The placenta as modulator of fetal prosperity

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THE PLACENTA AS MODULATOR

OF FETAL PROSPERITY

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Escrito nos raros intervalos de folga de uma carreira fatigante, este livro, que a princípio se resumia à história da Campanha de Canudos, perdeu toda a atualidade, remorada a sua publicação em virtude de causas que temos por escusado apontar.
Euclides da Cunha, *Os Sertões,* 1902

Geschreven in de weinige vrije uren van een drukbezet leven, heeft dit boek, dat aanvankelijk bedoeld was een geschiedenis van de Campagne van Canudos te zijn, alle actualiteit verloren nadat publicatie ervan werd vertraagd om redenen die wij het niet nodig achten te vermelden. (Vertaling door August Willemsen, 2001)

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List of abbreviations

11β-HSD-1	11β-hydroxysteroid dehydrogenase-1
11β-HSD-2	11β-hydroxysteroid dehydrogenase-2
BW	Birth weight
CP	Cerebral Palsy
CTG	Cardiotocography
D1	Iodothyronine Deiodinase type 1
D2	Iodothyronine Deiodinase type 2
D3	Iodothyronine Deiodinase type 3
ecNOS	Endothelial Nitric Oxide Synthase
EST	Expressed Sequence Tag
FGR	Fetal Growth Restriction
hCG	Human Chorionic Gonadotrofin
HELLP	Hemolysis, Elevated Liver enzymes and Low Platelet syndrome
HPA axis	Hypothalamic-Pituitary-Adrenal gland axis
HPT axis	Hypothalamic-Pituitary-Thyroid axis
HTM	Human Transcriptome Map
ISSHP	International Society for the Study of Hypertension in Pregnancy
LCHAD	Long Chain 3 Hydoxyacyl coA Dehydrogenase
PE	Preeclampsia
PR	Placental Ratio
PW	Placental weight
PWR	Placental Weight Ratio
sqPCR	Semi-quantitative Polymerase Chain Reaction
T_3	Triiodothyronine
T_4	Thyroxin
fT_4	Free Thyroxin
rT_3	Reverse-Triiodothyronine
TBG	Thyroxin Binding Globulin
TPOab	Thyroid Peroxidase Antibodies
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone

Chapter 1:

Introduction

Introduction, Part 1: Placental development and Function

Historical Perspective

This thesis provides evidence for the important role of the placenta during pregnancy. The importance of normal placental development for normal fetal development has not always been as evident as it nowadays seems. The history of knowledge of placental development and function reflects the expansion in knowledge of human biology in general, from mythical beliefs and classical views to anatomical, functional and molecular biological evidence based data. Reverence to the prominent role of the placenta for mankind is reflected in an ancient creation myth of Mande-speaking people of southern Mali[1]:

"Mangala tried to maintain this perfect creation, but chaos crept in; one of the male twins became ambitious and tried to escape from the egg. This chaotic character is called Pemba. He is a trickster figure who symbolizes the mischievousness of humans. Pemba's first trick was to steal a piece of the womb's placenta and throw it down. This action made the earth."

In contrast to these mythical beliefs, the Greek scientist Hippocrates in the fifth century B.C. strongly held to the view that the fate of the fetus was related to sickness of the mother and suffering due to delivery.[2]

Until the eighteenth century this paradigm predominated and scientists presumed that the placenta did not have a particular function. It was supposed to be the place where the mother's blood entered the umbilical cord to reach the fetus. In 1750 William Hunter was first to break this belief when he proposed that the function of the placenta is that of an exchange organ.[3] When he acquired the body of a pregnant woman who had died near term, he was able to carefully examine the pregnant womb, and inject coloured wax into the uterine and umbilical blood vessels (Figure1). This demonstrated the independence of the maternal and fetal circulation. His observations were first published in 1774 in "The anatomy of the Human Gravid Uterus." He held a fashionable obstetric practice in London and was appointed Physician-Extraordinary to Her Majesty, Charlotte of Mecklenburg-Strelitz, wife of King George III.[3] It took until the second half of the nineteenth century to prove the functional concept of the placenta as an exchange organ. This was done by Sir Joseph Barcroft (1872-1947), who measured fetal blood volume and placental blood flow as well as oxygen and carbon dioxide pressures across the placenta in pregnant sheep.[4] His interest in the influence of these parameters on fetal growth made him an experimental biologist far ahead of his time.In the 1970's a wealth of information on endocrine regulation of pregnancy was generated by G.C. Liggins, through his work on animal models of parturition,[5;6] hormonal influences of fetal lung maturation[7] and fetal adrenal function.[8] His work showed that the placenta is far more than

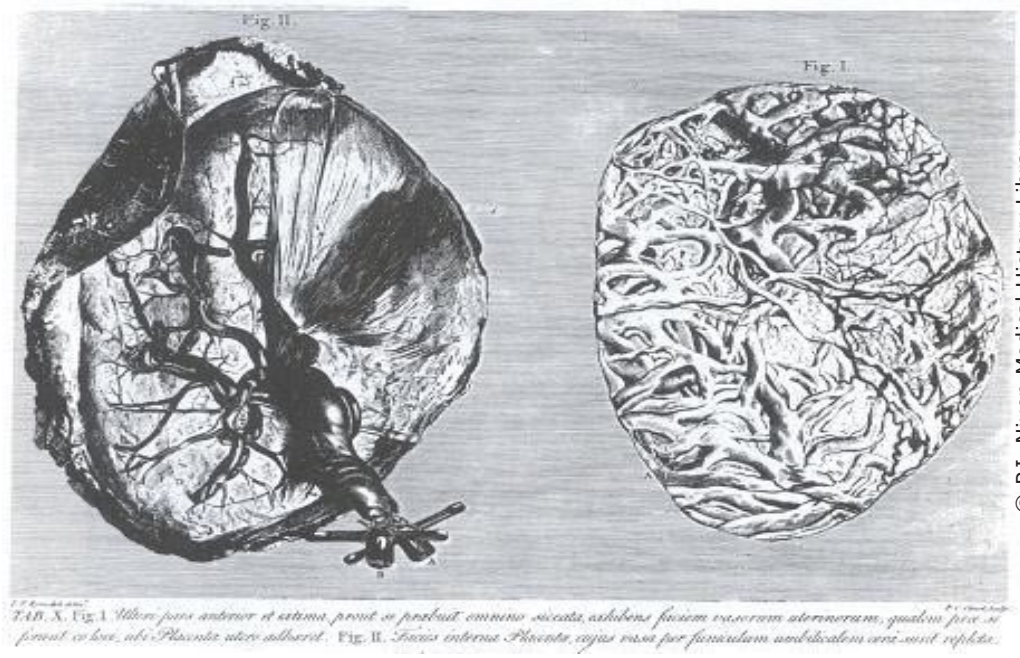

Figure 1: Figure I and II from plate X of "The anatomy of the Human Gravid Uterus" published by William Hunter in 1774, depicting the vasculature of the placenta. The maternal and fetal circulations are injected with coloured dye.
The legend states: TAB X.Fig I: *Uteri pars anterior et extima, prout se praebuit siccata, exhibens faciem vasorum uterinorum, qualem prae se ferunt eo loci, ubi Placenta utero adhaeret.* A view of the outside of the forepart of the womb, as it appeared when quite dry, exhibiting a specimen of the uterine vessels at the part where the placenta adhered. Fig II: *Facies interna Placentae, cujus vasa par funiculum umbilicalem cera sunt repleta.* The inside of the placenta, which was injected by the umbilical vessels.

an exchange organ. It boosted an enormous amount of research dedicated to the influence of the placenta on fetal development and maternal adaptation to pregnancy. Currently the placenta is seen as a pluripotent organ with active roles in remodelling of maternal physiology, maintenance of pregnancy and fetal homeostasis, hormonal control of fetal growth and development as well as regulator of key processes of maturation and parturition.[9]

Placental Structure

The human placenta is formed as early as 1-3 weeks post conception at the site of interaction of trophoblast cells and decidua.[10] Trophoblast cells form the main placental constituent and are unique to gestation. Histologically, we distinguish cytotrophoblast cells (mononucleate) and syncytiotrophoblast cells (multinucleate). Functionally, we specifically annotate those trophoblast cells that infiltrate through the endometrium into the maternal tissue (extravillous or intermediate trophoblast cells). All trophoblast cells stem from embryonal origin. Decidual cells that form the lining of the placenta and are in direct contact with the maternal myometrium are from maternal origin.

Subtypes according to zonation
In relation to the degree of zonation of placental tissue and formation of a single discrete organ within the uterus, placentas are classified into four major types (Figure 2).

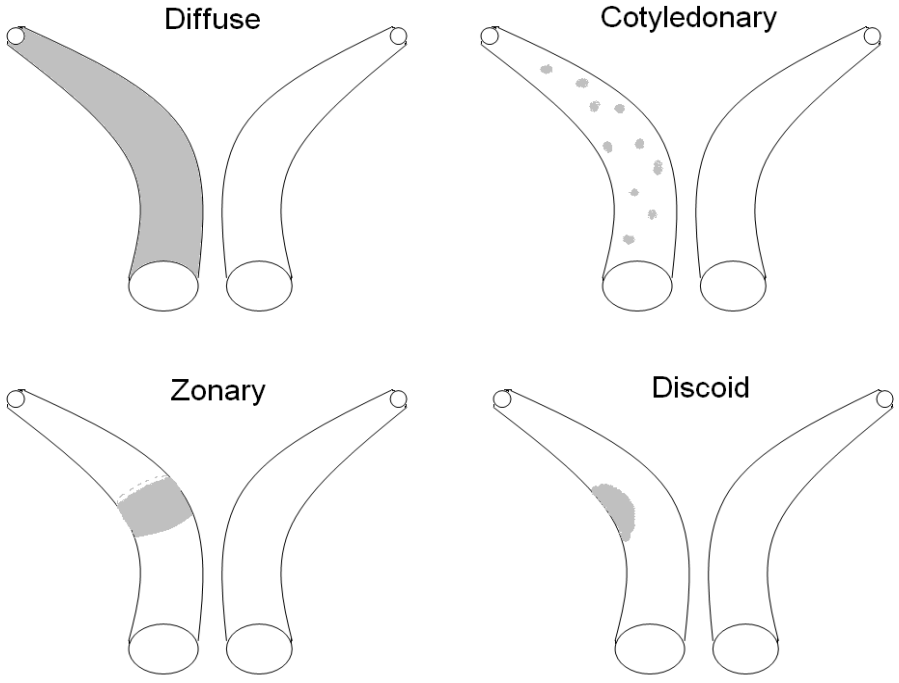

Figure 2: Placental subtypes according to zonation.
Depicted is a bicornate uterus, as this is the most common uterine form among mammals. Trophoblast cells, representing the predominant placental cell type are schematically represented by shaded areas.

In a diffuse type placenta, as in horse, pig, camel and whale, the placental trophoblast cells are distributed over the entire inner uterine surface. In cotyledonary placentas, specific for ruminants as sheep and cows, placental tissue is restricted to specific areas of the endometrium, called caruncles, that are dispersed over the whole inner uterine surface. On the interface of the endometrium and the fetal membranes the caruncles are covered by chorion, called cotyledon.

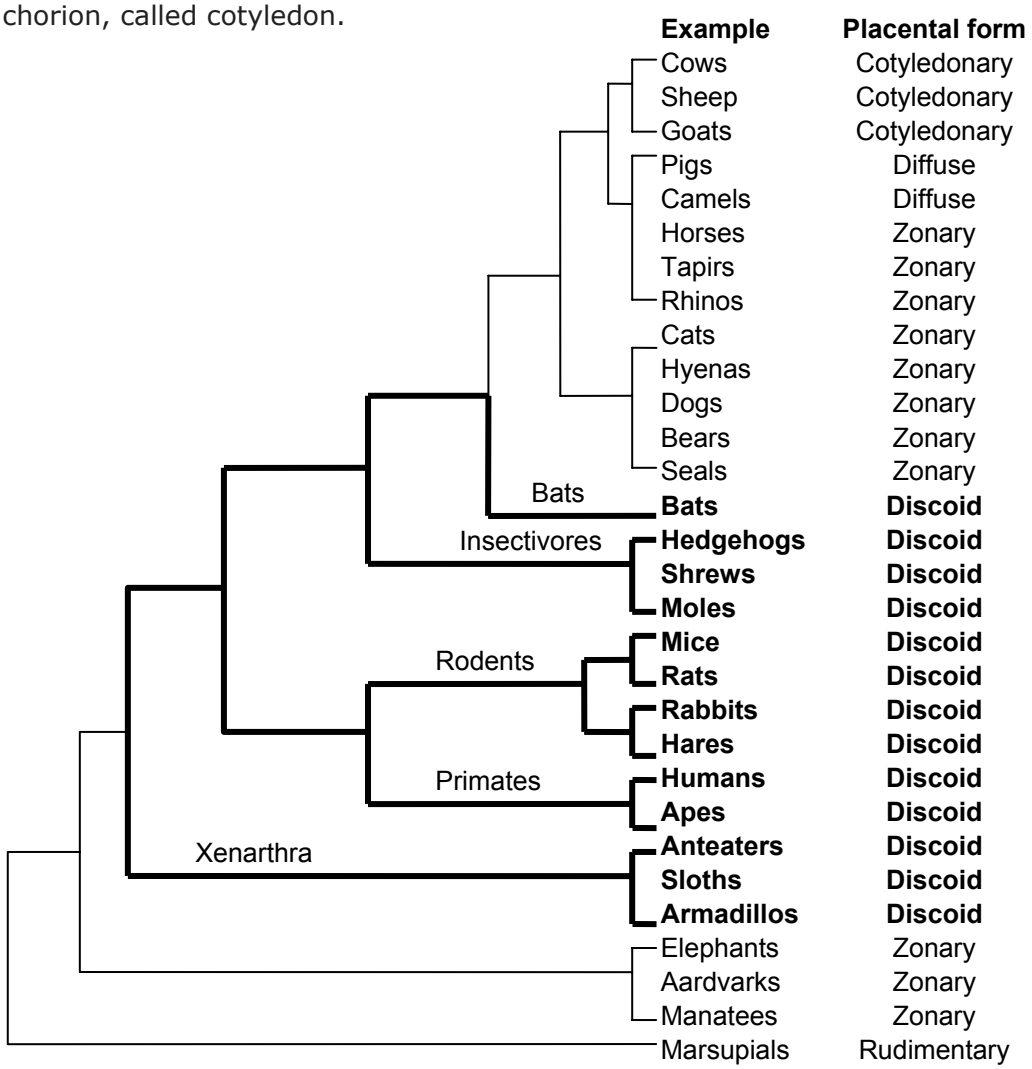

Figure 3: Placental forms of various species representing different taxonomic categories of the placenta-bearing mammalian phylogeny according to DNA homology.[11] Formation of the discoid placental form is seen in bats, insectivores, rodents, primates and Xenarthra. Xenarthra means "strange joints", as these animals' vertebral joints have extra articulations and are unlike those of any other mammals.

Zonary placentas show further concentration of placental trophoblast cells to form an equatorial band. The type with the most restricted zonation is the discoid type where there is a single plate. This is the human placental subtype. In Figure 3, taxonomic categories of the placenta-bearing mammalian phylogeny are arranged according to DNA homology.[11] The discoid placental form of humans is shared, amongst other species, by the other primates and by rodents.

Subtypes according to cellular structure

When placentas are categorized by cellular structure, they can either be epitheliochorial, endotheliochorial or hemochorial, as depicted in Figure 4. Across species there is no general concurrence between the degree of zonation (the different forms depicted in Figure 2) and the three placental cellular structures. In all species the placenta consists of both a maternal and a fetal component. In epitheliochorial placenta, as in sheep and the other ruminants, maternal and fetal blood are separated by maternal endothelium, maternal connective tissue, the endometrium, the trophoblast, fetal connective tissue and fetal endothelium (Figure 4, left panel). This implies shallow implantation, with an intact endometrium, as the placenta forms at the inner surface of the uterine wall. Sub-endometrial development is observed in the endotheliochorial placenta, found in carnivores. In these species, the endometrium is destroyed during placentation and maternal and fetal blood are separated by maternal endothelium, maternal connective tissue, trophoblast, fetal connective tissue and fetal endothelium.

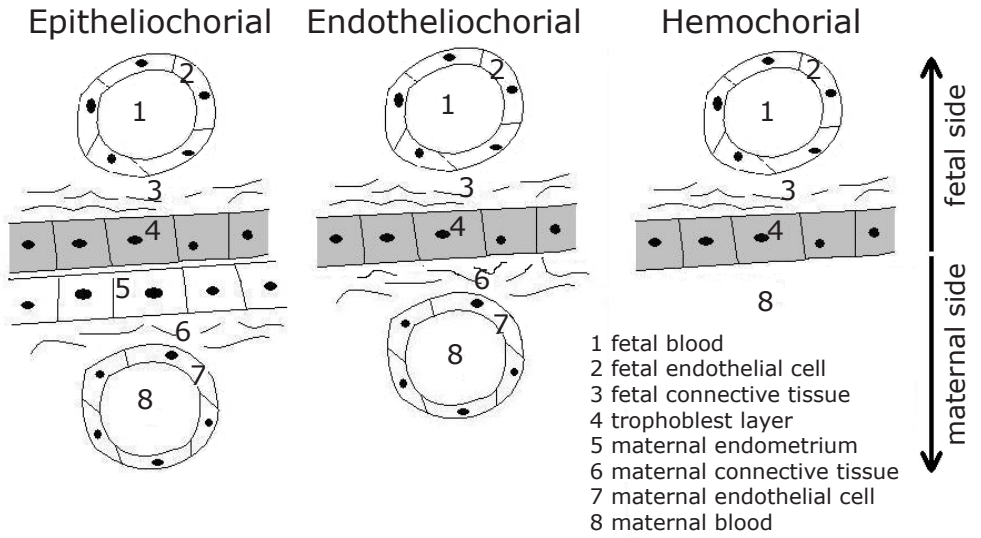

Figure 4: Schematic representation of placental cellular structure of three placental subtypes.

Based upon cellular structure, human as well as rodent placenta is characterized as hemochorial, where maternal blood is separated from fetal blood only by trophoblast cells, fetal connective tissue and fetal endothelium. This placental structure exhibits the greatest extent of uterine tissue lysis, with the maternal endometrium, connective tissue and maternal endothelium all being lost. Unique for this type is that maternal blood is in direct contact with trophoblast cells. Since trophoblast cells are from embryonic origin this implies very close contact between two genetically different systems.
While human placenta is characterized by a monolayer of syncytiotrophoblast cells, rodent placenta has three layers: two layers of syncytiotrophoblast cells and one cytotrophoblast cell layer. Additionally, rodents have a Choriovitelline (yolk sac) placenta, implying the yolk sac persists until term. Transfer of immunoglobulins via the yolk sac confers passive immunity to the fetus.[12;13]

In summary, human placenta is of the discoid hemochorial type, with the most extreme form of trophoblast zonation. Functionally the human placenta depends on the most extensive tissue replacement of the uterine wall, myometrium and maternal vascular endothelium as invasion of trophoblast cells into these structures in humans is far deeper than in most other mammalian species. This implies a high demand of maternal adaptation in the formation of a functional Fetal-Maternal-Placental Unit.

Placental development

Implantation and Invasion
Gestation starts with fertilization of an ovum. Seven to eight days post human conception the blastocyst nidates into the maternal endometrium.(Figure 5, top panel) Buds of cytotrophoblast cells invade into endometrium, myometrium and spiral-shaped maternal blood vessels, thereby widening these maternal vessels into trumpet-like forms (Figure 5, lower panels). From the 6th to 18th week, villi are formed, which are lined by syncytiotrophoblast cells as well as a continuous layer of cytotrophoblast cells. The cytotrophoblast cell layer functions as a stem cell pool, continually differentiating either into syncytiotrophoblast by fusion, or migrating as extra villous trophoblast. On the apex of villi, the cytotrophoblast cell layer is formed into cytotrophoblast cell columns. The process of invasion continues into the second trimester, and is complete at 18 weeks.[14]
The interstitial invasive trophoblast cells migrate and invade into the uterine tissue and anchor the placenta to the uterus. The syncytiotrophoblast vacuolizes and finally forms lacunae, ultimately to contain maternal blood. After extensive resorption of decidual epithelium, basement membrane and maternal vascular endothelium the maternal blood is able to circulate in

direct contact to syncytiotrophoblast. The function of cytotrophoblast cells, apart from entering endometrium and maternal blood vessels, is to form and sustain the placental villi after losing their invasive capacity by differentiation. The function of syncytiotrophoblast is covering the widening villous tree as exchange surface.

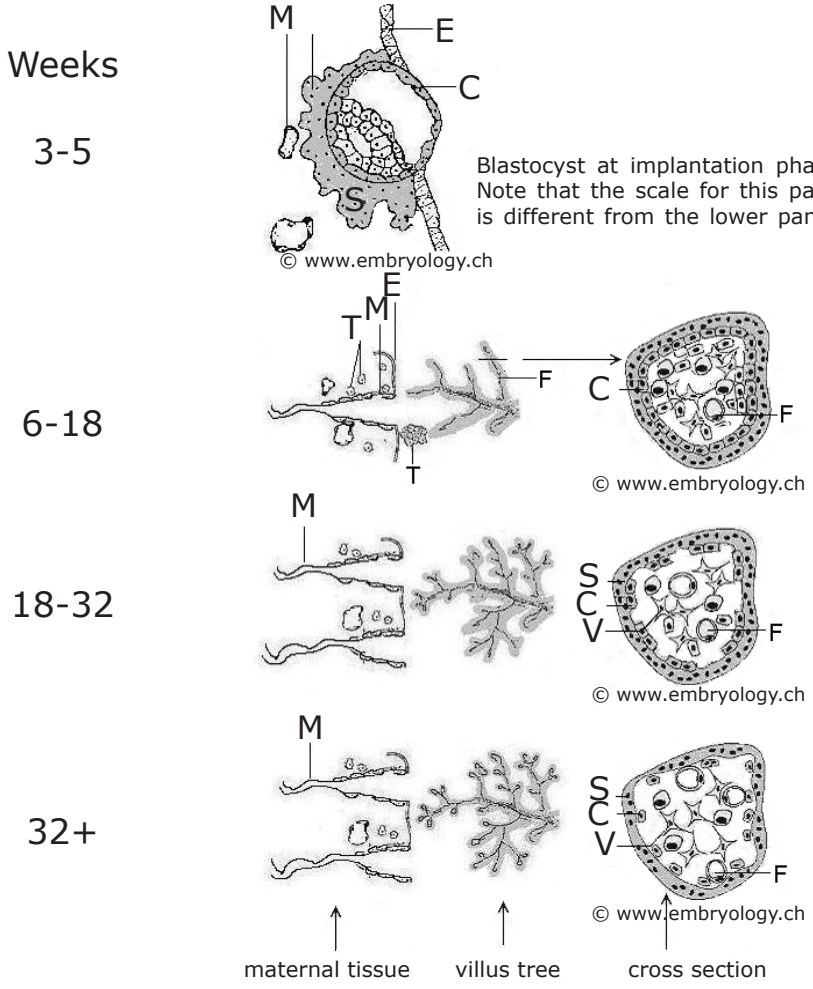

Figure 5: Functional developmental relations of placental cell types.
Upper panel: Blastocyst at implantation phase. Lower three panels: schematic representation maternal uterine tissue and trophoblast villus and its cross section at 6-18 weeks, at 18-32 weeks and at 32 weeks gestation until term. Grey shading represents trophoblast localization. C = cytotrophoblast cells, S = syncytiotrophoblast, T = extravillous trophoblast cells, M = maternal blood vessel, E = Maternal endometrium, F = fetal blood vessel, V = villous mesenchyme cell

The immature villi are covered by syncytiotrophoblast as well as cytotrophoblast cells until 18 weeks of gestation. The villous stroma is compact, composed of mesenchymal cells with centrally located fetal capillaries. After 18 weeks, mature placental villi are covered only by syncytiotrophoblast as the majority of the cytotrophoblast cell layer starts to disappear. Physiologic changes during the process of differentiation of placental cell types continues through the second trimester with division and elongation of villi,[15] proliferation and dilatation of capillaries.[16] Towards term, there is thinning of the trophoblast cell layer, predominantly by disappearance of most of the cytotrophoblast cells (Figure 5, bottom panel).[17] This results in a fall in resistance of the feto-placental vasculature which can be observed clinically by ultrasound Doppler.[18;19] This decrease in resistance is generally accepted as an indicator of normal placental development as it favours placental perfusion to comply with the increasing demands with advancing gestation.

Placental growth

There are several reports in literature on normal placental weight.[20-24] It has been established that placental weight increase accelerates after 12 weeks gestation and slows down again after 36 weeks.[20](see Figure 6)

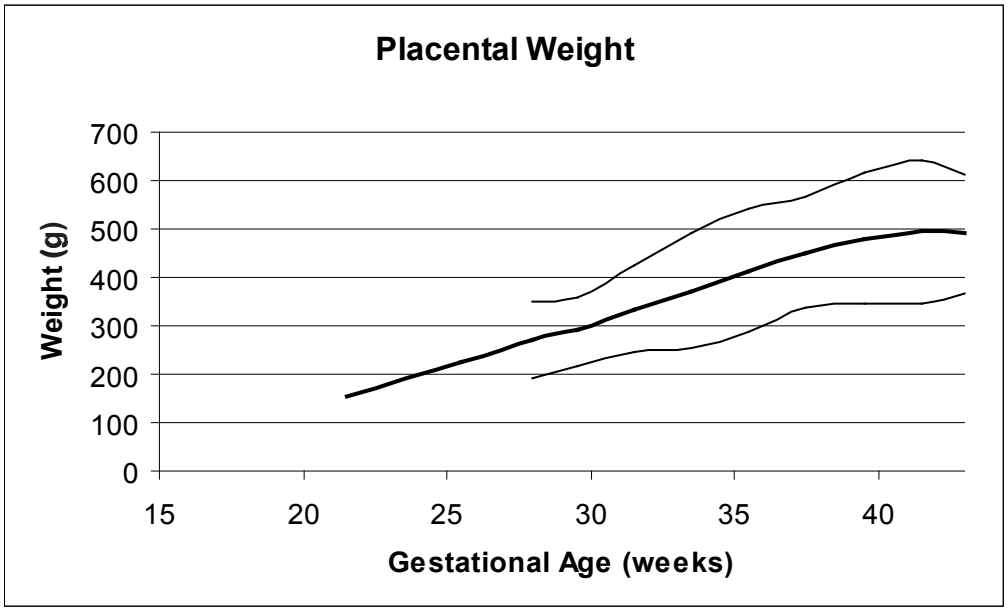

Figure 6: Relation between Placental weight (90th, 50th and 10th percentile) and gestational age. Figure composed of data 22 to 28 weeks[25] and 28 to 43 weeks[26] according to Schuler-Maloney.

Placental growth can be affected by all conditions that impinge on maternal supply and lead to hypoxia, hypoglycaemia or hypoproteinaemia. These conditions are anaemia,[23] living at high altitude,[27;28] maternal cardiac or pulmonary failure, malnutrition[29] and vascular pathology.[30] The most common uterine factor affecting placental growth is impaired uterine vascular adaptation[31-33] related to preeclampsia: this is discussed in Part 2 of the Introduction "The placenta as the basis of gestational and fetal disease". Other uterine factors affecting placental growth are uterine malformations, primigravidity[34], an unfavourable implantation site and impaired vascular supply. Other factors associated with impaired placental growth are placental mosaicisms in chromosomal disorders,[35;36] infection and infarction[37].
In general, impaired placental growth directly affects fetal growth. The only conditions where fetal growth is adversely affected without the concomitant decreased placental growth are umbilical cord anomalies, like abnormal insertion[38;39] and coiling.[40-42]

Although placenta and fetus develop under the same maternal influences, the Placental Ratio (PR), calculated as weight of the placenta divided by birth weight, has a non-linear relation with gestational age.[21] Fetal weight normally has a maximal absolute increase between 32 and 37 weeks of gestation, while the placenta gains most of its weight between 20 and 34 weeks. As the fetus grows relatively more rapidly than the placenta, the PR decreases with advancing gestation and normally amounts 15% of neonatal birth weight at term.[21] Population studies show racial differences in placental weight,[43;44] but the PR at term is 15% for all ethnic groups.[43]
The PR is not an accurate clinical marker of fetal growth restriction,[45] but is useful for functional comprehension and research purposes. A normal PR with a normal neonatal birth weight is a sign of adequate placental function. A normal or low PR in case of fetal growth restriction is typically found in case of placental dysfunction.[46] This does not imply that fetal growth restriction is always accompanied by a low PR. Interestingly, in smoking mothers[47] and mothers suffering from anaemia[34] the PR is high, in spite of fetal growth restriction. When maternal smoking is stopped in the first trimester there are high PRs with an even *normal* fetal weight.[48;49] Large placental size can reflect compensatory growth to support fetal growth in a hostile environment. In animal models a similar increased placental weight has been reported as a result of maternal undernutrition during placentation[50]. Generally, placental size is maximum when constraints to maternal nutrition are discontinued after placentation is completed,[51;52] suggesting a programming of placental size in the early phase of pregnancy. Human data from the 1944-1945 Dutch famine cohort[53] support this hypothesis. Birth weight of children born to mothers exposed to the famine solely in the 1st trimester of pregnancy was

normal, in spite of an increase in placental weight. A further finding in this cohort, that increased placental weight at birth is associated with an increased risk of hypertension in adult life is in line with two other observations from long term follow up studies.[54;55] Exposure to famine during pregnancy generally correlates with poor health, while the exact timing of the exposure determines which specific organ system is affected.[56]
In summary, both external and placental factors compromise fetal growth. The external and placental factors have opposite effects on placental development.

Functional Properties of Human Placenta

Transport and Metabolism

The most important placental function is to ensure maternal-fetal exchange of nutrients, gases and electrolytes.[57] The bulk of maternal-fetal exchange is via the placenta; other routes as transamniotic passage or transfer via amniotic fluid are unlikely to be of great importance. For most substances, the placenta is not much of a barrier: water and electrolytes, glucose, fatty acids, catecholamines, immunoglobulins and water-soluble vitamins readily cross the placenta. Exchange of oxygen and carbon dioxide is by diffusion. Amino acid transport across the placenta has all the characteristics of active transport, since it occurs against a high fetal-maternal gradient. The placenta is active in the metabolism of carbohydrates, fat and amino acids. This requires extensive placental oxygen consumption which has been shown to be as high as in liver or brain.[58]

Endocrine function of the Fetal-Maternal-Placental Unit

The remodelling and maintenance of homeostasis during pregnancy requires input of not only placenta but a concerted action between placenta, mother and fetus, commonly known as the Fetal-Maternal-Placental unit. This paragraph describes transport and metabolism of the major endocrine factors in the Fetal-Maternal-Placental unit.

In general, 97 – 99.9% of the total serum hormone pool is bound to carrier proteins[59] of which the levels change during the course of pregnancy.[60] For all steroid hormones as well as thyroid hormone metabolites it is the free hormonal concentration which determines the biological action, via their respective nuclear hormone receptors[61] and changes in the total hormone pool during pregnancy should always be considered in the light of changing levels of hormone binding proteins.

The Fetal-Maternal-Placental unit is capable of synthesizing and secreting a broad range of growth factors, protein- and steroid hormones many of which are also produced by other endocrine organs, pituitary and brain.[62] Due to the endocrine resemblance with the latter, the placenta has even been addressed as *the third brain during pregnancy*.[63] Table 1 summarizes the broad range of endocrine factors produced by the placenta.

Steroidogenesis in pregnancy is a good example of the tripartite efforts of the Fetal-Maternal-Placental unit. Maternal cholesterol is metabolized by placental desmolase and 3-β-Hydroxysteroid Dehydrogenase (3βHSD), daily yielding substantial amounts (250-350 mg) of progesterone. Most of this enters the maternal circulation, but in the fetal compartment this progesterone is the substrate for the production of androgens and estrogens. Progesterone is converted to androgens by the fetal P_{450}-17α-hydroxylase and 17,20-lyase,

for which the placenta itself is deficient. The fetal produced androgens are in turn aromatised to estrogens in the placenta.

Glucocorticoids

In extra-uterine life, the Hypothalamus-Pituitary-Adrenal gland axis (HPA axis) is an integrative part of the stress system, where the adrenal cortisol secretion is regulated by a negative feedback system and all hormones involved are produced by discrete organs. The human placenta is an important source of both CRH and ACTH that act in a positive feed-forward mechanism on fetal adrenal cortisol production, bypassing the hypothalamic part of the fetal hypothalamus-pituitary-adrenal gland axis. This mechanism is predominant in the 5 weeks preceding parturition.[64;65] This has led to the model of a placental clock, determining the duration of pregnancy. The increase in maternal plasma CRH, mainly due to the abundant production in placenta, saturates the CRH-binding proteins with a consequent rise in free CRH that acts as a parturition trigger.[66;67] Overall corticoids are of key importance for regulation of the fetal environment. Total maternal serum cortisol increases to 3 times pre-pregnancy levels at term.[68] The fetus is protected from high levels of maternal cortisol by the abundant presence of 11β-hydroxysteroid dehydrogenase-2 (11β-HSD-2) which oxidises cortisol to the bio-inactive metabolite cortisone.[69;70] The net increase of bioactive glucocorticoid metabolites with advancing gestation is an oestrogen dependent process. [71] The changing balance between the expression of the glucocorticoid inactivating enzyme11β-HSD-2 (that decreases) and the glucocorticoid activating enzyme 11β-hydroxysteroid dehydrogenase -1 (that increases) results in an increase of bioactive glucocorticoids. In contrast to hydrocortisone, dexamethasone and betamethasone are not degraded by 11ß-HSD-2. From a clinical perspective, this is of particular importance since

Table 1, opposite page: The shaded left side of the table describes what is known of placental expression, regulation and function. The non-shaded right side of the table describes the non-pregnant expression and regulation. The most striking discrepancies in regulation between the pregnant and the non pregnant state are indicated. CRH = Corticotropin Releasing Hormone; Prost = Prostaglandins; IL1α/β = Interleukin 1α/β; ACTH = Adrenocorticotropin; Cort = Cortisol; 11-β HSD1 = 11-β Hydroxysteroid Dehydrogenase 1; 11-β HSD2 = 11-β Hydroxysteroid Dehydrogenase 2; TRH = Thyrotropin Releasing Hormone; TSH = Thyroid Stimulating Hormone; BAT = brown adipose tissue; D_2 = Deiodinase 2; D_3 = Deiodinase 3; INH = Inhibin A/B; FSH = Follicle Stimulating Hormone; P = Progesterone; hCG = Human Chorionic Gonadotropin; 3βHSD = 3-β-Hydroxysteroid Dehydrogenase; GH = Growth Hormone; PRL = Prolactin; IGF1 = insulin-like growth factor 1 (somatomedin C); IGF2 = insulin-like growth factor 2 (somatomedin A); hPL = Somatomammotropin (Placental Lactogen) ; PAPPA = Pregnancy associated protein A

Table 1: Major endocrine factors in the Fetal-Maternal-Placental unit.

	During pregnancy		In General			
Factor produced in placenta	Function	Regulation through	Expressed in	Regulation through	Function	
CRH	Maturation	Prost, IL1α, IL1β	Hypothalamus	Cort	Stress response	Adrenal gland Axis
ACTH	Maturation	CRH	Pituitary	CRH, Cort	Stress response	
Cort	Maturation		Adrenal cortex	ACTH	Stress response	
11-β HSD1	-	Estrogen	Liver	Cort	Reduction of Cortisone	
11-β HSD2	Maturation	Estrogen	Liver	Cort	Oxidation of Cortisol	
TRH	Maturation		Hypothalamus		Release of TSH	Thyroidal Axis
TSH	-	TRH	Pituitary	TRH, T_4	Release of Thyroxin	
D_2	Increase of bioactive thyroid hormone metabolites	-	Brain, pituitary, muscle, BAT	T_4, rT_3	Increase of bioactive thyroid hormone metabolites	
D_3	Decrease of bioactive thyroid hormone metabolites	-	Brain, muscle, skin	T_4, T_3	Decrease of bioactive thyroid hormone metabolites	
INH	-	-	Ovarian follicle	FSH	-	Gonadal Axis
Aromatase	Converts fetal androgens to placental estrogens	-	-	-	-	
P	Substrate for fetal androgen production	hCG	Corpus Luteum	-	Maintenance luteal phase	
3βHSD	Cholesterol metabolism	-	-	-	-	
Desmolase	Cholesterol metabolism	-	-	-	-	
GH	Growth factor	GHRH	Pituitary	GHRH	Growth	Growth Hormone Axis
PRL	Growth factor	TRH	Pituitary	TRH	Lactation	
IGF1	Growth factor	GH	Various cell types	GH	mediates growth-promoting effects	
IGF2	Growth factor	GH	Various cell types	GH	mediates growth-promoting effects	
hCG	Growth factor	-	-	-	-	Pregnancy specific
hPL	Growth factor	-	-	-	-	
PAPPA	Metalloproteinase	-	-	-	-	

prophylactic use of antenatal glucocorticoids in the event of threatening preterm delivery for prevention of respiratory distress syndrome[72] has become one of the most undisputed and widespread perinatal interventions.[73;74] There is however a need for caution in administrating glucocorticoids, as it has been shown that in humans neurodevelopmental outcome is adversely affected by postnatal administration.[75;76] Also at repeated antenatal administration of glucocorticoids there is concern about adverse neurodevelopmental outcome[77] while evidence of longer-term benefits remains insufficient.[78;79]

Thyroid hormones

Thyroxin (T_4), the principal product of the thyroid gland, is produced under the classic negative feedback-controlled Hypothalamic-Pituitary-Thyroid axis (HPT axis). The hypothalamic Thyrotropin Releasing Hormone (TRH) stimulates the synthesis and release of Thyroid Stimulating Hormone (TSH), which in turn stimulates thyroid hormone secretion by the thyroid.

T_4 is metabolised by Iodothyronine Deiodinases type 1, 2 and 3, that specifically deiodinate the inner or outer ring. Iodothyronine Deiodinase type 1 (D1) and Iodothyronine Deiodinase type 2 (D2) can both deiodinate the outer ring producing bioactive T_3 and clearing the inactive metabolite rT_3. D1 is mainly expressed in liver, kidney and thyroid[80] where it is mainly responsible for maintenance of plasma levels of T_3. Additionally, D1 can deiodinate the inner ring. D2 is expressed in thyroid, muscle, brown adipose tissue, pituitary, brain and placenta,[81] where it is responsible mainly for local provision of T_3. Iodothyronine Deiodinase type 3(D3) only has inner ring deiodination capacity and is present in brain, muscle, skin, fetal liver, the pregnant uterus and in placenta[82;83], especially in syncytiotrophoblast and cytotrophoblast cells.[84] Inner ring deiodination lowers the concentration of bioactive T_3 by converting T_4 to rT_3[85] and T_3 to T_2.

The intracellular concentration of bioactive T_3 is the resultant of the tissue specific levels of D2 and D3, but also of transport of plasma T_4 and T_3 into the cell. Brain, and also liver highly express MCT8, a member of the monocarboxylate transporter family, which mainly transports T_3 into the cell.[86] Apart from uptake and deiodination, there are several other processes that influence the levels of bioactive thyroid hormone metabolites such as sulfation, glucuronidation, oxidative deamination, decarboxylation and ester link cleavage.[87] The bioactive thyroid hormone metabolite T_3 exerts its function through nuclear thyroid hormone receptors. At least three forms of thyroid hormone receptors (TRα1, TRβ1 and -β2) have thyroid hormone binding properties.[61] Interaction of thyroid hormone with the thyroid hormone receptor results in the regulation of transcription of thyroid hormone target genes. These genes encode proteins involved in both developmental as well as basic metabolic processes.

Optimum thyroid hormone levels are indispensable especially for development of the central nervous system[88] as they induce maturation of different cell types i.e. neurons, astrocytes, oligodendrocytes and microglia.[88] Thyroid hormones are essential for fetal development and are produced by the human fetus as early as 12 weeks.[79] We know from both animal and human studies that fetal coelomic fluid, amniotic fluid and brain tissue contain significant amounts of free T_4, before the onset of fetal thyroid hormone production.[89;90] This is presumed to be of importance since the α1, β1 and -β2 receptor[91] as well as iodothyronine deiodinases[83;92] are expressed in human brain in significant amounts as early as 7 to 8 weeks gestation. The only possible source of thyroid hormone for the fetus so early in gestation is the mother. Evidence that thyroid hormones are able to cross the placental barrier comes from the work of Vulsma et al[93] who showed that cord blood from fetuses who are unable to synthesize thyroid hormones, due to thyroid agenesis or a total iodide organification defect, contains substantial amounts of thyroid hormone. At term this is considered to be 30-40% of the fetal thyroid hormone pool. After 16 weeks, there is significant thyroid hormone secretion from the fetal thyroid, and serum T_4 levels increase and reach a plateau at 35 weeks.[94;95] At all times serum levels of bioactive thyroid hormone are lower in the fetus compared to the mother.[96]

In pregnant women, the function of TSH is mimicked by hCG that cross-reacts with the TSH receptor. Mirroring the hCG rise in the first trimester, TSH levels are relatively low in this period.[97;98] During pregnancy, the most prominent change in maternal thyroid function is a rise in mean total T_4 from 100 nmol/L to 140 nmol/L as a result of the oestrogen-induced TBG production by the liver.[97;99] Free T_3 only increases slightly, whereas free T_4 even shows some decrease.[100] Placental deiodination of T_4 to rT_3 by D_3 is a dynamic process and is considered the main regulator of the maternal-fetal T_4 transfer.[101] D3 activity per cell decreases during pregnancy, but due to placental growth total placental activity increases.[82;102] It is assumed that placental D3 activity prevents the untimely exposure of the developing embryo to excessive levels of thyroid hormone.[95] The intracellular trophoblast requirement for T_3 is supplied by D2 activity, which at all stages of pregnancy is 200-fold less than D3 activity.[82]

Since thyroid hormone provision to fetal cells is dependent on both the maternal and the fetal thyroid function, neurodevelopmental disabilities are most severe in combined maternal and fetal hypothyroidism. The most dramatic example is that of endemic cretinism where both mother and fetus are profoundly hypothyroid as a result of iodine deficiency.[103] Fetal thyroid hormone deficiency can cause neurodevelopmental defects, especially if this starts early in development before the onset of fetal thyroid hormone production.[96;104] Even mild maternal hypothyroxinemia during pregnancy is

associated with adverse neurodevelopmental outcome in the offspring.[105-107] As thyroid disease shows higher prevalence among women in child-bearing age, a current hot topic is whether thyroid hormone function should be evaluated during early pregnancy, aimed at achieving thyroid hormone levels in the high normal range, by treatment if necessary.[106;108;109] Some authors favour defining a pregnancy-specific TSH reference range by lowering the upper limit from 4.0 mU/L to 2.5 mU/L.[110] Since optimal timing and nature of screening is still not established, others advocate case finding of subclinical hypothyroidism in risk groups.[108] On the other side of the spectrum, also fetal hyperthyroidism is not without risk. It can result in permanent neurological and skeletal injury,[111;112] and long term neurodevelopmental studies of the children are needed to establish the benefit of treating pregnant women with thyroid hormone.

Influence of glucocorticoids on fetal and placental thyroid function
In vivo evidence of interaction of the HPT- and Hypothalamic-Pituitary-Adrenal gland axis (HPA axis) was established by the combined glucocorticoid and thyroid hormone treatment in pregnant sheep that has a supra-additive effect on pulmonary maturation.[113;114] The extent of HPA- and HPT axes cross-talk is species-specific and depends on the developmental stage.[115] From animal experiments there are numerous examples but for obvious reasons the data for humans with basically normal thyroid and adrenal function are limited. It has been shown that glucocorticoids directly influence the HPT axis. Apart from the expected suppression of the HPA axis[116] due to the physiological negative feedback system there is evidence of a similar suppressive effect of glucocorticoids on the HPT axis.[117;118] Physiological levels of corticosterone are known to inhibit TSH secretion.[119] In euthyroid subjects, a five-day course of Dexamethasone increases rT_3 serum levels.[120]
In clinical practice, antenatal glucocorticoids have temporary suppressive influences on fetal breathing movements,[121] variability of heart rhythm[122] and fetal behaviour[123] and there is a measurable effect on brain perfusion.[124] Interestingly, suppressed thyroid function can have the same effects, suggesting that the glucocorticoid effect may in part be mediated by thyroid hormones. There are currently no studies substantiating this point, but there are data showing a negative effect of postnatal dexamethasone on thyroid hormone metabolism.[125] On the molecular level this could be explained by induction of D3[115], or alternatively by suppression of D1.[126] Both would lead to a lowering of the bioactive metabolites, with influences both in fetus and placenta.

In summary, the placenta is able to modulate key processes regarding the Hypothalamic-Pituitary axes during pregnancy.

Introduction Part 2: The Placenta as the Basis of Gestational and Fetal Disease

Gestational Hypertensive Disorders

There is ample clinical evidence[127] that trophoblast cells are able to induce gestational hypertensive disorders as preeclampsia (PE), eclampsia, pregnancy-induced hypertension and Hemolysis, Elevated Liver enzymes and Low Platelet (HELLP) – Syndrome (defined as platelet count < 100 x 10^9 /l, aspartate aminotransferase ≥ 70 U/l and/or lactate dehydrogenase ≥ 600 U/l).
Gestational hypertensive disorders are highly associated with fetal growth restriction, that in turn is the resultant of placental dysfunction.[21;128;129] The exact molecular basis is unknown.

Preeclampsia

Preeclampsia is a syndrome with a highly variable expression typically occurring in the second half of pregnancy and is observed in approximately two to three percent of pregnancies.[130;131] It is a major cause of maternal[132] and fetal[133] morbidity and mortality, especially in the 25 percent of cases when the disease is severe, and when it occurs in pregnancies less than 34 weeks gestation. The syndrome is defined as new onset hypertension and proteinuria in a previously normotensive woman. According to the statement of the International Society for the Study of Hypertension in Pregnancy (ISSHP), the operational definition of preeclampsia is a diastolic blood pressure ≥ 110 mmHg on any occasion or a diastolic blood pressure ≥90 mmHg on two separate occasions at least four hours apart in combination with proteinuria ≥ 0.3 g/24 hr., that develops after 20 weeks gestation.[134] Other international organisations use more or less similar definitions.[13]
There are multiple predisposing factors for preeclampsia. Although preeclampsia is defined as pregnancy-induced hypertension, pre-existing hypertension is a major predisposing factor to develop superimposed preeclampsia. Primiparity, black race, obesity,[137] diabetes,[138] insulin resistance,[139] pre-existing vascular diseases such as systemic lupus erythematosus,[140;141] pre-existing thrombophilic disorders as protein S deficiency, activated protein C resistance, hyperhomocysteinemia or anticardiolipin antibodies[142] are other weakly predisposing factors. There are data reporting an incidence of 50 percent pre-existing renal disease in preeclampsia patients.[143] The predictive value of all of these factors is not clearly established.[131]
The clinical signs alerting the obstetrician that preeclampsia might develop is the gradual increase of blood pressure, although there are several biochemical

abnormalities (such as haemoconcentration, hyperuricemia[144]) that precede the clinical disease for weeks. Proteinuria is a late sign and generalised oedema usually becomes apparent in the latter part of the third trimester and progresses until and after delivery. Additionally, signs of severe disease include central nervous system manifestations such as headaches, blurred vision, scotomata and, rarely, cortical blindness. Right upper quadrant or epigastric pain is indicative of liver involvement.

The preeclamptic spectrum varies from very mild to life-threatening with first symptoms presenting very early, in the latter half of the second trimester, to very late during delivery or even in the early postpartum period. In order to define clinically relevant forms of preeclampsia, Ness and Roberts[145] and von Dadelszen[146] proposed a classification describing two forms in this clinical spectrum: early- and late-onset preeclampsia.

Both for the early- and late-onset forms of preeclampsia the presence of placental tissue is a prerequisite for the disease.[127] A fetus is not required, which is illustrated by the fact that hypertension and proteinuria occur with a high incidence in hydatiform mole.[147] Placental localisation in the uterus is also not required since preeclampsia can develop during an abdominal pregnancy. A case report describes the persistence of preeclampsia until removal of the placenta, 99 days after delivery of the fetus from an abdominal pregnancy.[148]
Clinically, the most prominent difference between the early-onset and late-onset class is the presence or absence of fetal growth restriction. In a nation-wide study in Norway, including over 670,000 births, neonates born after early-onset preeclampsia were lighter, shorter and leaner than neonates from either late-onset preeclampsia or normotensive pregnancies.[149]
Early-onset preeclampsia is seen as the consequence of poor placentation that is causal to the impairment of placental and fetal growth.[145;146] It is generally recognized that early-onset preeclampsia has most serious consequences for neonatal morbidity[150] and is accompanied by high recurrence rates.[151;152] Furthermore, early-onset preeclampsia is associated with maternal dislipidemia.[153]
In late-onset preeclampsia, placental dysfunction is not pronounced as in early-onset preeclampsia. Clinical symptoms in late-onset preeclampsia are modulated by generalized maternal endothelial dysfunction that is triggered by pre-existing maternal endothelial disease[145;146;154] such as diabetes, renal disease, obesity. This predisposition has been termed the metabolic syndrome of pregnancy.[155;156]

Pathogenesis

There are several processes essential to the pathogenesis of preeclampsia: impaired trophoblast invasion leading to defective placentation, placental oxidative stress and systemic endothelial activation. Figure 7 summarizes them in the simplified form of a cascade. As can be seen in the figure, the processes involved in the pathogenesis are local as well as systemic. Various steps in this cascade have joint effects on others, and in combination with either pre-existent maternal endothelial dysfunction or imbalance in clotting factors they lead to the complex spectrum of gestational hypertensive disorders, ranging from late-onset preeclampsia to early-onset preeclampsia and HELLP syndrome.

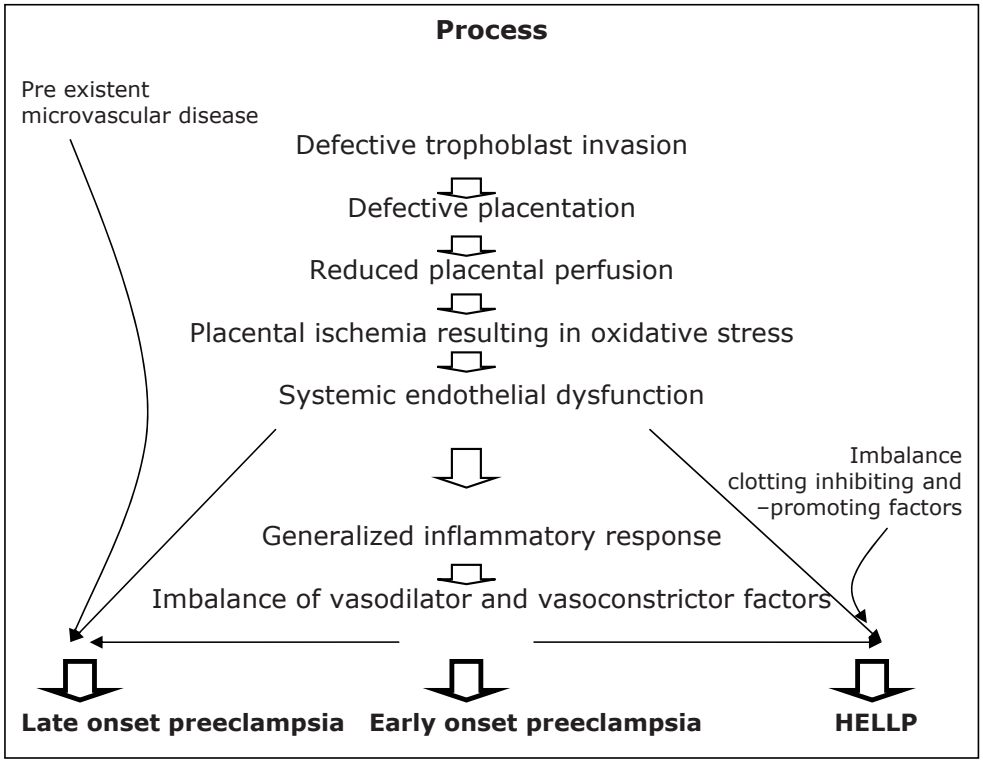

Figure 7: Pathogenesis of Preeclampsia.

Summary of the processes that attribute to the syndrome of early- and late-onset preeclampsia, as well as HELLP. Figure modified from Redman.[157]

Defective trophoblast invasion and abnormal placentation

Immune maladaptation is currently the leading theory with respect to abnormal placentation in preeclampsia. The importance of immune factors

is demonstrated by the presence of antibodies against endothelial cells and immune complexes in uterine spiral arteries, kidneys, liver and skin of preeclamptic women.[158] The placenta is in part an allograft.[159] Fetal trophoblast cells are in very close contact with blood from the genetically different maternal system, inducing local immunological factors.[158] As described on page 14 (Placental development, Implantation and Invasion) cytotrophoblast invasion into endometrium, myometrium and spiral-shaped maternal blood vessels is essential to normal placentation. The maternal lymphocytes encountered by the trophoblast cells invading the placental bed are mainly natural killer cells. They recognize the unique HLA combination displayed by the invading trophoblast cells, thereby permitting deep invasion into an immunologically different system. In preeclampsia this invasion is abnormally shallow and fails to modify the maternal spiral arteries.[127;160] Microscopically, the preeclamptic placenta indeed shows loss and distortion of villi, focal syncytial necrosis, decreased syncytial activity, cytotrophoblast hyperplasia as well as degeneration of cytotrophoblast cells and the presence of small fetal capillaries with bulbous endothelial cells.[161-163] The general concept, that prior exposure to allografts protects against a rejection reaction also holds for preeclampsia as the risk decreases in subsequent pregnancies, whether the initial pregnancy was carried to term or ended in abortion.[164] Blood transfusion and increased exposure to semen[165] (e.g. length of cohabitation, use of oral contraceptives) are also associated with a decreased risk of preeclampsia. On the other hand, artificial donor insemination[166] and oocyte donation[167] both lead to an increased risk.

Placental ischemia resulting in oxidative stress

The relatively high resistance in abnormally developed spiral arteries results in placental ischemia at the end of the first trimester of pregnancy.[168] Ischemia causes the formation of reactive oxygen species[169] which are normally offset by the activity of local anti-oxidants as glutathione S-transferase. Preeclamptic women can have increased amounts of circulating fatty acids that in turn are a target for reactive oxygen species, initiating a self-propagating chain reaction.[170;171] Based on this biological principle and the hypothesis that preeclamptic women might suffer from a deficient anti-oxidant system, clinical trials evaluating the effect of anti-oxidant therapy(vitamin C and vitamin E) have been performed. Although antioxidant therapy is able to change biochemical parameters in women at risk of preeclampsia,[172] it is unable to decrease the incidence of preeclampsia and has unexpected negative effects with respect to fetal growth.[173]

Systemic endothelial dysfunction

Oxygen radicals generated in the placenta are currently seen as the

main causal agents to the generalized endothelial damage[174] typical of preeclampsia.[175;176] Renal endothelial damage results in proteinuria,[177] liver cell damage is reflected by the elevation of aminotransferase enzymes and vascular constriction and oedema in the brain results in headaches and visual disturbances, with eclampsia and cerebral haemorrhage as very severe consequences.[30] The resulting imbalance of endothelium-derived vasodilators prostacyclin and nitric oxide with vasoconstrictor factors further aggravate peripheral vasoconstriction and hypertension.[178] Additional imbalance in clotting factors can lead to disseminated intravascular coagulopathy, clinically known as HELLP syndrome.[179] Table 2 on page 30 describes some of the mediators of generalized endothelial activation. Strikingly, all the factors that predispose for preeclampsia are also risk factors for endothelial diseases, atherosclerosis in particular, and it has been reported that women with a preeclamptic pregnancy have an increased risk of cardiovascular disease in later life.[180-182] More insight into the pathogenesis of preeclampsia will therefore help understanding vascular disease later in life.

The molecular basis of preeclampsia

In order to identify genes causal to the development of preeclampsia or HELLP syndrome, quite a number of polymorphisms and mutations in relevant genes have been investigated. Table 2 summarizes genetic analyses published in relation to gestational hypertensive disorders between 1989 and 2007 categorised according to their putative role in the pathogenesis of preeclampsia. Even though involvement of the cascade of processes discussed in the previous paragraphs is substantiated, no strong single causal factor in any of these processes has been identified.[183]

A population-based Swedish cohort study[184] on the recurrence of preeclampsia in second pregnancies demonstrated that over 50% of the preeclamptic phenotype is due to genetic factors. This observation is in contrast with the lack of concordance in the occurrence of preeclampsia between pregnant monozygotic twins.[185;186] While the majority of cases of preeclampsia and HELLP syndrome are not familial, the collection of rare familial cases in a number of geographic areas has enabled linkage studies. These studies identified chromosomal regions on 2p12[187], 2p13[188], 2p25[189], 4q[190], 7q33[191], 9p13[189], and 10q22[192] as susceptibility loci. Strikingly, there is little concordance between studies and analysis of candidate genes in these regions has not been very successful in identifying causal genes. One exception is an inactivating mutation of the long chain 3 hydoxyacyl coA dehydrogenase (LCHAD) that causes HELLP syndrome when a homozygous fetus is present.[193] These mutations are not frequent in pregnancies complicated by HELLP syndrome

and can explain only a very small proportion of cases.[194] The maternal inheritance of a variant of the STOX1 gene, common within the population, has been reported as causal to Dutch familial preeclampsia.[195]

Process	**Genes Considered**
Immune adaptation	HLA-DR, HLA-DQB1, HLA-DPB1, HLA-G, IGF2, IGF2R, IgG-CRHC, IL1, MMP1, TNFα
Placentation and Angiogenesis	ADAM12, ERVWE1, FOS, GCM1, INHB, IGFBP3, ITGA1, ITGB, JUN, MBL2, MMP7, MMP9, MMP10, MMP13, MMP15, NKB, sENG, sFlt, STOX1, TGFb, VEGF
Placental ischemia and oxidative stress	CASP10, CAT, EPHX1, GSH-Px, GSTP1, HIF1, LDHAL4, LDHB, LPL, LPLR, MTHFR, SOD, TNFRSF25, VLDLR
Endothelial activation and cytokine production	ALOX5, APOC3, apoE, CD14, CEACAM8, COX2, CTLA4, EDN, FSTL3, GNB3, IFNG, $IL_{1a/b,}$ $IL_{6,}$ $IL_{8,}$ $IL_{10,}$ LIPC, LIPE, LPL, LTA, NFKB, NO, NPY, TNFα, TNFαR
Vasodilator and vasoconstriction factors	ACE, AGT, AGTR1a/b, AGTRL1, ANP, APLN, BDKRB1, CAPN10, CALCRL, DDAH1, EDN1, ecNOS, HPGD, KLK1, NOS2A PCS, PTGS2, pro-ANF, PTHrP, RAMP1, REN, SCNN1B, TBX
Clotting factors	APC, F2, F7, F11, FVL, PAI1, PAR1, Plasminogen,THBD
Miscellaneous	ADIPOQ, COX1, LCHAD, LEP, LEPR, LIPC, HSD11B2, LIPC, PPARG, VDR

Table 2: Genes that have been reported in relation to preeclampsia. Extracted from the Pub Med search Gene[title/abstract] AND Preeclampsia[MeSH] between 1989 and March 2008.

Strategies to Elucidate the Molecular Basis of Pleeclampsia

Impediments to preeclampsia research

A main impediment to preeclampsia research is the lack of placenta material from the first trimester, the time the basis of the disease is established. Changes in placentation, and the cascade of oxidative- and inflammatory damage most likely take place early during gestation and not by the time of delivery, when placental bed biopsies are usually taken.[176] As preeclampsia is a very heterogeneous disease[131], it is extremely important that the criteria on which subjects are recruited in clinical studies are clearly defined and strictly applied. If this is not the case, the non-uniformly defined patient populations limit the interpretation and association of findings. Characterization of the disease phenotype should be done preferably according to the ISSHP criteria[134] and the diagnosis should be made only after delivery. As can be seen in Table 3 overleaf, not all studies fully meet generally accepted disease criteria. The complex inheritance pattern of susceptibility for preeclampsia and the interactions with environmental factors demonstrate that, with rare exceptions, preeclampsia is not a monogenic disease.[196] Illustrative of the complexity of genetic research is the large number of contradictory reports on the contribution of individual genes to the pathogenesis of preeclampsia.

The fact that different studies compare different determinants prohibits generalization of studies. To exemplify, studies on endothelial nitric oxide synthase (ecNOS), published in relation to preeclampsia are summarized in Table 3. The study populations are heterogeneous, varying from gestational hypertension or moderate late-onset to severe early-onset preeclampsia to cases with placental abruption. This, in combination with use of either fetal (placental) or maternal material investigating either DNA, RNA or protein, makes it difficult to generalize the findings. When these studies are pooled and evaluated, there is no increased risk of preeclampsia, neither under a recessive nor a dominant genetic model, as has been shown in a recent meta-analysis.[197]

Animal models

Although there are several animal (sheep, monkeys and rodents) models, none properly reflects the pathophysiological mechanisms of human preeclampsia. The oldest model in sheep is a pregnancy-induced hypertension model, provoked by a 4-day fasting period during early gestation.[198] Apart from hypertension, this condition bears little resemblance to the human preeclampsia syndrome. Moreover, both placental morphology and the placentation process of epitheliochorial placentas, (see Figure 4 page 13) in sheep are quite distinct from human. Arterial occlusion in rhesus monkeys, which have discoid placentas of hemochorial structure just like humans,

Study	Study Population	Clinical PE Criteria	Material	Biomarker	Result
Nasiell 1998[198] Sweden	13 nonsevere PE, 7 PE and SGA, 8 SGA, 41 control pregnancies	ISSHP	Placental tissue	RNA level of ecNOS / Total nucleic acids	ecNOS level significantly higher in complicated- than in normal pregnancies
Yoshimura 2000[199] Japan	80 severe PE, 35 nonsevere PE, 37 superimposed and 170 pregnant control women	NHBPEP	Maternal DNA	Glu298Asp ecNOS variant	T allele more frequent in subjects with severe PE than in either nonsevere PE superimposed PEor control
Faxen 2001[200] Sweden	8 nonsevere PE and 12 from normal pregnancies	ISSHP	Placental tissue	mRNA level of ecNOS / RNA GAPDH	mRNA expression was significantly higher in both myometrium and placenta
Tempfer 2001[201] Germany	24 severe PE and 24 nonpregnant women, with no history of hypertension	ISSHP	Maternal DNA	Glu298Asp ecNOS variant	No association
Orange 2003[202] Australia	14 nonsevere PE 4 gestational hypertension 12 pregnant control women	ASSHP	Placental tissue	Immunohistochemistry, antibody specific for ecNOS	No significant difference in ecNOS in either villous or decidual staining intensity
Hakli 2003[203] Finland	132 nonsevere PE 113 pregnant control women	ISSHP	Maternal DNA	Glu298Asp ecNOS variant	No association
Yoshimura 2003[204] Bangladesh	112 severe PE and 119 control women	NHBPEP	Maternal DNA	Glu298Asp ecNOS variant	No association of homozygote TT with PE
Landau 2004[205] USA	64 non-severe PE 397 pregnant control women	AmCOG	Maternal DNA	Glu298Asp ecNOS variant	No association
Ohta 2004[206] Japan	131 pregnant women with PE 327 pregnant control women	ISSHP	Maternal DNA	Glu298Asp ecNOS variant	No association
Serrano 2004[207] Colombia	322 PE and 522 control women	NHBPEP	Maternal DNA	Glu298Asp ecNOS variant	TT variant more frequent in PE
Wang 2004[208] Australia	15 severe PE, 5 nonsevere PE, 5 normotensive controls	ASSHP	RNA samples from HUVEC cells	RNA level of ecNOS / RNA actin	Decreased RNA level in PE samples
Hillerman 2005[209] South Africa	50 severe PE 50 abruptio placentae 50 pregnant control women	NHBPEP	Maternal DNA	Glu298Asp ecNOS variant	T allele more frequent in the abruption group than control group and in PE and abruption compared to PE alone

Table 3: Characteristics of 12 studies of endothelial nitric oxide synthase (ecNOS) in relation to preeclampsia published 1998-2005. AmCOG = American College of Obstetrics and Gynecology. ASSHP = Australasian Society for the Study of Hypertension in Pregnancy. ISSHP = International Society for the Study of Hypertension in Pregnancy. NHBPEP = National High Blood Pressure Education Program(USA). PE = preeclampsia. SGA= small for gestational age.

induces placental dysfunction with hypertension and fetal growth restriction as a consequence.[199] This model mimics placental ischemia and its consequences, but does not affect renal function.
There are several rodent models in which the placentation process is influenced by reduced uterine perfusion[200;201] or the administration of endogenous agents. Endotoxin[202], desoxycorticosterone acetate[203], nitric oxide synthase inhibitor[204] and the angiogenesis inhibitors TNP470[205] or Suramin[206] each produce a variety of preeclampsia-like symptoms and fetal growth restriction. Pregnancy-associated hypertension[207] and fetal growth restriction[208] are present in spontaneous hypertensive and heart failure (SHHF) rats, who have chronic hypertension, insulin resistance, and renal impairment as they age. Rats injected with sFLT and sENG develop hypertension and proteinuria or even HELLP syndrome.[209] Transgenic models also exist: $p52^{Kip2}$ mice[210] have hypertension, proteinuria and coagulation disorders, whereas pregnant BHP5 mice[211] have hypertension, late-gestational proteinuria and progressive renal glomerulosclerosis with growth restriction in the litter. Although rodents have a hemochorial discoid placental structure resembling human, there are several differences between humans and rodents. In human, rat and mouse gestation takes respectively 280, 22 and 20 days while implantation takes place respectively at day 6, 5 and 4. Therefore, in human preeclamptic pregnancy, placenta and fetus are faced with progression of the disease for the major part of gestation, while in rodents this time span is relatively short.

Cell culture and tissue culture
Since the placenta becomes available after delivery, it is then accessible for gene expression studies and primary cell culture. Trophoblast- or endothelial cell cultures are ideally suited to investigate physiological pathways under controlled conditions.[212] As an example, this type of experiment demonstrated secretion of TNFα from hypoxic trophoblast cells mediating the inflammatory response.[213] Cell culture can never be the equivalent for the *in vivo* situation. The fact that the cellular composition of the placenta changes dramatically during gestation, makes this more true for placenta.

Studies on gene expression
Every cell in the human body has the same genetic repertoire, consisting of genes located on chromosomes. The protein-coding sequences (exon sequences) that are usually dispersed over a gene are divided by intervening sequences (intron sequences). During RNA splicing the exon sequences are fused, rendering mature mRNA molecules of which the open reading frame is translated into protein.[214] (Figure 8, overleaf)

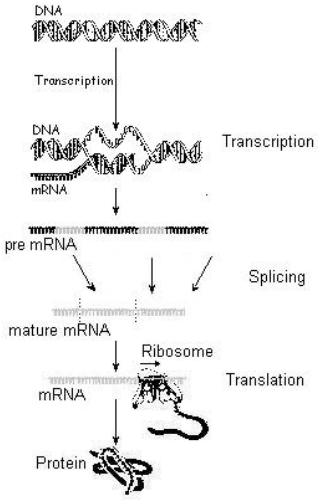

Figure 8: Schematic representation of RNA splicing.
Intron sequences are depicted in black, exon sequences are grey.

Basal common cellular processes occur in all cell types and are dependent on proteins encoded by housekeeping genes. As for all tissues and organs, placental tissue-specific gene expression defines the proteins that form the basis for its growth, development and function.[215]
Depending on the number of samples in which the transcription level has to be determined and the total number of genes that need to be investigated, several molecular biology techniques are available. Northern blot analysis requires relatively large amounts of RNA and is suitable to analyse the semi quantitative expression level of a few genes. The expression level of a specific single gene can be assessed by the far less material- and time-consuming semi-quantitative polymerase chain reaction (PCR),[216] or real-time PCR techniques.[217]
The development of high-throughput biotechnology techniques such as microarray analysis and serial analysis of gene expression (SAGE), in combination with major advances in bioinformatics has opened ways to investigate complex diseases at the RNA level.

A microarray is a solid surface to which tens of thousands DNA spots, representing genes, are attached. Hybridization of fluorescent labelled RNA derived from individual cell cultures or tissue samples allows the monitoring of the expression level of genes present on the array. This way, an array yields expression signals from tens of thousands of genes over series of individual samples.[218] Arrays can contain previously identified genes but also Expressed Sequence Tags (ESTs), partial cDNA sequences derived by

sequencing clones from cDNA libraries. The main limitation of the microarray technique is that only previously selected transcripts or ESTs are analyzed. Secondly, genes expressed at lower levels or in a small subset of cells are likely to be missed in this multigene technique.[219] Finally, the vast amount of data also cause a very unfavourable signal to noise ratio, hindering the identification of truly important genes.[220]
With respect to placental function, microarray technology has been used in different stages of cultured trophoblast cells[221], establishing different processes of differentiation. Also, there are models comparing gene expression in decidua and villi in placental samples using microarray technology [222] as well as models of trophoblast invasion[223] and placental hypoxia.[224] Microarray analysis in preeclamptic placentas has suggested up regulation of glycogen phosphorylase[225] as well as subsets of apoptosis-[226;227] calcium metabolism-related[228] protein matabolism-related[229] and obesity-related genes[230]. Differential regulation of tumour suppressor and growth regulatory genes are reported to play a role in the pathogenesis of severe early-onset preeclampsia.[231] Recently also genes related to an imbalance of reactive oxygen metabolites, abnormal trophoblast invasion, disorders of lipoprotein metabolism[232] and growth factors[233] have been found specifically differentially regulated in preeclampsia.

Serial Analysis of Gene Expression (SAGE)
The SAGE technique[234] is a tool to generate complete expression profiles at the transcriptional level. The traditional generation of a SAGE library starts with a milligram of mRNA from the tissue or cell type of interest. High-quality double stranded cDNA is synthesized using biotinated oligo-dT, captured and cleaved with the anchoring enzyme NlaIII, yielding 3` cDNA fragments with an average size of 265 bp. After ligation to specific linkers, the cDNA pool is digested with BsmF1 that recognizes a sequence in the linker and cuts a variable number of nucleotides downstream, generating an mRNA-specific SAGE tag. These SAGE tags are ligated to ditags, PCR amplified, concatenated and sequenced.(Figure 9) The concatenation of tags in a serial fashion makes SAGE a high-throughput technique for a limited number of samples. The resultant SAGE library consists of a list of ten-to-hundred thousands of 10 base pair SAGE tags that each occur 1 or more times. The quantitative properties are based on simply counting the number of times a tag occurs. The qualitative properties are based on the fact that a 10 base-pair sequence adjacent to the most 3` CATG site of a mRNA molecule contains sufficient information to uniquely identify the corresponding transcript. Since alternative splicing, alternative polyA adenylation and polymorphisms result in multiple tags corresponding to a single gene, tag to gene allocation is actually the most difficult step in SAGE library analysis.

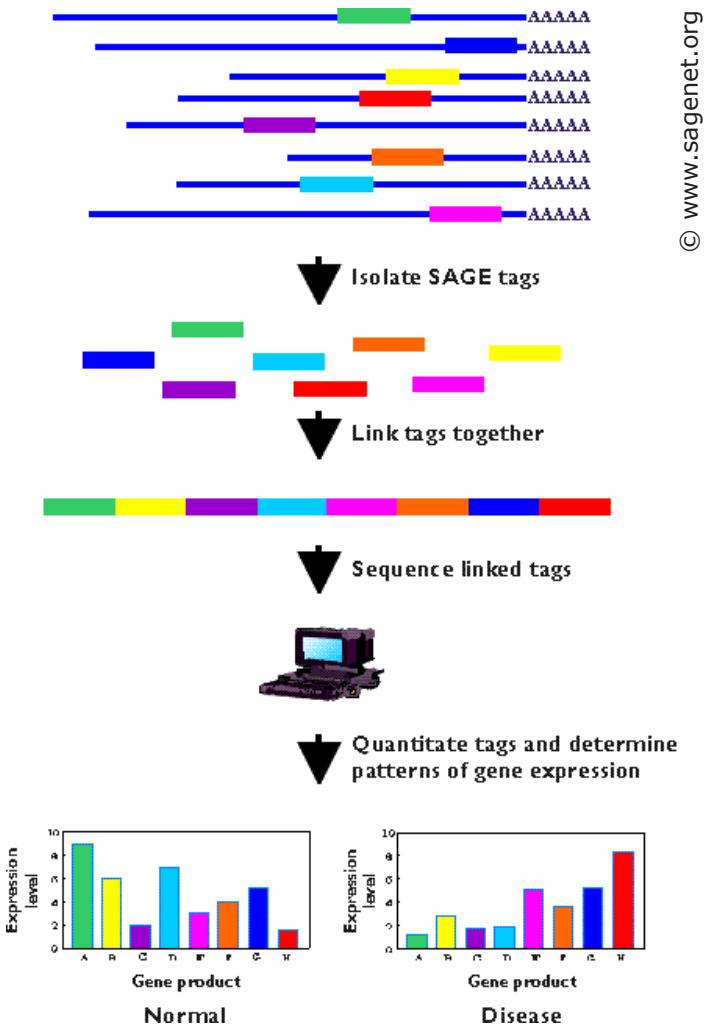

Figure 9: Schematic representation of the SAGE method.

Typically, each SAGE library also contains tags that can not be linked to a transcript with known or inferred function: the so-called NoMatch tags. They are the leads to identify novel genes and transcripts.
The public availability of tens of SAGE libraries of different human tissues enables the recognition of tissue (i.c. placenta-) specific transcripts.
SAGE is the only available tool to generate complete yet comprehensive expression profiles without prior availability of transcript information. It provides the opportunity, with disease phenotype-related No Matches as leads, to discover novel disease-specific genes. This concept has been extensively validated for cancer research.[235]

Aim and scope of this Thesis

With optimal placental function as prerequisite for fetal prosperity, this thesis describes the relation of placental development with maternal gestational and fetal disease. (Chapter 1) The consequences of placental function are investigated by the analysis of placental weight birth weight in Chapter 2 and 3. Since the placenta has an important role in hormone metabolism and hormonal interaction between mother and fetus, we describe thyroid hormone interactions with respect to fetal growth restriction and gestational hypertensive disorders in Chapter 4 and 5. Molecular biological techniques and bioinformatics are used to study gene expression relevant to characterization of PE and HELLP syndrome in Chapter 6 and to develop gestational age specific placental profiles in Chapter 7.

Reference List

1. www.fandm.edu/departments/Anthropology/Bastian/ANT269/cosmo.html
2. Angeletti LR. De octimestri partu and pathology of the last trimester of pregnancy. Med.Secoli. 1990;2:75-92.
3. Thornton JL, Want PC. William Hunter (1718-1783) and his contributions to obstetrics. Br.J.Obstet.Gynaecol. 1983;90:787-94.
4. Young M. Classics revisited: researches on pre-natal life by Sir Joseph Barcroft. Placenta 1992;13:607-12.
5. Liggins GC. Parturition in the sheep and the human. Basic Life Sci. 1974;4:423-43.
6. Liggins GC. Initiation of labour. Biol.Neonate 1989;55:366-75.
7. Liggins GC, Howie RN. A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. Pediatrics 1972;50:515-25.
8. Liggins GC. The role of the hypothalamic-pituitary-adrenal axis in preparing the fetus for birth. Am.J.Obstet.Gynecol. 2000;182:475-77.
9. Kingdom,J.C.; Jauniaux,E.R.; Shaughn O'Brien,P.M. The Placenta: Basic Science and Clinical Practice. London: RCOG Press, 2000.
10. Boyd J, Hamilton WJ. The human placenta. Cambridge: W.Heffer & Sons, 1970.
11. Murphy WJ, Eizirik E, Johnson WE, Zhang YP, Ryder OA, O'Brien SJ. Molecular phylogenetics and the origins of placental mammals. Nature 2001;409:614-18.
12. Georgiades P, Ferguson-Smith AC, Burton GJ. Comparative developmental anatomy of the murine and human definitive placentae. Placenta 2002;23:3-19.
13. Parr MB, Parr EL. Permeability of the primary decidual zone in the rat uterus: studies using fluorescein-labeled proteins and dextrans. Biol.Reprod. 1986;34:393-403.
14. Pijnenborg R, Dixon G, Robertson WB, Brosens I. Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. Placenta 1980;1:3-19.
15. Mayhew TM. Fetoplacental angiogenesis during gestation is biphasic, longitudinal and occurs by proliferation and remodelling of vascular endothelial cells. Placenta 2002;23:742-50.
16. Mayhew TM, Barker BL. Villous trophoblast: morphometric perspectives on growth, differentiation, turnover and deposition of fibrin-type fibrinoid during gestation. Placenta 2001;22:628-38.
17. Mayhew TM. Thinning of the intervascular tissue layers of the human placenta is an adaptive response to passive diffusion in vivo and may help to predict the origins of fetal hypoxia. Eur.J.Obstet.Gynecol.Reprod.Biol. 1998;81:101-09.
18. Newnham JP, Kelly RW, Roberts RV, MacIntyre M, Speijers J, Johnson T et al. Fetal and maternal Doppler flow velocity waveforms in normal sheep pregnancy. Placenta 1987;8:467-76.
19. Acharya G, Wilsgaard T, Berntsen GK, Maltau JM, Kiserud T. Reference ranges for serial measurements of blood velocity and pulsatility index at the intra-abdominal portion, and fetal and placental ends of the umbilical artery. Ultrasound Obstet.Gynecol. 2005;26:162-69.
20. Winick M, Coscia A, Noble A. Cellular growth in human placenta. I. Normal

placental growth. Pediatrics 1967;39:248-51.
21. Kloosterman GJ. On intrauterine growth. Int.J.Gynaecol.Obstet. 1970;8:895-912.
22. Hamilton WJ, Grimes DH. Growth relationship between the foetus and the placenta. Proc.R.Soc.Med. 1970;63:496-98.
23. Naeye RL. Causes and consequences of placental growth retardation. JAMA 1978;239:1145-47.
24. Pinar H, Sung CJ, Oyer CE, Singer DB. Reference values for singleton and twin placental weights. Pediatr.Pathol.Lab Med. 1996;16:901-07.
25. Schuler-Maloney D, Lee S. Development of the placenta: a synopsis. The placenta: to know me is to love me. A reference guide for gross placental examination. De Moines, Iowa: DSM Pathworks; 1998. p. 46-51.
26. Schuler-Maloney D, Lee S. Gross examination. The placenta: to know me is to love me. A reference guide for gross placental examination. De Moines, Iowa: DSM Pathworks; 1998. p. 57-58.
27. Moore LG, Young D, McCullough RE, Droma T, Zamudio S. Tibetan protection from intrauterine growth restriction (IUGR) and reproductive loss at high altitude. Am.J.Hum.Biol. 2001;13:635-44.
28. Moore LG, Shriver M, Bemis L, Hickler B, Wilson M, Brutsaert T et al. Maternal adaptation to high-altitude pregnancy: an experiment of nature--a review. Placenta 2004;25 Suppl A:S60-S71.
29. Mahajan SD, Singh S, Shah P, Gupta N, Kochupillai N. Effect of maternal malnutrition and anemia on the endocrine regulation of fetal growth. Endocr. Res. 2004;30:189-203.
30. VanWijk MJ, Kublickiene K, Boer K, VanBavel E. Vascular function in preeclampsia. Cardiovasc.Res. 2000;47:38-48.
31. Rogers MS, Needham PG. Unicornuate uterus and reproductive performance. Aust.N.Z.J.Obstet.Gynaecol. 1985;25:144-45.
32. Poma PA. Intrauterine growth retardation associated with uterine malformations. J.Natl.Med.Assoc. 1982;74:745-48.
33. Andrews MC, Jones HW, Jr. Impaired reproductive performance of the unicornuate uterus: intrauterine growth retardation, infertility, and recurrent abortion in five cases. Am.J.Obstet.Gynecol. 1982;144:173-76.
34. Heinonen S, Taipale P, Saarikoski S. Weights of placentae from small-for-gestational age infants revisited. Placenta 2001;22:399-404.
35. Grati FR, Miozzo M, Cassani B, Rossella F, Antonazzo P, Gentilin B et al. Fetal and placental chromosomal mosaicism revealed by QF-PCR in severe IUGR pregnancies. Placenta 2005;26:10-18.
36. Redaelli S, Sala E, Roncaglia N, Colombo C, Crosti F, Villa N et al. Severe intrauterine growth restriction and trisomy 15 confined placental mosaicism: a case report and review of literature. Prenat.Diagn. 2005;25:140-47.
37. Salafia CM, Minior VK, Pezzullo JC, Popek EJ, Rosenkrantz TS, Vintzileos AM. Intrauterine growth restriction in infants of less than thirty-two weeks' gestation: associated placental pathologic features. Am.J.Obstet.Gynecol. 1995;173:1049-57.
38. Nordenvall M, Ullberg U, Laurin J, Lingman G, Sandstedt B, Ulmsten U.

Placental morphology in relation to umbilical artery blood velocity waveforms. Eur.J.Obstet.Gynecol.Reprod.Biol. 1991;40:179-90.
39. Loos RJ, Derom C, Derom R, Vlietinck R. Determinants of birthweight and intrauterine growth in liveborn twins. Paediatr.Perinat.Epidemiol. 2005;19 Suppl 1:15-22.
40. Machin GA, Ackerman J, Gilbert-Barness E. Abnormal umbilical cord coiling is associated with adverse perinatal outcomes. Pediatr.Dev.Pathol. 2000;3:462-71.
41. Georgiou HM, Rice GE, Walker SP, Wein P, Gude NM, Permezel M. The effect of vascular coiling on venous perfusion during experimental umbilical cord encirclement. Am.J.Obstet.Gynecol. 2001;184:673-78.
42. Degani S, Leibovich Z, Shapiro I, Gonen R, Ohel G. Early second-trimester low umbilical coiling index predicts small-for-gestational-age fetuses. J.Ultrasound Med. 2001;20:1183-88.
43. Perry IJ, Beevers DG, Whincup PH, Bareford D. Predictors of ratio of placental weight to fetal weight in multiethnic community. BMJ 1995;310:436-39.
44. Sivarao S, Vidyadaran MK, Jammal AB, Zainab S, Goh YM, Ramesh KN. Weight, volume and surface area of placenta of normal pregnant women and their relation to maternal and neonatal parameters in Malay, Chinese and Indian ethnic groups. Placenta 2002;23:691-96.
45. Williams LA, Evans SF, Newnham JP. Prospective cohort study of factors influencing the relative weights of the placenta and the newborn infant. BMJ 1997;314:1864-68.
46. Bortolus R, Chatenoud L, Di Cintio E, Rossi P, Benzi G, Surace M et al. Placental ratio in pregnancies at different risk for intrauterine growth. Eur.J.Obstet. Gynecol.Reprod.Biol. 1998;80:157-58.
47. Lao TT, Wong WM. Placental ratio and intrauterine growth retardation. Br.J.Obstet.Gynaecol. 1996;103:924-26.
48. MacArthur C, Knox EG. Smoking in pregnancy: effects of stopping at different stages. Br.J.Obstet.Gynaecol. 1988;95:551-55.
49. Jaddoe VWW, Verburg BO, de Ridder MAJ, Hofman A, Mackenbach JP, Moll HA et al. Maternal smoking and fetal growth characteristics in different periods of pregnancyt. The generation R study. Am.J.Epidemiol. 2008;165:1207-15.
50. McCrabb GJ, Egan AR, Hosking BJ. Maternal undernutrition during mid-pregnancy in sheep: variable effects on placental growth. J.Agricult.Sci. 1992;118:127-32.
51. Wallace JM, Aitken RP, Milne JS, Hay WW, Jr. Nutritionally mediated placental growth restriction in the growing adolescent: consequences for the fetus. Biol.Reprod. 2004;71:1055-62.
52. Redmer DA, Wallace JM, Reynolds LP. Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. Domest. Anim Endocrinol. 2004;27:199-217.
53. Godfrey K, Robinson S, Barker DJ, Osmond C, Cox V. Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. BMJ 1996;312:410-14.
54. Barker DJ, Bull AR, Osmond C, Simmonds SJ. Fetal and placental size and risk of hypertension in adult life. BMJ 1990;301:259-62.

55. Moore VM, Miller AG, Boulton TJ, Cockington RA, Craig IH, Magarey AM et al. Placental weight, birth measurements, and blood pressure at age 8 years. Arch.Dis.Child 1996;74:538-41.
56. Roseboom TJ, Van Der Meulen JH, Ravelli AC, Osmond C, Barker DJ, Bleker OP. Perceived health of adults after prenatal exposure to the Dutch famine. Paediatr.Perinat.Epidemiol. 2003;17:391-97.
57. Hamilton WJ, Boyd JD. Observations on the human placenta. Proc.R.Soc. Med. 1951;44:489-96.
58. Hay WW, Jr. The placenta. Not just a conduit for maternal fuels. Diabetes 1991;40 Suppl 2:44-50.
59. Oppenheimer JH, Schwartz HL, Strait KA. Thyroid hormone action. In: Braverman LE, Utiger RD, editors. The Thyroid. Philadelphia: Lippincott; 1996. p. 162-89.
60. Hamrahian AH, Oseni TS, Arafah BM. Measurements of serum free cortisol in critically ill patients. N.Engl.J.Med. 2004;350:1629-38.
61. Oppenheimer JH, Schwartz HL, Mariash CN, Kinlaw WB, Wong NC, Freake HC. Advances in our understanding of thyroid hormone action at the cellular level. Endocr.Rev. 1987;8:288-308.
62. Krieger DT. Placenta as a source of 'brain' and 'pituitary' hormones. Biol. Reprod. 1982;26:55-71.
63. Yen SS. The placenta as the third brain. J.Reprod.Med. 1994;39:277-80.
64. Liggins GC. The role of cortisol in preparing the fetus for birth. Reprod.Fertil. Dev. 1994;6:141-50.
65. Challis JR, Matthews SG, Van Meir C, Ramirez MM. Current topic: the placental corticotrophin-releasing hormone-adrenocorticotrophin axis. Placenta 1995;16:481-502.
66. McLean M, Bisits A, Davies J, Woods R, Lowry P, Smith R. A placental clock controlling the length of human pregnancy. Nat.Med. 1995;1:460-63.
67. McLean M, Smith R. Corticotrophin-releasing hormone and human parturition. Reproduction. 2001;121:493-501.
68. Challis JR, Nancekievill EA, Lye SJ. Possible role of cortisol in the stimulation of cortisol-binding capacity in the plasma of fetal sheep. Endocrinology 1985;116:1139-44.
69. Beitins IZ, Bayard F, Ances IG, Kowarski A, Migeon CJ. The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. Pediatr.Res. 1973;7:509-19.
70. Murphy BE, Clark SJ, Donald IR, Pinsky M, Vedady D. Conversion of maternal cortisol to cortisone during placental transfer to the human fetus. Am.J.Obstet. Gynecol. 1974;118:538-41.
71. Pepe GJ, Burch MG, Albrecht ED. Estrogen regulates 11 beta-hydroxysteroid dehydrogenase-1 and -2 localization in placental syncytiotrophoblast in the second half of primate pregnancy. Endocrinology 2001;142:4496-503.
72. Crowley P. Prophylactic corticosteroids for preterm birth. Cochrane.Database. Syst.Rev. 2000;CD000065.
73. ACOG committee opinion. Antenatal corticosteroid therapy for fetal maturation. Committee on Obstetric Practice. American College of Obstetricians and

Gynecologists. Int.J.Gynaecol.Obstet. 1999;64:334-35.
74. Brocklehurst P, Gates S, McKenzie-McHarg K, Alfirevic Z, Chamberlain G. Are we prescribing multiple courses of antenatal corticosteroids? A survey of practice in the UK. Br.J.Obstet.Gynaecol. 1999;106:977-79.
75. Stark AR, Carlo WA, Tyson JE, Papile LA, Wright LL, Shankaran S et al. Adverse effects of early dexamethasone in extremely-low-birth-weight infants. National Institute of Child Health and Human Development Neonatal Research Network. N.Engl.J.Med. 2001;344:95-101.
76. Baud O. Postnatal steroid treatment and brain development. Arch.Dis.Child Fetal Neonatal Ed 2004;89:F96-100.
77. Wapner RJ, Sorokin Y, Mele L, Johnson F, Dudley DJ, Spong CY et al. Long-term outcomes after repeat doses of antenatal corticosteroids. N.Engl.J.Med. 2007;357:1190-98.
78. Baud O. Antenatal corticosteroid therapy: benefits and risks. Acta Paediatr. Suppl 2004;93:6-10.
79. Crowther CA, Harding JE. Repeat doses of prenatal corticosteroids for women at risk of preterm birth for preventing neonatal respiratory disease. Cochrane. Database.Syst.Rev. 2007;CD003935.
80. Kohrle J, Brabant G, Hesch RD. Metabolism of the thyroid hormones. Horm. Res. 1987;26:58-78.
81. Hidal JT, Kaplan MM. Characteristics of thyroxine 5'-deiodination in cultured human placental cells. Regulation by iodothyronines. J.Clin.Invest 1985;76:947-55.
82. Koopdonk-Kool JM, de Vijlder JJ, Veenboer GJ, Ris-Stalpers C, Kok JH, Vulsma T et al. Type II and type III deiodinase activity in human placenta as a function of gestational age. J.Clin.Endocrinol.Metab 1996;81:2154-58.
83. Richard K, Hume R, Kaptein E, Sanders J, van Toor H, de Herder W et al. Ontogeny of iodothyronine deiodinases in human liver. J.Clin.Endocrinol. Metab 1998;83:2868-74.
84. Huang SA, Dorfman DM, Genest DR, Salvatore D, Larsen PR. Type 3 iodothyronine deiodinase is highly expressed in the human uteroplacental unit and in fetal epithelium. J.Clin.Endocrinol.Metab 2003;88:1384-88.
85. Koerner D, Schwartz HL, Surks MI, Oppenheimer JH. Binding of selected iodothyronine analogues to receptor sites of isolated rat hepatic nuclei. High correlation between structural requirements for nuclear binding and biological activity. J.Biol.Chem. 1975;250:6417-23.
86. Friesema EC, Ganguly S, Abdalla A, Manning Fox JE, Halestrap AP, Visser TJ. Identification of monocarboxylate transporter 8 as a specific thyroid hormone transporter. J.Biol.Chem. 2003;278:40128-35.
87. Wu SY, Green WL, Huang WS, Hays MT, Chopra IJ. Alternate pathways of thyroid hormone metabolism. Thyroid 2005;15:943-58.
88. Bernal J, Guadano-Ferraz A, Morte B. Perspectives in the study of thyroid hormone action on brain development and function. Thyroid 2003;13:1005-12.
89. Contempre B, Jauniaux E, Calvo R, Jurkovic D, Campbell S, de Escobar GM. Detection of thyroid hormones in human embryonic cavities during the first trimester of pregnancy. J.Clin.Endocrinol.Metab 1993;77:1719-22.

90. Calvo RM, Jauniaux E, Gulbis B, Asuncion M, Gervy C, Contempre B et al. Fetal tissues are exposed to biologically relevant free thyroxine concentrations during early phases of development. J.Clin.Endocrinol.Metab 2002;87:1768-77.
91. Chan S, Kachilele S, McCabe CJ, Tannahill LA, Boelaert K, Gittoes NJ et al. Early expression of thyroid hormone deiodinases and receptors in human fetal cerebral cortex. Brain Res.Dev.Brain Res. 2002;138:109-16.
92. Kester MH, Martinez dM, Obregon MJ, Marinkovic D, Howatson A, Visser TJ et al. Iodothyronine levels in the human developing brain: major regulatory roles of iodothyronine deiodinases in different areas. J.Clin.Endocrinol.Metab 2004;89:3117-28.
93. Vulsma T, Gons MH, de Vijlder JJ. Maternal-fetal transfer of thyroxine in congenital hypothyroidism due to a total organification defect or thyroid agenesis. N.Engl.J.Med. 1989;321:13-16.
94. Ballabio M, Nicolini U, Jowett T, Ruiz de Elvira MC, Ekins RP, Rodeck CH. Maturation of thyroid function in normal human foetuses. Clin.Endocrinol.(Oxf) 1989;31:565-71.
95. Germain DL, Hernandez A, Schneider MJ, Galton VA. Insights into the role of deiodinases from studies of genetically modified animals. Thyroid 2005;15:905-16.
96. Burrow GN, Fisher DA, Larsen PR. Maternal and fetal thyroid function. N.Engl. J.Med. 1994;331:1072-78.
97. Glinoer D. The regulation of thyroid function in pregnancy: pathways of endocrine adaptation from physiology to pathology. Endocr.Rev. 1997;18:404-33.
98. Ballabio M, Poshychinda M, Ekins RP. Pregnancy-induced changes in thyroid function: role of human chorionic gonadotropin as putative regulator of maternal thyroid. J.Clin.Endocrinol.Metab 1991;73:824-31.
99. Skjoldebrand L, Brundin J, Carlstrom A, Pettersson T. Thyroid associated components in serum during normal pregnancy. Acta Endocrinol.(Copenh) 1982;100:504-11.
100. Berghout A, Endert E, Ross A, Hogerzeil HV, Smits NJ, Wiersinga WM. Thyroid function and thyroid size in normal pregnant women living in an iodine replete area. Clin.Endocrinol.(Oxf) 1994;41:375-79.
101. Mortimer RH, Galligan JP, Cannell GR, Addison RS, Roberts MS. Maternal to fetal thyroxine transmission in the human term placenta is limited by inner ring deiodination. J.Clin.Endocrinol.Metab 1996;81:2247-49.
102. Stulp MR, de Vijlder JJ, Ris-Stalpers C. Placental iodothyronine deiodinase III and II ratios, mRNA expression compared to enzyme activity. Mol.Cell Endocrinol. 1998;142:67-73.
103. Morreale de Escobar G, Obregon MJ, Escobar del Rey. Is neuropsychological development related to maternal hypothyroidism or to maternal hypothyroxinemia? J.Clin.Endocrinol.Metab 2000;85:3975-87.
104. Macfaul R, Dorner S, Brett EM, Grant DB. Neurological abnormalities in patients treated for hypothyroidism from early life. Arch.Dis.Child 1978;53:611-19.
105. Pop VJ, Kuijpens JL, van Baar AL, Verkerk G, van Son MM, de Vijlder JJ et al. Low maternal free thyroxine concentrations during early pregnancy are associated with impaired psychomotor development in infancy. Clin.

Endocrinol.(Oxf) 1999;50:149-55.
106. Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J et al. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. N.Engl.J.Med. 1999;341:549-55.
107. Auso E, Lavado-Autric R, Cuevas E, Del Rey FE, Morreale dE, Berbel P. A moderate and transient deficiency of maternal thyroid function at the beginning of fetal neocorticogenesis alters neuronal migration. Endocrinology 2004;145:4037-47.
108. Fliers E, Wiersinga WM. [Screening pregnant women for hypothyroidism: as yet only in high risk groups]. Ned.Tijdschr.Geneeskd. 2003;147:1159-61.
109. Pop VJ, Vulsma T. Maternal hypothyroxinaemia during (early) gestation. Lancet 2005;365:1604-06.
110. Dashe JS, Casey BM, Wells CE, McIntire DD, Byrd EW, Leveno KJ et al. Thyroid-stimulating hormone in singleton and twin pregnancy: importance of gestational age-specific reference ranges. Obstet.Gynecol. 2005;106:753-57.
111. Oppenheimer JH, Schwartz HL. Molecular basis of thyroid hormone-dependent brain development. Endocr.Rev. 1997;18:462-75.
112. Zimmerman D. Fetal and neonatal hyperthyroidism. Thyroid 1999;9:727-33.
113. Liggins GC, Schellenberg JC, Manzai M, Kitterman JA, Lee CC. Synergism of cortisol and thyrotropin-releasing hormone in lung maturation in fetal sheep. J.Appl.Physiol 1988;65:1880-84.
114. Fraser M, Liggins GC. The effect of cortisol on thyroid hormone kinetics in the ovine fetus. J.Dev.Physiol 1989;11:207-11.
115. Van der Geyten S., Darras VM. Developmentally defined regulation of thyroid hormone metabolism by glucocorticoids in the rat. J.Endocrinol. 2005;185:327-36.
116. Arnold JD, Bonacruz G, Leslie GI, Veldhuis JD, Milmlow D, Silink M. Antenatal glucocorticoids modulate the amplitude of pulsatile cortisol secretion in premature infants. Pediatr.Res. 1998;44:876-81.
117. Ahlquist JA, Franklyn JA, Ramsden DB, Sheppard MC. The influence of dexamethasone on serum thyrotrophin and thyrotrophin synthesis in the rat. Mol.Cell Endocrinol. 1989;64:55-61.
118. Rubello D, Sonino N, Casara D, Girelli ME, Busnardo B, Boscaro M. Acute and chronic effects of high glucocorticoid levels on hypothalamic-pituitary-thyroid axis in man. J.Endocrinol.Invest 1992;15:437-41.
119. Pamenter RW, Hedge GA. Inhibition of thyrotropin secretion by physiological levels of corticosterone. Endocrinology 1980;106:162-66.
120. LoPresti JS, Eigen A, Kaptein E, Anderson KP, Spencer CA, Nicoloff JT. Alterations in 3,3'5'-triiodothyronine metabolism in response to propylthiouracil, dexamethasone, and thyroxine administration in man. J.Clin.Invest 1989;84:1650-56.
121. Patrick J, Challis J, Campbell K, Carmichael L, Richardson B, Tevaarwerk G. Effects of synthetic glucocorticoid administration on human fetal breathing movements at 34 to 35 weeks' gestational age. Am.J.Obstet.Gynecol. 1981;139:324-28.
122. Senat MV, Minoui S, Multon O, Fernandez H, Frydman R, Ville Y. Effect of

dexamethasone and betamethasone on fetal heart rate variability in preterm labour: a randomised study. British Journal of Obstetrics and Gynaecology 1998;105:749-55.
123. Mulder EJH, Derks JB, Visser GHA. Antenatal corticosteroid therapy and fetal behaviour: A randomised study of the effects of betamethasone and dexamethasone. British Journal of Obstetrics & Gynaecology 1997;104:1239-47.
124. Ohlsson A, Bottu J, Govan J, Ryan ML, Myhr T, Fong K. The effect of dexamethasone on time averaged mean velocity in the middle cerebral artery in very low birth weight infants. Eur.J.Pediatr. 1994;153:363-66.
125. Williams FL, Ogston SA, van Toor H, Visser TJ, Hume R. Serum thyroid hormones in preterm infants: associations with postnatal illnesses and drug usage. J.Clin.Endocrinol.Metab 2005;90:5954-63.
126. Menjo M, Murata Y, Fujii T, Nimura Y, Seo H. Effects of thyroid and glucocorticoid hormones on the level of messenger ribonucleic acid for iodothyronine type I 5'-deiodinase in rat primary hepatocyte cultures grown as spheroids. Endocrinology 1993;133:2984-90.
127. Redman CW. Current topic: pre-eclampsia and the placenta. Placenta 1991;12:301-08.
128. Baird D, Thomson A, Billewicz W. Birth weights and placental weights in pre-eclampsia. J.Obstet.Gynaecol.Br.Emp. 1957;64:370-72.
129. Pietrantoni M, O'Brien WF. The current impact of the hypertensive disorders of pregnancy. Clin.Exp.Hypertens. 1994;16:479-92.
130. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science 2005;308:1592-94.
131. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. Lancet 2005;365:785-99.
132. Schuitemaker N, van Roosmalen J, Dekker G, van Dongen P, van Geijn H, Bennebroek GJ. Confidential enquiry into maternal deaths in The Netherlands 1983-1992. Eur.J.Obstet.Gynecol.Reprod.Biol. 1998;79:57-62.
133. Kochanek KD, Murphy SL, Anderson RN, Scott C. Deaths: final data for 2002. Natl.Vital Stat.Rep. 2004;53:1-115.
134. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). Hypertens.Pregnancy. 2001;20:IX-XIV.
135. Brown MA, Hague WM, Higgins J, Lowe S, McCowan L, Oats J et al. The detection, investigation and management of hypertension in pregnancy: full consensus statement. Aust.N.Z.J.Obstet.Gynaecol. 2000;40:139-55.
136. Davey DA, MACGILLIVRAY I. The classification and definition of the hypertensive disorders of pregnancy. Am.J.Obstet.Gynecol. 1988;158:892-98.
137. Bodnar LM, Ness RB, Markovic N, Roberts JM. The risk of preeclampsia rises with increasing prepregnancy body mass index. Ann.Epidemiol. 2005;15:475-82.
138. Kaaja RJ, Greer IA. Manifestations of chronic disease during pregnancy. JAMA 2005;294:2751-57.
139. Mikola M, Hiilesmaa V, Halttunen M, Suhonen L, Tiitinen A. Obstetric outcome in women with polycystic ovarian syndrome. Hum.Reprod. 2001;16:226-29.
140. Houser MT, Fish AJ, Tagatz GE, Williams PP, Michael AF. Pregnancy and

systemic lupus erythematosus. Am.J.Obstet.Gynecol. 1980;138:409-13.
141. Wolfberg AJ, Lee-Parritz A, Peller AJ, Lieberman ES. Association of rheumatologic disease with preeclampsia. Obstet.Gynecol. 2004;103:1190-93.
142. van Pampus MG, Dekker GA, Wolf H, Huijgens PC, Koopman MM, von Blomberg BM et al. High prevalence of hemostatic abnormalities in women with a history of severe preeclampsia. Am.J.Obstet.Gynecol. 1999;180:1146-50.
143. Jones DC, Hayslett JP. Outcome of pregnancy in women with moderate or severe renal insufficiency. N.Engl.J.Med. 1996;335:226-32.
144. Cnossen JS, de Ruyter-Hanhijarvi H, van der Post JA, Mol BW, Khan KS, Ter RG. Accuracy of serum uric acid determination in predicting pre-eclampsia: a systematic review. Acta Obstet.Gynecol.Scand. 2006;85:519-25.
145. Ness RB, Roberts JM. Heterogeneous causes constituting the single syndrome of preeclampsia: a hypothesis and its implications. Am.J.Obstet.Gynecol. 1996;175:1365-70.
146. von Dadelszen P, Magee LA, Roberts JM. Subclassification of preeclampsia. Hypertens.Pregnancy. 2003;22:143-48.
147. Page EW. The relation between hydatid moles, relative ischemia of the gravid uterus, and the placental origin of eclampsia. Am.J.Obstet.Gynecol. 1939;37:291-93.
148. Piering WF, Garancis JG, Becker CG, Beres JA, Lemann J, Jr. Preeclampsia related to a functioning extrauterine placenta: report of a case and 25-year follow-up. Am.J.Kidney Dis. 1993;21:310-13.
149. Rasmussen S, Irgens LM. Fetal growth and body proportion in preeclampsia. Obstet.Gynecol. 2003;101:575-83.
150. Paruk F, Moodley J. Maternal and neonatal outcome in early- and late-onset pre-eclampsia. Semin.Neonatol. 2000;5:197-207.
151. Sibai BM, Mercer B, Sarinoglu C. Severe preeclampsia in the second trimester: recurrence risk and long-term prognosis. Am.J.Obstet.Gynecol. 1991;165:1408-12.
152. Lain KY, Krohn MA, Roberts JM. Second pregnancy outcomes following preeclampsia in a first pregnancy. Hypertens.Pregnancy. 2005;24:159-69.
153. Clausen T, Djurovic S, Henriksen T. Dyslipidemia in early second trimester is mainly a feature of women with early onset pre-eclampsia. BJOG. 2001;108:1081-87.
154. Duckitt K, Harrington D. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. BMJ 2005;330:565.
155. Ness RB, Markovic N, Bass D, Harger G, Roberts JM. Family history of hypertension, heart disease, and stroke among women who develop hypertension in pregnancy. Obstet.Gynecol. 2003;102:1366-71.
156. Rodie VA, Freeman DJ, Sattar N, Greer IA. Pre-eclampsia and cardiovascular disease: metabolic syndrome of pregnancy? Atherosclerosis 2004;175:189-202.
157. Redman CW, Sargent IL. Placental debris, oxidative stress and pre-eclampsia. Placenta 2000;21:597-602.
158. Dekker GA, Sibai BM. The immunology of preeclampsia. Semin.Perinatol. 1999;23:24-33.

159. Redman CW.Immunology of preeclampsia.Semin.Perinatol.1991;15:257-62.
160. Moffett-King A. Natural killer cells and pregnancy. Nat.Rev.Immunol. 2002;2:656-63.
161. Jones CJ, Fox H. An ultrastructural and ultrahistochemical study of the human placenta in maternal pre-eclampsia. Placenta 1980;1:61-76.
162. Brosens IA, Robertson WB, Dixon HG. The role of the spiral arteries in the pathogenesis of preeclampsia. Obstet.Gynecol.Annu. 1972;1:177-91.
163. Dekker GA, Sibai BM. Etiology and pathogenesis of preeclampsia: current concepts. Am.J.Obstet.Gynecol. 1998;179:1359-75.
164. Eras JL, Saftlas AF, Triche E, Hsu CD, Risch HA, Bracken MB. Abortion and its effect on risk of preeclampsia and transient hypertension. Epidemiology 2000;11:36-43.
165. Dekker GA, Robillard PY, Hulsey TC. Immune maladaptation in the etiology of preeclampsia: a review of corroborative epidemiologic studies. Obstet. Gynecol.Surv. 1998;53:377-82.
166. Smith GN, Walker M, Tessier JL, Millar KG. Increased incidence of preeclampsia in women conceiving by intrauterine insemination with donor versus partner sperm for treatment of primary infertility. Am.J.Obstet.Gynecol. 1997;177:455-58.
167. Salha O, Sharma V, Dada T, Nugent D, Rutherford AJ, Tomlinson AJ et al. The influence of donated gametes on the incidence of hypertensive disorders of pregnancy. Hum.Reprod. 1999;14:2268-73.
168. Jauniaux E, Hempstock J, Greenwold N, Burton GJ. Trophoblastic oxidative stress in relation to temporal and regional differences in maternal placental blood flow in normal and abnormal early pregnancies. Am.J.Pathol. 2003;162:115-25.
169. Hung TH, Skepper JN, Charnock-Jones DS, Burton GJ. Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. Circ.Res. 2002;90:1274-81.
170. Mylonas C, Kouretas D. Lipid peroxidation and tissue damage. In Vivo 1999;13:295-309.
171. Cester N, Staffolani R, Rabini RA, Magnanelli R, Salvolini E, Galassi R et al. Pregnancy induced hypertension: a role for peroxidation in microvillus plasma membranes. Mol.Cell Biochem. 1994;131:151-55.
172. Chappell LC, Seed PT, Kelly FJ, Briley A, Hunt BJ, Charnock-Jones DS et al. Vitamin C and E supplementation in women at risk of preeclampsia is associated with changes in indices of oxidative stress and placental function. Am.J.Obstet.Gynecol. 2002;187:777-84.
173. Poston L, Briley AL, Seed PT, Kelly FJ, Shennan AH. Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. Lancet 2006;367:1145-54.
174. Burton GJ, Jauniaux E. Placental oxidative stress: from miscarriage to preeclampsia. J.Soc.Gynecol.Investig. 2004;11:342-52.
175. Shanklin DR, Sibai BM. Ultrastructural aspects of preeclampsia. I. Placental bed and uterine boundary vessels. Am.J.Obstet.Gynecol. 1989;161:735-41.
176. Roberts JM. Endothelial dysfunction in preeclampsia. Semin.Reprod.

Endocrinol. 1998;16:5-15.
177. Takacs P, Kauma SW, Sholley MM, Walsh SW, Dinsmoor MJ, Green K. Increased circulating lipid peroxides in severe preeclampsia activate NF-kappaB and upregulate ICAM-1 in vascular endothelial cells. FASEB J. 2001;15:279-81.
178. Wang YP, Walsh SW, Guo JD, Zhang JY. The imbalance between thromboxane and prostacyclin in preeclampsia is associated with an imbalance between lipid peroxides and vitamin E in maternal blood. Am.J.Obstet.Gynecol. 1991;165:1695-700.
179. Redman CW, Sacks GP, Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. Am.J.Obstet.Gynecol. 1999;180:499-506.
180. Fisher KA, Luger A, Spargo BH, Lindheimer MD. Hypertension in pregnancy: clinical-pathological correlations and remote prognosis. Medicine (Baltimore) 1981;60:267-76.
181. Wilson BJ, Watson MS, Prescott GJ, Sunderland S, Campbell DM, Hannaford P et al. Hypertensive diseases of pregnancy and risk of hypertension and stroke in later life: results from cohort study. BMJ 2003;326:845.
182. Smith GC, Pell JP, Walsh D. Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129,290 births. Lancet 2001;357:2002-06.
183. Goddard KA, Tromp G, Romero R, Olson JM, Lu Q, Xu Z et al. Candidate-gene association study of mothers with pre-eclampsia, and their infants, analyzing 775 SNPs in 190 genes. Hum.Hered. 2007;63:1-16.
184. Cnattingius S, Reilly M, Pawitan Y, Lichtenstein P. Maternal and fetal genetic factors account for most of familial aggregation of preeclampsia: a population-based Swedish cohort study. Am.J.Med.Genet.A 2004;130:365-71.
185. Thornton JG, Onwude JL. Pre-eclampsia: discordance among identical twins. BMJ 1991;303:1241-42.
186. Salonen RH, Lichtenstein P, Lipworth L, Cnattingius S. Genetic effects on the liability of developing pre-eclampsia and gestational hypertension. Am.J.Med. Genet. 2000;91:256-60.
187. Moses EK, Lade JA, Guo G, Wilton AN, Grehan M, Freed K et al. A genome scan in families from Australia and New Zealand confirms the presence of a maternal susceptibility locus for pre-eclampsia, on chromosome 2. Am.J.Hum. Genet. 2000;67:1581-85.
188. Arngrimsson R, Sigurard tS, Frigge ML, Bjarnad ttir RI, Jonsson T, Stefansson H et al. A genome-wide scan reveals a maternal susceptibility locus for pre-eclampsia on chromosome 2p13. Hum.Mol.Genet. 1999;8:1799-805.
189. Laivuori H, Lahermo P, Ollikainen V, Widen E, Haiva-Mallinen L, Sundstrom H et al. Susceptibility loci for preeclampsia on chromosomes 2p25 and 9p13 in Finnish families. Am.J.Hum.Genet. 2003;72:168-77.
190. Harrison GA, Humphrey KE, Jones N, Badenhop R, Guo G, Elakis G et al. A genomewide linkage study of preeclampsia/eclampsia reveals evidence for a candidate region on 4q. Am.J.Hum.Genet. 1997;60:1158-67.
191. Guo G, Lade JA, Wilton AN, Moses EK, Grehan M, Fu Y et al. Genetic susceptibility to pre-eclampsia and chromosome 7q36. Hum.Genet. 1999;105:641-47.

192. Lachmeijer AM, Arngrimsson R, Bastiaans EJ, Frigge ML, Pals G, Sigurdardottir S et al. A genome-wide scan for preeclampsia in the Netherlands. Eur.J.Hum. Genet. 2001;9:758-64.
193. Tyni T, Ekholm E, Pihko H. Pregnancy complications are frequent in long-chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency. Am.J.Obstet.Gynecol. 1998;178:603-08.
194. den Boer ME, Ijlst L, Wijburg FA, Oostheim W, van Werkhoven MA, van Pampus MG et al. Heterozygosity for the common LCHAD mutation (1528g>C) is not a major cause of HELLP syndrome and the prevalence of the mutation in the Dutch population is low. Pediatr.Res. 2000;48:151-54.
195. van Dijk M, Mulders J, Poutsma A, Konst AA, Lachmeijer AM, Dekker GA et al. Maternal segregation of the Dutch preeclampsia locus at 10q22 with a new member of the winged helix gene family. Nat.Genet. 2005.
196. Thornton JG, Sampson J. Genetics of pre-eclampsia. Lancet 1990;336:1319-20.
197. Yu CK, Casas JP, Savvidou MD, Sahemey MK, Nicolaides KH, Hingorani AD. Endothelial nitric oxide synthase gene polymorphism (Glu298Asp) and development of pre-eclampsia: a case-control study and a meta-analysis. BMC.Pregnancy.Childbirth. 2006;6:7.
198. Nasiell J, Nisell H, Blanck A, Lunell NO, Faxen M. Placental expression of endothelial constitutive nitric oxide synthase mRNA in pregnancy complicated by preeclampsia. Acta Obstet.Gynecol.Scand. 1998;77:492-96.
199. Yoshimura T, Yoshimura M, Tabata A, Shimasaki Y, Nakayama M, Miyamoto Y et al. Association of the missense Glu298Asp variant of the endothelial nitric oxide synthase gene with severe preeclampsia. J.Soc.Gynecol.Investig. 2000;7:238-41.
200. Faxen M, Nisell H, Kublickiene KR. Altered mRNA expression of ecNOS and iNOS in myometrium and placenta from women with preeclampsia. Arch. Gynecol.Obstet. 2001;265:45-50.
201. Tempfer CB, Dorman K, Deter RL, O'Brien WE, Gregg AR. An endothelial nitric oxide synthase gene polymorphism is associated with preeclampsia. Hypertens.Pregnancy. 2001;20:107-18.
202. Orange SJ, Painter D, Horvath J, Yu B, Trent R, Hennessy A. Placental endothelial nitric oxide synthase localization and expression in normal human pregnancy and pre-eclampsia. Clin.Exp.Pharmacol.Physiol 2003;30:376-81.
203. Hakli T, Romppanen EL, Hiltunen M, Helisalmi S, Punnonen K, Heinonen S. Endothelial nitric oxide synthase polymorphism in preeclampsia. J.Soc. Gynecol.Investig. 2003;10:154-57.
204. Yoshimura T, Chowdhury FA, Yoshimura M, Okamura H. Genetic and environmental contributions to severe preeclampsia: lack of association with the endothelial nitric oxide synthase Glu298Asp variant in a developing country. Gynecol.Obstet.Invest 2003;56:10-13.
205. Landau R, Xie HG, Dishy V, Wood AJ, Stein CM, Smiley RM. No association of the Asp298 variant of the endothelial nitric oxide synthase gene with preeclampsia. Am.J.Hypertens. 2004;17:391-94.
206. Ohta K, Kobashi G, Hata A, Yamada H, Minakami H, Fujimoto S et al. Association between a variant of the glutathione S-transferase P1 gene

(GSTP1) and hypertension in pregnancy in Japanese: interaction with parity, age, and genetic factors. Semin.Thromb.Hemost. 2003;29:653-59.
207. Serrano NC, Casas JP, Diaz LA, Paez C, Mesa CM, Cifuentes R et al. Endothelial NO synthase genotype and risk of preeclampsia: a multicenter case-control study. Hypertension 2004;44:702-07.
208. Wang Y, Gu Y, Zhang Y, Lewis DF. Evidence of endothelial dysfunction in preeclampsia: decreased endothelial nitric oxide synthase expression is associated with increased cell permeability in endothelial cells from preeclampsia. Am.J.Obstet.Gynecol. 2004;190:817-24.
209. Hillermann R, Carelse K, Gebhardt GS. The Glu298Asp variant of the endothelial nitric oxide synthase gene is associated with an increased risk for abruptio placentae in pre-eclampsia. J.Hum.Genet. 2005;50:415-19.
210. Thatcher CD, Keith JC, Jr. Pregnancy-induced hypertension: development of a model in the pregnant sheep. Am.J.Obstet.Gynecol. 1986;155:201-07.
211. Combs CA, Katz MA, Kitzmiller JL, Brescia RJ. Experimental preeclampsia produced by chronic constriction of the lower aorta: validation with longitudinal blood pressure measurements in conscious rhesus monkeys. Am.J.Obstet. Gynecol. 1993;169:215-23.
212. Isler CM, Bennett WA, Rinewalt AN, Cockrell KL, Martin JN, Jr., Morrison JC et al. Evaluation of a rat model of preeclampsia for HELLP syndrome characteristics. J.Soc.Gynecol.Investig. 2003;10:151-53.
213. Chang EY, Barbosa E, Paintlia MK, Singh A, Singh I. The use of N-acetylcysteine for the prevention of hypertension in the reduced uterine perfusion pressure model for preeclampsia in Sprague-Dawley rats. Am.J.Obstet.Gynecol. 2005;193:952-56.
214. Faas MM, Schuiling GA, Baller JF, Visscher CA, Bakker WW. A new animal model for human preeclampsia: ultra-low-dose endotoxin infusion in pregnant rats. Am.J.Obstet.Gynecol. 1994;171:158-64.
215. Ianosi-Irimie M, Vu HV, Whitbred JM, Pridjian CA, Nadig JD, Williams MY et al. A rat model of preeclampsia. Clin.Exp.Hypertens. 2005;27:605-17.
216. Mayr AJ, Lederer W, Wolf HJ, Dunser M, Pfaller K, Mortl MG. Morphologic changes of the uteroplacental unit in preeclampsia-like syndrome in rats. Hypertens.Pregnancy. 2005;24:29-37.
217. Rutland CS, Mukhopadhyay M, Underwood S, Clyde N, Mayhew TM, Mitchell CA. Induction of intrauterine growth restriction by reducing placental vascular growth with the angioinhibin TNP-470. Biol.Reprod. 2005;73:1164-73.
218. Nash P, Wentzel P, Lindeberg S, Naessen T, Jansson L, Olovsson M et al. Placental dysfunction in Suramin-treated rats--a new model for pre-eclampsia. Placenta 2005;26:410-18.
219. Sharkey LC, McCune SA, Yuan O, Lange C, Fray J. Spontaneous pregnancy-induced hypertension and intrauterine growth restriction in rats. Am.J.Hypertens. 2001;14:1058-66.
220. Fernandez CL, Carbajo RM, Munoz RM. Intrauterine growth restriction in spontaneously hypertensive rats. Hypertens.Pregnancy. 2004;23:275-83.
221. Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat.Med.

2006;12:642-49.
222. Kanayama N, Takahashi K, Matsuura T, Sugimura M, Kobayashi T, Moniwa N et al. Deficiency in p57Kip2 expression induces preeclampsia-like symptoms in mice. Mol.Hum.Reprod. 2002;8:1129-35.
223. Davisson RL, Hoffmann DS, Butz GM, Aldape G, Schlager G, Merrill DC et al. Discovery of a spontaneous genetic mouse model of preeclampsia. Hypertension 2002;39:337-42.
224. Arbogast BW, Leeper SC, Merrick RD, Olive KE, Taylor RN. Which plasma factors bring about disturbance of endothelial function in pre-eclampsia? Lancet 1994;343:340-41.
225. Hung TH, Charnock-Jones DS, Skepper JN, Burton GJ. Secretion of tumor necrosis factor-alpha from human placental tissues induced by hypoxia-reoxygenation causes endothelial cell activation in vitro: a potential mediator of the inflammatory response in preeclampsia. Am.J.Pathol. 2004;164:1049-61.
226. Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular biology of the cell. New York: Garland Publishing, 1989.
227. Cross JC, Baczyk D, Dobric N, Hemberger M, Hughes M, Simmons DG et al. Genes, development and evolution of the placenta. Placenta 2003;24:123-30.
228. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985;230:1350-54.
229. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res. 1996;6:986-94.
230. Southern EM. DNA microarrays. History and overview. Methods Mol.Biol. 2001;170:1-15.
231. Yang IV, Chen E, Hasseman JP, Liang W, Frank BC, Wang S et al. Within the fold: assessing differential expression measures and reproducibility in microarray assays. Genome Biol. 2002;3:research0062.
232. Ioannidis JP. Microarrays and molecular research: noise discovery? Lancet 2005;365:454-55.
233. Aronow BJ, Richardson BD, Handwerger S. Microarray analysis of trophoblast differentiation: gene expression reprogramming in key gene function categories. Physiol Genomics 2001;6:105-16.
234. Chen HW, Chen JJ, Tzeng CR, Li HN, Chang SJ, Cheng YF et al. Global analysis of differentially expressed genes in early gestational decidua and chorionic villi using a 9600 human cDNA microarray. Mol.Hum.Reprod. 2002;8:475-84.
235. Bilban M, Head S, Desoye G, Quaranta V. DNA microarrays: a novel approach to investigate genomics in trophoblast invasion--a review. Placenta 2000;21 Suppl A:S99-105.
236. Soleymanlou N, Jurisica I, Nevo O, Ietta F, Zhang X, Zamudio S et al. Molecular evidence of placental hypoxia in preeclampsia. J.Clin.Endocrinol. Metab 2005;90:4299-308.
237. Tsoi SC, Cale JM, Bird IM, Kay HH. cDNA microarray analysis of gene expression profiles in human placenta: up-regulation of the transcript encoding muscle subunit of glycogen phosphorylase in preeclampsia. J.Soc.Gynecol.Investig. 2003;10:496-502.

238. Pang ZJ, Xing FQ. DNA microarrays detect the expression of apoptosis-related genes in preeclamptic placentas. J.Perinat.Med. 2004;32:25-30.
239. Pineles BL, Romero R, Montenegro D, Tarca AL, Han YM, Kim YM et al. Distinct subsets of microRNAs are expressed differentially in the human placentas of patients with preeclampsia. Am.J.Obstet.Gynecol. 2007;196:261-66.
240. Hansson SR, Chen Y, Brodszki J, Chen M, Hernandez-Andrade E, Inman JM et al. Gene expression profiling of human placentas from preeclamptic and normotensive pregnancies. Mol.Hum.Reprod. 2006;12:169-79.
241. Durand S, Abadie P, Angeletti S, Genti-Raimondi S. Identification of multiple differentially expressed messenger RNAs in normal and pathological trophoblast. Placenta 2003;24:209-18.
242. Reimer T, Koczan D, Gerber B, Richter D, Thiesen HJ, Friese K. Microarray analysis of differentially expressed genes in placental tissue of pre-eclampsia: up-regulation of obesity-related genes. Mol.Hum.Reprod. 2002;8:674-80.
243. Heikkila A, Tuomisto T, Hakkinen SK, Keski-Nisula L, Heinonen S, Yla-Herttuala S. Tumor suppressor and growth regulatory genes are overexpressed in severe early-onset preeclampsia--an array study on case-specific human preeclamptic placental tissue. Acta Obstet.Gynecol.Scand. 2005;84:679-89.
244. Zhou R, Zhu Q, Wang Y, Ren Y, Zhang L, Zhou Y. Genomewide Oligonucleotide Microarray Analysis on Placentae of Pre-Eclamptic Pregnancies. Gynecol. Obstet.Invest 2006;62:108-14.
245. Nishizawa H, Pryor-Koishi K, Kato T, Kowa H, Kurahashi H, Udagawa Y. Microarray analysis of differentially expressed fetal genes in placental tissue derived from early and late onset severe pre-eclampsia. Placenta 2007;28:487-97.
246. Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. Science 1995;270:484-87.
247. Polyak K, Riggins GJ. Gene discovery using the serial analysis of gene expression technique: implications for cancer research. J.Clin.Oncol. 2001;19:2948-58.

Chapter 2:

Ted (G.J.) Kloosterman: On Intrauterine Growth, The Significance of Prenatal Care. Studies on Birth Weight, Placental Weight and Placental Ratio

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Abstract

Kloosterman studied 80.000 birth weights and 30.000 placental weights in relation to gestational age at birth, fetal sex, maternal parity, and perinatal mortality. He concluded that pregnancies with heavier placentas last longer. He also concluded that from about 30 weeks of gestational weeks onwards, children from primiparous women as compared to those from multiparous women, and twin children as compared to singleton children are relatively growth retarded, most likely related to prior relatively poor placental growth. Obviously, poor fetal growth is not the *cause*, but the *result* of poor placental growth.
Future early detection of poor placental growth may prospect poor fetal growth, and may even allow for early interventions to improve fetal outcome.

From 1954 until 1970 Ted Kloosterman published several articles on the significance of birth weight, placental weight, and Placental Ratio.[1-4] His 1970 keynote paper 'On intrauterine growth', was based on a group of 80,000 consecutive singleton pregnancies of women, visiting two clinics in Amsterdam (the Training School for Midwives and the University Clinic at the University of Amsterdam) between 1931 and 1967.[4] From these women, 30,000 consecutive placentas were processed and weighed by one single and devoted person, miss Huidekoper, a midwife and scientific co-worker of Kloosterman. According to a standard protocol, directly after birth, all blood clots were removed, the membranes were trimmed off along the edge of the placenta, the umbilical cord was cut within 2 cm and ligated after being allowed to bleed freely. The placenta was placed in a 10% formalin solution and weighed within a week. Insertion of the umbilical cord, and the macroscopic stage of 'infarction', observed on placental slices were registered.[1-4]

In those days a heated discussion took place on the significance of a small placenta with respect to the fetal growth. According to Fox and Gruenwald, a small placenta, being a *fetal* organ, just as a small fetal liver, was a manifestation rather than a cause of poor fetal growth.[5;6] They therefore saw 'no reason why the practice of routinely weighing the placenta should be continued'. Kloosterman on the other hand argued that a baby is small *because* the placenta is small.[4] In the next paragraphs we will provide the data on which Kloosterman based his arguments.

Birth weight, placental weight and Placental Ratio in relation to gestational age

From cross-sectional birth weight data, fetal growth seems to decelerate after 38 weeks. Kloosterman concluded that, as 4 weeks after birth acceleration of growth of the newborn occurs, the cause of the intrauterine deceleration of growth must be a limitation of the maternal supply line; that is the placenta and the mother.

Data on placental weight suggest a constant rate of growth during pregnancy unto and even beyond term, which he considered unlikely for an organ at the end of its lifespan.

At 20 weeks, the mean Placental Ratio (PR), i.e. placental weight divided by birth weight, is found to be about 35%, and subsequently to decrease unto 15% at 38 weeks, as the fetus grows more rapidly than the placenta. Oddly enough, after the 38th week, the Ratio did remain on the same level.

Kloosterman thought it very unlikely that the fetus decreases its growth very dramatically after 38 weeks, or that the placenta will accelerate its growth at the end of its lifespan. His elegant explanation for these findings was, that in

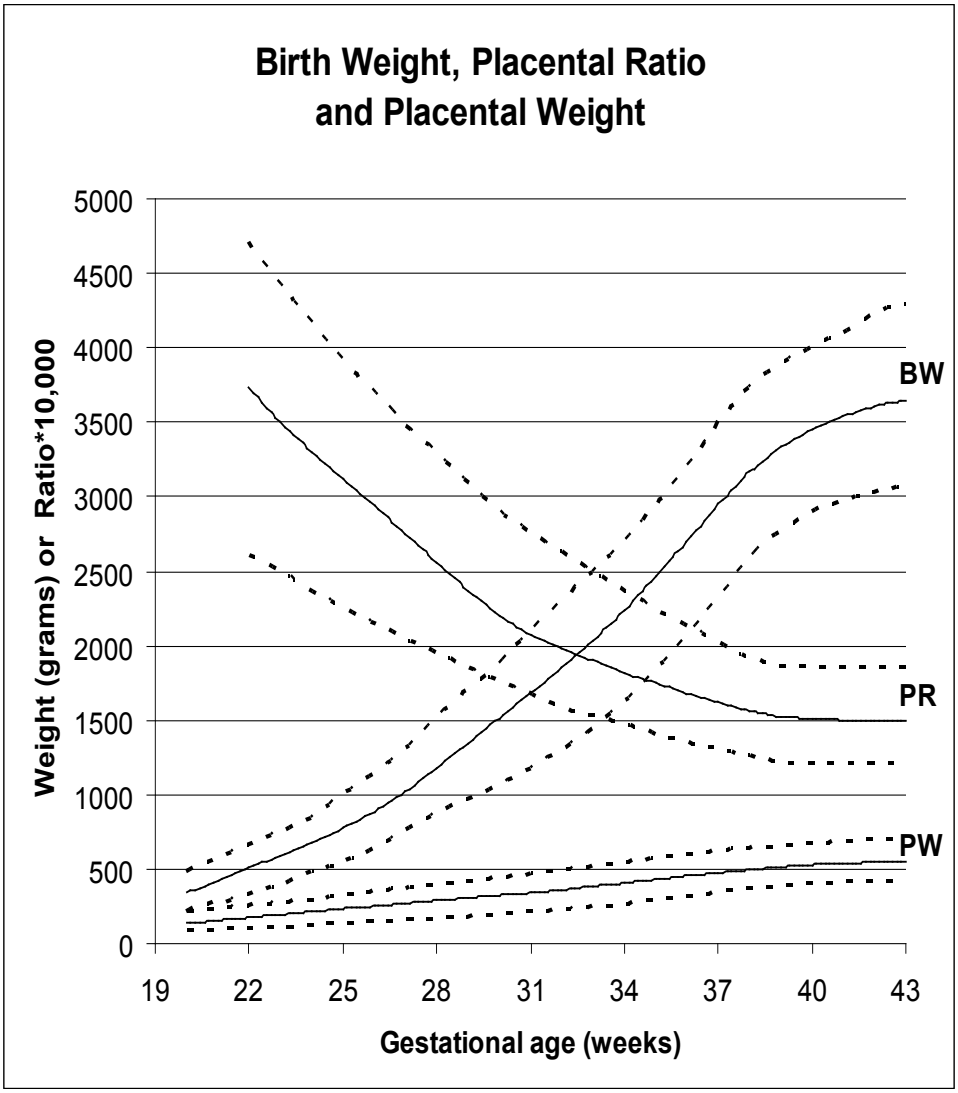

Figure 1: Birth Weight, Placental Weight and Placental Ratio.
Mean (continuous line) and 10th and 90th centiles of birth weight (BW), placental weight (PW) and Placental Ratio (PR) in singletons, by gestational age, according to Kloosterman's key article 'On Intrauterine Growth'; Figure 1, and Tables 5 and 7.[4]

the presence of a relatively heavier placenta, pregnancies last longer, which means that the placental 'growth curve', based on cross-sectional data, does not reflect real longitudinal growth.[2-4]

Influences of fetal sex, maternal parity

Boys are by 24 weeks of pregnancy, heavier than girls, the mean difference at 40 weeks being 140 g. Placental weights of boys and girls are the same throughout pregnancy,[1-4] Kloosterman concluded that boys, being heavier, demand more from their placentas as compared to girls. As expected, boys show indeed a relatively higher perinatal mortality, as compared to girls, especially after 40 weeks of gestational age.[2] Children from multiparous women, from about 32 weeks onwards, are heavier than children from primiparous women, the mean difference at 40 weeks being 200 g.[1-4] In multiparous women, the placentas are also heavier than in primiparous women, already from 25 weeks onwards.[1,4]

Kloosterman concluded that multiparous women offer, through remodelling of the maternal vasculature in former pregnancies, a more favourable environment for both placental development and placental function in next pregnancies.[2-4]

Taken together, these data suggest that the influence of fetal sex with respect to intrauterine growth is limited to the fetus and not extended to the placenta. This also suggests that, compared to children from multiparous women, children from primiparous women are relatively growth retarded, most likely due to a prior relatively poor placental growth. It is therefore not surprising that children from primiparous women show higher perinatal mortality, as compared to those from multiparous women, especially at and after 40 weeks of gestational age.[2;4]

Influence of litter size

Kloosterman found that, in comparison to singletons, the mean birth weight of twin children is found to be smaller, from about the 32nd week, the difference at 39 to 40 weeks being about 600 g.[3;7;8] Where the mean placental weight of twin children as compared to singleton children is found to be consistently smaller from 22 weeks to term, they concluded that, compared to singleton children, twin children are relatively growth retarded, most likely due to a prior relatively poor placental growth. These observations, though from cross-sectional data, confirm Kloosterman's concept, that smaller placentas are the *cause* and not the result of poor fetal development.[4]

McKeown and Record[9] and Gruenwald[10] who found higher PRs in twins as compared to singletons, concluded that relatively more placental tissue is available to the twin fetus and therefore, the restriction of fetal growth in twins (after 32 weeks) must be due to influences outside the placenta. In

contrast, Kloosterman's co-workers found lower PRs in twins, up to 37 to 38 weeks, and therefore the placenta may very well limit fetal growth in twins. After 38 weeks, in twins, the PRs were found to be relatively higher as compared to singletons, which was explained by twin pregnancies, related to relatively heavier placentas, last longer.[7;8]

The small placenta
Kloosterman's first publication on intrauterine growth dealt with 'the significance of the placenta in obstetrical mortality', and was based on the first 2000 singleton placentas, examined as described above. The lowest perinatal mortality of 1.6% was found in placental weight groups of 500 to 800 g. In lower placental weight groups a much higher perinatal mortality was met: 43.2% among the 200-300 g group, and as high as 97% among the less than 200 g group, the majority being ante partum mortality in that last group.[1]
In 1983, his co-worker miss Huidekoper published an additional study on 'the significance of the small placenta', based on 227 singleton pregnancies with placentas of 100-200 g, born in the University Clinic at the University of Amsterdam, in 1958-1981.[11] These very small placentas were found to be related to higher incidences of preeclampsia, placental infarction, lower birth weight, and perinatal mortality, especially ante partum mortality.

Comments
The historic significance of the work of Kloosterman is the study of consecutive birth weights, according to carefully assessed gestational ages, related to placental weights, measured according to a rigorous protocol. Even today this set of data is the largest ever reported. A limitation of his studies is that inferences are made on longitudinal fetal and placental growth, based on a cross-sectional design.

Of high interest are Kloosterman's observations on the relationship between fetal and placental growth. His cross-sectional curves demonstrate that poor placental development, as observed in primiparous women versus multiparous women, and in twins versus singletons, precedes poor fetal growth by many weeks of gestation. These observations seem to confirm his conclusion, that small placentas may be very well the cause, and not the consequence of poor fetal growth.
Kloosterman's observations point to the importance of the first half of pregnancy with respect to placental development. Most likely even the first trimester of pregnancy, being essential for early placental development, defines the course of pregnancy, and the outcome of pregnancy.

Recent ultrasound studies on the relationship between early (12-26 weeks) placental volume and fetal growth seem to confirm this suggested sequence of events: poor placental growth precedes poor fetal growth, lower second trimester placental volumes and lower placental growth rates are good predictors of lower birth weights, and even lower first trimester placental volume seems to be related to lower birth weight at term.[12-15] In daily practice, estimation of placental size by ultrasound, and other techniques, is still a technical problem and very time consuming. Improved ultrasound or other visual techniques may enable to estimate placental size in early pregnancy, in normal clinical settings and with enough accuracy. Future early detection of poor placental size, may prospect poor fetal growth, and may even allow for early interventions to improve fetal outcome.

Reference List

1. Kloosterman GJ, Huidekoper BL. The significance of the placenta in obstetrical mortality; a study of 2000 births. Gynaecologia. 1954;138:529-50.
2. Kloosterman GJ. Prolonged pregnancy. Gynaecologia. 1956;142:372-88.
3. Kloosterman GJ. Prevention of prematurity. Ned.Tijdschr.Verloskd.Gynaecol. 1966;66:361-79.
4. Kloosterman GJ. On intrauterine growth. Int.J.Gynaecol.Obstet. 1970;8:895-912.
5. Fox H. Pathology of the Placenta. London: W.B.Saunders, 1978.
6. Gruenwald P. The supply line of the fetus; definitions relating to fetal growth. In: Gruenwald P, editor. The Placenta. Lancaster: Medical and Technical Publishing Co.; 1983. p. 4-5.
7. van Bilderbeek, J. Twins. 1960. University of Amsterdam.
8. Bleker OP, Breur W, Huidekoper BL. A study of birth weight, placental weight and mortality of twins as compared to singletons. Br.J.Obstet.Gynaecol. 1979;86:111-18.
9. Mc Keown T, Record R. The influence of placental size on foetal growth in man, with special reference to multiple pregnancy. J.Endocrinol. 1953;9:418-26.
10. Gruenwald P. Environmental influences on twins apparent at birth. A preliminary study. Biol.Neonate 1970;15:79-93.
11. Huidekooper BL. The too small placenta. In: Treffers PE, Schutte MF, Bleker OP, et al, editors. Progress and Vision.(in Dutch). Utrecht/Antwerp: Bohn Scheltema Holkema; 1983. p. 109-16.
12. Wolf H, Oosting H, Treffers PE. A longitudinal study of the relationship between placental and fetal growth as measured by ultrasonography. Am.J.Obstet. Gynecol. 1989;161:1140-45.
13. Clapp JF, III, Rizk KH, Appleby-Wineberg SK, Crass JR. Second-trimester placental volumes predict birth weight at term. J.Soc.Gynecol.Investig. 1995;2:19-22.
14. Metzenbauer M, Hafner E, Hoefinger D, Schuchter K, Philipp K. [Associations between birth weight and placental volume in the first trimester]. Z.Geburtshilfe Neonatol. 2002;206:138-41.
15. Hafner E, Metzenbauer M, Hofinger D, Munkel M, Gassner R, Schuchter K et al. Placental growth from the first to the second trimester of pregnancy in SGA-foetuses and pre-eclamptic pregnancies compared to normal foetuses. Placenta 2003;24:336-42.

Chapter 3:

Birth weight ratio is a valuable clinical and research tool for fetal growth restriction

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Submitted

Abstract

Background: Accurate assessment of fetal growth is a principal tool in antenatal care and individual results are usually compared to gestational age-specific centile curves. Reports of obstetrical outcomes in research populations generally use absolute birth weights, and secondarily dichotomize birth weights according to the 10th centile for gestational age. Use of dichotomized data at the extremes discards important information.

Objectives: We coin the term 'Birth Weight Ratio', as the ratio of observed birth weight divided by the mean birth weight of the population-specific reference growth curve

Methods: To demonstrate the advantages of the Birth Weight Ratio, we explored the data from a recent randomized trial in a patient group with early preterm hypertensive complications of pregnancy. The functionality of the Birth Weight Ratio was explored in this patient group.

Results: At delivery women had a median gestational age of $31^{2}/_{7}$ weeks (range $26^{1}/_{7}$ -$38^{3}/_{7}$). The practical advantages were 1. improved classification of growth restriction as the scale is continuous and not dichotomized. 198 (92%) of infants had a birth weight below the 10th centile. If necessary, tailored dichotomization remains possible; 2. less influence by outliers in the reference curve, especially at lower gestational ages; the difference between Birth Weight Ratios calculated by two different charts is smaller than the differences between centile scores, and less dependent of weight. This shows that the Birth Weight Ratio is more suitable for international comparison of studies on fetal growth restriction.

Conclusion: We advocate to give Birth Weight Ratio a priority position to complement gestational age and absolute birth weights as predictors in reports on obstetric research populations.

Introduction

Gestational age and fetal growth restriction at birth are important determinants for perinatal mortality and morbidity. Accurate assessment of fetal growth in relationship with gestational age is therefore a principal tool in antenatal care. Results are usually compared to centile curves, which are calculated from cross-sectionally acquired data of newborns and dichotomized according to the 10th centile for gestational age. We coin the term 'Birth Weight Ratio' as the ratio of observed birth weight divided by the mean birth weight of the population-specific reference growth curve. Values above 1 indicate 'larger for gestational age than average' and values below 1 indicate 'smaller for gestational age than average'. We argue it complements birth weight and gestational age in clinical practice, and can validly replace dichotomization by centile score or another alternative, standard deviation score. We would like to discuss three theoretical arguments

I. No data reduction

Use of the Birth Weight Ratio transforms data into another continuous variable, as opposed to ordinal classes when a percentile is used for classification. This has the advantage that the original value can always be deducted from the transformed value without loss of information, which is not possible when ordinal classes are used. Although, by use of a z-score, percentiles might be used as a semi-continuous parameter, it is customary to dichotomize by the 10th centile, or alternatively the 5th or 2.3rd centile. Dichotomization can be a valuable simplification, despite the fact that there are no specific cut-offs that delineate normal from abnormal growth.[1] However, in tertiary care centers, the 10th centile of birth weight as a threshold classifies a majority of premature deliveries with fetal growth restriction. Thus, in groups at the extremes (e.g. severe fetal growth restriction) important information is lost.

II. Minimized disturbing influence from small sample size (or outliers in reference curves)

The Birth Weight Ratio makes use of the mean of a population reference curve. Many population-specific curves have been constructed from cross-sectional data. In a statistically normal distribution (even if skewed, as birth weights are), the numbers of observations are largest at the mean and outliers have little influence on the mean. This applies especially to low gestational age groups, where the number of cases in most charts is small, and lower or higher centile limits are therefore prone to bias. For these reasons, when relating an individual observation to a reference curve, comparisons using the mean are more reliable.

III. Superior options for statistical analysis

In contrast to weight centiles, the Birth Weight Ratio is expressed on a continuous and linear scale. This allows more powerful statistical analysis. The interpretation of absolute birth weights is gestational age-specific as 1,100 grams may be 'normal' for 28 weeks but 'abnormal' for 30 weeks, 'significantly abnormal' for 32 weeks and 'very abnormal' for 34 weeks. While birth weight in the latter three cases is below the 10th centile, the Birth Weight Ratios are 0.92, 0.72, 0.59 and 0.49 respectively. By use of the Birth Weight Ratio important information is retained for analysis. This provides better opportunity to assess the quantitative correlation and dose-effect dependency between growth restriction and possible causative influences or consequences. Birth Weight Ratio opens opportunities for the uniform interpretation of research results of tertiary care centers in meta-analyses.

Clinical application

To demonstrate the advantages of the Birth Weight Ratio as described above, we explored the data from a recent randomized trial comparing the outcomes of temporizing management with or without plasma volume expansion.[2] Women with early preterm hypertensive complications of pregnancy and a singleton pregnancy participated in this study (n=216). At delivery women had a median gestational age of $31^{2}/_{7}$ weeks (range $26^{1}/_{7}$ -$38^{3}/_{7}$). The primary endpoint of the study was an infant neurological test at 40 weeks corrected age.[3] This test was performed in 177 of 180 infants alive at term age. It was abnormal in 11 cases. For this study we defined Abnormal Neurological Outcome as the composite measure of death up to one year or abnormal neurological test (n=48). As a secondary composite short term outcome we defined Adverse Neonatal Outcome as death up to one year, major cerebral ultrasound abnormality (intracerebral hemorrhage >grade 2, periventricular leucomalacia > grade 1, or hydrocephalus) or bronchopulmonary dysplasia (additional oxygen after 36 weeks corrected age).[4-6] Fifty-five infants were classified as having Adverse Neonatal Outcome. Death until one year had occurred in 38 cases.

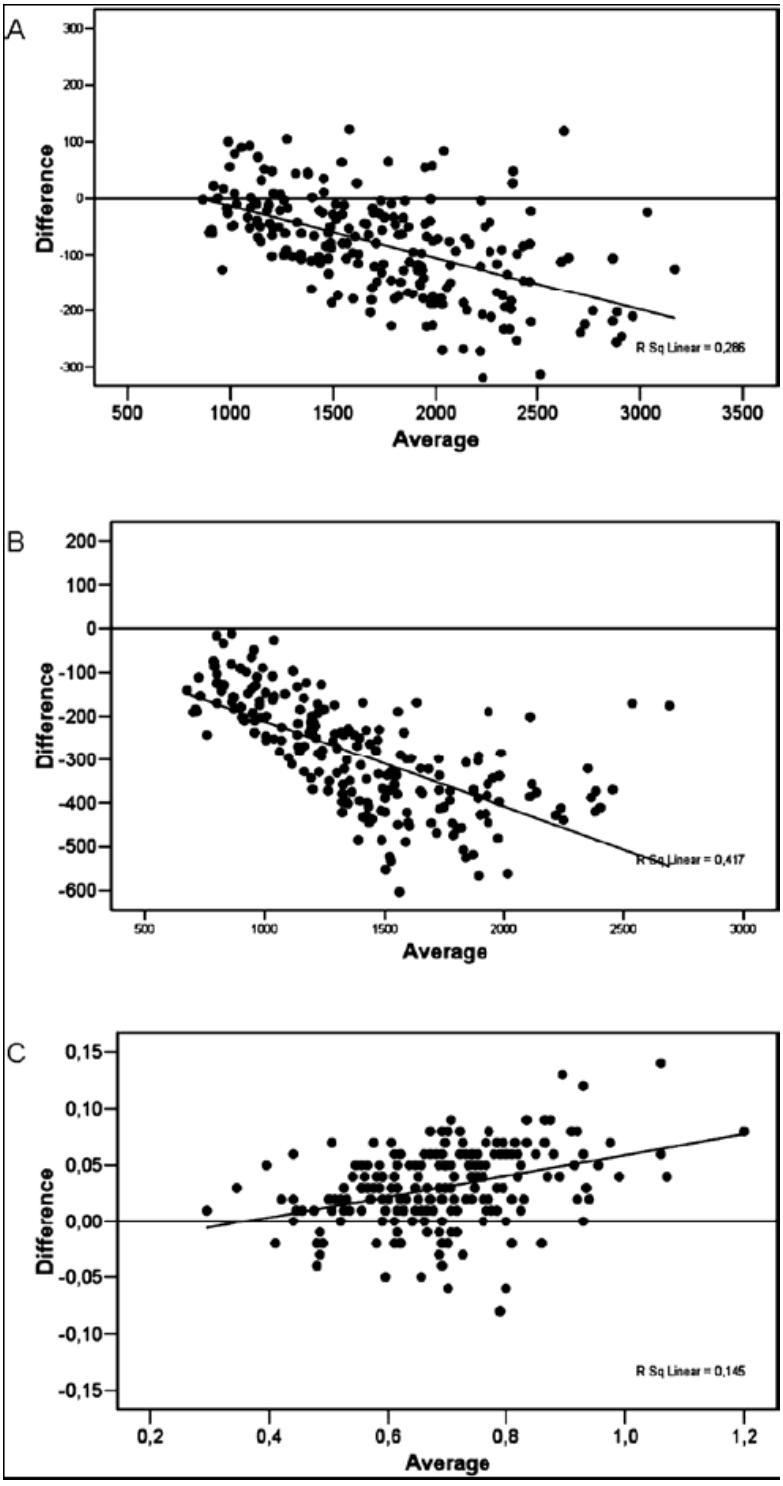
In the trial, the customized birth weight chart of Gardosi was used for reference of birth weight. This birth weight chart adjusts for gestational age, infant sex and maternal physiological variables (weight, length, parity end ethnic descent).[7] For the analysis presented here this chart was compared to the Kloosterman chart that is commonly used in The Netherlands.[8] Statistical analysis was performed with SPSS 12.0.2 (Chicago, U.S.A).

I. No data reduction:
At birth, 198 (92%) of infants had a birth weight below the 10th centile (the 10th centile corresponds to a Birth Weight Ratio of 0.86). Median Birth Weight Ratio of the study population was 0.67 (range 0.29-1.16). Obviously Birth Weight Ratio describes the severity of the fetal growth restriction in the study population more effectively than a 10th centile distribution.

II. Minimized disturbing influence from small sample size or outliers in reference curves:
The mean difference between the 50th centile weight of Kloosterman and Gardosi in the study population was -82 gram (SD 89, range -94 to -70).
In Figure 1A the difference between the 50th centile weight of the Kloosterman and the Gardosi chart is plotted against the average of both measures (Bland-Altman plot). The mean difference for the 10th centile of both charts was -289 (SD 128, range -306 to -272), as depicted in Figure 1B. The 50th centile and the 10th centile weights diverge substantially, with smaller weights in the Kloosterman chart.

In addition, this difference increases with higher birth weight and is larger for the 10th centile weight than for the 50th centile. In contrast, the difference between Birth Weight Ratios calculated by the Kloosterman chart or the Gardosi chart is small (mean 0.030, SD 0.034, range 0.025-0.035), and less dependent of weight, as depicted in Figure 1C. This demonstrates that even if birth weight charts show differences at the extremes the Birth Weight Ratios are more comparable, which makes this measure suitable for international comparison of studies on fetal growth restriction.

III. Superior options for statistical analysis
By use of discriminant analysis we defined the optimum cut-off for the Birth Weight Ratio to predict Abnormal Neurological Outcome and Abnormal Neonatal Outcome. A Birth Weight Ratio below 0.65 proved to be the optimum cut-off to predict Abnormal Neurological Outcome (odds ratio of 2.6 [95% CI 1.3 – 4.9; P = 0.005; sensitivity 60%, specificity 63%]) and Abnormal Neonatal Outcome (odds ratio of 2.6 [95% CI 1.4-4.9; P = 0.003; sensitivity 60%, specificity 63%]). When using the 10th centile of the Gardosi weight chart for prediction the odds ratios were respectively 2.5 (95% CI 0.5 - 11; P = 0.38; sensitivity 96%, specificity 10%) and 2.9 (95% CI 0.7-13; P = 0.16; sensitivity 96%, specificity 10%). This example shows that with a continuous measure, the statistics can be easily adapted to best fit the needs for each specific situation.



Discussion

The Birth Weight Ratio is not a new parameter,[9;10] but so far did not receive formal acclaim.[11;12] To avoid any misunderstanding: Birth Weight Ratio should not replace, but complement gestational age and absolute birth weights as predictors in reports on obstetric research populations. We demonstrated that application of a Birth Weight Ratio allows for better data description, less dependency on the type of birth weight chart and more appropriate statistics than any centile threshold.

Although dichotomisation in itself may be valuable, we believe, along with other authors,[1] there is a need for a continuous measure of the quantity of difference between observed weight and the mean or median for gestational age. Based on statistical presumption the use of multiples of the standard deviation or a semi-continuous percentile distribution might be preferred. However, in clinical practice these measures are scarcely used, probably because they require complex tables. The advantage of the Birth Weight Ratio is that it is easy to implement and that it allows simple statistics due to its linear nature. The Birth Weight Ratio may also be a valuable tool in counseling parents in clinical practice: "Your baby weighs about 75% of what is average for its gestational age" may better convey the message to parents than "Your baby is more than 2.3 standard deviations from what is average for its gestational age" or "… below the 1st centile of our reference chart".

Conclusion

We think sufficient arguments exist to give Birth Weight Ratio a priority position: it improves comparability, it is reliable, it is easy to understand, easy to use, and provides additional insight. In our opinion, editors of medical journals should encourage that reports on high-risk perinatal populations contain the Birth Weight Ratio.

Figure 1, opposite: Agreement of different Birth Weight measures
1A: Bland-Altman plot of agreement between the 50 th centile Kloosterman birth weight chart and the 50 th centile birth weight from the Gardosi chart in the study population.
1B: Bland-Altman plot of agreement between 10th centile birth weight from the Kloosterman chart with the 10th centile birth weight from the Gardosi chart in the study population.
1C: Bland-Altman plot of agreement between Birth Weight Ratio calculated from the Kloosterman chart with Birth Weight Ratio calculated from the Gardosi chart in the study population.

Reference List

1. Steer PJ. The Management of Large and Small for Gestational Age Fetuses. Semin.Perinatol. 2004;28:59-66.
2. Ganzevoort W, Rep A, Bonsel GJ, Fetter WPF, van Sonderen L, de Vries JIP et al. A randomised controlled trial comparing a temporising management strategy with plasma volume expansion with one without plasma volume expansion. Br. J.Obstet.Gynaecol. 2005;112:1358-68.
3. Prechtl HF, Beintema D. The Neurological Examination of the Full-Term Newborn Infant. London: Spastics International Medical Publications/William Heinemann Medical Books, 1964.
4. Volpe JJ. Intraventricular hemorrhage and brain injury in the premature infant. Diagnosis, prognosis, and prevention. Clin.Perinatol. 1989;16:387-411.
5. De Vries LS, Eken P, Dubowitz LM. The spectrum of leukomalacia using cranial ultrasound. Behav.Brain Res. 1992;49:1-6.
6. Shennan AT, Dunn MS, Ohlsson A, Lennox K, Hoskins EM. Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period. Pediatrics 1988;82:527-32.
7. Gardosi J, Chang A, Kalyan B, Sahota D, Symonds EM. Customised antenatal growth charts. Lancet 1992;339:283-87.
8. Kloosterman GJ. On intrauterine Growth - The significance of Prenatal Care. International Journal of Gynaecology and Obstetrics 1970;8:895-912.
9. Wilcox MA, Johnson IR, Maynard PV, Smith SJ, Chilvers CE. The individualised birthweight ratio: a more logical outcome measure of pregnancy than birthweight alone. Br.J.Obstet.Gynaecol. 1993;100:342-47.
10. Morley R, Brooke OG, Cole TJ, Powell R, Lucas A. Birthweight ratio and outcome in preterm infants. Arch.Dis.Child 1990;65:30-34.
11. Scherjon SA, Smolders-De Haas H, Kok JH, Zondervan HA. The "brain-sparing" effect: antenatal cerebral Doppler findings in relation to neurologic outcome in very preterm infants. American Journal of Obstetrics & Gynecology1993;169:169-75.
12. Chard T, Penney G, Chalmers J. The risk of neonatal death in relation to birth weight and maternal hypertensive disease in infants born at 24-32 weeks. European Journal of Obstetrics, Gynecology, & Reproductive Biology 2001;95:114-18.

Chapter 4:

Postnatal administration of dexamethasone for weaning off the ventilator affects thyroid function

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Abstract

Background: Very preterm neonates are at risk of hypothyroxinemia because of prematurity as well as because of neonatal disease. Hypothyroxinemia is associated with impaired developmental outcome. Preterm infants who cannot be weaned from the ventilator can be treated with dexamethasone. Glucocorticoid administration has been found to alter thyroid hormone parameters. Therefore, dexamethasone treatment in these infants might additionally impair their thyroid function, which could have consequences for developmental outcome.

Objective: To assess what changes in thyroid function occur in the first hours after initiating dexamethasone treatment in ventilated preterm infants.

Methods: Preterm infants, in whom the decision was taken to start dexamethasone treatment, were included. Thyroxin, T_3, rT_3, TSH and Cortisol were determined before and 6 to 9 after administration of the first dose of a postnatal dexamethasone course. Details of clinical condition were recorded at both time points.

Results: Sixteen very preterm infants were included at a median age of 20 days. While clinical condition was stable between start of dexamethasone and 6-9 hours thereafter, TSH and T_3 levels decreased significantly. ReverseT_3 levels significantly increased, resulting in a decrease of the T_3/rT_3 Ratio. There was no statistically significant effect on the levels of T_4.

Conclusion: Postnatal dexamethasone administration negatively affects thyroid function in the preterm infant with severe chronic lung disease.

Introduction

Dexamethasone treatment in ventilated preterm infants has been demonstrated to rapidly reduce requirements for oxygen and ventilation, decreasing the incidence of chronic lung disease.[1] In contrast, dexamethasone treatment has also been identified as an independent risk factor for delayed psychomotor development.[2-7] These data from follow-up studies are in agreement with observations from animal experiments,[8;9] in which abnormal neuronal growth as well as increased white matter damage[10] is observed after dexamethasone treatment.

Normal brain development is dependent on sufficient and continuous provision of thyroid hormone.[11] Preterm neonates are at risk of hypothyroxinemia, especially in case of respiratory and infectious diseas.[12] Follow-up of preterm neonates shows a higher prevalence of developmental disabilities, when postnatal thyroid hormone levels have been lower,[13-15] and this is reason for an ongoing debate on the necessity of thyroid hormone supplementation.[16] Dexamethasone is found to alter thyroid function in adult human[17] as well as in animal experiments. These effects comprise a direct Hypothalamic-Pituitary-Thyroid axis effect,[18] but also thyroid hormone metabolism is influenced, evidenced by altered Deiodinase activity in cultured brain cells[19] as well as in the chicken embryo.[20;21] Interestingly, dexamethasone can both increase[22] and decrease[23] Deiodinase type III (D3), depending on species, tissue localization and developmental stage. Therefore dexamethasone might either result in more as in less bio-active hormone (T_3). In a recent paper antenatal glucocorticoids were associated with higher T_4 concentrations in the first week after birth[24], while Williams et al found that postnatal dexamethasone administration was associated with significantly lower fT_4 and T_3 concentrations in the third week of life.[25] The aim of the present study was to assess direct effects of dexamethasone treatment on thyroid function in ventilated, very preterm infants.

Patients and Methods

This prospective exploratory study, performed between September 1st 2000 and June 1st 2004, included preterm neonates admitted to the NICU ward of the Academic Medical Center who could not be weaned from the ventilator and had an indication for a course of dexamethasone according to the attending neonatologist. Our preliminary estimates of eligible infants, based on department records, were of about 20 dexamethasone-treated infants per year, our intended study size. However, as is also the experience in other studies investigating postnatal glucocorticoids,[26] the inclusion rate dropped about 4-fold because of increasing clinical concerns about the harmful effects on brain development of dexamethasone treatment. This caused our inclusion phase to take almost 4 years, while the decision to treat infants with dexamethasone was increasingly restricted to those with the most severe respiratory complications. A three weeks tapering course of

dexamethasone was prescribed with a starting dose of 0.25 mg/kg/day, the total dexamethasone dose of a full course being 4 mg/kg. This decision was generally taken when despite optimal treatment of fluid balance and possible infectious comorbidity, there was no change in ventilatory settings and the chest X ray showed signs of chronic lung disease. After informed consent of the parents, preterm infants were enrolled. The study protocol was approved by the institutional review board of the Academic Medical Center.

Blood Samples
A baseline blood sample, within 2 hours before administration of the first dose of dexamethasone, and a second sample 6 to 9 hours after the first dose of dexamethasone was taken. One ml of peripheral blood was collected, either from capillary puncture or from an arterial line. Blood was centrifuged immediately and stored at −20°C until assay. In some cases the amount of blood was limited and hormone analysis was incomplete. In each sample T_3, rT_3, T_4, TSH and Cortisol were determined; in case of a limited amount of plasma priority was given in the order mentioned.

Clinical Data
Clinical data on ventilation, circulatory support, patent ductus arteriosus, cerebral damage and medication, at baseline and at the time of the second blood sample, as well as obstetric and neonatal data at birth were collected. Follow up data included assessment of neurodevelopmental outcome until 3 years of age, using the Bayley Scales of Infant Development II and neurological examination.

Assays
T_4, T_3 and rT_3 were measured by in-house RIA methods.[27] Detection limits were 5.0, 0.3 and 0.03 nmol/l, respectively; intra-assay coefficients of variation were 2-4%, 3-4% and 4-5%, respectively, and interassay coefficients of variation were 3-6%, 7-8% and 5-9%, respectively. TSH was measured by time-resolved fluoroimmunoassay (Delfia hTSH Ultra, Wallac Oy, Turku, Finland). Detection limit was 0.01 mU/l, intra-assay coefficient of variation was 1-2% and inter-assay coefficient of variation was 3-4%. Cortisol was measured by ELISA (Immulite analyzer, DPC, Los Angeles, CA, U.S.A.). Detection limit was 50 nmol/l, intra-assay and inter-assay coefficient of variation at 200 nmol/L were 6.4% and 9 %, respectively, and 5.8% and 7%, respectively, at 370 nmol/l.

Statistical Analysis
Data were analyzed using the statistical program SPSS 11.5.1 for Windows (SPSS Inc., Chicago, Ill). Hormone values before and after administration of dexamethasone were compared using Wilcoxon signed ranks test as these values turned out not to be normally distributed, as indicated by one sample Kolmogorov-Smirnov test. Due to availability of plasma, two Cortisol values ("< 50 nmol/l"), one T_3 value ("< 0.6 nmol/l") as well as two

TSH determinations (one was "< 0.04 mU/l" and, due to lack of material for a dilution step the other was classified as "< 0.1 mU/l") could only be determined by approximation, mentioning threshold values. Therefore, the Wilcoxon signed ranks tests were performed once with the target value equaling this threshold value, and a repeated, substituting the target value by 50% of this threshold value. As the substitution did not affect the rank, both approaches gave an identical result. Clinical parameters before and after initiation of treatment were compared using Wilcoxon signed ranks test, whereas clinical parameters of subgroups according to the T_3 / rT_3 Ratio were compared using Mann Whitney U test. Linear regression analysis and Pearson correlation were used in correlations of continuous variables. P-values < 0.05 were considered statistically significant.

Results

In the study period, 24 infants were eligible for the study. Four infants could not be included because dexamethasone administration had started before consent could be obtained. Two parent couples declined consent. Finally, eighteen infants were enrolled in the study. In one baby, blood was erroneously taken 90 minutes after dexamethasone administration, results were excluded. In another infant, the small amount of blood of a second sample taken 9 hours after dexamethasone administration only permitted determination of TSH and Cortisol, his results were also omitted from the Tables and the analysis.

Table 1 shows the obstetric and neonatal data at admission of the 16 babies analyzed. All infants were born at or less than 29 weeks gestational age. The age at which dexamethasone was started differed considerably (9-48 days after birth with 81% of the infants at 13-27 days after birth). In most infants dexamethasone administration started in the afternoon, while the second sample was generally taken in the evening.

Table 2 shows the median hormone values and range before and after the first dose of dexamethasone. TSH decreased statistically significantly after dexamethasone administration. We observed no statistically significant change of T_4. There was a significant decrease of T_3 and a significant increase of rT_3 after initiation of dexamethasone, resulting in a statistically significant decrease in the T_3 / rT_3 Ratio. Postmenstrual age had a significant positive correlation (Pearson r = 0.57, p=0.02) with the T_3 / rT_3 Ratio before treatment, in concordance with literature.[28] Figure 1 depicts the changes of the T_3 / rT_3 Ratio before and after Dexamethasone treatment as a function of postmenstrual age. Eleven out of fifteen T_3 / rT_3 Ratios decreased, in four infants the T_3 / rT_3 Ratio increased. Review of clinical neonatal variables in these four infants showed significantly lower T_3 levels (mean 0.4 vs. 1.0 nmol/L, p=0.002) and lower systolic blood pressures at baseline (mean 35 vs. 52 mmHg, p=0.02).

Clinical Characteristics at birth	N = 16	
GA at birth (weeks)	$27^{2}/_{7}$	($24^{5}/_{7}$ - 29)
Mode of delivery Vaginal / Caesarean	7 / 9	
Birth weight (g)	828	(615 – 1470)
Antenatal Corticosteroids	15	
Apgar 5	8	(2-10)
Intubated < 1 hour after birth	12	
Surfactant treatment	11	
Characteristics upon inclusion		
Age at first dose (days)	20	(9 - 48)
PMA (weeks)	$29^{5}/_{7}$	($27^{3}/_{7}$-$34^{3}/_{7}$)
Body weight (g)	905	(710 -1630)
Weight increase since birth (g)	113	(-135 – 380)
Duration of ventilation (days)	17	(4 – 33)
Ventilation: HFO / Conventional	13 / 3	
Patent Ductus Arteriosus	6	†
Receiving Dopamine	3	
Receiving Dobutamine	1	
Cerebral abnormalities on ultrasound	8	#

Table 1: Clinical Characteristics at birth, and upon inclusion

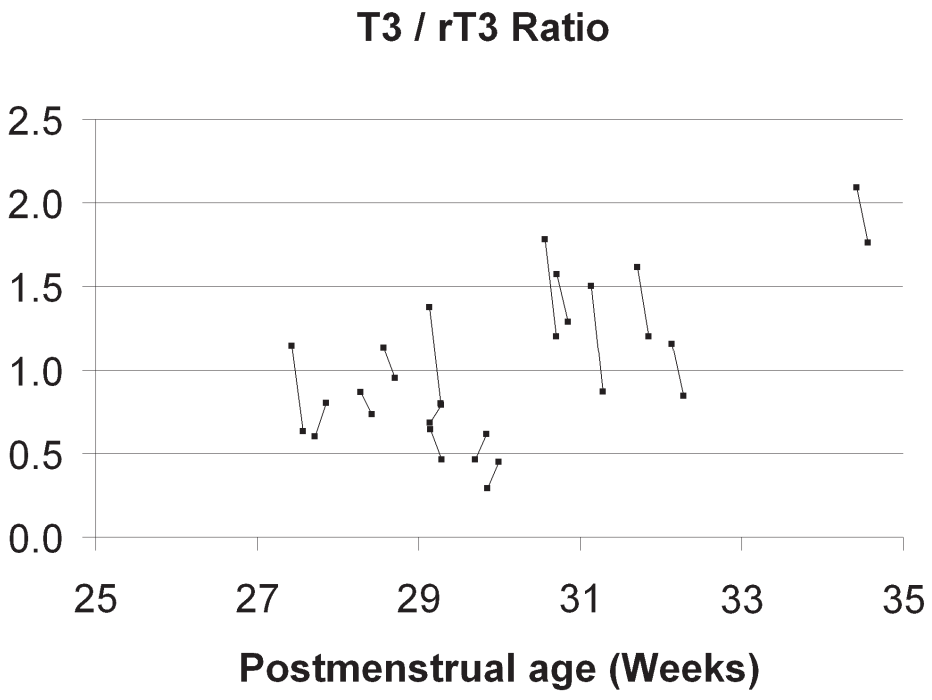
Values are median (range) or N, as appropriate. † Including one infant that underwent operative closure of ductus 3 days before dexamethasone course. # One infant with Periventricular Leukomalacia (grade 1) four infants with Intraventricular Hemorrhage grade 3 and three infants with Subependymal Hemorrhage (IVH grade 1).

Cortisol levels statistically significantly decreased by almost 50%, as expected.[29] In order to be certain that the thyroid hormone changes found were not caused by changes of the clinical condition,[30] we compared circulatory and ventilatory parameters of the infants before and at the time of collection of the second blood sample. Blood pressure values rose after start of dexamethasone therapy (median systolic pressure 49 and 52, and median diastolic pressure 27 and 35 mmHg before and after treatment, respectively, p= 0.003). Ventilatory parameters before initiation of dexamethasone and at the time of collection of the second blood sample did not change. The dosage of dopamine, which was administered to 3 infants, as well as dobutamine (administered to one infant) remained unaltered at the time of collection of the second sample, compared to dosage before initiation of dexamethasone. Table 3 shows individual clinical data as well as follow up data until 3 years of age. Only in one infant no follow up data were available. Of the remaining 15 children outcome was good in only three. Three infants died during or shortly after dexamethasone treatment, two infants had disabling Cerebral Palsy (CP), 5 infants had mild neurological abnormalities or non-disabling CP in combination with mental and/or motor delay and another two infants showed some motor or mental delay or behaviour problems with normal neurological examination.

	N	before	N	after	P value†
TSH	16	2.55 (<0.1 – 6.8)	15	1.2 (<0.04 – 3.3)	0.017
T_4	15	70 (35-120)	15	70 (30 - 95)	NS
T_3	16	0.85 (0.3 – 1.55)	15	0.75 (0.4 – 1.5)	0.022
rT_3	16	0.78 (0.5 – 1.7)	16	0.95 (0.5 – 1.85)	0.025
T_3 /rT_3	16	1.14 (0.29–2.1)	15	0.80 (0.45 – 1.4)	0.012
Cortisol	14	150 (<50 - 720)	12	80 (50 - 510)	0.009

Table 2: Hormone levels before and after Dexamethasone.
Values are median (range). T_4, T_3, rT_3 and Cortisol in nmol/l ; TSH in mU/l
† Wilcoxon signed ranks test

Figure 1: Changes in the T_3 / rT_3 Ratio before and after Dexamethasone treatment



Increase or decrease of T_3 / rT_3 Ratio at baseline and after the first dose of Dexamethasone in relation to postmenstrual age.

Table 3: Individual Characteristics at birth, during hospital admission at and after inclusion.

Infant	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p
Birth characteristics																
Gestational age at birth (weeks)	27 5/7	27 3/7	29	27 6/7	27 2/7	26 1/7	26	27 6/7	25	27 1/7	26 3/7	27 4/7	26 1/7	24 5/7	28 2/7	27 2/7
Birth weight (g)	615	645	1350	1145	740	945	810	715	820	1150	880	615	835	980	1470	680
BWR	0.94	0.93	0.68	1.02	0.89	0.63	1.04	1.11	0.91	0.52	0.93	1.30	1.21	0.62	0.94	0.93
Clinical data before starting dexamethasone treatment																
Time on ventilator (days)	13	15	19	18	26	4	14	13	13	10	14	33	20	26	27	23
Surfactant treatment	N	Y	Y	Y	Y	Y	N	Y	N	Y	N	Y	Y	Y	Y	Y
Cerebral abnormalities on ultrasound	IVH	IVH	SEH	SEH	-	-	-	-	-	IVH	-	FL	-	IVH PVL	SEH	-
PDA requiring treatment	M	-	-	-	M	-	-	M	-	-	S	-	-	M	-	M
Data at start dexamethasone treatment																
Age (days)	15	22	19	19	27	9	22	13	19	8	19	48	21	27	27	24
Body weight (g)	715	710	1350	1290	845	810	1190	800	820	1150	1000	740	965	1145	1630	830
Receiving Dopamine	Y	N	N	Y	N	N	Y	N	N	N	N	N	N	N	N	N
TSH (mU/L)	< 0.1	0.36	2.1	1.4	0.76	2.7	0.39	2.4	4.4	4.8	4.5	6.8	2.85	4.9	0.32	2.7
T_3 (nmol/L)	0.35	0.6	1.05	1.55	1.2	0.8	0.9	0.3	0.3	0.65	1.1	1.15	0.65	1.3	0.75	1.1
rT_3 (nmol/L)	1.2	1.7	0.65	0.87	0.8	0.7	1.4	0.65	0.5	0.75	0.8	0.55	0.95	1.15	0.65	0.7
Clinical course during dexamethasone treatment																
Time to extubation (days)	NA	7	4	9	3	1	8	2	4	8	1	18	3	3	25	1
Died during or shortly after dexamethasone treatment	+							+						+		
Abnormal neurodevelopmental outcome at age 3 years	NA	+	+++	++	++	+	N	NA	++	++	N	NA	+++	NA	N	++

Cerebral abnormalities on ultrasound: FL = Flaring; SHE = Subependymal haemorrhage; IVH = Intraventricular haemorrhage; PVL = Periventricular leukomalacia. Surfactant treatment, Receiving Dopamine, Died during or shortly after dexamethasone treatment: Y = yes; N = no PDA requiring treatment: Patent ductus arteriosus requiring treatment, M = Medical treatment; S = Surgical treatment. Abnormal neurodevelopmental outcome at age 3 years: Abnormal outcome was defined as +++ severely abnormal if infant had disabling (non-ambulant) CP,
++ abnormal if there were mild neurological abnormalities or non-disabling CP in combination with mental and/or motor delay >3months,
+ mildly abnormal if neurological examination was normal but if there was motor or mental delay or behaviour problems,
N normal if none of the above occurred.
NA: not applicable.
The four infants the T_3 / rT_3 Ratio increased instead of decreasing were infant **a, h, i** and **m**.

Discussion

In this study we were able to demonstrate clinically relevant and statistically significant changes of dexamethasone on thyroid function. Two regulatory thyroid mechanisms play a role. The first was on the pituitary level, indicated by lower TSH levels. This suppressive effect is in accordance with literature reports on effects of glucocorticoids.[31;32] The second effect was on the level of peripheral thyroid hormone metabolism reflected in changes in T_3 and rT_3. Normally after birth a gradual decrease of rT_3 and a increase of plasma T_3 is seen in the first week of life, compatible with a decrease in D3 activity.[28] The changes we found (decrease of T_3, increase of rT_3) were opposite, implicating inactivation of thyroid hormone. It is unlikely that these changes are a consequence of decreased production of thyroid hormone, as T_4 levels did not differ. Most probably this effect was caused by stimulation of D3. Alternatively this can be explained by inhibition of Deiodinase type 1, but this is less likely as levels of T_4 remained unchanged. It is well known that D3 tissue levels in premature infants are still high,[33] even more so in ill premature infants.[34]

Also in the chicken embryo animal model, dexamethasone was shown to stimulate D3.[20;21] This contrasts to findings under experimental conditions where dexamethasone leads to suppression of D3.[35] Whether dexamethasone leads to induction or suppression of D3 apparently depends, apart from species and tissue, on ontogenetic phase.[22;23;33] Although all of the found changes could also have been caused by deterioration of the clinical condition, we could not find such a change in the time window between start of dexamethasone and 6 to 9 hours thereafter. On the contrary, we recorded a stable respiratory course accompanied by a somewhat higher blood pressure. The four infants in which the low T_3 / rT_3 Ratio raised after dexamethasone treatment had comparatively low T_3 levels, possibly indicating an already increased activity of D3 at baseline.[34] Our study is the first to demonstrate direct thyroid hormone-related side effects of dexamethasone. The study design, in which each infant served as its own control permitted to find the above described effects.

In a next step, it must be examined whether the identified acute changes are persistent, and how they change during the dexamethasone course, and beyond. Since development of the preterm brain is dependent on continuous provision of thyroid hormone concerns seem justified, as another study demonstrated dexamethasone to decrease T_3-, but also fT_4 concentrations in premature infants at day 14 of life.[25] In the animal model it has been shown that D3 is abundantly present in brain astrocytes,[36] therefore local thyroid hormone delivery in brain tissue could be impaired during dexamethasone administration. In that case the side effect we found could be one of the mechanisms by which dexamethasone can impair psychomotor development. Indeed neurodevelopmental outcome of our study group was not normal in most infants.

Our study design is not appropriate to make any statement on the causal factors. Dexamethasone is likely to play a role, however in combination with the severe illness all these infants experienced. Current practice in prescribing dexamethasone has changed, not only has the dose been decreased, dexamethasone is to a greater extent prescribed as a last rescue medicine.

In summary, we have demonstrated possible harmful effects of dexamethasone on thyroid hormone regulation and metabolism in preterm infants with severe respiratory disease. If these effects persist throughout the complete dexamethasone course, they aggravate the already present transient hypothyroxinemia of prematurity.

Reference List

1. Halliday HL, Ehrenkranz RA, Doyle LW. Moderately early (7-14 days) postnatal corticosteroids for preventing chronic lung disease in preterm infants. Cochrane Database Syst.Rev. 2003;CD001144.
2. Yeh TF, Lin YJ, Huang CC, Chen JY, Lin CH, Lin HC et al. Early dexamethasone therapy in preterm infants: a follow-up study. Pediatrics 1998;101:e7.
3. O'Shea TM, Kothadia JM, Klinepeter KL, Goldstein DJ, Jackson BG, Weaver RG, III et al. Randomized placebo-controlled trial of a 42-day tapering course of dexamethasone to reduce the duration of ventilator dependency in very low birth weight infants: outcome of study participants at 1-year adjusted age. Pediatrics 1999;104:15-21.
4. Shinwell ES, Karplus M, Reich D, Weintraub Z, Blazer S, Bader D et al. Early postnatal dexamethasone treatment and increased incidence of cerebral palsy. Arch.Dis.Child Fetal Neonatal Ed 2000;83:F177-F181.
5. Romagnoli C, Zecca E, Luciano R, Torrioli G, Tortorolo G. A three year follow up of preterm infants after moderately early treatment with dexamethasone. Arch.Dis.Child Fetal Neonatal Ed 2002;87:F55-F58.
6. Stoelhorst GM, Rijken M, Martens SE, van Zwieten PH, Feenstra J, Zwinderman AH et al. Developmental outcome at 18 and 24 months of age in very preterm children: a cohort study from 1996 to 1997. Early Hum.Dev. 2003;72:83-95.
7. Yeh TF, Lin YJ, Lin HC, Huang CC, Hsieh WS, Lin CH et al. Outcomes at school age after postnatal dexamethasone therapy for lung disease of prematurity. N.Engl.J.Med. 2004;350:1304-13.
8. Weichsel ME, Jr. The therapeutic use of glucocorticoid hormones in the perinatal period: potential neurological hazards. Ann.Neurol. 1977;2:364-66.
9. Gramsbergen A, Mulder EJ. The influence of betamethasone and dexamethasone on motor development in young rats. Pediatr.Res. 1998;44:105-10.
10. Kauffman KS, Seidler FJ, Slotkin TA. Prenatal dexamethasone exposure causes loss of neonatal hypoxia tolerance: cellular mechanisms. Pediatr.Res. 1994;35:515-22.
11. Bernal J, Guadano-Ferraz A, Morte B. Perspectives in the study of thyroid hormone action on brain development and function. Thyroid 2003;13:1005-12.
12. van Wassenaer AG, Kok JH, Dekker FW, de Vijlder JJ. Thyroid function in very preterm infants: influences of gestational age and disease. Pediatr.Res. 1997;42:604-09.
13. Lucas A, Morley R, Fewtrell MS. Low triiodothyronine concentration in preterm infants and subsequent intelligence quotient (IQ) at 8 year follow up. BMJ 1996;312:1132-33.
14. Reuss ML, Paneth N, Pinto-Martin JA, Lorenz JM, Susser M. The relation of transient hypothyroxinemia in preterm infants to neurologic development at two years of age. N.Engl.J.Med. 1996;334:821-27.
15. Briet JM, van Wassenaer AG, Dekker FW, de Vijlder JJ, van Baar A, Kok JH. Neonatal thyroxine supplementation in very preterm children: developmental outcome evaluated at early school age. Pediatrics 2001;107:712-18.
16. La Gamma EF, van Wassenaer AG, Golombek SG, Morreale de EG, Kok JH, Quero J et al. Neonatal Thyroxine Supplementation for Transient Hypothyroxinemia of Prematurity : Beneficial or Detrimental? Treat.Endocrinol. 2006;5:335-46.
17. Chopra IJ, Williams DE, Orgiazzi J, Solomon DH. Opposite effects of dexamethasone on serum concentrations of 3,3',5'-triiodothyronine (reverse T3) and 3,3'5-triiodothyronine (T3). J.Clin.Endocrinol.Metab 1975;41:911-20.

18. Ahlquist JA, Franklyn JA, Ramsden DB, Sheppard MC. The influence of dexamethasone on serum thyrotrophin and thyrotrophin synthesis in the rat. Mol.Cell Endocrinol. 1989;64:55-61.
19. Courtin F, Chantoux F, Gavaret JM, Toru-Delbauffe D, Jacquemin C, Pierre M. Induction of type II 5'-deiodinase activity in cultured rat astroglial cells by 12-O-tetradecanoylphorbol-13-acetate: dependence on glucocorticoids. Endocrinology 1989;125:1277-81.
20. Darras VM, Kotanen SP, Geris KL, Berghman LR, Kuhn ER. Plasma thyroid hormone levels and iodothyronine deiodinase activity following an acute glucocorticoid challenge in embryonic compared with posthatch chickens. Gen.Comp Endocrinol. 1996;104:203-12.
21. Van der Geyten S., Buys N, Sanders JP, Decuypere E, Visser TJ, Kuhn ER et al. Acute pretranslational regulation of type III iodothyronine deiodinase by growth hormone and dexamethasone in chicken embryos. Mol.Cell Endocrinol. 1999;147:49-56.
22. Van der Geyten S., Darras VM. Developmentally defined regulation of thyroid hormone metabolism by glucocorticoids in the rat. J.Endocrinol. 2005;185:327-36.
23. Forhead AJ, Jellyman JK, Gardner DS, Giussani DA, Kaptein E, Visser TJ et al. Differential effects of maternal dexamethasone treatment on circulating thyroid hormone concentrations and tissue deiodinase activity in the pregnant ewe and fetus. Endocrinology 2007;148:800-05.
24. Martin CR, Van Marter LJ, Allred EN, Leviton A. Antenatal glucocorticoids increase early total thyroxine levels in premature infants. Biol.Neonate 2005;87:273-80.
25. Williams FL, Ogston SA, van Toor H, Visser TJ, Hume R. Serum thyroid hormones in preterm infants: associations with postnatal illnesses and drug usage. J.Clin.Endocrinol.Metab 2005;90:5954-63.
26. Doyle LW, Davis PG, Morley CJ, McPhee A, Carlin JB. Outcome at 2 years of age of infants from the DART study: a multicenter, international, randomized, controlled trial of low-dose dexamethasone. Pediatrics 2007;119:716-21.
27. Wiersinga, W. M. The peripheral conversion of thyroxine (T_4) into triiodothyronine (T_3) and reverse triiodothyronine (rT_3). Thesis, University of Amsterdam 1979.
28. Williams FL, Simpson J, Delahunty C, Ogston SA, Bongers-Schokking JJ, Murphy N et al. Developmental trends in cord and postpartum serum thyroid hormones in preterm infants. J.Clin.Endocrinol.Metab 2004;89:5314-20.
29. Petersen MC, Nation RL, McBride WG, Ashley JJ, Moore RG. Pharmacokinetics of betamethasone in healthy adults after intravenous administration. Eur. J.Clin.Pharmacol. 1983;25:643-50.
30. McIver B, Gorman CA. Euthyroid sick syndrome: an overview. Thyroid 1997;7:125-32.
31. Samuels MH, Luther M, Henry P, Ridgway EC. Effects of hydrocortisone on pulsatile pituitary glycoprotein secretion. J.Clin.Endocrinol.Metab 1994;78:211-15.
32. Coiro V, Volpi R, Capretti L, Speroni G, Pilla S, Cataldo S et al. Effect of dexamethasone on TSH secretion induced by TRH in human obesity. J.Investig. Med. 2001;49:330-34.
33. Richard K, Hume R, Kaptein E, Sanders J, van Toor H, de Herder W et al. Ontogeny of iodothyronine deiodinases in human liver. J.Clin.Endocrinol. Metab 1998;83:2868-74.

34. Pavelka S, Kopecky P, Bendlova B, Stolba P, Vitkova I, Vobruba V et al. Tissue metabolism and plasma levels of thyroid hormones in critically ill very premature infants. Pediatr.Res. 1997;42:812-18.
35. Hernandez A. Structure and function of the type 3 deiodinase gene. Thyroid 2005;15:865-74.
36. Pallud S, Ramauge M, Gavaret JM, Lennon AM, Munsch N, St Germain DL et al. Regulation of type 3 iodothyronine deiodinase expression in cultured rat astrocytes: role of the Erk cascade. Endocrinology 1999;140:2917-23.

Chapter 5:

Transient hypothyroxinemia in severe hypertensive disorders of pregnancy

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Abstract

Objective: Assess whether and to what extent thyroid function is affected in pregnant women with early and severe hypertensive disorders and their newborns.

Methods: Patients were 80 women with preeclampsia, hemolysis, elevated liver enzymes and low platelet count syndrome or gestational hypertension combined with fetal growth restriction in the 24th to 34th week of singleton pregnancies. Maternal thyroid hormone levels and thyroid peroxidase antibodies (TPOab) were determined at admission and three months post term. Neonatal levels were determined from cord blood at delivery. Maternal hypothyroxinemia was defined as fT_4 value below 9 pmol/l.

Results: At admission 26 (33%) women in the Study Group had fT_4 levels below 9 pmol/l, with spontaneous normalization during pregnancy. There were, however, no statistically significant differences between thyroid hormone values in women in the study group compared to 10 normotensive pregnant women in their third trimester. Three months post term 97.5% of patients had thyroid hormone levels in the normal range. TPOab were elevated in 10% of women post partum.
Their infants, born at a median gestational age of $30^6/_7$ weeks, had lower cord blood fT_4 and TSH values compared to preterm infants of the comparison group, appropriate for gestational age. Cord blood fT_4 had no correlation with gestational age or maternal fT_4, but there was a significant correlation of cord blood fT_4 with umbilical artery pH.

Conclusion: Women with severe hypertensive disorders of pregnancy may have transiently lower fT_4 levels, without evidence of a thyroid disorder. Their neonates have lower fT_4 levels at birth unrelated to maternal fT_4, but related to prenatal acidosis.

Introduction

Sufficient provision of thyroid hormone in the first trimester of pregnancy is essential for normal fetal brain development.[1-3] There is growing evidence, however, suggesting that maternal thyroid hormone levels remain important until term.[4-6] In the debate on benefits of screening for hypothyroidism in pregnancy[7;8] the question on the optimal freeT_4 (fT_4) level remains unanswered,[9] as reports on thyroid function in normal pregnancy are scarce.[10;11]

Preeclamptic patients are at particular risk. Several reports describe an association between preeclampsia and maternal thyroid dysfunction,[12-16] and low birth weight[17], some authors even suggested maternal thyroid function abnormalities to be a causal factor.[18-21] Free T_4 is generally found to be lower in umbilical cord samples from neonates born from preeclamptic pregnancies than in infants from normotensive pregnancies, but reports remain inconclusive.[13;14;17;22-26] A low fT_4 can be a consequence of lower maternal thyroid hormone levels, however, it can also be caused by impaired transfer of T_4 due to placental insufficiency or fetal disease due to fetal growth restriction (FGR) and fetal acidosis.

The aim of the present study was to assess whether and to what extent thyroid function is impaired in women with severe and early hypertensive disorders in pregnancy, whether autoimmunity is involved, and to what extent neonatal thyroid function is affected.

Patients and Methods

This prospective cohort study was performed between April 1st 2001 and June 1st 2003, in a subset of women with severe hypertensive disorders of pregnancy, participating in the Preeclampsia Eclampsia TRial of Amsterdam.[27] This randomized clinical intervention trial in women with severe hypertensive disorders of pregnancy was carried out in two tertiary care perinatal centers in Amsterdam from September 1st 2000 to June 1st 2003. In our study of thyroid function only the subset of women and neonates admitted in one of the centers (Academic Medical Center) were included. As blood collection, for thyroid hormone parameters at admission, preceded the study intervention data from both treatment arms were joined for our further analyses.

The study protocol was approved by the institutional review board of the Academic Medical Center. After informed consent, women were included upon admission if they were in the 24th to 34th week of a singleton pregnancy with either pregnancy induced hypertension in combination with fetal growth restriction (diastolic blood pressure >90 mmHg and estimated fetal weight < 5th centile or abdominal circumference <10th percentile) or severe preeclampsia (diastolic blood pressure > 110 mmHg and proteinuria > 0.3 g/L) or HELLP-syndrome (lactate dehydrogenase (LDH) > 600 U/L, aspartate aminotransferase > 70 U/L, Platelet Count < 100 x 10^9/L). After informed consent, patients were randomly allocated to a temporizing management

strategy with or without plasma volume expansion. Randomization was preformed by use of a designated palmtop computer with random number generation software. In all patients, obstetric management aimed to improve fetal prognosis through increasing gestational age at birth. Pregnancy was prolonged until deterioration of fetal or maternal condition necessitated delivery.[28] In the absence of normal reference values for thyroid hormone in pregnancy, apart from comparing thyroid function results with our local reference ranges for the specific assays, they were also compared with earlier described results in a prospectively followed group of 10 healthy women who became pregnant by artificial insemination because of male infertility.[10] Baseline, non-pregnant thyroid function was also known in these women. None of these pregnant women showed any sign of hypertensive disorders.

Maternal Blood Samples at Admission
At admission, maternal blood samples were collected for determination of T_4, T_3, Thyroid Stimulating Hormone(TSH), freeT_4(fT_4), Thyroxin Binding Globulin(TBG), reverse-T_3(rT_3) and Thyroid Peroxidase antibodies (TPOab). Normal values of fT_4 in pregnancies are not known.[29;30] Therefore, we defined low maternal thyroxin levels by the lower limit of our laboratory (i.e. nonpregnant) reference range: fT_4 of <9 pmol/l.A second sample was taken if fT_4 was < 9 pmol/l or TSH was < 0.4 or > 4 mU/l. If fT_4 or TSH in the second sample were also outside these limits, the patient was referred to an endocrinologist for further evaluation.

Neonatal Blood Samples
At birth, an umbilical cord blood sample for determination of T_4, T_3, TSH, fT_4, TBG and rT_3 was collected. If the amount of cord blood after determination of arterial pH was limited and thyroid hormone analysis was incomplete, preference was given to determining fT_4, TSH, T_4 and T_3. We compared results with data previously collected in our hospital using the same assays, in a group of 114 premature infants below 30 weeks gestational age (mean gestational age 28 1/7 weeks ± 8 days) of whom 90% were appropriate for gestational age.[31]

Maternal Blood Sample Post Partum
Three months after term date, at a scheduled visit, a maternal post partum blood sample was obtained for determination of T_4, T_3, TSH, fT_4, TBG, rT_3 and TPO-antibodies. Values were compared with reference values of the laboratory. If one of these determinations was outside the reference range, the patient was further evaluated by an endocrinologist for thyroid disorders.

Assays
Blood was centrifuged immediately and stored at –20°C until assay. T_4, T_3 and rT_3 were measured by in-house RIA methods.[32] Detection limits were 5.0, 0.3 and 0.03 nmol/l, respectively; intra-assay coefficients of variation

were 2-4%, 3-4% and 4-5%, respectively, and interassay coefficients of variation were 3-6%, 7-8% and 5-9%, respectively. FreeT_4 was measured by time-resolved fluoroimmunoassay (Delfia fT_4, Wallac Oy, Turku, Finland). Detection limit was 2 pmol/l, intra-assay coefficient of variation was 4-6% and inter-assay coefficient of variation was 5-8%. TSH was also measured by time-resolved fluoroimmunoassay (Delfia hTSH Ultra, Wallac Oy, Turku, Finland). Detection limit was 0.01 mU/l, intra-assay coefficient of variation was 1-2% and inter-assay coefficient of variation was 3-4%. Anti-TPO antibodies were measured by chemiluminescence immunoassay (LUMI-test anti-TPO, BRAHMS, Berlin, Germany). Detection limit was 30kU/l, intra- and inter-assay coefficient of variation were 3-7% and 8-12%, respectively. Thyroxin Binding Globulin was determined by commercial radioimmunoassay (Eiken Chemical Co, Tokyo, Japan). Detection limit was 30 nmol/l, intra- and interassay coefficient of variation were 2-4% and 4-6% respectively.

Placental Insufficiency Parameters

We used three measures of severity of placental insufficiency. Ultrasound Doppler examination of a free loop of umbilical artery was performed twice weekly, and the most recent Pulsatility Index, calculated from the flow velocity profile, was recorded. Secondly, we used Birth Weight Ratio as a measure for dysmaturity. The Birth Weight Ratio is the observed birth weight divided by the expected weight at the corresponding gestational age according to the customized antenatal growth chart.[33] By definition, an infant with the appropriate weight for gestational age is to have a BWR of 1, whereas a birth weight ratio less than 0.86 corresponds to a birth weight less than the 10th percentile (SGA). The net weight of the placenta was measured after birth after removal of cord and membranes; centile values were calculated by means of Dutch reference curves, stratified for parity and gender of the neonate.[34]

Statistical Analysis

Data were analyzed with the statistical program SPSS 10.0.7 for Windows (SPSS Inc., Chicago, Illinois, USA). Patient characteristics as well as hormone values were checked for normal distribution as indicated by one sample Kolmogorov-Smirnov test. All analyses were done for the whole group and within subgroups according to low fT_4 or admission diagnosis. Groups were compared using the Student *t* test and the Chi square test. One way ANOVA was used to compare groups according to admission diagnosis. Linear regression analysis and Pearson correlation were used in correlations of continuous variables. Multivariable regression analysis was performed by the enter model, with the significant factors of the univariate analyses, combined with gestational age and maternal fT_4. The statistical power to detect a clinically significant difference of 1.5 pmol/l in maternal fT_4 level between our study group and the comparison group was calculated as 80% (effect size = 0.84; N=10 compared with N=80; α=0.05).

Results

The mothers
In the study period, 80 women were included. Table 1 shows the demographic and obstetric data at admission. There were 69 live-born babies and 11 stillbirths. Median gestational age at birth was $30^{6}/_{7}$ weeks (range $26^{1}/_{7}$ - $36^{6}/_{7}$ weeks), birth weights ranged 525–2310 grams (median 1100) and BWR ranged 0.36 -0.88 (median 0.63): all babies but one were SGA. Median umbilical cord artery pH was 7.20, ranging 6.94 – 7.44. Table 2 shows mean maternal thyroid hormone levels at admission. There were no statistically significant differences between the study group and the comparison group, although TT_4, fT_4, T_3 and TBG were lower than in the comparison group, in combination with a higher TSH.

		Study Group
N		80
Maternal Age	years	30.1 (4.7)
Nulliparity		56 (70)
Maternal Weight	kg	68.6 (14)
Gestational Age at admission	weeks	29 $24^{2}/_{7}$–$33^{5}/_{7}$
Systolic Blood Pressure	mmHg	156 (19)
Diastolic Blood Pressure	mmHg	101 (10)
Admission Diagnosis*		
- HELLP		18 (23)
- Severe preeclampsia		23 (29)
- Gestational hypertension and FGR		39 (49)
Interval admission-birth	days	10 (0-44)
Lowest Recorded Platelet Count	10^{9}/l	119 (76)
Highest Recorded Proteinuria	g/24 hrs	8.3 (7.7)

Table 1:Maternal characteristics at admission and during observation
Values are mean (+/- standard deviation) median (range) or n(%), as appropriate. *****During clinical observation, in the Severe Preeclampsia Group 9 patients developed HELLP Syndrome whereas in the FGR Group 11 patients developed HELLP Syndrome. At birth 38 patients (48%) had HELLP, 15 (19%) had Severe Preeclampsia, and 27 (34%) had FGR.

However, in 26 patients (33%) fT_4 was <9 pmol/l, their mean fT_4 was 8.0 (±0.66) pmol/l, ranging 6.8–8.9 pmol/l. In the Comparison Group, 2 patients (20%) had fT_4 levels < 9 pmol/l. On reassessment one to three weeks later a mean fT_4 of 10.5 (±2.8) and a TSH of 3.9 (±2.8) were found. Only one patient developed a specific thyroid disorder(see post partum section) When

we compared clinical characteristics on admission (systolic and diastolic blood pressure, admission diagnosis, ultrasound Doppler PI of the umbilical artery, highest level of proteinuria, lowest platelet count during observation, gestational age at delivery, interval admission-delivery, birth weight, BWR, Apgar score at 5`, placenta weight centile) of women with low fT_4 to women with normal fT_4 values, no statistically significant differences were found. TPO antibodies were elevated in 7 patients at admission, but there was no significant relationship between the presence of TPOantibodies and fT_4 below 9 pmol/l (p= 0.22, χ^2 = 1.5, df=1). In univariate regression analyses, T_3 and T_4 levels had significant positive correlations with TBG levels, which is expected, as T_3 and T_4 are predominantly bound to TBG. Concentrations of this binding protein, subsequently, were significantly lower in women with a higher quantity of proteinuria (Pearson R = -0.33, p =.006), suggesting that lower T_4 and T_3 concentrations are due to loss of binding protein.

		Study Group	Comparison Group* [10]
N		80	10
TT_4	nmol/l	147 (33)	158 (26)
TT_3	nmol/l	2.7 (0.8)	2.88 (0.5)
TSH	mU/l	3.1 (3.9)	1.4 (0.6)
TBG	nmol/l	733 (140)	805 (72)
fT_4	pmol/l	9.8 (1.7)	10.2 (1.6)
rT_3	nmol/l	0.38 (0.13)	0.32 (0.08)
TPOab			
low/undetectable <70	kU/l	69 (86)	
elevated 70 – 2240	kU/l	7 (9)	
missing		4 (5)	
Ratio T_3/rT_3		7.9 (3.2)	9.5 (3.0)

Table 2: Maternal Hormone Levels at admission

Values are mean (sd) or n (%), as appropriate. *Ten women in the Comparison Group[10] had a mean age of 33.2(±3.5) years; 2(20%) were nulliparous; had a mean body weight of 79(±11) kg; median gestational age at testing was $35^3/_7$weeks, ranging $29^2/_7$–$41^1/_7$.

Postpartum maternal blood samples, taken 14 to 27 (mean 22) weeks after delivery were obtained in all patients but 1, who was lost to follow-up. Table 3 lists the results of maternal thyroid hormone levels at three months post term date. TPO antibodies were elevated in 8 patients (10%). There were two patients (2.5%) with abnormal thyroid hormone parameters. One patient, with a low fT_4 level during hospitalization, was diagnosed with Graves' Hyperthyroidism. The second patient had suppressed TSH (0.08 mU/l), elevated TT_3 (2.75 nmol/l) and normal T_4 (150 nmol/l) and fT_4 (15.7 pmol/l), suggesting T_3 hyperthyroidism. All other patients with a prior low fT_4 now

		Study Group	Normal Values
N		79	
TT_4	nmol/l	117 (27)	70 -150
T_3	nmol/l	2.0 (0.7)	1.3 – 2.7
TSH	mU/l	1.8 (1.2)	0.4 – 4
TBG	nmol/l	405 (125)	200 – 650
FT_4	pmol/l	13.7 (3.9)	10 – 22
rT_3	nmol/l	0.25 (0.08)	0.11 – 0.44
Ratio T_3/rT_3		8.5 (2.7)	
TPO ab low / undetectable < 70	kU/l	71 (90)	
elevated 150 - >3000	kU/l	8 (10)	

Table 3: Maternal hormone levels, 3 months after term date

Values are mean (sd) or n (%), as appropriate. Reference levels are normal lab values. One patient was lost to follow up.

	N		Study Group	Comparison Group([31]) n =114
TT_4	38	nmol/l	96 (37)	87 (30)
TT_3	37	nmol/l	0.87 (0.48) †	0.6 (0.3)
TSH	43	mU/l	7.9 (3.0) †	10.4 (11)
TBG	38	nmol/l	389 (114)	353 (96)
fT_4	46	pmol/l	11 (3.7) †	14.7 (4.5)
rT_3	33	nmol/l	2.9 (0.9)	3.1 (1.3)

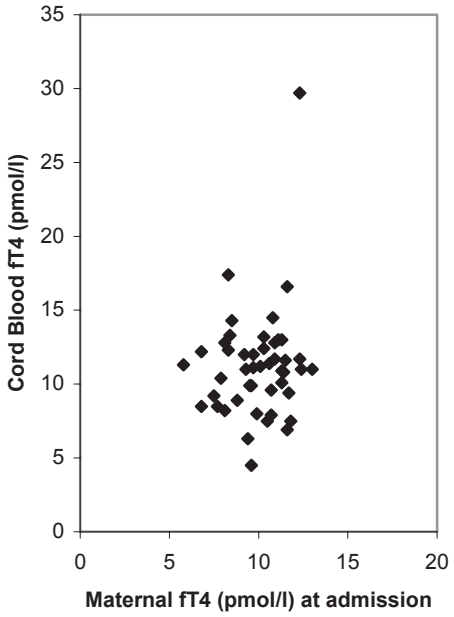
Table 4: Cord blood hormone levels of live-born neonates

Values are mean (sd). †$p < 0.001$ vs. cord blood of reference group, Student t test. As gestational age in the study group was 3 weeks higher than in the comparison group, higher concentrations of TT_4, fT_4, TT_3 and TBG anticipated in the study group.

had normal thyroid function.

Umbilical cord blood thyroid hormones were assessed in of 46 (67%) of live-born neonates. Results are shown in Table 4. As gestational age in the study group was 3 weeks higher than in the comparison group, higher concentrations of T_4, fT_4, T_3 and TBG were anticipated in the study group. Free T_4, however, was significantly lower in this group than in cord blood of the comparison group. There was no correlation between maternal and cord blood fT_4, as shown in Figure 1 (Pearson R = 0.17, p= 0.27).

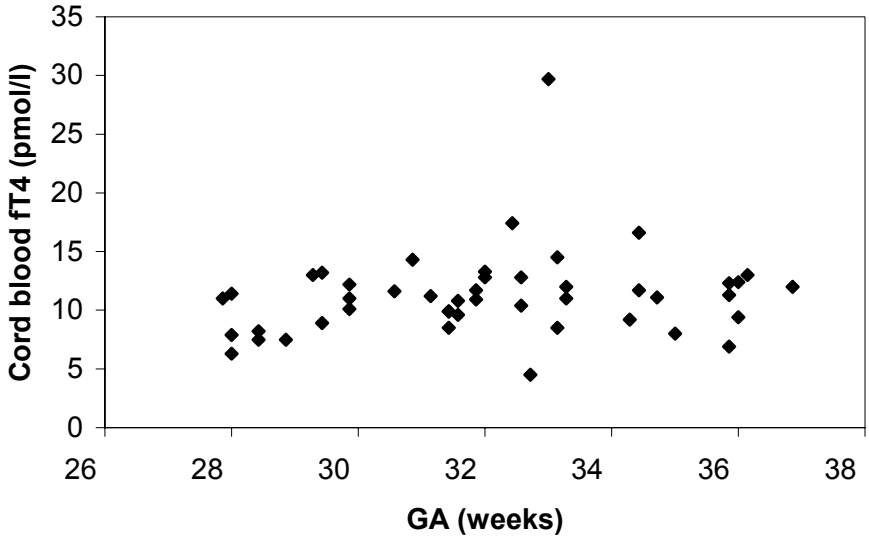
Figure 1: Cord blood fT_4 in relation to maternal fT_4



Cord blood freeT_4 values for 46 infants, measured at birth, by maternal blood fT_4 values, measured at admission. Pearson R = 0.17, p= 0.27

Contrary to normal pregnancies, in these growth restricted neonates, there was no relation between fT_4 levels and gestational age (Figure 2: Pearson R = 0.18, p= 0.22). On further univariate testing of perinatal factors, only umbilical artery pH (Pearson R = 0.48, p= 0.001) and gender (Pearson R = -0.33, p= 0.03) were significant determinants and gestational age, maternal fT_4, Doppler PI, BWR, placenta weight centile and treatment by plasma volume expansion were not. Multivariate linear regression analysis showed that umbilical cord fT_4 was significantly dependent on umbilical artery pH and gender, and was only slightly influenced by gestational age and maternal fT_4.

Figure 2: Cord blood fT_4 in relation to gestational age



Cord blood freeT_4 values for 46 infants by gestational age at birth. Pearson R = 0.18, p= 0.22

Discussion

This observational study of maternal and neonatal thyroid function was carried out in women referred to a tertiary care center because of early and severe hypertensive disorders of pregnancy. Although we did not find statistically significant differences with thyroid hormone levels of a group of 10 healthy women, TT_4, fT_4, and T_3 were somewhat lower, consistent with literature[29]. Moreover, 33% of patients had fT_4 concentrations below the lower limit of our local reference range of 9 pmol/l. These women had no identifiable maternal disorder or specific clinical course. In contrast to some authors,[14-16;23] we found total T_4 and T_3 to be of limited clinical value in assessing thyroid function in preeclampsia, as they reflect low TBG due to proteinuria. The observed fT_4 levels spontaneously changed to normal at reassessment during pregnancy and more so three months after term, at the scheduled post partum visit.
In the light of the present discussion on the necessity for screening thyroid function in pregnancy, it is therefore pivotal that the normal lower limit of fT_4 levels is identified, especially since no pathophysiological pathway has been determined to explain the observed fT_4 values in our study group. Notably, two subjects(20%) in the comparison group of healthy women had a third trimester fT_4 level below 9 pmol/l.
We anticipated a high prevalence of thyroid disorders and thyroid autoimmunity in the women in our study. However, three months post term specific thyroid abnormalities were diagnosed in only 2 women (2.5%), which is the normal prevalence of post partum thyroid disease in the non-preeclamptic population.[35]

In this study, umbilical fT_4 levels in the neonates were lower than in the comparison group, a finding in concordance with literature.[13;14;17;22;26] This low umbilical fT_4 was not related with maternal fT_4, therefore it is not likely to result from decreased maternal supply of fT_4 or impaired transfer. According to our data, these low fT_4 levels are due to prenatal acidosis as a result of utero placental insufficiency.
In the present study we were not able to investigate the duration of low fT_4 levels in utero. In a cordocentesis study, high fetal TSH and low fT_4 levels were found to be correlated to PO_2 levels in FGR fetuses without signs of fetal distress, suggesting slowly advancing chronic hypothyroxinemia.[36] The present study confirms the general concern about adequate fT_4 supply in FGR fetuses. It raises the question whether low fT_4 is just a derivative of intrauterine malnutrition. It could well be an independent cause of impaired brain development and the observed impairment of neuropsychological development in infants who were born growth restricted.[37;38]
These data stress the importance of an adequate follow up of growth restricted preterm infants, as they are at risk of hypothyroxinemia, and follow-up of preterm neonates shows a high prevalence of developmental disorders, especially after low postnatal thyroid hormone levels.[39]

In summary, we have demonstrated that transient hypothyroxinemia is common in women with severe hypertensive disorders, but is not associated with an increased incidence of thyroid disorders. The neonates show low fT_4 and TSH levels, unrelated to maternal fT_4 levels. These lower fT_4 levels are most likely caused by fetal acidosis. Follow-up is in progress and will reveal whether developmental outcome is associated with low fT_4 levels of mother, low fT_4 levels of the neonate, or merely with the deleterious consequences of fetal growth restriction itself.

Reference List

1. Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J et al. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. N.Engl.J.Med. 1999;341:549-55.
2. Calvo RM, Jauniaux E, Gulbis B, Asuncion M, Gervy C, Contempre B et al. Fetal tissues are exposed to biologically relevant free thyroxine concentrations during early phases of development. J.Clin.Endocrinol.Metab 2002;87:1768-77.
3. Pop VJ, Brouwers EP, Vader HL, Vulsma T, van Baar AL, de Vijlder JJ. Maternal hypothyroxinaemia during early pregnancy and subsequent child development: a 3-year follow-up study. Clin.Endocrinol.(Oxf) 2003;59:282-88.
4. Morreale dE, Obregon MJ, Escobar dR. Is neuropsychological development related to maternal hypothyroidism or to maternal hypothyroxinemia? J.Clin. Endocrinol.Metab 2000;85:3975-87.
5. Blazer S, Moreh-Waterman Y, Miller-Lotan R, Tamir A, Hochberg Z. Maternal hypothyroidism may affect fetal growth and neonatal thyroid function. Obstet. Gynecol. 2003;102:232-41.
6. Smallridge RC, Ladenson PW. Hypothyroidism in pregnancy: consequences to neonatal health. J.Clin.Endocrinol.Metab 2001;86:2349-53.
7. Spong CY. Subclinical hypothyroidism: should all pregnant women be screened? Obstet.Gynecol. 2005;105:235-36.
8. Casey BM, Dashe JS, Wells CE, McIntire DD, Byrd W, Leveno KJ et al. Subclinical hypothyroidism and pregnancy outcomes. Obstet.Gynecol. 2005;105:239-45.
9. Pop VJ, van Baar AL, Vulsma T. Should all pregnant women be screened for hypothyroidism? Lancet 1999;354:1224-25.
10. Berghout A, Endert E, Ross A, Hogerzeil HV, Smits NJ, Wiersinga WM. Thyroid function and thyroid size in normal pregnant women living in an iodine replete area. Clin.Endocrinol.(Oxf) 1994;41:375-79.
11. Glinoer D. The regulation of thyroid function in pregnancy: pathways of endocrine adaptation from physiology to pathology. Endocr.Rev. 1997;18:404-33.
12. Osathanondh R, Tulchinsky D, Chopra IJ. Total and free thyroxine and triiodothyronine in normal and complicated pregnancy. J.Clin.Endocrinol. Metab 1976;42:98-104.
13. Fetter WP, Waals-Van de Wal CM, Van Eyck J, Samson G, Bongers-Schokking JJ. Thyroid hormone concentrations in preterm infants born to pre-eclamptic women with placental insufficiency. Acta Paediatr. 1998;87:186-90.
14. Lao TT, Chin RK, Swaminathan R, Lam YM. Maternal thyroid hormones and outcome of pre-eclamptic pregnancies. Br.J.Obstet.Gynaecol. 1990;97:71-74.
15. Basbug M, Aygen E, Tayyar M, Tutus A, Kaya E, Oktem O. Correlation between maternal thyroid function tests and endothelin in preeclampsia-eclampsia. Obstet.Gynecol. 1999;94:551-55.
16. Qublan HS, Al Kaisi IJ, Hindawi IM, Hiasat MS, Awamleh I, Hamaideh AH et al. Severe pre-eclampsia and maternal thyroid function. J.Obstet.Gynaecol. 2003;23:244-46.
17. Kaya E, Sahin Y, Ozkececi Z, Pasaoglu H. Relation between birth weight and thyroid function in preeclampsia-eclampsia. Gynecol.Obstet.Invest 1994;37:30-33.

18. Davis LE, Leveno KJ, Cunningham FG. Hypothyroidism complicating pregnancy. Obstet.Gynecol. 1988;72:108-12.
19. Leung AS, Millar LK, Koonings PP, Montoro M, Mestman JH. Perinatal outcome in hypothyroid pregnancies. Obstet.Gynecol. 1993;81:349-53.
20. Millar LK, Wing DA, Leung AS, Koonings PP, Montoro MN, Mestman JH. Low birth weight and preeclampsia in pregnancies complicated by hyperthyroidism. Obstet.Gynecol. 1994;84:946-49.
21. Mecacci F, Parretti E, Cioni R, Lucchetti R, Magrini A, La Torre P et al. Thyroid autoimmunity and its association with non-organ-specific antibodies and subclinical alterations of thyroid function in women with a history of pregnancy loss or preeclampsia. J.Reprod.Immunol. 2000;46:39-50.
22. Narin N, Kurtoglu S, Basbug M, Caksen H, Kafali M, Durak AC et al. Thyroid function tests in the newborn infants of preeclamptic women. J.Pediatr. Endocrinol.Metab 1999;12:69-73.
23. Martin CR, Van Marter LJ, Allred EN, Leviton A. Growth-restricted premature infants are at increased risk for low thyroxine. Early Hum.Dev. 2001;64:119-28.
24. Gemer O, Shenhav S, Segal S, Tur-Kaspa I. Thyroid hormone levels in cord blood of infants with acidemia at birth. Eur.J.Obstet.Gynecol.Reprod.Biol. 2000;93:53-55.
25. Pereira DN, Procianoy RS. Effect of perinatal asphyxia on thyroid-stimulating hormone and thyroid hormone levels. Acta Paediatr. 2003;92:339-45.
26. Belet N, Imdat H, Yanik F, Kucukoduk S. Thyroid function tests in preterm infants born to preeclamptic mothers with placental insufficiency. J.Pediatr. Endocrinol.Metab 2003;16:1131-35.
27. Ganzevoort W, Rep A, Bonsel GJ, De Vries JI, Wolf H. A randomized trial of plasma volume expansion in hypertensive disorders of pregnancy: influence on the pulsatility indices of the fetal umbilical artery and middle cerebral artery. Am.J.Obstet.Gynecol. 2005;192:233-39.
28. Schiff E, Friedman SA, Sibai BM. Conservative management of severe preeclampsia remote from term. Obstet.Gynecol. 1994;84:626-30.
29. Glinoer D. The regulation of thyroid function during normal pregnancy: importance of the iodine nutrition status. Best.Pract.Res.Clin.Endocrinol. Metab 2004;18:133-52.
30. Pop VJ, Vulsma T. Maternal hypothyroxinaemia during (early) gestation. Lancet 2005;365:1604-06.
31. Van Wassenaer AG, Kok JH, Dekker FW, de Vijlder JJ. Thyroid function in very preterm infants: influences of gestational age and disease. Pediatr.Res. 1997;42:604-09.
32. Wiersinga WM, Chopra IJ. Radioimmunoassay of thyroxine (T4), 3,5,3'-triiodothyronine (T3), 3,3',5'-triiodothyronine (reverse T3, rT3), and 3,3'-diiodothyronine (T2). Methods Enzymol. 1982;84:272-303.
33. Gardosi J, Chang A, Kalyan B, Sahota D, Symonds EM. Customised antenatal growth charts. Lancet 1992;339:283-87.
34. Arts, N. F. Th., Galjaard, H., Kloosterman, G. J., and Treffers, P. E. De pathologische zwangerschap. De voortplanting van de mens. Leerboek voor obstetrie en gynaecologie. 7th edition, 1985. 340-341. Centen Weesp, the Netherlands.

35. Pearce EN, Farwell AP, Braverman LE. Thyroiditis. N.Engl.J.Med. 2003;348:2646-55.
36. Thorpe-Beeston JG, Nicolaides KH, Snijders RJ, Felton CV, McGregor AM. Thyroid function in small for gestational age fetuses. Obstet.Gynecol. 1991;77:701-06.
37. Cheng SW, Chou HC, Tsou KI, Fang LJ, Tsao PN. Delivery before 32 weeks of gestation for maternal pre-eclampsia: neonatal outcome and 2-year developmental outcome. Early Hum.Dev. 2004;76:39-46.
38. Many A, Fattal A, Leitner Y, Kupferminc MJ, Harel S, Jaffa A. Neurodevelopmental and cognitive assessment of children born growth restricted to mothers with and without preeclampsia. Hypertens.Pregnancy. 2003;22:25-29.
39. Reuss ML, Paneth N, Pinto-Martin JA, Lorenz JM, Susser M. The relation of transient hypothyroxinemia in preterm infants to neurologic development at two years of age. N.Engl.J.Med. 1996;334:821-27.

Chapter 6:

Seven Transcripts Characterize HELLP syndrome

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Abstract

The human placenta is prerequisite for the development of gestational hypertensive diseases like early-onset preeclampsia (PE) and Hemolysis, Elevated Liver enzymes and Low platelets (HELLP) syndrome. Both syndromes are associated with extensive maternal and perinatal mortality, and morbidity with life long consequences.

We aimed to investigate differences in gene expression between placental tissue obtained from normotensive pregnant women and women with PE and HELLP syndrome.

Firstly, comparison of Serial Analysis of Gene Expression profiles of a 28 weeks' control placenta (available after idiopathic premature delivery) to a HELLP/PE placenta matched for gestational age identified 404 differentially expressed transcripts.

Secondly, using sqPCR, the expression levels of 37 of these transcripts were analyzed in placentas of 36 pregnant women, 22 with preeclampsia and HELLP syndrome.

Thirdly, nearest centroid classification determined the HELLP specific molecular signature consisting of the upregulated expression of genes encoding the *vascular endothelial growth factor receptor* (*FLT1*), *leptin* (*LEP*), *pappalysin 2* (*PAPPA2*), and *WW domain containing transcription regulator 1* (*WWTR1*) combined with down regulated expression of the genes encoding *cadherin-associated protein* (*CTNNAL*), glutathione S-transferase pi (*GSTP1*) and calgranulin A (*S100A8*). This set discriminates HELLP placenta from control and PE placenta with a 24% misclassification rate (95%CI 8.3 to 41.9%), independent from known risk factors like parity and ethnicity.

The transcripts involved correspond to diverse molecular pathways, exemplifying the multigenic molecular basis of the disorder. This distinct placental molecular signature suggests that HELLP is not a PE variant but a separate disease entity. Our data may prove fundamental for the further molecular analysis of PE and HELLP syndrome.

Introduction

Gestational hypertensive disorders as preeclampsia (pregnancy-induced hypertension combined with proteinuria, PE) and Hemolysis, Elevated Liver enzymes and Low platelets (HELLP) syndrome, complicate 2 to 7% of all pregnancies. They are a major obstetrical problem and contribute extensively to maternal and perinatal morbidity and mortality in the Western world.[1-3] Despite reports describing HELLP in patients with normal or minimally elevated blood pressure without proteinuria, HELLP syndrome is categorized as a gestational hypertensive disorder and seen as the more severe variant of PE.[2,4] HELLP and PE become clinically manifest during the second (early onset form) or third trimester (late onset form) of pregnancy but the initiating event occurs much earlier in gestation. In case of HELLP and PE, aberrant restructuring of the uterine spiral arteries by invading trophoblast between week 8 and 12 of gestation ultimately causes poor placental perfusion and placental ischemia.[2] Angiogenic factors, cell adhesion proteins, immunological factors, matrix metalloproteinases and their inhibitors are all anticipated to play a role in this crucial process of spiral artery dilatation.[5,6]

It is well documented that the placenta is prerequisite for the development of HELLP syndrome and PE. The heterogeneity of the disorder, the limited number of familial cases and the at least 8 week window between the molecular initiation and clinical manifestation of the disease, have hampered identification of factors that contribute to the molecular cause, diagnosis and treatment of HELLP and PE.[7,8]

Disease specific transcriptional signatures have proven essential to classify malignancies at the molecular level guiding the development and use of targeted therapeutics.[9,10] In analogy we defined the disease specific aberrant expression profile of the HELLP/PE placenta after non-selective comparison of control versus HELLP/PE transcriptomes by serial analysis of gene expression (SAGE) analysis.[11] SAGE is an unbiased and technically uncomplicated high-throughput genomics technique where individual mRNA molecules are represented by 10 basepair tags that are localized downstream of the most 3' CATG sequence in each mRNA. Expression levels are determined by straightforward counting of the number of times a tag sequence occurs rather than by measuring relative signal intensities.

Comparison of our 28-week gestational age placenta SAGE libraries resulted in a differential expression profile providing the opportunity to define a composite multigenic disease specific signature. We successively developed a molecular signature for gestational hypertensive disease by analyzing the differential SAGE expression profile using sqRT-PCR for 37 known genes in placental tissue of 22 PE and/or HELLP patients as well as 14 normotensive controls.

Methods

Definitions
Clinical/biochemical definitions used are HELLP (platelet count < 100 x 109 /l, aspartate aminotransferase ≥ 70 U/l and/or lactate dehydrogenase ≥ 600U/l), PE (blood pressure ≥ 140/90 mm Hg and proteinuria > 0.3 g / 24 hr) and severe PE (PE with a diastolic blood pressure ≥ 110 mm Hg).[12]

Patients
SAGE index and control patients were matched for race, parity, gestational age at delivery, mode of delivery and gender of the fetus.

The HELLP/PE patient is a Caucasian nulliparous woman with no previous history of hypertension or renal disease, referred because of fetal growth restriction and clinical symptoms of PE and HELLP syndrome (blood pressure 150/100 mmHg and protein excretion in urine of 17.6 g/24 hrs, platelet count nadir 40 x 109 /L, aspartate aminotransferase maximum 1350 U/L, lactic dehydrogenase maximum 1831 U/L). She received magnesium sulphate as eclampsia prophylaxis, paracetamol and morphine because of severe abdominal pain and one course of betamethasone to enhance fetal lung maturation. Because of an abnormal fetal non-stress test (CTG) a caesarean section was performed at 28 $^{5}/_{7}$ weeks gestation, thirteen days after admission.
The 780 gram female neonate had no clinical signs of asphyxia at or after birth (5`Apgar Score: 8) and was admitted to the neonatal intensive care for 30 days, ventilated and treated with surfactant because of respiratory distress syndrome. She was released after an additional stay of 28 days in the pediatric department of the local hospital. Further follow up until the corrected age of 1 year was normal. As routine follow up for HELLP patients, three months post term date, the mother tested negative for thrombophilia, renal disease and hypertension.

The control patient was referred because of imminent premature delivery and to enhance fetal lung maturation she was treated with tocolytics and betamethasone. Two days after admission, at 28$^{1}/_{7}$ weeks gestation a caesarean section was performed because of abnormal fetal non-stress test. Neither before nor after delivery did the mother have signs of infection. The 1220 gram neonate had no signs of asphyxia at birth (5`Apgar score: 10; umbilical artery pH 7.24) and was admitted to the neonatal intensive care for seven days, without need for ventilatory or circulatory support. Her clinical course was relatively uneventful. A right sided choanal atresia was operatively corrected at age 8 months. Further follow up until the uncorrected age of 1 year is normal.
Additionally tissues from 21 HELLP/PE and 13 Control placentas were collected. (For clinical data see Supplementary Data on page 117).

Tissue selection, preparation, RNA isolation and cDNA synthesis
The institutional review board of the Academic Medical Center approved the study protocol. Placental tissue was obtained with informed consent. For SAGE library construction, a macroscopically viable (non-infarcted) cotyledon of trophoblast tissue obtained from the maternal side and was directly frozen in liquid nitrogen. All other samples were frozen within 2 hours after birth. All samples were kept at -80°C until use. After homogenization under liquid nitrogen total RNA was extracted using TRIzol (Gibco BRL). RNA integrity was checked by gel electrophoresis and mRNA was extracted using PolyA Tract mRNA Isolating System III (Promega).

Construction and analysis of SAGE libraries
Libraries were constructed using the I-SAGE kit (Invitrogen, Groningen, The Netherlands) from 5 µg placenta mRNA. SAGE clones were sequenced with Big Dye Terminator V.1.1 Cycle Sequencing (Applied Biosystems) using T7 primers (Sigma), run on an ABI377XL Automatic Sequencer (Perkin-Elmer Corp., Norwalk, CT) and analyzed using Sequence Analysis 3.0 software. After exclusion of linker artefacts and duplicate ditags SAGE data were analyzed using SAGE 2000 software version 4.5. Cancer Genome Anatomy Project Tag to Gene list "Hs_short.best_gene" at http://cgap.nci.nih.gov/SAGE was used for identification. As this database is known to misclassify tags that hit on multiple genes, tag to gene allocation of these allocations was cross validated with the NCBI Tag to Gene list "SAGEmap_tag_ug-full" at ftp://ftp.ncbi.nlm.nih.gov/pub/sage/map/Hs/NlaIII/ Unequivocal allocation was not possible if multiple genes are identified with the tag following the most 3` CATG before the poly(A) tract or in case of highly repetitive tags. Differentially regulated transcripts were linked to their chromosomal location using the Human Transcriptome Map (HTM) http://bioinfo.amc.uva.nl/htmseq/controller[13] Gene Ontology classes were identified at http://www.ebi.ac.uk/ego/?&format=nomenu and linked to all differentially expressed genes, not accepting NAS and NR classes of evidence.

Semi quantitative RT-PCR
A subset of 37 transcripts with manually checked proper tag-to-gene annotation was analyzed by semi quantitative RT-PCR. For *ADAM12, CGA, CKAP2, COL3A1, COL4A2, CRH, CTNNAL1, CYR61, DUSP1, EFEMP1, EPAS1, FLT1, GSTP1, IGF1, IGF2, ILK, INHBA, ITGA5, ITGA6, LEP, MAP4K3, MGST2, NUDC, PA2G4, PAPPA2, PRL, PSG7, S100A8, SERPINA3, SPP1, TIMP2, TIMP3, TNFSF10, TRIM28, TRIP12, VDAC1* and *WWTR1* the log linear phase for the corresponding primer sets (primer sequences and amplification conditions available on request) was determined and amplicons were quantified by One D Scan® and normalized to *Eukaryotic Translation Elongation Factor 1 alpha 1* (*EEF1A1,* expression level 0.33 ± 0.24% in 22 SAGE libraries from non-tumor human tissues) expression after size correction, essentially as described by Bakker.[14]

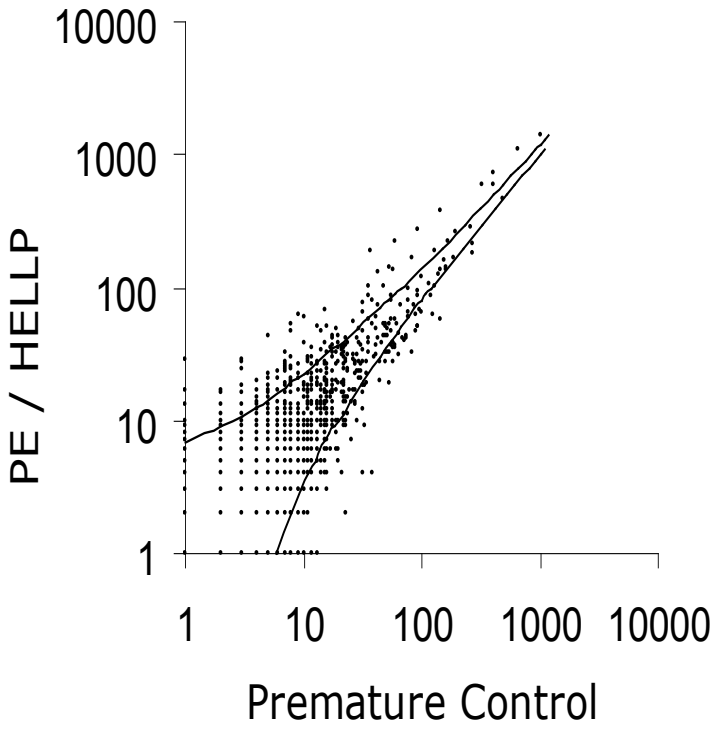

Figure 1: Tag abundance distribution in the Premature Control and HELLP/PE placenta SAGE libraries.
Each dot represents an individual SAGE tag for which the tag count in both libraries is displayed, on the X (Premature Control library) and Y (HELLP/PE library) axis. Tag abundance is depicted on logarithmic scales and each dot can represent more than one tag with identical counts in the two libraries. Plotted lines represent upper and lower threshold for differences in level of expression by two-sided Z test, p value < 0.05.

Statistical Analysis
SAGE data were analyzed using the USAGE[15] statistical program. Differences in level of expression were assessed by two-sided Z test,[16] p values < 0.05 were considered significant.

To determine the classification model we randomly divided all samples (*N=36*), patients (n_1*=22*) and the control group (n_2*=14*), in a training (of size *A*) and test set (of size *N-A*) such that the proportions of samples of each class in these sets are equal to their proportions in the complete dataset. For the training set the average expression profiles of the Control class and the PE/HELLP class is determined by calculating the average expression levels for each of the *p* transcripts over the samples in each class. Next, the Pearson correlation between the expression profile of each sample and the average class profiles is calculated. The correlation coefficients for the HELLP/PE class reflect the chance that the sample is from the HELLP/PE class. Samples with a correlation above the threshold are classified as HELLP/PE. This threshold is chosen at the correlation coefficient value that minimizes the number of misclassified samples from the training set. Similarly, one defines the correlation threshold from the samples that are ordered according to the control group and determines the number of misclassified sample. The model with the fewest number of misclassifications is selected (see Figure 2).
The model is now used to classify the test samples. To determine the average misclassification error and the 95% confidence intervals (95% CI) we generate 200 random samples from the complete dataset, which results in 200 training and test sets of size *A* and *N-A* respectively for which the classification model is built and the classification error is determined. Since the size of the training set affects the misclassification rate this procedure is repeated for training set sizes of *A=15* to *A=31*.
The subset of transcripts selected to participate in the model is determined by leave-one-out cross-validation of the training set within the sampling loop. First, the transcripts are sorted according to their absolute Pearson correlation with the outcome based on all but one training samples. Subsequently, the *p* most correlating transcripts are selected and the classification model is constructed from the *A-1* training samples. The left out sample is then classified with this model. By leaving out every sample once from the training set and classifying the left out sample with the constructed model, the cross-validation error of the model with *p* transcripts is determined. This error is then determined for *p* in the range from three to 37 transcripts. The model with the lowest cross-validation error is selected and determines the number of transcripts to be included in the model.
The final model is built from the complete dataset (*N=36*) for a training set size (*A=24*) that resulted in the best model. To determine the number of transcripts for the final model we summed the classification errors for the 200 models with equal numbers of included transcripts. From this, we determined the size *p* of the model that resulted in the fewest misclassification errors. This model included seven genes. Subsequently, we selected the *p=7*

Table 1: Most frequently occurring annotated tags in the Premature Control and HELLP/PE placenta SAGE libraries.

Symbol	Title	Tag count premature control	Tag count HELLP/PE	UniGene cluster	Tag sequence
HBG1	Hemoglobin, gamma A	1024	1399	Hs.295459	ATGCAGAGCT
HBA1	Hemoglobin, alpha 1	655	1095	Hs.449630	CTTCTTGCCC
	unequivocal allocation not possible	494	470		ACTTTTTCAA
HBG1	Hemoglobin, gamma A	411	598	Hs.295459	ATTCAGAGCT
CSH1	Chorionic somatomammotropin hormone 1 (placental lactogen)	400	731	Hs.406754	GTGCAGTGCC
HBA1	Hemoglobin, alpha 1	327	590	Hs.449630	CCCAACGCGC
RPS29	Ribosomal protein S29	272	184	Hs.156367	ATAATTCTTT
	unequivocal allocation not possible	268	217		CCCATCGTCC
TPT1	Tumor protein, translationally-controlled 1	265	284	Hs.374596	TAGGTTGTCT
	unequivocal allocation not possible	197	257		TTCATACACC
	unequivocal allocation not possible	188	167		CACCTAATTG
	unequivocal allocation not possible	167	219		AAAAAAAAAA
IGF2	Insulin-like growth factor 2 (somatomedin A)	162	144	Hs.523414	CTTGGGTTTT
NT5C2	5'-nucleotidase, cytosolic II	161	138	Hs.591920	CCTGTAATCC
PAFAH2	Platelet-activating factor acetylhydrolase 2, 40kDa	154	160	Hs.590913	GTGAAACCCC
DLK1	Delta-like 1 homolog (Drosophila)	147	58	Hs.533717	ATACAGAATA
PSG9	Pregnancy specific beta-1-glycoprotein 9	146	378	Hs.502092	AGTATTCATA
B2M	Beta-2-microglobulin	144	135	Hs.534255	GTTGTGGTTA
TSPAN4	Tetraspanin 4	140	123	Hs.437594	GGATTTGGCC
CCNB1IP1	Cyclin B1 interacting protein 1	131	191	Hs.107003	CCACTGCACT
PSG9	Pregnancy specific beta-1-glycoprotein 9	92	278	Hs.502092	TACCACATTT
TFPI2	Tissue factor pathway inhibitor 2	82	170	Hs.438231	TGCTTTTAAC
	unequivocal allocation not possible	59	222		TCTCCATACC
CGA	Glycoprotein hormones, alpha polypeptide	37	190	Hs.119689	GGCTGCTGCT

The top 20 of most frequently occurring tags is shaded. Tag to Gene allocation was done using CGAP Hs_short.best_gene for identification and cross validated with the NCBI SAGEmap_tag_ug-full. Unequivocal allocation was not possible if multiple genes are identified with the tag following the most 3` CATG before a poly AAAA tract or in case of highly repetitive tags.

transcripts that were selected most often in the 200 models.
After calculating standard normal deviates for all samples per gene, two-way hierarchical Clustering was performed with no prior filtering by centered correlation using Cluster software version 2.11 (Eisen Lab, Stanford University CA, USA http://rana.lbl.gov/downloads/Cluster/).

Results

The Premature Control and HELLP/PE placental transcriptomes
From two 28-week gestational age placentas, a control and a HELLP/PE placenta, 38,257 respectively 41,704 tags were sequenced corresponding to 14,513 respectively 14,330 unique tags (Supplementary Table 1, Available on doi:10.1016/j.placenta.2008.02.007
In both libraries 72% of unique tags allocate to a gene with known or inferred function, 16 % correspond to an EST sequence whereas the remaining 12% (of which 235 occur in both libraries) do not match any cDNA sequence. The libraries are 98.3% similar when taking into account all tags (Figure 1) and the top 20 most frequently occurring unique transcripts in both libraries have 16 tags or transcripts (80%) in common (Table 1). With an expression level of 6.7% in the premature control and 9.4% in the HELLP/PE library, 4 out of 6 most frequent unique transcripts represent hemoglobins suggesting that human placenta, like previously shown for mice placenta,[17] is a major hematopoietic organ. Northern Blot analysis of matched umbilical cord and maternal blood samples excluded the possibility that the observed Hemoglobin expression is due to contamination from nucleated red blood cells from either fetal or maternal blood (data not shown).
Of the 404 significantly differentially expressed tags 180 are up- and 224 are down regulated in HELLP/PE placenta compared to the premature control and 370 can be annotated to a known gene (although 20 not unequivocally). The 350 unequivocally annotated differentially expressed transcripts (displayed in Supplementary Table 2, Available on doi:10.1016/j.placenta.2008.02.007) are dispersed over the humane genome (Figure 4) with only eight transcripts localized on previously reported PE loci.[18-21] Differential expression in the cell adhesion Gene Ontology Class accounts for 6.3% of the absolute differentially expressed tag count, substantiating the contribution of aberrant cell adhesion to the pathogenesis of HELLP/PE. Twenty-three differentially regulated transcripts relate to the immune response Gene Ontology classes representing 4.2% of the absolute sum of differences. *HLA-C* and –G (both expressed relatively low) and the higher expressed *HLA–E* (with absolute expression levels of 10 vs. 4) are not differentially expressed in our SAGE libraries. Only 2.5% (18 transcripts) relate to the oxidative stress Gene Ontology classes.
Signature determination
To distinguish inter-individual changes in gene expression from those associated with disease, semi quantitative RT-PCR experiments were performed 36 placenta tissue samples. Based on differential expression

of the corresponding tag (see supplementary Table 2), 25 transcripts belonging to diverse functional classes or processes based on NCBIs Gene Ontology were selected; *ADAM12* (metallopeptidase activity/cell adhesion), CGA (hormone activity/cell-cell signaling), *COL3A1* (extracellular matrix structural constituent/circulation), COL4A2 (extracellular matrix structural constituent/negative regulation of angiogenesis), CRH (neuropeptide hormone activity/parturition), *CTNNAL1* (cadherin binding/cell adhesion), CYR61 (heparin binding/cell proliferation), *EFEMP1* (calcium ion binding/ visual perception), *EPAS1* (transcription coactivator activity/response to hypoxia), *FLT1* (vascular endothelial growth factor activity/patterning of blood vessels), *IGF2* (growth factor activity/insulin receptor signaling pathway)*, INHBA* (activin inhibitor activity/cell cycle arrest), *ITGA6* (calcium ion binding/integrin-mediated signaling pathway), *LEP* (hormone activity/ energy reserve metabolic process), *MGST2* (glutathione transferase activity/ cell-cell signaling), *NUDC* (protein binding/cell proliferation), *PAPPA2* (metallopeptidase activity/cell differentiation), PSG7 (molecular function/ female pregnancy), S100A8 (calcium ion binding/inflammatory response), *SERPINA3* (chemotrypsin inhibitor activity/inflammatory response), *TIMP2* (metalloendopeptidase inhibitor activity/negative regulation of cell proliferation), TIMP3 (metalloendopeptidase inhibitor activity /induction of apoptosis by extracellular signals), *TRIM28* (transcription co-activator activity/transcription), *TRIP12* (thyroid hormone receptor binding/protein ubiquitination) and *WWTR1* (transcription regulator activity/transcription).

Not corresponding to differentially regulated tags in our SAGE libraries, but included based on their reported association with PE were GSTP1 (tagcount 6 versus 10), *IGF1* (tagcount 99 versus 67) and ILK (tagcount 10 versus 8). As technical controls *CKAP2* (tagcount of 30 versus 18), *DUSP1* (tagcount 11 versus 21), *ITGA5* (tagcount 3 versus 7), *MAP4K3* (tagcount 2 versus 3), *PA2G4* (tagcount 5 versus 5), *PRL* (tagcount 1 versus 4), *SPP1* (tagcount 12 versus 13), *TNFSF10* (tagcount 8 versus 12) and *VDAC1* (tagcount 10 versus 9) were included. Relative amplicon intensity reflected the expression levels established by SAGE tagcount. Except for *CTNNAL1*, *PSG7* and *TRIM28*, differential mRNA expression levels assessed by semi quantitative RT-PCR are consistent with the SAGE data.

To extract the most predictive set of transcripts defining the PE/HELLP transcriptional signature we applied the nearest centroid classification method (Figure 2). This method predicts cancer outcome more realistically than the over-optimistic clustering models that often do not classify patients better than chance.[22] We derived classification models for a range of training set sizes of which all but the most extreme cases classified better than random.
The final classification model used has a training set size of $^2/_3$ of the subjects (i.e. 24 out of 36 samples) and 200 random splits of the complete data set into training- and test set. A classification model becomes significant when

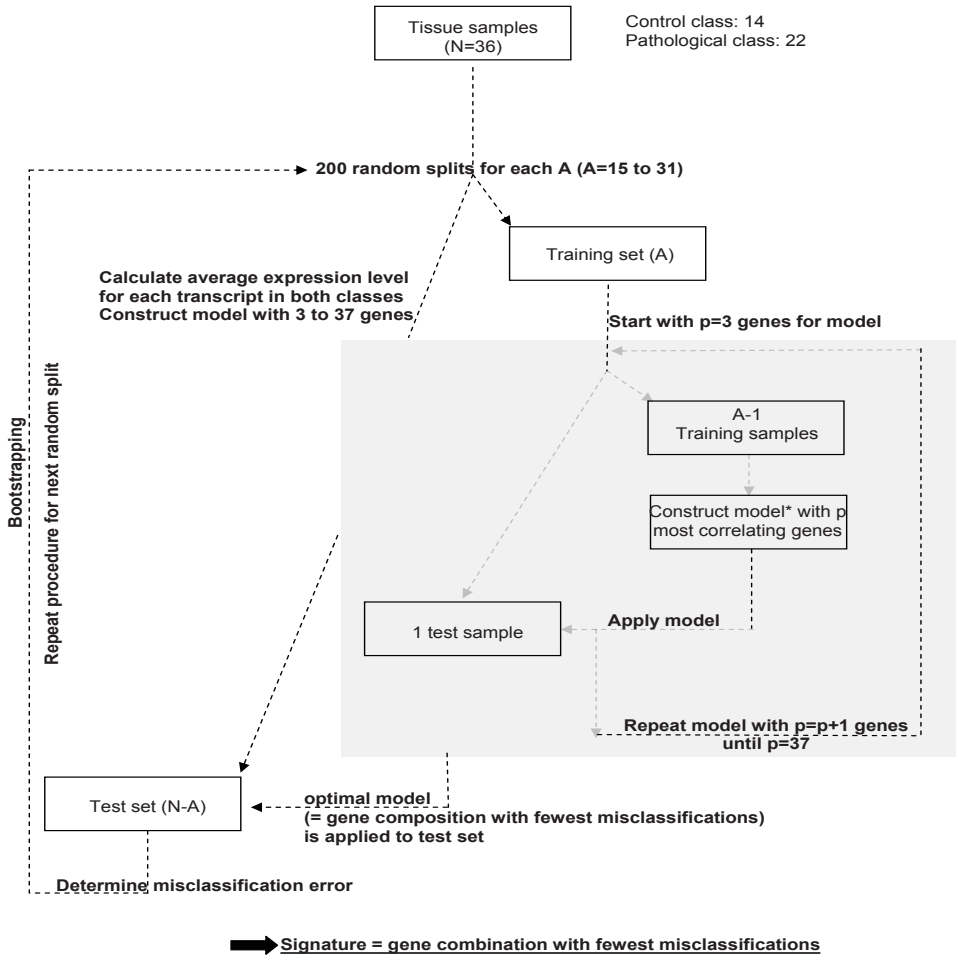

Figure 2: Signature determination by the nearest centroid classification method

Shaded area represents the leave-one-out-cross-validation procedure in which every sample is used once as a test sample. In total A different models are constructed with p genes. Each model is defined by average gene expression profile for both classes of tissues. The cross-validation procedure is applied to the nearest centroid statistical model that is constructed from the p most correlating genes to determine the performance of the model. A statistical model is made for p=3 to p=37 genes. The final model constitutes the model with p genes that gave the fewest number of misclassifications.

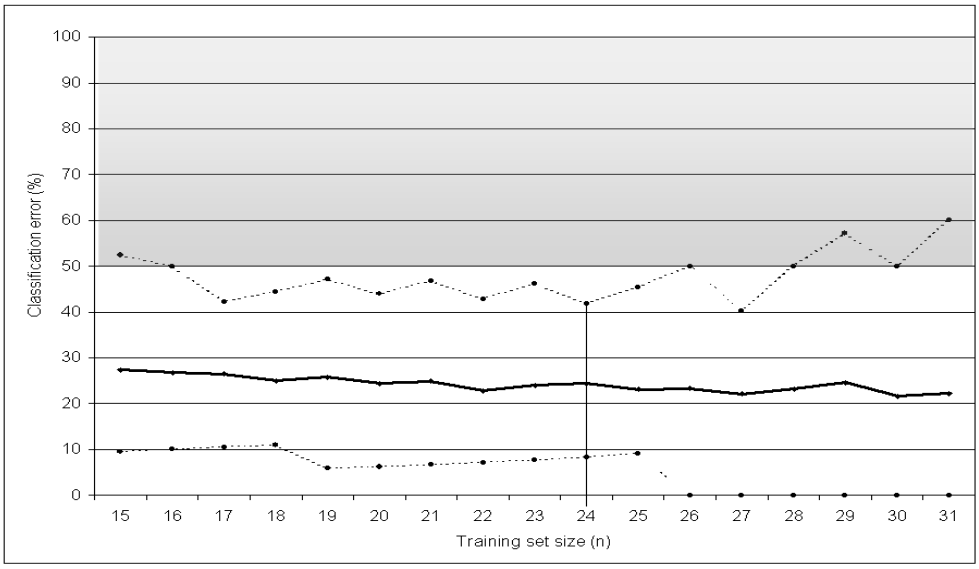

Figure 3: Proportion of misclassifications in the test sets as a function of corresponding training-set sizes.

The uninterrupted line indicates the average misclassification errors. Dotted lines denote the 95% confidence intervals. The model with a training set size of 24 samples (containing seven genes) which was finally used is indicated by the vertical line.

the 95% CI drops below 50%. Ordering our model by correlation with the normal expression profile led to a mean misclassification rate of 24.4% and an upper limit of the 95% CI of 41.9%. In the final classification model, the four classification errors render the molecular signature a sensitivity of 91% with a specificity of 86%.(Figure 5, on page 115). Figure 3 displays the misclassification error in the test sets for different training-set sizes.

The final set with optimal performance ranking consists of seven transcripts that were selected most often in the 200 models: *CTNNAL1, FLT1, GSTP1, LEP, PAPPA2, S100A8* and *WWTR1*. Strikingly, cluster analysis of placentas and selected transcripts uniquely segregates the HELLP placental transcriptional signature from both the severe PE and the Control signature. HELLP is distinguished by up regulation of *FLT1, LEP, PAPPA2, WWTR1* combined with down regulation of *CTNNAL1, GSTP1, S100A8* (Figure 6). Neither individual factors as ethnicity or parity nor any of the clinical or treatment parameters account for these differences (for clinical data see Supplementary Data).

Discussion

We analyzed and compared SAGE libraries from a 28 weeks' gestation control and a 28 weeks' gestation HELLP/PE placenta. From both tissues, we obtained around 40,000 SAGE tags corresponding to approximately 14,000 different transcripts. Illustrative of the high similarity of both tissue samples is the fact that the libraries are over 98% similar and that only 404 tags are statistically differentially expressed. Of the differential expression profile, 2.5% of tags allocate to transcripts encoding proteins belonging to the oxidative stress Gene Ontology classes, 6.3% correspond to the cell adhesion and 4.2% correspond to the immune response Gene Ontology classes. In number and diversity with respect to pathway analysis and Gene Ontology this is comparable to recent microarray analysis that identified 366 differentially regulated genes in PE.[23]

Although the control placenta, from a pregnancy that terminated at 28 weeks' gestation because of fetal distress cannot be termed 'normal', there was no evidence of infection, congenital abnormalities, maternal disease or any other explanation for the premature birth. Since the mode of delivery was equal, an effect of parturition on the expression profile is unlikely. We therefore agree with Zhou et al that gestational age matched preterm placenta is a proper control for comparative purposes in PE research.[24]

Downstream sqRT-PCR analysis of 36 placental tissue samples and nearest centroid classification of the 37 analyzed genes defined a molecular signature that distinguishes HELLP placenta not only from normal placenta tissue but to our surprise also from PE placental tissue. The maternal disease HELLP syndrome that is caused by defective placentation early during gestation is generally described as 'just one pattern of presentation or a particular potent form' of severe PE.[7,25,26] This study provides the first placental molecular basis for the evolving clinical opinion that advocates HELLP as a distinct disease entity.[27]

Our molecular signature that discriminates HELLP placenta from control and PE was identified by the nearest centroid classification method and has a 24% misclassification rate with an upper limit of the 95% CI of 41.9%. For a study using high throughput genomics that depends on bioinformatical analysis a classification model is considered significant when the 95% CI drops below 50%.[22] Our HELLP signature consists of 7 transcripts (encoding *cadherin-associated protein* (*CTNNAL1*), *leptin* (*LEP*), *vascular endothelial growth factor receptor* (*FLT1*), *glutathione S-transferase pi* (*GSTP1*), *pappalysin 2* (*PAPPA2*), *calgranulin A* (*S100A8*) and *WW domain containing transcription regulator 1* (*WWTR1*)) that belong to distinct Gene Ontology classes reflecting the diverse pathways implicated in the initiation and progression of HELLP syndrome.

Four of these seven genes (*FLT1, GSTP1, LEP* and *PAPPA2*) have been previously associated with PE, although not specifically with HELLP syndrome. Elevated maternal serum levels of the soluble form of *FLT1* (*sFLT1*) decrease the circulating levels of VEGF and PlGF and inhibit VEGF- and PlGF-induced vasodilatation.[28] Down regulation of *GSTP1* reflects the decreased capacity of the glutathione/glutathione S-transferase detoxification system and increased oxidative stress.[29] *LEP* regulates the fetal energy reserve metabolism[30] and is an angiogenic factor induced by hypoxia in adipose tissue.[31] In contrast to the increased *LEP* expression in preeclamptic placentas, increased LEP expression is not observed in placentas from women with growth-restricted pregnancies without PE or HELLP[32] indicating that the increased *LEP* expression that is part of the HELLP signature is not due to fetal growth restriction that is present in our patient cohort (see Supplementary Data). *PAPPA2* is very homologous to the metalloproteinase pregnancy-associated plasma protein-A (PAPPA). Increased serum levels of PAPPA have been reported for growth restricted pregnancies and PE[33] fitting the increased expression of *PAPPA2* in our HELLP signature.

The altered expression *of WWTR1, CTNNAL1* and *S100A8* has not been associated with placental function or dysfunction previously. They unexpectedly contribute to the distinction between HELLP and PE in addition to the distinction from normotensive pregnancy. *WWTR1* facilitates the formation of protein scaffolds and regulates the activity of transcription factors.[34] Inactivation in mice leads to renal cysts.[35] Identifying the transcription factors of which the activity is regulated by *WWTR1* in placenta, and the consequences of the increased *WWTR1* expression for placenta dysfunction need further investigation.

Expression of the adhesion molecule CTNNAL1 is down regulated in HELLP placenta. Interestingly, the gene resides on the locus that maps to hypertensive nephropathy.[36] S100 proteins are a family of low-molecular weight calcium-binding proteins. S100A8 is important for inflammatory activation and leukocyte trafficking[37] and is the endogenous ligand of Toll-like receptor 4.[38]

At this time, one can only speculate that the known functions of these genes make them candidates for the pathogenesis of HELLP syndrome. Clinical research does not allow identification of any cause effect relationship. On the other hand, our findings fit easily with the numerous theories on the pathophysiology of gestational hypertensive disease: abnormal immune or inflammatory processes, failure of trophoblast invasion, disequilibrium in angiogenic proteins, increased oxidative stress, endothelial dysfunction and aberrations in calciotrophic hormones.[1,6,39,40] Microarray analysis[23,41] further substantiate that gestational hypertensive disease like preeclampsia results from of a cascade of pathophysiological changes[1] following a relatively early placentation defect[6] due to aberrant angiogenesis, failure of proper trophoblast invasion and the engagement of multiple cellular targets in the late phase of the disease.

Our data do not support the role of placental pro-inflammatory cytokines in the development of generalized maternal endothelial cell activation[42] since *TNF* and *tumor necrosis factor alpha converting enzyme* (*ADAM17*) are not differentially regulated. The equal expression levels of the unique *HLA-C, -E* and *–G* combination in our gestational age matched placentas argues against differential HLA trophoblast expression[6,43] as one of the immunological components in the development of PE or HELLP syndrome, although it is feasible that altered expression levels that contribute to the initial molecular events around 12 weeks' gestation are not present at a later stage of the disease in this case 28 weeks' gestation. Unfortunately this is inaccessible for investigation in humans but part of the differential expression profile relates to key-events in early placentation,[1,6,44] suggesting that they are not solely resultant but also causal to the disease.

In conclusion, we identified a heterogenic placental HELLP molecular signature consisting of the combined altered expression of *CTNNAL1, FLT1, GSTP1, LEP, PAPPA2, S100A8* and *WWTR1*. Our data show why up-to-now no single comprehensive HELLP or PE marker has been identified and why all association studies of preeclampsia, with part of the cases complicated by HELLP syndrome, are frustrated by poor predictive value.[45]

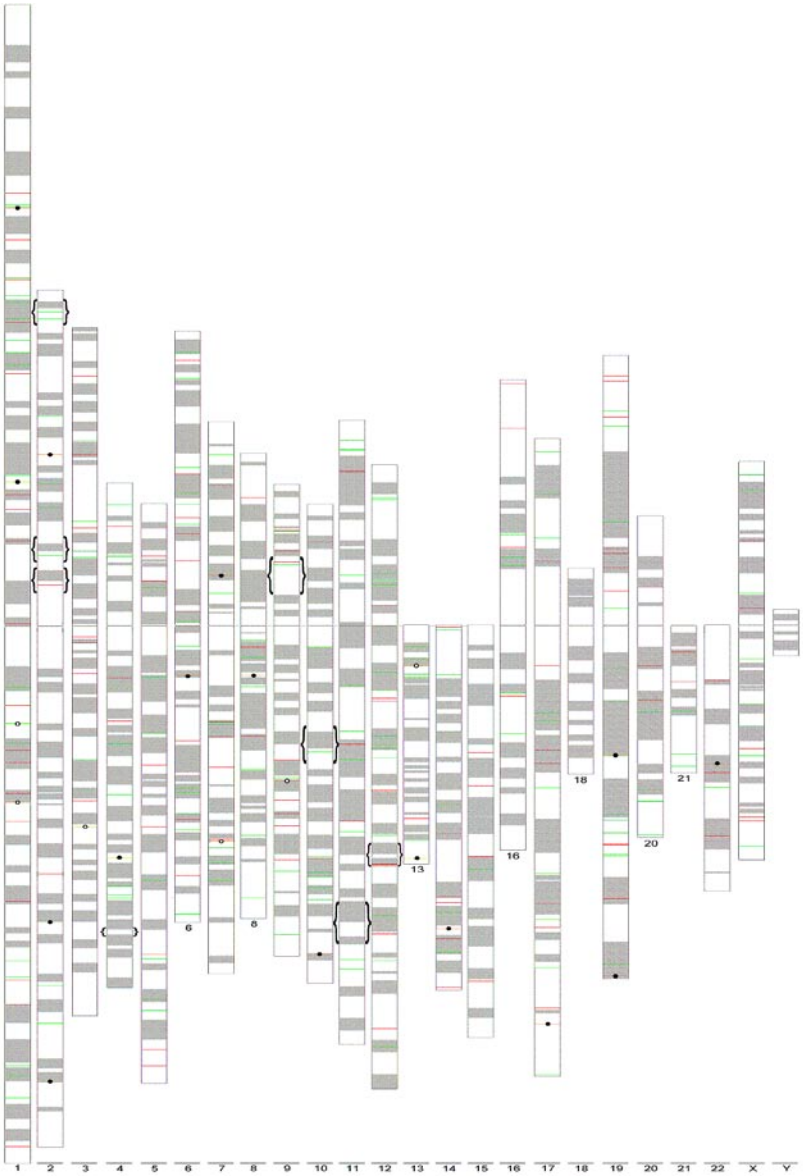

Figure 4: Human transcriptome map, showing the chromosomal localization of statistically differentially regulated transcripts.

Red indicates up regulation in the HELLP/PE placenta SAGE library, green down regulation. Genes, selected for the validation experiment are shown in yellow (down regulated in HELLP/PE) and orange (up regulated in HELLP/PE) and indicated by dots (● and **O**), where open dots represent the 6 significantly differentially regulated genes that contribute to the 7 gene signature. Positive G staining is indicated by the grey bands. PE or HELLP associated loci are indicated between brackets.

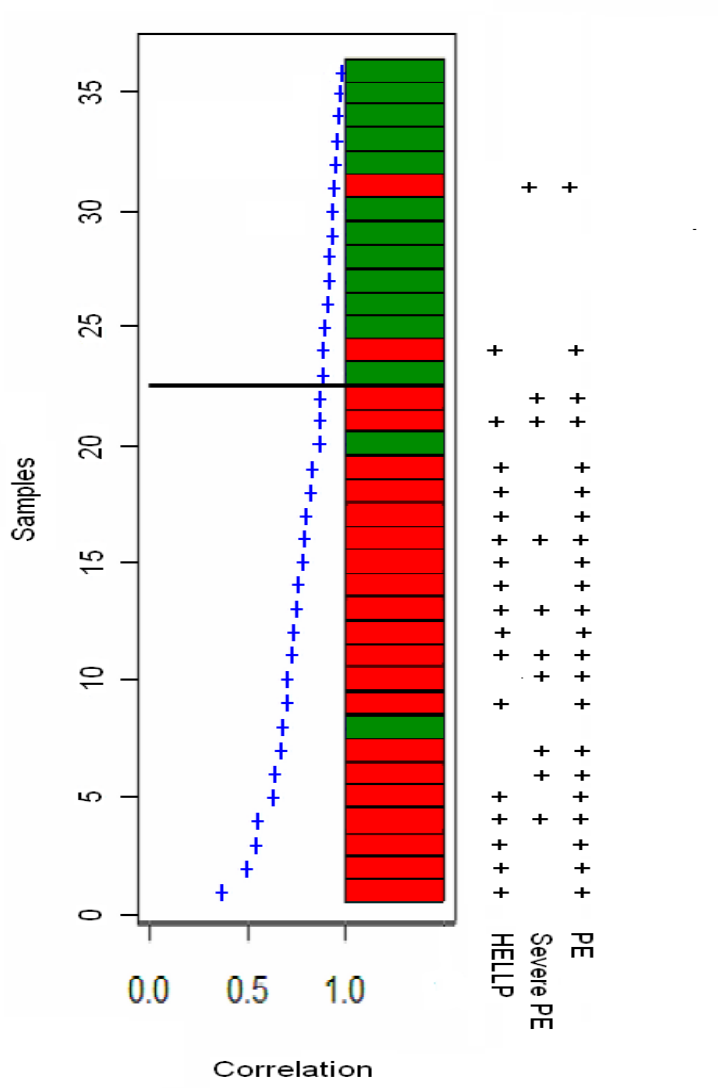

Figure 5: Final classification model

Result of the classification model by nearest centroid classification. On the X axis: Pearson correlation of the classification model for each sample with the Control profile. On the Y axis: 36 placenta samples. Control samples are depicted by green boxes, PE/HELLP samples by red. Clinical conditions relevant for each placenta sample are indicated in the right panel. Samples are ordered according to their correlation with the Control profile. The threshold (horizontal line) is chosen such that it minimizes the classification error. Two samples from the Control group were classified as HELLP and two HELLP samples (red) were classified as control.

Supplementary Data: Maternal characteristics during clinical observation and characteristics of the neonates at birth.

Age: Maternal age at delivery; Steroids: Antenatal corticosteroids administered; Ethnicity: A = African origin, C = Caucasian, I = Indian Subcontinent, M = Mediterranean; GA adm: Gestational Age at admission; Syst BP Adm: Systolic Blood Pressure measured at admission; Diast BP Adm: Diastolic Blood Pressure measured at admission; Aldomet/Nifedipine/Labetalol/Ketanserin/MgSO4: antihypertensives applied. GA: Gestational Age at Birth; Mode of delivery: C = Caesarean Section, V = Vaginal Delivery; BWR: Birth Weight Ratio = Observed Weight / Expected Weight according to the Customized Antenatal Growth Chart. Platelets: Lowest recorded Platelet count during observation. Proteinuria: Highest recorded proteinuria, quantified in any 24 hour period during observation; ASAT: Highest Aspartate Aminotransferase recorded during observation, Normal lab values ≤ 40 U/L; Patients whose placenta tissues were used for SAGE are marked bold.

Supplementary Table 1

Tags and Counts of all annotated transcripts in the 28 weeks Premature Control and the 28 weeks HELLP/PE placenta SAGE library. Available on doi:10.1016/j.placenta.2008.02.007

Supplementary Table 2

Tag sequence, Tag Counts, Gene description, Chromosomal Localization and Function of the 350 significantly differentially up- or down regulated unequivocally annotated transcripts in the Premature Control and HELLP/PE placenta SAGE library. Available on doi:10.1016/j.placenta.2008.02.007

Supplementary Data, Legend opposite

St Nr	Rank Fig 6	Age	Steroids	Ethnicity	Gravida	Para	Height	Weight	BMI	GAadm	Syst BP Adm	Diast BP Adm	Aldomet	Nifedipine	Labetalol	Ketanserin	MgSO4	Gender	GA	Mode of delivery	Birth Weight	BWR	Apgar 5	Platelets	Proteinuria	ASAT
4407	1	20.2	No	A	1	0				42 2/7								f	42 2/7	C	4165		10			
4411	2	29.8	No	C	2	1				38 2/7								f	38 2/7	C	3740		9			
4402	3	31.3	No	C	2	1				36 4/7								m	36 4/7	V	3130		10			
4401	4	26.1	No	M	3	2				38 2/7								m	38 2/7	V	3810		10			
4404	5	29.8	No	C	1	0				39 1/7								f	39 1/7	C	3370		10			
4408	6	28.1	No	M	1	0				40 6/7								f	40 6/7	V	2660		10			
3304	7	31.3	Yes	C	1	0	170	63	21.8	29 3/7								m	29 4/7	C	1395	0.90	9			
4406	8	31.3	No	M	5	3				38 2/7								f	38 2/7	V	2640		10			
3305	9	25.8	Yes	C	1	0	168	66	23.4	32								m	32	V	1835	0.94	10			
4409	10	31.4	No	C	3	2				39 5/7								f	39 5/7	C	3785		9			
1204	11	32.2	No	C	1	0	171	72	24.6	30 1/7	183	118	yes	yes			yes	m	36	C	2090	0.73	10	203	14.6	27
3302	**12**	**32.8**	**Yes**	**C**	**3**	**0**	**176**	**65**	**21.0**	**27 6/7**								**f**	**28 1/7**	**C**	**1220**	**1.01**	**10**			
3306	13	26.5	No	C	1	0	170	83	28.7	30								m	30	C	1070	1.17	8			
4403	14	36.2	No	C	2	1				40								f	40	V	3020		10			
1111	15	32.0	Yes	C	1	0	168	61	21.6	27 5/7	185	112	yes	yes	yes	yes		f	32 3/7	C	1235	0.62	10	106	18.2	42
4405	16	26.2	No	I	2	1				39 6/7								f	39 6/7	C	2850		10			
1182	17	29.1	Yes	C	3	2	170	55	19.0	31 4/7	150	105	yes				yes	f	32	C	1080	0.55	9	32	6.3	336
1129	18	27.7	Yes	C	1	0	163	61	23.0	31 2/7	155	95				yes	yes	f	33 2/7	C	1485	0.73	10	54	9.2	1110
1131	19	25.5	Yes	C	1	0	168	53	18.8	29 1/7	150	95				yes	yes	f	29 6/7	C	715	0.59	4	28	22.5	2085
1208	20	29.9	No	C	1	0	173	60	20.0	29 6/7	130	80						m	35 6/7	C	2310	0.86	9	33	0.5	703
1113	21	27.2	Yes	C	1	0	161	73	28.2	27 6/7	145	95	yes					m	29 6/7	C	1050	0.70	6	38	24.0	232
1096	22	28.1	Yes	C	1	0	164	49	18.2	33 2/7	170	105	yes				yes	m	34 2/7	C	1690	0.74	7	83	8.4	44
1073	23	26.2	No	C	3	1	176	78	25.2	33	160	115	yes	yes				f	35 6/7	C	2060	0.71	9	112	10.3	54
1074	24	34.5	Yes	C	1	0	164	61	22.7	31 3/7	160	120						m	31 4/7	C	1525	0.83	8	38	9.7	103
1126	25	29.1	No	C	1	0	173	80	26.7	29 4/7	180	100						f	29 4/7	C	855	0.58	10	19	0.0	1060
1114	26	22.1	No	A	1	0	169	53	18.6	32 6/7	125	95		yes		yes		f	34 5/7	C	1470	0.65	8	80	12.6	263
1155	27	33.3	No	M	1	0	159	52	20.6	32 4/7	195	120	yes	yes	yes	yes		f	32 4/7	C	1675	0.88	10	61	13.7	116
1154	**28**	**23.7**	**Yes**	**C**	**1**	**0**	**174**	**68**	**22.5**	**26 6/7**	**150**	**100**					**yes**	**f**	**28 5/7**	**C**	**780**	**0.60**	**8**	**40**	**17.6**	**1350**
1214	29	31.0	Yes	C	1	0	180	93	28.7	33 1/7	170	110	yes	yes	yes	yes	yes	f	33 1/7	C	1330	0.58	7	135	11.0	34
1152	30	34.2	Yes	C	3	2	154	54	22.8	31 3/7	170	115	yes			yes		f	31 5/7	C	835	0.44	10	264	0.1	29
1090	31	19.6	Yes	A	1	0	173	85	28.4	32	155	115	yes	yes	yes	yes		m	36 6/7	C	1860	0.62	10	74	19.4	110
1095	32	38.3	Yes	A	1	0	158	58	23.2	30 6/7	160	100						m	31 3/7	C	1005	0.59	7	59	0.5	160
1082	33	27.5	Yes	C	1	0	162	55	21.0	31 1/7	150	100	yes	yes				m	34 3/7	C	1600	0.67	9	72	12.1	262
1065	34	27.2	Yes	M	3	1	155	65	27.1	32 1/7	160	110	yes	yes			yes	m	36 1/7	C	1800	0.65	10	80	12.2	222
1072	35	32.8	Yes	C	6	3	169	67	23.5	31 3/7	160	90	yes					m	31 6/7	C	1565	0.78	8	55	1.7	175
1083	36	33.4	Yes	C	1	0	173	80	26.7	30	200	110	yes	yes		yes		f	33	C	1250	0.57	9	75	5.8	306

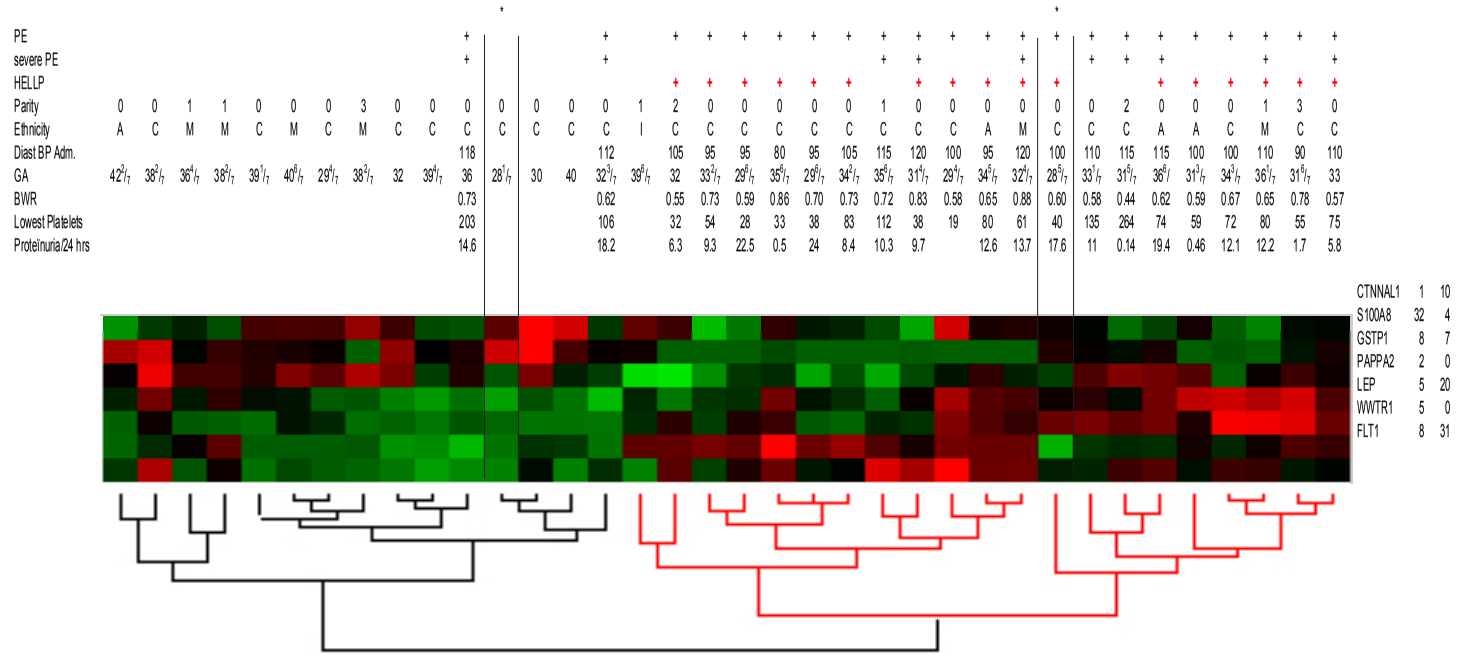

Figure 6: Cluster map of the seven transcript placental HELLP syndrome molecular signature

Cluster map shows standard normal deviate calculated per gene as transcription levels measured by sqRT-PCR relative to EEF1A1 for genes defined by nearest centroid classification and leave-one-out cross validation. From top to bottom: seven genes predicting the HELLP/PE transcriptional signature. From left to right: placenta samples (from 14 Control and 22 PE and HELLP patients). Tissues used for SAGE are boxed and marked with *. Red indicates up regulation, green down regulation, black no change. Two way Cluster analysis was performed by centered correlation with average linkage and the resultant cluster tree is shown in the bottom panel. SAGE tag counts, summated per gene after validation, are shown in the right panel. PE, Severe PE, HELLP: clinical conditions indicated by '+'. HELLP: Platelet Count < 100 x 10^9 /l and Aspartate Aminotransferase ≥ 70 U/l and/or Lactic Dehydrogenase ≥ 600U/l; PE blood pressure ≥ 90/140 mm Hg with proteinuria; Severe PE: Diastolic blood pressure ≥ 110 mm Hg and proteinuria. Ethnicity: A = African origin, C = Caucasian, I = Indian Subcontinent, M = Mediterranean Diast BP Adm: Diastolic Blood Pressure measured at admission. GA: Gestational Age at Birth; BWR: Birth Weight Ratio = Observed Weight / Expected Weight according to the Customized Antenatal Growth Chart. Lowest Platelets: Lowest recorded Platelet count during observation. Proteinuria / 24 hr: Highest recorded proteinuria, quantified in any 24 hour period during observation.

Reference List

1 Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. Lancet 2005;365:785-99.
2 Sibai BM. The HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets): much ado about nothing? Am J Obstet Gynecol 1990;162:311-6.
3 Ganzevoort W, Rep A, de Vries JI, et al. Prediction of maternal complications and adverse infant outcome at admission for temporizing management of early-onset severe hypertensive disorders of pregnancy. Am J Obstet Gynecol 2006;195:495-503.
4 Baxter JK, Weinstein L. HELLP syndrome: the state of the art. Obstet Gynecol Surv 2004;59:838-45.
5 Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. Placenta 2006;27:939-58.
6 Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science 2005;308:1592-4.
7 Martin JN, Jr., Rose CH, Briery CM. Understanding and managing HELLP syndrome: the integral role of aggressive glucocorticoids for mother and child. Am J Obstet Gynecol 2006;195:914-34.
8 Oudejans CB, van DM, Oosterkamp M, et al. Genetics of preeclampsia: paradigm shifts. Hum Genet 2007;120:607-12.
9 Bild AH, Yao G, Chang JT, et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature 2006;439:353-7.
10 Liu R, Wang X, Chen GY, et al. The prognostic role of a gene signature from tumorigenic breast-cancer cells. N Engl J Med 2007;356:217-26.
11 Velculescu VE, Zhang L, Vogelstein B, et al. Serial analysis of gene expression. Science 1995;270:484-7.
12 Brown MA, Lindheimer MD, de SM, et al. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). Hypertens Pregnancy 2001;20:IX-XIV.
13 Versteeg R, van Schaik BD, van Batenburg MF, et al. The human transcriptome map reveals extremes in gene density, intron length, GC content, and repeat pattern for domains of highly and weakly expressed genes. Genome Res 2003;13:1998-2004.
14 Bakker O, Razaki H, de JJ, et al. Expression of the alpha 1, alpha 2, and beta 1 T3-receptor mRNAs in the fasted rat measured using competitive PCR. Biochem Biophys Res Commun 1998;242:492-6.
15 van Kampen AH, van Schaik BD, Pauws E, et al. USAGE: a web-based approach towards the analysis of SAGE data. Serial Analysis of Gene Expression. Bioinformatics 2000;16:899-905.
16 Kal AJ, van Zonneveld AJ, Benes V, et al. Dynamics of gene expression revealed by comparison of serial analysis of gene expression transcript profiles from yeast grown on two different carbon sources. Mol Biol Cell 1999;10:1859-72.
17 Alvarez-Silva M, Belo-Diabangouaya P, Salaun J, et al. Mouse placenta is a major hematopoietic organ. Development 2003;130:5437-44.
18 Arngrimsson R, Sigurard tS, Frigge ML, et al. A genome-wide scan reveals a

maternal susceptibility locus for pre-eclampsia on chromosome 2p13. Hum Mol Genet 1999;8:1799-805.
19 Lachmeijer AM, Arngrimsson R, Bastiaans EJ, et al. A genome-wide scan for preeclampsia in the Netherlands. Eur J Hum Genet 2001;9:758-64.
20 Laivuori H, Lahermo P, Ollikainen V, et al. Susceptibility loci for preeclampsia on chromosomes 2p25 and 9p13 in Finnish families. Am J Hum Genet 2003;72:168-77.
21 Moses EK, Lade JA, Guo G, et al. A genome scan in families from Australia and New Zealand confirms the presence of a maternal susceptibility locus for pre-eclampsia, on chromosome 2. Am J Hum Genet 2000;67:1581-5.
22 Michiels S, Koscielny S, Hill C. Prediction of cancer outcome with microarrays: a multiple random validation strategy. Lancet 2005;365:488-92.
23 Hansson SR, Chen Y, Brodszki J, et al. Gene expression profiling of human placentas from preeclamptic and normotensive pregnancies. Mol Hum Reprod 2006;12:169-79.
24 Zhou Y, Bianco K, Huang L, et al. Comparative analysis of maternal-fetal interface in preeclampsia and preterm labor. Cell Tissue Res 2007;329:559-69.
25 Brown MA, Hague WM, Higgins J, et al. The detection, investigation and management of hypertension in pregnancy: full consensus statement. Aust N Z J Obstet Gynaecol 2000;40:139-55.
26 O'Brien JM, Barton JR. Controversies with the diagnosis and management of HELLP syndrome. Clin Obstet Gynecol 2005;48:460-77.
27 Sibai BM. Diagnosis, controversies, and management of the syndrome of hemolysis, elevated liver enzymes, and low platelet count. Obstet Gynecol 2004;103:981-91.
28 Widmer M, Villar J, Benigni A, et al. Mapping the theories of preeclampsia and the role of angiogenic factors: a systematic review. Obstet Gynecol 2007;109:168-80.
29 Zusterzeel PL, Visser W, Peters WH, et al. Polymorphism in the glutathione S-transferase P1 gene and risk for preeclampsia. Obstet Gynecol 2000;96:50-4.
30 Mise H, Sagawa N, Matsumoto T, et al. Augmented placental production of leptin in preeclampsia: possible involvement of placental hypoxia. J Clin Endocrinol Metab 1998;83:3225-9.
31 Cao Y. Angiogenesis modulates adipogenesis and obesity. J Clin Invest 2007;117:2362-8.
32 Laivuori H, Gallaher MJ, Collura L, et al. Relationships between maternal plasma leptin, placental leptin mRNA and protein in normal pregnancy, pre-

eclampsia and intrauterine growth restriction without pre-eclampsia. Mol Hum Reprod 2006;12:551-6.
33 Smith GC, Stenhouse EJ, Crossley JA, et al. Early pregnancy levels of pregnancy-associated plasma protein a and the risk of intrauterine growth restriction, premature birth, preeclampsia, and stillbirth. J Clin Endocrinol Metab 2002;87:1762-7.
34 Kanai F, Marignani PA, Sarbassova D, et al. TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. EMBO J 2000;19:6778-91.
35 Hossain Z, Ali SM, Ko HL, et al. Glomerulocystic kidney disease in mice with a targeted inactivation of Wwtr1. Proc Natl Acad Sci U S A 2007;104:1631-6.
36 Chung KW, Ferrell RE, Ellis D, et al. African American hypertensive nephropathy maps to a new locus on chromosome 9q31-q32. Am J Hum Genet 2003;73:420-9.
37 Roth J, Vogl T, Sorg C, et al. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. Trends Immunol 2003;24:155-8.
38 Vogl T, Tenbrock K, Ludwig S, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med 2007;13:1042-9.
39 Shah DM. Preeclampsia: new insights. Curr Opin Nephrol Hypertens 2007;16:213-20.
40 Sargent IL, Germain SJ, Sacks GP, et al. Trophoblast deportation and the maternal inflammatory response in pre-eclampsia. J Reprod Immunol 2003;59:153-60.
41 Winn VD, Haimov-Kochman R, Paquet AC, et al. Gene expression profiling of the human maternal-fetal interface reveals dramatic changes between midgestation and term. Endocrinology 2007;148:1059-79.
42 Hung TH, Charnock-Jones DS, Skepper JN, et al. Secretion of tumor necrosis factor-alpha from human placental tissues induced by hypoxia-reoxygenation causes endothelial cell activation in vitro: a potential mediator of the inflammatory response in preeclampsia. Am J Pathol 2004;164:1049-61.
43 Moffett-King A. Natural killer cells and pregnancy. Nat Rev Immunol 2002;2:656-63.
44 Roberts JM, Cooper DW. Pathogenesis and genetics of pre-eclampsia. Lancet 2001;357:53-6.
45 Conde-Agudelo A, Villar J, Lindheimer M. World Health Organization systematic review of screening tests for preeclampsia. Obstet Gynecol 2004;104:1367-91.

Chapter 7:

Gene expression patterns in human placenta during gestation

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Manuscript in preparation

Abstract

Objective: Functional Serial Analysis of Gene Expression (SAGE) based analysis of gestation stage-specific gene expression profiles in undissected human placentas.

Methods: Three SAGE libraries from human placenta, derived from 1st, 2nd, and 3rd trimester were compared using two sided Z test. Statistically significantly differentially expressed transcripts were ordered using K means clustering and functionally annotated.

Results: 669 tags show statistical differential expression in at least one of the libraries. Tag-to-gene allocation reliably annotates 524 tags to 442 unique genes. Clustering results in 3 clusters with expression levels statistically significantly up- or down regulated in the 1st, 2nd and 3rd trimester of pregnancy. Functional annotation displayed a relative overrepresentation of transcripts encoding factors involved in protein translation in the 1st trimester.

Conclusion: Comparison of SAGE expression libraries through gestation did not identify major trimester specific pathways with the exception of extensive upregulation of transcripts encoding ribosomal proteins in the first trimester.

Introduction

A healthy placenta is the basis for successful pregnancy outcome. In humans, during a 9 months life span the placenta undergoes rapid growth, differentiation and maturation. The placenta regulates fetal homeostasis, controls fetal growth, development and maturation and is a key regulator in the process of parturition.[1] To cope with these demands, adaptation of placental function during gestation is elemental and the placenta redirects maternal endocrine, immune and metabolic functions to the embryo's advantage.[2]

During the first trimester, implantation and placentation involve the concerted action of cell-adhesion molecules, cytokines, angiogenic factors, growth factors, extracellular matrix metalloproteinases, hormones and transcription factors.[3] Almost all detailed knowledge on implantation comes from animal models since human material is usually not available. Transgenic and mutant mouse models have identified several factors essential to mouse implantation.[4;5] Immune modulation is expected to be one of the key functions of placenta, as from the maternal point of view fetal tissue is a semi-allograft. The normal maternal immune response needs to be subverted in order to avoid rejection.[6] The formation of vascular connections is essential as the fetus needs access to the maternal circulation and at the end of the first trimester vasculogenesis and angiogenesis have resulted in a fully formed effective vascular plexus.[7]

In the second trimester organogenesis of the human embryo is complete and from this point on maturation and accelerated growth are the main focuses. To cope with the increasing fetal demands for exchange of nutrients, respiratory gasses and excess metabolites, the placenta needs an extending surface area. The process of differentiation of placental cell types continues with division and elongation of villi proliferation and dilatation of capillaries.[8]

In the third trimester the fetal growth rate is stable until 37 weeks of gestation with the concomitant large transplacental exchange that is the sum of the capacity of the placental vascular bed and the resultant uterine and umbilical blood flows.[9] In contrast, the placenta decreases its growth rate at 34 weeks and towards term there is thinning of the trophoblast cell layer, predominantly by disappearance of most of the cytotrophoblast cells. In contrast to e.g. mice where the timing of birth is closely linked to the maturation of the lungs, the length of human pregnancy is controlled by the placental CRH-ACTH-Cortisol system, that operates collateral to the Hypothalamus-Pituitary-Adrenal gland axis.[10]

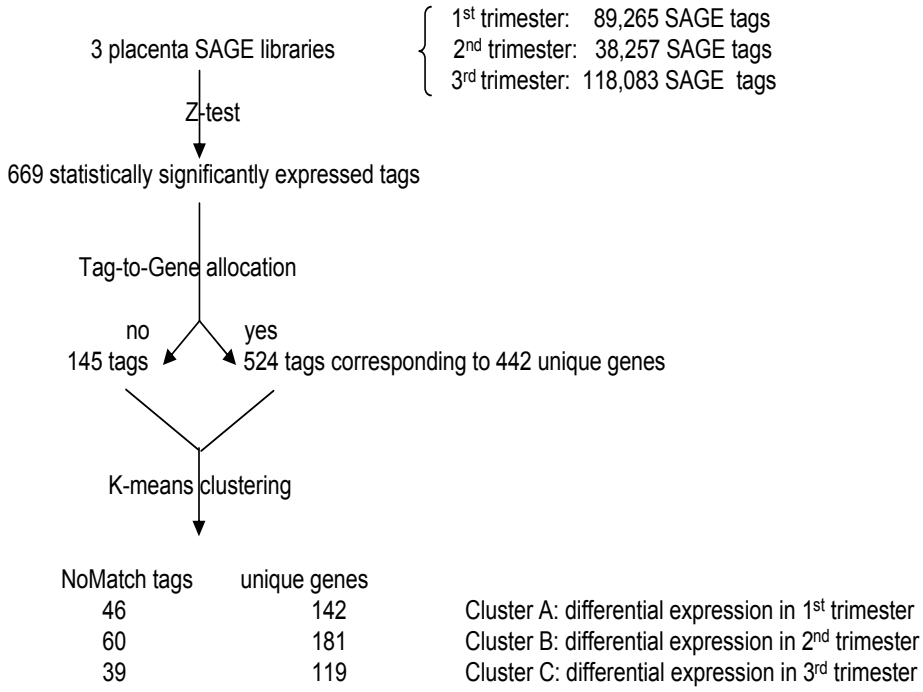

Figure 1: Analysis of SAGE libraries.

Flowchart demonstrating the analysis of 1st, 2nd and 3rd trimester human placenta Serial Expression of Gene Analysis (SAGE) libraries.

Several studies focusing on single pathways or specific placental cell types are available[4;9;11] and our perspective on placental expression of genes can be further advanced by gene expression profiling techniques. Several high throughput studies are reported, focussing on pathological versus control placenta[12-18] specific placental subsections[19;20] or the effect of labour.[21] Up to now, no gestational age specific profiles have been described[13;19;22] and we opted for analysis of global gene expression profiles of undissected human placentas through gestation. Expression profile generation without making any prior assumption as to what factors or pathways are elemental, need high-throughput techniques as microarray analysis and Serial Analysis of Gene Expression (SAGE).[23]

Apart from providing large scale and quantitative transcriptome analysis, SAGE provides the additional opportunity to identify novel transcripts in the form of SAGE tags that cannot be annotated to a known gene (NoMatch

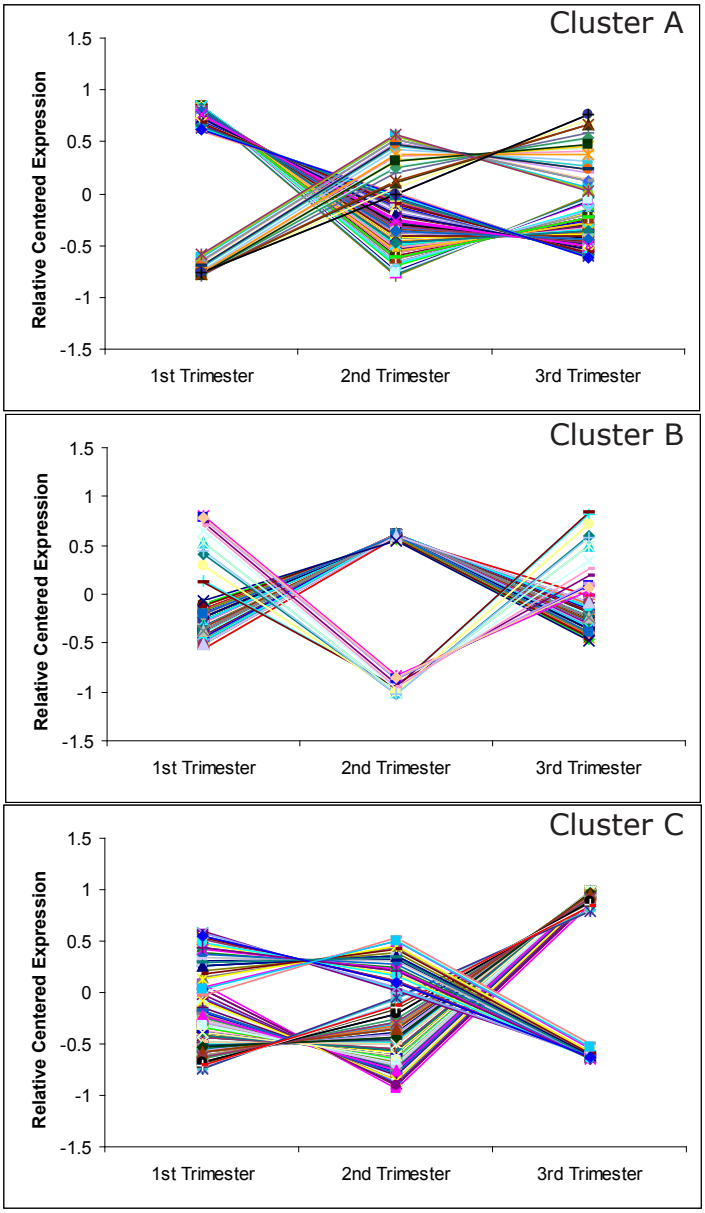

Figure 2: Graphical representation of gene expression profiles in human placenta during gestation.

Lines represent unique genes of which the expression level at three measurement times are linked. The Y-axis shows the relative centered expression of SAGE tag counts normalized to the size of the smallest SAGE library. One line can represent more than one unique gene with identical relative centered expression in the libraries.

tags). Before initiating further downstream analysis of NoMatch tags aimed at identifying the corresponding gene and protein, it is valuable to have some indication as to which pathway or gene family the NoMatch gene might belong. As gene expression clusters tend to be significantly enriched for specific functional categories clustering can be used to infer a functional role for unknown genes in the same cluster.[24] For partitioning purposes, K-means clustering outperforms hierarchical clustering[24] and we used this method to analyze gene expression profiles of undissected human placenta's of 12, 28 and 40 weeks gestational age that were generated by SAGE.

Material and methods

SAGE Libraries

Three SAGE libraries from human placenta are available. The placenta of 12 weeks gestational age is from an elective abortion and has a total tag count of 89,265 corresponding to 26,325 unique tags; the data are publicly available at CGAP http://cgap.nci.nih.gov/SAGE/SAGELibraryFinder

The second trimester SAGE library, which contains 38,257 total tags corresponding to 14,513 unique tags, was made from a placenta of 28 weeks gestational age from a Caucasian nulliparous woman referred because of imminent premature delivery. At $28^{1}/_{7}$ weeks gestational age a caesarean section was performed because of an abnormal fetal non-stress test (CTG) of the female fetus. The premature delivery is considered idiopathic since neither before nor after delivery did the mother have signs of infection or other obstetrical complications.[18]

The third trimester SAGE library was from placenta tissue of a full term uncomplicated pregnancy of a female neonate, containing 118,083 total tags corresponding to 33,222 unique tags, publicly available at the CGAP site.

Statistical Analysis

Differences in level of expression were assessed by two-sided Z test.[25] To correct for false discovery rate (FDR) in these multiple comparisons, tags were first ranked according to highest Z level. A significance threshold t was then calculated for each tag with rank i : t = i * 0.05 / N (N = total number of comparisons). If the corresponding p value was below the threshold, the transcript was considered statistically significantly differentially expressed.

Differentially expressed tags were mapped to their corresponding gene using the Automatic Correspondence of Tags and Genes web-tool.[26]

K-means Clustering

Tag counts were normalized to the size of the smallest SAGE library. All statistically significantly differentially expressed tags were used for K means

clustering.[27] Using Cluster software version 2.11 (Eisen Lab, Stanford University CA, USA) at http://rana.lbl.gov/downloads/Cluster/.
Data were centered and normalized per tag. Subsequently, tags were organized by correlation into 3 to 6 clusters according to Pearson correlation, Spearman rank correlation, Euclidian distance and Absolute correlation. Transcripts in Cluster A and C were ranked by decreasing absolute normalized tag count difference between (for cluster A) the 1st and the 2nd and (for cluster C) the 2nd and the 3rd trimester. Transcripts in cluster B were ranked by the absolute normalised tag count difference in 2nd trimester and the average tag count in the 1st and 3rd trimester.

Pathway Analysis and Functional Annotation
For pathway analysis, the list of gene names was reduced by pooling identical genes. From the GeneGo Metacore database (http://www.genego.com), selective pathway-specific gene lists were extracted and linked to the list of 442 genes. Manual functional annotation of individual transcripts was extracted from Entrez Gene and PubMed at http://www.ncbi.nlm.nih.gov.

Results and Discussion

Analysis of SAGE libraries and clustering of data
Overall, the three placenta SAGE libraries are highly similar. Two-sided Z testing of the three human placenta SAGE libraries of the 1st, 2nd and 3rd trimester that have over 55,000 unique tags results in the identification of 669 tags that are statistically differentially expressed in at least one of the libraries. Tag-to-gene allocation can reliably annotate 524 tags to 442 unique genes. (Figure 1) One of the advantage points of SAGE is that a library usually contains SAGE tags that cannot be reliably annotated (NoMatch tags). They correspond to either tags that result from previously undocumented single nucleotide polymorphisms, sequence errors, splice variants or polyA adenylation sites[28] or they correspond to novel genes.[29]
To identify genes specifically up- or down regulated either the 1st, 2nd or 3rd trimester of pregnancy, gene expression profiles were generated using K-means clustering. As the use of differential initial centroid positions can yield different cluster results,[24] each algorithm was run several times with different random seeds. The most stable result (77 out of 100 clustering runs) was obtained using absolute uncentered correlation, resulting in 3 mutually exclusive clusters of gene expression. Cluster A, B and C contain genes with expression levels statistically significantly up- or down regulated in the 1st, 2nd and 3rd trimester of pregnancy, respectively.(Figure 2) Cluster A, B and C contain respectively 142, 181 and 119 transcripts. NoMatch tags are assigned to clusters in relatively identical proportions. (Figure 1)

Mapping to functional classes
Mapping to functional classes by GeneGo resulted in the linking of approximately 30% of the transcripts in each cluster to the pathways specified in Table 1.

Pathway	(number of genes in pathway)	Cluster A	Cluster B	Cluster C
Development - Common Pathways	(697)#	14	19	14
Translation	(135)*	32	4	12
Cell Adhesion	(308)#	9	10	10
Development-Angiogenesis	(192)#	4	6	7
Hypoxia	(137)#	5	3	0
Oxidative stress	(77)#	2	8	3
Energy+Lipids+Steroids+Carbohydrates	(578)+	9	9	2
Apoptosis	(333)#	5	8	7

Table 1: Functional annotation of clustered genes using GeneGo process and map listings Number of genes annotated to each pathway in the specific clusters is listed. *GeneGo Process Network, #GeneGo Map Regulatory Process, +GeneGo Map Metabolic Map (http://www.genego.com).

There is no striking enrichment for specific functional categories in any of the clusters, with the exception of the high amount of transcripts involved in translation in cluster A. The vast majority of significantly differentially expressed transcripts encoding ribosomal proteins shows increased expression in the 1st trimester along with decreased expression in the 3rd trimester. Ribosomal proteins combined with ribosomal RNA form the ribosome that is responsible for translation of the genetic code into protein. Differential expression of RPL26 and RPL27 mRNAs has been previously reported in normal placentas of 17 weeks gestational age compared to hydatidiform mole.[13] Studies in mouse placenta have shown upregulation of transcripts corresponding to ribosomal proteins at embryonal day 12.5, roughly comparable to 2nd trimester in human.[30]
Although we expected that clustering would identify specific functional categories, this was not the case, and our modelling of placental gene transcription cross gestation does not infer new information that contributes to functional annotation of NoMatch tags in the same cluster.

Highlighted transcripts

We manually attributed function to the top 20 most significantly differentially regulated genes in each cluster. (Table 3) Cluster A and B are headed by high transcription levels of hemoglobins. This supports for human placenta the finding previously done in mice, that the placenta is a major hematopoietic organ.[31] Cluster A shows differential expression of HBG1, encoding the hemoglobin gamma chain that with the hemoglobin alpha chain (HBA) forms fetal hemoglobin (HbF). HBG1 expression is lower in the 1st trimester and markedly increases in the 2nd trimester. The transcript for HBA2 is prominent in cluster B and highly expressed in the 2nd trimester. During gestation, the balance between HBG1 and HBG2 changes, with HBG2 expression predominantly expressed at birth.[32] HBG2 is not selected based on differential regulation, but its expression gradually increases across gestation with a decrease in the HBG1/HBG2 tag count ratio from 35 in the 2nd to 7 in the 3rd trimester (data not shown).

The second ranking in cluster A, with relatively high expression in the 1st trimester, is CGA encoding the alpha subunit of all anterior pituitary glycoprotein hormones including the placental growth factor hCG.[33] The hCG beta chain (CGB5) expression is also relatively high expressed in the 1st trimester with tag counts of respectively 7,0 and 0 in 1st, 2nd and 3rd trimester placenta, respectively.

Many factors involved in extracellular matrix formation and remodelling feature in all three clusters, indicating that this process in important during all phases of pregnancy. Cluster A shows that COL1A1, COL1A2 and SERGEF are highly expressed in the 1st trimester, while in the 2nd trimester there is prominent expression of CFL1 and CRK together with down regulation of KISS1 and TFP (Cluster B). The 3rd trimester is characterized by high expression of ADAM12, ADAMTSL, MT3, PAPPA, SERPINE1 and TIMP2 while LGALS1 is downregulated. Apart from their obvious role in extracellular matrix modulation that is an aspect of growth, these genes are also essential for vasculogenesis and angiogenesis.[34] This is reflected by the capacity of endothelial cells to adhere to each other and to form new tubes.[8] Angiopoietin-2 (ANGPT2) has been reported in terminal villous capillaries at term[7] but is predominantly present in our 1st trimester SAGE library. Integrin alpha V (ITGAV) has been studied in cell culture of extra villous trophoblast as an angiogenesis promoting factor[35] and in our profiles an up regulation is seen in the 3rd trimester.

The human pregnancy-specific glycoprotein (PSG) genes comprise a family of 11 highly conserved members whose expression is maximal in placental cells and marginal in other cell types. They may be involved in the modulation of

the innate and adaptive immune response in mouse[36] while their function in humans is not yet established. PSG1, PSG4 and PSG9 all show decreased expression in the 2nd trimester.

Corticotrophin releasing hormone (CRH) has been named the placental clock.[10;37] As pregnancy advances an exponential rise in maternal plasma CRH levels of placental origin is seen. In women who deliver prematurely this increase occurs earlier. As can be seen in cluster B, we observe an interestingly high CRH expression in the 2nd trimester placenta. This patient was admitted with imminent premature delivery that probably is related to the high 2nd trimester CRH expression.

We show a number of annotated transcripts highly differentially expressed in placenta during human gestation to which currently no function can be attributed: GRIN2C, EMID2, PCBD2, PLAC1, PLAC4, SVEP1 TncRNA and TAGLN2. Increased TncRNA expression at the maternal-fetal interface towards term has been observed before.[20] High expression of TAGLN2 at the mouse implantation site has been reported[5] although in our profiles an up regulation is seen towards term.

Limitations of the study

Our method of functional annotation using the GeneGo Metacore database resulted in functional allocation of 30% of transcripts. In our study functional annotation is hampered by the fact that the database content is publication-

directed and the majority of content is included based on research on neoplasms or cardiovascular disease.
The initial comparison of SAGE expression profiles uses only three placentas and the resultant differential expression pattern will be a combination of gestational age related and interindividual differences. Our results show that the expression profiles are highly similar but at this point some contamination of our results by interindividual differences cannot be excluded. Specific subsections of term placenta show differential expression using microarray analysis.[19] The tissue samples used for cross gestational analysis reported in this study were not dissected because the changing composition of placenta tissue during gestation does not allow this type of comparison.

Gender specific placental gene expression has been documented[19] and both the neonates born in the 2nd and 3rd trimester are female. The first trimester placenta SAGE library shows expression of the Y-chromosomal genes RPS4Y, DBY and EIF1AY, making it likely that in this case the embryo was male. Our study design does not permit identification of gender specific transcripts.

Conclusion

SAGE analysis and K-means clustering of 1st, 2nd and 3rd trimester placenta tissue does not reflect established pathways of placenta growth and development. Extensive up regulation of ribosomal proteins is the most striking feature in the first trimester.

	expression up- or down regulated	Angiogenesis	Development	Metabolism	Apoptosis	Cell Adhesion	Hypoxia	Oxidative Stress	Translation
cluster A: in the 1st trimester	↑	ANGPT2	CGA	ALG9	FOS		BNIP3		FAU
			COL1A1	ATP5L	INHBA	COL1A1			RPL4, 8, 9, 11,
			COL1A2	B4GALT5		COL1A2			13, 15, 18A, 23A,
		FOS	FOS	GAPDH		COL3A1	FOS		27, 28, 30, 31,
			HSPA8	NDUFA4		FLOT2	HSPA8	HSPA8	35, 36, 39
			IGFBP3	UQCR					RPLP0
			INHBA	UQCRH					RPS 2, 3, 3A, 4Y1
		JUN	JUN	UQCRQ	JUN	JUN			5, 7. 8, 9, 14, 18,
			MESDC2			MMP2			19, 20, 23, 25, 28
			SPP1			SPARC			
	↓		CCND1				HBG1		
		GNG11	GNG11		GNG11	GNG11		GNG11	
			IGFBP1				HBB		
			IGFBP5			PPP1CB			
cluster B: in the second trimester	↑	CALM1	CALM1	ATP5E	CALM1	CAV1		GPX1	RPL6
			CAV1	CYP39A1		CFL1	HBA2		RPL26
		DUSP1	CSNK1A1	PLCB4		COL4A2	HSPA5		RPLP1
		CRK	CRK	PPAP2B		CRK			RPS4X
		GNAI2	GNAI2	PPT1	GNAI2	GNAI2	GNAI2	GNAI2	
		GNAZ	GNAZ	RPN2	GNAZ	GNAZ		GNAZ	
			HSPA5	SULT1A1				HSPA5	
		PLCB4	HSP90AA1	SULT1E1	HSP90AA1			HSP90AA1	
			HSP90AB1		HSP90AB1			HSP90AB1	
			HSP90B1					HSP90B1	
			IBSP						
			IGF1R		IGF1R	IGF1R			
			IGF2			IGF2			
			LEP			TIMP1			
			MGLL		PRKDC				
			PLCB4						
			VDR						
			VIM						
			YWHAB		YWHAB				
	↓			HSD3B1		VCL			
cluster C: in the 3rd trimester	↑	ADAM12	ADAM12					ADAM12	
			CSH1	CYP19A1	CHEK2	ACTN4			
			DAB2	HSD11B2					
		EGFR	EGFR		EGFR	EGFR		EGFR	
		EPAS1	FN1			FN1			
		FLT4	FURIN			MYH9			
		FN1	IL1R1			SERPINE1			
		ITGAV	PRKAR1A		PRKAR1A	SERPINE2			
			PRKCZ		PRKCZ	TIMP2			
			SERPINE1			TIMP3			
	↓		EGR1		EGR1				RPL2, 5, 21
		GNG5	GNG5		GNG5	GNG5		GNG5	RPL32, 34, 38
			RPS6			MYL6			RPS6, 12, 16, 17
			SMAD2		SMAD2				RPS24, 29

Table 2: Gene names with corresponding GeneGo annotation. Genes attributes to cluster A, B, or C either with upregulated (upwards arrow) or downregulated expression in respectively the 1st, 2nd or 3rd trimester of pregnancy are listed according to their GeneGo annotation. Some genes are annotated to more than one pathway.

Table 3A: Top 20 of most differentially expressed genes in cluster A.

	Gene symbol	1st trimester normalized SAGE tag count	2nd trimester normalized SAGE tag count	3rd trimester normalized SAGE tag count	Function
CLUSTER A	HBG1	27	1435	549	Hemoglobin gamma chain, specific for fetal hemoglobin (HbF), important for oxygen transport
	CGA	327	41	14	Common alpha subunit of the anterior pituitary glycoprotein hormones
	GRIN2C	326	15	80	Glutamate receptor, mediates neurotransmitter action, function outside central nervous system unknown.
	MRPL44	155	5	17	Mitochondrial ribosomal protein, contributes to protein synthesis within the mitochondrion.
	COL1A2	117	36	20	Involved in integrin mediated cell adhesion, part of the fibrillar collagen found in most connective tissues.
	HBB	0	58	53	Hemoglobin beta chain, component of adult hemoglobin, important for oxygen transport
	RPL37A	124	34	34	Ribosomal protein, component of the 60S subunit, contributes to protein synthesis.
	SERGEF	60	5	5	Modulates secretion of proteoglycans, a maior component of the extracellular matrix
	EEF1A1	158	80	36	Eukaryotic translation elongation factor 1 α1, delivers aminoacyl tRNAs to the ribosome
	EMID2	48	3	0	EMI domain containing 2, function unknown
	PAGE4	201	98	80	P antigen family, member 4, found in fetal and reproductive tissues and tumors, function unknown
	RPL15	75	22	8	Ribosomal protein, component of the 60S subunit, contributes to protein synthesis.
	RPS19	147	56	67	Ribosomal protein, component of the 40S subunit, contributes to protein synthesis.
	COL1A1	98	41	19	Collagen type I alpha 1, fibril-forming collagen found in most connective tissues
	RPS18	98	45	20	Ribosomal protein, component of the 40S subunit, contributes to protein synthesis.
	FOS	66	24	10	v-fos Oncogene, regulator of cell proliferation, differentiation and transformation
	RPL31	92	44	20	Ribosomal protein, component of the 60S subunit, contributes to protein synthesis.
	RPS3A	112	57	36	Ribosomal protein, component of the 40S subunit, contributes to protein synthesis.
	RPL30	106	57	28	Ribosomal protein, component of the 60S subunit, contributes to protein synthesis.
	SPP1	42	12	2	Osteopontin, constraining factor on haemopoietic stem cell proliferation

Official gene symbol, SAGE tag count normalized to the smallest library and function, manually extracted from PubMed and Entrez Gene, are displayed.

Table 3B: Top 20 of most differentially expressed genes in cluster B.

	Gene Symbol	1st trimester normalized SAGE tag count	2nd trimester normalized SAGE tag count	3rd trimester normalized SAGE tag count	Function
CLUSTER B	HBA2	21	990	216	Hemoglobin alpha 2 chain, important for oxygen transport
	KISS1	555	34	218	KiSS-1 metastasis-suppressor, involved in cell matrix adhesion, regulator of the gonadotropic axis
	PSG1	419	109	505	Pregnancy specific beta-1-glycoprotein 1, contributes to immunomodulation during pregnancy
	PSG4	328	92	471	Pregnancy specific beta-1-glycoprotein 4, low levels correlate with complications of pregnancy
	TFPI2	530	181	360	Tissue factor pathway inhibitor 2, inhibits trypsin, plasmin and tissue factor. Involved in matrix remodelling
	IGF2	64	203	79	Insulin-like growth factor 2, involved in development and growth. Paternally expressed gene
	PFTK2	0	59	0	PFTAIRE protein kinase 2, Serine/threonine kinase involved in signal transduction.
	CRH	1	62	2	Corticotropin Releasing Hormone, increases at the onset of parturition.
	S100P	123	47	175	S100 calcium binding protein P, regulates cellular processes as cell cycle progression and differentiation
	PSG9	64	8	66	Pregnancy specific beta-1-glycoprotein 9, thought to be involved in membrane transport processes
	RAD23B	33	95	18	RAD23 homolog B, involved in nucleotide excision repair and ubiquitin mediated proteolysis.
	DLK1	54	147	71	Delta-like 1 homolog, paternally expressed gene, mediating immune response
	PCBD2	29	98	36	Pterin-4 alpha-carbinolamine dehydratase/cofactor of HNF 1α2, function unknown
	QSOX1	42	0	17	Quiescin Q6 sulfhydryl oxidase 1, may be important for proliferation, associated with quiescence
	RPLP1	17	50	4	Ribosomal protein, component of the 60S subunit, contributes to protein synthesis.
	TFPI	44	7	40	Tissue factor pathway inhibitor, regulator of plasmin-mediated matrix remodeling.
	H3F3A	5	33	2	H3 histone, family 3A, nuclear protein responsible for nucleosome structure
	CFL1	18	66	24	Cofilin 1, important for cytoskeletal proteins
	PTMA	2	27	1	Prothymosin alpha, involved in the formation, maintenance or functioning of the mitotic spindle
	CRK	17	50	10	v-crk Sarcoma virus CT10 homolog, phagocytosis of apoptotic cells and cytoskeletal reorganization

Official gene symbol, SAGE tag count normalized to the smallest library and function, manually extracted from PubMed and Entrez Gene, are displayed.

Table 3C: Top 20 of most differentially expressed genes in cluster C.

	Gene Symbol	1st trimester normalized SAGE tag count	2nd trimester normalized SAGE tag count	3rd trimester normalized SAGE tag count	Function
CLUSTER C	PAPPA	12	38	172	Pregnancy-associated plasma protein A, metalloproteinase, cleaves insulin like binding proteins
	TncRNA	42	30	144	Trophoblast-derived noncoding RNA
	SMAD2	229	268	107	SMAD family member 2, involved in apoptotic activin A signalling.
	RPS29	234	272	120	Ribosomal protein, component of the 40S subunit, contributes to protein synthesis.
	TAGLN2	12	3	65	Transgelin2, homolog of th transgelin, an early marker of differentiated smooth muscle.
	GPC3	24	37	106	Glypican 3, control of cell division and growth regulation by influencing IGF2 interactions with IGF2R
	CSH1	45	4	96	Chorionic somatomammotropin hormone 1, growth factor.
	TIMP2	16	32	91	TIMP metallopeptidase inhibitor 2, endogenous inhibitor of angiogenesis, inactivates metalloproteinases
	PLAC1	2	5	42	Placenta-specific 1, associated to trophoblast differentiation
	RPS12	55	53	10	Ribosomal protein, component of the 40S subunit, contributes to protein synthesis
	PLAC4	14	9	54	Placenta-specific 4, function unknown
	SERPINE1	5	27	62	Plasminogen activator inhibitor type 1, prevents the inactivation of plasminogen, promoting coagulation
	ERVWE1	29	9	64	Syncytin, involved in fusion of the cytotrophoblast cells to form the syncytial layer of the placenta
	SVEP1	2	4	29	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1, function unknown
	MT3	4	2	28	Metallothionein 3, growth inhibitory factor induced by hypoxia
	ADAMTSL4	8	3	33	ADAMTS-like 4, involved in cellular adhesion and angiogenesis
	LGALS1	31	52	9	Galectin 1, modulator of cell-cell and cell-matrix interactions
	RPL32	131	115	63	Ribosomal protein, component of the 60S subunit, contributes to protein synthesis
	ADAM12	25	24	64	Meltrin alpha, disintegrin and metalloprotease involved in growth and differentiation
	IL1R1	3	0	23	Interleukin1 receptor 1, involved in cytokine induced immune and inflammatory responses

Official gene symbol, SAGE tag count normalized to the smallest library and function, manually extracted from PubMed and Entrez Gene, are displayed.

Reference List

1. Kingdom J, Jauniaux E, Shaughn O'Brien PM. The Placenta: Basic Science and Clinical Practice. London: RCOG Press, 2000.
2. Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. Science 1994;266:1508-18.
3. Anthony RV, Limesand SW, Jeckel KM. Transcriptional regulation in the placenta during normal and compromised fetal growth. Biochem.Soc.Trans. 2001;29:42-48.
4. Cross JC, Baczyk D, Dobric N, Hemberger M, Hughes M, Simmons DG et al. Genes, development and evolution of the placenta. Placenta 2003;24:123-30.
5. Ma XH, Hu SJ, Ni H, Zhao YC, Tian Z, Liu JL et al. Serial analysis of gene expression in mouse uterus at the implantation site. J.Biol.Chem. 2006;281:9351-60.
6. Moffett A, Loke YW. The immunological paradox of pregnancy: a reappraisal. Placenta 2004;25:1-8.
7. Leach L, Babawale MO, Anderson M, Lammiman M. Vasculogenesis, angiogenesis and the molecular organisation of endothelial junctions in the early human placenta. J.Vasc.Res. 2002;39:246-59.
8. Mayhew TM. Fetoplacental angiogenesis during gestation is biphasic, longitudinal and occurs by proliferation and remodelling of vascular endothelial cells. Placenta 2002;23:742-50.
9. Reynolds LP, Redmer DA. Angiogenesis in the placenta. Biol.Reprod. 2001;64:1033-40.
10. McLean M, Bisits A, Davies J, Woods R, Lowry P, Smith R. A placental clock controlling the length of human pregnancy. Nat.Med. 1995;1:460-63.
11. Godfrey KM. The role of the placenta in fetal programming-a review. Placenta 2002;23 Suppl A:S20-S27.
12. Reimer T, Koczan D, Gerber B, Richter D, Thiesen HJ, Friese K. Microarray analysis of differentially expressed genes in placental tissue of pre-eclampsia: up-regulation of obesity-related genes. Mol.Hum.Reprod. 2002;8:674-80.
13. Durand S, Abadie P, Angeletti S, Genti-Raimondi S. Identification of multiple differentially expressed messenger RNAs in normal and pathological trophoblast. Placenta 2003;24:209-18.
14. Feng HC, Tsao SW, Ngan HY, Kwan HS, Shih SM, Xue WC et al. Differential gene expression identified in complete hydatidiform mole by combining suppression subtractive hybridization and cDNA microarray. Placenta 2006;27:521-26.
15. Hansson SR, Chen Y, Brodszki J, Chen M, Hernandez-Andrade E, Inman JM et al. Gene expression profiling of human placentas from preeclamptic and normotensive pregnancies. Mol.Hum.Reprod. 2006;12:169-79.
16. Zhou R, Zhu Q, Wang Y, Ren Y, Zhang L, Zhou Y. Genomewide Oligonucleotide Microarray Analysis on Placentae of Pre-Eclamptic Pregnancies. Gynecol. Obstet.Invest 2006;62:108-14.
17. Nishizawa H, Pryor-Koishi K, Kato T, Kowa H, Kurahashi H, Udagawa Y. Microarray analysis of differentially expressed fetal genes in placental tissue derived from early and late onset severe pre-eclampsia. Placenta

2007;28:487-97.
18. Buimer, M., Keijser, R., Jebbink, J. M., Wehkamp, D., van Kampen, A. H. C., Boer, K., van der Post, J. A., and Ris-Stalpers, C. Seven placental transcripts characterize HELLP-syndrome. Placenta . 2008. In Press.
19. Sood R, Zehnder JL, Druzin ML, Brown PO. Gene expression patterns in human placenta. Proc.Natl.Acad.Sci.U.S.A 2006;103:5478-83.
20. Winn VD, Haimov-Kochman R, Paquet AC, Yang YJ, Madhusudhan MS, Gormley M et al. Gene expression profiling of the human maternal-fetal interface reveals dramatic changes between midgestation and term. Endocrinology 2007;148:1059-79.
21. Cindrova-Davies T, Yung HW, Johns J, Spasic-Boskovic O, Korolchuk S, Jauniaux E et al. Oxidative stress, gene expression, and protein changes induced in the human placenta during labor. Am.J.Pathol. 2007;171:1168-79.
22. Pidoux G, Gerbaud P, Laurendeau I, Guibourdenche J, Bertin G, Vidaud M et al. Large variability of trophoblast gene expression within and between human normal term placentae. Placenta 2004;25:469-73.
23. Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. Science 1995;270:484-87.
24. D'haeseleer P. How does gene expression clustering work? Nat.Biotechnol. 2005;23:1499-501.
25. Kal AJ, van Zonneveld AJ, Benes V, van den Berg M., Koerkamp MG, Albermann K et al. Dynamics of gene expression revealed by comparison of serial analysis of gene expression transcript profiles from yeast grown on two different carbon sources. Mol.Biol.Cell 1999;10:1859-72.
26. Galante PA, Trimarchi J, Cepko CL, de Souza SJ, Ohno-Machado L, Kuo WP. Automatic correspondence of tags and genes (ACTG): a tool for the analysis of SAGE, MPSS and SBS data. Bioinformatics. 2007;23:903-05.
27. D'haeseleer P. How does gene expression clustering work? Nat.Biotechnol. 2005;23:1499-501.
28. Pauws E, van Kampen AH, van de Graaf SA, de Vijlder JJ, Ris-Stalpers C. Heterogeneity in polyadenylation cleavage sites in mammalian mRNA sequences: implications for SAGE analysis. Nucleic Acids Res. 2001;29:1690-94.
29. Moreno JC, Bikker H, Kempers MJ, van Trotsenburg AS, Baas F, de Vijlder JJ et al. Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism. N.Engl.J.Med. 2002;347:95-102.
30. Gheorghe C, Mohan S, Longo LD. Gene expression patterns in the developing murine placenta. J.Soc.Gynecol.Investig. 2006;13:256-62.
31. Alvarez-Silva M, Belo-Diabangouaya P, Salaun J, eterlen-Lievre F. Mouse placenta is a major hematopoietic organ. Development 2003;130:5437-44.
32. Huisman TH, Schroeder WA, Felice A, Powars D, Ringelhann B. Anomaly in the gamma chain heterogeneity of the newborn. Nature 1977;265:63-65.
33. Speroff L, Glass RH, Kase NK. The Endocrinology of Pregnancy. Clinical Gynecologic Endocrinology and Infertility. Baltimore, Maryland, USA: Williams & Wilkins; 2008.
34. Rhodes JM, Simons M. The extracellular matrix and blood vessel formation: not just a scaffold. J.Cell Mol.Med. 2007;11:176-205.

35. Fukushima K, Miyamoto S, Tsukimori K, Kobayashi H, Seki H, Takeda S et al. Tumor necrosis factor and vascular endothelial growth factor induce endothelial integrin repertories, regulating endovascular differentiation and apoptosis in a human extravillous trophoblast cell line. Biol.Reprod. 2005;73:172-79.
36. Motran CC, Diaz FL, Montes CL, Bocco JL, Gruppi A. In vivo expression of recombinant pregnancy-specific glycoprotein 1a induces alternative activation of monocytes and enhances Th2-type immune response. Eur.J.Immunol. 2003;33:3007-16.
37. Smith R. Parturition. N.Engl.J.Med. 2007;356:271-83.

Chapter 8:

Discussion

Most obstetricians and paediatricians would agree that examination of the placenta can often explain abnormal pregnancy outcome.[1] 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.[2] 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,[3] 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.[4] Moreover, FGR is also found with normal and high PRs.[5;6]

We used the cohort of pregnancies described in Chapter 5 to assess the relation of PR with gestational age.(Figure 1) 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.

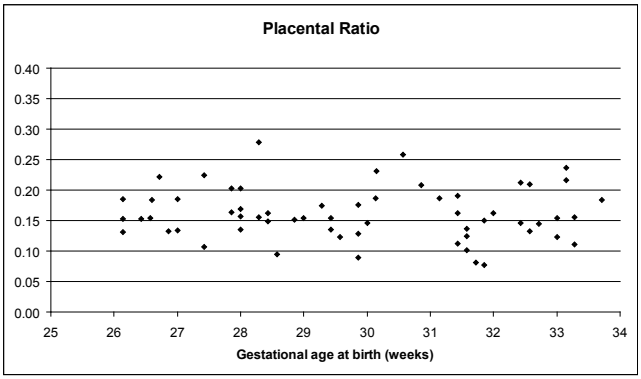

Figure 1: Placental Ratio (placental weight divided by fetal weight) as a function of gestational age at birth in the cohort described in Chapter 5. There is no significant linear trend.

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.

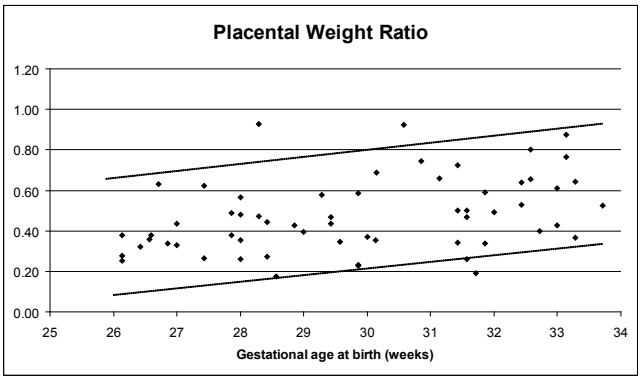

Figure 2: Placental weight ratio (placental weight divided by expected placental weight) as a function of gestational age at birth in the cohort described in Chapter 5. Trend: Pearson R = 0.38, B = 0.03, p = 0.003; The lines are the 95% confidence intervals.

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 ultrasound[8-10] 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,[13] the measurement of placental perfusion by echo planar imaging[14] 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[15-17] and as an early marker to predict PE.[18] Predictive performance of this single parameter in isolation is not enough to rigorously apply this diagnostic test in clinical practice.[18] Therefore we agree with Romero that prediction on the basis of a single determinant will be inferior to a combination of markers.[19] 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,[20] 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.[21] Antenatally administered glucocorticoids are associated with increased plasma T_3 and rT_3 in the fetal sheep.[22] We found a different pattern: decreased plasma T_3 and increased rT_3 in severely ill preterm neonates.

Interactions between glucocorticoids and thyroid hormones are even more complex if studied in different tissues. In rat pups, intracellular T_3 may be increased in liver and decreased in brain as result of dexamethasone administration, but an opposite effect can be seen before birth.[23]
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 fT_4 values last extensively in fetal life or if they persist after birth, they can have a negative impact on brain maturation.[20] 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.[24-26] The incidence of maternal thyroid dysfunction in our cohort of pregnant women

with PE was 2.5%, about equal to the 2% in the normal population.[27] Our data are also of interest to the discussion on screening on thyroid dysfunction in pregnancy.[28] 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.[29] Analyses of genome wide scans have revealed several chromosomal susceptibility loci for PE[29-34] and resulted in the association of the STOX1 Y153H variation with familial PE.[35] The role of STOX1 in PE is not clear since the initial results could not be confirmed by independent studies[36;37] and the maternal imprinting and monoallelic expression of the gene that was essential for the disease mechanism in fact does not occur.[38] 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[39] except an extremely rare mutation of LCHAD affecting fetal mitochondrial fatty acid oxidation, as described in the Introduction.[40] 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.

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.

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. [114] This applies especially to research with no highly specific a priori hypotheses, as described by Ioannidis.[115] 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[116] is of importance since labour has distinguishable effects of placental gene expression patterns.[117;118] 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[109] 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 are 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 placenta. This will allow the monitoring of placenta RNA levels in maternal serum in analogy to the studies of Lo.[119-121] 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.[51;103;122;123] It is however generally accepted that a combination of markers will be more appropriate for diagnostic purposes.[19]

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.[124] 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.[51] 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.[125]

Reference List

1. Benirschke K, Kaufmann P. Pathology of the human placenta. 2000. p. v.
2. Naeye RL. Do placental weights have clinical significance? Hum.Pathol. 1987;18:387-91.
3. Bortolus R, Chatenoud L, Di Cintio E, Rossi P, Benzi G, Surace M et al. Placental ratio in pregnancies at different risk for intrauterine growth. Eur.J.Obstet. Gynecol.Reprod.Biol. 1998;80:157-58.
4. Williams LA, Evans SF, Newnham JP. Prospective cohort study of factors influencing the relative weights of the placenta and the newborn infant. BMJ 1997;314:1864-68.
5. Lao TT, Wong W. The neonatal implications of a high placental ratio in small-for-gestational age infants. Placenta 1999;20:723-26.
6. Lao TT, Wong WM. Implications of a high placental ratio in pregnancies with appropriate-for-gestational age neonates. Gynecol.Obstet.Invest 2001;52:34-37.
7. Rep A, Ganzevoort W, Van Wassenaer AG, Bonsel GJ, Wolf H, De Vries JI. One-year infant outcome in women with early-onset hypertensive disorders of pregnancy. BJOG. 2008;115:290-98.
8. Bleker OP, Kloosterman GJ, Breur W, Mieras DJ. The volumetric growth of the human placenta: a longitudinal ultrasonic study. Am.J.Obstet.Gynecol. 1977;127:657-61.
9. Wolf H, Oosting H, Treffers PE. Second-trimester placental volume measurement by ultrasound: prediction of fetal outcome. Am.J.Obstet. Gynecol. 1989;160:121-26.
10. Metzenbauer M, Hafner E, Hoefinger D, Schuchter K, Stangl G, Ogris E et al. Three-dimensional ultrasound measurement of the placental volume in early pregnancy: method and correlation with biochemical placenta parameters. Placenta 2001;22:602-05.
11. Hafner E, Metzenbauer M, Hofinger D, Munkel M, Gassner R, Schuchter K et al. Placental growth from the first to the second trimester of pregnancy in SGA-foetuses and pre-eclamptic pregnancies compared to normal foetuses. Placenta 2003;24:336-42.
12. Wolf H, Oosting H, Treffers PE. A longitudinal study of the relationship between placental and fetal growth as measured by ultrasonography. Am.J.Obstet. Gynecol. 1989;161:1140-45.
13. Ong SS, Tyler DJ, Moore RJ, Gowland PA, Baker PN, Johnson IR et al. Functional magnetic resonance imaging (magnetization transfer) and stereological analysis of human placentae in normal pregnancy and in pre-eclampsia and intrauterine growth restriction. Placenta 2004;25:408-12.
14. Francis ST, Duncan KR, Moore RJ, Baker PN, Johnson IR, Gowland PA. Non-invasive mapping of placental perfusion. Lancet 1998;351:1397-99.
15. Scherjon SA, Smolders-DeHaas H, Kok JH, Zondervan HA. The "brain-sparing" effect: antenatal cerebral Doppler findings in relation to neurologic outcome in very preterm infants. Am.J.Obstet.Gynecol. 1993;169:169-75.
16. Scherjon SA, Oosting H, Smolders-DeHaas H, Zondervan HA, Kok JH.

Neurodevelopmental outcome at three years of age after fetal 'brain-sparing'. Early Hum.Dev. 1998;52:67-79.
17. Mires GJ, Williams FL, Leslie J, Howie PW. Assessment of uterine arterial notching as a screening test for adverse pregnancy outcome. Am.J.Obstet. Gynecol. 1998;179:1317-23.
18. Cnossen JS. Prediction of Preeclampsia by Ultrasound Doppler. Can.Med. Assoc.J. 2008;12.
19. Than NG, Romero R, Hillermann R, Cozzi V, Nie G, Huppertz B. Prediction of Preeclampsia - A Workshop Report. Placenta 2007.
20. Bernal J, Guadano-Ferraz A, Morte B. Perspectives in the study of thyroid hormone action on brain development and function. Thyroid 2003;13:1005-12.
21. Williams FL, Ogston SA, van Toor H, Visser TJ, Hume R. Serum thyroid hormones in preterm infants: associations with postnatal illnesses and drug usage. J.Clin.Endocrinol.Metab 2005;90:5954-63.
22. Forhead AJ, Jellyman JK, Gardner DS, Giussani DA, Kaptein E, Visser TJ et al. Differential effects of maternal dexamethasone treatment on circulating thyroid hormone concentrations and tissue deiodinase activity in the pregnant ewe and fetus. Endocrinology 2007;148:800-05.
23. Van der Geyten S., Darras VM. Developmentally defined regulation of thyroid hormone metabolism by glucocorticoids in the rat. J.Endocrinol. 2005;185:327-36.
24. Davis LE, Leveno KJ, Cunningham FG. Hypothyroidism complicating pregnancy. Obstet.Gynecol. 1988;72:108-12.
25. Leung AS, Millar LK, Koonings PP, Montoro M, Mestman JH. Perinatal outcome in hypothyroid pregnancies. Obstet.Gynecol. 1993;81:349-53.
26. Millar LK, Wing DA, Leung AS, Koonings PP, Montoro MN, Mestman JH. Low birth weight and preeclampsia in pregnancies complicated by hyperthyroidism. Obstet.Gynecol. 1994;84:946-49.
27. Boelaert K, Franklyn JA. Thyroid hormone in health and disease. J.Endocrinol. 2005;187:1-15.
28. Surks MI, Ortiz E, Daniels GH, Sawin CT, Col NF, Cobin RH et al. Subclinical thyroid disease: scientific review and guidelines for diagnosis and management. JAMA 2004;291:228-38.
29. Lachmeijer AM, Arngrimsson R, Bastiaans EJ, Frigge ML, Pals G, Sigurdardottir S et al. A genome-wide scan for preeclampsia in the Netherlands. Eur.J.Hum. Genet. 2001;9:758-64.
30. Harrison GA, Humphrey KE, Jones N, Badenhop R, Guo G, Elakis G et al. A genomewide linkage study of preeclampsia/eclampsia reveals evidence for a candidate region on 4q. Am.J.Hum.Genet. 1997;60:1158-67.
31. Arngrimsson R, Sigurard tS, Frigge ML, Bjarnad ttir RI, Jonsson T, Stefansson H et al. A genome-wide scan reveals a maternal susceptibility locus for pre-eclampsia on chromosome 2p13. Hum.Mol.Genet. 1999;8:1799-805.
32. Guo G, Lade JA, Wilton AN, Moses EK, Grehan M, Fu Y et al. Genetic susceptibility to pre-eclampsia and chromosome 7q36. Hum.Genet. 1999;105:641-47.
33. Moses EK, Lade JA, Guo G, Wilton AN, Grehan M, Freed K et al. A genome scan in families from Australia and New Zealand confirms the presence of a

maternal susceptibility locus for pre-eclampsia, on chromosome 2. Am.J.Hum. Genet. 2000;67:1581-85.
34. Laivuori H, Lahermo P, Ollikainen V, Widen E, Haiva-Mallinen L, Sundstrom H et al. Susceptibility loci for preeclampsia on chromosomes 2p25 and 9p13 in Finnish families. Am.J.Hum.Genet. 2003;72:168-77.
35. van Dijk M, Mulders J, Poutsma A, Konst AA, Lachmeijer AM, Dekker GA et al. Maternal segregation of the Dutch preeclampsia locus at 10q22 with a new member of the winged helix gene family. Nat.Genet. 2005.
36. Berends AL, Bertoli-Avella AM, de Groot CJ, van Duijn CM, Oostra BA, Steegers EA. STOX1 gene in pre-eclampsia and intrauterine growth restriction. BJOG. 2007;114:1163-67.
37. Kivinen K, Peterson H, Hiltunen L, Laivuori H, Heino S, Tiala I et al. Evaluation of STOX1 as a preeclampsia candidate gene in a population-wide sample. Eur. J.Hum.Genet. 2007;15:494-97.
38. Iglesias-Platas I, Monk D, Jebbink J, Buimer M, Boer K, van der PJ et al. STOX1 is not imprinted and is not likely to be involved in preeclampsia. Nat. Genet. 2007;39:279-80.
39. Goddard KA, Tromp G, Romero R, Olson JM, Lu Q, Xu Z et al. Candidate-gene association study of mothers with pre-eclampsia, and their infants, analyzing 775 SNPs in 190 genes. Hum.Hered. 2007;63:1-16.
40. Tyni T, Ekholm E, Pihko H. Pregnancy complications are frequent in long-chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency. Am.J.Obstet.Gynecol. 1998;178:603-08.
41. van Pampus MG, Dekker GA, Wolf H, Huijgens PC, Koopman MM, von Blomberg BM et al. High prevalence of hemostatic abnormalities in women with a history of severe preeclampsia. Am.J.Obstet.Gynecol. 1999;180:1146-50.
42. Kobashi G, Hata A, Shido K, Ohta K, Yamada H, Kato EH et al. Insertion/ deletion polymorphism of the angiotensin-converting enzyme gene and preeclampsia in Japanese patients. Semin.Thromb.Hemost. 2005;31:346-50.
43. Ward K, Hata A, Jeunemaitre X, Helin C, Nelson L, Namikawa C et al. A molecular variant of angiotensinogen associated with preeclampsia. Nat. Genet. 1993;4:59-61.
44. Levesque S, Moutquin JM, Lindsay C, Roy MC, Rousseau F. Implication of an AGT haplotype in a multigene association study with pregnancy hypertension. Hypertension 2004;43:71-78.
45. Konijnenberg A, Stokkers EW, van der Post JA, Schaap MC, Boer K, Bleker OP et al. Extensive platelet activation in preeclampsia compared with normal pregnancy: enhanced expression of cell adhesion molecules. Am.J.Obstet. Gynecol. 1997;176:461-69.
46. Belo L, Gaffney D, Caslake M, Santos-Silva A, Pereira-Leite L, Quintanilha A et al. Apolipoprotein E and cholesteryl ester transfer protein polymorphisms in normal and preeclamptic pregnancies. Eur.J.Obstet.Gynecol.Reprod.Biol. 2004;112:9-15.
47. Kumru S, Godekmerdan A, Kutlu S, Ozcan Z. Correlation of maternal serum high-sensitive C-reactive protein levels with biochemical and clinical parameters in preeclampsia. Eur.J.Obstet.Gynecol.Reprod.Biol. 2006;124:164-67.

48. Dong YL, Chauhan M, Green KE, Vegiraju S, Wang HQ, Hankins GD et al. Circulating calcitonin gene-related peptide and its placental origins in normotensive and preeclamptic pregnancies. Am.J.Obstet.Gynecol. 2006;195:1657-67.
49. Wang Y, Walsh SW. Antioxidant activities and mRNA expression of superoxide dismutase, catalase, and glutathione peroxidase in normal and preeclamptic placentas. J.Soc.Gynecol.Investig. 1996;3:179-84.
50. Kim YJ, Williamson RA, Murray JC, Andrews J, Pietscher JJ, Peraud PJ et al. Genetic susceptibility to preeclampsia: roles of cytosineto-thymine substitution at nucleotide 677 of the gene for methylenetetrahydrofolate reductase, 68-base pair insertion at nucleotide 844 of the gene for cystathionine beta-synthase, and factor V Leiden mutation. Am.J.Obstet.Gynecol. 2001;184:1211-17.
51. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP et al. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N.Engl.J.Med. 2006;355:992-1005.
52. Fiore G, Florio P, Micheli L, Nencini C, Rossi M, Cerretani D et al. Endothelin-1 triggers placental oxidative stress pathways: putative role in preeclampsia. J.Clin.Endocrinol.Metab 2005;90:4205-10.
53. Leach RE, Romero R, Kim YM, Chaiworapongsa T, Kilburn B, Das SK et al. Pre-eclampsia and expression of heparin-binding EGF-like growth factor. Lancet 2002;360:1215-19.
54. Zusterzeel PL, Peters WH, Visser W, Hermsen KJ, Roelofs HM, Steegers EA. A polymorphism in the gene for microsomal epoxide hydrolase is associated with pre-eclampsia. J.Med.Genet. 2001;38:234-37.
55. Malamitsi-Puchner A, Tziotis J, Evangelopoulos D, Fountas L, Vlachos G, Creatsas G et al. Gene analysis of the N-terminal region of the estrogen receptor alpha in preeclampsia. Steroids 2001;66:695-700.
56. Witsenburg CP, Rosendaal FR, Middeldorp JM, Van der Meer FJ, Scherjon SA. Factor VIII levels and the risk of pre-eclampsia, HELLP syndrome, pregnancy related hypertension and severe intrauterine growth retardation. Thromb. Res. 2005;115:387-92.
57. Watanabe H, Hamada H, Yamakawa-Kobayashi K, Yoshikawa H, Arinami T. Evidence for an association of the R485K polymorphism in the coagulation factor V gene with severe preeclampsia from screening 35 polymorphisms in 27 candidate genes. Thromb.Haemost. 2001;86:1594-95.
58. Steegers EA, Mulder TP, Bisseling JG, Delemarre FM, Peters WH. Glutathione S-transferase alpha as marker for hepatocellular damage in pre-eclampsia and HELLP syndrome. Lancet 1995;345:1571-72.
59. Tsoi SC, Cale JM, Bird IM, Kay HH. cDNA microarray analysis of gene expression profiles in human placenta: up-regulation of the transcript encoding muscle subunit of glycogen phosphorylase in preeclampsia. J.Soc.Gynecol.Investig. 2003;10:496-502.
60. O'Shaughnessy KM, Fu B, Downing S, Morris NH. Thrombophilic polymorphisms in pre-eclampsia: altered frequency of the functional 98C>T polymorphism of glycoprotein IIIa. J.Med.Genet. 2001;38:775-77.
61. Furugori K, Kurauchi O, Itakura A, Kanou Y, Murata Y, Mizutani S et al. Levels of

hepatocyte growth factor and its messenger ribonucleic acid in uncomplicated pregnancies and those complicated by preeclampsia. J.Clin.Endocrinol.Metab 1997;82:2726-30.
62. Takakuwa K, Honda K, Ishii K, Hataya I, Yasuda M, Tanaka K. Studies on the HLA-DRB1 genotypes in Japanese women with severe pre-eclampsia positive and negative for anticardiolipin antibody using a polymerase chain reaction-restriction fragment length polymorphism method. Hum.Reprod. 1999;14:2980-86.
63. Kilpatrick DC. Influence of human leukocyte antigen and tumour necrosis factor genes on the development of pre-eclampsia. Hum.Reprod.Update. 1999;5:94-102.
64. Schoof E, Girstl M, Frobenius W, Kirschbaum M, Dorr HG, Rascher W et al. Decreased gene expression of 11beta-hydroxysteroid dehydrogenase type 2 and 15-hydroxyprostaglandin dehydrogenase in human placenta of patients with preeclampsia. J.Clin.Endocrinol.Metab 2001;86:1313-17.
65. Caniggia I, Winter J, Lye SJ, Post M. Oxygen and placental development during the first trimester: implications for the pathophysiology of pre-eclampsia. Placenta 2000;21 Suppl A:S25-S30.
66. Kocyigit Y, Bayhan G, Atamer A, Atamer Y. Serum levels of leptin, insulin-like growth factor-I and insulin-like growth factor binding protein-3 in women with pre-eclampsia, and their relationship to insulin resistance. Gynecol. Endocrinol. 2004;18:341-48.
67. Tjoa ML, Oudejans CB, van Vugt JM, Blankenstein MA, van W, I. Markers for presymptomatic prediction of preeclampsia and intrauterine growth restriction. Hypertens.Pregnancy. 2004;23:171-89.
68. Lim KH, Zhou Y, Janatpour M, McMaster M, Bass K, Chun SH et al. Human cytotrophoblast differentiation/invasion is abnormal in pre-eclampsia. Am.J.Pathol. 1997;151:1809-18.
69. Krauss T, Kuhn W, Lakoma C, Augustin HG. Circulating endothelial cell adhesion molecules as diagnostic markers for the early identification of pregnant women at risk for development of preeclampsia. Am.J.Obstet. Gynecol. 1997;177:443-49.
70. Lyall F, Greer IA, Boswell F, Young A, Macara LM, Jeffers MD. Expression of cell adhesion molecules in placentae from pregnancies complicated by pre-eclampsia and intrauterine growth retardation. Placenta 1995;16:579-87.
71. Rinehart BK, Terrone DA, Lagoo-Deenadayalan S, Barber WH, Hale EA, Martin JN, Jr. et al. Expression of the placental cytokines tumor necrosis factor alpha, interleukin 1beta, and interleukin 10 is increased in preeclampsia. Am.J.Obstet.Gynecol. 1999;181:915-20.
72. Borekci B, Aksoy H, Al RA, Demircan B, Kadanali S. Maternal serum interleukin-10, interleukin-2 and interleukin-6 in pre-eclampsia and eclampsia. Am.J.Reprod.Immunol. 2007;58:56-64.
73. Pang ZJ, Xing FQ. Comparative study on the expression of cytokine--receptor genes in normal and preeclamptic human placentas using DNA microarrays. J.Perinat.Med. 2003;31:153-62.
74. Gratacos E, Filella X, Palacio M, Cararach V, Alonso PL, Fortuny A. Interleukin-

4, interleukin-10, and granulocyte-macrophage colony stimulating factor in second-trimester serum from women with preeclampsia. Obstet.Gynecol. 1998;92:849-53.
75. Agarwal R, Loganath A, Roy AC, Wong YC, Ng SC. Expression profiles of interleukin-15 in early and late gestational human placenta and in pre-eclamptic placenta. Mol.Hum.Reprod. 2001;7:97-101.
76. Hannke-Lohmann A, Pildner von SS, Dehne K, Benard V, Kolben M, Schmitt M et al. Downregulation of a mitogen-activated protein kinase signaling pathway in the placentas of women with preeclampsia. Obstet.Gynecol. 2000;96:582-87.
77. Saito S, Sakai M, Sasaki Y, Nakashima A, Shiozaki A. Inadequate tolerance induction may induce pre-eclampsia. J.Reprod.Immunol. 2007;76:30-39.
78. Chaiworapongsa T, Romero R, Yoshimatsu J, Espinoza J, Kim YM, Park K et al. Soluble adhesion molecule profile in normal pregnancy and pre-eclampsia. J.Matern.Fetal Neonatal Med. 2002;12:19-27.
79. Hung TH, Skepper JN, Charnock-Jones DS, Burton GJ. Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. Circ.Res. 2002;90:1274-81.
80. Lee H, Park H, Kim YJ, Kim HJ, Ahn YM, Park B et al. Expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in human preeclamptic placenta: possible implications in the process of trophoblast apoptosis. Placenta 2005;26:226-33.
81. Chappell LC, Seed PT, Briley A, Kelly FJ, Hunt BJ, Charnock-Jones DS et al. A longitudinal study of biochemical variables in women at risk of preeclampsia. Am.J.Obstet.Gynecol. 2002;187:127-36.
82. Kharb S, Gulati N, Singh V, Singh GP. Lipid peroxidation and vitamin E levels in preeclampsia. Gynecol.Obstet.Invest 1998;46:238-40.
83. Belo L, Caslake M, Santos-Silva A, Pereira-Leite L, Quintanilha A, Rebelo I. Lipoprotein(a): a longitudinal versus a cross-sectional study in normal pregnancy and its levels in preeclampsia. Atherosclerosis 2002;165:393-95.
84. Hayashi M, Ohkura T, Inaba N. Elevation of serum macrophage colony-stimulating factor before the clinical manifestations of preeclampsia. Am.J.Obstet.Gynecol. 2003;189:1356-60.
85. Campbell S, Rowe J, Jackson CJ, Gallery ED. Interaction of cocultured decidual endothelial cells and cytotrophoblasts in preeclampsia. Biol.Reprod. 2004;71:244-52.
86. Myers JE, Merchant SJ, Macleod M, Mires GJ, Baker PN, Davidge ST. MMP-2 levels are elevated in the plasma of women who subsequently develop preeclampsia. Hypertens.Pregnancy. 2005;24:103-15.
87. de Jager CA, Linton EA, Spyropoulou I, Sargent IL, Redman CW. Matrix metalloprotease-9, placental syncytiotrophoblast and the endothelial dysfunction of pre-eclampsia. Placenta 2003;24:84-91.
88. Pang ZJ, Xing FQ. Expression profile of trophoblast invasion-associated genes in the pre-eclamptic placenta. Br.J.Biomed.Sci. 2003;60:97-101.
89. Laigaard J, Sorensen T, Placing S, Holck P, Frohlich C, Wojdemann KR et al. Reduction of the disintegrin and metalloprotease ADAM12 in preeclampsia. Obstet.Gynecol. 2005;106:144-49.

90. Kosmas IP, Tatsioni A, Ioannidis JP. Association of C677T polymorphism in the methylenetetrahydrofolate reductase gene with hypertension in pregnancy and pre-eclampsia: a meta-analysis. J.Hypertens. 2004;22:1655-62.
91. Page NM, Woods RJ, Gardiner SM, Lomthaisong K, Gladwell RT, Butlin DJ et al. Excessive placental secretion of neurokinin B during the third trimester causes pre-eclampsia. Nature 2000;405:797-800.
92. Khatun S, Kanayama N, Belayet HM, Bhuiyan AB, Jahan S, Begum A et al. Increased concentrations of plasma neuropeptide Y in patients with eclampsia and preeclampsia. Am.J.Obstet.Gynecol. 2000;182:896-900.
93. Faxen M, Nisell H, Kublickiene KR. Altered mRNA expression of ecNOS and iNOS in myometrium and placenta from women with preeclampsia. Arch. Gynecol.Obstet. 2001;265:45-50.
94. Hakli T, Romppanen EL, Hiltunen M, Helisalmi S, Punnonen K, Heinonen S. Endothelial nitric oxide synthase polymorphism in preeclampsia. J.Soc. Gynecol.Investig. 2003;10:154-57.
95. Curtis NE, King RG, Moseley JM, Ho PW, Rice GE, Wlodek ME. Intrauterine expression of parathyroid hormone-related protein in normal and pre-eclamptic pregnancies. Placenta 1998;19:595-601.
96. Sasaki H, Roberts J, Lykins D, Fujii Y, Auclair D, Chen LB. Novel chemiluminescence assay for serum periostin levels in women with preeclampsia and in normotensive pregnant women. Am.J.Obstet.Gynecol. 2002;186:103-08.
97. Ilic D, Genbacev O, Jin F, Caceres E, Almeida EA, Bellingard-Dubouchaud V et al. Plasma membrane-associated pY397FAK is a marker of cytotrophoblast invasion in vivo and in vitro. Am.J.Pathol. 2001;159:93-108.
98. Conover CA, Bale LK, Overgaard MT, Johnstone EW, Laursen UH, Fuchtbauer EM et al. Metalloproteinase pregnancy-associated plasma protein A is a critical growth regulatory factor during fetal development. Development 2004;131:1187-94.
99. Gude NM, Stebbing PN, Wang L, Xue J, Brennecke SP, Lim AT. Relative abundance of placental pro-atrial natriuretic factor mRNA in normal pregnancy and pre-eclampsia. Gynecol.Obstet.Invest 2000;49:114-18.
100. Wetzka B, Charnock-Jones DS, Viville B, Cooper JC, Nusing R, Zahradnik HP et al. Expression of prostacyclin and thromboxane synthases in placenta and placental bed after pre-eclamptic pregnancies. Placenta 1996;17:573-81.
101. Tempfer CB, Schneeberger C, Huber JC. Applications of polymorphisms and pharmacogenomics in obstetrics and gynecology. Pharmacogenomics. 2004;5:57-65.
102. Shah DM. Role of the renin-angiotensin system in the pathogenesis of preeclampsia. Am.J.Physiol Renal Physiol 2005;288:F614-F625.
103. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF et al. Circulating angiogenic factors and the risk of preeclampsia. N.Engl.J.Med. 2004;350:672-83.
104. Langbein M, Strick R, Strissel PL, Vogt N, Parsch H, Beckmann MW et al. Impaired cytotrophoblast cell-cell fusion is associated with reduced Syncytin and increased apoptosis in patients with placental dysfunction. Mol.Reprod. Dev. 2008;75:175-83.

105. Antovic JP, Rafik HR, Antovic A, Blomback M, Bremme K. Does thrombin activatable fibrinolysis inhibitor (TAFI) contribute to impairment of fibrinolysis in patients with preeclampsia and/or intrauterine fetal growth retardation? Thromb.Haemost. 2002;88:644-47.
106. Wiwanitkit V. Correlation between thrombomodulin and severe preeclampsia: a summary. Clin.Appl.Thromb.Hemost. 2008;14:99-101.
107. Lala PK, Chakraborty C. Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury. Placenta 2003;24:575-87.
108. Kupferminc MJ, Peaceman AM, Aderka D, Wallach D, Socol ML. Soluble tumor necrosis factor receptors and interleukin-6 levels in patients with severe preeclampsia. Obstet.Gynecol. 1996;88:420-27.
109. Zhou Y, McMaster M, Woo K, Janatpour M, Perry J, Karpanen T et al. Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. Am.J.Pathol. 2002;160:1405-23.
110. Arbogast BW, Leeper SC, Merrick RD, Olive KE, Taylor RN. Which plasma factors bring about disturbance of endothelial function in pre-eclampsia? Lancet 1994;343:340-41.
111. Holthe MR, Staff AC, Berge LN, Lyberg T. Different levels of platelet activation in preeclamptic, normotensive pregnant, and nonpregnant women. Am.J.Obstet.Gynecol. 2004;190:1128-34.
112. Liu ET, Karuturi KR. Microarrays and clinical investigations. N.Engl.J.Med. 2004;350:1595-97.
113. Hom G, Graham RR, Modrek B, Taylor KE, Ortmann W, Garnier S et al. Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. N.Engl.J.Med. 2008;358:900-09.
114. Ruijter JM, van Kampen AH, Baas F. Statistical evaluation of SAGE libraries: consequences for experimental design. Physiol Genomics 2002;11:37-44.
115. Ioannidis JP. Microarrays and molecular research: noise discovery? Lancet 2005;365:454-55.
116. Hassan SS, Romero R, Tarca AL, Draghici S, Pineles B, Bugrim A et al. Signature pathways identified from gene expression profiles in the human uterine cervix before and after spontaneous term parturition. Am.J.Obstet. Gynecol. 2007;197:250-57.
117. Salomonis N, Cotte N, Zambon AC, Pollard KS, Vranizan K, Doniger SW et al. Identifying genetic networks underlying myometrial transition to labor. Genome Biol. 2005;6:R12.
118. Esplin MS, Fausett MB, Peltier MR, Hamblin S, Silver RM, Branch DW et al. The use of cDNA microarray to identify differentially expressed labor-associated genes within the human myometrium during labor. Am.J.Obstet.Gynecol. 2005;193:404-13.
119. Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RW, Panesar NS et al. mRNA of placental origin is readily detectable in maternal plasma. Proc.Natl.Acad.Sci. U.S.A 2003;100:4748-53.

120. Farina A, Chan CW, Chiu RW, Tsui NB, Carinci P, Concu M et al. Circulating corticotropin-releasing hormone mRNA in maternal plasma: relationship with gestational age and severity of preeclampsia. Clin.Chem. 2004;50:1851-54.
121. Lo YM, Tsui NB, Chiu RW, Lau TK, Leung TN, Heung MM et al. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. Nat.Med. 2007;13:218-23.
122. Levine RJ. Erratum. N.Engl.J.Med. 2006;355:1840.
123. Kalionis B, Moses E. Advanced molecular techniques in pregnancy research: proteomics and genomics--a workshop report. Placenta 2003;24 Suppl A: S119-S122.
124. Ioannidis JP. Commentary: grading the credibility of molecular evidence for complex diseases. Int.J.Epidemiol. 2006;35:572-78.
125. Morrish DW, Kudo Y, Caniggia I, Cross J, Evain-Brion D, Gasperowicz M et al. Growth factors and trophoblast differentiation-workshop report. Placenta 2007;28 Suppl A:S121-S124.

Summary

The Placenta as Modulator of Fetal Prosperity

Chapter 1 introduces placental development and function in various species and elaborates on features like placental weight relevant for gestational diseases as fetal growth restriction and preeclampsia.
The placenta is an endocrine organ, capable of synthesizing and secreting a broad range of growth factors, protein- and steroid hormones. Besides this, the placenta plays an important role in hormone metabolism and hormonal interaction between mother and fetus. Since thyroid hormone is crucial for fetal brain development, regulation of thyroid hormone metabolism during pregnancy is described.
In addition the role of placental tissue in the development of gestational hypertensive disease as preeclampsia is described. It is stated that no single genetic disorder will explain the full spectrum of preeclampsia. This complex genetic disease deserves a sophisticated approach that investigates gene expression in its full context. We propose the use of non-selective high-throughput analysis of gene expression in placental tissue using both publicly available and in-house made Serial Analysis of Gene Expression (SAGE) libraries.

In Chapters 2 and 3 of this thesis we evaluate the clinical significance of placental weight and birth weight. Placental and birth weight are closely related and are associated with obstetrical pathology and adverse neonatal outcome.

Placental weight is addressed in Chapter 2 and we illustrate the pivotal importance of placental function for fetal growth with so far unpublished data on placental weight. In this review we reinterpret the study of Kloosterman on 80.000 consecutive birth weights and 30.000 placental weights. We conclude with Kloosterman that pregnancies with heavier placentas last longer. Furthermore, it appears that birth weight of children from primiparous women compared to those from multiparous women and of twin children compared to singleton children is lower and that this difference is associated with smaller placentas. Therefore we conclude that the placenta is in control of fetal growth. This concept has motivated longitudinal investigations on placental volume and the relation of placental volume to fetal outcome.

In Chapter 3 we explore an alternative parameter in the classification of Birth Weight. Birth weight is an important obstetrical determinant for neonatal outcome since it is related to perinatal mortality, neonatal morbidity, but also childhood intelligence and future health. In daily practice, birth weights are dichotomized by defining growth restriction below a centile threshold,

for instance the 5th or 10th centile. The determination of centile thresholds, however, is subject to a considerable numbers of outliers in the reference curve at lower gestational ages. Most of the tertiary care study populations investigated in clinical studies indeed are of lower gestational ages. Birth Weight Ratio (BWR) is a parameter that does not suffer the shortcomings of centile thresholds. We define BWR as the ratio of observed birth weight divided by the mean birth weight of the reference growth curve.
We show that difference between BWRs calculated by either the Gardosi or the Kloosterman birth weight charts is smaller than the differences between centile scores, and less dependent of weight. This illustrates the dependency of centile scores on the type of chart. We demonstrate that application of the BWR allows for a more consistent classification of newborn weight. Obviously, the BWR is more appropriate to describe fetal growth restriction and increases discriminative powr for statistical analysis, since it is a continuous variable.

In Chapter 4 we investigate an aspect of the interaction between glucocorticoids and thyroid hormone in neonates. We studied preterm neonates receiving dexamethasone treatment for weaning off the ventilator as a model for thyroid hormone and glucocorticoid interaction because they may still partly show maturational aspects that normally occur at the end of term gestation. These infants are known to be at risk of hypothyroxinemia because of prematurity as well as because of severe neonatal disease. We found significantly decreased TSH and T_3 levels and significantly increased reverseT_3 levels after dexamethasone administration. We conclude that dexamethasone administration has an effect on thyroid function and on thyroid hormone metabolism. Whether this also occurs in the antenatal period has to be established in future research.

In Chapter 5 we assess whether and to what extent thyroid function is affected in pregnant women with early and severe hypertensive disorders and in their growth restricted newborns. These women were enrolled in a randomized clinical intervention trial evaluating the use of plasma volume expansion. We observed low fT_4 levels in 33% of these women at admission followed by a spontaneous normalization during pregnancy in almost all of

them. We found no correlation between cord blood fT_4 and maternal fT_4, and in contrast to other studies, we found no gestational age effect of cord blood fT_4 levels. We conclude that women with severe hypertensive disorders of pregnancy may transiently have lower fT_4 levels without evidence of a thyroid disorder. Their neonates have lower fT_4 levels not related with their mothers' thyroid hormone status.

Chapter 6 reports the data generated by SAGE on placental tissue obtained from a normotensive pregnancy and a pregnancy with PE and HELLP syndrome. Comparison of SAGE profiles identified differentially expressed transcripts, a subset of which is validated on 36 placental tissue samples. Further downstream analysis and nearest centroid classification results in a 7 gene molecular placental signature specific for HELLP syndrome. This distinct placental molecular signature indicates that HELLP is not a PE variant but a separate disease entity and partly explains why studies aimed at identifying a common cause for gestational hypertensive disorders lack statistical power. The transcripts involved (CTNNAL, FLT1, GSTP1, LEP, PAPPA2, S100A8 and WWTR1) correspond to diverse molecular pathways, exemplifying the multigenic molecular basis of the disorder.

Chapter 7 describes genes that are differentially expressed during three time points in gestation, 12, 28 and 40 weeks of gestational age. Using K-means clustering we assign the placental transcripts to 3 mutually exclusive clusters of gene expression profiles with predominant expression in the first, second and third trimester. Mapping to gene ontology classes shows that genes involved in protein synthesis are relatively overrepresented in the first trimester. The gene ontology classes representing processes like development, angiogenesis, cell adhesion and apoptosis are rather evenly distributed over the three clusters. The prospected sequence of gestation specific events was not reflected in the clustered expression profiles. Extensive up regulation of ribosomal proteins is the most striking feature in the first trimester.

Chapter 8 discusses the main findings of this thesis and indicates aims for future research.

Samenvatting

The Placenta as Modulator of Fetal Prosperity

In Hoofdstuk 1 wordt de placenta ontwikkeling en placentafunctie in verschillende diersoorten beschreven. De placenta is een endocrien orgaan, dat in staat is tot synthese en secretie van een grote variëteit aan groeifactoren, peptide- en steroïd-hormonen. Daarnaast speelt de placenta een belangrijke rol in het metabolisme van hormonen en in de hormonale wisselwerking tussen moeder en kind. Omdat schildklierhormoon essentieel is voor een goede hersenontwikkeling van de foetus, wordt extra aandacht besteed aan de regulatie van schildklierhormoon. Verder wordt de rol van de placenta in het ontstaan van zwangerschapsgerelateerde ziekten als hypertensie en preeclampsie beschreven. Ook het gewicht van de placenta komt hierbij aan bod en het wordt duidelijk gemaakt dat een enkelvoudig gendefect nooit het volledige spectrum van het preeclampsie-syndroom zal kunnen verklaren. Voor onderzoek naar deze genetisch complexe aandoening is een meer verfijnde benadering noodzakelijk, die gen expressie patronen in hun context beschrijft. Wij kiezen voor *Serial Analysis of Gene Expression* (SAGE), een analysemethode van zeer omvangrijke genetische databanken. Deze hebben we deels zelf gemaakt, maar er zijn ook banken gebruikt die publiek beschikbaar zijn via Internet.

De hoofdstukken 2 en 3 bespreken het klinische belang van het placenta gewicht en geboortegewicht van het kind. Placenta gewicht en geboortegewicht zijn onderling sterk gerelateerd, afwijkingen van zowel placenta gewicht als geboortegewicht zijn gerelateerd aan obstetrische problematiek als hypertensie, preeclampsie en vroeggeboorte en aan een slechte conditie van het kind bij geboorte.

Placenta gewichten worden besproken in hoofdstuk 2. We illustreren het belang van de placenta voor de groei van de foetus aan de hand van tot nu toe ongepubliceerde historische gegevens over het placenta gewicht. In dit overzicht herinterpreteren we de gegevens van Kloosterman aangaande 80.000 opeenvolgende geboorte gewichten en 30.000 placenta gewichten. We concluderen samen met Kloosterman dat zwangerschappen met grotere placenta's langer behouden blijven. Verder blijkt dat het geboorte gewicht van eerst geborenen lager is dan het gewicht van kinderen uit volgende zwangerschappen. Deze resultaten illustreren dat de placenta controle uitoefent op de foetale groei. Dit concept heeft geleid tot meer onderzoek, onder andere naar longitudinale metingen van het placenta volume in de zwangerschap, en naar de relatie van placenta volume met de conditie van het kind bij geboorte.

Geboortegewicht is een heel belangrijke determinant voor de conditie van het kind omdat het een sterke relatie heeft met sterfte rond de geboorte, morbiditeit tijdens eventuele couveuseopname, maar ook intelligentie op de kinderleeftijd en de gezondheidstoestand op volwassen leeftijd. In de dagelijkse praktijk worden geboortegewichten opgedeeld in twee groepen aan de hand van een percentiel drempelwaarde, gewoonlijk de 5^{e} of 10^{e} percentiel. Het bepalen van de juiste percentiel is nogal onzeker en wordt bemoeilijkt door uitbijters in de referentiegroep, vooral bij lagere zwangerschapsduren. Het grootste deel van de studiepopulatie van derde lijns centra wordt geboren bij deze lagere zwangerschapsduur.

In hoofdstuk 3 beoordelen we een aan geboortegewicht gerelateerde parameter om de pasgeborene beter te kunnen classificeren als groeivertraagd. De *Birth Weight Ratio* (BWR) (letterlijk vertaald: geboortegewicht ratio) is een parameter die niet beïnvloed wordt door afwijkingen als bestaand bij de percentiel drempelwaardes. We definiëren de BWR als het quotiënt van het gemeten geboortegewicht en het verwachte geboortegewicht, als er geen sprake zou zijn van groeiachterstand. Wij laten zien dat het gebruik van de BWR een consistentere classificatie van het geboortegewicht geeft. Het is ook duidelijk dat BWR een meer geëigende maat is voor het beschrijven van groeiachterstand, en een groter discriminerend vermogen voor statistische analyses heeft, omdat het een continue variabele is.

Hoofdstuk 4 onderzoekt de interactie tussen corticosteroïden en schildklierhormoon bij vroeggeboren baby's op de neonatale intensive care, die behandeld worden met dexamethason om ze van de beademing af te kunnen krijgen. De kinderen in onze studiegroep hebben een risico van een schildklierhormoon tekort, zowel ten gevolge van de vroeggeboorte als ten gevolge van hun de ziekte die ze doormaken. We vonden een significante verlaging van TSH en T_3 spiegels na dexamethason toediening. We hebben geconcludeerd dat dexamethason toediening een effect heeft op zowel de schildklierfunctie als het metabolisme van schildklierhormoon. Of dit ook optreedt bij antenataal toegediende corticosteroïden moet nog worden onderzocht.

In hoofdstuk 5 onderzoeken we hoe en in welke mate de schildklierfunctie beïnvloed wordt bij zwangere vrouwen met ernstige hypertensieve aandoeningen in de zwangerschap waaronder preeclampsie, en bij hun pasgeborenen. De vrouwen participeerden in een gerandomiseerde klinische trial die het effect bestudeerde van plasma volume expansie op de zwangerschapsuitkomst. Bij opname constateerden we verlaagde vrij T_4 waardes in 33% van de

vrouwen, maar bij bijna allemaal trad nog tijdens de zwangerschap een spontane normalisering van de schildklierfunctie op. We vonden geen relatie van moederlijk vrij T_4 met het vrije T_4 in navelstrengbloed. In tegenstelling tot andere studies vonden we geen hogere schildklierhormoon spiegels in navelstrengbloed naarmate de zwangerschapsduur bij geboorte toe nam. We concluderen dat vrouwen met ernstige hypertensieve aandoeningen in de zwangerschap tijdelijk verlaagde schildklier hormoon spiegels kunnen hebben, maar dat dit niet betekent dat ze een schildklier aandoening hebben. Het lagere vrij T_4 in het navelstrengbloed van de pasgeborenen wordt niet veroorzaakt door het lagere moederlijke vrij T_4.

Hoofdstuk 6 beschrijft onze SAGE experimenten en analyses, waarin genexpressie in placenta weefsel van een normotensieve zwangerschap vergeleken wordt met een zwangerschap gecompliceerd door preeclampsie en HELLP. De vergelijking van deze SAGE profielen identificeerde een aantal differentieel gereguleerde transcripten, waarvan een subset werd gevalideerd op een panel van 36 placenta monsters. Verdere analyses met behulp van de *nearest centroid classification* methode resulteert in een profiel van 7 genen, specifiek voor het HELLP syndroom. Dit specifieke patroon laat zien dat HELLP geen variant van preeclampsie is, maar een afzonderlijke ziekte entiteit. Het verklaart ook waarom studies die gericht zijn op het ophelderen van een gemeenschappelijke oorzaak van preeclampsie en HELLP tekort schieten. De transcripten van het profiel (CTNNAL, FLT1, GSTP1, LEP, PAPPA2, S100A8 en WWTR1) vertegenwoordigen zeer verschillende moleculaire functies, dit is in overeenstemming met het multi-genetische karakter van de aandoening.

In hoofdstuk 7 beschrijven we genen die differentieel gereguleerd zijn in het 1ste, 2de en 3de trimester van de zwangerschap. Met behulp van *K-means clustering* identificeren we 3 patronen van gen expressie, met afwijkende expressie in ofwel het 1ste, ofwel het 2de, ofwel het 3de trimester. Toewijzing van een functie aan deze genen laat zien dat de transcripten die verband houden met eiwitsynthese relatief oververtegenwoordigd zijn in het eerste trimester. De functionele groepen die processen als differentiatie, vaatvorming, celadhesie en apoptose beschrijven zijn gelijk verdeeld over de drie clusters en de upregulatie van ribosomale eiwitten in het eerste trimester blijft het meest opvallende fenomeen.

In hoofdstuk 8 worden de voornaamste bevindingen van het proefschrift bediscussieerd, en worden perspectieven voor toekomstig onderzoek geschetst.

Alfirevic Z, Boer K, Brocklehurst P, Buimer M, Elbourne D, Kok JH, Tansey S. Two trials of antenatal thyrotropin-releasing hormone for fetal maturation: stopping before the due date. British Journal of Obstetrics and Gynaecology 1999;106:898-906.

Briët JM, van Sonderen L, Buimer M, Boer K, Kok JH. Neurodevelopmental outcome of children treated with antenatal thyrotropin-releasing hormone. Pediatrics 2002;110:249-53.

Buimer M, Van Wassenaer AG, Ganzevoort W, Wolf H, Bleker OP, Kok JH. Transient hypothyroxinemia in severe hypertensive disorders of pregnancy. Obstet.Gynecol. 2005;106:973-79.

Bleker OP, Buimer M, van der Post JA, van der Veen F. Ted (G. J.) Kloosterman: On Intrauterine Growth. The Significance of Prenatal Care. Studies on Birth Weight, Placental Weight and Placental Index. Placenta 2005;27:1052-4.

Iglesias-Platas I, Monk D, Jebbink J, Buimer M, Boer K, van der Post JA, Hills F, Apostolidou S, Ris-Stalpers C, Stanier P, Moore GE. STOX1 is not imprinted and is not likely to be involved in preeclampsia. Nat. Genet. 2007;39:279-80.

Buimer M, Keijser R, Jebbink JM, Wehkamp D, van Kampen AHC, Boer K, van der Post JA, Ris-Stalpers C. Seven placental transcripts characterize HELLP-syndrome. Placenta 2008;29:444-53

Buimer M, van Wassenaer AG, Kok, JH. Postnatal administration of dexamethasone for weaning off the ventilator affects thyroid function. Neonatology;*In Press.*

Buimer M, Lok CAR, Nieuwland R, Ris-Stalpers C, van der Post JA. Placental corticotrophin-releasing hormone mRNA and microparticles in maternal plasma are not measures of placental shedding of debris. J.Thromb.Haemost. 2008;*[Epub ahead of print]*.

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