in conjunction. SAGE enabled us to analyze differential gene expression in multiple pathways, without bias or presuppositions.
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The use of SAGE may however entail some problems, since the immense amount of data generated requires a prudent bioinformatics approach as there are pitfalls to both the statistical analysis as well as interpretation of results. This applies especially to research with no highly specific a priori hypotheses, as described by Ioannidis. We think we have overcome both pitfalls thanks to our study design.

Our initial comparison of SAGE expression profiles uses only one affected placenta and compares it to one control. The resultant differential expression pattern will be a combination of disease related and interindividual differences. This characteristic of SAGE is sometimes overcome by pooling mRNAs of different patient samples, but this carries the risk of regression to the mean and losing statistical power. We opted for downstream analysis of multiple tissue samples to separate the interindividual changes from those related to disease.

By definition, the control placenta tissue for an early preeclamptic tissue should also be premature, as the placental expression profile does not only differ due to disease, but also due to gestational age, as shown in chapter 7. Additionally the mode of delivery is of importance since labour has distinguishable effects of placental gene expression patterns. Our index PE/HELLP patient was delivered by Caesarean section and we were in the most lucky possession of a placenta sample from a premature Caesarean delivery of a patient without any hypertensive disorder. These are rare, particularly in the total absence of signs of infection.

As input material, we used a full thickness non-infarcted cotyledon of trophoblast tissue obtained from the maternal side. Ideally, analysis should have been performed on the extravillous trophoblast as this is the cell type believed to be primarily affected in PE but harvesting these is not feasible in human research. Since the clinical presentation of PE evolves in a time frame of at least 10 weeks, by definition analysis of preeclamptic placental tissue will always be performed at a late clinical stage. Currently, in human PE research there is no way to overcome this problem and only detailed investigation of those pathways resulting in the aberrant expression profile will be able to segregate initial causal events from secondary phenomena. Our data indicate that HELLP syndrome is not just a severe variant of PE and illustrate why it is important to describe phenotypes in detail. This explains why cohort study results are probably confounded when patients with PE as well as HELLP are included. Additionally we show the strength of multigenic molecular profiles above the study of single factors. Also in our study there
is no single factor that fully correlates with the disease phenotype. Another strength of our study compared to for instance microarray analysis is the 72 differentially expressed tags that cannot be annotated to a specific gene. Twenty of these tags correspond to more than one gene. The other 52 leads to yet unassigned genes with relevance for placental function that might contribute to novel, disease specific signatures. Before being able to transform the placental expression signature to a profile suitable for early diagnosis during pregnancy it will be necessary to focus on genes expressed predominantly in placentia. This will allow the monitoring of placenta RNA levels in maternal serum in analogy to the studies of Lo.

Studies on increased circulating levels of soluble fms-like tyrosine kinase receptor-1 (sFlt-1) and soluble endoglin (sEng) in preeclamptic women have shown that they can significantly contribute to the early detection of PE. It is however generally accepted that a combination of markers will be more appropriate for diagnostic purposes.

Apart from discerning a disease specific molecular profile, the combining of our SAGE data with publicly available placenta SAGE libraries enabled the definition of gestational age specific expression profiles using K-means clustering. The sequence of important events in placental development and function through gestation; from cell growth and extra cellular matrix rearrangement in the first trimester to angiogenesis, prevention of oxidative stress and metabolism in the second and preparation for birth involving apoptosis was not reflected in the clustered expression profiles. Extensive up regulation of ribosomal proteins is the most striking feature in the first trimester. In our clustering we did not visualize specific gene expression patterns that can aid the functional annotation of NoMatch genes.

Concluding, we made clear that the complex interactions of genetic factors, from which some are yet unidentified, require assessment by novel techniques: a complex disease requires a complex explanatory model. The approach to a multifactor model is different from the classical disease aetiology model. In future molecular biological research for PE, we should take an approach appropriate for complex diseases. We need to expand our disease signature on solid knowledge, this implies we first replicate evidence of markers that are proposed to contribute to the early detection of PE. We need to build tissue banks with adequate numbers of each phenotype to validate results. The use of signatures will also support our general knowledge as they help to integrate complex interactions into a multifactor paradigm, which should be our research goal over the next few years.
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