

*Jouko Järvenpää*

PLACENTAL ANGIOGENESIS  
AND ANGIOGENESIS  
RELATED RISK FACTORS  
IN SEVERE PRE-ECLAMPSIA

FACULTY OF MEDICINE,  
INSTITUTE OF CLINICAL MEDICINE, DEPARTMENT OF OBSTETRICS AND GYNECOLOGY,  
INSTITUTE OF DIAGNOSTICS, DEPARTMENT OF CLINICAL CHEMISTRY,  
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*JOUKO JÄRVENPÄÄ*

**PLACENTAL ANGIOGENESIS AND  
ANGIOGENESIS RELATED RISK  
FACTORS IN SEVERE PRE-ECLAMPSIA**

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of the Faculty of Medicine of the University of Oulu, for  
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Supervised by  
Professor Markku Ryyänen  
Docent Eeva-Riitta Savolainen

Reviewed by  
Docent Risto Kaaja  
Docent Hannele Laivuori

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## **Järvenpää, Jouko, Placental angiogenesis and angiogenesis related risk factors in severe pre-eclampsia**

Faculty of Medicine, Institute of Clinical Medicine, Department of Obstetrics and Gynecology, Institute of Diagnostics, Department of Clinical chemistry, Biocenter Oulu, University of Oulu, P.O. Box 5000, FI-90014 University of Oulu, Finland

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### ***Abstract***

The incidence of pre-eclampsia (PE) is 2–7% in different populations and in the worst cases PE may threaten the survival of both mother and newborn; its pathogenesis is not resolved. Field literature today considers PE an angiogenic disorder. Coordinated vascularization is essential for placental development.

We wanted to find novel factors in the etiology of PE, and focused our attention on angiogenesis, inherited thrombophilia and folate-homocysteine metabolism. Homocysteine inhibits endothelial cell proliferation, which is closely related to angiogenesis. We performed gene expression profiling of placental tissue using microarray chips, studied the prevalence of factor V Leiden (FVL), prothrombin (F5) G20210A and methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism in patients with severe pregnancy complications and normal controls, compared the expression of the placental adiponectin, leptin and their receptor genes and the relationship of each to trophoblast apoptosis and further, studied the effect of folic acid fortified mineral water on plasma homocysteine concentration during pregnancy.

Gene expression profiling revealed downregulation of nine and upregulation of four genes. Interestingly, in one PE patient with cord compression during delivery the profile resembled that observed in normals. The expression level of the leptin and the adiponectin receptor 1 (ADIPOR1) genes was significantly higher in PE. No other significant expression changes were observed. The rate of apoptosis was higher in patients with PE. The FVL prevalence was 9.5%, in PE cases and 1.8% in the controls; a difference of 7.7%, (95% CI 2.0–13.4%). No statistical difference was found in other polymorphisms. Maternal serum folate concentration increased in our intervention group, but decreased in the control group ( $p < 0.05$ ). The plasma homocysteine concentrations decreased more in the intervention group ( $p < 0.001$ ).

The expression of angiogenesis-related placental genes can be altered in PE and cord compression cases. The activity of adipocytokine genes in PE may mean that they have a role in placental angiogenesis and apoptosis. Women with FVL may have an increased risk of PE. Fortified mineral water will help us to ensure that especially pregnant women achieve adequate folate intake.

**Keywords:** adipocytokines, angiogenesis, apoptosis, food fortification, genes, homocysteine, placenta, pre-eclampsia, thrombophilia



*To my mother Helvi,  
to my grandmothers Hilma and Inni,  
to my family*



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Oulu, August 2008

Jouko Järvenpää

## Abbreviations

AC	abdominal circumference
ACA	anticardiolipin antibody
ADIPOR1	adiponectin receptor 1
ADIPOR2	adiponectin receptor 2
ANG1	angiopoietin 1
ANG2	angiopoietin 2
ANG4	angiopoietin 4
ANPEP	alanyl aminopeptidase
ADPN	adiponectin gene
ATR	atrium
AUC	area under the curve
BAECs	bovine aortic endothelial cells
Bm	biparietal diameter
BMI	body mass index
CAM	chorioallantoic membrane
cDNA	complementary deoxyribonucleic acid
CER	cerebellum
CI	confidence interval
CM	cisterna magna
COL18A1	collagen, type XVIII, alpha1
cRNA	complementary ribonucleic acid
ECGF1	endothelial cell growth factor 1 (platelet derived)
EPAS1	endothelial PAS domain protein 1
F2	coagulation factor II (thrombin)
FL	femur length
FLT1	fms-related-tyrosine kinase
F5	coagulation factor V
FVL	factor V Leiden
GLM	general linear model
HC	head circumference
HELLP	hemolysis, elevated liver enzymes and low platelet count
HIF1A	hypoxia-inducible factor 1, alpha subunit
HIF2A	hypoxia-inducible factor 2, alpha subunit
HMEC-1	human microvessel endothelial cell-1
IUGR	intrauterine growth restriction

JAG1	jagged 1
KDR/FLK1	vascular endothelial growth factor 2, kinase insert domain receptor
LEPRA	leptin receptor A
LEPRB	leptin receptor B
LEPRC	leptin receptor C
LEPRD	leptin receptor D
LEP	leptin gene
MTHFR	5,10-methylenetetrahydrofolate reductase
NO	nitric oxide
ODBP	observed difference between prevalences
OR	odds ratio
PALLD	palladin, cytoskeletal associated protein
PCR	polymerase chain reaction
PDGFA	platelet derived growth factor alpha polypeptide
PE	pre-eclampsia
PI	pulsatility index
PLGF	placenta growth factor
ROS	V-ROS avian UR2 sarcoma virus oncogene homolog 1; ROS 1
SERPINI2	serineprotease inhibitor, grade F, member 1
sFLT1	soluble- fms-like tyrosine kinase 1
SIGLE10	immunoglobulin superfamily, number 10
siRNA	small interfering RNA (silencing RNA)
src	V-SRC-avian sarcoma (Smidt- Rubbin A2) viral oncogene; SRC
STBMs	syncytiotrophoblast membrane microparticles
TF	tissue factor
TIE1	protein receptor tyrosine kinaseTie 1 (human)
TIE2	protein receptor tyrosine kinase, endothelial specific Tie 2 (human)
TNFSF12	tumor necrosis factor ligand superfamily, member 12
VEGF	vascular endothelial growth factor
VTE	venous thromboembolism

## List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals

- I Järvenpää J, Päckilä M, Savolainen ER, Perheentupa A, Järvelä I & Ryyänen M (2006) Evaluation of factor V Leiden, prothrombin and methylenetetrahydrofolate reductase gene mutations in patients with severe pregnancy complications in northern Finland. *Gynecol Obstet Invest* 62: 28-32.
- II Järvenpää J, Schwab U, Lappalainen T, Päckilä M, Niskanen L, Punnonen K & Ryyänen M (2007) Fortified mineral water improves folate status and decreases plasma homocysteine concentration in pregnant women. *J Perinat Med* 35: 108-114.
- III Järvenpää J, Vuoristo JT, Savolainen ER, Ukkola O, Vaskivuo T, Ryyänen M (2007) Altered expression of angiogenesis-related placental genes in pre-eclampsia associated with intrauterine growth restriction. *Gynecol Endocrinol* 23: 351-315.
- IV Järvenpää J, Vuoristo JT, Ukkola O, Hirvikoski P, Savolainen E-R, Raudaskoski T & Ryyänen M (2008) Cord compression may rapidly influence the expression of placental angiogenic genes in pre-eclampsia. *Placenta* 29: 436–438.
- V Järvenpää J, Vuoristo JT, Santaniemi M, Ukkola O, Savolainen E-R, Tapanainen J, Jääskeläinen M, Kesäniemi A & Ryyänen M (2008) Adiponectin induced placental cell apoptosis could be mediated via ADIPOR1- receptor in pre- eclampsia with IUGR. Manuscript.



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# 1 Introduction

Pre-eclampsia (PE) is a syndrome characterized by hypertension and proteinuria. It is a relatively common pregnancy disorder that originates in the placenta. The reported incidence varies between 2–7 in different populations (Roberts & Cooper 2001, Zhong *et al.* 2001). PE causes variable maternal and fetal problems. The neonates delivered to mothers with PE are at higher risk of being small for gestational age (SGA) than those delivered to women with normal pregnancies (Odegard *et al.* 2000, Xiao *et al.* 2003). In the worst cases PE may threaten the survival of both mother and newborn (Redman & Sargent 2005). The pathogenesis is still far from being resolved. Field literature today considers PE as an angiogenic disorder. Mechanisms of the disease implicated in this syndrome include uteroplacental ischemia and endothelial dysfunction, increased trophoblast deportation with apoptosis/necrosis, oxidative stress and exaggerated systemic inflammatory response (Table1).

**Table 1. Mechanisms of the disease implicated in PE.**

Mechanism	References
Uteroplacental ischemia	Redman & Sargent 2005, Roberts & Gammill 2005, Sibai <i>et al.</i> 2005, Sheppard & Bonnar 1981
Endothelial cell dysfunction	Fisher 2004, Kaufmann <i>et al.</i> 2003, Pijnenborg <i>et al.</i> 1991
Increased trophoblast deportation	Johansen <i>et al.</i> 1999, Redman & Sargent 2000, Sargent <i>et al.</i> 2003
Apoptosis/necrosis	Huppertz <i>et al.</i> 2003, Mor <i>et al.</i> 1998, Neale <i>et al.</i> 2003
Oxidative stress	Bowen <i>et al.</i> 2001, Burton & Jauniaux. 2004, Chamy <i>et al.</i> 2006, Chappell <i>et al.</i> 2002, Hubel 1999, Roberts & Gammill 2005, Rogers <i>et al.</i> 2006, Sharma <i>et al.</i> 2006, Walsh 1998, Wiktor <i>et al.</i> 2004
Exaggerated systemic inflammatory response	Gervasi <i>et al.</i> 2001, Redman <i>et al.</i> 1999, Sacks <i>et al.</i> 1998

The risk factors observed in the patients with PE include increased insulin resistance, hyperlipidemia, excess thrombin generation and thrombophilia, and widespread endothelial damage/dysfunction resulting in multiple organ damage (Table2).

**Table 2. The risk factors observed in the patients with PE.**

Risk factor	References
Insulin resistance	Kaaja <i>et al.</i> 1999, Seely & Solomon 2003, Thadhani <i>et al.</i> 2004, Wolf <i>et al.</i> 2002
Hyperlipidemia	Clausen <i>et al.</i> 2001, Enquobahrie <i>et al.</i> 2004, Hubel <i>et al.</i> 1998
Excess thrombin generation and thrombophilia	Cadroy <i>et al.</i> 1993, Chaiworapongsa <i>et al.</i> 2002b, Hayashi <i>et al.</i> 2002, Weiner <i>et al.</i> 1985
Widespread endothelial damage/dysfunction resulting in multiple organ damage.	Chaiworapongsa <i>et al.</i> 2002a, Friedman <i>et al.</i> 1995, Roberts <i>et al.</i> 1991

Coordinated vascularization of the placenta is essential for proper placental development and involves the processes of vasculogenesis (new blood vessel formation) and angiogenesis (growth of blood vessels) (Cross *et al.* 1994). Normal placentation is thought to involve a two-stage process including the formation of a branching network of vessels within the chorionic villi of fetal origin, followed later by modification of the existing vascular network and transformation from a high resistance relatively hypoxic vascular bed into a low resistance circuit with increased oxygen tension (Cross *et al.* 1994, Kaufmann *et al.* 2004, Leiser *et al.* 1985). A certain amount of hypoxia is needed to stimulate placental blood vessel formation but continued hypoxia or insufficient uteroplacental oxygenation is believed to be responsible for the molecular events leading to the clinical manifestations of PE (Soleymanlou *et al.* 2005a)

In normal pregnancies maternal decidual arteries undergo an extreme vasodilation to increase blood flow in the intervillous space (Craven *et al.* 1998). Later on, placental cytotrophoblasts invade the arterial walls of the endo- and myometrium and undergo transformation of their phenotype to acquire endothelial cell characteristics, which is critical to endovascular invasion (Zhou *et al.* 1997). The normal endovascular invasion of trophoblast cells and spiral artery remodeling are fundamental to normal vasculature development and the functioning of the placenta.

Our interest was to try to find novel factors in the etiology and development of PE. We particularly focused our attention on angiogenesis, inherited thrombophilia and folate metabolism. From that point of view we studied the prevalence of three heritable thrombophilic factors: factor V Leiden (FVL), prothrombin G20210A and 5,10-methylenetetrahydrofolate reductase (MTHFR C677T) polymorphism in patients with severe pregnancy complications: severe PE, stillbirth, IUGR, placental abruption and fetal death, and in normal controls.

In an attempt to identify novel secreted factors playing a pathological role in PE, we performed gene expression profiling of placental tissue from women with and without PE using micro array chips. We compared the expression differences of the adiponectin, and adiponectin receptor genes, and leptin and leptin receptor genes in the placenta between patients with severe PE and normal controls. We also wanted to study the possible relationship of adiponectin and leptin to placental cell apoptosis, and we furthermore wanted to study the effect of a new nutritional principle, fortified mineral water, on serum and erythrocyte folate concentrations, serum vitamin B12 concentrations and plasma homocysteine concentrations during pregnancy.



## 2 Review of literature

### 2.1 Definitions and Classification of PE

The definition of PE includes hypertension, proteinuria and certain signs and symptoms such as headache, visual disturbances, epigastric pain, and pulmonary edema. Hypertension in PE in the previously normotensive patient is defined as a systolic blood pressure greater than 140 mmHg and diastolic blood pressure greater than 90 mmHg on two successive measurements 4–6 hours apart, and daily proteinuria over 0.3 g. Despite these concrete criteria, hypertension or proteinuria may be absent in women that develop the most serious complications, such as hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome and eclampsia (Weinstein 2005). Diagnostic criteria for severe PE include at least one of the following as classified in Table 3. (Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy 2000, ACOG Committee on Practice Bulletins-Obstetrics 2002)

**Table 3. Diagnosis of severe pre-eclampsia.**

---

Severe pre-eclampsia is diagnosed by the presence of one or more of the following
A systolic pressure higher than 160 mmHg or diastolic pressure of 110 or higher on two occasions six or more hours apart in a pregnant woman who is on bed rest
Proteinuria, with excretion of 5 g or more of protein in a 24- hour urine specimen or 3+ or greater on two random samples collected four or more hours apart
Oliguria, with excretion of less than 500 ml of urine in 24 hours
Pulmonary edema or cyanosis
Impaired liver function
Visual or cerebral disturbances
Pain in the epigastric area or right upper quadrant
Decreased platelet count
Intrauterine growth restriction

---

Eclampsia is a serious complication of pregnancy and is characterised by convulsions. Usually eclampsia occurs after onset of PE though sometimes no pre-eclamptic symptoms are recognisable. The convulsions may appear before, during or after labour, though cases of eclampsia after just 20 weeks of pregnancy have been recorded. HELLP syndrome is a life-threatening obstetric complication considered by many to be a variant of PE (Martin *et al.* 1999, Weinstein 1982,

Weinstein 2005). Both conditions occur during the later stages of pregnancy, or sometimes after childbirth.

## **2.2 Mechanisms of PE**

### **2.2.1 Placental factors and endothelial dysfunction**

PE can be expressed in pregnancies without fetus as is the case in hydatiform mole. The etiology of PE still remains unknown, but maternal endothelial dysfunction and shallow placentation are hallmarks of the disease (Roberts & Lain 2002). Reduced placental perfusion is an essential factor. In normal pregnancies maternal decidual arteries undergo vasodilation to increase blood flow in the intervillous space (Craven *et al.* 1998). Later on, placental cytotrophoblasts invade the uterus and undergo transformation of their phenotype to acquire endothelial cell characteristics, which could be critical to endovascular invasion (Zhou *et al.* 1997). The normal endovascular invasion of trophoblast cells and spiral artery remodeling are impaired in PE. Thus, increased vascular resistance and reduced placental perfusion ensue, which are important early features of PE (Roberts 1998). The primary event could be a failure of trophoblasts to obtain an endothelial phenotype (Zhou *et al.* 1997). On the other hand reduced placental perfusion can also be a consequence of excessive placental size as in multiple pregnancies and in diabetes (Gifford *et al.* 1990). The risk of PE is increased also in association with some chromosomal abnormalities in the fetus, such as trisomy 13 and triploidy (Rijhsinghani *et al.* 1997, Tuohy & James 1992).

Endothelial dysfunction has been considered an essential phenomenon in the pathophysiology of PE (Roberts 1998). The circulating factor secreted by the placenta and the cause of the widespread endothelial dysfunction in PE have not yet been identified. Several candidates have been suggested, including homocysteine, tumor necrosis factor alpha (TNF- $\alpha$ ), soluble Fas ligand, anti-phospholipid antibodies, and oxidized lipid products, but none of these have been confirmed unequivocally in subsequent work (Page *et al.* 2000, Roberts & Cooper 2001). Although manifestations of PE are diverse and heterogenous, a limited number of critical pathological events are required for the development of the disease. Pang and Xing found that apoptosis related genes were more highly expressed in PE placentas than in controls (Pang & Xing 2004c).

Placental soluble fms-like tyrosine kinase 1 (sFlt1), also named vascular endothelial growth factor receptor-1 (VEGF-R1), has been proposed as a possible circulating endothelial damaging factor in PE (Maynard *et al.* 2003). sFlt1 mRNA is upregulated in PE placentas, possibly due to placental hypoxia (Maynard *et al.* 2003). Placental cytotrophoblast sFlt1 secretion and sFlt1 concentrations in amniotic fluid are elevated in PE (Vuorela *et al.* 2000, Zhou *et al.* 2002). sFlt1 acts as a potent VEGF and placental growth factor (PLGF) antagonist by binding VEGF and PLGF molecules, thereby reducing free circulating concentrations of VEGF and PLGF. Maynard and other groups have demonstrated elevated concentrations of sFlt1 in maternal serum in pregnancies complicated by PE compared to control pregnancies. (He *et al.* 1999, Maynard *et al.* 2003, Shibuya 2001) It has been suggested that circulating sFlt1 contributes to the pathogenesis of PE by binding circulating VEGF and PLGF, thereby opposing physiological vasorelaxation and contributing to the development of hypertension. Maynard also demonstrated reduced free VEGF and PLGF concentrations in maternal serum in PE. (Maynard *et al.* 2003)

### **2.2.2 Maternal factors**

Only one third of newborns of pre-eclamptic mothers are growth-retarded (Eskenazi *et al.* 1993). On the other hand, pre-eclamptic-like changes in the placenta are also seen in pregnancies complicated with intrauterine growth restriction without PE (Khong *et al.* 1986). Thus placental factors alone cannot account for the disease. Many factors, e.g. dyslipidemia in diabetes and renal changes in hypertension could have direct effects on endothelial cells (Table 2). Hypertensive pregnant women have hyperlipidemic sera, hyperinsulinemia and increased insulin resistance, with their ensuing influence on endothelium (Endresen *et al.* 1992, Kaaja *et al.* 1999)

**Table 4. Maternal factors predisposing to pre-eclampsia.**

Factor	Odds ratio (95 % confidence interval) or relative risk	References
Family history of pre-eclampsia	2- to 5- fold	Chesley & Cooper 1986
Collagen vascular disease	3- 4- fold	Kaaja <i>et al.</i> 1990
Migraine	2.4 (1.4–4.2)	Marcoux <i>et al.</i> 1992
Gestational diabetes mellitus	3.11 (1.61–6.00)	Ros <i>et al.</i> 1998
Insulin-dependent diabetes mellitus	5.58 (2.72–11.43)	Ros <i>et al.</i> 1998
Hyperthyroidism (uncontrolled)	4.7 (1.1–19.7)	Millar <i>et al.</i> 1994
Chronic hypertension	9- fold	Rey & Couturier 1994
Primiparity	3.8 (2.8–5.2)	Mittendorf <i>et al.</i> 1996
Elevated systolic blood pressure (during early pregnancy)	1.7 (1.3–2.2)	Sibai <i>et al.</i> 1997
Elevated diastolic blood pressure (during early pregnancy)	1.7 (1.3–2.2.)	Sibai <i>et al.</i> 1997
Polycystic ovary syndrome	5- 6- fold	de Vries <i>et al.</i> 1998
Renal disease	7.2 (4.2– 12.5)	Fink <i>et al.</i> 1998
Obesity	5.2 (2.4– 11.5)	Ros <i>et al.</i> 1998
Maternal low birth weight	5.2. (1.2– 21.5)	Innes <i>et al.</i> 1999
Maternal preterm birth	3.6 (1.3– 10.3)	Innes <i>et al.</i> 1999

### 2.2.3 Familial tendency

A familial tendency has been well established (Arngrimsson *et al.* 1990, Dawson *et al.* 2002, Skjaerven *et al.* 2005). In their classic study Chesley and Cooper found PE in 26% of the daughters and 16% in granddaughters of pre-eclamptic mothers (Chesley & Cooper 1986). PE is a heterogenous condition, which suggests that many common alleles may act as susceptibility genes. Major dominant gene models with reduced penetrance or multifactorial inheritance have been considered as the best working hypotheses (Arngrimsson 2005). However, the exact mode of inheritance and the interactions between maternal and fetal genotype have not been elucidated (Bernard & Giguere 2003).

Laivuori performed a genomewide scan in 15 multiplex families recruited predominantly in the Kainuu province in central eastern Finland. They found two loci that exceeded the threshold for significant linkage: chromosome 2p25, and 9p13. These loci may harbor susceptibility genes for PE. They hypothesized that, in a founder population, the genetic background of PE might also show reduced heterogeneity. (Laivuori *et al.* 2003)

#### **2.2.4 Graft versus host reaction – inflammation**

Redman and his group suggest that PE is an ultimate end of normal host versus graft reaction which occurs in pregnancy. In his opinion during pregnancy host versus graft reaction causes an inflammation process where cellular microparticles are ubiquitously shed from cell membranes or secreted as endocytic vesicles called exosomes.(Redman *et al.* 1999) Shed cellular microparticles are  $\geq 100\text{nm}$  in size and are generated during apoptosis or necrosis. In contrast, secreted exosomes are smaller ( $< 100\text{nm}$ ), express more limited protein content and are released from late endosomes. In PE the number of cellular microparticles increases and includes not only particles derived from platelets, endothelium and various leukocytes but also from syncytiotrophoblast-derived microparticles. Syncytiotrophoblast membrane microparticles (often called STBMs) interact with both immune and endothelial cells. Thus they may contribute to the systemic inflammatory response of both normal and pre-eclamptic pregnancies. Moreover, trophoblast-derived exosomes may contribute to the downregulation of T cell activity that has been repeatedly observed during pregnancy. Deletion of activated T cells which express Fas-ligand by Fas-expressing exosomes derived from trophoblasts may contribute to the immunoregulation necessary for normal pregnancy.(Redman & Sargent 2005) On the other hand, trophoblasts have been rarely found in the peripheral circulation and no correlation has been found between trophoblast numbers and either the severity of PE, the extent of placental pathology or the inhibitory effect of uterine and peripheral vein plasma on endothelial growth *in vitro*. Thus, it is speculated that increased trophoblast deportation in PE is secondary to the structural and functional changes occurring in the placenta, rather than directly linked with the circulating endothelial cell damaging factor in PE.(Johansen *et al.* 1999.)

#### **2.2.5 Apoptosis**

Apoptosis is an active component of normal placental development; it is a critical step during villi formation. Apoptosis removes aging trophoblast cells, which are thereafter replaced by new cell population (Mayhew 2001). Trophoblast cell apoptosis increases towards term in normal pregnancy. Pang and Xing and other scientists have found that expression of apoptosis related genes were more highly expressed in PE placentas than in controls, possibly leading to defective

trophoblast invasion and subsequently lowered placental function. (DiFederico *et al.* 1999, Mayhew 2001, Pang & Xing 2004b, Reister *et al.* 2001.)

DNA-cleavage is a hallmark for apoptosis and the assays which measure the prelytic DNA fragmentation are used for the determination of apoptotic cell death. The methods used to assess DNA strand breaks are based on labelling/ staining the cellular DNA. The labelled/ stained DNA is subsequently analyzed by flow cytometry, fluorescence microscopy or light microscopy. In addition, individual cell death may be studied by assays that measure alterations in plasma membranes e.g. asymmetry, permeability.

### **2.2.6 Placental angiogenesis**

Redman points to the important factor, angiogenesis, in the etiology of PE (Redman *et al.* 1999, Redman & Sargent 2005). The key mechanism in PE can be defective placentation and the major players are the angiogenic genes, and their receptors and antagonists in the placenta (Zhou *et al.* 2002, Zhou *et al.* 2003, Zhou *et al.* 2003). The first step in the formation of the placenta is decidualization. This is followed by extensive angiogenesis, which is an essential step in the maturation of new blood vessels in pregnancy. In normal placentation the maternal spiral arteries invade the decidua and myometrium with the assistance of fetal cytotrophoblasts. During this invasion procedure, trophoblast cells transform from an epithelial phenotype to an endothelial phenotype. This process is called pseudovasculogenesis.(Damsky & Fisher 1998).+ The next step is transformation of the maternal spiral arterioles from small caliber resistance vessels to large caliber capacitance vessels allowing adequate maternal blood flow to the placenta for the assistance of migrating trophoblasts.

## **2.3 Placental angiogenesis and oxygen tension**

Early placental development occurs in a hypoxic environment. Oxygenation increases with the development of placental vasculature; when this process fails the hypoxic state continues. This continued hypoxic state leads to failure of the fetal trophoblasts to properly invade the maternal myometrium and spiral arterioles. (Brosens *et al.* 1972) The cardinal pathologic feature noted in the preeclamptic placenta is perturbation of trophoblast invasion into the myometrium and a reduction in the remodeling of the endomyometrial

vasculature from high to low resistance vessels (Figure 1) (Brosens *et al.* 1972, Luttun & Carmeliet 2003).

### **2.3.1 VEGF and its receptors**

VEGF and PLGF constitute a family of regulatory peptides capable of controlling blood vessel formation and permeability by interacting with two endothelial tyrosine kinase receptors, FMS-like tyrosin kinase (FLT1) and vascular endothelial growth factor receptor 2 (FLK1) (Shibuya 2001). The expression of VEGF is upregulated by hypoxia. In angiogenesis VEGF is a major contributor in the process where large caliber capacitance vessels are formatted and migrate. VEGF is also a stimulator of endothelial cells. Nitric oxide (NO) is considered to be an important contributor to the angiogenic response because inhibition of NO significantly reduces the effects of angiogenic growth factors.

### **2.3.2 FMS- like tyrosin kinase (oncogene FLT)**

Oncogene FLT1 belongs to the avian sarcoma virus (src) gene family. Like other members of this family, it shows tyrosine protein kinase activity that is important for the control of cell proliferation and differentiation (Fong *et al.* 1995, Satoh *et al.* 1987). FLT1 acts as a potent VEGF and PLGF antagonist by binding VEGF and PLGF molecules, thereby reducing free circulating concentrations of VEGF and PLGF. It has been suggested that circulating FLT1 may contribute to the pathogenesis of PE by binding circulating VEGF and PLGF, thereby opposing physiological vasodilatation and contributing to the development of hypertension (Figure 1) (Maynard *et al.* 2003).

Maynard has demonstrated elevated concentrations of FLT1 in pre-eclamptic maternal serum compared with control pregnancies and up regulated FLT1 in placentas of pre-eclamptic mothers (Maynard *et al.* 2003). The excess of FLT1 gene product, sFLT1, in early pregnancy possibly inhibits angiogenesis, contributing with other factors to placental insufficiency. The placental cytotrophoblast secretion of sFLT1 and sFLT1 concentration in amniotic fluid are elevated in pre-eclampsia (Vuorela *et al.* 2000). On the other hand, an elevated maternal plasma but not amniotic fluid concentration of sFlt-1 at the time of mid-trimester genetic amniocentesis has been found to be a risk factor for pre-eclampsia (Park *et al.* 2005).

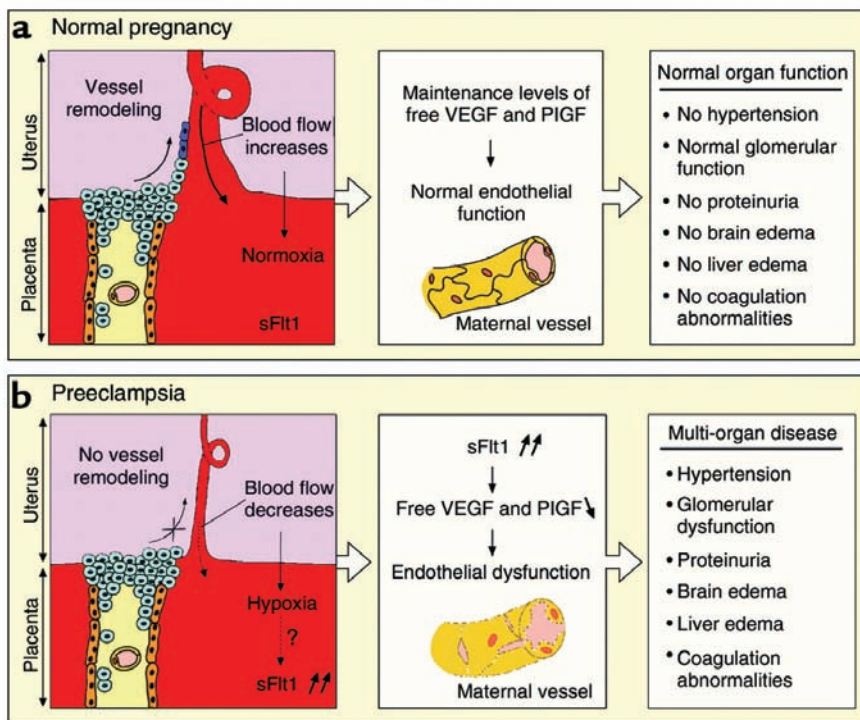


Fig. 1. sFLT, VEGF and PLGF in normal and hypoxic placentas. (Luttun & Carmeliet 2003).

### 2.3.3 Angiopoietins

The family of angiopoietins, especially angiopoietin 1 (ANG1) and angiopoietin 2 (ANG2), are required for the formation of mature blood vessels. The mouse knock out studies have demonstrated that Ang1 and Ang2 are protein growth factors which act by binding their receptors, Tie-1 and Tie-2. The cell signals are transmitted mostly by Tie-2; though some papers show physiological signaling via Tie-1 as well. (Thurstun 2003) These receptors are tyrosine kinases. Thus, they can initiate cell signaling when ligand binding causes a dimerization that initiates phosphorylation on key tyrosines. Comparing the actions of VEGF system with those of the angiopoietin system the former appears to play a key role in vessel sprouting and new vessel initiation, whereas angiopoietins have a role in the remodeling and maturation phases (Thurstun 2003).

### **2.3.4 Adiponectin and adiponectin receptors**

The adipose tissue, an important and perhaps the largest endocrine organ in the body, secretes adiponectin and other endocrine hormones and several angiogenic growth factors including vascular VEGF, TNF $\alpha$ , and leptin (Bouloumie *et al.* 1998, Claffey *et al.* 1992, Frater-Schroder *et al.* 1987, Sierra-Honigmann *et al.* 1998). The adiponectin gene (ADPN) and its receptors (ADIPOR1 and ADIPOR2) are members of the adipocytokine family, which play an important role in regulation of angiogenesis. Bråkenhielm found that adiponectin inhibits endothelial cell proliferation and migration, which means it is a negative regulator of angiogenesis (Brakenhielm *et al.* 2004). On the other hand adiponectin can function to stimulate angiogenesis in response to ischemic stress by promoting AMP-activated kinase signaling. Therefore, adiponectin may be useful in the treatment for obesity-related vascular deficiency diseases. (Shibata *et al.* 2004)

Adiponectin is also an important regulator of the sensitivity of tissues to insulin (Ukkola & Santaniemi 2002). It has become evident that also fetal growth is to a great extent controlled by the actions of insulin and insulin like growth factor (Yamauchi *et al.* 2001). Adiponectin levels are decreased in obesity, insulin-resistant diabetes, and coronary vascular disease (Guerre-Millo 2002).

The presence of adiponectin in the fetal tissue and circulation has been demonstrated (Corbetta *et al.* 2005). In addition, both ADPN and its receptors, ADIPOR1 and ADIPOR2, are abundantly expressed in the placenta, suggesting that adiponectin may have a physiological function during pregnancy (Caminos *et al.* 2005, Yamauchi *et al.* 2003). Paradoxically, increased maternal plasma concentrations of adiponectin have been reported in PE (Ramsay *et al.* 2003). Haugen, using Northern blot analysis and real-time RT-PCR, could not detect any ADPN expression in placenta biopsies from the pre-eclamptic subjects or from normal controls (Haugen *et al.* 2006).

### **2.3.5 Leptin and leptin receptors**

Leptin stimulates angiogenesis resulting in maintaining energy homeostasis and its serum concentrations correlate highly with the amount of body fat (Considine *et al.* 1996, Mantzoros & Moschos 1998). Leptin may facilitate lipid release from the fat stores to maintain energy homeostasis (Sierra-Honigmann *et al.* 1998). Women have higher plasma leptin levels than men at any degree of adiposity (Saad *et al.* 1997). Insulin increases leptin secretion (Boden *et al.* 1997,

Kolaczynski *et al.* 1996, Malmstrom *et al.* 1996, Utriainen *et al.* 1996) and in insulin resistance syndrome independent of adiposity, plasma levels are high (Haffner & Miettinen 1997, Segal *et al.* 1996). During pregnancy the maternal serum leptin levels are elevated about two to three times compared to the non pregnant state and the concentrations are highest during the second trimester of pregnancy (Sattar *et al.* 1998, Sivan *et al.* 1998). This extra production of leptin could be derived from adipocytes but placental trophoblasts must also be taken into account as a source of leptin production (Masuzaki *et al.* 1997, Mise *et al.* 1998). In PE the serum leptin levels of the mother are higher and the LEP gene is upregulated in the placenta (Iwagaki *et al.* 2004). There are four known leptin receptors: LEPR<sub>A</sub>, LEPR<sub>B</sub>, LEPR<sub>C</sub> and LEPR<sub>D</sub> (Meller *et al.* 2006, Tartaglia *et al.* 1995). Hauge, using Northern blotting, detected Leptin mRNA expression in the placenta which was threefold higher in the PE than in the normal controls (Haugen *et al.* 2006). Meller *et al.* found in their study that compared with controls, significant increases in leptin mRNA expression were seen in placentas from pre-eclamptic patients on the maternal and fetal sides, and in placentas from chronic hypertensive mothers only on the fetal side (Meller *et al.* 2006).

### **2.3.6 Other angiogenesis related genes in the placenta**

There are few published reports on possible gene actions in the placentas of PE mothers; the knowledge of gene actions as presented in current literature is not well established, and some reasoning may be speculative (Table 1).

**Table 5. Placental angiogenetic related genes.**

Gene	Name	Entrez Gene ID	Function	Influence	References
ECGF1	Platelet derived endothelial growth factor	1890	Acts only in endothelial cells.	Vessel proliferation.	+ Hagiwara <i>et al.</i> 1991
JAG1	Jagged 1	843177	Signaling gene		+ Siekmann & Lawson 2007, Siekmann <i>et al.</i> 2008
PALLD	Palladin	23022	Signaling gene controlling cell shape, adhesion, and contraction		+ Bang <i>et al.</i> 2001
COL18A1	Collagen XVIII A1	80781	Cleavage product endostatin, which inhibits angiogenesis		- Sudhakar <i>et al.</i> 2003, van Wijngaarden <i>et al.</i> 2004
TNFSF12	Tumor necrosis factor ligand superfamily, member 12	8742	Induction of VEGF- production		+ Hayashi <i>et al.</i> 2006
VEGF	Vascular endothelial growth factor	30682	Blood vessel formation and permeability		+ Demir <i>et al.</i> 2007
ANPEP	Alanyl amino- peptidase	290	Local modulation of placental blood pressure		Sato 2004
PDGFA	Platelet derived growth factor, alpha polypeptide	5154	Stimulation of cell growth in mouse fibroblasts. Function in humans is unclear	?	
SERPIN I2	Serineprotease inhibitor, grade F, member 1	100009515	Cooperation with urokinase and tissue-type plasminogen leads to extra cellular matrix degeneration and trophoblast migration		+ Chelbi <i>et al.</i> 2006
EPAS1(HIF2A)	Endothelial PAS domain protein 1	2034	Important regulator of vascularization, perhaps involving the regulation of endothelial cell gene expression in response to hypoxia		+ Sood <i>et al.</i> 2006, Tian <i>et al.</i> 1997
FLT1	FM-like tyrosine kinase	2321	Receptor for VEGF, inhibitor of VEGF response.		- Kendall <i>et al.</i> 1996, Maynard <i>et al.</i> 2003
SIGLE10	Immunoglobulin superfamily, number 10	9308	Possible co-factor with sFLT1		-

Gene	Name	Entrez Gene ID	Function	Influence	References
ANG4	Angiopoietin 4	219033	Not clear	+?	Valenzuela <i>et al.</i> 1999)
F5	FV-deficiency	2153	sFit1- expression enhancing in early pregnancy	-	Lockwood <i>et al.</i> 2007)
F2	Prothrombin mutation	2147	sFit1- expression enhancing in early pregnancy	-	Lockwood <i>et al.</i> 2007)
MTHFR	Methylenetetrahydrofolate reductase	4524	Homocysteine production	-	Nagai <i>et al.</i> 2001
ADPN	Adiponectin	9370	Activation of apoptosis and inhibition of the cell cycle	-	Dieudonne <i>et al.</i> 2006
LEP	Leptin	3952	Growth signal involving a tyrosine kinase-dependent intracellular pathway	+	Park <i>et al.</i> 2001

### **2.3.7 Placental gene expression studies in PE**

Microarray analysis is a powerful tool to identify differentially expressed genes in specific tissues. The placenta has been the target tissue for transcriptional profiling in PE and thousands of genes can be assayed simultaneously. One of the most up-regulated genes has been the leptin gene. The augmented production of leptin has been suggested to be a consequence of reduced placental perfusion and hypoxia (Mise *et al.* 1998) but additional contributing factors to placental leptin expression are likely. Laivuori investigated the relationship between placental LEP expression, placental leptin protein concentration and maternal plasma leptin concentration among control pregnant women, women with PE and women with growth-restricted infants. Placental leptin expression, placental protein and maternal plasma concentration were higher in PE than in the controls but not in women with growth-restricted infants. Placental leptin expression and placental protein were higher in the preterm pre-eclamptic subjects, whereas maternal leptin was higher in the term PE subjects. This study suggests that despite similar failed placental bed vascular remodelling in PE and IUGR, LEP expression is higher only in preterm PE. (Laivuori *et al.* 2006) In microarray studies different expression of placental glycogen phosphorylase, acid phosphatase 5, calmodulin 2, v-rel reticuloendotheliosis viral oncogene homolog A, as well as cytokines associated, trophoblast-invasion associated, metabolism related and apoptosis related genes have been reported (Hansson *et al.* 2006, Pang & Xing. 2003a, Pang & Xing 2003b, Pang & Xing 2004a, Pang & Xing 2004b, Tsoi *et al.* 2003).

Microarray has great potential as a screening method but a critical attitude is needed when interpreting the results, which could be only partly overlapping if we use the same microarray platform again to find differentially expressed genes (Catherino & Segars 2003, Tsibris *et al.* 2002, Wang *et al.* 2003). Moreover, several results have been found to be different when a second microarray has been performed by the same researchers (Catherino & Segars 2003). Data analysis is lacking in retrospect and more specific methods like reverse transcriptase-polymerase chain reaction (RT-PCR) or real time RT-PCR are needed to verify the results.

Pooling of all samples from one group has been done in a number of studies (Pang & Xing 2003a, Pang & Xing 2003b, Pang & Xing 2004a, Pang & Xing 2004b, Reimer *et al.* 2002, Tsoi *et al.* 2003), to reduce costs, to compensate for low amounts of starting material or to reduce variation between individual

samples. Pooling RNA from different subjects results in permanent loss of information and has been shown to result in loss of sensitivity and an increase in false positive results (Chappell & Morgan 2006).

## **2.4 Angiogenesis related risk factors in PE**

Risk factors for PE include primiparity, extremes of age, molar pregnancy, previous personal or familial history, maternal obesity, insulin resistance, diabetes, hyperlipidemia, hypertension, kidney disease and thrombophilias which may be markers for underlying vascular disease (Duckitt & Harrington 2005, Said & Dekker 2003, Thadhani *et al.* 1999, Thadhani *et al.* 2004, Wolf *et al.* 2001, Wolf *et al.* 2002). PE can be expressed in pregnancies without fetus as is the case in hydatiform mole.

There are reports that in pregnancies associated with oocyte donation when the fetus is genetically totally different from the mother and when the man has fathered a pre-eclamptic pregnancy earlier, the risk of PE is increased (Lie *et al.* 1998, Soderstrom-Anttila *et al.* 1998). Barrier contraceptive use and donor insemination could predispose to PE, although contradicting data also exists on this point (Klonoff-Cohen *et al.* 1989, Smith *et al.* 1997). It is equally clear that the placental and maternal constitutional factors have an influence on the pathogenesis of PE (Roberts & Lain 1998). The risk of PE is increased also in some chromosomal abnormalities such as trisomies and triploidy (Rijhsinghani *et al.* 1997, Tuohy & James 1992).

### **2.4.1 Inherited thrombophilias**

The physiological changes of pregnancy produce a hypercoagulable state that increases the risk of venous thromboembolism (VTE) (Bremme 2003). Women with inherited or acquired thrombophilias are, during pregnancy, at particularly high risk of VTE, which may also occur in the placental vessels (Greer 2003). Fatal pulmonary embolism remains the most common cause of mortality among pregnant women in many Western countries (Greer 1999). Inherited thrombophilias are significant risk factors and combined with pregnancy they can lead to impaired angiogenesis, thrombosis formation and impaired function of the placenta (Brenner & Kupferminc 2003, Salafia *et al.* 2005).

Lockwood demonstrated that isolated decidual cells express sFLT-1 mRNA, suggesting that they can synthesize sFLT-1. Moreover, in first trimester decidual

cells, thrombin enhanced both sFLT-1 mRNA levels, as measured by quantitative RT-PCR, and levels of secreted sFLT-1 protein, as measured by enzyme-linked immunosorbent assay. The thrombin antagonist hirudin blocked this effect, demonstrating that active thrombin is required. Emphasizing the specificity of the thrombin response, neither interleukin-1beta nor TNF $\alpha$  affected sFLT-1 expression in the decidual cells. In contrast to first trimester decidual cells, thrombin did not affect sFLT-1 levels in cultured term decidual cells. In early pregnancy, thrombin may act as an autocrine/paracrine enhancer of sFLT-1 expression by decidual cells to promote PE by interfering with local vascular transformation. (Lockwood *et al.* 2007)

The main heritable thrombophilias nowadays comprise abnormalities of anticoagulant proteins- antithrombin, protein C and Protein S. Deficiency states of the natural anticoagulants: antithrombin, protein C and protein S, are relatively rare, accounting for around 10% of cases (Martinelli *et al.* 1998). Much more common and important are gene defects of factor V Leiden, prothrombin genes and hyperhomocysteinemia due to MTHFR C677T polymorphism. Thrombophilia is clearly associated with early-onset and severe PE, placental abruption and PE with IUGR (Jarvenpaa *et al.* 2006, Lin & August. 2005).

### *Factor V Leiden mutation*

Factor V Leiden mutation, a specific G to A substitution at nucleotide 1691 within the factor V gene, is considered one of the most important causes of genetic thrombophilias. Its mean prevalence is 2–7% in Caucasian populations (Walker 1997). The prevalence in Northern Finland is 3% (Pastinen *et al.* 2001). The mutation occurs in the factor V gene at the site where protein C acts. Therefore the protein C cannot be activated and as the consequence the factor V cannot be broken down, leading to the hypercoagulable effect (Figure 2). The mutation is inherited as dominant. It has been hypothesized that the mutation may have conferred an evolutionary survival advantage by reducing menstrual blood loss and postpartum hemorrhage (Clark 2003).

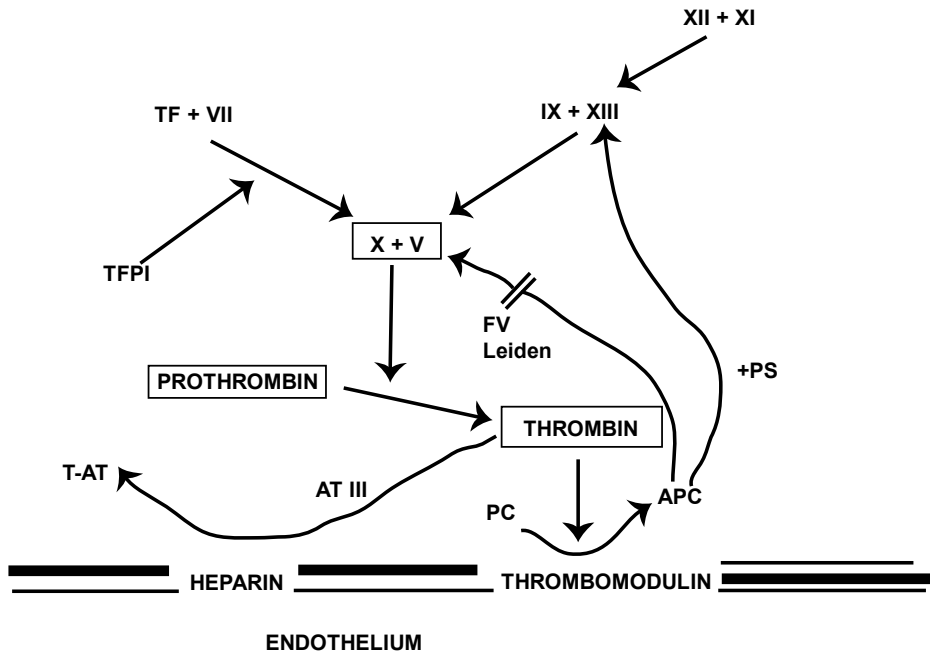


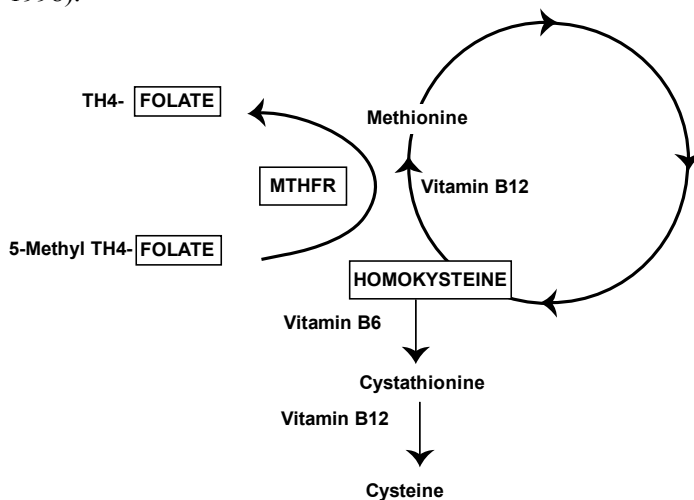
Fig. 2. The regulation of thrombin There are five factors regulating the production and effect of thrombin: 1) antithrombin III (ATIII) neutralizes thrombin and other enzymes in the clotting system, 2) protein C (PC) inactivates the clotting factors V and VIII, 3) protein S (PS) is a cofactor of protein C, 4)thrombomodulin (together with thrombin) activates protein C (APC) and 5) tissue factor pathway inhibitor (TFPI) inhibits the effect of clotting factor VII and tissue factor complex. T-AT= thrombine- antithrombine complex. (Kaaja *et al.*, Duodecim 1999; 115:1235–43).

### *Prothrombin G20210A mutation*

Second congenital thrombophilic factor is the mutation of the prothrombin gene leading to variant prothrombin G20210A, which is associated with elevated plasma prothrombin concentration (Figure 2) and a threefold risk of venous thrombosis (Poort *et al.* 1996). The prevalence of this mutation is about 2% in Western Europe and 1% in the general population in Northern Finland (Lockwood *et al.* 2007, Pastinen *et al.* 2001).

### MTHFR C677T polymorphism

MTHFR catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine (Figure 3). Individuals homozygous for the MTHFR C677T polymorphism had significantly elevated plasma homocysteine levels. Thus, the C677T polymorphism may represent an important genetic risk factor in vascular disease (Frosst *et al.* 1995). Alternatively, hyperhomocysteinemia may reflect abnormal methionine metabolism that affects the methylation of DNA and cell membranes (Blom 1998). Elevated homocysteine levels may result from low levels of folic acid, vitamin B<sub>6</sub>, or vitamin B<sub>12</sub> (Figure 3). Klerk *et al.* concluded in meta-analysis that impaired folate metabolism, resulting in high homocysteine levels, is causally related to increased risk of coronary heart disease (Klerk *et al.* 2002). The definitive cause is unknown, but high homocysteine levels have been found to cause direct endothelial injury by increasing oxidative stress, by lowering the production of nitric oxide (NO) and by accumulating the oxidation of low density lipoproteins in the endothelium (Rao *et al.* 1997, Selhub & D'Angelo 1998).



**Fig. 3. Homocysteine-methionine cycle.**

Homocysteine has been reported to inhibit endothelial cell proliferation, which is closely related to angiogenesis. However, the relationship between homocysteine and angiogenesis has been unknown. To clarify whether homocysteine would

inhibit angiogenesis in vitro and in vivo, Nagai examined the effect of homocysteine on tube formation by bovine aortic endothelial cells (BAECs) and by human microvessel endothelial cell-1 (HMEC-1) in vitro, and on angiogenesis in vivo using the chorioallantoic membrane (CAM) assay, as well as on BAEC proliferation and migration. Homocysteine, but not cysteine, inhibited BAEC proliferation, migration, and tube formation in a dose-dependent manner. It was suggested that homocysteine inhibited angiogenesis by preventing proliferation and migration of endothelial cells. (Nagai *et al.* 2001)

MTHFR gene C677T polymorphism heterozygosity may be of no clinical significance, but homozygosity or multiple mutations of that gene is followed by abnormal homocysteine metabolism leading to hyperhomocysteinemia and low folate status. Moreover, several genetic alterations in enzymes involved in homocysteine metabolism have been found (Boers *et al.* 1985, Boers *et al.* 1985, Engbersen *et al.* 1995, Frosst *et al.* 1995). About 10% of individuals in Western European populations are homozygous for this genetic variant (Greer 1999, McColl *et al.* 2000). The incidence of MTHFR C677T polymorphism in Finland is not known. Sohda showed that the homozygosity for C677T polymorphism was significantly associated with PE (Sohda *et al.* 1997). The Japanese study found no association but the authors hypothesized that the lack of association may be due to dietary folate intake in their population (Kobashi *et al.* 2000).

Homozygosity for the C677CT allele of the MTHFR gene is more prevalent in venous thromboembolism although there are conflicting reports (Zalavras *et al.* 2002). In meta-analysis of 22 case-control studies including 3387 white adult patients found a significant association between ischemic stroke and the C677T allele of the MTHFR gene (Casas *et al.* 2004). Hyperhomocysteinemia due to homozygosity for this variant predisposes to deep-vein thrombosis in the general population. It remains unclear whether hyperhomocysteinemia of different causes entails the same risk of thrombosis. Nevertheless, it is well known that vitamin supplementation lowers homocysteine concentrations in almost all subjects with hyperhomocysteinemia, regardless of the underlying cause.

#### **2.4.2 Acquired thrombophilias**

Antiphospholipid antibody (APA) syndrome is the most common acquired thrombophilia of pregnancy. It can be diagnosed when  $\beta_2$  glycoprotein 1 or immunoglobulin G or immunoglobulin M level are elevated, or when lupus anticoagulant is present (Lee *et al.* 2003). The most important antibodies

associated with thrombosis are anticardiolipin antibodies (ACA) and lupus anticoagulant (LA). The prevalence of those antibodies in the general obstetrics population is around 2%, nearly the same as in healthy young control subjects (Petri 2000). Other forms of acquired thrombophilia are uncommon or of unknown clinical significance. Other causes of acquired thrombophilia during pregnancy are ulcerative colitis, diabetes mellitus, nephritic syndrome and ovarian hyperstimulation. Ovarian stimulation for in vitro fertilization is associated with increased risk of thrombosis (Chan & Dixon 2008).



### **3 Aims of the study**

Our interest was to try to find novel factors in the etiology and development of PE. We particularly focused attention on angiogenesis, inherited thrombophilia and folate metabolism in order to find out new targets for research into PE prevention.

We wanted:

1. to identify novel secreted factors playing a pathological role in PE; we performed gene expression profiling of placental tissue from women with and without severe PE using Affymetrix U133 + 2.0 micro arrays.
2. to open discussion on why the placental gene expression profile of the angiogenic genes in a severely pre-eclamptic patient with cord compression was different from the others in the severely pre-eclamptic group.
3. to compare the expression of the adipocytokines: adiponectin, leptin, and their receptor genes in the placenta between patients with severe PE and normal controls and to get insights into their possible relationship to placental cell apoptosis.
4. to compare the prevalences of three important heritable thrombophilic, angiogenic risk factors, FV-Leiden, prothrombin G20210A mutations and MTHFR C677T polymorphism in patients with severe pregnancy complications, namely fetal death, placental abruption, severe PE and IUGR and in normal controls in Northern Finland.
5. to investigate the effect of a new nutritional principle, fortified mineral water, on serum and erythrocyte folate concentrations, on serum vitamin B12 concentrations, and on one endothelial risk factor, plasma homocysteine concentrations during pregnancy.



## 4 Subjects and Methods

### 4.1 Subjects

The study subjects were collected during the period 1999–2004 from Oulu University Hospital and from the city of Oulu. After recruiting the study patient, we always collected the next voluntary parturient with completely normal pregnancy outcome as the control. The case and control patients were all Finns. The criterion for severe PE was the presence of blood pressure over 160/110 mmHg with consistent proteinuria, or blood pressure over 140/90 with proteinuria over 5g per day. IUGR was defined as when the fetal weight was below the 10<sup>th</sup> percentile for gestational age and when occurring with PE it was considered as a sign of severe PE (Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy 2000). The definitions of placental abruption was diagnosed after delivery when over 1/3 of the placenta was abrupted, and stillbirth when the fetus died in uterus after 22 week of gestation.

We recorded maternal age, body mass index (BMI), parity, diabetes, pregnancy complications, and the delivery. From the newborn the birthweight, Apgar scores, and fetal umbilical artery blood acid base values were recorded.

**Table 6. Study population**

Study	N total	Cases	Controls
I	237	126	111
II	72	40	32
III	5	2	3
IV	4	1	3
V	30	14	16

### 4.2 Methods

#### 4.2.1 Placental biopsies (III,IV,V)

Placental biopsies were taken during caesarean section immediately after the delivery of the placenta about two centimeters from the naval insertion and immediately frozen in nitric oxide liquid. Only one biopsy was taken. The results of the microarray analyses were verified by RT-PCR from the RNA extracted

from the same biopsy. In studies III, IV and V, the method of delivery in all patients and controls was caesarean section. To eliminate labor-associated changes in placental gene expression, we used in studies III and V only placental samples obtained from the caesarean section performed before labour.

#### **4.2.2 Blood collection and DNA Analysis (I)**

We investigated the mutations in the study and control groups. Venous blood was drawn into EDTA-containing tubes and stored frozen at  $-20^{\circ}\text{C}$ , and DNA was isolated using standard methods. Factor V Leiden G1691A and prothrombin (F2) gene G20210A polymorphisms were analysed using the ThromboType<sup>®</sup> test and MTHFR C677T using the Geno Type<sup>®</sup> MTHFR test (Hain Lifescience GmbH, Germany) based on multiplex amplification with biotinylated primers and reverse hybridization.

#### **4.2.3 RNA isolation (III–IV)**

Frozen samples from the placentas were homogenized with a BD Medimachine automated disaggregation system (BD Biosciences, San Jose, CA, USA) in PAXGene processing tubes (Qiagen GmbH, Hilden, Germany). Total RNA was isolated with the PAXgene blood RNA kit (Qiagen GmbH, Hilden, Germany). The manufacturer's protocol was modified by extending the proteinase K treatment time to 35 minutes. The RNA integrity was ensured with the RNA Experion automated electrophoresis system (Bio-ad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

#### **4.2.4 Gene Chip protocol**

Experimental procedures for Gene Chip were performed according to the Affymetrix Gene Chip Expression Analysis Technical Manual. In essence, using 8  $\mu\text{g}$  of total RNA as a template, double-stranded DNA was synthesized by means of the One-cycle cDNA synthesis kit (Affymetrix) and T7-(dT)24 primer. The DNA was purified using the Gene Chip Sample Cleanup Module (Qiagen). In vitro transcription was performed to produce biotin labeled cRNA using an IVT labeling kit (Affymetrix) according to the manufacturer's instructions. Biotinylated cRNA was cleaned with a Gene Chip Sample Cleanup Module (Qiagen), fragmented to 35 to 200 nt, and hybridized to GeneChip Human

Genome U133 Plus 2.0 arrays which contain approximately 55000 human transcripts. After being washed, the array was stained with streptavidin-phycoerythrin (Molecular Probes). The staining signal was amplified using biotinylated anti-streptavidin (Vector Laboratories) and the second staining with streptavidin-phycoerythrin, and the array was then scanned on the Gene Chip Scanner 3000. The expression data was analyzed using the Affymetrix Gene Chip Operating System (Affymetrix) and dChip (Li & Wong 2001) software.

#### 4.2.5 Real-time PCR (III–V)

The microarray analysis results were verified using Taqman (III–IV) and Syber Green (V) real-time PCR for the FLT1, COL18A1, JAG1 (III–IV), and Leptin, ADIPOR1 and ADIPOR2 (V) genes (Table8). In studies III and IV, the first strand of cDNA was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase. The quantitative PCR reactions were performed with an ABI 7700 Sequence Detection System using TaqMan chemistry. The amplicons were detected using bifunctional fluorogenic probes. The results were normalized to 18S RNA quantified from the same samples. In study V, The first strand of cDNA was synthesized using DyNAmo cDNA synthesis kit (Finnzymes) following the manufacturer’s instructions. The quantitative PCR reactions were performed with a Stratagene MX3005P using DyNAmo Flash SYBR Green qPCR Kit (Finnzymes). The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Table 7. Real time PCR primers.**

Gene	Forward primers	Reverse primers and Taqman probes
FLT1	TCAGCTACTGGGACACCGG	CCTGAACTAGATCCTGTGAGAAGCA 5'-Fam- TCCTGCTGTGCGCGCTGCTC-Tamra-3'
COL18A1	CAGGTGAGCCCCATGCA	TCCGAATGCCCCATCTGA 5'-Fam- TGCCTGGACGAGGAAGGCGATG-Tamra-3'
JAG1	ACACACACTCAGCCTCTGAGGAC	GGGTTTTTGATCTGGTTCAGCT 5'-Fam-ACACCACCAACAACGTGCGGGA-Tamra-3'
18 S RNA	TGGTTGCAAAGCTGAAACTTAAAG	AGTCAAATTAAGCCGCAGG 5'-Vic-CCTGGTGGTGCCCTTCCGTCA-Tamra-3'
LEPT	ACACAAAACCCTCATCAAGAC	AGCTCTTAGAGAAGGCCAGCAC
ADIPOR1	CTTCTACTGCTCCCCACAGC	GACAAAGCCCTCAGCGATAG
ADIPOR2	GGCATGTCCCCTCTCTTGCA	TGTGTCCAAATGTTGCCTGT
GAPDH	TCCACCACCCTGTTGCTGTAG	GACCACAGTCCATGCCATCACT

#### **4.2.6 Apoptosis (V)**

Apoptosis was analysed from paraffin embedded tissue sections by using in situ DNA 3'-end labelling. The apoptosis detection kit (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, Chemicon international, inc. Temecula, California, USA) was used as previously described (Vaskivuo *et al.* 2000). Briefly, tissue sections were deparaffinized in xylene and hydrated through series of alcohols. Sections were then pre-treated with 20µg/ml proteinase K and endogenous peroxidase activity was quenched in 3% hydrogen peroxide in PBS-buffer. Samples were incubated in reaction buffer with terminaldeoxynucleotidyl transferase (TdT) enzyme for 1 hour at 37 °C. Color reaction was visualized with diaminobenzene (DAB) and counterstained with hematoxylin.

#### **4.2.7 Laboratory analyses (II)**

Venous blood samples were obtained after a 12-hour overnight fast. Laboratory samples for routine hematology (blood count, serum thyroid stimulating hormone, glutamyltransferase, creatinine, urate) were analyzed at the Clinical Chemistry Laboratory, Oulu University Hospital. Plasma glucose was analyzed at the Clinical Research Unit, University of Oulu, using commercial reagents (Granutest 100 Glucose, Merck, Germany) by the enzymatic photometric method with the Kone Pro Analyser (Kone Corporation, Finland).

Body weight was measured twice at each visit with a digital scale (Scale Seca 707, Vogel & Falke GmbH & Co, and Germany). Blood pressure was measured after ten minutes' rest in a sitting position with a mercury sphygmomanometer (Mercury 300, Speidel & Keller GmbH & Co, and Germany) three times on every visit. The mean values of the measurements were used in the analyses.

Plasma homocysteine was analyzed at the Department of Clinical Chemistry, Kuopio University Hospital by AXSYM Homocysteine FPIA (Abbott Laboratories, USA).

The other analyses were carried out at the Laboratory of Clinical Chemistry, Oulu University Hospital. Serum vitamin B12, folate and erythrocyte folate were analyzed by the automated chemiluminescence system (Advia Centaur, Bayer Corporation, New York, USA).

#### **4.2.8 Pregnancy monitoring (II)**

Because this fortified mineral water was a new nutritional principle, the pregnancies were monitored carefully. We performed ultrasound examinations in the 12th, 21st, 25th and 32nd weeks of gestation. The growth of the babies was monitored carefully including biparietal measurement (Bm), head circumference (HC), abdominal circumference (AC), femur length (FL), cerebellum diameter (CER), posterior fossa (CM) and the back horn of the lateral ventricle (ATR). 12<sup>th</sup> week examinations included dating and Doppler velocimetry of the uterine arteries, and later on we also included velocimetry of the umbilical and mean cerebral arteries and of the ductus venosus of the fetus. The pulsatility index (PI) was calculated from the Doppler measurements.

#### **4.2.9 Diet (II)**

The subjects were requested to stop or minimize their alcohol and tobacco consumption, and keep their physical activity and possible use of vitamins and nutrient supplements constant during the study. The diet was monitored by means of a 4-day food record during the run-in period and during the intervention period. The subjects recorded their dietary intake for four consecutive days, including one weekend day or the person's day off from work. Oral and written instructions regarding how to fill in the records were given to all subjects. At the study visits, the food records were checked by the nurse for completion of the data. The food records provided the data for calculations using the Micro-Nutrica® dietary analysis software (The Social Insurance Institution, Turku, Finland). The intake of folate, vitamins B6 and B12 was calculated from those records. Furthermore, intakes of energy, energy nutrients, cholesterol and dietary fiber were also calculated.

### **4.3 Statistical analysis (I,II,V)**

The clinical and laboratory data were downloaded into a computer and the results analysed using the Statistical Package for Social Science program (SPSS Inc., Chicago, Illinois, USA). Odds ratios (OR), observed difference between prevalences (ODBP) and 95% confidence intervals (CI) were calculated (study I).

The results in study II were given as group means and standard deviations. Normal distribution of variables was checked by the Kolmogorov-Smirnov test.

The equality of variances between the groups was tested with the Levene's test for homogeneity of variance. The general linear model (GLM) was used to test the changes in the continuous variables during the intervention. When the analysis indicated that the overall change in a variable with time or the time and group interaction was significant ( $p < 0.05$ ), further analysis was carried out by means of the t-test. For variables which were not normally distributed, the Mann-Whitney' U-test and the Friedman and Wilcoxon tests were used. Intakes of cholesterol and vitamin D, serum folate and vitamin B12 concentrations were logarithmically transformed for analyses. The sign test was used to compare smoking habits, alcohol consumption and physical activity between the groups. The area under the curve (AUC) was calculated from the Doppler velocimetry indices and the statistical analysis was carried out by means of the t-test as well as the analysis of the PCR and Apoptosis results in study V.

## 5 Results

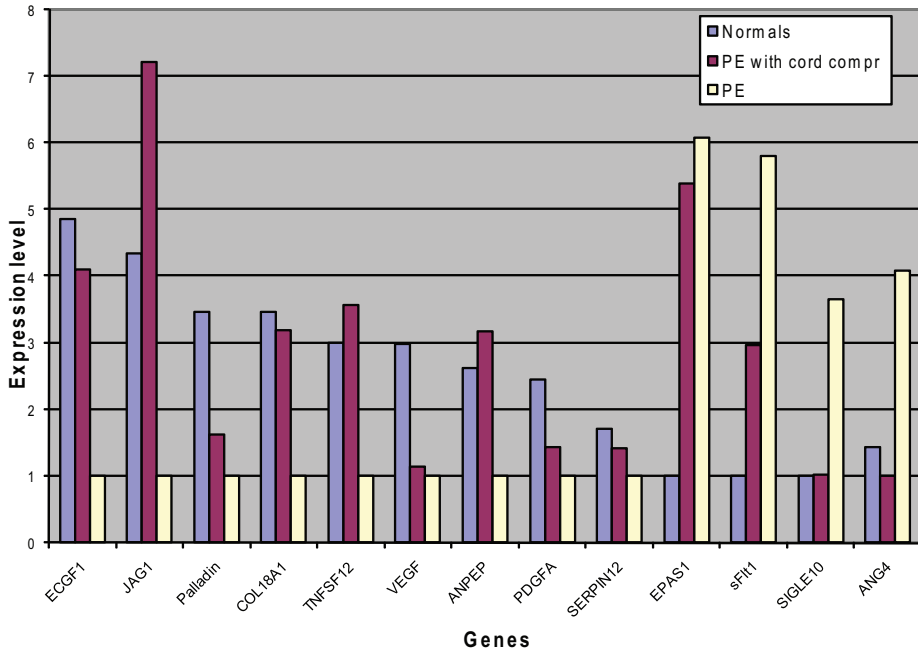
### 5.1 Placental angiogenesis and severe PE

Gene expression profiling revealed down regulation of nine angiogenesis related genes in the study group compared to the control group. On the other hand, the level of four genes was up regulated in the study group compared to the control group (Table3). Real-time RT-PCR is used to validate the observed trends seen in the micro array experiments. For this we chose 3 genes, JAG1, COL18A1 and FLT1, from the final gene list of 13 genes, and these genes had at least a 1.9-fold increase or decrease in expression in the PE patients compared to the controls. Thus all 3 genes chosen were validated successfully in the real time PCR experiments and were in accordance with the gene expression results. In the PE case with cord compression, the angiogenesis related gene expression profile more closely resembled that observed in normal placentas (Figure 4).

**Table 8. Changes between gene expression in normal and PE placentas.**

Gene	Average Signal intensity, controls	Average Signal intensity, PE	Fold change
ECGF1	216.93	44.75	-4.85
JAG1	659.92	152.46	-4.33 <sup>a</sup>
Palladin (PALLD)	1574.21	455.61	-3.46
COL18A1	1504.57	434.44	-3.46 <sup>a</sup>
TNFSF12	176.97	59.24	-2.99
VEGF	1296.39	436.37	-2.97
ANPEP	313.55	119.93	-2.61
PDGFA	2351.35	960.19	-2.45
SERPINI2	3705.6	1885.67	-1.97
EPAS1 (HIF2A)	109.74	667.42	6.08
FLT1	886.5	4275.94	4.82 <sup>a</sup>
SIGLE10	54.86	161.01	2.93
ANG4	82.27	195	2.37

<sup>a</sup> indicates verification by Real-time PCR. Average fold changes derived from Real-time PCR were -21.79, -5.96, and +1.65, respectively.



**Fig. 4. Relative expression levels of angiogenesis related genes. The lowest expression has been placed on level 1.**

## **5.2 Placental adipocytokines, apoptosis and PE**

The placental expression level of the leptin and ADIPOR1 genes was significantly higher in the pre-eclamptic mothers than in the controls. No significant changes were observed in the expression levels of the adiponectin, ADIPOR2, and four variants of leptin receptor genes. The expression level of the adiponectin was low in all of the studied samples. The real-time PCR results were in accordance with the microarray results. The rate of apoptosis was higher in the placentas of the patients with PE.

## **5.3 Thrombophilias and PE**

The prevalence of the Leiden mutation was 9.5%, in the study group and 1.8% in the control group. The odds ratio (OR) was 5.7 and the observed difference

between prevalences 7.7%, (95% CI 2.0 – 13.4%). No statistical difference was found in the prothrombin and MTHFR C677T polymorphism between the groups.

#### **5.4 Maternal plasma homocysteine concentration, folic acid, B6 and B12 vitamin supplementation during pregnancy**

At baseline, the mean serum and erythrocyte folate and serum vitamin B12 concentrations were similar. The mean folate intake was 274 µg/day in the controls and 255 µg/day in the intervention group. The intake did not differ significantly between the two groups. Women in the control group consumed more carbohydrates and energy while women in the intervention group consumed more saturated fatty acids. Serum folate concentration increased during the intervention period in the intervention group, but decreased in the control group ( $p < 0.05$ ). Erythrocyte folate concentrations increased more in the intervention than in the control group ( $p = 0.001$ ). There were no significant differences in serum vitamin B12 concentrations and also no differences in plasma homocysteine concentrations at baseline, and the homocysteine concentrations decreased more in the intervention group ( $p < 0.001$ )



## 6 Discussion

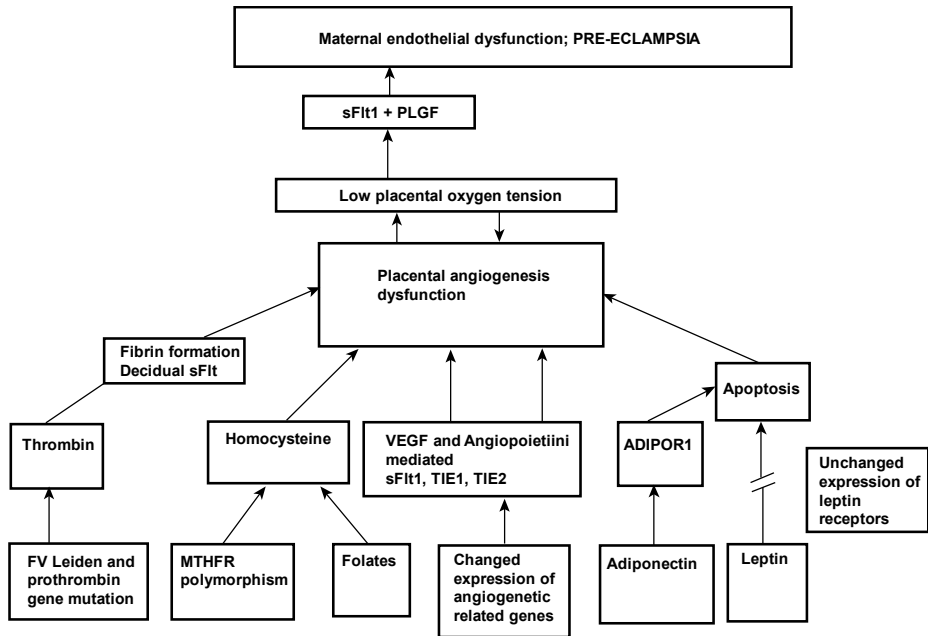
### 6.1 Placental angiogenesis

Gene expression profiling revealed down regulation of nine and up regulation of four angiogenesis related genes in the study group compared to the control group. Most of the genes known to increase angiogenesis were downregulated and two out of three genes decreasing angiogenesis were upregulated (Table 9). These results suggest that also in late PE pregnancy placental angiogenesis is deteriorated.

**Table 9. Upregulated and downregulated genes.**

Influence on angiogenesis	Upregulated genes	Downregulated genes	Total
Increase	2	6	8
Decrease	2	1	3
Unknown	0	2	2
Total	4	9	13

The vasculature of the feto-placental unit is highly angiogenic and impairment in angiogenesis can lead to placental insufficiency, followed by severe pregnancy complications (Nasu *et al.* 2003). There are few published reports on possible gene actions in the placentas of pre-eclamptic mothers; the knowledge of gene actions as presented in current literature is not well established, and some reasoning may be speculative. The basic changes in gene expressions and disturbance in the developing placental vessels could happen in very early pregnancy in decidual tissue followed by the low placental oxygen tension during the second trimester leading to PE later in pregnancy (Figure 5) (Lockwood *et al.* 2007).



**Fig. 5. Factors related to impaired angiogenesis in PE.**

We focused on the subjects with PE complicated by IUGR in the present study, The placental low oxygen tension has been suggested to play a major role in this form of PE. Hypoxia in second trimester results from insufficient adaptation of the spiral arteries in the decidual and intramyometrial portions of the endometrium. This hypoxic state may disrupt the angiogenic process and may well also be one reason for the development of PE and could create placental insufficiency. Hypoxia can lead to an increase in the expression of genes associated with or regulated by hypoxia as EPAS1= hypoxia-inducible factor 2, alpha subunit (HIF2A) in our study (McCarthy *et al.* 2007)(Figure 4). So it is possible that IUGR plays a role in our results and perhaps it would be better to compare gene expression profiles between subjects without IUGR. On the other hand Rajakumar *et al.* have shown that placental hypoxia-inducible factor 1, alpha subunit (HIF1A), HIF2A and membrane and soluble VEGF receptor-1 proteins are not increased in normotensive pregnancies complicated by late-onset intrauterine growth restriction. Thus, the placentas from women with PE and normotensive women with late onset IUGR could be fundamentally different at the molecular level. (Rajakumar *et al.* 2007)

Serum levels of angiogenic and anti-angiogenic factors alter with gestational age; in the same manner the gene expression may change too with the progress of pregnancy. The mean time difference of delivery (and sampling) between our patients and controls was 18 days (11–21 d), which might also influence our results. Besides that our study comprised only five patients. The study was based on clinical settings and it is not easy to get controls with elective caesarean section before the 38th week of pregnancy. Overweight could have an impact on the serum levels of various proteins and it is one of the risk factors in PE (Williams *et al.* 1999). Differences in nutritional state and their impact on protein expressions might either mask or suggest an alteration in protein expression. In our study, one control's BMI was 35 kg/m<sup>2</sup>; all the other controls and subjects had a BMI of 25 kg/m<sup>2</sup> or lower in early pregnancy with normal weight gain (8–15 kg) during pregnancy.

## **6.2 Angiogenesis and cord compression**

In our cord compression case with PE, the angiogenic gene expression profile resembled that observed in normal controls. Our hypothesis is that cord compression may change a hypoxic environment in the PE placenta to normoxic and thus have effects on gene expression. Certain reports support this speculation. Hopf has confirmed that angiogenesis requires oxygen, and in ischemic tissue an intermittent oxygen exposure can satisfy the need for oxygen (Hopf *et al.* 2005). Trophoblasts can react rapidly to restitution of normal oxygen tension by readopting their reduced metabolism (Esterman *et al.* 1997). Accidental cord compression during delivery blocks the fetal blood flow momentarily and in pre-eclamptic hypoxic placentas the restitution of normal oxygen tension could be rapid. Also in our case the expression change of EPAS1= HIF2A gene towards the expression of normal controls supports our hypothesis (Figure 4). The minimal expression changes in the normal placenta with cord compression may mean that the changes occur only in the hypoxic placenta, not in the normal functioning placenta.

It is likely that the trial of labor affected the gene expression studied. However, in the pre-eclamptic cord compression case the duration of intense contractions was only two hours. So we can speculate that the effect of a short period of labor on the gene expressions might not be dramatic. Besides that, we do not know the effect of possible high intravascular pressure on placental gene expression during delivery complicated by cord compression.

### 6.3 Adipocytokines, apoptosis and PE

Our study demonstrates the expression of ADPN, LEP, and their receptor genes in the placenta. This means that adipocytokines may have a role in the development of placental vasculature. In the microarray study in women with severe PE the ADPN gene was present, although the expression level was very low, and absent in the controls. Since adiponectin is a negative regulator of angiogenesis, ADPN expression may reflect the inhibition of placental angiogenesis leading to disturbed vascularisation. On the other hand it is indicated that adiponectin can function to stimulate angiogenesis in response to ischemic stress by promoting AMP-activated kinase signaling (Shibata *et al.* 2004). So ADPN and its receptor gene expression may also represent a counter-regulatory mechanism aimed at reducing endothelial damage and insulin resistance.

In the PE group the LEP expression was high in the placenta but the expression of the leptin receptor genes did not differ from those of the healthy controls. The placental oxygen tension in PE is low. Meissner has shown by *in-vitro* study that hypoxia-induced high leptin production does not protect trophoblasts from apoptosis (Meissner *et al.* 2005). The low expression level of the leptin receptor genes noticed in our study could have some role in that phenomenon.

Adiponectin mediates antiproliferative and apoptotic responses and a leptin proliferative response in breast cancer cells, which express adiponectin receptors and respond to human recombinant adiponectin by reducing their growth (Dieudonne *et al.* 2002, Dieudonne *et al.* 2006). These findings suggest that adiponectin and leptin can act *in vivo* as paracrine/endocrine growth factors, the former inhibiting and the latter activating the growth of epithelial cells. Dieudonne have also demonstrated that the antiproliferative effect of adiponectin involves activation of apoptosis and inhibition of the cell cycle (Dieudonne *et al.* 2006).

Adiponectin inhibits the growth and peritoneal metastasis of gastric cancer through its specific membrane receptors ADIPOR1 and ADIPOR2; the downregulation of either ADIPOR1 or ADIPOR2 receptor by specific siRNA significantly suppresses the inhibitory effect of growth in cancer cells, and moreover a local injection of adiponectin effectively suppressed the development of peritoneal metastasis of cancer (Ishikawa *et al.* 2007).

Recent findings suggest the possibility of a conserved pathway through which adiponectin may regulate the induction of apoptosis (Narasimhan *et al.* 2005). In

humans, adiponectin has been shown to induce apoptosis in endothelial cells and breast cancer cells (Brakenhielm *et al.* 2004, Kang *et al.* 2005). Interestingly, our results indicate that ADIPOR1 expression is significantly increased in PE, providing a possible route for adiponectin to trigger apoptosis placental trophoblast cells. This is supported by our results and previous studies which indicate increased trophoblast apoptosis during PE (Soleymanlou *et al.* 2005b).

Comparing the results of different gestational ages are problematic, especially concerning placental apoptosis (Cobellis *et al.* 2007, DiFederico *et al.* 1999, Reister *et al.* 2001). In a clinical setting it is difficult to locate a control with exactly the same gestational age. The mean time difference between the patients and the controls was 19 days (14–24). One should also bear in mind the accuracy of the gestational dating.

## **6.4 Thrombophilias**

In general, there are conflicting findings about the association between thrombophilic mutations and severe pregnancy complications. Why are the results of the cited studies contradictory? First of all, the prevalence of inherited thrombophilic mutations varies markedly between different populations. If the prevalence is low, then a much larger study population is needed to investigate an association. Secondly, there might be a combined effect with other unknown inherited or acquired prothrombotic risk factors either systemic or operating locally at the placental level. Those effects may cause or exclude an association. Thirdly, acquired thrombophilias may interact with inherited thrombophilias to enhance the risk and lead to a significant association.

### **6.4.1 Factor V Leiden**

We found a significant difference in the prevalence of FVL-mutation between our complicated pregnancies and our controls. Other investigators have also obtained similar results. Kupfermink and his colleagues found that FVL appeared in 20% of pregnancies complicated with PE, IUGR, stillbirth or placental abruption and in 6% of normal pregnancies (Kupfermink *et al.* 1999). Agorastos *et al.* reported that the FVL mutation was detected in 22% of women with similarly complicated pregnancies and in 4% of controls.(Agorastos *et al.* 2002). On the other hand, De Groot found no differences in the frequency of factor V Leiden between the women who had PE and their control subjects.(De Groot *et al.* 1999). A meta-

analysis found a significant association only in women with early onset severe PE (Morrison *et al.* 2002).

There are contradictory findings about the association between thrombophilic mutations and IUGR. In our study, despite using the 10<sup>th</sup> percentile for the definition of IUGR, we found a significant difference in the prevalence of FVL in mothers with IUGR (7.2%) and in our controls (1.7%). This might be due to the fact that 72% of our IUGR newborns were below the 5<sup>th</sup> percentile. Similarly Kupferminc and Martinelli reported a significant association between maternal thrombophilic gene defects and IUGR in infants (Kupferminc *et al.* 1999, Martinelli *et al.* 2001). On the contrary Infante-Rivard found no such association, but the progress of the pregnancy of severe PE mothers is not known in this case (Infante-Rivard *et al.* 2002). In normotensive women with IUGR the rate of inherited thrombophilias was not increased (McCowan *et al.* 2003). In this study FVL mutation was related to PE complicated by IUGR, but not to IUGR pregnancies without PE.

Findings on the association between the FVL-mutation and placental abruption or stillbirth seem to be consistent. In our materials the incidence of the FVL mutation was 22% in women with placental abruption compared to 1.7% in controls. Similarly Kupferminc found the FVL-mutation in 25% of women with abruption placenta or stillbirth (Kupferminc *et al.* 1999). Moreover, studies with 17% and 49% prevalences of the FVL-mutation were reported by Preston F in women who delivered stillborn infants (Preston *et al.* 1996).

#### **6.4.2 Prothrombin G20210A mutation**

We did not find any difference in the prevalence of the prothrombin G20210A mutation between the study group and the control group. Neither did Agorastos find any association between complicated pregnancies and the prothrombin mutation.(Agorastos *et al.* 2002). Similar results were obtained for F2 in the study of de Groot (De Groot *et al.* 1999). Morrison pointed out in their meta-analysis that it is not yet clear whether severe PE and other complications are associated with prothrombin or MTHFR polymorphism (Morrison *et al.* 2002).

#### **6.4.3 MTHFR C677T polymorphism**

No correlation was found between pregnancy complications and the MTHFR polymorphism. This finding is supported by the studies of both Ogunyemi and

Kaiser (Kaiser *et al.* 2001, Ogunyemi *et al.* 2002) The latter concluded that the MTHFR polymorphism is not associated with the development of PE in the Australian population. Similarly, Laivuori observed that the carrier status for the 677TT genotype of the MTHFR-gene does not predispose to PE in the Finnish population (Laivuori *et al.* 2000). In our study the prevalence was 1.8%, in line with the newborn prevalence of 1.5–4.2 in Finland (Pastinen *et al.* 2001).

## **6.5 Homocysteine, folic-acid and B vitamin supplementation during pregnancy**

It has been shown in non-pregnant subjects that fortified mineral water decreases the concentration of plasma homocysteine and increases serum and erythrocyte folate and serum vitamin B12 concentrations in normohomocysteinemic subjects (Tapola *et al.* 2004). The metabolism of homocysteine is different during pregnancy: plasma homocysteine decreases during pregnancy, presumably due to hemodilution and increased consumption of methionine (Andersson *et al.* 1992).

Dietrich showed in their work that even after folic acid fortification of all cereal-grain products in the U.S., less than 10% of women of childbearing age reached the recommended erythrocyte folate concentration of greater than 906 nmol/L that has been shown to be associated with a significant reduction in neural tube defect risk (Dietrich *et al.* 2005). That is why they recommend that women of childbearing age take extra folic acid supplements to reach the appropriate erythrocyte folate levels. In our study the erythrocyte folate levels were 967.3 nmol/L after intervention and 701.4 nmol/L in our controls, so we can say that in this small group the fortified mineral water worked at least as well as mandatory cereal-grain fortification, although the primary aim of our study was not NTD-prevention.

Fortified mineral water increased both serum and erythrocyte folate concentrations and decreased homocysteine concentrations significantly in the intervention group during the intervention period, and the difference was also significant between the groups. This could ameliorate endothelial function and even affect angiogenesis as homocysteine has been reported to inhibit endothelial cell proliferation, which is closely related to angiogenesis. Erythrocyte folate concentration increased also in the controls, which is in concordance with the study by Holmes and the phenomenon is probably a normal physiological change during pregnancy: in early pregnancy the folates are accumulated in the fetal erythrocytes, to be liberated later in pregnancy in response to the fetal

requirements (Ek & Magnus 1981, Holmes *et al.* 2005). Folic acid has been shown to decrease homocysteine concentrations and the reduction in concentration is dependent on the baseline homocysteine concentration; subjects with high initial concentration respond to folic acid treatment with a larger reduction (Bronstrup *et al.* 1998, Tapola *et al.* 2004, Ubbink *et al.* 1993). Our study population was normohomocysteinemic.

The fortified mineral water did not cause an increase in serum vitamin B12 concentration, which is in discordance with the study by Brönstrup (Bronstrup *et al.* 1998). Vitamin B12 and folic acid are cofactors of methionine-synthase-enzyme, catalyzing the formation of methionine from homocysteine. In a previous meta-analysis, vitamin B12 combined with folic acid was associated with an additional reduction in homocysteine concentration compared to folic acid alone (Rajkovic *et al.* 1997). In Brönstrup's study the homocysteine concentration decreased more with the folic acid-B12 combination (400µg+6µg) (Bronstrup *et al.* 1998). Folate administration alone might mask vitamin B12 deficiency (Lopez-Quesada *et al.* 2004, Nelen *et al.* 2000).

Folic acid and dietary calcium could also have preventive effects against hypertensive disorders during pregnancy (Hofmeyr *et al.* 2006, Wen *et al.* 2008). The growth of the babies was similar in both groups apart from Bm and HC in the study group at the 21st week, but since there was no difference in Bm and HC later in pregnancy, the difference could be due to chance.

## 7 Summary and conclusions

1. The expression of angiogenesis-related placental genes can be altered in PE associated with IUGR. The conditions in the placenta in PE differ from those in normal pregnancy. In PE the placental cells are living in a hypoxic environment which can affect gene expressions. Down or up regulation of certain genes may also mean a normal physiological process at this stage of pregnancy. There must also be compensatory mechanisms which can partially explain our results.
2. The accidental cord compression during delivery blocks the fetal blood-flow momentarily and especially in pre-eclamptic hypoxic placentas the changes in oxygen tension can be rapid, leading to the gene expression changes.
3. The placental LEP and ADIPOR1 genes are upregulated in severe PE but the expression of ADPN is low. The activity of the placental adipocytokine and some of their receptor genes in severe PE may mean that they have an important role in angiogenesis and regulation of placental vasculature and placental function. Placental apoptosis induced by high serum levels of maternal adiponectin could be mediated via the ADIPOR1 receptor.
4. Thrombophilic risk factors are common and can be found in 15% to 25% of Caucasian populations. Since pregnancy is an acquired hypercoagulable state, women harboring thrombophilia may present with clinical symptoms of vascular complications for the first time during gestation or at the postpartum period. Women with thrombophilia may have, perhaps via placental SFL1 stimulated by thrombine in very early pregnancy, an increased risk of placental vascular complications including PE, intrauterine growth restriction, and placental abruption.
5. Mineral water fortified with folic acid, vitamins B6, B12 and D and calcium decreases plasma homocysteine concentrations, and increased serum and erythrocyte folate concentrations in normohomocysteinemic pregnant women. Folate intake of women of fertile age is below the recommended level in Finland. Fortified mineral water will increase the number of products fortified with folic acid and therefore help to ensure that especially pregnant women achieve the desired increase in folate (and calcium) intake without increasing caloric intake, especially in countries without a mandatory folic acid fortification policy.



## 8 Weaknesses of the study

1. Comparing the results of different gestational ages are problematic, especially concerning placental apoptosis (Cobellis *et al.* 2007, DiFederico *et al.* 1999, Reister *et al.* 2001). In a clinical setting it is difficult to locate a control with exactly the same gestational age. The mean time difference between the patients and the controls was 19 days (14–24) in the study V. One should also bear in mind the accuracy of gestational dating.
2. The placental biopsies in our study were taken during the third trimester of pregnancy and that is why the expression changes in angiogenesis related genes could be almost partly consequences of the disease; the primary changes could have happened in early pregnancy. Besides that we took only one biopsy and because the placenta is heterogenic in relation to the expression of genes, especially the genes sensitive to hypoxia, the method could cause a bias in the study.
3. The number of case mothers in studies III and IV was low and that is why the results could only be considered as suggestive.
4. In the study we studied only three thrombophilic factors; the knowledge about all the other thrombophilic factors is lacking.



## 9 Future perspectives

In recent years the scientific community has focused on improving its ability to predict severe PE, although no predictive interventions have been proven to be efficient. Several predictive biochemical markers—including PLGF, sFlt1, endothelin, plasma protein 13, and pregnancy associated plasma protein-A—have been evaluated, but none is currently in routine clinical use.

Markers of endothelial dysfunction may serve as predictors of the syndrome in women that develop PE because many are often elevated weeks before observance of clinical manifestations. Nursing research and future practice may incorporate findings from microarray analyses to identify susceptibility to and prevent the disease, to diagnose early, and to design and monitor personalized therapies. The most effective and clinically useful method to predict subsequent pre-eclampsia continues to evolve using historical, clinical, biophysical, and biochemical modalities. Also early/mid pregnancy maternal uterine artery doppler velocimetry and mean arterial pressure measurements seem to be important.

If we could identify a high risk group, still several questions would remain unanswered. What is the right window for the possible intervention: before conception or immediately when the pregnancy test is positive? May first trimester intervention already be too late? Should all women of reproductive age be screened for pre-eclamptic risk factors before conception? If the results are positive, what should the clinician do with this information? It is unknown whether attenuation or elimination of these risk factors with treatment before conception will reduce the risks of PE or IUGR. Thus, future research is needed to identify which patients may benefit from routine screening and which screening methods are useful.

The basic problem is that we do not exactly know what happens in the placenta and decidua at the time of nidation; eg the exact nature of immunological adaptation and oxygen tension in the placenta is unknown. We also need to understand more about the physiological and pathological phenomena in early pregnancy, especially in angiogenesis in the decidua and placenta during and immediately after nidation. Only thereafter we will be able to conduct randomized trials to evaluate the benefits of various interventions before conception.



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# Appendix 1 Tables of the study I

**Table 1. Characteristics of the study and control subjects.**

Characteristics	Study group		Control group	
	N=126		N=111	
Age	30 years		26 years	
Gestational diabetes	8		6	
Insulin-dependent diabetes	3		1	
Primipara	66	50%	38	32%
Pregnancies, mean	2.5		2.0	
Weight	64.3 kg		66.1 kg	
Height	163.6 cm		165.0 cm	
Body Mass Index	24		24	
Delivery, week	33+6		39+5	
Normal delivery	21	17%	71	82%
Vacuum extraction	1	0.7%	6	5%
Cesarean	96	78%	8	7%
Newborn's weight	1913 g		3575 g	
Umbilical artery blood sample (pH)	7.26		7.28	

**Table 2. The association of inherited thrombophilic mutations and severe pregnancy complications.**

Complication	Total	FVL	F II	MTHFR	Combined	Total %
Severe pre-eclampsia	82	8		5	1	14 17.0
IUGR	75 <sup>a</sup>	6	1	3		10 14.5
Placental abruption	9 <sup>b</sup>	2		1		3 33.3
Fetal death	10 <sup>c</sup>	1				1 10.0

<sup>a</sup>39 patients had also severe pre-eclampsia <sup>b</sup>2 patients had also severe pre-eclampsia, 3 patients had also IUGR. <sup>c</sup>1 patient had also severe pre-eclampsia, 4 patients had IUGR and one patient had both IUGR and severe preeclampsia, 2 patients had also abruption and one had both IUGR and abruption

**Table 3. The prevalence of inherited thrombophilic mutations. Mutations of Factor V Leiden and prothrombin are heterozygous and methylene-tetrahydrofolate reductase homozygous.**

Mutation	Study group		Control group		Odd ratio	Observed difference between prevalences 95% CI
	N = 126		N = 111			
FVL +/-	12	9.5%	2	1.8%	5.7	7.7% (CI 2.0–13.4)
Prothrombin +/-	1	0.8%	1	0.9%	0.9	-0.11% (CI -2.4–2.2)
MTHFR,C 677T +/-	7	5.6%	5	4.5%	1.1	1.1% (CI -4.5–6.6)
Total	20	15.9%	8	7.2%	2.4	8.7% (CI 0.7–16.7)



## Original articles

- I Järvenpää J, Päckilä M, Savolainen ER, Perheentupa A, Järvelä I & Ryyänen M (2006) Evaluation of factor V Leiden, prothrombin and methylenetetrahydrofolate reductase gene mutations in patients with severe pregnancy complications in northern Finland. *Gynecol Obstet Invest* 62: 28-32.
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- III Järvenpää J, Vuoristo JT, Savolainen ER, Ukkola O, Vaskivuo T, Ryyänen M (2007) Altered expression of angiogenesis-related placental genes in pre-eclampsia associated with intrauterine growth restriction. *Gynecol Endocrinol* 23: 351-315.
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- V Järvenpää J, Vuoristo JT, Santaniemi M, Ukkola O, Savolainen E-R, Tapanainen J, Jääskeläinen M, Kesäniemi A & Ryyänen M (2008) Adiponectin induced placental cell apoptosis could be mediated via ADIPOR1- receptor in pre- eclampsia with IUGR. Manuscript.

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965. Ikäheimo, Pekka (2008) Suomalaisen aikuisen astma – kysely- ja rekisteritutkimus vuonna 2000
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