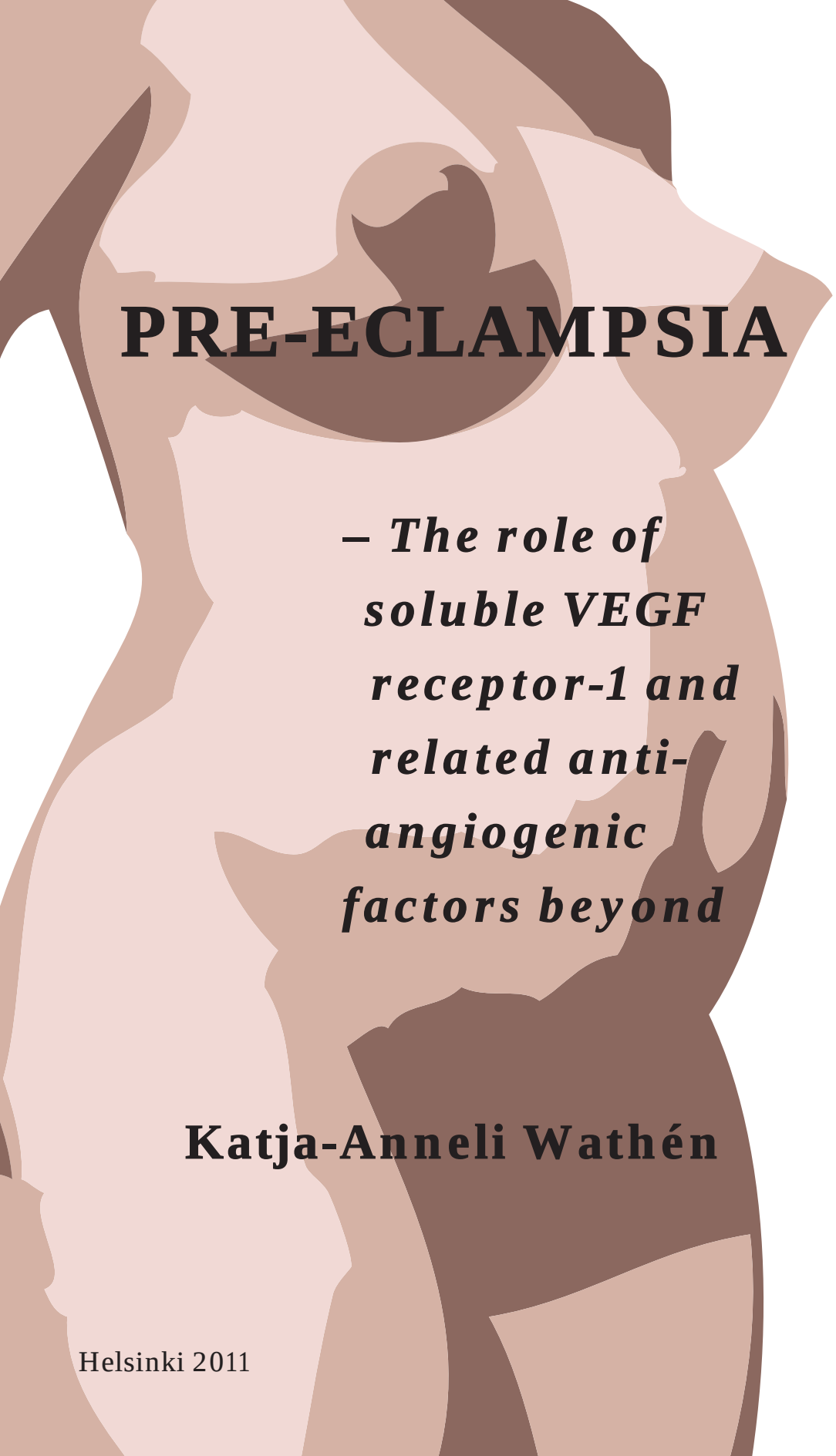

PRE-ECLAMPSIA

– The role of soluble VEGF receptor-1 and related anti-angiogenic factors beyond

Katja-Anneli Wathén

Helsinki 2011

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The role of soluble VEGF receptor-1 and related anti-angiogenic factors beyond

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To Onni & Tenho

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– ja tälle koko Sirkusperheelleni.

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Contents

List of Original Publications

This thesis is based on the following original publications, which have been referred to in the text by their Roman numerals.

I Wathén KA, Tuutti E, Stenman UH, Alfthan H, Halmesmäki E, Finne P, Ylikorkala O, and Vuorela P. Maternal Serum-Soluble Vascular Endothelial Growth Factor Receptor-1 in Early Pregnancy Ending in Preeclampsia or Intrauterine Growth Retardation. J Clin Endocrinol Metab. 2006; 91 (1): 180-4.

II Kämäräinen M, Soini T, Wathén KA, Leinonen E, Bützow R, Stenman UH, and Vuorela P. Smoking and sVEGFR-1: Circulating maternal concentrations and placental expression. Mol Cell Endocrinol. 2009; 299 (2): 261-5.

III Wathén KA, Ylikorkala O, Andersson S, Alfthan H, Stenman UH, and Vuorela P. Maternal serum endostatin at gestational weeks 16–20 is elevated in subsequent pre-eclampsia but not in intrauterine growth retardation. Acta Obstet Gynecol Scand. 2009; 88 (5): 593-8.

IV Leinonen E, Wathén KA, Alfthan H, Ylikorkala O, Andersson S, Stenman UH, and Vuorela P. Maternal Serum Angiopoietin-1 and -2 and Tie-2 in EarlyPregnancy Ending in Preeclampsia or Intrauterine Growth Retardation. J Clin Endocrinol Metab. 2010; 95 (1): 126-33.

V Wathén KA, Sarvela J, Stenman F, Stenman UH, and Vuorela P. Changes in serum concentrations of soluble vascular endothelial growth factor receptor-1 after pregnancy. Hum Reprod. 2011; 26 (1): 221-6.

Abbreviations

Ang-1, -2	Angiopoietin-1, -2
ANOVA	analysis of variance
AUC	area under the curve
CI	confidence interval
ELISA	enzyme-linked immunosorbent assay
Flt-1, -4	fms-like tyrosine kinase-1, -4 (see VEGFR-1 and -3)
hCG	human chorionic gonadotropin
HIF-1α, -1β	hypoxia-inducible transcription factor-1α, -1β
HSPG	heparan sulfate proteoglycan
IUGR	intrauterine growth retardation
NO	nitric oxide
NRP-1, -2	neuropilin-1, -2
OR	odds ratios
PAPP-A	pregnancy-associated plasma protein-A
PBS	phosphate buffered saline
P/GF	placental growth factor
ROC curve	receiver operating characteristic –curve
SD	standard deviation
SGA	small for gestational age
sTie-1, -2	soluble Tie-1, -2 (see also Tie-1 and Tie-2)
sVEGFR-1, -2	soluble VEGFR-1, -2 (see also VEGFR-1, -2)
Tie-1	tyrosine kinase with immunoglobulin and epidermal growth factor-like extracellular domains
Tie-2	tyrosine kinase with epidermal growth factor homology
VEGF-A,-B,-C,-D,-E,-F	vascular endothelial growth factor -A, -B, -C, -D, -E, -F
VEGFR-1, -2, -3	vascular endothelial growth factor receptor-1, -2, -3

Definitions

Apgar score	Measure of a newborn's immediate well-being determined by evaluating it on the basis of five criteria (breathing, pulse rate, reflexes, muscle tone and skin color) using scale from 0 to 2, the maximum score being 10 which represents an optimal condition.
Birth	Process resulting in infant with a gestational age of ≥ 22+0 weeks or weight ≥ 500 g.
CBA/J x DBA/2	Mouse model of immunologically mediated pre-eclampsia (DBA/J –mated CBA/J – female mice).
Eclampsia	Occurrence of seizures in pregnant women diagnosed with pre-eclampsia and unrelated to other origins.
Ex vivo	Experimentation or measurements performed in/on tissue in artificial environment outside the organism with minimum alteration of natural conditions. "*Ex vivo*" – conditions allow experimentation under more controlled conditions than often possible in "*in vivo*" –experiments (see below).
HELLP syndrome	Complication of pre-eclampsia with hemolysis, elevated liver-enzymes and low platelet count.
In utero	'In the uterus' – Expression referring to unborn child (in legal context) or state of embryo / fetus (biological context).
In vitro	Experimentation conducted using components of organism, isolated from their usual biological context, in order to permit a more detailed or more convenient analysis than the one conducted with whole organisms.
In vivo	Experimentation conducted with a whole, living organism in its normal, intact state, as opposed to partial or dead organism.
IUGR	'Intrauterine growth retardation' – failure of the fetus / newborn to achieve genetically determined individual growth potential *in utero.*
LBW	'Low birthweight' – live-born infant with a birthweight of < 2500 g.
locus	Position that a given gene occupies on a chromosome. (plural: "loci")
Maternal mortality	Number of maternal deaths during pregnancy or within the following 6 weeks divided by 100000 live born children. Accidental or violent deaths are excluded from the total.
Nullipara	Woman who has never given birth to a child.
PBS	'Phosphate buffered saline' – isotonic medium solution for cell cultures and washing of tissue sections, containing the following chemicals in the given concentrations (pH = 7.4): 0.14 M NaCl, 2.7 M KCl, 0.01 M Na_2HPO_4, and 1.76 M KH_2PO_4.
Perinatal mortality	Number of stillbirths and early neonatal deaths during the first week of life divided by number of births during the same time period (usually statistical year). 'Neonatal mortality' is derived similarly from number of deaths during the first 4 weeks of life.
Premature infant	Infant born < 37+0 weeks of gestation. Newborn with a weight of ≤ 1500 g or a gestational age of < 32+0 weeks at birth is referred to as 'small premature infant'.
Preterm delivery	Length of gestation < 37+0 weeks at delivery.
Primipara	Woman who is pregnant / has been delivered of a child for the first time.
Puerperium	State of a mother during childbirth or immediately thereafter extending for about six weeks, until the return of a normal uterine size. Synonymic to 'postpartum period', whereas 'postnatal period' is an equivalent term referring to the infant.
SGA	'Small for gestational age' – small infant who has failed to achieve a gender-specific weight threshold, usually defined as the 10^{th} percentile of the population.
Stillbirth	Birth of a child showing no evidence of life (breathing, heartbeat, pulsation of umbilical cord or movement of voluntary muscles) but fulfilling the criteria of 'birth'.
Term delivery	Length of gestation ≥ 37+0 and > 42+0 weeks at delivery.
VEGF Trap	Soluble VEGF receptor –like fusion protein containing the domain 2 of VEGFR-1 and the domain 3 of VEGFR-2, fused with an Fc fragment (AVE0005 or aflibercept).

Abstract

Pre-eclampsia is a pregnancy complication that affects about 5% of all pregnancies. It is known to be associated with alterations in angiogenesis-related factors, such as vascular endothelial growth factor (VEGF). An excess of anti-angiogenic substances, especially the soluble receptor-1 of VEGF (sVEGFR-1), has been observed in maternal circulation after the onset of the disease, probably reflecting their increased placental production. Smoking reduces circulating concentrations of sVEGFR-1 in non-pregnant women, and in pregnant women it reduces the risk of pre-eclampsia. Soluble VEGFR-1 acts as a natural antagonist of VEGF and placental growth factor (P*l*GF) in human circulation, holding a promise for potential therapeutic use. In fact, it has been used as a model to generate a fusion protein, "VEGF Trap", which has been found effective in anti-angiogenic treatment of certain tumors and ocular diseases.

In the present study, we evaluated the potential use of maternal serum sVEGFR-1, Angiopoietin-2 (Ang-2) and endostatin, three central anti-angiogenic markers, in early prediction of subsequent pre-eclampsia. We also studied whether smoking affects circulating sVEGFR-1 concentrations in pregnant women or their first-trimester placental secretion and expression *in vitro*. Last, in order to allow future discussion on the potential therapy based on sVEGFR-1, we determined the biological half-life of endogenous sVEGFR-1 in human circulation, and measured the concomitant changes in free VEGF concentrations. Blood or placental samples were collected from a total of 268 pregnant women between the years 2001–2007 in Helsinki University Central Hospital for the purposes above. The biomarkers were measured using commercially available enzyme-linked immunosorbent assays (ELISA).

For the analyses of sVEGFR-1, Ang-2 and endostatin, a total of 3 240 pregnant women in the Helsinki area were admitted to blood sample collection during two routine ultrasound-screening visits at 13.7 ± 0.5 (mean ± SD) and 19.2 ± 0.6 weeks of gestation. Of them, 49 women later developing pre-eclampsia were included in the study. Their disease was further classified as mild in 29 and severe in 20 patients. Isolated early-onset intrauterine growth retardation (IUGR) was diagnosed in 16 women with otherwise normal medical histories and uncomplicated pregnancies. Fifty-nine women remaining normotensive, non-proteinuric and finally giving birth to normal-weight infants were picked to serve as the control population of the study.

Maternal serum concentrations of Ang-2, endostatin and sVEGFR-1, were increased already at 16–20 weeks of pregnancy, about 13 weeks before the clinical manifestation of pre-eclampsia. In addition, these biomarkers could be used to identify women at risk with a moderate precision. However, larger patient series are needed to determine whether these markers could be applied for clinical use to predict pre-eclampsia.

Intrauterine growth retardation (IUGR), especially if noted at early stages of pregnancy and not secondary to any other pregnancy complication, has been suggested to be a form of pre-eclampsia compromising only the placental sufficiency and the fetus, but not affecting the maternal endothelium. In fact, IUGR and pre-eclampsia have been proposed to share a common vascular etiology in which factors regulating early placental angiogenesis are likely to play a central role. Thus, these factors have been suggested to be involved in the pathogenesis of IUGR. However, circulating sVEGFR-1, Ang-2 and endostatin concentrations were unaffected by subsequent IUGR at early second trimester. Furthermore, smoking was not associated with alterations in maternal circulating sVEGFR-1 or its placental production.

The elimination of endogenous sVEGFR-1 after pregnancy was calculated from serial samples of eight pregnant women undergoing elective Caesarean section. As typical for proteins in human compartments, the elimination of sVEGFR-1 was biphasic, containing a rapid half-life of 3.4 h and a slow one of 29 h. The decline in sVEGFR-1 concentrations after mid-trimester legal termination of pregnancy was accompanied with a simultaneous increase in the serum levels of free VEGF so that within a few days after pregnancy VEGF dominated in the maternal circulation. Our study provides novel information on the kinetics of endogenous sVEGFR-1, which serves as a potential tool in the development of new strategies against diseases associated with angiogenic imbalance and alterations in VEGF signaling.

Introduction

Angiogenesis, defined as growth of new vessels sprouting from pre-existing ones, is essential in fetal development, but aside from pathologic and trauma conditions, it is mainly limited to the female reproductive cycle in adults. Pathologic angiogenesis is characterized as either excessive or inadequate neovascularization. It is a crucial pathophysiological event in malignant, ischemic, infectious, immune and inflammatory disorders (Carmeliet. 2005). Angiogenesis is tightly regulated by a number of pro- and anti-angiogenic factors, and vascular endothelial growth factor (VEGF) is a key mediator of several angiogenic signals.

In human physiology, the most remarkable scene of angiogenesis is the formation of the placenta and fetus. Successful implantation, placentation and the following gestation are dependent on vascular development and adaptation. Both VEGF and its soluble receptor-1 (sVEGFR-1), acting as a natural antagonist of VEGF, are highly expressed in the placenta throughout the gestation (Vuorela et al. 1997, Sharkey et al. 1993, Clark et al. 1996, Vuorela et al. 2000). Beginning from early pregnancy, sVEGFR-1 is secreted by the trophoblasts, and it can be detected as constantly elevating concentrations in the maternal circulation already about a month following the conception (Levine et al. 2004, McKeeman et al. 2004, Powers et al. 2005, Molskness et al. 2004).

Pre-eclampsia affects about 5% of all pregnancies, and it is the most common cause of maternal and perinatal mortality and morbidity in the developing countries (Khan et al. 2006, Duley. 2009). The pathophysiology of the disease has been characterized as placental hypoperfusion and ischemia, starting already at the implantation and early pregnancy. The etiology of pre-eclampsia has been intensively studied, but still the final cause remains unknown. So far, the delivery of the placenta is the only definitive cure for the disorder (Datta. 2004).

Pre-eclampsia is characterized by an angiogenic imbalance, which has been proposed to first act on the placental level, but subsequently become evident also on the systemic level. As a result, there is an overall maternal endothelial dysfunction, which has been associated with the clinical manifestations of the syndrome (Roberts et al. 1989, de Groot and Taylor. 1993). An excess of anti-angiogenic factors, especially sVEGFR-1, has been found in the maternal circulation after the onset of the disease, probably reflecting an increased placental production of this pregnancy –induced protein (Levine et al. 2004, Chaiworapongsa et al. 2004, Koga et al. 2003, Maynard et al. 2003). Indeed, the vascular endothelium is considered as the target organ of the biochamical alterations in the placenta during pre-eclampsia.

Besides the angiogenic imbalance, the underlying events in the placental morphology have been proposed to be associated with some other etiologies. Most notably, genetic and immunological factors have been shown to contribute to the risk of pre-eclampsia (Chappell and Morgan. 2006, Dekker and Robillard. 2007). Interestingly, smoking has been observed to reduce the risk of pre-eclampsia in pregnant women (Conde-Agudelo and Belizan. 2000). In non-pregnant women, it affects circulating sVEGFR-1 concentrations, which have been noted to be lower than in non-smokers (Belgore et al. 2000, Schmidt-Lucke et al. 2005).

Intrauterine growth retardation (IUGR), especially if noted at early stages of pregnancy and not secondary to any other pregnancy complication, has been suggested to be a form of pre-eclampsia not affecting the maternal endothelium (Regnault et al. 2002). Based on poor remodeling of the placental bed spiral arteries, which have been detected in both IUGR and pre-eclampsia (Khong et al. 1986), they have been proposed to share a common vascular etiology (Regnault et al. 2002, VanWijk et al. 2000, Myatt. 2002). Thus, factors regulating angiogenesis in the early placenta are likely to play a central role also in the pathogenesis of IUGR.

As pre-eclampsia is associated with increases in anti-angiogenic substances, the phenomenon could possibly be detectable in the maternal circulation already in early pregnancy preceding the development of the symptoms, perhaps even providing a predictive tool for identifying women at risk. The protective effect of smoking in pre-eclampsia might also be mediated by the angiogenic imbalance. Furthermore, such principles could hold a promise for future interventions on the angiogenesis-related factors.

Literature Review

1. Angiogenesis

Aside from pathologic and traumatic conditions, blood vessel development is mostly limited to the reproductive track in adults. Female reproductive organs, the ovaries and the uterus, undergo sequential growth and regression during the menstrual cycle, maintaining their functions with a careful balance in the vascular systems (Carmeliet. 2005). Successful implantation, placentation and the following gestation are dependent on vascular development and adaptation.

1.1. Angiogenesis and vasculogenesis

Vasculogenesis includes the formation of a primitive vascular capillary network, achieved by mesoderm-derived precursor cells, with differentiation and proliferation. This primitive network is further remodeled to a complexed circulatory system by creating vascular sprouts from pre-existing microvessels. This process is referred to as angiogenesis, which begins with capillary sprouting and culminates in the formation of a new microcirculatory bed composed of arterioles, capillaries, and venules (Risau. 1997).

1.2. Mechanisms and regulation of angiogenesis

The initiation of angiogenesis consists of three processes: break down of the basement membrane of the existing vessel, migration of their endothelial cells towards an angiogenic stimulus, and proliferation of the endothelial cells (Schoenwolf et al. 2009).

Angiogenesis occurs by at least the four following ways (Risau. 1997, Schoenwolf et al. 2009):

1. sprouting - mostly during neovascularization of vascular tissue,
2. intussusception – internal division of the vessel by endothelial cells resulting in the vessel split in two,
3. elongation or widening – lengthways growth of the vessel without formation of new vascular junctions, and
4. incorporation of circulating endothelial cells into pre-existing vessels.

Numerous factors regulate the angiogenic process in human tissues. They comprise peptide growth factors, hormones, cytokines, chemical substances and physical stimuli (Risau. 1997).

2. Uncomplicated pregnancy

2.1. Beginning of pregnancy

The gestation period from the beginning of previous menstruation until birth usually lasts for 280 days, accounting for 40 gestational weeks. The first 10 weeks of pregnancy is referred to as the embryonic period which is followed by the fetal period. The 9-month gestation is divided into three 3-month trimesters (Schoenwolf et al. 2009).

Fertilization occurs at the beginning of the third week of the following pregnancy. After fertilization, the zygote travels from the uterine tube going through rapid mitotic divisions. Finally, the formed blastocyst enters the uterine cavity, which serves as the implantation site and is essential for normal pregnancy.

2.1.1. Implantation

About the 20th day of preganancy, the trophoblast cells of the blastocyst begin to invade the maternal endometrium by penetrating between the endometrial cells. These trophoblasts later fuse to form an expanding peripheral layer of multinucleated syncytiotrophoblasts, called the placental syncytium. The trophoblasts from the wall of the blastocyst remain unicellular, thereafter referred to as cytotrophoblasts, which act as a stem cell population. On about the 23rd day the blastocyst is deeply embedded in the endometrium, which initiates the lacunar stage of trophoblast development. Implantation is considered complete three days later, around the 12th day post-conception, at the end of the 4th gestational week (Schoenwolf et al. 2009).

2.1.2. Formation of placenta

The development of the placenta begins as the implanting blastocyst induces a response in the uterine endometrium called the decidual reaction, decidualization. Cells in the endometrial stroma accumulate lipid and glycogen and the endometrium thickens, becoming also a highly vascularized tissue, thereafter called “decidua”.

During the first weeks the embryo is nourished through simple diffusion. However, later on, due to its rapid growth, it needs a more powerful gas and nutrient exchange system, which is made possible by the development of the uteroplacental circulation system. The early placenta develops under hypoxic conditions (Schoenwolf et al. 2009).

Already upon the implantation, the cytotrophoblasts proliferate to form finger-like outgrowths giving rise to the villous trees of the placenta. The outgrowths, containing only cytotrophoblasts, appear soon after the first signs of primitive uteroplacental circulation. These structures are called primary chorionic stem villi, which are further transformed into secondary chorionic stem villi during the early 5th week of pregnancy. The process is initiated by proliferating extraembryonic mesoderm, which invades the cores of primary villi.

At the end of the 5th week the villus mesoblast, mesodermal core of secondary villus, differentiates into connective tissue and blood vessels. They connect up with the embryonic blood vessels, forming the basis of tertiary stem villi, which contain differentiated blood vessels (Schoenwolf et al. 2009). All subsequent vascularized villi are subtypes of them (Kingdom et al. 2000). From there on, a total of four layers must be passed in order for gases, nutrients, and waste products to diffuse through the maternal and fetal blood: capillary endothelium of the villus, loose connective tissue surrounding the endothelium, cytotrophoblast and syncytiotrophoblast.

The mesenchymal cells hold a potential to further differentiate into angioblasts, endothelial cells and hematopoietic stem cells. The cells associated with the vasculature are derived from mesenchymal cells, which have first differentiated from the extraembryonic mesoderm. In addition, mesenchyme-derived macrophages, called Hofbauer cells, express angiogenic growth factors in the mesenchyme of the villi for the paracrine initiation of vasculogenesis (Schoenwolf et al. 2009).

2.1.3. Development of uteroplacental circulation

In the uteroplacental circulation system the circulations of the mother and that of the embryo become closer to each other, allowing exchange of gases and metabolites via diffusion. During pregnancy the uterine circulation is highly adaptive to the growing metabolic demands of the fetus. Maternal blood flows into the intervillous spaces through the spiral arterioles, which branch from the uterine arteries. The deoxygenated blood leaves the placenta via the uterine venules located in the periphery of the intervillous space. The placental blood flow can rise up to 6 000 mL in a minute and the pressure in the spiral arteries to 70 mmHg from which it falls to only 10 mmHg in the intervillous space. The blood in the maternal side of the placenta is exchanged up to three times per minute.

The development of the uteroplacental circulation starts at the middle of the 4th week of gestation when irregular spaces appear in the syncythiothrophoblast and they soon join together to form lacunae. These lacunae are originally filled with tissue fluids and uterine secretions, but later on they anastomose with maternal capillaries. A week later fetal blood vessels and tertiary stem villi, projecting into the trophoblastic lacunae, begin to develop.

Maternal capillaries near the syncytiotrophoblasts expand to form maternal sinusoids, and the lacunae are fulfilled with blood around the beginning of the 6th week of pregnancy. At the end of the pregnancy the lacunae communicate with each other forming a connected system delimited by the syncytiotrophoblast and referred to as the intervillous space.

The anastomoses between trophoblastic lacunae and maternal sinusoids continue to develop during the next few days, when the cytotrophoblasts begin to proliferate locally and form the villous structures of the placenta. The first blood vessels are completed by the end of the 7th week establishing the uteroplacental circulation (Schoenwolf et al. 2009).

2.1.4. Development of fetal vasculature

Formation of the fetal vasculature starts at the early 5th week of gestation. After differentiation, the endothelial cells organize into tiny capillaries, which then lengthen and interconnect. This *de novo* formation of blood vessels, vasculogenesis, supplies the primary vascular network in the yolk sac, connecting stalk and the chorionic villi already by the end of the 5th week. This network transporting fetal erythrocytes further develops, growing and spreading by different mechanisms throughout the embryo (Schoenwolf et al. 2009, Demir et al. 1989). Later, during the first trimester, new fetal vessels are formed within the villi by branching angiogenesis.

Figure 1. The placental circulation and the fetoplacental unit.

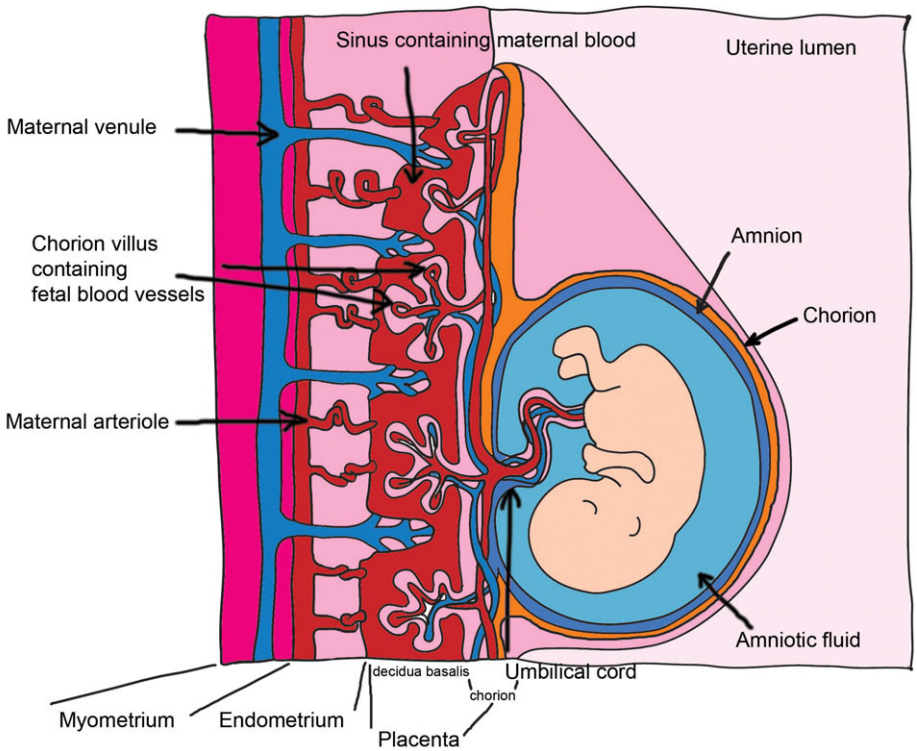


Figure 1. The placenta is a combination of maternal and embryonic tissues. It transports nutrients, respiratory gases and waste products between the mother and the fetus from the 6th week of gestation until birth. During the first events of implantation, the cytotrophoblasts start to proliferate to form finger-like outgrowths giving rise to the villous trees of the placenta. Only about a week from the implantation, at late 4th week of pregnancy, the trophoblastic lacunae within the syncytiotrophoblasts anastomose with maternal capillaries initiating the development of the uteroplacental circulation. The first blood vessels are completed by the end of the 7th week establishing the uteroplacental circulation. Simultaneously, also the fetal blood vessels have begun to develop from the 5th week of gestation.

Maternal blood enters the intervillous space via spiral arterioles, and flows to the blood sinus surrounding the chorionic villi due to the relatively high pressure. As the pressure decreases, the deoxygenated blood leaves the placenta through the endometrial venules.

Deoxygenated fetal blood enters the placenta via two umbilical arteries, which branch radially to form chorionic arteries at the junction of the umbilical cord and the placenta. The following vessels travel embedded in the chorion, which is the fetal part of the placenta. They further branch into cotyledon arteries and eventually form an extensive arteriocapillary venous system, passing through the capillaries of the chorionic villi projecting into the maternal portion of the placenta, the decidua, bringing the fetal blood extremely close to maternal blood.

Fetal and maternal blood are separated by "placental barrier" the thinnest part of which contains only the syncytiotrophoblast layer, the merged basal membrane and the endothelium of the fetal vessels, the latter of which retains the fetal blood inside them while the maternal blood flushes the chorionic villi with faily high blood pressure. This is the site of oxygen and nutrient transport, after which fetal blood leaves the placenta via the umbilical vein, leading back to the fetus. Materials are exchanged between the fetal capillary bed and the maternal sinus by diffusion, active transport and selective absorption.

2.2. Adaptation of maternal and uteroplacental circulation

The placental villi reach their maximal length during the first trimester. During the second trimester, the placenta exhibits dramatic growth accompanied by a substantial increase in the vascular volume. During the third trimester, except for the last gestational weeks, the villi also form numerous slender side branches called mature intermediate villi, and they further start to produce small secondary branches called terminal villi. This process results in changes in the villous vascular architecture, which then develops via non-branching angiogenesis, i.e. increasing the size of the existing vessels. As the gestation proceeds, these terminal capillaries dilate focally to form large sinusoids in order to optimize the diffusional gas exchange between the fetal and maternal circulations (Schoenwolf et al. 2009).

2.2.1. Remodeling of spiral arteries

The remodeling of the maternal spiral arteries takes place towards the end of the first trimester. During this process the extravillous, so-called “endovascular” cytotrophoblast cells invade these narrow, highly coiled, muscular vessels of the decidua and the inner third of the myometrium (Burton et al. 2009, Jauniaux and Greenough. 2007). They transform into endothelial-phenotype cells expressing typical endothelial markers and replace the maternal endothelium of the spiral arteries in a process called “pseudovasculogenesis” (Zhou et al. 1997b).

Due to the trophoblast invasion, the maternal spiral arteries are widened to form high-caliber ("high-flow"), low-resistance channels, which are no longer responsive to vasomotor influences. Concomitantly, a mixture of fibrinoid and fibrous tissue replaces the normal musculoelastic tissue around the vessels. During the early second trimester, the trophoblasts have invaded about 40-60 spiral arterioles of which 12-15 may be called major arteries.

2.2.2. Hemodynamic and cardiovascular adaptation

Pregnancy has been referred to as “the time of special physiological stress” during female life. The demands of the growing fetus are mediated via the placenta, which serves as the pathway of nutrient supply, gas exchange and metabolite transfer. The adaptation of the maternal physiology to these needs is ensured by hormonal changes beginning from fertilization and continuing until delivery.

Maternal adaptation to pregnancy is associated with tremendous changes namely in the cardiovascular system, as the circulation becomes hyperdynamic. This phenomenon is characterized by increases in plasma volume, heart rate and cardiac output, and reductions in blood pressure and vascular resistance. The increase in maternal blood volume begins in the first trimester, increases rapidly during the second trimester and continues, although much more moderately, until term (Duvekot and Peeters. 1994).

Cardiac output begins to increase in early pregnancy initiated by an increase in the heart rate and followed by an elevation in the stroke volume (Hunter and Robson. 1992). Cardiac output tends to rise until the middle of pregnancy (Duvekot and Peeters. 1994), eventually being nearly 50% more than that before the pregnancy (Hunter and Robson. 1992). The increase in the heartbeat, however, remains less than 20% of its non-pregnant level (Datta. 2004).

The maternal systemic blood pressure declines after the beginning of pregnancy, reaching a minimum during the second trimester (Duvekot and Peeters. 1994). After that, the blood pressure usually returns to its baseline level at term (Duvekot and Peeters. 1994). The diastolic pressure is usually reduced more than the systolic one, and the reduction is typically about 5-10 mmHg at the second trimester. Because of the hemodynamic changes above, peripheral vascular resistance lowers as pregnancy advances (Duvekot and Peeters. 1994).

There is an increase in the myocardial contractility during the entire pregnancy, causing the development of mild ventricular hypertrophy in the heart (Duvekot and Peeters. 1994, Hunter and Robson. 1992). The most visible alterations in cardiovascular parameters have been detected within 2 weeks postpartum, and only a minor ventricular hypertrophy persists 5 months after pregnancy (Duvekot and Peeters. 1994)

Renal plasma flow is elevated due to vasodilatation in the kidneys. As a result, the glomerular filtration rate increases in early pregnancy slightly declining towards term (Jeyabalan and Conrad. 2007). Minor excretion of proteins (<300 mg / 24h) into urine is a normal finding during pregnancy (Taylor and Davison. 1997).

Blood flow to the uterus, kidneys and breasts increase during the gestation. Uterine vascular resistance is lower than that of the systemic circulation, thus allowing the blood flow in uterus and the placenta to be elevated to a major extend.

2.3. Parturition and puerperium

At term, around 40 weeks of gestation, the uterine starts to contract rhythmically ending in the delivery of the fetus. The placenta first separates from the decidual tissue after which it is expelled. Myometrial contractions act to constrict the uterine vessels to prevent excessive bleeding. Upon birth of the placenta, its hormonal production naturally ends and the previous products disappear from the maternal circulation according to their characteristic half-lives. For example, the steroid and protein hormone concentrations have generally reached their typical non-pregnant concentrations during about 48 to 72 hours after delivery, such as in the case of human chorionic gonadotropin (hCG; Korhonen et al. 1997).

3. Pre-eclampsia

3.1. General aspects

Pre-eclampsia is a pregnancy complication characterized by hypertension and proteinuria. The risk of developing this systemic syndrome is about 2–8% (Duley. 2009). Especially in the developing countries, pre-eclampsia is a major cause of maternal morbidity and mortality, and worldwide it is one of the leading contributors to adverse perinatal outcomes, such as prematurity and intrauterine growth retardation (Khan et al. 2006, Duley. 2009). Other hypertensive disorders in pregnancy comprise pre-existing hypertension and gestational hypertension. Pre-eclampsia is commonly considered as a placental disorder, although despite decades of research its definitive etiology is still unknown. In fact, the delivery of the placenta remains the only known cure for pre-eclampsia so far (Datta. 2004).

In Finland, the percentage of pregnant women diagnosed with pregnancy induced hypertension, proteinuria, pre-eclampsia or eclampsia based on the hospital records from 2000, was 2.6% of all women giving birth at the same year (Gissler et al. 2002). However, according to a large national population based study in 2000–2001, which was conducted among over 3 500 women aged 18 or older, the lifetime occurrence of pre-eclampsia had been about twice higher, 5.0% (Koponen and Luoto. 2004). In the same study, gestational hypertension had been present at some point in nearly 19% of the women, and the percentage was highest in the youngest (age < 30 years; 22%) and the low-educated (21%) subgroups. In a tertiary referral center of Kuopio University Hospital nearly 22 000 singleton, structurally and chromosomally normal pregnancies were compared to those of female doctors and teachers during an almost 12-year period (Heinonen and Saarikoski. 2002). The study found a 3.2% overall risk of pre-eclampsia which was interestingly much lower among the doctors (0.9%). In the same population, the incidence of pre-eclampsia was measured to be unaltered with the marital status of the pregnant women being 3.3% among the unmarried and 3.0% among the married women (Raatikainen et al. 2005).

3.1.1. Clinical features and diagnosis of pre-eclampsia

Pre-eclampsia is a heterogeneous disorder manifesting after 20 weeks of pregnancy with a wide spectrum of clinical presentations (Datta. 2004, Steegers et al. 2010). Pre-eclampsia is defined as new-onset hypertension and proteinuria during the mid- or late gestation (Schroeder and American College of Obstetricians and Gynecologists. 2002). However, how to define the syndrome of pre-eclampsia in the best way, and how to differentiate between the mild and severe forms of the disease, has been debated. The updated diagnostic criteria of pre-eclampsia according to American College of Obstetricians and Gynecologists are shown in Table 1 (Steegers et al. 2010,

Table 1. Diagnostic criteria of pre-eclampsia.

Hypertension	Measured at least twice and 4-6 hours apart After 20 weeks of pregnancy and returning normal within 3 months postpartum Systolic blood pressure ≥ 140 mmHg and/or Diastolic blood pressure ≥ 90 mmHg
Proteinuria	≥ 300 mg per 24 hours / Urine dipstick ≥ +1

Schroeder and American College of Obstetricians and Gynecologists. 2002)

According to some of the classification systems, proteinuria is not obligatory for the diagnosis of pre-eclampsia (Datta. 2004). Furthermore, in case of superimposed disease, the diagnostic criterion for proteinuria can be also defined as "significant increase from the baseline" (Steegers et al. 2010).

The classification of "severe" pre-eclampsia varies, but one of the most commonly used criteria are either the blood pressure exceeding ≥ 160/110 mmHg and/or proteinuria ≥ 5 g during a 24-hour collection (Datta. 2004, Steegers et al. 2010). More detailed diagnostic signs of severe pre-eclampsia are defined in Table 2.

Table 2. Diagnosis of severe pre-eclampsia.

Severe pre-eclampsia is commonly diagnosed by the presence of one or more of the signs and symptoms listed in the table. The list has been modified after the practice guidelines by American College of Obstetricians and Gynecologists in 2002 and its update by Steegers et al. in 2010.

Blood pressure	• Systolic blood pressure ≥ 160 mmHg (and/or) • Diastolic blood pressure ≥ 110 mmHg
Proteinuria	• ≥ 3 - 5 g per 24 h (or) • Urine dipstick ≥ +++ in two random samples collected 4 h apart (or) • Sudden oliguria (urine excretion ≤ 500 mL / 24h) – especially with elevation in previous creatinine level

Other end-organ damage	
Central nervous system disturbance: headache, altered vision	Epigastric or right upper quadrant abdominal pain
Fetal compromise: IUGR, oligohydramnios	Thrombocytopenia (platelet count >100 000 mm^3)
Pulmonary edema / Cyanosis	Liver dysfunction / HELLP syndrome

HELLP = hemolysis, elevated liver-enzymes and low platelet count
IUGR = intrauterine growth retardation

A serious end phase of pre-eclampsia is, by definition, "eclampsia", characterized as presence of tonic-clonic seizures, also known as eclamptic convulsions (Datta. 2004, Zeeman. 2009). In addition, pre-eclampsia can be accompanied with other neurological manifestations including headache, visual disturbances, lethargy and confusion, or even more serious defects such as hemorrhagic complications, strokes or loss of consciousness (Zeeman. 2009). The incidence of eclampsia in Finland during 1990-1994 was 2.4 per 10 000 pregnant women, accounting for a total of 77 women in Finland (Salmi et al. 1999). Eight of them had serious complications, but none of them died. However, the perinatal mortality amongst their infants was 5%.

Pre-eclampsia can be classified according to the time of its onset. The subtype is called early-onset pre-eclampsia if the symptoms arise before 32 gestational weeks, and late-onset if they occur

after 37 weeks of pregnancy. Early-onset pre-eclampsia is associated with a 20-fold increase in maternal mortality compared with the late-onset form of the disease (Steegers et al. 2010).

3.1.2. Risk factors of pre-eclampsia

Pre-eclampsia affects about 5% of all pregnancies, the incidence varying between 2-8% depending on the measured population and the methods used (Duley. 2009, Duley et al. 2001, Saftlas et al. 1989). A number of risk factors for pre-eclampsia have been introduced in epidemiological studies. These predisposing factors include maternal chronic disease and special characteristics, genetic background and immunological exposure, many of which are also known to be associated with the pathophysiology or the inheritance of pre-eclampsia. (Datta. 2004, Chappell and Morgan. 2006, Dekker and Robillard. 2007). A modified list of the most common risk factors for it is shown in Table 3. (Datta. 2004, Barton and Sibai. 2008, Dekker and Sibai. 2001, Duckitt and Harrington. 2005).

Table 3. Risk factors of pre-eclampsia.

Factors predisposing to pre-eclampsia comprise of maternal special characteristics and chronic disease, genetic background and immunological exposure. Many of them also play a central role in the pathophysiology of pre-eclampsia.

Maternal pre-existing medical condition	Chronic hypertension / chronic renal disease / cardiovascular disease
	Autoimmune disease / antiphospholipid
	Thrombophilia (acquired or congenital)
	Obesity / insulin resistance / IDDM (gestational of type-1)
Personal and family history	Increasing maternal age / prolonging interval between pregnancies
	Nulliparity / teenage pregnancy
	Personal or family history of pre-eclampsia
	Ethnicity (Hispanic, African-American) / low maternal birthweight
	Adverse outcome in previous pregnancy (IUGR, abruption placenta, fetal death)
Pregnancy-associated factors	Multiple gestation / hydatidiform moles
	Urinary tract infection
	Congenital or chromosomal anomalies (trisomy 13, triploidy) / hydrops fetalis
Partner-related factors	Nulliparity / primipaternity
	Change of partner / teenage pregnancy
	Partner who has fathered pre-eclamptic pregnancy in another women
	Limited sperm exposure / donor insemination / IVF / use of donated oocytes

IDDM = insulin-dependent diabetes mellitus
IUGR = intrauterine growth retardation
IVF = *in vitro* fertilization

3.1.3. Genetics of pre-eclampsia

There is a wide spectrum of plausible evidence that the cause of pre-eclampsia is at least partly genetic, and the underlying patterns of its heritable aspects have been intensively studied (Roberts and Cooper. 2001). A familial predisposition to pre-eclampsia has been confirmed in a number of studies which have observed a 2–5 -fold increase in the risk of the disease among first-degree relatives to women with pre-eclampsia (Chappell and Morgan. 2006).

There is no universally accepted susceptibility gene for pre-eclampsia, but genome-wide linkage analyses of the affected families have yielded three loci with significant linkage and many additional loci that could be associated with pre-eclampsia to a minor extend (Chappell and Morgan. 2006). The three main loci have been identified in chromosomes 2p12, 2p25 and 9p13 (Arngrimsson et al. 1999, Laivuori et al. 2003).

Studies on more than 50 candidate genes, many of which have been suggested on the basis of the typical pathophysiological features of the established disease, have been reported, but only eight of them seem to account for about 70% of the publications concerning this matter (Chappell and Morgan. 2006). Furthermore, the results of these candidate gene studies are both conflicting and inconclusive. However, by combining data from the genetic studies it has been estimated that the genetic factors contribute to more than half of the risk of pre-eclampsia.

3.1.4. Prevention and treatment of pre-eclampsia

As the etiology of pre-eclampsia remains unclear, the only truly effective primary prevention method would be contraception (Dekker and Sibai. 2001, Briceno-Perez et al. 2009). However, manipulation of some of the known risk factors of pre-eclampsia can, be used as potential primary preventive strategies, at least theoretically. Such factors include, for instance, limited sperm exposure, paternal-dependent risk factors, obesity and insulin resistance.

Trials on secondary prevention, meaning break-off the disease process before its clinical manifestation, have been based on the known pathophysiological mechanisms of pre-eclampsia, mainly focusing on specific drugs (aspirin and other antiplatelet agents, progesterone, nitric oxide), nutritional supplies or dietary restrictions (calcium, antioxidants, fish oil, low dietary salt, folic acid, garlic, protein and energy supplementation or restrictions) and lifestyle choices (rest, exercise etc.) (Dekker and Sibai. 2001, Briceno-Perez et al. 2009). However, based on the latest Cochrane database systematic review and other meta-analyses, antiplatelet agents, largely low-dose aspirin, have only moderate benefits in prevention of pre-eclampsia, preterm birth, and adverse effect of them in fetal growth (Duley et al. 2007, Askie et al. 2007) as the risk of pre-eclampsia is lowered by 10-14%. The benefits of calcium supplementation have been observed only among mothers with high risk of pre-eclampsia or low dietary calcium intake (Dekker and Sibai. 2001, Briceno-Perez et al. 2009), although in them, calcium supplementation seems to halve the risk of the syndrome (Hofmeyr et al. 2010). Of the other methods or agents tested for secondary prevention of pre-eclampsia, there has been not enough evidence, or it has been insufficient in quality, to draw positive conclusions (Briceno-Perez et al. 2009).

By far the most common interventions considering pre-eclampsia have been associated with tertiary prevention. This refers to various kinds of attempts to lower the risk of complications caused by the disease, thus, being somewhat synonymous to “treatment” (Dekker and Sibai. 2001). The scope in the antenatal care of pre-eclampsia is to prolong the pregnancy, maximizing fetal viability, and to protect the mother from the adverse effects of the manifestations of the syndrome, most commonly hypertension (Datta. 2004). In fact, antihypertensive agents are the most commonly used medication in manifest pre-eclampsia (Datta. 2004). They protect the mother from the potential adverse effects of uncontrolled hypertension, especially intracerebral hemorrhage (Datta. 2004, Steegers et al. 2010). In addition, intravenous $MgSO_4$ can be used for both prevention and management of eclamptic convulsions (Datta. 2004, Steegers et al. 2010). Despite of many pharmacological advantages, timed delivery still remains of special importance in the tertiary prevention of pre-eclampsia (Datta. 2004, Steegers et al. 2010, Briceno-Perez et al. 2009).

3.2. Mechanisms of pre-eclampsia

The etiology of pre-eclampsia has been intensively searched, but a final explanation remains to be stated. Although commonly accepted as a key step in the pathogenesis of pre-eclampsia, the placental origin as a definitive cause of the disease has been challenged by several theories seeking for the underlying sources for the placental failure. Some observations have been suggested that mainly genetic factors (both fetal and maternal genes) would determine the risk of pre-eclampsia whereas environmental factors have been suggested to mainly interact with the genetic ones, determining the predisposition and severity of the disease (Datta. 2004, Chappell and Morgan. 2006). Interestingly, pre-eclampsia has been observed to be namely a disorder of the nullipara (Makkonen et al. 2000), supporting the theory of immune maladaptation as the main mediator of its pathogenesis (Dekker and Robillard. 2007). Recently, many of the studies on the pathogenesis of pre-eclampsia have focused on the angiogenic imbalance, which has been found as one of the leading disturbances in the placental level, possibly due to abnormal, initial placentation.

3.2.1. Placental vascular remodeling

Pre-eclampsia has been observed to take place only in the presence of the placenta. The pathophysiology of the disease has been characterized as placental hypoperfusion and ischemia, beginning from the implantation and early pregnancy. The most typical morphological changes noted in the uteroplacental vasculature of pre-eclamptic women is the impaired vascular remodeling of the spiral arteries, caused by abnormal trophoblast invasion and taking place toward the end of the first trimester (Figure 2, next page ➔). In pre-eclampsia, this process has been observed rudimentary leading to a cascade of its characteristic pathophysiological events (Brosens et al. 1972).

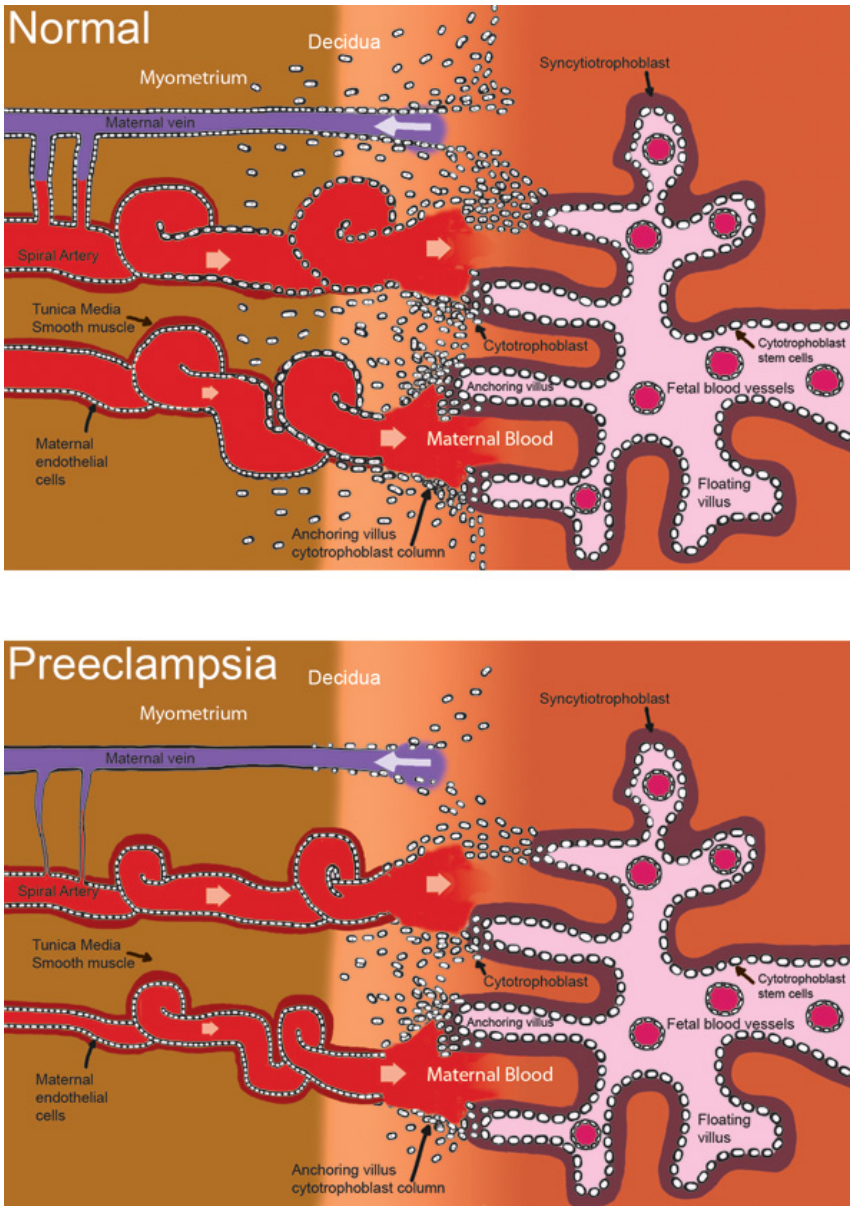
In pre-eclampsia, the cytotrophoblasts fail to mimic a vascular adhesion phenotype (Zhou et al. 1997a) and their invasion is shallow, limited to the superficial layer of the decidua (Figure 2.). They fail to transform the maternal spiral arteries to high-caliber capacitance vessels, and they remain small-caliber resistance vessels. Consequently, the placental morphology is characterized as narrow myometrial arteries and minimal arteriovenous shunts in women with subsequent pre-eclampsia. These placental vessels are unable to provide adequate perfusion considering the demands of the growing fetus. Consequently, the failed trophoblast invasion and the impaired vascular remodeling lead to hypoperfusion of the placenta (Brosens et al. 1972).

Figure 2. (Next page ➔). In normal pregnancy, the remodeling of the maternal spiral arteries is caused by the “endovascular” cytotrophoblast cells, which invade the narrow, highly coiled, musculo-elastic spiral arteries of the decidua, until the inner third of the myometrium (top). As a result, the maternal spiral arteries are widened to form high-caliber low-resistance channels, which are no longer responsive to vasomotor influences. During the invasion, this specific population of trophoblasts transforms into endothelial phenotype cells, which replace the maternal endothelium of the spiral arteries in a process called “pseudovasculogenesis”.

The abnormal trophoblast invasion and the following impaired vascular remodeling of the maternal spiral arteries are characteristic of pre-eclampsia (bottom). The cytotrophoblasts fail to mimic a vascular adhesion phenotype resulting in shallow invasion and failure in the transformation of the maternal spiral arteries into high-caliber capacitance vessels instead of small-caliber resistance vessels. Consequently, the placental morphology is characterized as narrow myometrial arteries and minimal arteriovenous shunts in women designated to develop pre-eclampsia.

The impaired vascular remodeling lead to placental hypoperfusion and following disturbances in providing adequate perfusion for demand of the growing fetus. Furthermore, the insufficient placentation has been suggested to lead to a cascade of typical pathophysiological events known as the maternal disease of pre-eclampsia.

Figure 2. The impaired trophoblast invasion and remodeling of the spiral arteries in the pathophysiology of pre-eclampsia.



3.2.2. Maternal endothelial dysfunction

The failure in remodeling the placental bed in early pregnancy and the subsequent placental ischemia has been suggested to lead to generation releases some vasoactive substances into the maternal circulation which further act on maternal endothelium (Roberts et al. 1989, de Groot and Taylor. 1993). This has been postulated to result in an endothelial cell disorder throughout the vasculature and lead to the multi-organ failure, which can be detected in pre-eclampsia (Roberts et al. 1989, de Groot and Taylor. 1993). This vascular endothelium is considered as the

target organ of the placental pathophysiological changes in the disease.

The most important endothelial cell functions associated with pre-eclampsia are increased vascular tone, lipid uptake, intravascular coagulation, vascular permeability, and inadequate immunological response (de Groot and Taylor. 1993). The clinical symptoms of pre-eclampsia reflect these malfunctions of the maternal endothelial cells (Roberts et al. 1989, de Groot and Taylor. 1993). Most often the endothelial cell dysfunction is described as a cascade of coagulation, vasoconstriction and intravascular fluid redistribution, together of which manifest as the clinical syndrome of pre-eclampsia finally leading to vasoconstriction and end-organ ischemia (Roberts et al. 1989).

3.2.3. Hemodynamic changes

Arterial blood pressure and peripheral vascular resistance decrease during normal pregnancy. However, because of the overt vasoconstriction associated with the clinical symptoms of pre-eclampsia, the systemic vascular resistance remains higher whereas the cardiac output stays lower in women with this pregnancy complication (Datta. 2004). Women with subsequent pre-eclampsia represent altered vasorelaxation independent of the endothelium (Khan et al. 2005). Furthermore, there is a slight increase in the blood pressure preceding the onset of the manifest symptoms of the disease.

3.2.4. Edematous changes in kidney and brain

Pre-eclampsia and eclampsia are associated with a global hypoperfusion of organs. Disordered maternal endothelium is abundant in the kidney, representing one of the main characteristic pathologic steps of pre-eclampsia: glomerular endotheliosis.

Eclampsia is often characterized as cerebral edema and intracerebral parenchymal hemorrhage. The cerebral edema, however, is independent of the severity of hypertension, and instead of being caused by the blood pressure elevation it has been suggested to result from the endothelial dysfunction (Zeeman. 2009).

3.3. Prediction of pre-eclampsia

Despite of decades of attempts to identify the women “at risk” of pre-eclampsia, it remains a critical aim for research. Many potential tests have been assessed for their ability to detect the women at risk of pre-eclampsia already during the early pregnancy. These setting have commonly assessed placental perfusion or vascular resistance, and serum or urine markers of placental distress, renal and endothelial dysfunction, oxidative stress, and insulin resistance. Other measured compounds have been fetal or placental –derived products and hormones (Steegers et al. 2010, Carty et al. 2008). A subset of angiogenic factors has recently been under extensive research as potential contributor to the pathogenesis and thereby offering a predictive tool for subsequent pre-eclampsia.

Only a few of the nearly 30 tests evaluated in one of the lately published reviews reached specificity above 90% (Meads et al. 2008). No single test, however, met the clinical standards for a predictive test, which was concluded also by authors of another systematic review (Conde-Agudelo et al. 2004). Although new maternal circulating candidates, such as soluble endoglin, activin-A, inhibin-A, pregnancy-associated plasma protein-A (PAPP-A), and placental protein 13 (PP13), have been introduced for potential screening purposes, it has become obvious that a single biomarker is unlikely to predict all forms of such a multifactorial syndrome as pre-eclampsia (Thangaratinam et al. 2008).

Recently, it has been proposed that a combination of tests, such as ultrasound screening (uterine artery Doppler waveforms, placental thickness and homogeneity) and analyses of circulating markers associated with placental insufficiency would increase the utility of former assessments (Steegers et al. 2010). As an example, a logistic regression analysis combining uterine artery pulsatility index, mean arterial pressure, serum PAPP-A, serum placental growth factor (P*l*GF), body-mass index (BMI), and the presence of nulliparity or previous pre-eclampsia has been observed to have high sensitivity and specificity in prediction of early onset pre-eclampsia (Steegers et al. 2010). In addition,

ratios of factors associated with the possible underlying angiogenic imbalance in pre-eclampsia distinguish between the women with subsequent disease later in pregnancy (Steegers et al. 2010, Carty et al. 2008).

3.4. Intrauterine growth retardation (IUGR)

Adequate fetal growth is dependent of four principal determinants. First, each fetus has a unique genetic growth potential associated with parental genetic properties. The respective growth potential is later modified by the placental function as well as maternal and fetal health. If all of the last three variables are unaffected during the pregnancy, the fetus will achieve the genetically determined growth potential.

3.4.1. Classification of disturbances in fetal growth

The term "small for gestational age" (SGA) refers to a small infant, failed to achieve a gender-specific weight threshold, most often defined as the 10th percentile of the referred population (Bamberg and Kalache. 2004). However, this definition will lead to the fact that about 10% of healthy population is classified as SGA. To avoid over-treatment, it is important to distinguish between the small but healthy fetuses and the ones with compromised fetal growth. Therefore, customized fetal weight centiles have been studied as a more precise tool for assessing potential growth disturbances *in utero* (Gardosi. 2009).

Importantly, different from the population-based SGA fetuses, a real compromise in fetal growth should be diagnosed with intrauterine growth retardation (IUGR). In this subgroup, the neonates fail to achieve the growth potential they were assumed according to the principals above (Baschat. 2004). These infants are usually also SGA, but not as a rule (Bamberg and Kalache. 2004).

In developing countries, the valid information of the duration of gestation is often impossible to obtain, and the incidence of low birthweight (LBW) is often used as a proxy to represent the occurrence of IUGR. Worldwide, almost 16% of newborns were classified as LBW infants during 1997–2001 (UNICEF. 2004). However, up to 96% of them are born in developing countries where the incidence of LBW has been estimated to be 2.5 times that of industrialized countries (UNICEF. 2004).

In Finland, SGA infants accounted for 2.0% of the infants born after singleton pregnancies during 2007–2008 (Vuori and Gissler. 2010). At this time period, the percentage of LBW infants (< 2501 g) was 3.2% in singleton and 4.4% in all pregnancies with representative percentages for preterm births of 4.5% and 5.7%. The occurrence of very low birthweight (VLBW; < 1501 g) newborns was 0.9% including also twins and triplets. Perinatal mortality in Finland has long been one of the lowest in the world, being 5.0 per 1000 children (Vuori and Gissler. 2010).

3.4.2. Diagnosis and risk factors of IUGR

Detection of the possible disturbances in fetal growth is essential, as it may improve the antenatal outcome of the fetus that is at risk of complications from the growth retardation. The main approach is to separate IUGR resulting from placental disease from fetuses with underlying etiologies or those constitutionally small ones. Thus, the Doppler investigation of fetoplacental blood flow accompanied with the ultrasound assessment of amniotic fluid volume and fetal anatomy and growth play a central role in the diagnosis (Baschat. 2004).

Growth retardation implies that fetal growth is compromised by factors originating in the mother and her environment, the placenta or the fetus. Besides fetal chromosomal and structural abnormalities, impaired placental vascular development accounts for most of IUGR diagnoses in otherwise healthy, singleton pregnancies in the Western world (Baschat. 2004, Grivell et al. 2009).

Logically, IUGR is associated with increased perinatal mortality and morbidity. Furthermore, there might also be an increased risk of health problems in the adulthood of the offspring, such as type-2 diabetes, cardiovascular disease and metabolic syndrome (Barker. 2002).

3.4.3. Pathophysiology of IUGR and possible association with pre-eclampsia

Especially when starting early in gestation and not being secondary to any other pregnancy complication, IUGR has been suggested to be a form of pre-eclampsia affecting only the myometrium, placenta, and the fetus, although not causing any systemic effects on the maternal endothelium (Regnault et al. 2002). The hypothesis has been based on the findings on similar vascular defects: poor vascularization of placental bed spiral arteries has been detected in both of the conditions (Khong et al. 1986). Therefore, they have been postulated to share a common etiology associated with vascular defects (Regnault et al. 2002, VanWijk et al. 2000, Myatt. 2002). It also appears likely that factors regulating vascularization and angiogenesis in early placenta play a role in the pathophysiology of these diseases.

4. Smoking during pregnancy

4.1. Smoking during pregnancy

Smoking among pregnant women is associated with a number of adverse fetal and obstetrical complications (Higgins. 2002). These comprise placenta previa, placental abruption, premature rupture of the membranes, preterm birth, IUGR and sudden infant death syndrome (SIDS; Higgins. 2002, Andres and Day. 2000). Cigarette smoking during pregnancy is a major risk for pregnancy-related morbidity and mortality worldwide (Higgins. 2002, Andres and Day. 2000).

In Finland, the smoking rate during pregnancy in 1997 was counted amongst 15% of pregnant women (Jaakkola et al. 2001). Smoking was defined as cigarette smoking daily or occasionally at some point during pregnancy. The information retrieved from a questionnaire was highly comparable to that of the medical records and the Finnish Medical Birth Registry data on smoking during pregnancy. The prevalence of smoking during pregnancy in Finland in this study was similar to that observed ten years earlier, in 1987 (Jaakkola et al. 2001). According to the Finnish Medical Registry data, there had been no change in the percentage of children exposed to maternal smoking *in utero* during the next decade as its incidence was still 14.7% in 2008, and slightly higher (17.5%) when counting only the infants of primiparas (Vuori and Gissler. 2010). However, 30% of the mothers quit smoking after the first trimester in the same year. In the western countries, it has been approximated that about 10–20% of all women smoke during pregnancy (Andres and Day. 2000, Ananth and Cnattingius. 2007).

The adverse effects of smoking on pregnancy have been suggested to be produced mostly by nicotine, but also by the other numerous toxins detected in cigarette smoke, e.g. carbon monoxide, lead, selenium and cadmium (Leonardi-Bee et al. 2008, Pulkkinen. 1989). Fetal and neonatal nicotine exposure alone leads to major adverse postnatal health consequences (Bruin et al. 2010).

A list of the fetoplacental consequences of maternal smoking in early and late pregnancy is shown in Table 4. (Jauniaux and Burton. 2007).

Table 4. Placental and fetal effects of maternal smoking in early and late pregnancy. The list has been modified after Jauniaux et al. (2007).

Early pregnancy	Miscarriage Ectopic pregnancy Placenta previa and placenta previa-accreta Fetal orofacial clefts
Late pregnancy	Intrauterine growth retardation Placental insufficiency Placental abruption Premature rupture of the placental membranes Preterm delivery Perinatal death

4.2. Vascular effects of nicotine exposure

Exposure to cigarette smoke *in utero* is associated with later hypertension also in humans (Beratis et al. 1996, Blake et al. 2000). Based on animal studies, this phenomenon is likely to be mediated by nicotine (Gao et al. 2008). In rats, fetal and neonatal exposure to nicotine also causes increases in postnatal blood pressure due to endothelial dysfunction (Xiao et al. 2007) and/or changes in renal structure or function (Mao et al. 2009, Pausova et al. 2003). Administration of high doses of nicotine to animals during pregnancy has adverse consequences on both the maternal and fetal cardiovascular such as subsequent type II diabetes, obesity, hypertension and respiratory dysfunction (Bruin et al. 2010).

4.3. Placental effects of smoking

Nicotine can travel across the placenta, after which it accumulates in the amniotic fluid and the fetal blood. Also cotinine, the main metabolite of nicotine crosses the placenta (Pastrakuljic et al. 1998). Thus, the fetuses of smoking mothers are exposed to even relatively higher cotinine concentrations as compared to their mothers, and there is a positive correlation between maternal serum cotinine concentrations and those in fetal serum and amniotic fluid throughout pregnancy (Jauniaux et al. 1999).

Cadmium, Cd, is a common environmental pollutant, which is also present with high amounts in tobacco smoke. It has been observed to cause serious disturbances in human reproductive functions. During pregnancy, Cd is transferred to the fetus through the placenta, although it also accumulates in the placenta probably preventing the fetus from the toxic effects of the metal (Pulkkinen. 1989, Jauniaux et al. 1999).

Smoking results in many placental morphological changes related to the villous cytotrophoblast proliferation and differentiation (Genbacev et al. 1995, Genbacev et al. 2000, Genbacev et al. 2003) as well as the placental vasculature (Pfarrer et al. 1999, Zdravkovic et al. 2005). Some of the placental defects may result in reduction of the placental vascularization already in earlier stages of pregnancy. These morphological changes begin to develop in early pregnancy resulting in alterations in the biological functions of both the trophoblastic and fetal cells, mainly associated with synthetic (protein metabolism) and enzymatic activity (reviewed in Zdravkovic et al. 2005).

The dysregulation of the placental blood vessels in connection to maternal smoking has been assumed to be due to some compensatory mechanisms in the placental blood flow (Pfarrer et al. 1999, Zdravkovic et al. 2005). The relative volumes of maternal intervillous space and surface area of fetal capillaries are increased whereas both the relative and absolute volumes of the fetal capillaries are reduced, which is suggested to happen as a result of a decrease in mean capillary diameter rather than total length (Bush et al. 2000).

Interestingly, the alterations seen in smoking women are in contrast to the main adaptive changes associated with pregnancies with preplacental hypoxia, suggesting that the fetoplacental unit might be affected by some other factors connected to cigarette smoke. A modified list of the placental morphological changes associated with maternal smoking is shown in Table 5.

Table 5. Placental morphological changes associated with maternal smoking.
The table has been modified after Jauniaux and Burton (2007).

Increased	Trophoblastic and villous membrane thickness Thickness of the trophoblast basal membrane Necrosis of the syncytiotrophoblast Apoptosis in the syncytiotrophoblast Expression of pVHL, HIF, and VEGF Simulation of nicotinic acetylcholine receptors Vasoconstrictive response to endothelin-1
Decreased	Decrease in villous capillary volume fraction Decrease in total surfaces of syncytial knots Progesterone synthesis

pVHL = von Hippel-Lindau tumor suppressor protein HIF = hypoxia-inducible transcription factor
VEGF = vascular endothelial growth factor

4.4. Effects of smoking on perinatal outcome

Smoking has been referred to as the cause of about 15% of all preterm births and 20–30% of all infants born with a low birthweight, which are associated with increased risk of neonatal morbidity and mortality (reviewed by Andres and Day. 2000). Indeed, maternal tobacco use has been suggested to increase the overall perinatal mortality by 150%.

The direct effect of the cigarette smoke toxins on fetal cell proliferation and differentiation could explain the increased risk of miscarriage, IUGR, stillbirth, preterm birth and placental abruption reported by epidemiological studies (Higgins. 2002, Andres and Day. 2000). In the newborn, maternal smoking has been noted to associate with a decrease in birthweight, newborn fat mass, and changes in protein metabolism and enzyme.

4.5. Smoking and pre-eclampsia

In a systematic review of 28 cohort studies considering the association between cigarette smoking during pregnancy and pre-eclampsia, each study reported a negative connection between the incidence of pre-eclampsia and cigarette smoking during pregnancy (Conde-Agudelo and Belizan. 2000).

In a recent study on certain high-risk groups, smoking was associated with changes in the concentrations of pro-angiogenic factors during early pregnancy, but no obvious association with subsequent pre-eclampsia was observed (Jeyabalan et al. 2008). A more detailed review of the angiogenic factors in connection to smoking is given in Chapter 5 and the risk factors of pre-eclampsia are reviewed in Chapter 3.

5. Vascular endothelial growth factor (VEGF) family and soluble VEGF receptor-1

The human VEGF gene family comprises VEGF-A (usually referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (P*l*GF). Besides them, alternative non-mammalian homologs of VEGF, often referred to as viral VEGF (VEGF-E) and snake venom VEGF (VEGF-F), have been identified (Lyttle et al. 1994, Takahashi et al. 2004). All of the VEGFs above are dimeric glycoproteins. Each of the VEGF family members contains of a central VEGF homology domain (VHD) consisting of 92–96 amino acids (Yamazaki et al. 2009). They share 29–64% of their VHD regions, but the eight cysteine residues of the VHD can be seen in all members and they are highly conserved. Besides of the central VHD, each molecule has N and C – terminal extensions (Yamazaki et al. 2009).

Each of the VEGF family members binds, in unique ligand-specific manner, to some of the three tyrosine kinase receptors (RTK), known as the VEGF receptors. VEGFR-1 (also referred as fms –like tyrosine kinase-1, Flt-1), VEGFR-2 (kinase insert domain receptor, KDR), and VEGFR-3 (Flt-4). Of these endothelial cell membrane receptors, VEGFR-1 and -2 are usually distributed on vascular endothelial cells mediating a number of angiogenic signals, such as endothelial cell proliferation and migration. In contrast, VEGFR-3 is mainly expressed on the lymphatic endothelium taking part in lymphangiogenesis (Shibuya et al. 1999).

5.1. VEGF-A

VEGF is known as a central angiogenic molecule during development, adult physiology and pathology (Ferrara et al. 2003). It was earlier known as the vascular permeability factor (VPF) identified in 1983 in the supernatant of a quinea-pig tumor cell line by Senger and colleagues (reviewed in Ferrara 2009: History of Discovery). However, attempts to purify or sequence the protein were not successful.

Six years later, in 1989, Napoleone Ferrara isolated and identified a novel endothelial cell mitogen from medium conditioned by bovine pituitary follicular cells. He had begun the effort as a postdoctoral fellow in the early 1980s. Since this newly found protein displayed growth-promoting activity only on vascular endothelium, it was named as “vascular endothelial growth factor” (Ferrara and Henzel. 1989).

Ferrara and his colleagues continued their intensive research on VEGF, and soon, they isolated clones of the first three VEGF isoforms (Leung et al. 1989). Concomitantly, the earlier work on VPF had been followed up by another group of scientists, led by Daniel Connolly. They had finally advanced in further analyses on VPF, ending up in the cloning of the protein. For their great surprise, the human clone of VPF, described by the Connolly’s team, encoded a protein identical to $VEGF_{189}$.

5.1.1. Structure

The best known subtype of VEGF-A, $VEGF_{165}$, from here on referred to as VEGF, is a secreted, homodimeric, heparin-binding and glycosylated protein, and it is about 46 kDa of its molecular size (Ferrara et al. 2003). Due to alternative exon splicing, at least eight other VEGF isoforms varying by the number of amino acids have so far been identified: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$b, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$ (reviewed in Takahashi and Shibuya. 2005). Of these VEGF subtypes, VEGF121, $VEGF_{165}$ and $VEGF_{189}$ are the predominant ones, and they can be secreted by most cell types (Robinson and Stringer. 2001).

$VEGF_{165}$b, is a unique form of VEGF acting in an inhibitory fashion: it binds to VEGFR-2 with the affinity of $VEGF_{165}$ without resulting in the activation of the receptor and the subsequent downstream signaling cascade (Woolard et al. 2004). Different biological functions of VEGF isoforms have been detected in studies on isoform-specific VEGF knockout mice concluding that $VEGF_{165}$ is the main mediator of the actions of VEGF (reviewed in Ferrara. 2004).

5.1.2. Function

VEGF is known for its two main biological functions: First, it is able to stimulate vascular endothelial cell proliferation. It enhances mitogenesis and angiogenesis of the endothelial cells (Ferrara. 2004). Second, it has a capacity to increase vascular permeability. Indeed, VEGF was originally described as a permeability factor (Ferrara et al. 2003), and it was later described to cause vasodilatation inducing the endothelial nitric oxide synthase (eNOS) leading to an increase in nitric oxide production (Fukumura et al. 1997). Besides the two main features above, VEGF acts as a survival factor for the endothelium during both physiological and pathological angiogenesis.

VEGF is essential for embryonic development as homozygous VEGF knockout mice die at embryonic day E8–E9 due to failure in blood island formation, defects in vascular formation and disturbances in endothelial cell development (reviewed in Ferrara. 2004). Also the concentrations of VEGF during early development seem to be critical: even the lack of a single VEGF allele causes death of the mice *in utero* by E12.5 representing serious disturbances in the vascular system (reviewed in Ferrara. 2004).

5.1.3. Sites of expression

Vascular endothelial growth factor is expressed by various adult and fetal tissues as well as by various cultured cell types (Maharaj et al. 2006). Besides of these normal tissues, it is widely expressed in tumors (Hicklin and Ellis. 2005). During organogenesis, VEGF plays an essential role in the development in the formation of the cardiovascular system (Haigh. 2008). VEGF can be detected in fetal circulation at the delivery and in neonatal venous blood at least during the first postnatal days.

Throughout human life, VEGF is a major mediator of angiogenic signals. However, angiogenesis is restricted to certain physiological processes during adulthood. Thus, the expression pattern of VEGF is greatly altered in connection with these individual angiogenic events. In human physiology, VEGF is important especially during the female reproductive cycle in the uterus, ovary and breast, and in skeletal muscle response to exercise. The expression of VEGF is also up-regulated in relation to the angiogenic actions in wound healing and bone repair. (Maharaj et al. 2006)

In female reproduction, increased VEGF production has been associated with endometriosis (McLaren et al. 1996), ovarian hyperstimulation syndrome (Levin et al. 1998), and in patients with hydatidiform mole (Nomura et al. 1998). Of the numerous malignant tumors, which have been observed to express high amounts of VEGF, many are located in the female reproductive tract. For example, they include tumors of the ovary, endometrium, uterine cervix and the breasts (Doldi et al. 1996, Guidi et al. 1995, Guidi et al. 1997).

5.1.4. Regulation of expression

Hypoxia rapidly and reversibly up-regulates the gene expression of VEGF both *in vitro* and *in vivo* (reviewed in Takahashi and Shibuya. 2005, Ferrara. 2004). This up-regulation is mediated by hypoxia-inducible factor-1 (HIF-1), which is a transcriptional activator including two subunits, HIF-1α and HIF-1β (Semenza. 2002).

The expression of VEGF can also be increased by some other growth factors or nitric oxide (NO), which regulate the release of VEGF in a paracrine or autocrine fashion in response to local hypoxia (Ferrara. 2004). Transforming events, such as oncogenic mutation and existence of tumor promoters have also been shown to result in induction of VEGF up-regulation (Ferrara. 2004). Besides them, a variety of hormonal substances have been shown to induce VEGF expression in different contexts (reviewed in Ferrara. 2004). These hormones comprise, for example, the thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), sex steroids (estradiol and progestins), and the gonadotropins e.g. follicle-stimulating hormone (FSH), luteinizing hormone (LH), and human chorionic gonadotropin (hCG).

5.2. P*I*GF

Placental growth factor, a dimeric 46 kDa protein structurally closely related to VEGF, was identified 20 years ago (Maglione et al. 1991). P*I*GF$^{-/-}$ knockout mice are born without an apparent phenotype. Thus, it was first suggested that the loss of P*I*GF gene would not affect development, reproduction or the postnatal life. However, further studies have shown that under pathologic conditions, such as ischemia, these mice have major impairment in their ability to respond to the ischemic damage through angiogenesis and adaptive arteriogenesis. Thus, P*I*GF is now considered mainly arteriogenic of its properties (reviewed in Ribatti. 2008).

Following the alternative mRNA splicing of the primary transcript, four subtypes of human P*I*GF have been detected: P*I*GF$_{131}$, P*I*GF$_{152}$, P*I*GF$_{203}$, and P*I*GF$_{224}$, also referred to as P*I*GF -1, P*I*GF-2, P*I*GF-3 and P*I*GF-4, varying in terms of the number of amino acids, their secretion properties and their binding affinities.

P*I*GF-1 is considered as the main form of the protein. It mediates its activity via VEGF receptor-1 (see below), and unlike the other isoforms, it does not bind to heparan sulfate proteoglycans (HSPG) or the neuropilins (reviewed in Ribatti. 2008). P*I*GF contributes to angiogenesis by different mechanisms: First, acting directly on the endothelial cells via VEGFR-1, it can drive VEGF to bind and activate VEGFR-2 and stimulate the VEGF sensitivity of the cell. Second, P*I*GF takes part in angiogenesis as it recruits monocytes/macrophages, which play an important role in vessel growth, and it mobilizes hematopoietic progenitor cells from the bone marrow (reviewed in Ribatti. 2008).

P*I*GF gene is highly expressed at all stages of pregnancy, although the amount of P*I*GF increases toward the terminal development of the placenta (Khaliq et al. 1996, Persico et al. 1999). Both P*I*GF protein and mRNA have been observed in the trophoblastic bilayer as well as in the villous mesenchyme. Although first found in the placental tissue, P*I*GF mRNA has later been detected in a number of other organs including the heart, lung, skeletal muscle, thyroid and adipose tissue (Persico et al. 1999, Voros et al. 2005).

5.3. Other members of VEGF family

5.3.1. VEGF-B

Another member of the VEGF family, later named as VEGF-B because of its structural similarities with VEGF, was characterized in 1996 by two independent research groups (Grimmond et al. 1996, Olofsson et al. 1996). VEGF-B is also produced in two different secreted sybtypes, resulting in a 21 kDa, non-glycosylated HSPG-binding form (VEGF-B$_{167}$), and a 32 kDa, O-glycosylated and therefore freely diffusible form of the protein (VEGF-B$_{186}$; Olofsson et al. 1996). Unlike VEGF, hypoxia does not seem to upregulate the expression of VEGF-B (Olofsson et al. 1996, Lagercrantz et al. 1996).

VEGF-B is abundant in highly metabolic organs and tissues, such as the skeletal muscle, brown fat and the myocardium. VEGF-B mediates its actions via VEGFR-1 (Olofsson et al. 1998). However, there is no clear evidence for the mitogenicity of VEGF-B *in vivo*. It has been suggested to have important non-vascular roles, perhaps in the survival of smooth muscle cells by inhibiting apoptosis and inducing adequate inflammatory responses (reviewed in Cao. 2009).

5.3.2. VEGF-C

Like the other members of this protein family, VEGF-C is a secreted and further proteolysed homodimeric glycoprotein, approximately 21 kDa of its final size in the extracellular environment (Joukov et al. 1996, reviewed in Tammela et al. 2005). VEGF-C binds to VEGF receptor-2 and -3 (Joukov et al. 1996, Lee et al. 1996).

Of the members of VEGF family, VEGF-C and VEGF-D are best known for their ability to induce growth of the lymphatic vasculature in mice. However, to some extend they are also mitogenic for vascular endothelial cells during embryonic development (Tammela et al. 2005, Enholm et al. 1998, Enholm et al. 1998, Jeltsch et al. 1997, Joukov et al. 1998). Like VEGF-B, the expression of VEGF-C is unaffected by hypoxia (Enholm et al. 1998).

VEGF-C has been detected in various human tissues, including prolactin-secreting cells in the

anterior pituitary, heart, vascular smooth muscle cells, small intestine and thyroid gland, ovary, and placenta (Joukov et al. 1996, Partanen and Paavonen. 2001, Gu et al. 2006). In the placenta, VEGF-C has been detected in villous core endothelium as well as in invasive cytotrophoblasts during early gestation, but not at term (Gu et al. 2006, Zhou et al. 2002).

5.3.3. VEGF-D

VEGF-D was first isolated as a fibroblast growth factor (FGF), but was then noticed to have highly similar structural properties as the VEGF family (Orlandini et al. 1996, Yamada et al. 1997). It has many common features with VEGF-C as it also binds to VEGFR-2 and VEGFR-3, and they also share many details in their expression pattern. VEGF-D is, indeed, strongly expressed in the heart, muscle and small intestine (Joukov et al. 1996, Lee et al. 1996, Achen et al. 1998).

VEGF-D is present in mice and human embryonic lungs, thereby considered to be of special importance during vascularization of lung tissue at the last trimester of pregnancy (Tammela et al. 2005, Farnebo et al. 1999). In humans, VEGF-D can be detected in vascular smooth muscle cells, lungs, heart, small intestine, skeletal muscles, the innermost zone of adrenal cortex, and serotonin-positive enteroendocrine cells later, in the adulthood (Yamada et al. 1997). In the placenta, VEGF-D is localized in syncytiotrophoblasts and villous core endothelium (Gu et al. 2006).

In human disease, the lymphangiogenic growth factors VEGF-C and VEGF-D, and their receptors, have been shown to be associated with lymphedema, Kaposi´s sarcoma, vascular tumors and tumor metastases (Jussila and Alitalo. 2002).

5.3.4. VEGF-E and VEGF-F

VEGF homologues have also been found in the genomes of Orf viruses, which have been shown to have VEGF –like activities. VEGF-E refers to a group of these proteins, of which at least five have been described in the literature (reviewed by Joukov et al. 1998 and Shibuya. 2003). All forms of VEGF-E are able to selectively bind and activate VEGFR-2, which provides a unique tool for studies on the biological function of this specific subtype of the VEGF receptor (Shibuya. 2003). Furthermore, its affinity to VEGFR-2 is very high.

As reviewed by Yamazaki et al. 2009, snake venom has been found as an exogenous source of unique VEGF –like protein, recently named as "VEGF-F" (Yamazaki et al. 2009). Two different forms of VEGF-F, Vammin and VR-1, were the first snake venom VEGFs to be found. They are homodimeric, heparin-binding proteins, similar to other VEGF subtypes, although with distinct biochemical properties. Vammin and VR-1 bind only VEGFR-2 with high affinity. However, two novel forms of VEGF-F have been shown to bind VEGFR-1, although preferring VEGFR-2.

Of the snake venom VEGFs, at least Vammin and VR-1 have been noted to have hypotensive potential. Furthermore, they can induce vascular permeability, in which the response has been observed to be stronger than after stimulus enhanced by human VEGF. Besides those longer known VEGF-Fs, Yamazaki et al. showed that the venom -type VEGFs are widely found in several viper venoms, and they are highly variable in structure and function among species (Yamazaki et al. 2009).

5.4. VEGF –receptors

The members of the VEGF family bind to one or two of the three VEGF receptors with different affinity and selectivity. For example, VEGF binds both VEGFR-1 and -2, although the latter one with a 10-fold affinity, whereas VEGF-B and P*l*GF are specific to VEGFR-1. Upon binding their ligands, the VEGFRs dimerize and become phosphorylated as a positive response to the stimulus (Shibuya et al. 1999). A schematic presentation of the mammalian VEGF family, including also the soluble receptors (introduced above), is shown in Figure 3.

In addition to these receptor tyrosine kinase –type (RTK) receptors, some non-RTK –type receptors, neuropilin-1 (NRP-1) and heparan sulfate proteoglycans (HSPGs), have been found as co-receptors for VEGFs. Certain subtypes of VEGF, VEGF-B, and P*l*GF can also bind to NRP-1, NRP-2, or HSPG with their C-terminal regions, which results in modulation of the signaling via the VEGFRs (Ferrara. 2004).

Figure 3. The VEGF family.

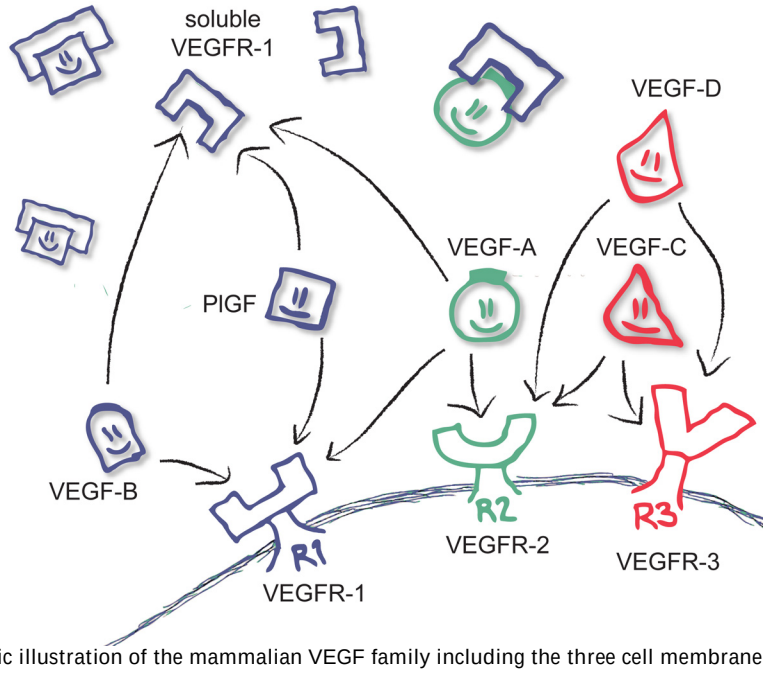


Figure 3. Schematic illustration of the mammalian VEGF family including the three cell membrane receptors named as VEGFR-1, VEGFR-2 and VEGFR-3, and their five ligands: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF. The figure demostrates the unique binding profile of each one of the ligands for one or two of the cell membrane receptors. In addition, VEGFR-1 and VEGFR-2 are known to occur in a soluble form *in vivo*, e.g. in the human circulation. The figure also represents the physiological role of soluble VEGFR-1 (sVEGFR-1) in antagonizing VEGF-A, VEGF-B and PlGF.

5.4.1. VEGFR-1

VEGFR-1, a 180 kDa membrane-bound protein, was the first VEGF receptor to be described in 1990 (de Vries et al. 1992). VEGFR-1 binds VEGF with high affinity but induces rather weak kinase activity (Shibuya. 2006). It also binds P*l*GF and VEGF-B, although with even weaker affinity than VEGF-A (Park et al. 1994, Sawano et al. 1996).

Knockout models of VEGFR-1 have revealed that these null-mutant mice die due to overgrowth and disorganization of blood vessels at E8.5–9.5 (Fong et al. 1995). Thus, during embryogenesis, it firstly acts as a negative regulator of angiogenesis by trapping free VEGF and thereby suppressing the accessibility of VEGF for its other receptor, VEGFR-2, on the endothelial cell surface (Shibuya. 2006).

Secondly, during adulthood, VEGFR-1 mediates paracrine signals on the endothelium. On the other hand, the VEGFR-1 on macrophages is likely to potentate the angiogenic actions mediated by VEGFR-2. Furthermore, VEGFR-1 mediates signals of vascular permeability with inflammation, and stimulates tumor growth and metastasis.

VEGFR-1 is expressed in endothelial cells and pericytes, as well as monocytes / macrophages and osteoblasts, placental trophoblasts, renal mesangial cells and also in some hematopoietic stem cells. VEGFR-1 has been observed in the placenta and in uterine smooth muscle cells. VEGFR-1 expression is increased at the sites of angiogenesis. Unlike VEGFR-2 and VEGFR-3, hypoxia up-regulates the VEGFR-1 gene, which is mediated by a HIF-1 –dependent mechanism (Gerber et al. 1997).

5.4.2. VEGFR-2

The second one of the three VEGF receptors, VEGFR-2, is structurally quite closely related to

VEGFR-1 (Terman et al. 1992). The mature receptor is a 230 kDa glycosylated protein (Sait et al. 1995, Takahashi and Shibuya. 1997), which has been shown to bind VEGF, VEGF-C and VEGF-D. Later on, the Orf-virus VEGFs, also called VEGF-E, have also been detected to be specific ligands for VEGFR-2. A growing amount of studies have led to the understanding that VEGFR-2 is actually the primary receptor mediating the activating signals of VEGF on the endothelium (Shibuya. 2006).

VEGFR-2 mediates most of the endothelial growth and survival signals whereas based on the actions of VEGFR-1, as described above, it is commonly discussed to have a "dual role in angiogenesis" (Shibuya. 2006, Zachary and Gliki. 2001). Null-mutation of VEGFR-2 in mice results in embryonic death at day E8.5-E9.5 due to lack of development of the blood islands, embryonic vasculature and hematopoietic cells (Shalaby et al. 1995). VEGFR-2 expression is down-regulated in the adult vascular endothelial cells, and is again up-regulated in the endothelium of angiogenic blood vessels (Partanen et al. 1999).

Based on animal studies, also VEGFR-2 is highly specific to vascular endothelium throughout the body, but besides the endothelium, VEGFR-2 have been localized on neuronal cells, osteoblasts, pancreatic duct cells, retinal progenitor cells, megakaryocytes and hematopoietic stem cells (Ferrara. 2004). During development VEGFR-2 is expressed by the primitive endoderm, embryonic angioblasts and in the blood islands as well as in angiogenic vessels (Kaipainen et al. 1993). However, it has also been detected in the human placenta (Dunk and Ahmed. 2001). Unlike VEGFR-1, the expression of VEGFR-2 is not regulated by hypoxia (Gerber et al. 1997).

5.4.3. VEGFR-3

In humans, VEGF-C and VEGF-D also bind to VEGFR-3 which is an approximately 195 kDa protein (Tammela et al. 2005). A shorter form of the receptor also exists, but it is not the main one seen in other tissues (Eriksson and Alitalo. 1999, Borg et al. 1995). During embryogenesis VEGFR-3 is expressed in both venous and lymphatic endothelia, but in adult humans it is restricted to lymphatic vessels and certain endothelial venules supporting the notion of a venous origin of lymphatic vessels (Tammela et al. 2005, Kaipainen et al. 1995). VEGFR-3 is also expressed in syncytiotrophoblasts in the human placenta (Tammela et al. 2005, Dunk and Ahmed. 2001). In mice, lack of VEGFR-3 causes intra-uterine death due to cardiovascular failure (Dumont et al. 1998).

5.4.4. Soluble VEGF receptors

A soluble form of VEGFR-1 (sVEGFR-1), comprising only the extracellular parts of the full-length membrane-bound receptor, has been found. It is expressed extremely abundantly in the human circulation and amniotic fluid during pregnancy (Kendall and Thomas. 1993, Kendall et al. 1996, Banks et al. 1998, Hornig et al. 2000). A similar soluble form of VEGFR-2 (sVEGFR-2) also exists, and it has been detected *in vivo* (Ebos et al. 2004). However, Chapters 5.5. and 5.6. focus on the first one on them, sVEGFR-1.

5.4.5. Neuropilin-1 and -2

Neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2) are about 120-130 kDa transmembrane glycoproteins, which modulate VEGF signaling, although the main ligands of the neuropilins have been found among the class 3 semaphorins. NRP-1 serves as a receptor for some members of the VEGF family, namely $VEGF_{165}$, VEGF-B, VEGF-E and P*l*GF -2, whereas NRP-2 mediates its actions targeting $VEGF_{145}$, VEGF-C, VEGF-D as well as $VEGF_{165}$ and P*l*GF. The neuropilins also interact directly with VEGFR-1 and -2, mediating their signal transduction (Staton et al. 2007).

Both of the neuropilins consist of three extracellular domains (a1/a2, b1/b2, and c), a transmembrane region and a short cytoplasmic domain. They can form homodimers between their c-domains. Soluble neuropilins consist only of the a1/a2 and b1/b2 domains and compete with the membrane-bound forms of the receptors for binding to semaphorins and VEGFs, thereby serving as alternative regulators of their availability.

Co-expression of NRP-1 and VEGFR-2 on endothelial cells increases the binding of $VEGF_{165}$ to VEGFR-2 about 4-fold, enhancing the $VEGF_{165}$ – mediated effects as compared to the cells expressing only VEGFR-2. Thus, NRP-1 acts as a co-receptor for $VEGF_{165}$, not only amplifying the binding but also the bioactivity of VEGFR-2, which seems to be due to increased signaling via VEGFR-2. $VEGF_{165}$, VEGFR-2 and NRP-1 may form complexes on endothelial cells potentiating the binding of $VEGF_{165}$ to VEGFR-2. In addition, also NRP-2 acts as a subtype-specific receptor to some of the VEGFs, including $VEGF_{165}$, but unlike NRP-1, it also binds to $VEGF_{145}$, VEGF-C and VEGF-D. Furthermore, both neuropilins bind P*l*GF-2, and NRP-1 also binds both of the VEGF-B subtypes. P*l*GF-2 and VEGF-B alone do not bind to VEGFR-2, but they serve as ligands for VEGFR-1, which can complex with both of the neuropilins. Therefore, VEGFR-1 has been suggested to take part in the neuropilin-modulated signaling. In addition to this, NRP-1 modulates angiogenesis complexing with VEGFR-1, which inhibits the interaction between NRP-1/$VEGF_{165}$.

The expression of NRP-1 and NRP-2 differ from each other in the adult vasculature: NRP-1 is expressed mainly by arterial and NRP-2 by venous and lymphatic endothelium. Both neuropilins are known to be over-expressed in the sites of physiological and pathological angiogenesis. However, either the pathway of their signal transduction, gene expression or their definitive role in angiogenesis is not completely understood.

5.5. Soluble VEGFR-1

Soluble VEGFR-1 is a variant of the cell membrane VEGFR-1 lacking its transmembrane and cytoplasmic domains (Kendall and Thomas. 1993). Similarly to the membrane-bound form, it binds both VEGF and P*l*GF (Park et al. 1994, Kendall and Thomas. 1993). Therefore, sVEGFR-1 acts as a natural inhibitor of VEGF and P*l*GF signaling in two different ways: First, by binding VEGF, the bioavailability of VEGF for VEGFR-2 is reduced, resulting in decreased binding and downstream signaling through its main angiogenic route (Kendall and Thomas. 1993). Second, sVEGFR-1 and VEGFR-2 are able to heterodimerize which causes a blockade of the signal transduction mediated by VEGFR-2 (Kendall et al. 1996). Based on this, sVEGFR-1 has been proposed as a “sink” for free VEGF (Hornig et al. 2000, Vuorela et al. 2000).

5.6. Soluble VEGFR-1 and its ligands during pregnancy

In human physiology, the most remarkable scenes for vasculo- and angiogenesis are the formations of the placenta and the fetus. Both VEGF and sVEGFR-1 are highly expressed in the placenta throughout pregnancy (Vuorela et al. 1997, Sharkey et al. 1993, Clark et al. 1996, Vuorela et al. 2000). Soluble VEGFR-1 is released by the early placental trophoblasts into maternal circulation where this can be detected as early as 30 days after conception (Molskness et al. 2004). Later on, its concentrations increase with advancing pregnancy (Levine et al. 2004, McKeeman et al. 2004, Powers et al. 2005). Meanwhile, also the circulating concentrations of total VEGF become higher (McKeeman et al. 2004), but those of the free and biologically active form decline already in early pregnancy (Levine et al. 2004, Molskness et al. 2004, Vuorela-Vepsalainen et al. 1999) and remain mainly undetectable when assessed near term (Levine et al. 2004, Maynard et al. 2003, Wikstrom et al. 2007, Tsatsaris et al. 2003). Following the first days postpartum, free VEGF can, again, be detected in the maternal circulation (Vuorela-Vepsalainen et al. 1999, Wikstrom et al. 2007), suggesting that sVEGFR-1 disappears in the absence of the placenta.

5.6.1. Expression of sVEGFR-1 and its ligands by the placenta

VEGF mRNA has been found in both the endo- and myometrium of the non-pregnant uterus (Charnock-Jones et al. 1993, Harrison-Woolrych et al. 1995, Torry et al. 1996). The endometrial immunostaining for VEGF has been observed during the entire menstrual cycle (Li et al. 1994).

VEGF and sVEGFR-1 are both produced by the placenta. Both mRNA and protein forms of VEGF have been seen in the decidua (Sharkey et al. 1993, Ahmed et al. 1995). The first-trimester placental expression of sVEGFR-1 protein has been localized in the villous cyto- and syncytiotrophoblasts and Hofbauer cells, as well as in decidual stromal, extravillous trophoblast, macrophages and glandular epithelial cells (Clark et al. 1996, Vuorela et al. 2000, Shiraishi et al. 1996). Some authors have also reported immunostaining for sVEGFR-1 in the stromal cells of the placental villi (Clark et al. 1996, Cooper et al. 1996), although the data is contradicting (Ahmed et al. 1995, Shiraishi et al. 1996, Jackson et al. 1994). Placental vascular endothelium has been observed to be negative for sVEGFR-1 in a number of studies (Clark et al. 1996, Vuorela et al. 2000, Ahmed et al. 1995, Shiraishi et al. 1996, Cooper et al. 1996, Jackson et al. 1994).

5.6.2. Circulating angiogenic and anti-angiogenic factors

The placenta produces VEGF and sVEGFR-1 into the circulations of both the fetus and the mother, but the production is much more abundant in the maternal side (Brownbill et al. 2007). Starting from the very early pregnancy, sVEGFR-1 is secreted by the trophoblasts, and it can be seen in constantly elevating concentrations in the maternal circulation as early as the second month following the conception (Levine et al. 2004, McKeeman et al. 2004, Powers et al. 2005, Molskness et al. 2004). This results in a concomitant decline in the levels of free VEGF, already at early pregnancy.

With advancing gestation, the levels of total circulating VEGF remain about 10-fold higher than those of its free form, which are nearly undetectable throughout pregnancy (Levine et al. 2004, Molskness et al. 2004, Vuorela-Vepsäläinen et al. 1999, Wikstrom et al. 2007). However, the circulating concentrations of the unbound VEGF begin to increase postpartum possibly because of the disappearance of its main binding protein in circulation, sVEGFR-1 (Vuorela-Vepsäläinen et al. 1999, Wikstrom et al. 2007). In fact, sVEGFR-1 has been suggested to rapidly disappear from the maternal circulation after the delivery (Powers et al. 2005, Koga et al. 2003, Maynard et al. 2003, Wikstrom et al. 2007).

The postpartum levels of sVEGFR-1 have been observed to be diminished when measured at 48 hours after delivery or to decline by 2.5% per hour when measured at delivery and once more within 48 hours (Powers et al. 2005, Maynard et al. 2003). As the placenta is also the primary source of circulating VEGF during pregnancy, also the total concentrations of VEGF decline after delivery (Hunter et al. 2000).

5.6.3. Soluble VEGFR-1 in association with maternal smoking

Cigarette smoke, nicotine and its major metabolite, cotinine, have been shown to up-regulate VEGF and VEGFR-1 gene expression in vitro (Wright et al. 2002, Conklin et al. 2002). In pulmonary arteries and bronchial mucosa of smokers the immunostaining for VEGF has been observed to increase (Santos et al. 2003, Calabrese et al. 2006). Similarly, the gene expressions of both VEGF and the receptor of its main signaling pathway, VEGFR-2, are increased in vivo in the rat trachea (Wright et al. 2002).

Smoking is associated with decreased circulating concentrations of sVEGFR-1 in men and in non-pregnant women (Belgore et al. 2000, Schmidt-Lucke et al. 2005). During pregnancy, serum concentrations of sVEGFR-1 in the second and third trimesters are reduced in smokers. Cigarette smoke reduces the secretion of sVEGFR-1 under hypoxic conditions in third-trimester placental tissue of healthy, non-smoking women (Mehendale et al. 2007).

6. Soluble VEGF receptor-1 in pre-eclampsia

During the past decade, a growing amount of studies have suggested that sVEGFR-1 plays an important role in the pathogenesis of pre-eclampsia. In 2003, a rat model was generated to demonstrate that administration of sVEGFR-1 into the circulation caused hypertension and proteinuria, known as hallmarks of pre-eclampsia (Maynard et al. 2003). The excess of sVEGFR-1 was accompanied with a concomitant decrease in the concentrations of its ligands, VEGF and P*l*GF. This finding led to an assumption that elevated circulating sVEGFR-1 is associated with the development of the clinical signs of pre-eclampsia, soon shown in several studies.

6.1. Placental expression of sVEGFR-1 in pre-eclampsia

VEGF ligands and receptors are highly expressed by the first trimester placenta. In immunohistochemistry, the expression of VEGF, P*l*GF and VEGFR-1 protein by invasive cytotrophoblasts is altered in pre-eclampsia (Zhou et al. 2002). Pre-eclampsia is associated with an up-regulation of sVEGFR-1 in the placental tissue (Maynard et al. 2003, Tsatsaris et al. 2003, Ahmad and Ahmed. 2004, Gu et al. 2008), which can be seen already at 11 weeks of gestation in chorionic villous biopsies in women with subsequent pre-eclampsia (Farina et al. 2008).

It has been suggested that the increase in the cytotrophoblastic production of sVEGFR-1 is caused by hypoxia and subsequent placental ischemia (Nagamatsu et al. 2004). Indeed, sVEGFR-1 production has been noted to increase in models of placental hypoxia both *in vitro* and *in vivo* (Nevo et al. 2006). A few years ago a new primate model of pre-eclampsia was generated to demonstrate that uteroplacental ischemia results in pre-eclampsia –like symptoms, which was accompanied with a remarkable increase in circulating sVEGFR-1 concentrations (Makris et al. 2007). In addition, the amniotic fluid sVEGFR-1 concentrations have been shown to increase in women with pre-eclampsia possibly reflecting the placental production of it (Vuorela et al. 2000).

The production of sVEGFR-1 by placental cytotrophoblasts was recently shown to comprise four different splice variants (named as sFlt1_v1, sFlt1_v2, sFlt_v3 and sFlt1_v4) of which the first three (sFlt1_v1-3) are up-regulated in pre-eclampsia as compared to healthy women (Heydarian et al. 2009). These splice variants are identical in their N-termini whereas each of them contains a unique C-terminus. As in uncomplicated pregnancies, a 100 kDa variant has been assessed as the most abundant one in both plasma and culture media from placental explants of pre-eclamptic women (Rajakumar et al. 2009). However, along with the previously known isoform, significantly higher amounts of a novel 145 kDa transcript was exhibited by women with pre-eclampsia.

6.2. Circulating concentrations of sVEGFR-1 in pre-eclampsia

Elevated production and release of sVEGFR-1 by the placenta leads to an excess of it in the maternal circulation. This up-regulation of sVEGFR-1 is associated with concomitant decrease in circulating concentrations of free VEGF and P*l*GF (Maynard et al. 2003). Several studies have shown increased maternal circulating concentrations of sVEGFR-1 in pre-eclampsia. Shortly after the rat model generated by Maynard et al. it was observed, that sVEGFR-1 concentrations are elevated in women with manifest symptoms already about 5 weeks before the onset of them (Levine et al. 2004, Chaiworapongsa et al. 2004, Koga et al. 2003, Maynard et al. 2003). Furthermore, the amount of sVEGFR-1 correlated with the severity of the disease (Chaiworapongsa et al. 2004). Earlier studies have also measured circulating sVEGFR-1 concentrations postpartum in connection with pre-eclampsia (Levine et al. 2004, Powers et al. 2005, Koga et al. 2003, Maynard et al. 2003, Wikstrom et al. 2007).

6.3. Hypothesis on the role of sVEGFR-1 in pre-eclampsia

At the placental level, sVEGFR-1 has been shown to inhibit placental cytotrophoblast invasion and differentiation in primary cytotrophoblast cultures (Zhou et al. 2002). Thus, it has been postulated that the inhibition of VEGF blocks the endothelial differentiation process of invasive cytotrophoblasts thereby contributing to the pathophysiology of pre-eclampsia. The leading mechanism for placental overproduction of sVEGFR-1 has been suggested to be hypoxia (as reviewed in Chapter 6.1.), although speculations on the role of inflammatory reasons have been made (Redman and Sargent. 2009).

The excess of sVEGFR-1 in the maternal circulation inhibits the actions of its ligands, VEGF and P*l*GF, leading to the general endothelial dysfunction seen as the classical symptoms of the maternal disease (hypertension, proteinuria and edema) (Maynard et al. 2003). The hypothesis has been tested in a number of settings, most notably, in the cell culture studies, where the endothelial dysfunction, induced by pre-eclamptic sera, could be reversed by removing sVEGFR-1 (Ahmad and Ahmed. 2004). Symptoms can be triggered also by administration of exogenous sVEGFR-1 to pregnant mice (Maynard et al. 2003).

In blood samples obtained from women with pre-eclampsia, sVEGFR-1 concentrations seem to correlate with the severity of the disease (Chaiworapongsa et al. 2004). The delivery of the placenta is followed by improvement of the signs of pre-eclampsia. Subsequently, the concentrations of sVEGFR-1 decrease postpartum, although sometimes in prolonged fashion in pre-eclamptic women, at the same time as the clinical symptoms cease (Levine et al. 2004, Powers et al. 2005, Koga et al. 2003, Maynard et al. 2003, Wikstrom et al. 2007). This supports the role of placental sVEGFR-1 behind the symptoms of pre-eclampsia.

Soluble VEGFR-1 concentrations are also elevated in connection with many known risk factors for pre-eclampsia: they have been noted to be higher in nulliparous women (Wolf et al. 2004), twin pregnancies (Maynard et al. 2008) and pregnancies with trisomy 13 (Bdolah et al. 2006).

6.4. Prediction of pre-eclampsia with sVEGFR-1

Along with the accumulating data on increased sVEGFR-1 concentrations antedating the clinical signs of pre-eclampsia, several attempts to identify women in risk of the disease have been made based on measurements of either sVEGFR-1 alone or in combination with other angiogenesis-related substances, such as P*l*GF. It appears to be proven that elevated sVEGFR-1 concentrations precede the clinical symptoms by at least 5 weeks (Levine et al. 2004), but according to growing amount of evidence, this time period can be further extended until the early second trimester, especially when sVEGFR-1 is assessed in ratio with other angiogenesis-related substances.

A systematic review of the literature on increased sVEGFR-1 and decreased P*l*GF in predicting pre-eclampsia was published in 2007 by Widmer et al. The recommendation of the authors was to conduct a rigorous prospective trial to assess the final clinical usefulness of these markers for screening purposes (Widmer et al. 2007). Interestingly, according to a review by Lapaire et al. in 2010, a novel automated measurement system for the ratio of sVEGFR-1 and P*l*GF might soon be available in a growing number of obstetric centers providing a supplementary tool for diagnostics of pre-eclampsia (Lapaire et al. 2010). However, no test has been proven effective in screening the risk of the disease.

6.5. Soluble VEGF receptor-1 in association with IUGR

IUGR is a common complication in especially early-onset pre-eclampsia, and it has long been recognized that pre-eclampsia and IUGR share many common clinical and pathologic features (closer described in Chapter 3.4.). Considering the anti-angiogenic factors, which have been intensively studied mostly in association with pre-eclampsia, there is some overlap between these two pregnancy complications.

Elevated maternal circulating concentrations have been suggested to be associated with IUGR

in several studies (Chaiworapongsa et al. 2008, Boutsikou et al. 2006, Savvidou et al. 2006, Shibata et al. 2005). Most of them, however, recognize that the increase in maternal circulating sVEGFR-1 concentrations is smaller than in case of pre-eclampsia (Savvidou et al. 2006, Schlembach et al. 2007).

Umbilical and neonatal blood (assessed at 1st and 4th postpartial days) had increased sVEGFR-1 concentrations as compared to appropriate for gestational age (AGA) full-term fetuses and neonates (Boutsikou et al. 2006). In detail, elevated sVEGFR-1 has been proposed to be present only in umbilical vein and not the arteries, suggesting that the fetus would not contribute to the angiogenic imbalance observed most clearly in the maternal circulation, where the concentrations of sVEGFR-1 are almost 20-fold of those in umbilical arteries (Wallner et al. 2007). The presence of pre-eclampsia does not seem to affect the umbilical venous or arterial sVEGFR-1 concentrations in IUGR (Schlembach et al. 2007).

6.6. New animal models of pre-eclampsia associated with sVEGFR-1

6.6.1. Catechol-O-methyltransferase (COMT) and 2-methoxyoestradiol

Soluble VEGFR-1 has been shown to be up-regulated in pregnant mice deficient of catechol-O-methyltransferase (COMT) (Kanasaki et al. 2008), which generates 2-methoxyoestradiol (2-ME) from hydroxyoestradiol. 2-ME is a potent inhibitor of angiogenesis (Fotsis et al. 1994, Mabjeesh et al. 2003). 2-ME inhibits the expression of HIF-1a resulting in subsequent down-regulation of sVEGFR-1 production in the placenta (Kanasaki et al. 2008).

The $Comt^{-/-}$ mice in the study by Kanasaki et al. present a pre-eclampsia-like phenotype, characterized as new-onset hypertension and proteinuria, accompanied by endothelial injury in the decidual arteries and renal glomeruli (Kanasaki et al. 2008). The symptoms of pre-eclampsia were reverted by 2-ME administration. The mice had elevated sVEGFR-1 concentrations in the circulation, which could also be avoided by the administration of 2-ME.

In this mouse model of pre-eclampsia, it seems evident that the disturbance in COMT / 2-ME –balance results in an increase in HIF-1a levels, followed by elevating sVEGFR-1 production and angiogenic imbalance. This, in turn, leads to placental insufficiency and finally to the manifest symptoms of pre-eclampsia in mice.

6.6.2. CBA/J x DBA/2 mouse model of pre-eclampsia

Recently, in October 2010, Ahmed and co-workers published their finding on a novel mouse model of pre-eclampsia (Ahmed et al. 2010). Complement activation, namely C5a, is a central event in the pathogenesis of placental and fetal injury in an antibody-independent mouse model (CBA/J x DBA/2 mice), which has previously been used as a model of recurrent miscarriage (Girardi et al. 2006). This complement activation results in dysregulation of the angiogenic factors necessary in normal placental development. Inhibition of this complement activation *in vivo* blocked the increase of sVEGFR-1 levels, seen in the placentas of miscarried or IUGR pregnancies, which could even rescue the pregnancies. Furthermore, monocytes stimulated *in vitro* immediately released sVEGFR-1 when treated with products of the complement cascade (Girardi et al. 2006).

The study by Giraldi et al. provided a link between complement activation and angiogenic imbalance, and a new insight into their role in the pathogenesis of pre-eclampsia. Based on that, Ahmed et al. postulated that DBA/J –mated CBA/J –female mice might also facilitate a mouse model of immunologically mediated pre-eclampsia (Ahmed et al. 2010). Indeed, they saw that the DBA/J –mated CBA/J –female mice spontaneously presented multiple features of human pre-eclampsia (albuminuria, endotheliosis). These symptoms were associated with adverse pregnancy outcome. Furthermore, the pre-eclamptic features occurred only in the first pregnancies.

The authors detected that the administration of pravastatin to these mice improves the antagonistic effect of sVEGFR-1 on VEGF signaling and the subsequent placental and fetal injury in the CBA/J x DBA/2 –mice (Ahmed et al. 2010). It increases the release of VEGF from trophoblasts ameliorating the symptoms of pre-eclampsia: pravastatin restores the glomerular injury and reduces the hypersensitivity to angiotensin II. As a result, it preserves pregnancy. The authors conclude that their finding may contribute to further development of therapeutic tools in pre-eclampsia.

7. VEGF-dependent angiogenesis in human disease

Angiogenesis is a normal process in growth, development, the female reproductive cycle, and wound healing. Pathologic angiogenesis is characterized as excessive, insufficient or abnormal neovascularization. It is a crucial event in malignant, ischemic, infectious, immune and inflammatory disorders (Carmeliet. 2005). Angiogenesis is tightly regulated by the balance of a number of pro- and anti-angiogenic factors, such as VEGF and the members of VEGF family.

7.1. Diseases associated with alterations in VEGF signaling

As the VEGF family plays a central role in both physiologic and pathologic angiogenesis, and it has been considered a key factor in a number of processes associated with human disease. Thus, the blockade of this pathway is a promising therapeutic strategy against many of them (Carmeliet. 2005, Hicklin and Ellis. 2005, Folkman. 2007). In addition, some endogenous anti-angiogenic factors, also of potential therapeutic use, have been identified (Folkman. 2004).

In solid tumors, the development of a sufficient vascular supply is crucial. Building of new vascular network is dependent on molecular interactions between tumor cells and their environment (endothelial cells, macrophages, fibroblasts, extracellular matrix etc.), which are needed to influence angiogenic mechanisms by releasing pro-angiogenic agents. It is estimated that up to 60% of human cancer cells express VEGF.

7.2 Therapeutic use of anti-VEGF agents

7.2.1. Overview of anti-VEGF agents

During the past decade, the growing understanding of the precise mechanisms of angiogenesis has led to the development of new therapeutic substances. Blocking the signaling of VEGF has been shown to inhibit both physiological and pathological angiogenesis in a remarkable number of settings. These new biological agents influence the process of VEGF –mediated neovascularization in many different ways, and the blockade of VEGF signaling is currently an established part of anti-angiogenic therapy, mostly used in oncology and ophthalmology (Carmeliet. 2005, Hicklin and Ellis. 2005, Folkman. 2007).

Anti-angiogenic therapy can be based on targeting of either the molecule or its receptors, the use of ribozymes to decrease receptor expression, or inhibitors of tyrosine kinase to block receptor activation (Folkman. 2007, Chu. 2009). The first VEGF-antagonists, brought into clinical use several years ago, were antibodies against human VEGF. The very first VEGF-antagonist, bevacizumab, was a chimeric murine / human antibody against human VEGF (Hurwitz et al. 2004). Bevacizumab was approved for clinical use in 2004 and, later on, several other monoclonal VEGF or VEGF receptor antibodies have been introduced (Folkman. 2007, Chu. 2009). These molecules antagonize VEGF receptors or their respective ligands.

Some studies have tested the blockade of VEGF receptors by tyrosine kinase inhibitors, which inhibit phosphorylation and subsequent signal transduction of the receptor. In addition, the VEGF signaling pathway has been disturbed by interfering intracellular VEGF mRNA, using ribozymes to decrease VEGF receptor expression, or by affecting signaling in the target cell. Also soluble VEGF receptor mutants or fusion-proteins have been generated.

7.2.2. VEGF Trap

Soluble VEGFR-1 has been suggested to be the predominant regulator of the bioavailability of circulating VEGF. Endogenous soluble VEGFR-1 serves as a potent inhibitor of the biological actions of VEGF (and P/GF). Because of these unique binding characteristics, as compared to VEGF-antibodies, sVEGFR-1 has been used as a model to generate a VEGF "trap" (VEGF Trap,

Regeneron Pharmaceuticals, Inc. NY, USA), later named as aflibercept (or AVE0005).

Aflibercept is a 115 kDa chimeric soluble VEGF receptor containing the domain 2 of VEGFR-1 and the domain 3 of VEGFR-2 fused to an Fc fragment (Holash et al. 2002). The Trap mimics the actions of endogenous sVEGFR-1 having a high affinity to both VEGF-A and P*l*GF, and it can also bind VEGF-B. Its high affinity to all the ligands of the naturally occurring VEGFR-1 distinguishes it from the VEGF antibodies which bind only VEGF-A (Chu. 2009, Riely and Miller. 2007).

VEGF Trap has been shown to be effective in treating some angiogenesis-associated malignant tumors and ocular diseases, and many other ones are extensively studied in multiple ongoing clinical trials analyzing its benefits (Folkman. 2007, Chu. 2009, Holash et al. 2002).

7.2.3. Side effects of anti-VEGF therapy

Hypertension is one of the most frequent side effects of systemic VEGF-blocking therapy. Its incidence and severity are dependent on the type of the drug, dose, and the schedule used in the administration of the agent (Izzedine et al. 2009, Gurevich and Perazella. 2009). Proteinuria is also a dose-related side effect occurring after anti-VEGF treatment and it may reflect severe glomerular damage (Izzedine et al. 2010). Interestingly, these side effects of inhibition of VEGF signaling resemble the main symptoms of pre-eclampsia; i.e. hypertension, proteinuria and edema (Chu. 2009, Launay-Vacher and Deray. 2009).

8. Endostatin

8.1. Structure, function and expression

Endostatin was identified in 1997, when a part of collagen XVIII was found to represent another endogenous anti-angiogenic substance (O'Reilly et al. 1997). Two different variants of collagen XVIII have been identified in humans (Saarela et al. 1998). The short variant of collagen XVIII is a component of vascular and epithelial basement membranes whereas the long one has been shown to be associated with the liver.

Endostatin is a 20 kDa product of a proteolytic cleavage of collagen XVIII, thereby not subject to post-translational modifications. It is produced from the basement-membrane collagen XVIII. In fact, it is a fragment of the C-terminal region of collagen XVIII (O'Reilly et al. 1997). Schematic illustrations of collagen XVIII and the formation of endostatin are shown in Figure 4.

Endostatin is known to suppress angiogenesis and branching morphogenesis in different settings. It inhibits vascular endothelial cell migration and proliferation *in vitro* (O'Reilly et al. 1997, Ramchandran et al. 2002, Yamaguchi et al. 1999, Becker et al. 2005) and the function of endothelial cells *in vivo* (O'Reilly et al. 1997). During embryogenesis, it also regulates branching morphogenesis of epithelial cells, which has been studied in an *ex vivo* culture model of rat uretic bud explants (Karihaloo et al. 2001).

Endostatin is called an “endogenous angiogenesis inhibitor” for a good reason: based on its ability to block tumor growth it is one of the first anti-angiogenic agents proven for clinical practice in cancer therapy (Fu et al. 2009). The functional receptor of endostatin on the vascular endothelium is $\alpha5\beta1$ (Wickstrom et al. 2002, Sudhakar et al. 2003).

Endostatin has been suggested to play a role in autocrine regulation of hypoxia-driven angiogenesis (Wu et al. 2001). The same genes regulate endostatin and VEGF, and they are expressed in the microvascular endothelial cells and pericytes (Wu et al. 2001, Nomura et al. 1995). However, opposite to VEGF, endostatin production has been shown to be suppressed under hypoxic conditions (Wu et al. 2001, Nomura et al. 1995).

Figure 4:

A. Linear structure of the parent molecule of endostatin, collagen XVIII, which consists of an N-terminal region, a series of collagen-like domains, and an NC1 domain.

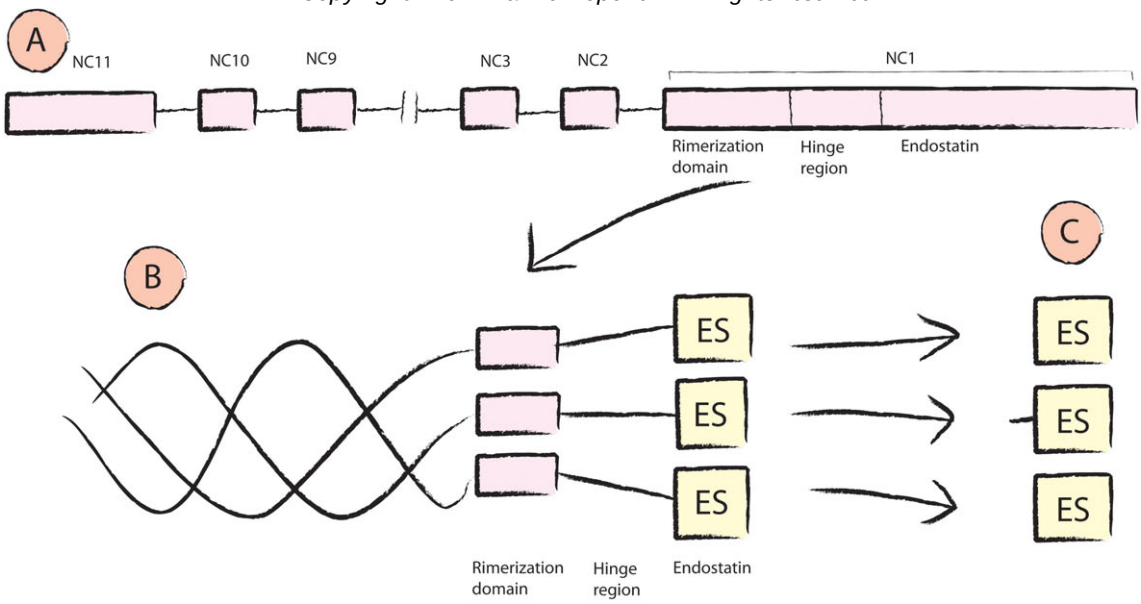
B. Illustration of collagen XVIII carboxyl-terminus including part of the COL1 domain and the full-length NC1 domain. The NC1 domain, containing approximately 300 amino acid residues, consists of three functionally different subdomains:

1. N-terminal, non-covalent trimerization domain, which is necessary for the association of trimers,
2. Hinge region containing multiple sites, which are sensitive to different proteases, and
3. Endostatin globular domain, covering a fragment of 20 kDa with anti-angiogenic activities.

The proteolytic cleavage within the protease-sensitive hinge region of NC1 domain results in a release of fragments with endostatin activity. At least two steps are involved in the generation of fragments of endostatin from collagen XVIII: a metal-dependent early step and an elastase activity-dependent final step.

C. Endostatin protein corresponds to the last 184 amino acid residues of the NC1 domain. Human endostatin protein monomer consists of a seven-stranded β-sheet at the center of the core structure. One side of the β-sheet is occupied by an α-helix ($\alpha1$), while the other side is occupied by a sequence of elaborate loops, a short antiparallel β-sheet, and a second, shorter α-helix ($\alpha2$). The protein structure also contains two disulfide bridges and a zinc-binding site located at the N-terminus of the protein immediately adjacent to the precursor cleavage site. Interestingly, the zinc-binding site of endostatin protein resembles the zinc site of matrix metalloproteinases.

Figure 4. Structure and generation of endostatin from collagen XVIII.



8.2. Endostatin in pregnancy

During human pregnancy, both the 20 kDa and the 26 kDa proteolytically produced forms of endostatin are localized in the villous tissue in all stages of gestation (Pollheimer et al. 2004). It has been suggested, that the stromal compartments of the villous placenta, as well as its vascular extracellular matrix, would represent the source of endostatin in regulation of angiogenesis during placental development. In the same study, endostatin was absent in all trophoblastic cells throughout gestation.

8.3. Endostatin in association with pre-eclampsia and IUGR

As previously suggested, endostatin may modulate trophoblast function (Pollheimer et al. 2004), which is inadequate in women later developing pre-eclampsia. In manifest pre-eclampsia, the endostatin concentrations in maternal circulation are increased (Hirtenlehner et al. 2003, Mahmound and Abdel-Raouf. 2006). However, there was no association between maternal serum endostatin and fetal IUGR. In umbilical and neonatal blood (the first and fourth postnatal days), however, endostatin concentrations seemed to be lower in neonates with IUGR as compared to those appropriate for gestational age (Malamitsi-Puchner et al. 2005). In addition, the concentrations of endostatin in healthy pregnant women are similar to those of non-pregnant women (Mahmound and Abdel-Raouf. 2006, Malamitsi-Puchner et al. 2005).

9. Angiopoietins and Tie receptors

9.1. Introduction to Angiopoietin family

The Angiopoietin family, comprising four ligands (Ang-1, Ang-2, Ang-3 and Ang-4) and two corresponding tyrosine kinase receptors (Tie-1 and Tie-2) plays an important role in the development of blood and lymphatic vasculature (reviewed in Thurston. 2003). They were identified in the middle of the 1990s and soon shown to play an essential role in vascular remodeling and maturation during angiogenesis (reviewed in Thomas and Augustin. 2009).

Angiopoietins are secreted dimeric glycoproteins with a molecular weight of approximately 75 kDa (Thurston. 2003). They are all either direct or indirect ligands for the Tie-2 receptor. For the Tie-1 receptor, however, no ligand has so far been found despite the ongoing research.

9.1.1. Angiopoietin-1

The first Angiopoietin to be discovered (Davis et al. 1996), Ang-1, has been shown to promote angiogenesis by recruiting periendothelial cells into blood vessels, thus, promoting structural integrity of blood vessels. Unlike VEGF, it does not directly induce endothelial cell proliferation (reviewed in Thomas and Augustin. 2009 and Thurston. 2003).

Ang-1 is crucial for vascular development: mice lacking it die *in utero* due to serious vascular defects (Suri et al. 1996). Over-expression of Ang-1 has been studied in transgenic mice under the control of the keratin 14 (K14) promoter. These mice are viable and healthy (Suri et al. 1998).

Ang-1 is expressed by a variety of tissues: during early human development, it is expressed by myocardial cells, whereas later on Ang-1 can be found mostly in smooth muscle and other perivascular cells (Davis et al. 1996, Suri et al. 1996, Maisonpierre et al. 1997, Gale et al. 2002). It has also been detected in association with some tumors and neuronal cells in the brain (Thomas and Augustin. 2009).

Ang-1 binds to and activates an endothelial cell -specific tyrosine kinase receptor (Tie-2; Maisonpierre et al. 1997). This signaling is crucial for postnatal hematopoiesis (Kukk et al. 1997), as well as in female reproduction, especially during the placental development (Dunk and Ahmed. 2001). Tie-2 is further introduced in Chapter 9.2.2.

9.1.2. Angiopoietin-2

Ang-2 is a competitive antagonist to Ang-1 at the Tie-2 receptor (Maisonpierre et al. 1997). It acts in an autocrine manner resulting mainly in anti-angiogenic effects by inhibiting the Ang-1 induced phosphorylation of Tie-2, which might cause destabilization of the vasculature. However, in non-endothelial cells Ang-2 can also act as an agonist to Tie-2 (Maisonpierre et al. 1997). During the past decade it has been established that Ang-2 modulates angiogenesis in a context and concentration dependent manner either acting as an agonist or an antagonist of Tie-2 (reviewed in Thomas and Augustin. 2009 and Thurston. 2003).

Mice deficient of Ang-2 survive embryogenesis, but they die after birth due to lymphatic defects (Gale et al. 2002). However, mice overexpressing Ang-2 die *in utero* due to serious vascular defects (Sato et al. 1995). Ang-2 also plays a central role in lymphangiogenesis during embryonic development (Gale et al. 2002).

Under physiological conditions, Ang-2 is expressed mainly by the endothelial cells at the sites of vascular remodeling (Maisonpierre et al. 1997), especially in the female reproductive tract, as in the villous placenta (Zhang et al. 2001) and the ovaries (Sugino et al. 2005). It is stored in Weibel-Palade bodies (WPB; Fiedler et al. 2004) where it can be rapidly released under cytokine activation of the endothelium (e.g. histamine, thrombin). Ang-2 expression is also increased under pathological conditions involving blood vessel remodeling, especially in tumors (Thomas and Augustin. 2009). Opposite to Ang-1, its expression is up-regulated by hypoxia (Mandriota and Pepper. 1998).

Ang-2 has been proposed to induce vascular sprouting and vessel destabilization in the presence of VEGF (Holash et al. 1999). At the sites of angiogenic sprouting, Ang-2 expression is up-regulated along with that of VEGF, whereas their expression is reduced together in association with vascular regression. Thus, it has been proposed that Ang-2 acts by interfering with Ang-1 –induced stabilization of the vessels, which makes them more sensitive to other angiogenic cytokines, especially VEGF (Maisonpierre et al. 1997, Stratmann et al. 1998). This theory has been tested in rodents using a pupillary membrane as an *in vivo* model created for studying the effects of different vascular modulators (Lobov et al. 2002). In this transient ocular microvessel network, Ang-2 promoted remarkable pro-angiogenic effects in the presence of exogenous VEGF, but when the VEGF was blocked by its soluble receptor, sVEGFR-1, Ang-2 induced endothelial cell apoptosis resulting in vascular regression (Lobov et al. 2002). In conclusion, Lobov et al. suggested that VEGF can convert the consequence of Ang-2 stimulation from anti- to pro-angiogenic.

9.1.3. Other members of angiopoietin family: Ang-3 and Ang-4

Two other Angiopoietins, Ang-3 and Ang-4, have been identified (Valenzuela et al. 1999). They are actually interspecies orthologs of the same Angiopoietin presenting an amino acid identity of 65% and characteristic protein structures (Valenzuela et al. 1999). Ang-3 is present in various mouse tissues, and it was first thought to have anti-angiogenic potential acting as a context –dependent antagonist of Tie-2 (Valenzuela et al. 1999).

Ang-4 is a human counterpart of mouse Ang-3, which has been observed to act as a relatively potent agonist to Tie-2 (Valenzuela et al. 1999, Lee et al. 2004). Ang-4 mRNA is present in high levels in human lungs (Valenzuela et al. 1999). A set of *in vivo* experiments on the biological effects of mouse Ang-3 and human Ang-4 revealed that also they can both act as agonists of Tie-2, but mouse Ang-3 has strong activity only on the endothelial cells of its own species (Lee et al. 2004). Ang-4 induces the survival and migration of primary cultured human endothelial cells (Lee et al. 2004).

9.2. Tie receptors

The two endothelial cell –specific receptors of the Angiopoietin family, Tie-1 and Tie-2, are expressed by both vascular and lymphatic vessels. They share identical domain structures of which the cytoplasmic parts are structurally quite close (76%) to each other, but of the extracellular regions, two thirds are unique (Mac Donald et al. 2006). A schematic presentation of the mammalian Angiopoietin family, including also the soluble receptors (introduced above), is shown in Figure 5.

9.2.1. Tie-1 receptor

Tie-1 receptor (also called as “tie”, tyrosine kinase with immunoglobulin and epidermal growth factor –like homology domains) is an orphan receptor, approximately 140 kDa of its molecular size (Partanen et al. 1992). It has been located in differentiating angioblasts and migrating endothelial cells during early embryonic development in mice (Korhonen et al. 1994). Mice lacking Tie-1 functions die around birth due to severe hemorrhages and edema, and they can be found with hyperactive endothelial cells suggesting that Tie-1 has a role in regulating endothelial quiescence (Sato et al. 1995, Puri et al. 1995).

Although in vivo studies point out its essential role in vascular development, no ligands have been found to Tie-1. It has been suggested to have a specific ligand-independent function involving shedding of the receptor and heterodimeric complex formation with Tie-2, and quite recently, also Ang-1 and Ang-4 have been shown to activate Tie-1 although they do not directly bind to it (Saharinen et al. 2005). A soluble form of Tie-1 (sTie-1) has been shown to be present in umbilical blood and maternal serum (Vuorela et al. 1998).

9.2.2. Tie-2 receptor

Both of the Angiopoietins, Ang-1 and Ang-2 bind to the same site of the Tie-2 receptor, which is an endothelial cell -specific tyrosine kinase receptor also referred to as “tek” (tunica interna endothelial cell kinase-2; Dumont et al. 1992, Fiedler et

al. 2003). Upon binding the Angiopoietins, Tie-2 dimerizes and becomes phosphorylated in tyrosine residues at its C-terminal (reviewed in Thomas and Augustin. 2009).

The endothelial cell membrane-bound Tie-2 receptor is approximately 150 kDa of its molecular size. Like Tie-1, it has an unusual N-terminal ligand-binding domain, a single transmembrane domain and an intra-cellular tyrosine kinase domain. This domain structure is highly conserved among vertebrates (Lyons et al. 1998), which serves as important evidence for its biological importance. Indeed, disrupting the Tie-2 function in transgenic mice models has been shown to result in early embryonic lethality due to vascular defects (Sato et al. 1995, Dumont et al. 1994).

Tie-2 receptor is widely expressed by mouse vascular endothelial cells already during early embryonic development (Sato et al. 1995, Korhonen et al. 1994). In adults, Tie-2 is also expressed by vascular and lymphatic endothelial cells and hematopoietic cells (Thomas and Augustin. 2009).

The Tie-2 receptor also interplays with the VEGF family. First, its signaling in the endothelial cells is restricted by VEGF. Second, Ang-1 mediated Tie-2 activation inhibits VEGF induced signaling by its main receptor (VEGFR-2). This leads to stabilization of endothelial cells (Gavard et al. 2008). Third, Ang-2 has also been shown to synergize VEGF by stimulating angiogenesis, although in the presence of VEGF it can also act as an agonist of Tie-2 (Visconti et al. 2002).

9.2.3. Soluble Tie-2 (sTie-2)

Soluble Tie-2 (sTie-2) is present in human circulation as a result of shedding of the extracellular region of the membrane-bound receptor. This shedding also reduces the amount of receptors available on the endothelial cell surface (Reusch et al. 2001).

Soluble Tie-2, approximately 75 kDa of its molecular size, binds both free Ang-1 and Ang-2, thereby decreasing their bioavailability for the membrane-bound receptor (Findley et al. 2007, Shantha Kumara et al. 2010). Some stimuli, e.g. the presence of VEGF, are able to induce shedding of the Tie-2 receptors leading to a decrease of active receptors on the cell surface (Findley et al. 2007). Data on the affinity of sTie-2 to its ligands are conflicting: one study has demonstrated an equal sTie-2 affinity to both Ang-1 and Ang-2 (Maisonpierre et al. 1997), but another one reported a twenty-time higher affinity to Ang-1 than to Ang-2 (Yuan et al. 2009).

Figure 5. The Angiopoietin family.

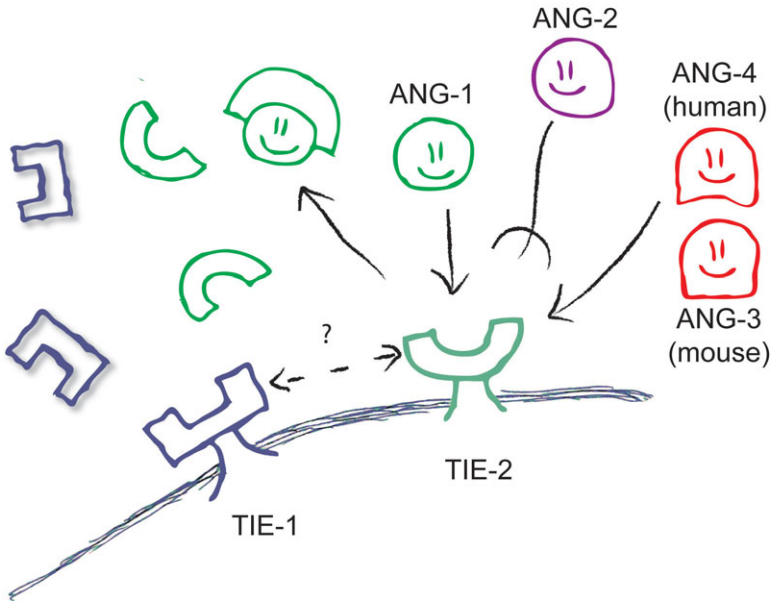


Figure 5. Illustration of the mammalian Angiopoietin family including the two cell membrane Tie receptors, Tie-1 and Tie-2, and their four ligands: Ang-1, Ang-2, Ang-3, and Ang-4. The figure demontrates binding and subsequent consequences of the Angiopoietins on Tie-2, either activating (arrows) or inhibiting it. No specific ligand is known for Tie-1, but it has been suggested to act in a ligand-independent manner and by complex formation with Tie-2. Ang-1 and Ang-4 have been shown to activate Tie-1 although they do not directly bind to it. In addition to the membrane-bound Tie receptors, both of them occur in soluble form (sTie-1 and sTie-2) in the human circulation. The figure also represents the physiological role of sTie-2 in sequestration of the Angiopoietins. In addition to Ang-1, sTie-2 can bind Ang-2, thereby decreasing their bioavailability for the cell surface receptor.

9.3. Angiopoietins and Tie receptors in pregnancy

Angiopoietin-1, Ang-2 and Tie-2, all expressed in the early placenta, are crucial for placental vascular development (Maisonpierre et al. 1997, Kayisli et al. 2006). While VEGF mediates vasculogenesis and has been proposed to guide fetoplacental vascular development, the Angiopoietins have been suggested to remodel the maternal vessels during placentation. They might also promote trophoblast growth and migration (Wulff et al. 2003). Besides the trophoblastic and vascular cells, the stromal macrophages have been shown to secrete both Ang-1 and Ang-2 (Geva et al. 2002).

In the placenta, Ang-1 and -2 and their common receptor, Tie-2, have been co-localized to the trophoblasts, which has been suggested to prove the autocrine role of the Angiopoietins in the placental development (Dunk et al. 2000). Ang-1 mRNA has been detected in the cytotrophoblast and syncytiotrophoblast bilayer (Geva et al. 2002, Dunk et al. 2000) as well as in perivascular cells (Geva et al. 2002, Buhimschi et al. 2010). Ang-2 is also secreted by smooth muscle and perivascular cells (Thomas and Augustin. 2009). Its expression is decreased by hypoxia (Zhang et al. 2001), and it is restricted to peri-

vascular cells of large term villi in the course of pregnancy (Dunk et al. 2000).

First, Ang-2 was detected in the placenta using Northern blotting and RNA in situ hybridization until early second trimester when it was located only in the syncytiotrophoblast layer of the chorionic villi (Goldman-Wohl et al. 2000). Later on, is has been shown to be expressed throughout gestation not only by the syncytiotrophoblasts, but also the cytotrophoblasts and even more extensively by the perivascular cells (Zhang et al. 2001, Geva et al. 2002, Dunk et al. 2000, Buhimschi et al. 2010). Besides, Ang-2 is also expressed by villous stromal and endothelial cells (Zhang et al. 2001, Buhimschi et al. 2010). Placental expression of Ang-2 decreases in the course of pregnancy (Zhang et al. 2001, Geva et al. 2002, Tseng et al. 2006). However, cultured villous explants of placentas, obtained from term elective Caesarean deliveries, have recently been shown to produce significant amounts of Ang-2 but not Ang-1 (Buhimschi et al. 2010).

Tie-2 is expressed by angiogenic cells and cytotrophoblasts of the human placenta (Zhang et al. 2001, Kayisli et al. 2006). The Tie-2 mRNA expression was earlier thought to be restricted to the first trimester as analyzed by in situ hybridization (Zhang et al. 2001), when its mRNA has also been detected in the fetal and maternal blood vessels, and during the early second trimester the Tie-2 staining was namely positive for endovascular trophoblasts invading the maternal spiral arteries (Goldman-Wohl et al. 2000). However, recently Tie-2 mRNA was present in the placenta also during the third trimester as analyzed by quantitative real time polymerase chain reaction (PCR; Buhimschi et al. 2010). The expression of Tie-2 in the placenta decreases in the course of pregnancy (Zhang et al. 2001, Kayisli et al. 2006, Geva et al. 2002, Tseng et al. 2006). Hypoxia does not affect placental Tie-2 expression (Zhang et al. 2001).

9.3.1. Circulating Ang-1, Ang-2 and sTie-2

Maternal serum Ang-2 concentrations have been reported to be higher at 10–13 weeks (Wang et al. 2007) and even at 32 weeks (Hirokoshi et al. 2007) than at 38 weeks of pregnancy (Malamitsi-Puchner et al. 2006). This indicates that their concentrations in the maternal circulation decrease with advancing pregnancy, although no data from longitudinal studies are available. Furthermore, the serum concentrations of Ang-2 decrease rapidly after delivery (Hirokoshi et al. 2007) indicating that the placenta is probably the main source of circulating Ang-2 during pregnancy. In amniotic fluid, Ang-1, Ang-2 and sTie-2 are all present throughout pregnancy (Buhimschi et al. 2010, Pacora et al. 2009).

9.4. Angiopoietins and Tie receptors in association with pre-eclampsia and IUGR

In women with pre-eclampsia, alteration in the angiopoietin family has been detected both in the placental and in the systemic level: For instance, the placental Ang-2 expression has been shown to be decreased (Zhang et al. 2001), as has been the case also with the maternal circulating Ang-2 levels (Hirokoshi et al. 2007, Hirokoshi et al. 2005) when analyzed at the third trimester. In pre-eclamptic women, the circulating concentrations of sTie-1 (Hirokoshi et al. 2005) are lower, whereas those of Ang-1 higher (Nadar et al. 2005), compared to the concomitant concentrations in healthy women. Besides them, the maternal sTie-2 concentrations have been assessed, although resulting in either lower (Gotsch et al. 2008) or similar levels (Hirokoshi et al. 2005) as compared to those in pre-eclamptic women.

Along with pre-eclampsia, pregnancy-induced hypertension is associated with an increase in circulating Ang-1, but not sTie-2, concentrations (Nadar et al. 2005). However, the circulating concentrations of Ang-2 have been shown to be decreased already at 10–13 weeks of pregnancy in women with subsequent IUGR (Wang et al. 2007), although the report did not specify whether the later IUGR was accompanied with pre-eclampsia or not.

Aims of Study

Pre-eclampsia is associated with alterations in angiogenesis-related markers in maternal circulation. An excess of anti-angiogenic substances, especially soluble VEGF receptor-1, has been observed in maternal circulation after the onset of pre-eclampsia, probably reflecting their increased placental production. Smoking reduces circulating concentrations of sVEGFR-1 in non-pregnant women, and in pregnant women it reduces the risk of pre-eclampsia.

The present study was designed...

1. ... to evaluate the potential use of maternal serum sVEGFR-1, Ang-2 and endostatin in early prediction of subsequent pre-eclampsia (Study I, III and IV),

2. ... to evaluate whether smoking affects sVEGFR-1 concentrations in pregnant women and to study its placental secretion and expression *in vitro* (Study II), and

3. ...to determine the biological half-life of endogenous sVEGFR-1 in human circulation, and elucidate the concomitant changes in free VEGF concentrations (Study V).

Material and methods

1. Subjects and specimens

The studies were approved by the Ethical Committee of Helsinki University Central Hospital. All women volunteered for the study and gave written informed consent. Clinical data were retrieved by chart review of files in Helsinki University Central Hospital, where all women were treated and delivered. Gestational age was determined by transvaginal ultrasound. All women were of Caucasian race and had singleton pregnancies. A summary of the blood and placental samples collected from the pregnant women (n=268) taking part in this study between the years 2001–2007 is given in Table 6.

1.1. Blood samples

1.1.1. Patient recruitment for blood sample collections

For the early pregnancy blood samples, the women were recruited at either preoperative visits before induced abortion during 13–17 weeks of gestation (V), or at routine ultrasound screening visits either at 12–15 weeks (I, III and IV) or at 19–20 weeks (II) of pregnancy. A closer description on the patient selections for studies I, III and IV is given in Chapter 1.1.2.

The women for the late pregnancy blood samples were recruited at antenatal visits offered for counseling due to their fear of vaginal delivery (II) or during the pre-operative visits before elective Caesarean sections (V).

1.1.2. Patient selection for serial blood samples in early pregnancy

A total of 3 240 pregnant women in the Helsinki area were admitted to a blood sample collection during two routine ultrasound screening visits at 13.7 ± 1 (mean ± SD) and 19.2 ± 1 weeks of gestation. The women, eventually included in the study, were clinically healthy, non-smoking, with singleton pregnancies and of Caucasian race. Women with any kind of underlying hypertensive disorder, renal disease, diabetes, or other pre-existing disease were excluded. The samples collected according to the selection protocol were used for studies I, III and IV.

According to the diagnostic criteria for pre-eclampsia (given in Chapter 3.1.1.), 71 women (2.2%) developed pre-eclampsia, although 22 of them were excluded due to the reasons given above. Of the remaining 49 women with subsequent pre-eclampsia, the disease was classified as mild in 29 and severe in 20 patients.

Isolated early-onset IUGR (defined in Chapter 3.4.), was suspected in 73 women due to the findings in uterine palpation and symphysis–fundus measurements, and the following fetal growth estimation by ultrasound examination at the outpatient clinic. Of the ones who were further followed and closer examined at the Department of Obstetrics and Gynecology, 16 women with otherwise normal medical histories and uncomplicated pregnancies substantially delivered newborns diagnosed with IUGR.

Fifty-nine women remaining normotensive, non-proteinuric and finally giving birth to neonates of normal weight (± 1 SD of national average at term) were picked amongst the samples collected at the same gestational age. These women served as the control population for the studies.

1.1.3. Serial sample collection after legal termination of pregnancy

The legal termination of pregnancy in Study V was performed surgically by uterine curettage at 15 ± 2 weeks of gestation due to trisomy 18 (n = 2), trisomy 21 (n = 1) or socioeconomic factors (n = 2). In the sampling procedure, serial blood samples from each woman were taken before, during and following the curettage, starting from an initial blood sampling 2 h before the administration of vaginal misoprostol. The second sample was collected at 1 h and the third one

at 4 h after the start of uterine contractions. The fourth blood sample was taken just before the operation, from when on the following samples were collected at 5, 10, 15, 20, 30, 40, 60, 120, 180, 360 and 720 min. Additional samples were drawn every 24 h for the next 6 days, and at 14 and 21 days after the curettage.

1.1.4. Serial sample collection after elective Caesarean section

A low level Caesarean section was performed due to either breech presentation of the fetus, two or more previous Caesareans, or fear of vaginal delivery, for eight women participating in Study V. In the sampling procedure, the first one of the serial blood samples, taken from each participant in connection with her elective Caesarean section, was drawn after placing the patient in a supine position. The second sample was taken 15 minutes later, following prehydration and initiation of spinal anesthesia, and the third one immediately prior to the surgery. Following the start of the operation, blood samples were collected at 15, 30, 60, 120, 180, 360, and 720 min. A few more samples were taken daily during the hospital stay (2 to 4 days).

1.1.5. Management of blood samples

Blood samples were collected from the antecubital vein, and if applicable, of contralateral to the arm used for fluid infusions (V). Plasma (II), or serum after and having clot at room temperature (I, III, IV and V), were separated by centrifugation. All samples were stored at -80°C until analyses.

1.2. Placental samples

1.2.1. Patient recruitment for placental sample collection

Placental samples (II) were collected by uterine curettage during a legal termination of pregnancy performed for psychosocial reasons at the first trimester, 9 ± 1 weeks of gestation.

1.2.2. Management of placental samples

Following the curettage, the placental samples were immediately set in sterile phosphate buffered saline (PBS) for < 2 h. The pH of the PBS solution was 7.4, and it was composed using the following substances in the given concentrations: 0.14M NaCl, 2.7M KCl, 0.01M Na_2HPO_4, and 1.76M KH_2PO_4. Thus, the PBS used for the placental samples was isotonic, able to maintain the osmolarity of the cells.

After a short incubation in PBS, the tissue samples were processed for cell culturing, dehydrated for paraffinization and subsequent use in immunohistochemical assessments, or split to proceed with both analyses.

Table 6. Summary of the blood and placental samples.
Samples for the study were collected from 268 pregnant women between 2001–2007 in the Department of Obstetrics and Gynecology, Helsinki University Central Hospital.

Type of Samples	Patient group	Gestational age (wk) at sampling	Number of women	Samples per woman	Study
Venous blood	Healthy non-smoking women	12 – 15	51	1	I, III, IV
		12 – 20	28	2	I, III, IV
		13 – 17	5	23	V
		16 – 20	36	1	I, III, IV
		19 – 20	19	1	II
		38 ± 3	22	1	II
		39 – 41	8	12 - 14	V
	Smokers	19 – 20	20		II
		35 ± 5	6		II
	Subsequent pre-eclampsia	12 – 15	44		I, III, IV
		12 – 20	26	2	I, III, IV
		16 – 20	36		I, III, IV
	Subsequent IUGR	12 – 25	11		I, III, IV
		12 – 20	5	2	I, III, IV
		16 – 20	10		I, III, IV
Placental tissue	Healthy women	9 ± 1	20		II
	smokers	9 ± 1	22		II

2. Laboratory methods

2.1. Detection of cytokine concentrations in peripheral blood

An established assay was available for each cytokine analyzed in the blood samples (I-V). Except for hCG (V), they were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Serum hCG was assessed using a two-site immunofluorometric assay (Delfia®, PerkinElmer Life and Analytical Sciences, Inc. – Wallac Oy, Finland). Detailed information on each assay used is given in Table 7.

The serum samples were diluted for Ang-1, Ang-2, endostatin and sTie-2 analyses (III, IV) according to the ratio given in Table 7. so that their concentrations would fall within the measurable range of the assay. To reduce the impact of inter-assay variation, samples from both the patients and the controls (I, II, III, IV) or consecutive samples, from each woman, when applicable (I, III, IV, V), were run in the same batch.

Table 7. Description of the assays used for analyses of cytokine concentrations in blood samples and in placental cell culture medium.

Protein	Intra-assay variation (%)	Inter-assay variation (%)	Detection limit	Assay	Dilution
Ang-1	> 3.3	> 6.4	63 ng/L	Quantikine® ELISA	50-fold
Ang-2	> 6.9	> 10.4	47 ng/L	Quantikine® ELISA	5-fold
hCG	3.1	3.9	2 IU/L	Delfia®	
endostatin	< 7.0	< 8.0	160 ng/L	Quantikine® ELISA	50-fold
sTie-2	> 5.0	> 8.3	156 ng/L	Quantikine® ELISA	10-fold
sVEGFR-1	3.2	7.7	5 ng/L	Quantikine® ELISA	
VEGF	5.0	8.2	5 ng/L	Quantikine® ELISA	

2.2. Laboratory methods for placental samples

2.2.1. Placental cell culture

Tissue samples from the first-trimester placentas of smoking (n = 19) and non-smoking (n = 11) women (II) were washed and prepared into small pieces of villi (2–5 mm^3), which were then incubated for 24 h. The culture media (Amnio-MAX™-C100 Supplement; Gibco® Products, Life Technologies, Carlsbad, CA, USA) were then collected for analysis of sVEGFR-1 concentrations.

Measurements for sVEGFR-1 were performed using the same commercially available ELISA as for peripheral blood samples, described in Chapter 2.1. and Table 7. Before analysis, the cell culture media were diluted 1:10 in order for them to fall within the measurable range of the sVEGFR-1 assay. The amount of sVEGFR-1 secreted was calculated from the measured concentration and the volume of medium. The ratio of total sVEGFR-1 to the weight of the cultured tissue was calculated.

2.2.2. Preparations and performance of immunohistochemistry

Placental tissue samples, described above, were used for immunolocalization of sVEGFR-1 expression during the first trimester. The amount of tissue allowed the analysis in samples of 21 smoking and 19 non-smoking women (II). Small pieces of placental tissues were fixed with formalin, embedded with paraffin, sectioned into 5 μm sections on a microtome, and transferred onto slides for the final analyses.

To begin the procedure, the tissue sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. They were then immersed in citrate buffer (pH 6) to break the protein cross-links formed by formalin fixation, and heated in a microwave oven (4 x 5 min, 550 W). The citrate solution unmasks the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections enhancing staining intensity of antibodies.

The slides were cooled at room temperature (RT; 20 min) and washed twice in PBS (pH 7.4). Endogenous peroxidase activity was removed at RT setting the sections in hydrogen peroxide (H_2O_2, Perhydrol; Merck, Darmstadt, Germany) in methanol (MeOH), containing 3.2 mL of H_2O_2 and 200 mL of MeOH, for 30 min. These reagents were washed using PBS (2 x 5 min), after which the tissue sections were treated with 100 μl of CAS-Block (Zymed® Laboratories Inc. San Francisco, CA, USA) at RT for 20 min. CAS-Block is a blocking agent which eliminates the need for species-specific serum blockers.

Each section was covered with 100 μL of primary antibody solution against VEGFR-1 (polyclonal rabbit anti-VEGFR-1, 0.5 μg/mL; R&D Systems, Inc, Minneapolis, MN, USA) or the negative control (goat IgG, 0.5 μg/mL; DAKO, Glostrup, Denmark). These primary antibodies were incubated at +4º C overnight, after which they were detected by an avidin-biotin-peroxidase –based method using the Vectastain ABC kit according to the manufacturer´s instructions (Vector Laboratories, Burlingame, CA, USA). The slides were washed in PBS (2 x 5 min) between each step after applying the primary antibodies.

The sections were first incubated for 30 min with 100 μL of biotinylated secondary antibody solution (anti-goat IgG in rabbit serum; Vector Laboratories) after which they were counterstained with hematoxylin (Mayer´s hemalum solution; Merck, Darmstadt, Germany) and mounted with Aquamount Improved mounting medium (BDH laboratory supplies, Poole, England). After the actual immunohistochemistry and staining with hematoxylin the slides were washed with sterile water.

2.2.3. Analysis of immunostaining

Following coding and processing the placental samples as described above, the immunostaining was analyzed by two investigators, who were blinded to the clinical data. The intensity of immunoreactivity was determined semi-quantitatively so that positively stained cells were classified into four categories shown in Table 8.

Table 8. Semi-quantitative classification of immunostaining of placental tissue.

The cells, stained positively in immunohistochemistry, were classified into four categories according to the intensity of the immunostaining. The analyses were performed by two investigators, who were blinded to the clinical data associated with the samples.

Classification category	Description of immunostaining
-	no staining
1+	weak but detectable
2+	moderate or distinct
3+	intense

3. Statistical methods

Several statistical tests were performed in each study to evaluate differences in the measured variables, mostly concentrations of Ang-1, Ang-2, endostatin, sTie-2 and sVEGFR-1 (I-V). The analyses were mainly performed using SPSS 13.0 for Windows® (SPSS Inc. Chicago, IL, USA).

Besides a variety of statistical tests performed on the data, a curve-fitting algorithm for multi-exponential decay (Microsoft Excel®; Microsoft Corporation, Redmond, WA, USA) was created for the half-life calculations (V). Details of the algorithm are described in Chapter 3.1.

An effective sample size was calculated manually to represent a statistical power of at least 80% within a 2-tailed α error of 0.05. A two-tailed p -value < 0.05 was considered significant. A summary of the statistical methods used is given in Table 9.

3.1. Calculation of half-lives

The half-lives for sVEGFR-1 were determined based on serum samples of eight healthy women during and after elective Caesarean sections (V). The calculations were performed with a curve-fitting algorithm for multi-exponential decay using Microsoft Excel® (provided by Microsoft Office®, Microsoft Corporation).

Soluble proteins in human compartments are known to represent a biphasic elimination pattern, probably attributable to a rapid initial phase caused by a clearance from the intravascular space and the following slower phase reflecting its removal from the extravascular space. Similarly, also the sVEGFR-1 concentrations were postulated to consist of two exponentially decaying components, a rapid and a slow one, and a constant background, which was used as a basis for interpretation of the data obtained from the sVEGFR-1 measurements.

The data were fitted to the following function:

$$A(t) = a_1 e^{-\left(\frac{t \ln 2}{T_1}\right)} + a_2 e^{-\left(\frac{t \ln 2}{T_2}\right)} + a_3$$

... where α_1 and α_2 denote the amplitudes of the decaying components, α_3 the amplitude of the constant background and T_1 and T_2 denote the corresponding half-lives.

3.2. Analyses of distribution and homogeneity of variance

The normality of distribution among continuous variables was analyzed either with visual observation (I, II, V) or Kolmogorov-Smirnov's test (III, IV). If the distribution was skewed, a logarithmic transformation corrected it in some cases (I, III, IV) allowing the use of a parametric test. After that, the homogeneity of variances in concentrations between the groups was analyzed using Levene's test (III, IV).

3.3. Comparisons of continuous variables: cytokine concentrations in blood and cell culture media samples

Based on the findings concerning the distribution and the homogeneity of variances, either

Kruskal-Wallis analysis of variance (ANOVA; I, III) or Oneway ANOVA (III, IV) was performed to compare different cytokine concentrations between the study groups, and when significant, Student's *t* –test was used for further analyses of differences in continuous variables, such as the concentrations, between the groups. Mann-Whitney U –test was used for the comparison of non-parametric continuous variables (II, V).

Differences in normally distributed and continuous variables within specific group of patients, such as concentrations in serial blood samples, were analyzed using paired-samples *t* –test (I, IV), whereas the changes in non-parametric continuous variables were determined by Wilcoxon's signed-rank test (V).

Correlations between clinical characteristics and cytokine concentrations (Study IV) were analyzed by univariate linear regression analysis (IV) or Spearman rank correlation (I, III, V), the latter being commonly used for continuous non-parametric variables. Parametric correlations were determined by Pearson's correlation (V).

3.4. Evaluation of risk for subsequent pre-eclampsia

The χ^2 test was used to determine differences in distributions between categorical variables. For example, odds ratios (OR) and 95% confidence intervals (95% CI) for detection of subsequent disease (according to an individual concentration above the median of a specific group of patients) were obtained from χ^2 test (I, IV), which could also be utilized in calculating trends for changing prevalence of a disease based on cytokine concentrations divided into quartiles (I).

Receiver operating characteristic (ROC) curve analysis was used to determine area under curve (AUC) for high cytokine concentrations in prediction of a subsequent disease (III, IV).

Table 9. Statistical analyses used in the study.

Analysis	Study
Descriptive statistics	I –V
Logarithmic transformation	I, III, IV
Power calculations	IV
Analysis of distribution or the homogeneity of variance	
Kolmogorov-Smirnov´s test	III, IV
Visual observation	I, II, V
Levene´s test for homogeneity of variances	IV
Comparison of data within groups	
Parametric tests	
Paired-samples *t* –test	I, IV
Non-parametric tests	
Wilcoxon's signed-rank test	V
Comparison of data between groups	
Parametric tests	
Student's *t* –test	I, II, III, IV
One-way ANOVA*	III, IV
Non-parametric tests	
Mann-Whitney U –test	II
Kruskal-Wallis analysis (ANOVA)	III
Correlation between different parameters	
Spearman rank correlation	I, III, IV, V
Pearson's correlation	V
Linear regression analysis (univariate)	III, IV
Diagnostic tests	
X^2 test	I, IV
ROC curve analysis	III, IV

*Including the Post hoc –test of Bonferroni, Duncan, Dunnett, and Tukey

ROC = Receiver operating characteristics curve

Results

1. First and second trimester concentrations of serum markers in pre-eclampsia

Soluble VEGFR-1, Ang-1, Ang-2, sTie-2 and endostatin were detectable in all maternal serum samples analyzed in the study. At the first sampling, 13.7 ± 1 (mean ± SD) weeks of pregnancy, their median concentrations were statistically similar between the study groups. Furthermore, the measured concentrations were unaffected by subsequent IUGR diagnosis at both samplings.

1.1. Concentrations at 2nd trimester (16–20 weeks of gestation)

At 19.2 ± 1 weeks of gestation, the women with subsequent pre-eclampsia had higher concentrations of sVEGFR-1 ($p = 0.005$), Ang-2 ($p = 0.006$) and endostatin ($p = 0.026$) than the women in the control group. This was also seen separately for sVEGFR-1 in women with later mild ($p = 0.043$) and severe ($p = 0.022$) pre-eclampsia, and for endostatin concentrations preceding later severe pre-eclampsia ($p = 0.041$). Ang-2 concentrations correlated with sVEGFR-1 concentrations ($r = 0.306$, $p = 0.007$). The median concentrations of the study groups, and the significant differences between them, at both 12–15 and 16–20 weeks of gestation in each of the study groups is shown in Table 10.

1.2. Changes in concentrations between serial samplings

Samples for sVEGFR-1 and endostatin analyses from both sampling points were available from 26 of 49 women with subsequent pre-eclampsia, 5 of 16 women with subsequent IUGR, and 28 of 59 healthy controls. Based on them, sVEGFR-1 concentration decreased by 15% ($p = 0.003$) from the first to the second sampling in the control group.

Serial samples for Ang-1, Ang-2 and sTie-2 analyses were available from 23 of 49 women in the pre-eclampsia group, 4 of 16 women in the IUGR group, and 27 of 59 women healthy controls. Of them, only the concentrations of sTie-2 showed a marginal, although significant decrease of by 4% ($p = 0.031$) in the control group with the advancing pregnancy. Such changes between the samplings were not seen in women with later pre-eclampsia or IUGR.

1.3. Prediction of pre-eclampsia

1.3.1. Odds ratios for sVEGFR-1 and Ang-2 concentrations

Women with sVEGFR-1 and/or Ang-2 concentrations above the median of the groups had an elevated risk of pre-eclampsia, as the odds ratio (OR) for sVEGFR-1 was 2.1 (0.8–5.6 = 95% confidence interval, CI) and that for Ang-2 was 2.9 (1.6–7.3). Severe pre-eclampsia was characterized with even higher ORs being 4.1 (1.1–15.6) for sVEGFR-1 and 4.3 (1.1–16.3) for Ang-2.

Combining the two proteins, sVEGFR-1 and Ang-2, the ORs were improved to 4.7 (1.5–14.4; $p = 0.007$) in later pre-eclampsia and to 7.5 (1.9–29.1; $p = 0.004$) for subsequent severe pre-eclampsia.

The quartile analysis also confirmed the value of sVEGFR-1 in identifying women at risk of pre-eclampsia ($p = 0.033$) and severe pre-eclampsia ($p = 0.018$). However, the ratios of Ang-1 to Tie-2, Ang-2 to Tie-2 or Ang-1 to Ang-2 concentrations did not increase the predictive value of the individual markers.

1.3.2. ROC analysis of Ang-2 and endostatin at 2nd trimester

For further validity of elevated Ang-2 and endostatin concentrations in screening of pre-eclampsia, a receiver operating characteristics

(ROC) curve was applied to the data received from the women in the control and pre-eclampsia groups. It was further used to calculate the area under the curve (AUC) for Ang-2 and endostatin for diagnosis of pre-eclampsia. The ROC curve was also used to determine the optimal cut-off value, giving the best combina-tion of sensitivity and specificity, of maternal serum endostatin concentrations in the prediction of subsequent disorder.

In the ROC analysis, the AUC for Ang-2 was 0.69 (95% CI = 0.57–0.82; p = 0.007) whereas that for endostatin was 0.64 (0.50–0.81) to identify subsequent pre-eclampsia.

The optimal cut-off values were determined and used for calculations of sensitivity and specificity of endostatin concentrations, which were 80% and 52% (cut-off value = 58.0 ng/L) in pre-eclampsia, and 8% and 65% (cut-off value = 65.5 ng/L) in the severe form of the disease.

2. Effects of smoking on sVEGFR-1 during pregnancy

2.1. Effects of maternal smoking on circulating sVEGFR-1 concentrations in pregnant women

Maternal plasma sVEGFR-1 concentrations increased from the second to the third trimester in both smoking ($p < 0.05$) and non-smoking ($p < 0.001$) women. However, the median concentration of sVEGFR-1 was independent on maternal smoking at both the second and the third trimester.

2.2. Effects of maternal smoking on placental sVEGFR-1 secretion

All placental tissue samples secreted sVEGFR-1 in measurable concentrations. The ratio of total sVEGFR-1 (ng) per tissue weight (as described in Methods, Chapter 2.2.1.) did not alter between the smokers and non-smoking women. Although the amount of sVEGFR-1 was higher in smokers, this difference was not statistically significant.

2.3. Effects of maternal smoking on placental sVEGFR-1 expression *in vitro*

In immunohistochemistry, we found sVEGFR-1 expression mainly in the villous cytotrophoblasts. The staining located in the cytotrophoblasts was negative in one of the samples in each group. Otherwise the intensity of immunostaining varied between 1+ (weak but detectable) and 3+ (intense) the pattern of which was independent on maternal smoking before the termination of pregnancy. The vascular endothelium and the stroma of the placental villi were negative for sVEGFR-1 staining whereas a few samples in both groups presented weak immunoreactivity in the villous syncytiotrophoblasts layer.

Table 10. Maternal serum concentrations of Ang-1, Ang-2, endostatin, sTie-2, and sVEGFR-1 at 12–15 ("first sampling", range) and 16–20 weeks of gestation ("second sampling") in women with subsequent pre-eclampsia (mild or severe) or IUGR compared to normotensive women.

		Controls	Pre-eclampsia				IUGR
			All	Mild	Severe	concomitant IUGR	
First sampling at 12–15 weeks of gestation							
Ang-1 (µg/L)	Number of samples	50	41	27	14	10	11
	Median	27.8	35.0	26.7	35.8	36.5	23.9
	IQR	18.3 – 34.9	22.0 – 42.2	17.1 – 42.4	26.2 – 43.4	26.4 – 50.8	22.0 – 35.2
Ang-2 (ng/mL)	Number of samples	50	41	27	14	10	11
	Median	33.2	38.8	38.8	38.8	31.5	30.0
	IQR	24.7 – 43.1	26.0 – 48.9	26.0 – 55.9	25.8 – 43.7	24.8 – 41.8	20.8 – 48.5
Endostatin (µg/L)	Number of samples	51	44	28	16	10	11
	Median	59	66	69	58	ND	68
	IQR	53-69	56-74	62-76	53-63	ND	44-78
sTie-2 (ng/mL)	Number of samples	50	41	27	14	10	11
	Median	18.2	17.8	17.5	18.6	18.5	19.3
	IQR	16.6 – 20.8	15.9 – 20.3	16.2 – 20.5	15.5 – 20.4	15.6 – 22.0	16.5 – 21.5
sVEGFR-1 (ng/L)	Number of samples	51	44	28	16	10	11
	Median	432	481	463	489	491	486
	IQR	236 – 575	384 – 628	364 – 662	411 – 591	381 – 578	293 – 582

Second sampling at 16–20 weeks of gestation							
Ang-1 (µg/L)	Number of samples	36	31	16	15	8	9
	Median	26.0	31.4	33.7	30.6	31.7	32.3
	IQR	16.6 – 34.6	23.9 – 35.0	23.7 – 35.6	23.9 – 32.9	25.3 – 51.9	23.7 – 38.3
Ang-2 (ng/mL)	Number of samples	36	31	16	15	8	9
	Median	17.7	25.0 [a]	23.5	29.0 [b]	26.6	31.0 [c]
	IQR	10.8 – 27.4	19.3 – 39.5	18.9 – 42.7	20.2 – 36.6	14.6 – 35.1	19.3 – 36.5
Endostatin (µg/L)	Number of samples	36	31	16	15	8	10
	Median	58	68 [d]	65	69 [e]	ND	56
	IQR	45 – 70	62 – 72	62 – 69	67 – 73	ND	45 – 71
sTie-2 (ng/mL)	Number of samples	36	31	16	15	8	9
	Median	18.1	17.1	17.4	17.1	15.8	18.2
	IQR	16.2 – 19.9	16.2 – 19.9	16.4 – 19.9	15.7 – 21.3	13.6 – 20.4	14.7 – 19.9
sVEGFR-1 (ng/L)	Number of samples	36	31	16	15	8	10
	Median	296	436 [f]	340 [g]	497 [h]	532	349
	IQR	184 – 508	282 – 699	285 – 750	235 – 699	235 – 988	261 – 487

p –values compared to controls: [a] p = 0.006 [b] p = 0.016 [c] p = 0.038 [d] p = 0.026 [e] p = 0.041 [f] p = 0.005 [g] p = 0.043 [h] p = 0.022		All	Mild	Severe	concomitant IUGR	
IQR = interquartile range	Controls	Pre-eclampsia				IUGR

p –values in comparison to controls are based on Student's *t* –test, and after logarithmic transformation if tested with skewed distribution.

3. Sequential changes in sVEGFR-1 concentrations after pregnancy

3.1. Disappearance of sVEGFR-1 from maternal circulation after elective Caesarean section

The baseline serum sVEGFR-1 concentration at term was 11 180 ng/L (6 380–17 480 ng/L; median, range) from where it declined slightly due to the fluid infusions. However, at 30 min (18–72 min) after the beginning of the operation it had increased by 20% (8–47%, p = 0.028) in six of the patients. At its highest, the median sVEGFR-1 concentration in maternal serum was 15 390 ng/L (6 910–17 680 ng/L) and a similar elevation in its levels was later seen also in the two other patients.

As there was no simultaneous increase in serum hCG, the half-lives of sVEGFR-1 were determined based on the measurements after this initial increase. The elimination of sVEGFR-1 was biphasic, comprising two exponentially decaying components that represent its biological half-lives, a rapid and a slow one (described in more detail in Figures 6 and 7). Furthermore, the calculated half-lives were similar to those of hCG observed in both the present and a previous study (Korhonen et al. 1997).

Figure 6. The two components (rapid and slow) of the biphasic half-life of circulating sVEGFR-1 and hCG after term pregnancy in eight healthy women.

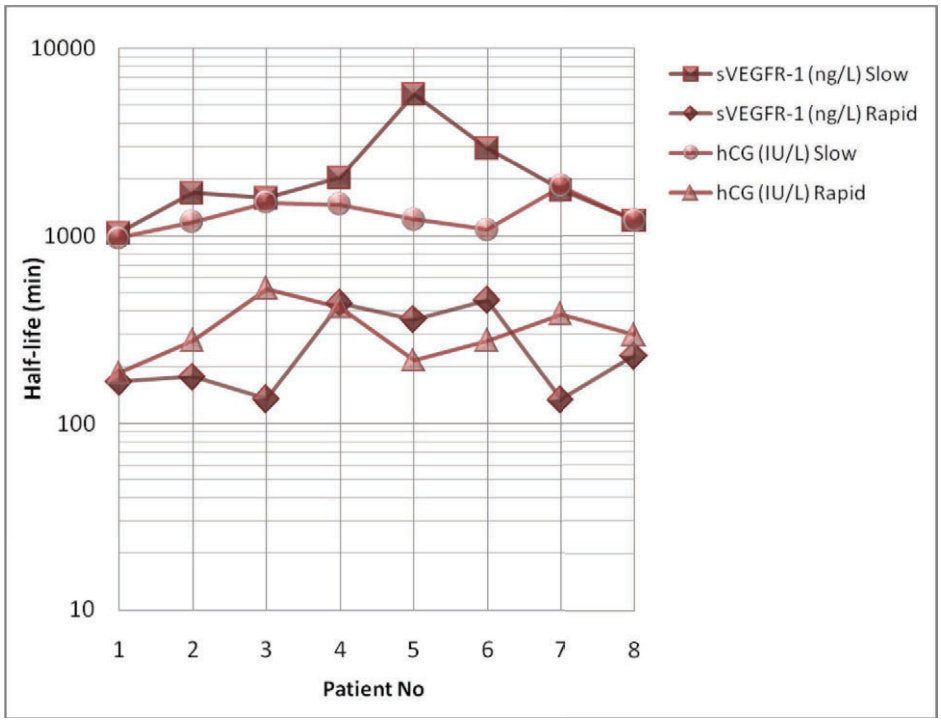


Figure 6. Individual half-lives of maternal serum sVEGFR-1 and hCG were calculated in the serum samples of eight healthy Finnish women undergoing elective Caesarean sections at 40 ± 0.4 (mean ± SD) weeks of pregnancy using a curve-fitting algorithm. The biphasic half-lives had two exponentially decaying components, the rapid and the slow one, here expressed in minutes, for both sVEGFR-1 and hCG. Their half-lives were quite similar to each other, but this notion was not statistically significant. However, a positive correlation was observed between the two half-life components of hCG ($r = 0.76$, $p = 0.028$; Spearman's rank correlation).

3.2. Sequential changes in sVEGFR-1 and concurrent VEGF levels after induced abortion

In the five pregnant women undergoing legal termination of pregnancy at 15 ± 2 (mean ± SD) weeks of gestation, the baseline concentrations of serum sVEGFR-1 and VEGF were 1 000 ng/L (200–1 430 ng/L; median, range) and 21 ng/L (14–28 ng/L), respectively. However, following the beginning of the contractions there was a 25% (12–29%, $p = 0.043$) increase in sVEGFR-1 concentration at 1 h, whereas no change in the amount of VEGF was seen. Such an elevation in the sVEGFR-1 levels was previously observed after the beginning of the Caesarean section, as described in Chapter 3.1., although the baseline of the 3rd –trimester sVEGFR-1 levels appeared about 10-fold higher compared to those during the later first trimester.

After this initial rise in serum sVEGFR-1 concentrations, the levels decreased simultaneously with an increase in free VEGF concentrations, with a highly significant negative correlation to each other ($r = -0.90$, $p < 0.0001$). A constant level for VEGF seemed to be reached between the 3rd and 6th day and that of sVEGFR-1 in 6 days after the operation as analyzed graphically from the curve drawn on the basis of the data.

Figure 7. Biphasic elimination of endogenous sVEGFR-1 from maternal circulation after term pregnancy.

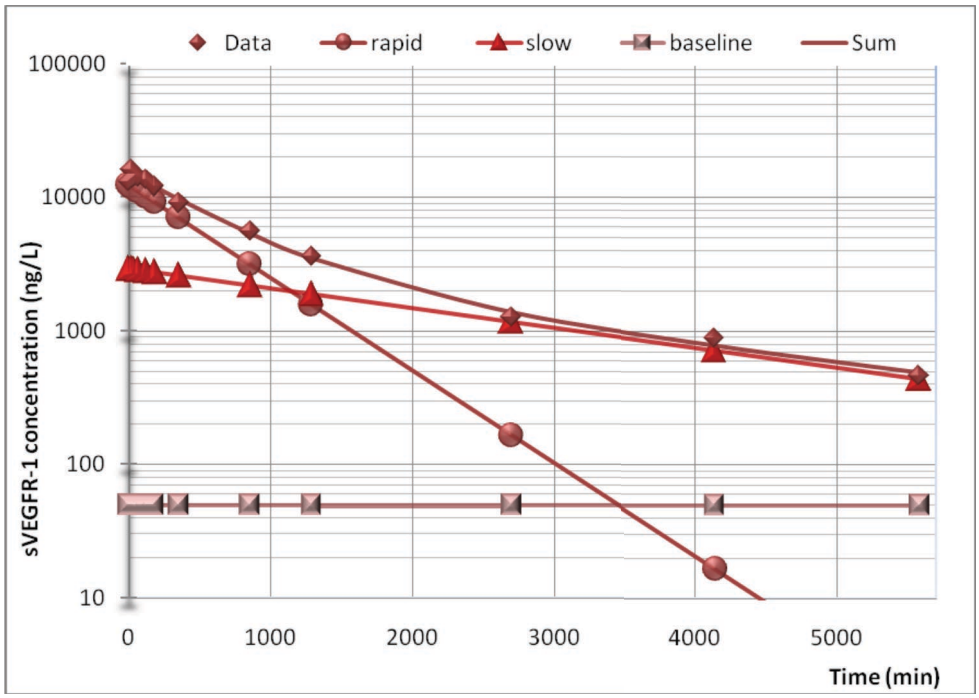


Figure 7. The elimination of endogenous sVEGFR-1 from maternal circulation during and after Caesarean delivery was composed of a rapid half-life of 3.4 h (2.2 – 7.5 h; median, range) and a slow one of 29 h (17–94 h). The figure represents these two exponential curves (using a logarithmic scale) of the biphasic disappearance of sVEGFR-1 in one of the patients (Patient 5). Her individual rapid half-life was 7.2 h (430 min) and the slow one 34 h (2 050 min). After an initial increase in sVEGFR-1 concentrations during the operation, her sVEGFR-1 concentration was 15 880 ng/L from where on the half-lives were calculated.

Discussion

1. Serum markers of pre-eclampsia in early pregnancy

On the basis of the different pathophysiological phenomena associated with pre-eclampsia, several circulating biochemical markers, exhibiting placental and endothelial dysfunction, activation of coagulation, or inflammation, have been tested for their ability to predict the disease. Among many others, such markers include fibronectin, fetal DNA, hemoglobin or hematocrit, hCG, estriol, leptin, plasminogen activator, P*l*GF, and uric acid (Meads et al. 2008, Chappell et al. 2002). Circulating concentrations of these biomarkers have been noted either to increase or decrease several weeks before the clinical manifestation of pre-eclampsia. However, in systematic reviews the evidence has been too week to make recommendations on their clinical use in screening (Meads et al. 2008, Conde-Agudelo et al. 2004). In addition, numerous clinical and biophysical tests have been introduced as promising candidates for risk assessments of pre-eclampsia. Nevertheless, neither the biochemical nor the other markers have been specific or predictive enough, and so far, no single test has reached the clinical standards of a screening test for pre-eclampsia (Meads et al. 2008, Conde-Agudelo et al. 2004).

Pre-eclampsia is associated with an angiogenic imbalance and alterations in related biomarkers in the maternal circulation. In this study, we found that the maternal serum concentrations of Ang-2, endostatin and sVEGFR-1, three central anti-angiogenic factors, were increased already at 16 – 20 weeks of pregnancy, preceding the onset of pre-eclampsia. Furthermore, they could be used to identify women at risk of the disorder with a moderate precision.

1.1. Concentrations of maternal serum sVEGFR-1, Ang-2 and endostatin at 1st and 2nd trimesters

The first one of the angiogenic-related markers chosen for this study was sVEGFR-1 which had become known as an inhibitor of the actions of VEGF and P*l*GF in a rat model and the accompanying experiments by Maynard et al. in 2003 (Maynard et al. 2003). The researchers had shown that administration of sVEGFR-1 into the circulation of pregnant rats caused the classic signs of pre-eclampsia: hypertension and proteinuria (Maynard et al. 2003). Similarly, sVEGFR-1 concentrations had been shown to be elevated in women with manifest symptoms and already about 5 weeks before the onset of them (Levine et al. 2004, Chaiworapongsa et al. 2004, Koga et al. 2003, Maynard et al. 2003). Furthermore, the amount of sVEGFR-1 correlated with the severity of the disease (Chaiworapongsa et al. 2004).

As assumed by the previous data, sVEGFR-1 seemed likely to reflect the placental events associated with pre-eclampsia. Thus, we hypothesized that similar excess might be present in the systemic level long before the development of the clinical syndrome. Indeed, based on our measurements, women with subsequent pre-eclampsia had higher serum sVEGFR-1 concentrations at 16–20 weeks of gestation ($p = 0.005$) than the group of healthy controls, which could also be seen in the subgroup analysis of women with mild ($p = 0.043$) or severe ($p = 0.022$) pre-eclampsia.

Similarly, we found that also Ang-2, an anti-angiogenic growth factor, is increased in maternal circulation at the early second trimester, 16–20 weeks of gestation, preceding the onset of pre-eclampsia ($p = 0.006$). The finding was controversial to the earlier data as placental Ang-2 expression and maternal circulating Ang-2 levels

have been shown to be decreased at the third trimester in connection to pre-eclampsia (Zhang et al. 2001, Hirokoshi et al. 2007, Hirokoshi et al. 2005). However, our results might reflect the overall elegant angiogenic balance at this specific stage of pregnancy differing from that of the third trimester, presented in the referred studies. It is also worth noting that in uncomplicated pregnancy, maternal serum Ang-2 concentrations seem to decrease with advancing pregnancy (Wang et al. 2007, Hirokoshi et al. 2007, Malamitsi-Puchner et al. 2006).

Besides sVEGFR-1 and Ang-2, we observed that circulating maternal endostatin concentrations were elevated at this point of early second trimester in association with subsequent pre-eclampsia ($p = 0.026$), and especially its severe form ($p = 0.041$). Our results are in line with the earlier data according to which circulating endostatin is increased in women with manifest pre-eclampsia (Hirtenlehner et al. 2003). In accordance with this previous study concerning the third trimester, we did not find any association between maternal serum endostatin and subsequent IUGR.

The studied anti-angiogenic markers have been suggested to be produced by the placenta. Therefore, we measured their serum concentrations around 14 weeks of gestation, when early placentation has taken place and steroid production has shifted from corpus luteum to the placenta, knowing that it has been suggested that some placental defect associated with pre-eclampsia might operate already at this point of pregnancy. We performed the second sampling at 19 gestational weeks, when pre-eclampsia should be evident on the placental level. None of the biomarkers were elevated preceding pre-eclampsia around 14 weeks of pregnancy, but about 5 weeks later, at the second sampling around 19 weeks of pregnancy, the subsequent syndrome had become evident in the systemic level. Thus, the placental events associated with the development of pre-eclampsia could be measured about 13 weeks before its clinical onset. This result is consistent with the assumptions that an excess of anti-angiogenic factors contribute to the etiology of pre-eclampsia.

1.2. Potential serum markers in prediction of subsequent pre-eclampsia

Besides the increased concentrations of the studied anti-angiogenic factors prior to the manifestation of clinical pre-eclampsia, we showed that their elevated concentrations at 16 - 20 weeks of pregnancy were associated with an increased risk of this disorder. Based on odds ratios, an elevated sVEGFR-1 concentration was assessed to hold an increased risk of pre-eclampsia. The same was observed also for Ang-2. In the ROC analysis, Ang-2 was also somewhat predictive of later pre-eclampsia. Using the optimal cut-off values determined from the curve drawn for endostatin, its sensitivity and specificity were 80% and 52%, and identifying the women presenting the severe form of the disease alone only slightly improved the specificity (80% and 65%, respectively). Thus, the predictive value of these individual markers can be pronounced as moderate at the best. All in all, studies based on larger population are needed to determine whether these markers could be used in clinical practice for the prediction of pre-eclampsia.

Interestingly, we noticed that combining the two biomarkers, sVEGFR-1 and Ang-2, somewhat improved the accuracy of these markers compared to using one of them alone. Similar findings have been reported on the ratio of sVEGFR-1 to P*l*GF in several studies (De Vivo et al. 2008, Moore Simas et al. 2007, Levine et al. 2006). As it has been shown that a blockade of both VEGF and P*l*GF is required to produce pre-eclampsia –like symptoms in pregnant rats, it is likely that namely the blockade of P*l*GF signaling plays an important role in the pathogenesis of sVEGFR-1 –mediated endothelial dysfunction (Maynard et al. 2003).

Soluble VEGFR-1 has been thought to play a key role in the pathogenesis of pre-eclampsia (Maynard et al. 2005). Upon the publication process of our results on sVEGFR-1 (Study I), a growing number of papers were published supporting our results. However, by 2007 there were only 10 of 184 studies, including ours, with sVEGFR-1 levels measured retrospectively at different gestational ages comparing the results

between the women with subsequent pre-eclampsia to the ones with uneventful pregnancies (reviewed by Widmer et al. 2007). This systematic review of the literature on increased sVEGFR-1 and decreased P*l*GF concentrations in predicting pre-eclampsia concludes that a prospective trial is still needed to evaluate the clinical usefulness of them in screening purposes (Widmer et al. 2007).

Lately, measurements of sVEGFR-1 and P*l*GF with novel commercial assays have been proposed as new promising tools in early screening of pre-eclampsia (Schiettecatte et al. 2010, Sunderji et al. 2010) and such an automated measurement systems are currently being investigated for clinical use (Lapaire et al. 2010). However, their predictive value may still fall under that acquired for proper diagnostic tests. As pre-eclampsia has been suggested to be a multifactorial disease, perhaps the best predictive tool could be created by combining a number of markers, including those of the activated coagulation or inflammation, with the ones reflecting the angiogenic imbalance. Importantly, assessing specifically the women already at risk of pre-eclampsia might bring new insight into the use of such combinations.

2. Effects of smoking on sVEGFR-1 during pregnancy

Cigarette smoking has been observed to decrease circulating sVEGFR-1 concentrations in men and in non-pregnant women (Belgore et al. 2000, Schmidt-Lucke et al. 2005). In addition, cigarette smoke, nicotine and its major metabolite, cotinine, have been shown to up-regulate VEGF and VEGFR-1 gene expression in vitro (Wright et al. 2002, Conklin et al. 2002). However, cigarette smoking during pregnancy reduces the risk of pre-eclampsia (Conde-Agudelo and Belizan. 2000). As smoking affects the expression of sVEGFR-1, which is increased in pre-eclampsia, we studied whether it affects circulating sVEGFR-1 concentrations in pregnant women. We then further evaluated whether the placental expression or secretion of sVEGFR-1 are also altered in connection to smoking.

Based on our results, maternal smoking did not statistically affect the plasma concentrations of sVEGFR-1. This finding is contrary to an earlier study reporting lower sVEGFR-1 concentrations in serum of smokers during the mid- and third trimesters (Powers et al. 2005), possibly reflecting different smoking habits between these populations or the small number of smokers in our study. However, there was a tendency towards decreased sVEGFR-1 concentration among smokers in the third trimester, but the small sample size (n = 6) might make the statistical significance impossible to detect. Interestingly, smoking was recently associated with alterations in the concentrations of pro-angiogenic factors during early pregnancy, but no obvious association with subsequent pre-eclampsia was observed (Jeyabalan et al. 2008). Our data also indicates that the protective effect of smoking against pre-eclampsia may be mediated by factors other than sVEGFR-1, but we cannot exclude its role in the disease process due to our indirect study setting.

Placental tissue has been observed to secrete sVEGFR-1 (Vuorela-Vepsalainen et al. 1999, Mehendale et al. 2007, Gu et al. 2008). In our study, the first-trimester placental sVEGFR-1 secretion in tissue samples collected from smoking women tended to be higher than in those of non-smoking women. However, this difference was not statistically significant. Nicotine and its stable metabolite cotinine, have toxic effects on the placenta (Zdravkovic et al. 2005) and maternal smoking affects placental development impairing the function of cytotrophoblasts (Jauniaux and Burton. 2007, Zdravkovic et al. 2005). Cigarette smoke also reduces the secretion of sVEGFR-1 under hypoxic conditions in third-trimester placental tissue of non-smoking women (Mehendale et al. 2007). Based on our results, we cannot exclude the effect of smoking on placental production of sVEGFR-1.

In our study, we detected sVEGFR-1 expression in first-trimester placental tissue mostly in the cytotrophoblast layer. In a minority of samples immunostaining was also seen in the syncytiotrophoblast layer and decidua. However, the pattern or the intensity of immunostaining were not observed to be altered in connection to maternal smoking, and the staining pattern was quite similar to that previously described by us and some other authors (Clark et al. 1996, Vuorela et al. 2000, Cooper et al. 1996). Further direct studies on samples from both smoking and non-smoking women with pre-eclampsia would give a closer view to the potential protective role of sVEGFR-1 in the placental level.

3. Biological half-life of endogenous sVEGFR-1 in human circulation and potential implications on anti-angiogenic therapy

3.1. Biphasic elimination of endogenous sVEGFR-1 from circulation

Circulating concentrations of sVEGFR-1 have been assessed postpartum in connection with pre-eclampsia, when its third-trimester levels are much higher compared to healthy women at the same gestational age (Levine et al. 2004, Powers et al. 2005, Koga et al. 2003, Maynard et al. 2003, Wikstrom et al. 2007). As the maternal sVEGFR-1 concentrations decrease rapidly within a week after delivery, also following uncomplicated pregnancies, this excess of circulating sVEGFR-1 is likely to be of placental origin (Powers et al. 2005, Koga et al. 2003, Maynard et al. 2003, Wikstrom et al. 2007). In fact, sVEGFR-1 was seen to decline within 48 h after delivery (Maynard et al. 2003), and it was reduced by 2.5% per hour according to two measurements at the time of delivery and after 48 h (Powers et al. 2005). However, a precise half-life for endogenous sVEGFR-1 was not determined in these studies.

Caesarean section provides a unique setting for analyzing the disappearance of endogenous sVEGFR-1 as the circulating concentrations of this pregnancy-associated protein are in this case markedly higher compared to those of non-pregnant stage. Furthermore, in this setting, the main source of sVEGFR-1, the placenta, is rapidly removed during the operation. Based our results, the disappearance of sVEGFR-1 is biphasic, representing two exponentially decaying components for the half-lives, a rapid half-life of 3.4 h and a slow one of 29 h.

In our setting, the fluid infusion preceding the Caesarean section was followed by a minor decrease in sVEGFR-1 concentrations. Additionally, a transient increase in the circulating sVEGFR-1 levels was seen right after the beginning of the operation, similar to the one seen in connection with the beginning of contractions during the induced abortion. This phenomenon might be due to the manipulation of the placenta, which contains sVEGFR-1 in huge amounts. Interestingly, there was no such rise in serum hCG levels, which were measured as a reference. In fact, the obtained half-lives of sVEGFR-1 resembled the ones of hCG in our study as well as in a previous study (Korhonen et al. 1997). Furthermore, we speculated that if there was a sufficiently long delay before the fitting procedure this transient increase would be attenuated and it would not affect the proper evaluation of the half-lives.

The half-lives of sVEGFR-1 were calculated after the initial increase in its concentrations. The disappearance of sVEGFR-1 was found biphasic, which is usual for proteins in human circulation. This has been suggested to reflect a rapid initial phase of elimination caused by the clearance of the protein from the intravascular space and a slower phase associated with the removal of it from the extravascular space. The route of elimination for sVEGFR-1 has not been determined, although according to its size, which is 110 kDa (Kendall and Thomas. 1993), it is too big to cross the glomerular barrier and enter the urine. Thus, it is probably mostly eliminated either by the liver and/or the reticuloendothelial system.

3.2. Sequential changes in sVEGFR-1 and VEGF after induced abortion

During pregnancy, the concentrations of free VEGF fall already at the beginning and remain low until term (Levine et al. 2004, Molskness et al. 2004, Vuorela-Vepsäläinen et al. 1999), probably as a result of binding to a naturally occuring antagonist, sVEGFR-1. This notion was further supported by our study indicating that the serum concentrations of sVEGFR-1 and free VEGF after legal termination of pregnancy present a highly negative correlation to each other. The circulat-

ing concentrations of free VEGF rise to its non-pregnant level postpartum possibly resulting from the disappearance of its binding protein, sVEGFR-1 (Vuorela-Vepsäläinen et al. 1999, Wikstrom et al. 2007). Our study provides confirming evidence that there is an excess of sVEGFR-1 during pregnancy, but shortly following the delivery free VEGF dominates in the maternal circulation. However, it is worth noting that although sVEGFR-1 has been proposed as the predominant regulator of the bioavailability of circulating VEGF, other soluble receptors in the VEGF family, sVEGFR-2 and soluble neuropilin-1 (sNRP-1), may also contribute to this regulation (Ebos et al. 2004, Gagnon et al. 2000). Furthermore, sVEGFR-1 itself has additional ligands as it also binds VEGF-B and P*l*GF.

3.3. Anti-angiogenic therapy and sVEGFR-1

There is an elegant balance between pro- and anti-angiogenic factors regulating a number of biological processes. For example, angiogenesis and the VEGF family play an essential role in tumor growth and metastasis. Therefore, the blockade of this pathway is a promising therapeutic strategy for inhibition of tumor growth (Carmeliet. 2005, Hicklin and Ellis. 2005, Folkman. 2007). Anti-VEGF therapy can target either the molecule or its receptors, decrease receptor expression or block the activation of the receptor (Folkman. 2007, Chu. 2009). Interestingly, VEGF-blocking therapy has side effects resembling the symptoms of pre-eclampsia (hypertension, proteinuria and edema). This phenomenon probably reflects similar mechanisms of the endothelial dysfunction in case of pre-eclampsia and the adverse effects of anti-angiogenic therapy (Chu. 2009, Launay-Vacher and Deray. 2009). Thus, data on the kinetics of endogenous sVEGFR-1 might also be valuable in further studies on pregnancy complications.

In fact, sVEGFR-1 has already been used as a model to generate a VEGF ”trap”, later named as aflibercept (or AVE0005). It is a 115 kDa chimeric receptor, which contains the domain 2 of VEGFR-1 and the domain 3 of VEGFR-2 fused to an Fc fragment (Holash et al. 2002). VEGF Trap mimics the action of endogenous sVEGFR-1 as it has a high affinity to both VEGF and P*l*GF. This substance has been shown to be effective in treating angiogenesis-associated cancers and ocular diseases in animal models, and there are multiple ongoing trials analyzing its potential use in clinical practice (Folkman. 2007, Chu. 2009, Holash et al. 2002). Opposite to VEGF antibodies, VEGF Trap binds all three ligands (VEGF-A, VEGF-B and P*l*GF) of the endogenous cell membrane VEGFR-1 and it has thereby been suggested to be superior to all anti-VEGF therapies (Chu. 2009, Riely and Miller. 2007). Yet, the adverse effects of these substances still somewhat limit the use of them in humans even if proven effective in animal models. Thus, the novel information on the kinetics of endogenous sVEGFR-1 could be of utility in the development of new therapeutic tools for diseases associated with altered expression of members of the VEGF family.

Conclusions

1. Pre-eclampsia is associated with an angiogenic imbalance reflecting alterations in anti-angiogenic markers in the maternal circulation. After the onset of the disease, circulating anti-angiogenic substances, especially sVEGFR-1, are found in excess probably reflecting increased placental production. In this study, maternal serum concentrations of Ang-2, endostatin and sVEGFR-1, three central anti-angiogenic markers, were increased already at 16–20 weeks of pregnancy, about 13 weeks before the clinical manifestation of pre-eclampsia. Furthermore, these biomarkers could be used with a moderate precision to identify women at risk of the disorder. However, larger patient series are needed to determine whether these markers could be applied for clinical use in the prediction of pre-eclampsia.

2. Smoking reduces circulating sVEGFR-1 concentrations in non-pregnant women, and in pregnant women it is likely to reduce the risk of pre-eclampsia. In spite of this, we did not find statistically significant support to our hypothesis that smoking would affect maternal circulating sVEGFR-1 or placental sVEGFR-1 expression in vitro. However, we cannot exclude the possible effect of smoking on placental secretion of sVEGFR-1 during the first trimester, a result which would be in line with the previous notions of altered cytotrophoblast function associated with maternal tobacco exposure.

3. The disappearance of endogenous sVEGFR-1 after pregnancy is biphasic representing a rapid half-life of 3.4 h and a slow one of 29 h. The decline in sVEGFR-1 concentrations after mid-trimester legal termination of pregnancy is accompanied with a simultaneous increase in the serum levels of free VEGF. Our study provides novel information on the kinetics of endogenous sVEGFR-1, which could be of interest in the development of new therapeutic tools in diseases associated with alteration in the members of VEGF family.

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~ ~ ~

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~ ~ ~

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~ ~ ~

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~

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I love You, El Hombre,
from the bottom of my heart
– Я люблю тебя!
And exactly the way You are.

~

Я без тебя не пою –
Я ведь тебя так люблю –
Я без тебя не живу – Жить без тебя не могу

Я без тебя горько плачу –
Я от тебя слез не прячу –
Я без тебя пропадаю – Имя твое повторяю.

Люблю, люблю, люблю, тебя люблю –
Люблю, люблю, люблю, тебя люблю –
Тебя люблю!

~ ~ ~

(Татьяна Буланова: "Тебя Люблю")
(Part of Song "I love You" by Tatyana Bulanova)

~ ~ ~ ~ ~

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~ ~ ~

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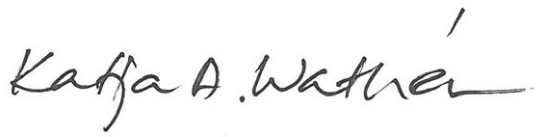
~ ~ ~

Helsinki, May 2011



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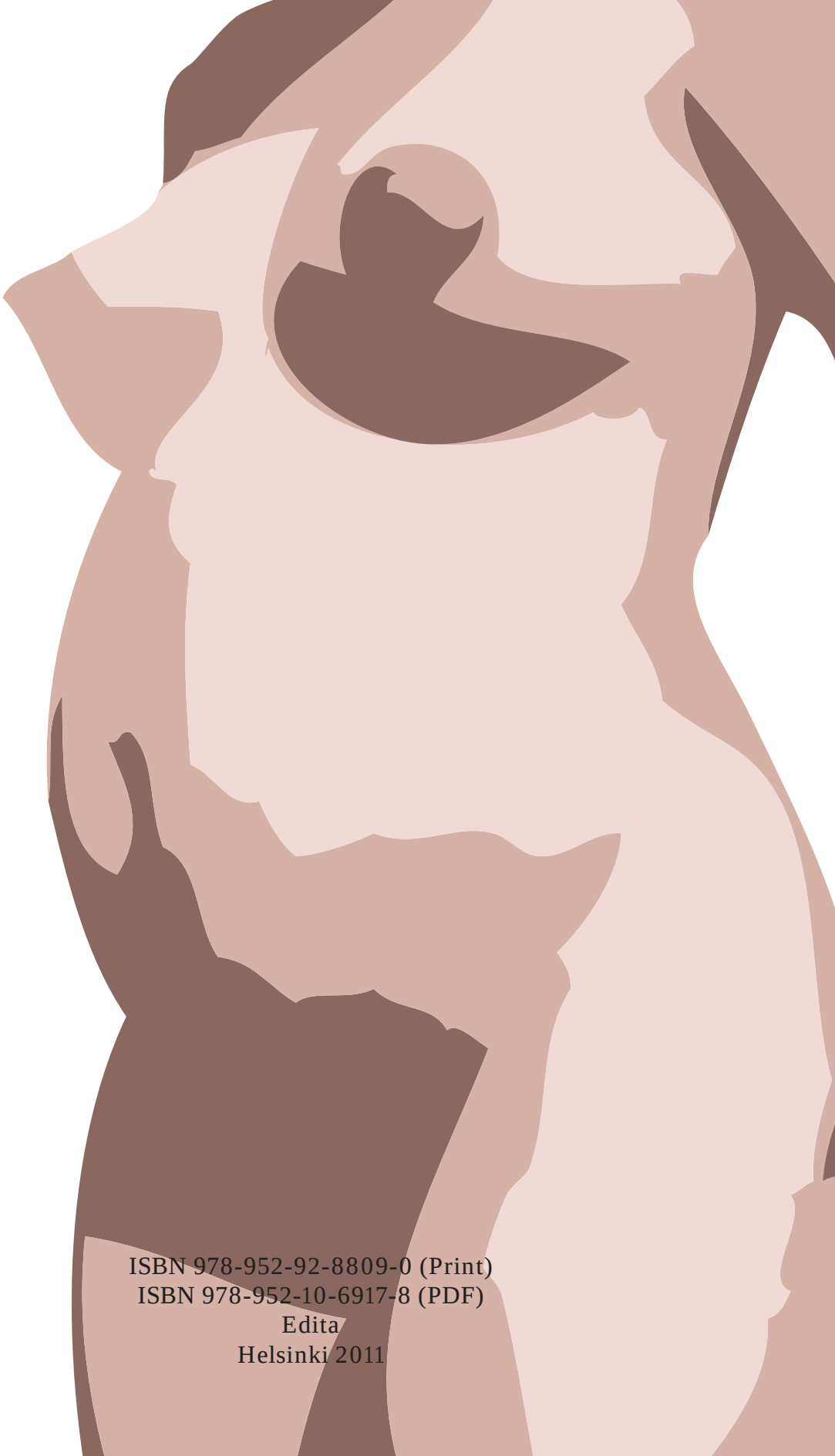

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