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ACADEMIC DISSERTATION
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Tampere University, Finnish Centre for Alternative Methods
Tampere University Hospital, Department of Obstetrics and Gynecology, Finland

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To women with pre-eclampsia

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Anita Virtanen

ABSTRACT

Pre-eclampsia is a condition unique to human pregnancy. It is characterized by new-onset hypertension and widespread endothelial dysfunction. The exact aetiology of pre-eclampsia is unsolved, but knowledge of its pathophysiology has increased in recent decades. Currently, pre-eclampsia is considered as a placental disease. One of the pathogenic mechanisms is thought to be imbalanced angiogenesis in the maternal circulation. That has been found weeks before the onset of clinical disease and has been seen to correlate with the severity of the disease. Angiogenic factors have been widely studied in recent years with the aim of finding a method to predict subsequent pre-eclampsia, or therapeutic targets. So far, the only curative treatment for pre-eclampsia remains delivery of the foetus and placenta.

This thesis covers four studies, in all of which we have utilized a human cell-based tissue model to study angiogenic properties of pre-eclampsia. The vasculogenesis/angiogenesis model was developed in the Finnish Centre for Alternative Methods (FICAM) which is a centre of expertise for alternative methods to animal experimentation.

In the first study, we collected maternal blood and umbilical-cord blood samples from eleven primiparous women with pre-eclampsia and ten primiparous controls in 2011–2014. Maternal blood samples were taken near delivery and umbilical blood samples were taken after childbirth. Sera from pre-eclamptic women strongly inhibited angiogenesis whereas in the control group there was no inhibition. Umbilical blood samples were inhibitory after both pre-eclampsia and normal pregnancy, and there was no difference between the groups.

It the second study, we assessed early gestational angiogenic properties and longitudinal changes in angiogenic capacity of sera from women with healthy and pre-eclamptic pregnancies by using *in vitro* and immunoassay tests. The study population consisted of six primiparous women who subsequently developed pre-eclampsia and six healthy controls. In the first trimester, maternal sera from both groups had a stimulatory effect on angiogenesis *in vitro* and levels of angiogenic proteins did not differ between the groups.

The aim of the third and fourth studies was to elucidate the effects of three concentrations of metformin (Study III) and pravastatin (Study IV) on angiogenesis with and without maternal sera. For this we recruited twenty pregnant women in 2017-2018. Maternal serum samples were obtained from women with early-and late-onset pre-eclampsia, intrauterine growth restriction and healthy pregnancies. At therapeutic concentrations, neither of the drugs enhanced angiogenesis. In contrast, metformin at a high concentration had a strong inhibitory effect on angiogenesis in every group. Pravastatin at the lowest concentrations along with maternal sera from early-onset pre-eclamptic pregnancies had a stimulatory effect on angiogenesis in some women.

In conclusion, we showed that a human cell-based vasculogenesis/angiogenesis model can be utilized to study angiogenic properties in pre-eclampsia as well as interactions between maternal sera and therapeutic agents. In addition, the data obtained from the *in vitro* assay offer a holistic perspective to the angiogenic capacity of serum. In contrast to previous studies, which have hypothesized that metformin and pravastatin therapies restore angiogenic balance in pre-eclampsia, the results of this study suggest that at therapeutic concentrations neither of these drugs improve angiogenesis markedly.

TIIVISTELMÄ

Pre-eklampsia on raskauden aikainen sairaus, johon liittyy verenpaineen nousu ja yleistynyt endoteelin toimintahäiriö. Sitä esiintyy eläinkunnassa ainoastaan ihmisillä. Pre-eklampsian perimmäistä syytä ei täysin tunneta, mutta viime vuosina tutkimustieto sen tautimekanismeista on huomattavasti lisääntynyt. Nykyään pre-eklampsiaa pidetään istukkasairautena. Sen kliinistä taudinkuvaa edeltää useiden viikkojen epätasapaino verisuonikasvua säätelevien tekijöiden välillä ja tämän on todettu korreloivan sairauden vakavuuteen. Verisuonikasvutekijöitä on viime vuosina tutkittu laajasti sekä mahdollisena ennusteellisina tekijöinä sairauden kehittymiselle että lääkehoidon kohteena. Toistaiseksi pre-eklampsian ainoa parantava hoito on synnytys.

Väitöskirja koostuu neljästä osatyöstä, joissa on tutkittu seerumin verisuonikasvuominaisuuksia pre-eklampsiassa. Kaikissa osatöissä on käytetty ihmissoluperäistä kudosviljelymallia. Käytetty verisuonimalli on kehitetty Tampereen yliopiston alaisuudessa toimivassa asiantuntijakeskuksessa FICAM:ssa (Finnish Centre For Alternative Methods), joka on edelläkävijä ei-eläinkokeellisten kudos- ja elinmallien kehityksessä.

Ensimmäiseen osatyöhön osallistui 21 naista, joista 11 oli sairastunut pre-eklampsiaan ja 10 toimi terveenä vertailuryhmänä. Tutkittavien rekrytointi tapahtui vuosina 2011-2014. Kaikilta naisilta otettiin lähellä synnytysajankohtaa seeruminäyte, ja synnytyksen yhteydessä napanuorasta seeruminäytteet. Seeruminäytteistä tutkittiin verisuonikasvuominaisuuksia verisuonimallia hyödyntäen. Tutkimuksessa todettiin pre-eklampsiaan sairastuneen naisen seerumin estävän verisuonikasvua. Terveillä vertailuryhmän naisilla vastaavaa löydöstä ei todettu. Molemmissa ryhmissä sikiön napaseerumi oli verisuonikasvua estävää eikä ryhmien välillä todettu eroa.

Toisessa osatyössä tutkittiin ensimmäiseen osatyöhön osallistuneiden naisten seerumin verisuonikasvuominaisuuksia alkuraskaudessa ja siinä tapahtuvia muutoksia raskauden edetessä. Molemmissa ryhmissä oli kuusi naista. Verisuonimallin lisäksi verisuonikasvuominaisuuksia tutkittiin määrittämällä yksittäisten verisuonikasvutekijöiden pitoisuuksia seerumissa. Kaikki seeruminäytteet lisäsivät verisuonten kasvua eikä ryhmien välillä ollut eroa

alkuraskauden näytteissä. Myöskään verisuonikasvutekijöiden määrissä ei todettu ryhmien välillä eroa alkuraskaudessa.

Kolmanteen ja neljänteen osatyöhön osallistui 20 raskaana olevaa naista. Seeruminäytteitä kerättiin neljästä eri potilasryhmästä (varhainen pre-eklampsia, myöhäinen pre-eklampsia, sikiön kasvuhidastuma ja terve) vuosina 2017-2018. Kolmannessa osatyössä määritettiin metformiinin ja neljännessä pravastatiinin vaikutusta verisuonikasvuun kolmella eri lääkepitoisuudella. Terapeuttisilla pitoisuuksilla kumpikaan lääke ei merkittävästi vaikuttanut verisuonikasvuun yksinään tai yhdessä äidin seerumin kanssa. Terapeuttisen annoksen ylittävillä pitoisuuksilla metformiini esti verisuonikasvua kaikissa ryhmissä. Kahdella matalimmalla pitoisuudella pravastatiini sen sijaan lisäsi seerumin verisuonikasvua osalla varhaista pre-eklampsiaa sairastavista naisista.

Väitöstyö osoittaa, että käytetty ihmissoluperäinen verisuonimalli soveltuu pre-eklampsian verisuonikasvuominaisuuksien tutkimiseen. Verisuonimallia käyttäen saadaan kokonaisvaltainen käsitys tutkittavan aineen verisuonivaikutuksesta, ja malli soveltuu myös lääkeaineiden verisuonivaikutusten tutkimiseen. Tässä tutkimuksessa määritettiin pravastatiinin ja metformiinin vaikutusta verisuonikasvuun, koska molemmilla lääkeaineilla on ajateltu olevan korjaava vaikutus pre-eklampsiassa usein esiintyvään verisuonikasvutekijöiden epätasapainoon. Tämän tutkimuksen perusteella kumpikaan lääkeaine ei merkittävästi parantanut seerumin verisuonikasvuominaisuuksia terapeuttisilla pitoisuuksilla.

CONTENTS

ABBREVIATIONS

ACOG	American College of Obstetricians and Gynecologists
BMI	body mass index
BP	blood pressure
ECs	endothelial cells
ET-1	endothelin-1
FDA	Food and Drug Administration
FGF-β	fibroblast growth factor β
FICAM	Finnish Centre for Alternative Methods
hASC	human adipose stromal cell
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HO-1	heme oxygenase 1
HUVEC	human umbilical vein endothelial cell
IUGR	intrauterine growth restriction
ISSHP	International Society for the Study of Hypertension in Pregnancy
LDL	low density lipoprotein
MMP-2	matrix metalloproteinase-2
PBS	phosphate buffered saline
PE	pre-eclampsia
PCOS	polycystic ovary syndrome
PlGF	placental growth factor
RT	room temperature
sEng	soluble endoglin
sFlt-1	soluble fms-like tyrosine kinase-1
STB	syncytiotrophoblast
TNF-α	tumour necrosis factor α
VCAM-1	vascular cell adhesion molecule 1
VEGF-A	vascular endothelial growth factor A
VEGFR	vascular endothelial growth factor receptor
vWF	von Willebrand factor

ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by the Roman numerals I–IV.

Publication I **Virtanen A**, Toimela T, Tihtonen K, Sarkanen JR, Huttala O, Heinonen T, Uotila J. Strong inhibitory effect of pre-eclampsia serum on angiogenesis detected *in vitro* by human cell-based angiogenesis tests. Pregnancy Hypertens. 2016 Oct;6(4):367-373.

Publication II **Virtanen A**, Huttala O, Tihtonen K, Toimela T, Heinonen T, Uotila J. Angiogenic capacity in pre-eclampsia and uncomplicated pregnancy estimated by assay of angiogenic proteins and an *in vitro* vasculogenesis/angiogenesis test. Angiogenesis. 2019 Feb;22(1):67-74.

Publication III **Virtanen A**, Huttala O, Tihtonen K, Toimela T, Heinonen T, Laivuori H, Uotila J. Therapeutic doses of metformin do not have impact on angiogenesis in presence of sera from pre-eclamptic, IUGR and healthy pregnancies. Pregnancy Hypertens. 2020 Oct;22:7-13.

Publication IV **Virtanen A**, Huttala O, Tihtonen K, Toimela T, Heinonen T, Laivuori H, Uotila J. Angiogenic effect of pravastatin alone and with sera from healthy and complicated pregnancies studied by *in vitro* vasculogenesis/angiogenesis assay. J Vasc Res. 2021 Feb;11:1-9.

1 INTRODUCTION

Pre-eclampsia complicates 2–8% of all pregnancies worldwide (ACOG Practice Bulletin, 2020). It is characterized by new-onset hypertension, endothelial dysfunction and signs of end-organ dysfunction, most often affecting the liver and kidneys (Steegers et al., 2010). Although most women with pre-eclampsia experience mild symptoms, it is still one of the leading causes of maternal and foetal morbidity and even mortality, especially in developing countries (Hutcheon et al., 2011). Pre-eclampsia has commonly been subclassified into early- and late-onset forms (von Dadelszen et al., 2003). Early-onset pre-eclampsia occurs before 34 weeks of pregnancy and it is mainly considered as a placental disease, whereas the late-onset form occurs after 34 weeks and it is more associated with maternal metabolic factors (Ogge et al., 2011). Although the pathogenic mechanisms behind the disorders may be different, the endpoint of both forms is multi-organ endothelial dysfunction.

Angiogenesis is a process by which new blood vessels form from existing ones. It is tightly regulated by pro- and anti-angiogenic molecules. Controlled angiogenesis is crucial for many physiological conditions as well as for normal pregnancy outcome (Carmeliet, 2003). Both excess and insufficient angiogenesis contribute to the pathogenesis of various diseases. Abnormal angiogenesis has been implicated in the development and spread of malignant tumours decades before the discovery of its involvement in pre-eclampsia. In the late 1980s Roberts and colleagues hypothesized that shallow trophoblast invasion in early gestation and subsequent reduction in placental perfusion lead to placental ischaemia, and release of a damaging factor into the maternal circulation (Roberts et al., 1989). This factor was later recognized as soluble fms-like tyrosine kinase 1 (sFlt-1) (Maynard et al., 2003). In 2004 Levine and colleagues presented evidence that sFlt-1 concentrations begin to increase five weeks before the onset of clinical pre-eclampsia and the rise in sFt-1 levels is accompanied by decreases in the circulating concentrations of free placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) (Levine et al., 2004). The angiogenic imbalance theory of pre-eclampsia was introduced. Since then, angiogenesis has been widely studied, first to solve the exact pathophysiology of this "disease of theories", and later to find a predictive method or curative treatment for pre-

eclampsia. Despite increasing evidence, the exact pathophysiology of pre-eclampsia is still unknown, and the only curative treatment remains delivery.

Angiogenesis-related diseases would optimally be studied *in vivo,* and various animal models of pre-eclampsia have been developed (Sunderland et al., 2011). However, there is growing evidence that animal and human tissue biochemistry is more different than has been previously thought, and the results obtained from animal experiments do not necessarily predict human reactions (Bracken, 2009). *In vitro* models offer an alternative method for testing the modulators of angiogenesis. Recent advances in *in vitro* technologies offer opportunities to improve modelling of human conditions, and human cell-based *in vitro* models are increasingly being used in studies of medical disorders and in the development of new therapies (López et al., 2018).

The main objective of this study was to test the suitability of a human adipose stromal cell/human umbilical vein endothelial cell (hASC/HUVEC) *in vitro* model for evaluating angiogenic properties of maternal and umbilical sera. We were particularly interested in angiogenic alteration in pre-eclamptic pregnancies, but additionally we evaluated angiogenesis in cases of intrauterine growth restriction and in healthy pregnancies. Another major objective was to study the angiogenic effects of metformin and pravastatin, since they both have been suggested to reverse the angiogenic balance in pre-eclamptic pregnancies. In addition, in order to assess the direct effects of these drugs on angiogenesis, we investigated their interaction with maternal sera from healthy and complicated pregnancies.

2 REVIEW OF THE LITERATURE

2.1 Angiogenesis and vasculogenesis

Vasculogenesis and angiogenesis are two distinct mechanisms of blood-vessel formation (Risau, 1997). In human fetoplacental development, vasculogenesis starts at 21 and continues until 32 days post conception (Charnock-Jones et al., 2004). During vasculogenesis undifferentiated mesodermal cells are recruited to form primitive vascular networks during embryogenesis, and it occurs in parallel with the formation of blood cells (hematopoiesis) (Schmidt et al., 2007). Hemangioblastic stem cells give rise to angioblasts that are the progenitors of endothelial cells. VEFG is the major regulator controlling the differentiation and behaviour of endothelial cells during embryonic development, but particularly during the first steps of vasculogenesis also fibroblast growth factor (FGF) is important mediator (Charnock-Jones et al., 2004). Previously, vasculogenesis was thought to be limited to embryonic development, but currently it is known that bone marrow-derived progenitor cell-mediated vasculogenesis also exists in adults (Tepper et al., 2005).

Angiogenesis is defined as a process of creating new blood vessels from pre-existing vasculature (Flamme et al., 1997). Angiogenesis is involved in many physiological and pathological conditions, as presented in Table 1 (Carmeliet, 2003) (Salajegheh, 2016). It is an essential process in growth and development of the embryo and placenta, and it plays a central role in wound healing, the menstrual cycle and tissue repair (D'Alessio et al., 2015). Tumour angiogenesis is defined as proliferation of a network of blood vessels which supply a tumour with nutrients and oxygen for growth and spread (Kerbel, 2008). Therefore, inhibition of angiogenesis is an important target in cancer therapy (Jayson et al., 2016). Uncontrolled angiogenesis is also involved in other (non-malignant diseases) such as rheumatoid arthritis and psoriasis (Szekanecz & Koch, 2007). A new and interesting field in the treatment of cancer and other diseases is control of angiogenesis, i.e. normalization of the vasculature (Goel et al., 2011) (Carmeliet & Collen, 2000).

2.1.1 Mechanism and regulation of angiogenesis

There are two types of angiogenesis; sprouting and intussusceptive (Potente et al., 2011). Sprouting angiogenesis is more understood than intussusceptive angiogenesis since it has been recognized earlier and it is easier to visualize (Adair & Montani, 2010) (Mentzer & Konerding, 2014). The stages of sprouting are 1) dissolution of basement membrane, 2) proliferation of endothelial cells (ECs) into surrounding matrix, 3) migration of ECs toward the angiogenic stimulus, 4) reorganization of ECs to form tubules, 5) interconnection with other sprouts and 6) vessel stabilization (Potente et al., 2011) (Eilken & Adams, 2010) (Adair & Montani, 2010). Sprouting angiogenesis is initiated by hypoxia. Intussusceptive angiogenesis, also known as splitting angiogenesis, is the formation of a new blood vessel by splitting a single vessel into two. The splitting happens when two opposing capillary walls establish a zone of contact in the capillary lumen. The endothelial junctions are reorganized, the basement membrane perforated and capillaries are divided by tissue pillars into two vessels (Nowak-Sliwinska et al., 2018). Since intussusceptive angiogenesis is an intravascular process, it is not visualized by conventional light microscope. The basic principles of intussusceptive angiogenesis are well described, but the exact cellular and molecular mechanisms are not yet characterized (Nowak-Sliwinska et al., 2018). Intussusceptive angiogenesis is considered to be a fast and effective mechanism compared with sprouting angiogenesis (Adair & Montani, 2010).

Angiogenesis is a complex and tightly coordinated process that is regulated by stimulatory and inhibitory molecules. The balance between pro- and anti-angiogenic factors is important because both excessive formation of blood vessels and their insufficient development can lead to adverse consequences (Matsumoto & Claesson-Welsh, 2001). Members of the vascular endothelial growth factor (VEGF) family are the most important inducers of angiogenesis. The VEGF family includes VEGF A, B, C, D, and placental growth factor (PlGF) (Holmes & Zachary, 2005). VEGFs are produced in response to hypoxia mainly by endothelial cells. They bind to three tyrosine kinase receptors on the cell surface. These receptors are called vascular endothelial growth factor receptors (VEGFR1-3) or alternatively fms-like tyrosine kinase (Flt-1-3) receptors (Matsumoto & Claesson-Welsh, 2001)(Fig. 1). PlGF is an important pro-angiogenic factor in placental formation and in early embryonic development (De Falco, 2012). The main sources of PlGF are placental trophoblasts, and its level in the maternal circulation increases along with gestation, reaching a peak at 29–32 weeks (Levine et al., 2004). PlGF binds to Flt-1 with higher affinity compared with VEFG-A or VEGF-B (De Falco, 2012). Besides VEFGs, other

molecules also stimulate angiogenesis, such as fibroblast growth factor, angiopoietin, tumour necrosis factor-alpha and interleukin 8 (Ucuzian et al., 2010) (Koch et al., 1992). Soluble fms-like tyrosine kinase-1 (sFlt-1) is a circulating anti-angiogenic protein which is a splice variant of the Flt-1 receptor lacking the transmembrane domain. Its main source is the placenta, but it is additionally produced by endothelial cells and monocytes. It has anti-angiogenic activity as it binds free VEGF and PlGF and prevents them from binding to transmembrane receptors (Stepan et al., 2004)(Fig. 1). Recently sFlt-1 has been extensively studied in association with pre-eclampsia, but it is also an important regulator of blood-vessel formation in embryogenesis (Ambati et al., 2006). Overall, angiogenic factors do not simply control angiogenesis, they also maintain normal endothelial structure and function. Soluble endoglin (sEng) is an anti-angiogenic glycoprotein that binds circulating transforming growth factor beta (TGF-β). Endoglin is expressed in vascular endothelial cells and placental trophoblasts (Venkatesha et al., 2006).

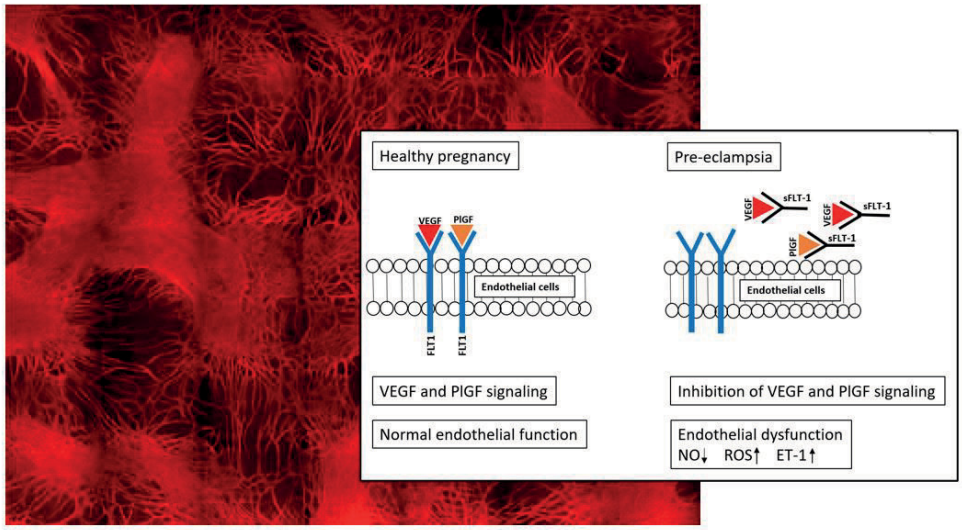

Figure 1. Immunofluorescence image of enhanced tubule formation (angiogenesis) and simplified illustration of disrupted angiogenic balance in pre-eclampsia. NO, nitric oxide; ROS, reactive oxidative species; ET-1, endothelin 1. Immunofluorescence image obtained from Outi Huttala.

Table 1. Physiological and pathological conditions of angiogenesis. (Carmeliet et al. 2003, Salajegheh 2016)

Physiological	Pathological (increased/decreased angiogenesis)
Embryogenesis	Tumour growth and metastasis (increased)
Formation of placenta	Diabetic retinopathy (increased)
Wound healing	Haemangioma (increased)
Fracture repair	Rheumatoid arthritis (increased)
Regeneration of endometrium	Atherosclerosis (increased)
after menstruation	Gastric ulcerations (decreased)
	Crohn's disease (increased)
	Psoriasis (increased)

2.1.2 Vascular homeostasis and endothelial function

The vascular system has a crucial role in ensuring that oxygen and nutrients reach every organ and tissue, and waste products are removed. Vascular endothelium, the innermost layer of the blood vessel (tunica intima), is located at the interface between blood and surrounding tissues. The middle layer (tunica media) contains smooth muscle and it comprises the contractile part of the vessel wall, and the outmost layer (tunica adventitia) is made of connective tissue (Pugsley & Tabrizchi, 2000). Although the general structure of blood vessels is maintained throughout the body, each organ has unique demands on the vasculature. Vascular homeostasis is the balance between vascular injury and vascular repair. Healthy state of vascular system is maintained by constant adjustment of several biochemical and physiological pathways (Bondareva & Sheikh, 2020). Vascular endothelium maintains vessel stability and proper function and it is the most important regulator of vascular homeostasis. Endothelial cells release substances controlling vascular relaxation and contraction as well as enzymes controlling blood clotting, immune function, inflammatory processes and platelet adhesion (Pearson, 2000). Normal endothelial function is essential for vascular system to respond varying demands of the tissues. Endothelial dysfunction is characterized by imbalanced vascular tone, elevated levels of reactive oxygen species (ROS) and proinflammatory factors consequently leading to disruption of endothelial permeability (Deanfield et al., 2007). Endothelial dysfunction has been mostly studied in connection with cardiovascular diseases, and it is known to precede the development of atherosclerosis (Rajendran et al., 2013).

However, it is also associated with several other diseases and pathological states such as pre-eclampsia, diabetes and septic shock (Chambers et al., 2001) (Tabit et al., 2010) (Lee & Slutsky, 2010). There is little data on endothelial function in normal pregnancy, but reduced peripheral vascular resistance in middle gestation is thought to be mediated by endothelium-dependent factors such as nitric oxide synthesis. There is evidence that in normal pregnancy vascular flow mediated dilatation increases until 32 weeks of gestation, with a fall at 36 weeks (Quinton et al., 2007).

2.1.3 *In vitro* models as research tools

In vitro (within the glass) studies are performed outside of a living organism whereas *in vivo* (within the living) refers to work that is performed in a living organism including animal models and human clinical trials. Angiogenesis can be assessed through both *in vivo* and *in vitro* methods.

Animal models have played an important role in many medical advances, and continue to be an essential part of medical research (Matthews, 2008). However, the 3Rs principles (replacement, reduction, refinement) were launched as early as in the late 1950s (Russell, 1995). Following 3R guidance, whenever possible the use of animals should be 1) replaced by alternative methods, 2) fewer animals should be used to obtain sufficient data to answer the research hypothesis, and 3) procedures should be modified to minimize pain and distress of an animal used in science (Aske & Waugh, 2017). Although animal tests have improved our understanding of human disease and the biological effects of many substances, they also have several limitations (Bracken, 2009). Animal data does not always predict the human response, since animal species have different metabolic pathways that can lead to variation in efficacy and toxicity of a tested drug, for example (Schmidt et al., 2010). The most well-known failure of animal testing concerns thalidomide. Preclinical tests in animals did not show signs of teratogenic risk, but maternal exposure to thalidomide caused severe birth defects (Vargesson, 2015). The teratogenic effects of thalidomide were later associated with its anti-angiogenic properties (Franks et al., 2004). To study angiogenesis, various animal models have been created. In comparison with *in vitro* models, they are considered to be more expansive and complex to apply (Staton et al., 2009).

In vitro tests are commonly used in the detection of toxic properties of drugs and chemicals. However, recent advances in *in vitro* technologies offer opportunities to improve modelling of human conditions, and human cell-based *in vitro* models are

increasingly being used in studies of medical disorders and development of new therapies (Roggen, 2011). Following EU legislation there is growing pressure to replace animal-based testing with alternative methods. This has led to the development of novel microphysiological systems such as organs-on-chips, tissue-chips and 3D modelling that can express human-specific interactions between drugs and organs (Hammad, 2013) (Wu et al., 2020). Even though there have been great advances in *in vitro* technologies, we are still far from full replacement of animal experiments by *in vitro* systems (Adler et al., 2011). The challenges in *in vitro* methods include difficulties in capturing interaction between different cell types, changes in up/down regulation of genes when primary cells are isolated from their normal microenvironment, and difficulties in extrapolating results from *in vitro* to *in vivo* (Ghallab, 2013). In medical research, at least for now, *in vitro* methods should not be considered to be substitutes for animal models; rather, they should be used as adjuncts to animal experiments. For example, in the development of therapies for cartilage-defect repair, both *in vitro* and *in vivo* methods are utilized. *In vitro* testing provides information about safety, but in order to assess regenerative capabilities, and immune responses associated with implantation, the use of animal models is necessary (Moran et al., 2016).

2.2 Angiogenesis in uncomplicated pregnancy

Vasculogenesis and angiogenesis are involved in every step of placental development. During the first and second weeks after fertilization the blastocyst forms an inner and outer cell mass. The placenta develops from the outer cell mass, the trophectoderm, which subsequently differentiates into syncytiotrophoblast (STB) and cytotrophoblast cells (Torry et al., 2007). Implantation takes place eight to nine days after conception. Adequate uterine vascularity at the implantation site is needed for successful attachment of the embryo to the uterine wall, and it has been hypothesized that embryo-derived factors mediate these vascular changes in the decidua (Torry et al., 2007). Following implantation, trophoblasts invade the decidualized endometrium and migrate into maternal spiral arteries. Early placental development is initiated in a hypoxic environment, and it is a highly regulated process with a predominance of pro-angiogenic biomarkers such as PlGF and VEGF (Reynolds et al., 2010). These factors are not secreted only by endothelial cells, but also by the trophoblast. Angiogenesis in the placenta requires proliferation,

migration and differentiation of endothelial cells in the same manner as it occurs in any other organ (Kaufmann et al., 2004). Both sprouting and intussusceptive angiogenesis have been described in the placenta, the former occurring before 24 weeks of gestation and the latter after that (Chen & Zheng, 2014). Although angiogenesis is particularly important in the first weeks of pregnancy, it continues to occur throughout gestation as the placental vasculature further expands. The major source of angiogenic biomarkers is the trophoblasts, although they are also secreted from other cells. The balance between pro- and anti-angiogenic biomarkers changes with advancing gestational age. At the end of pregnancy, maternal sera have a more anti-angiogenic nature than in early gestation, but the alteration in angiogenic balance is less pronounced than in pregnancies complicated by pre-eclampsia (Levine et al., 2004). Since angiogenic biomarkers are mainly produced in the placenta, rapidly after delivery levels of angiogenic biomarkers normalize to resemble the non-pregnant state.

2.3 Disturbed angiogenesis in pregnancy

2.3.1 Pre-eclampsia

Pre-eclampsia is a hypertensive disorder of pregnancy in which genetic, immunological and angiogenic factors interact (Lokki et al., 2017) (Ahn et al., 2011) (Levine & Karumanchi, 2005). Current definition of pre-eclampsia by the American College of Obstetricians and Gynecologists (ACOG) (ACOG Practice Bulletin, 2020) and by the International Society for the Study of Hypertension in Pregnancy (ISSHP) (Tranquilli et al., 2014) is presented in Table 2. In Finland, the 10th edition of International Statistical Classification of Diseases and Related Health Problems (ICD) is used for classification of hypertensive disorders in pregnancy (WHO, n.d.). The World Health Organization has already published the 11th version of ICD, but it comes into effect not until in 2022. The classification of hypertensive disorders in pregnancy in ICD-10 and ICD-11 is presented in Table 3. Pre-eclampsia affects approximately 2–8% of pregnancies and remains one of the major causes of maternal and neonatal morbidity worldwide (Khan et al., 2006) (Duley, 2009). Although several medical conditions, such as chronic hypertension, diabetes, renal diseases and obesity, are known to increase the risk of pre-eclampsia, prediction and prevention of the disease is still challenging (Burton et al., 2019) (Maynard & Karumanchi,

2011). Low-dose acetylsalicylic acid and calcium supplementation in women with a low-calcium diet have proven to work as preventive measure in high risk pregnancies (Roberge et al., 2013) (Hofmeyr et al., 2018), but most cases of pre-eclampsia occur in healthy nulliparous women. Pre-eclampsia is generally divided into two main subtypes, early- and late-onset pre-eclampsia. Late-onset pre-eclampsia occurs after 34 weeks, and has been considered to be a 'maternal' disease due to its association with maternal metabolic factors (Lisonkova & Joseph, 2013). It is the most frequent form of pre-eclampsia, and usually a less severe disease than the early-onset form. Early-onset pre-eclampsia occurs before 34 weeks, and it is associated with a substantial risk of intrauterine growth restriction (Steegers et al., 2010). It is regarded as 'placental' pre-eclampsia as it appears to be caused by placental dysfunction as a result of deficient placentation in early pregnancy (Phipps et al., 2019). Multi-organ endothelial dysfunction, due to disturbed angiogenesis and systemic vascular inflammation, is the endpoint of both forms of pre-eclampsia, although the pathogenic mechanisms behind the disorders may be different (van der Merwe et al., 2010). Previously, pre-eclampsia was considered to be a pregnancy-limited disease that resolves at birth. The pathogenesis of pre-eclampsia is still not completely understood. However, it is known that pre-eclampsia is a more multidimensional disease than was thought earlier, and it seems to affect long-term health of both mother and offspring (Lykke et al., 2009) (Kajantie et al., 2009). A history of pre-eclampsia, especially recurrent or the early-onset form of the disease, is recognized as an independent risk factor of cardiovascular disease (Mosca et al., 2011) (Staff et al., 2016). It has been suggested that pre-eclampsia can worsen pre-existing cardiovascular risk factors or even induce a de novo risk (Staff et al., 2016). Despite increasing understanding of the pathophysiology of the disease, there is still no effective prevention or curative treatment of pre-eclampsia apart from delivery. New management strategies are directed to reverse or arrest the pathological processes of pre-eclampsia or to prevent its occurrence (El-Sayed, 2017). One of the main targets is to restore angiogenic balance and thus maintain normal endothelial function (Armaly et al., 2018).

Table 2. Diagnostic criteria of pre-eclampsia by ISSHP and ACOG

ISSHP (2014)

Hypertension developing after 20th weeks of gestation and the coexistence of one or more of the following new-onset conditions:
1. Proteinuria 2. Other maternal organ dysfunction: - renal insufficiency (creatinine ≥90 μmol/L) - liver involvement (elevated transaminases and/or severe right-upper quadrant or epigastric pain) - neurological complications (examples include eclampsia, altered mental status, blindness, stroke, clonus, severe headaches, persistent visual scotomata) - haematological complications (thrombocytopenia, DIC, haemolysis) 3. Uteroplacental dysfunction - foetal growth restriction
Hypertension is defined as systolic blood pressure ≥140 and/or diastolic blood pressure ≥90 mmHg. DIC, disseminated intravascular coagulation.

ACOG (2017)

Hypertension occurring on two occasions at least 4 hours apart after 20 weeks of gestation in a woman whose blood pressure has previously been normal, and the coexistence of one of the following conditions:
1. Proteinuria 2. Thrombocytopenia: Platelet count less than 100,000/μL 3. Renal Insufficiency: Serum creatinine concentration greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal disease. 4. Impaired liver function: Elevated blood levels of liver enzymes to twice normal concentrations 5. Pulmonary oedema 6. Cerebral or visual symptoms
Hypertension is defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or systolic blood pressure ≥160 mmHg or diastolic blood pressure ≥110 mmHg confirmed within a short interval.

Table 3. Classification of hypertensive disorders in pregnancy (ICD-10 and ICD-11)

ICD-10

Oedema, proteinuria and hypertensive disorders in pregnancy, childbirth and the puerperium	
O10	Pre-existing hypertension
O11	Pre-eclampsia superimposed on chronic hypertension
O12	Gestational (pregnancy-induced) oedema and proteinuria without hypertension
O13	Gestational (pregnancy-induced) hypertension
O14	Pre-eclampsia
	O14.0 Mild to moderate pre-eclampsia
	O14.1 Severe pre-eclampsia
	O14.2 HELLP syndrome
	O14.9 Pre-eclampsia, unspecified
O15	Eclampsia
O16	Unspecified maternal hypertension

HELLP, haemolysis, elevated levels of liver enzymes and low platelet count

ICD-11

Oedema, proteinuria and hypertensive disorders in pregnancy, childbirth and the puerperium	
JA20	Pre-existing hypertension BP (>140/90 mmHg) prior to the 20th week of pregnancy, or persisting >12 weeks postpartum.
JA21	Pre-eclampsia superimposed on chronic hypertension BP (>140/≥90mmHg) developing >20 weeks of gestation in the presence of either proteinuria or thrombocytopenia, elevated creatinine or liver transaminases, or neurological conditions or fetal growth restriction in a female diagnosed with pre-existing hypertension
JA22	Gestational oedema or proteinuria without hypertension
JA23	Gestational hypertension Hypertension (>140mmHg and/or ≥90mmHg) >20 weeks gestation or <1 week postpartum
JA24	Pre-eclampsia Same as JA21, but without week limit or pre-existing hypertension
JA25	Eclampsia Seizure or convulsions during pregnancy or the puerperium that are often associated with high BP, anxiety, epigastric pain, severe headache, blurred vision, proteinuria, and oedema
JA2Z	Unspecified residual category

BP, blood pressure

2.3.1.1 Pre-eclampsia and angiogenesis in early gestation

Pre-eclampsia, especially its early-onset form, is considered as a two-stage disorder in which the first stage takes place during early placental development (Chaiworapongsa et al., 2014). The role of vasculogenesis in the pathogenesis of pre-eclampsia is unknown. In the first weeks of pregnancy remodelling of the spiral arteries begins, when vascular smooth muscle and endothelial cells lining the vessels are replaced by cytotrophoblast. This converts the normally narrow and rigid uterine vessels into dilated structures which lose their susceptibility to vasoconstriction (Fig. 2) (Lyall, 2005). In pregnancies destined to develop pre-eclampsia, reduced cytotrophoblast invasion prevents appropriate spiral artery remodelling, leading to impaired placentation (Chaiworapongsa et al., 2014) (Brosens et al., 2011). Angiogenic proteins have been widely studied in early gestation in order to identify women destined to develop pre-eclampsia later on (McElrath et al., 2012) (Myers et al., 2013). Investigations concerning combinations of angiogenic proteins, other biomarkers and uterine artery Doppler measurements have resulted in better performance than single biomarkers in the prediction of pre-eclampsia (Kenny et al., 2014) (Akolekar et al., 2013). Generally speaking, risk assessment of pre-eclampsia is still challenging, and use of algorithms that combine maternal factors and biomarkers are of poor prognostic value, especially in identifying women who will develop late-onset pre-eclampsia (Chaiworapongsa et al., 2014) (Myers et al., 2013). The predictive performance of angiogenic biomarkers for identifying women at risk of developing pre-eclampsia increases with advancing gestational age, and when trying to predict early rather than late-onset disease (Kusanovic et al., 2009).

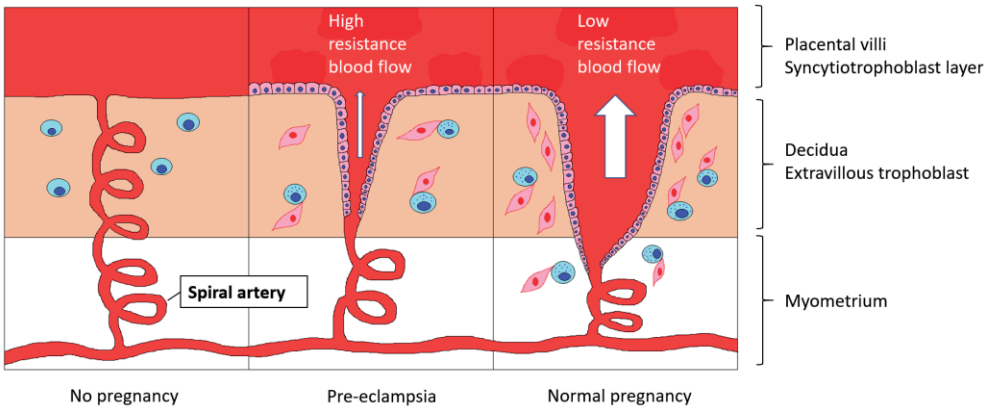

Figure 2. Uterine spiral artery remodelling in pre-eclampsia and normal pregnancy. Modified from Bell E., Nature reviews immunology 2004 (Bell, 2004).

2.3.1.2 Angiogenesis and clinical presentation of pre-eclampsia

In 1989 Roberts and colleagues proposed that pre-eclampsia represents a maternal endothelial disorder. They hypothesized that shallow trophoblast invasion and subsequent reduction in placental perfusion leads to placental ischaemia, and release of a damaging factor(s) into the maternal circulation. The unknown circulating factor was hypothesized to cause endothelial dysfunction and activation of the coagulation cascade, blood pressure abnormalities, and loss of fluid from the intravascular space (Roberts et al., 1989). More than a decade later, this factor was recognized as a soluble fms-like tyrosine kinase 1 (sFlt-1) (Maynard et al., 2003), and soon after that the angiogenic imbalance theory of pre-eclampsia was presented (Fig. 3) (Levine et al., 2004). Currently, it is known that sFlt-1 concentrations begin to increase approximately five weeks before the onset of clinical pre-eclampsia. The increase in sFt-1 levels is accompanied by decreases in circulating free PlGF and VEGF concentrations (Maynard & Karumanchi, 2011). In pregnancies destined to develop pre-eclampsia, a low level of PlGF has been documented as early as several weeks before symptomatic disease (Romero et al., 2008). The decrease in free PlGF levels is mainly a consequence of an increased amount of sFlt-1 in the maternal circulation, but there is also evidence of reduced placental secretion as the STB reduces the expression of the PlGF mRNA under hypoxic conditions (Shore et al., 1997) (Redman & Staff, 2015). The key part of the placenta is the inner part of the villi, the

STB layer, which is in contact with maternal blood. The increasing demand for blood flow with advancing pregnancy leads to inadequate uteroplacental perfusion. Subsequent hypoxia and oxidative stress cause dysfunction in STB cells that stimulates release of multiple vasoactive factors (Redman & Staff, 2015). As a result, widespread damage to the maternal vascular endothelium occurs, and clinical symptoms of pre-eclampsia appear (Maynard et al., 2003). The second stage of pre-eclampsia is regarded as a clinical manifestation of the disease (Steegers et al., 2010). Alterations in levels of angiogenic proteins has been observed to correlate with disease severity and earlier onset of pre-eclampsia (Rana et al., 2012). Use of the ratio of sFlt-1/PlGF has been suggested as a potential predictive method to identify or rule out pre-eclampsia (Stepan et al., 2015). A ratio greater than 85 is associated with adverse outcomes and imminent delivery within two weeks in cases of early-onset pre-eclampsia, whereas a cut-off value of 38 is appropriate for ruling out pre-eclampsia (Stepan et al., 2015) (Caillon et al., 2018).

Another anti-angiogenic protein, soluble endoglin, is expressed in the STB and on the surface of endothelial cells. Soluble endoglin levels increase markedly, beginning two to three months before the onset of pre-eclampsia, and the levels correlate with disease severity (Levine et al., 2006) (Leanos-Miranda et al., 2019).

During normal pregnancy the levels of PlGF rises until the beginning of the third trimester when it peaks at around 29–32 weeks and declines thereafter (Levine & Karumanchi, 2005). The placenta also produces increasing amounts of sFlt-1 and sEng toward the end of normotensive pregnancies, but significantly higher amounts are produced from hypoxic placentas in those pregnancies affected by pre-eclampsia (Levine & Karumanchi, 2005). It has been postulated that elevated levels of anti-angiogenic factors are not biomarkers of pre-eclampsia but of STB cellular stress, as their levels also increase in healthy pregnancies toward term, reflecting restricted placental capacity (Redman & Staff, 2015).

Altogether, several biochemical markers have been studied in the context of pre-eclampsia. Summary of most studied angiogenic biomarkers and their mechanism is presented in Table 4.

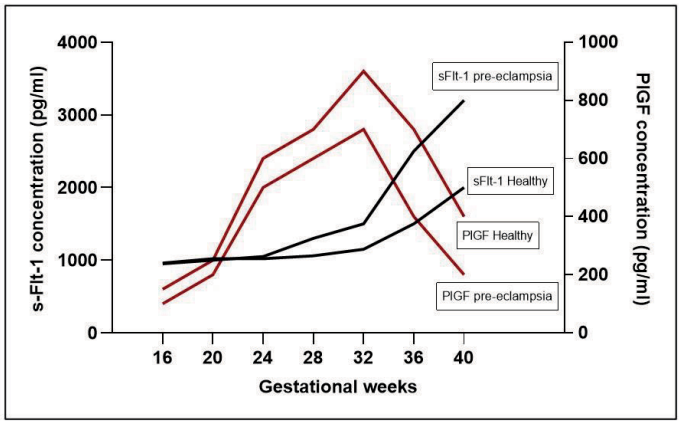

Figure 3. Mean concentrations of sFlt-1 and PlGF in pre-eclampsia and healthy pregnancy. Modified from nested case-control study of Levine et al., N Eng J Med 2004.

Table 4. Mechanism of stimulatory and inhibitory angiogenic biomarkers that have been associated with pre-eclampsia (Levine et al. 2004, Thissier-Levy 2013, Karakus et al. 2016, Adu-Gyamfi et al. 2019, Jiang et al 2021)

Angiogenic biomarker	Mechanism
Stimulatory	
VEGF/PlGF	Stimulation of proliferation, migration and sprouting of ECs. Regulation of permeability of ECs
TNF-α	Induction and enhancement the expression of pro-angiogenic molecules such as VEGF and IL-8 in ECs
ANG-1	Mediation of migration, adhesion and survival of ECs
ANG-2	Context-dependent impact on angiogenesis.
FGF	Stimulation of proliferation and migration of ECs and remodeling extracellular matrix
PDGF	Induction of recruitment of perivascular cells
Inhibitory	
sFlt-1	VEGF and PlGF binding, and subsequently blocking their angiogenic effect on VEGFR
sEng	Pro-angiogenic molecule TGF-beta binding, and subsequently decreasing its levels in circulation
Endostatin	Suppression of EC adhesion, migration and proliferation
Angiostatin	Suppression of EC adhesion, migration and proliferation
Vasostatin	Suppression of EC adhesion

EC, endothelial cell; VEGF, vascular endothelial growth factor; PlGF, placental growth factor; TNF-α, tumour necrosis factor alpha; IL-8; interleukin-8; Ang1 and 2, angiopoietin 1 and 2; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; sEng, soluble endoglin

2.3.1.3 Angiogenesis after pre-eclamptic pregnancy

Delivery of the placenta ends the production of sFlt-1 and sEng, and by 48 hours postpartum the anti-angiogenic effect in maternal serum has ceased (Maynard et al., 2003). However, it has been observed that the clearance rates of sFlt-1 is much longer in women with pre-eclampsia compared with healthy women (Powers et al., 2005). The authors speculate that the slower decrease could be due to either slower excretion or a continued higher rate of production of sFlt-1. The normal mechanism by which sFlt-1 is cleared from the body may be disrupted or there is another source of sFlt-1 production in women with pre-eclampsia. Although resolution of the anti-angiogenic environment begins shortly after birth, recovery from endothelial damage occurs over a longer time period (Weissgerber et al., 2016). Persistent vascular dysfunction has been observed in the early postpartum period (Blaauw et al., 2005) as well as years after pre-eclamptic pregnancy (Aykas et al., 2015) (Henriques et al., 2014). Women who have had early-onset pre-eclampsia have an eightfold higher risk of death from cardiovascular disease compared with women who not had pre-eclampsia and whose pregnancies have continued to term (Irgens et al., 2001). Pre-eclampsia and cardiovascular disease share several pathological findings such as hypertension, endothelial dysfunction, inflammation and oxidative stress (Lisowska et al., 2018). Pre-existing vascular endothelial dysfunction is currently viewed as a key common factor shared between pre-eclampsia and pre-eclampsia-related cardiovascular disease.

2.3.1.4 Angiogenesis and offspring

Data concerning angiogenic biomarkers in infants born to mothers with pre-eclampsia is scarce and limited only to preterm neonates. Hentges and colleagues reported that sFlt-1 levels are higher and those of VEGF lower in blood samples collected within 72 hours after birth from preterm neonates born to mothers with pre-eclampsia versus healthy pregnancies (Hentges et al., 2015). Comparable results have been published in connection with full-term neonates who have suffered from intrauterine growth restriction (IUGR), since sFlt-1 levels have been observed to be higher at the first and fourth postnatal day in comparison with appropriate-for-gestational-age infants (Boutsikou et al., 2006). Angiogenic biomarkers in umbilical

blood might reflect the angiogenic profile in the foetal circulation, and they have been evaluated in the association with postnatal growth and pre-eclampsia (Voller et al., 2014) (Staff et al., 2005). Nevertheless, umbilical blood has not been investigated to the same extent as maternal sera. In a preterm birth study, cord-blood VEGF levels were positively correlated, and those of sFlt-1 negatively correlated with both absolute birth weight and birth weight for gestational age percentiles (Voller et al., 2014). The association between angiogenic biomarkers in umbilical blood and pre-eclampsia remains unclear (Sezer et al., 2012) (Staff et al., 2005). Some investigators have reported elevated levels of sFlt-1, and decreased levels of PlGF, whereas others have not found any differences compared with normal pregnancies (Tsao et al., 2005) (Paredes et al., 2017) (Staff et al., 2005).

Davis and colleagues reported that offspring of mothers with pre-eclampsia had increased blood pressure in childhood and in early adulthood (Davis et al., 2012). A recent systemic review concerning the association between hypertensive disorders of pregnancy and cardiometabolic outcomes in childhood, revealed higher blood pressure in children exposed to pregnancy-induced hypertension, but not in children exposed to pre-eclampsia (Jansen et al., 2019). Children born to women with pre-eclampsia have noticed to have disturbed endothelial function in both the pulmonary and systemic circulation (Jayet et al., 2010) (Kvehaugen et al., 2011). However, in both studies a possible confounding factor was restricted foetal growth, since an adverse intrauterine environment is associated with disturbed metabolism in childhood (Barker et al., 1993) (Gluckman et al., 2008).

2.3.2 Intrauterine growth restriction

Pre-eclampsia and intrauterine growth restriction (IUGR) share many clinical and pathophysiological features (Maynard & Karumanchi, 2011). IUGR is a common complication of early-onset pre-eclampsia, whereas in late-onset disease, growth of the foetus is generally normal. The placental bed of women with pre-eclampsia associated with IUGR is similar to that which has been described in women with pre-eclampsia. It is characterized by non-transformed spiral arteries and acute atherosis (Brosens et al., 2011). It is unknown why some women with placental insufficiency manifest pre-eclampsia, whereas others have IUGR without pre-eclampsia (Maynard & Karumanchi, 2011). Results concerning angiogenic factors in IUGR pregnancy are inconsistent: some investigators have reported changes in sFlt-

1, sEng, and PlGF similar to those seen in pre-eclampsia (Crispi et al., 2006) (Laskowska et al., 2012), whereas others have not found such changes or have reported less-pronounced alterations in angiogenic factors (Romero et al., 2008) (Jeyabalan et al., 2008).

2.4 Pharmacological agents modulating angiogenesis

Drugs that modulate angiogenesis are in clinical use in oncology and ophthalmology. Angiogenesis is exaggerated both in tumour growth and metastasis as well as in retinal neovascularization. Therefore, the focus of the treatments of these diseases is on inhibition of angiogenesis, not on stimulation. The most well-known of these anti-angiogenic drugs is bevacizumab, which targets VEGF-A (Yoo & Kwon, 2013). After Levine and colleagues published the finding of changed angiogenic balance in pre-eclampsia in 2004 (Levine et al., 2004), therapeutic studies have mainly been focused on restoring normal angiogenesis. However, there are also other research directions such as those concerning therapies that improve endothelial dysfunction and attenuate generalized vasoconstriction. Genetic variation in pre-eclampsia is a growing area of investigation, and there is a publication on RNA therapy targeted on suppression of placental overexpression of sFlt-1 (Roberge et al., 2013).

Thadhani and colleagues investigated apheresis as an optional procedure in the management of pre-eclampsia since an excess amount of sFlt-1 in the maternal circulation is likely to be one of the major drivers of the disease. Apheresis is commonly used in the treatment of familial hypercholesterolaemia, including during gestation, but in the context of pre-eclampsia it is used to remove excess sFlt-1 from the maternal circulation. In a small cohort study, apheresis therapy extended the duration of pregnancy by eight to 15 days compared with a delay of three days in an untreated comparison group. No adverse effects were reported (Thadhani et al., 2016). However, the results must be interpreted with caution since the reduction in sFlt-1 levels was short-lived and repeated treatments were needed to maintain reduced concentrations (Easterling, 2016).

Studies in animal models of pre-eclampsia have investigated VEGF-therapy (Li et al., 2007) (Gilbert et al., 2010). It attenuates hypertension and proteinuria, but there is also evidence of VEGF toxicity at high doses. There are reports of oedema formation and heart abnormalities, which might be a result of increased vascular permeability (Miquerol et al., 2000). Another potential therapeutic option to correct

the angiogenic imbalance in pre-eclampsia is PlGF therapy. In animal models of pre-eclampsia, recombinant human PlGF therapy has resulted in reduced levels of free sFlt-1 and decreased blood pressure (Spradley et al., 2016) (Makris et al., 2016). Although toxic effects have not been reported, there are still several uncertainties concerning the therapy such as timing and duration of exposure and the concentration to which PlGF should be restored (Eppler et al., 2002) (Chau et al., 2017).

The pharmaceutical agents that are considered to affect angiogenesis in pre-eclampsia, and whose use has proceeded to preclinical and clinical studies are pravastatin, metformin and esomeprazole. In addition, sildenafil citrate has been investigated in several studies, but its pharmacological effect is targeted on vasodilatation rather than restoration of angiogenic balance. In this study pravastatin and metformin are included.

2.4.1 Metformin

2.4.1.1 Pharmacodynamic properties and therapeutic indications of metformin

Metformin is a synthetic biguanide. It is a hydrophilic base, and its passive diffusion through the cell membrane is low. It does not bind to plasma proteins, and the mean oral bioavailability is 55 ± 16%. It is absorbed predominantly from the small intestine through several transporters. The elimination half-life of metformin is approximately five hours (Graham et al., 2011). Metformin lowers both basal and postprandial plasma glucose levels. It suppresses excessive hepatic glucose production, through a reduction in gluconeogenesis (Gong et al., 2012). Metformin increases glucose intake in peripheral tissues, and decreases intestinal absorption of glucose.

Metformin is an oral antidiabetic drug. The labelled indication for metformin use is a management of type 2 diabetes mellitus when hyperglycaemia cannot be managed with diet and exercise alone (Inzucchi et al., 2012). It can be used in monotherapy or in combination with other types of antidiabetic medication. Metformin is used as an "off label" medication in prevention of type 2 diabetes in patients with impaired glucose tolerance, in treatment of gestational diabetes (Farrar et al., 2017) and in a second-line therapy for menstrual irregularities in women with polycystic ovary syndrome (PCOS) (Creanga et al., 2008).

2.4.1.2 Metformin use in pregnancy

Metformin is commonly used during pregnancy in the treatment of type 2 and gestational diabetes. Metformin is less expensive and easier to administer than insulin and it is associated with less weight gain and fewer severe hypoglycaemic events (Lindsay & Loeken, 2017). However, many women undergoing metformin therapy require supplemental insulin treatment (Rowan et al., 2008). Metformin crosses the placenta, and the foetus is exposed to concentrations from negligible to as high as maternal plasma concentrations (Eyal et al., 2010). An increased risk of birth defects or adverse foetal or neonatal outcome has not been observed (Cassina et al., 2014), but there is evidence of increased weight of offspring in childhood after metformin therapy during pregnancy (van Weelden et al., 2018). Also, in a Finnish randomized controlled trial it was found that children exposed to metformin during pregnancy were heavier and taller at 18 months than those exposed to insulin, but there were no differences in cognitive skills between the groups (Ijas et al., 2015).

2.4.1.3 Metformin and pre-eclampsia

De Leo and colleagues reported that gestational hypertension was less common in women with PCOS using metformin throughout pregnancy compared to normal pregnant controls (De Leo et al., 2011). Meta-analysis of five randomized controlled trials showed that women with gestational diabetes treated with metformin versus insulin had lower incidence of pregnancy-induced hypertension, and the beneficial effect of metformin on hypertension was proposed to be related to less weight gain and a lower maternal inflammatory response (Gui et al., 2013). However, the association between metformin use and a lower risk of pre-eclampsia is ambiguous (Gui et al., 2013) (Alqudah et al., 2018). Among obese women without pregestational diabetes, metformin treatment from 12 to 18 weeks of gestation until delivery decreased the incidence of pre-eclampsia (Syngelaki et al., 2016). There is some evidence that metformin improves endothelial dysfunction, and promotes angiogenesis (Brownfoot et al., 2016), but there are also opposite findings of anti-angiogenic activity of metformin (Evans et al., 2005). The impact of metformin on angiogenesis has been studied mainly in cancer research, since epidemiological evidence suggests that diabetic cancer patients treated with metformin have a better outcome (Coyle et al., 2016). So far only one study group has evaluated the angiogenic capacity of metformin in human tissues obtained from pre-eclamptic women (Table 5). In a study by Brownfoot and colleagues, placental villous explants

and omental tissue were collected from women with pre-eclampsia. Explants of omental vessels were cultured with and without sFlt-1, and a reduction in angiogenic sprouting, caused by sFlt-1, was rescued by the addition of metformin (Brownfoot et al., 2016). The double-blind, randomized placebo-controlled study of efficacy of metformin in the treatment of preterm pre-eclampsia is still ongoing, and the results are not yet available (Cluver et al., 2019).

Table 5. Studies on the association between metformin and angiogenesis in pre-eclampsia.

Authors/year of publication	Type of study	Results after metformin treatment
Brownfoot et al., 2016	Placental villous explants	Metformin significantly improved the function of placental endothelial cells in pre-eclampsia, it dilated and ameliorated injured vessels, and promoted angiogenesis.
Wang et al., 2019	Mouse model of pre-eclampsia	Metformin promoted the expression of VEGF and MMP-2 in pregnant mice with a high-fat diet, and further improved shallow placental implantation in pre-eclamptic mice.
Brownfoot et al., 2020	Primary human placenta	Combination low-dose metformin and sulfasalazine reduced sFlt-1 secretion from the placenta.

MMP-2, matrix metalloproteinase-2; sFlt-1, soluble fms-like tyrosine kinase-1; VEGF, vascular endothelial growth factor

2.4.2 Pravastatin

2.4.2.1 Pharmacodynamic properties and therapeutic indications of pravastatin

Pravastatin belongs to a group of drugs called 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, also known as "statins". Pravastatin produces its lipid-lowering effect in two ways. Firstly, it inhibits HMG-CoA reductase resulting in decrease of the synthesis of intracellular cholesterol. Secondly, pravastatin inhibits low-density lipoprotein cholesterol (LDL) production by inhibiting the hepatic synthesis of very-low-density lipoprotein cholesterol (VLDL), the precursor of LDL-cholesterol (Ahmed et al., 2019). Pravastatin has a short elimination half-life (2 hours), and it is a hydrophilic statin unlike most of the statins (Egom & Hafeez, 2016).

The indications for pravastatin therapy are hypercholesterolaemia and mixed dyslipidaemia as an adjunct to diet when other non-pharmacological treatments are inadequate. Pravastatin is used as a primary prevention in reduction of cardiovascular mortality and morbidity in patients with moderate or severe hypercholesterolaemia and at high risk of a first cardiovascular event (FDA, 2012).

2.4.2.2 Pravastatin use in pregnancy

Statins are rated as category X drugs by The Food and Drug Administration (FDA) in the United States. According to instructions, statins should only be used in those fertile-aged women who are highly unlikely to conceive and have been informed of the potential hazards. If woman gets pregnant while taking statins, therapy should be discontinued. The major concern of using statins during pregnancy is inhibition of foetal cholesterol synthesis (Porter, 2003). However, foetal anomalies reported in pregnant women with statin therapy are associated with lipophilic statins, and adverse pregnancy outcomes have not been reported following exposure to pravastatin (Edison & Muenke, 2004) (Bateman et al., 2015) (Taguchi et al., 2008). It has been observed that transplacental transfer of pravastatin is limited (Zarek et al., 2013), and the concentrations of pravastatin in the umbilical cord following maternal treatment are below the limit of detection (Costantine et al., 2016).

Pravastatin crosses the placenta in both maternal to foetal and foetal to maternal directions and the transfer has been even higher in the foeto-maternal direction (Nanovskaya et al., 2013).

2.4.2.3 Pravastatin and pre-eclampsia

Pre-eclampsia shares many risk factors and pathological similarities, such as angiogenic imbalance, oxidative stress, endothelial injury and inflammation, with adult cardiovascular disease (Chen et al., 2014). The benefit of statin use for the prevention of cardiovascular disease is well-documented (Scandinavian Simvastatin Survival Study Group, 1994) (Cheung et al., 2004). Statins are generally used for hypercholesterolaemia, but there is evidence that they also have cholesterol-independent protective effects on the vascular endothelium, so-called pleiotropic effects (Calabro & Yeh, 2005) (Ludman et al., 2009). Owing to similarities in pathogenic mechanisms, statins have been proposed for potential use in prevention of pre-eclampsia (Cindrova-Davies, 2014). Various studies have investigated associations between pravastatin and angiogenesis (Table 6). In mouse models of pre-eclampsia, treatment with pravastatin has reduced maternal s-Flt-1 levels, lowered blood pressure and improved the vascular profile (Fox et al., 2011) (Saad et al., 2014) (Kumasawa et al., 2011). In addition, pravastatin administration has increased PlGF levels, and together with decreased s-Flt-1 levels, restored angiogenic balance (Kumasawa et al., 2011). Pravastatin treatment has also ameliorated pre-eclamptic symptoms by increasing the release of nitric oxide (NO), which initiates vasodilatation in the vasculature (Fox et al., 2011). However, not all studies support the findings of restored angiogenesis. Bauer and colleagues demonstrated decreased endothelial tube formation in pravastatin-treated healthy rats (Bauer et al., 2013), and Weis and associates showed that low concentrations of statins enhanced angiogenesis, and promoted vasculogenesis, whereas high statin concentrations inhibited angiogenesis (Weis et al., 2002) (Urbich et al., 2002). Although pilot trial of Constantine and associates showed an association between pravastatin and an improved angiogenic profile in pregnancies at high risk of pre-eclampsia (Costantine et al., 2016), in a randomized placebo-controlled trial pravastatin given to women with early-onset pre-eclampsia did not reduce maternal plasma s-Flt-1 levels (Ahmed et al., 2019). This finding would suggest lack of an angiogenic effect of pravastatin.

Table 6. Studies on the association between pravastatin and angiogenesis in pre-eclampsia.

Author and year of publication	Type of study	Results after pravastatin treatment
Costantine et al., 2010	Mouse model of pre-eclampsia	Pravastatin improved vascular reactivity by decreasing sFlt-1 level
Kumasawa et al., 2011	Mouse model of pre-eclampsia	Hypertension and proteinuria eased, foetal growth restriction improved, sFlt-1 levels decreased, PlGF levels increased, and the endothelial-cell proliferation improved
Fox et al., 2011	Mouse model of pre-eclampsia	Pravastatin promoted nitric oxide synthase expression and improved vascular function, suggesting that the protective effects of pravastatin may act via a cholesterol-independent pathway
Bauer et al., 2013	Rat model of placental ischaemia-induced hypertension	Increased blood pressure and urinary protein levels were decreased, but the angiogenic potential in rodents serum did not improve
Brownfoot et al., 2014	Pre-eclamptic placental explants	Pravastatin did not decrease sEng production
Saad et al., 2014	Mouse model of pre-eclampsia	Pravastatin prevented the rise in circulating anti-angiogenic factors
Brownfoot et al., 2015	Primary human tissues from women with pre-eclampsia	Pravastatin reduced sFlt-1 secretion from primary endothelial cells, purified cytotrophoblast cells, and placental explants from women with preterm pre-eclampsia
Costantine et al., 2016	A pilot randomized controlled trial	No safety risks were associated with pravastatin use. More favourable angiogenic profile was observed
Chaiworapongsa et al., 2016	Case report	Increased level of anti-angiogenic factor was corrected with pravastatin therapy. Treatment resulted in a live birth infant at 34 weeks of gestation who had normal growth
Brownfoot et al., 2016	Primary human cells, and preterm pre-eclamptic placental explants	Simvastatin was more potent inhibitor of sFlt-1 secretion from endothelial and trophoblast cells and from the placenta in women with pre-eclampsia compared with pravastatin or rosuvastatin
Balan et al., 2017	Human placental cotyledon model and placental explants cultures	The concentrations of PlGF, sFlt-1 and sEng were not significantly altered by pravastatin therapy
Garrett et al., 2018	Mouse model of pre-eclampsia	Pravastatin therapy prevented pre-eclampsia-associated cardiovascular complications in both mother and offspring
Ahmed et al., 2019	A randomized placebo-controlled trial	Pravastatin therapy did not lower maternal plasma sFlt-1 levels once early-onset pre-eclampsia had developed

APS, antiphospholipid syndrome; sEng, soluble endoglin; MAP, mean arterial pressure; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1

3 AIMS OF THE STUDY

1) To test the suitability of a human cell-based vasculogenesis/angiogenesis assay in the study of angiogenesis in pre-eclampsia and in other pregnancy conditions. To study how the angiogenic capacity of maternal serum assessed by an *in vitro* test is associated with known pro- and anti-angiogenic factors.

2) To study whether sera from pre-eclamptic women exhibit different angiogenic effects compared with sera from healthy controls and to explore the angiogenic properties of umbilical blood after pre-eclamptic and normotensive pregnancies.

3) To determine if differences in the angiogenic properties of maternal sera already exist in the first trimester samples when comparing healthy and subsequently pre-eclamptic women, utilising the hASC-HUVEC assay.

4) To evaluate the effects of metformin and pravastatin on angiogenesis by human cell-based vasculogenesis/angiogenesis assay.

5) To determine the direct effects of metformin and pravastatin on angiogenesis, and to study the interactions between those drugs and maternal sera in four study groups (early-onset pre-eclampsia, late-onset pre-eclampsia, IUGR and healthy pregnancy) by utilizing the hASC-HUVEC assay.

4 MATERIALS AND METHODS

4.1 Study design

All studies (I–IV) were *in vitro* trials. Since the vasculogenesis/angiogenesis assay has not been previously utilized to study in connection with maternal or umbilical sera, these trials were considered as pilot studies.

4.2 Study population

4.2.1 Studies I and II

Eleven primiparous previously healthy women with pre-eclampsia and ten primiparous controls with uncomplicated single pregnancies were recruited to Study I during the period of 2011–2014 at the Department of Obstetrics and Gynecology, Tampere University Hospital.

The Study II population consisted of six primiparous women with pre-eclampsia and six controls. The women were same as in Study I, but we included only those women whose blood samples from first-trimester screening for infectious diseases were stored at the National Institute for Health and Welfare.

The definition of pre-eclampsia was based on the International Society for the Study of Hypertension in Pregnancy (ISSHP) in 2000: systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg on at least two occasions 4 h apart after 20 weeks of gestation in previously normotensive women, with proteinuria of 300 mg or more in 24 h (Brown et al, 2001). Pre-eclampsia was defined as severe if HELLP (haemolysis, elevated levels of liver enzymes and low platelet count) syndrome, eclampsia or exceptionally high blood pressure (>160 mmHg systolic or >110 mmHg diastolic) appeared.

4.2.2 Studies III and IV

A total of 20 pregnant women were recruited to Studies III and IV. Five pregnant women had early-onset pre-eclampsia, five had late-onset pre-eclampsia, and five had IUGR. Five healthy pregnant women served as controls. The women were recruited to the study at the Department of Obstetrics and Gynaecology, Tampere University Hospital, in 2017–2018.

Pre-eclampsia was diagnosed as hypertension and proteinuria occurring after 20th gestational weeks. Hypertension was diagnosed as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and proteinuria as urinary excretion of ≥300 mg protein in a 24-h specimen. Pre-eclampsia was defined as early-onset when diagnosis was set before 34th gestational weeks and late-onset when at equal to or later than 34th gestational weeks. IUGR was defined as a foetal abdominal circumference below the 10th percentile or estimated foetal weight below the 10th percentile in ultrasonographic examination. The control group consisted of healthy women with uncomplicated pregnancies without any medication.

Maternal serum samples were obtained within four days prior to delivery at the time of admission into hospital.

4.3 Cell and tissue samples

Human adipose-tissue samples were obtained from waste materials of surgical operations. Human umbilical cords were obtained from Caesarean sections from healthy women. Both sample types were obtained from Tampere University Hospital with written informed consent from patients. Each donor gave either an adipose tissue sample or an umbilical cord sample.

4.3.1 Isolation and culture of human umbilical vein endothelial cells

HUVECs used in the study were isolated from donated umbilical cords. The umbilical cord was separated from the placenta. The cords were stored in Tissue Storage Solution (Miltenyi biotech) at +4 °C until utilized (max. one day). The umbilical vein was cannulated with a 20G needle. The vein was perfused with PBS (phosphate-buffered saline) to wash out blood and later the vein was infused with

0.05% collagenase I. The umbilical cord was incubated in a water bath at 37 °C for up to 20 min. After incubation, the collagenase solution containing HUVECs was flushed from the cord by infusing the vein with PBS. HUVECs were cultured in EGM-2 medium, and the medium was changed every 2–3 days. Before use, cells were tested for mycoplasma contamination. The seeding density of HUVECs was 4000 cells/cm^2. Culture media compositions are presented in Table 7.

Table 7. Culture media compositions

	Study I	Study II	Study III/IV
Medium name	**Content**	**Content**	**Content**
Fibroblast culture medium	EBM-2 Basal medium 2% Foetal bovine serum 2 mM L-glutamine 10 ng VEGF/ml (inhibition test) or 2.5 ng VEGF/ml (stimulation test) 1 ng FGF-β/ml (inhibition test) or 0.25 ng FGF-β/ml (stimulation test)		
hASC culture medium		DMEM/F12 10% human serum 2 mM L-Glutamine	DMEM/F12 10% human serum 1% L-Glutamine
hASC-HUVEC test medium	DMEM/F12 2.56 mM L-glutamine 0.1 nM 3,3',5-triiodo-L-thyronine sodium salt ITSTM Premix: 1.15 µM: 6.65 µg insulin/ml 6.65 µg transferrin/ml 6.65 ng selenious acid/ml 1% Bovine serum albumin 2.8 mM Sodium pyruvate 200 µg Ascorbic acid/ml 0.5 µg Heparin/ml 2 µg Hydrocortisone/ml 10 ng VEGF/ml 1 ng FGF-β/ml	DMEM/F12 2.56 mM L-glutamine 0.1 nM 3,3',5-triiodo-L-thyronine sodium salt ITSTM Premix: 6.65 µg insulin/ml 6.65 µg transferrin/ml 6.65 ng selenious acid/ml 1% Bovine serum albumin 2.8 mM Sodium pyruvate 200 µg Ascorbic acid/ml 0.5 µg Heparin/ml 2 µg Hydrocortisone/ml 2.5 ng VEGF/ml 0.25 ng FGF-β/ml	DMEM/F12 2.56 mM L-glutamine 0.1 nM 3,3',5-triiodo-L-thyronine sodium salt ITSTM Premix: 6.65 µg insulin/ml 6.65 µg transferrin/ml 6.65 ng selenious acid/ml 1% Bovine serum albumin 2.8 mM Sodium pyruvate 70 µg Ascorbic acid/ml 175 ng Heparin/ml 0.7 µg Hydrocortisone/ml 3.5 ng VEGF/ml 0.35 ng FGF-β/ml

EBM-2, endothelial basal medium; VEGF, vascular endothelial growth factor; FGF-β, fibroblast growth factor beta; DMEM/F12, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12

4.3.2 Isolation and culture of human adipose stromal cells

Isolation of hASCs was performed by mechanically cutting adipose tissue into small pieces and enzymatically digesting them with 0.15% collagenase I in DMEM/F12. The digested tissue was then centrifuged, and filtered through 100-µm and 40-µm filters. The cells were seeded in 75 cm^2 flasks in hASC culture medium (Table 7), and next day washed with PBS. The medium was changed every 2–3 days. Cells were tested for mycoplasma contamination before experimental use. The seeding density of hASCs was 20 000 cells/cm^2.

4.4 Angiogenesis assays

Two co-cultures were used: fibroblast-HUVEC (Study I) and hASC-HUVEC (Study I-IV). The fibroblast-HUVEC test was used to show basic effects of angiogenesis. The hASC-HUVEC test offered the possibility of evaluating the effects of both angiogenesis and vasculogenesis. The flow of the hASC-HUVEC vasculogenesis/angiogenesis assay experiments is presented in Figure 4.

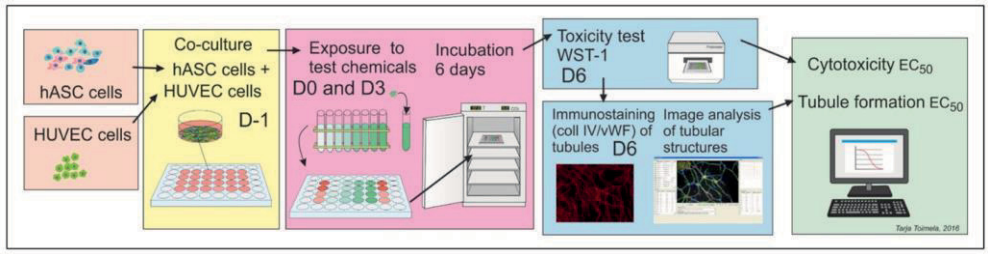

Figure 4. Schematic presentation of steps involved in the hASC-HUVEC vasculogenesis/angiogenesis assay experiments. Figure drawn by Tarja Toimela.

4.4.1 Co-culture establishment and study protocol

At first BJ fibroblasts (human foreskin fibroblasts), HUVECs and hASCs were cultured separately. Next, the HUVEC cells were carefully seeded on top of BJ fibroblasts or hASCs at a cell density of 4000 cells/cm^2. Two different growth-factor concentrations were used in the fibroblast-HUVEC test to induce either strong

vascular formation (to reveal possible inhibitory effects of test samples on vascular formation) or to induce only moderate vascular formation, where stimulation of vascular formation may also take place. The hASC-HUVEC test was carried out using vasculogenesis/angiogenesis test medium (Table 7). During both *in vitro* tests, the co-cultures were exposed to patient serum-samples at a dilution of 1:15 and cultured for a further six days with one replenishment of the growth medium. To rule out possible basal cytotoxicity caused by the serum samples used in this study, WST-1 (Water Soluble Tetrazolium) cytotoxicity assays were performed simultaneously with fibroblast-HUVEC and hASC-HUVEC angiogenesis assays. After culture the amount of living cells (viability) was evaluated.

To evaluate the effects of metformin or pravastatin on tubule formation, drugs were added to the vasculogenesis/angiogenesis test media alone and along with patient-serum samples on day one and replenished once during the six days of culture. The studied concentrations of metformin were 5 μg/ml, 50 μg/ml and 600 μg/ml. The two lowest concentrations correspond to therapeutic levels of metformin and the highest was greater. The studied concentrations of pravastatin were 20, 1000 and 8000 ng/ml. The lowest concentration corresponds to the therapeutic level, and the two highest were over the reported therapeutic concentration of pravastatin. High concentrations of the drugs were used to ensure sufficiently high concentrations of free metformin and pravastatin in the *in vitro* model, since concentrations in the media of cell cultures may not always directly correspond to concentrations in vivo. Serum samples alone, or with three concentrations of metformin or pravastatin, were tested for cytotoxicity, as determined by WST-1 assays, using 80% viability compared with unexposed controls as the non-cytotoxic limit.

4.4.2 Immunocytochemical staining

Following the WST-1 assays, the cells were fixed with 70% ethanol and immunostained for vWf (von Willebrand factor) to detect endothelial cells, and collagen IV to detect the basement membranes of the tubules. For visualization of the tubules, fluorescent secondary antibodies against the primary antibodies were applied: anti-rabbit tetramethylrhodamine isothiocyanate for vWf and anti-mouse fluorescein 5-isothiocyanate for collagen IV.

4.4.3 Microscopic analysis of tubule formation

In vitro tubule formation was used for quantitative analysis of angiogenesis. Endothelial cells form capillary-like structures and those tubules were imaged and analysed using an automated image-analysis platform (Cell-IQ, CM-Technologies, Tampere, Finland) (Fig. 1). Tubule formation was quantified on the basis of the intensity of the tubular network formed (tubule length and branching). The obtained result was compared with the positive tubule-formation control (highest level of tubule formation induced with stimulatory factors). Values were first normalized to the in-plate control to remove variation between the plates. In Studies III and IV, the resulting values were compared with the mean value of the healthy group to see the effect of serum between the groups. To determine the effect of drugs on the tubules, the normalized values were compared with the unexposed tubules cultured with serum from the corresponding patient. These results were expressed as percentages of positive tubule formation.

4.5 Immunoassays

The serum samples were analysed for the concentrations of several angiogenic and inflammatory key proteins (Studies II–IV). The concentrations of heme oxygenase 1 (HO-1), endothelin-1 (ET-1) and angiopoietin 2 (Ang2) were determined by using ELISA kits (Studies III & IV). HO-1 (sensitivity 0.78 ng/ml), ET-1 (sensitivity 0.41 pg/ml)(ADI-EKS-800 and ADI-900-020A, Enzo life sciences, Farmingdale, NY, USA) and Ang2 assays (sensitivity 10 pg/ml; ELH-angiopoietin 2, Raybiotech, Norcross, GA, USA) were performed according to the manufacturers´ instructions as follows: In the HO-1 and Ang2 ELISAs standards and samples were incubated at room temperature (RT) for 30 min and 2.5 h, respectively, and in the ET-1 ELISA they were incubated overnight at +4°C followed by removal of the liquids from the plates. Antibody solutions were added and incubation carried out for 1 h for HO-1 and Ang2 and for 30 min for ET-1, at RT. Horseradish peroxidase conjugate was added to the HO-1 and Ang2 plates (ET-1 antibody was already conjugated) and incubation carried out for 30 min and 45 min, respectively, at RT. Next, TMP substrate was added and incubation carried out with slow shaking in the dark at RT for 15 to 30 min, followed by addition of stop solution. Measurements were performed at 450 nm with a Varioskan Flash Multimode Reader (Thermo Fischer Scientific, Vantaa, Finland).

The concentrations of tumour necrosis factor alpha (TNF-α), soluble fms-like tyrosine kinase-1 (sFlt-1), placental growth factor (PlGF), vascular endothelial growth factor A (VEGF-A), vascular cell adhesion molecule 1 (VCAM-1), soluble endoglin (sEng) and interleukin-6 (IL-6) were determined in all samples using ProcartaPlex assays (Thermo Fisher Scientific) according to the manufacturer's instructions (Studies II–IV). Briefly, samples were centrifuged at 1000 × g for 10 min before use in the assay. The samples were not diluted. Antigen standards and magnetic beads were prepared according to the manufacturers' instructions. Samples and standards were then added to the beads and incubated with shaking for two hours at RT. The detection antibodies were added and incubated for 30 min at RT. To stain the proteins, streptavidin-PE was added and incubated for 30 min at room temperature, after which the beads were analysed in reading buffer. The results were analysed with Bio-plex200 (Bio-Rad, California, USA) and Bio-Plex Manager™ 6.0 software (Bio-Rad). Concentrations were obtained in pg/ml. If the results were lower or higher than the detection rate of the immunoassay, a fixed concentration was used in analysis of the results. The concentration used for sEng was 2550 pg/ml when the results were over and for PlGF it was 1 pg/ml when the results were lower than the lowest detection rate (Study II). The results of the VEGF and PlGF assays can also include the bound versions of these growth factors. Hence the results should be considered as total concentrations.

4.6 Statistical analysis

Study I. The results are presented as mean values with standard deviations (SDs) when normally distributed, and as medians and ranges when not normally distributed. The Statistical Package for the Social Sciences (SPSS) was used for statistical analysis. Differences between the study groups were analysed by independent-samples t-tests or by the Mann–Whitney U-test. Differences were considered significant at $p < 0.05$. Figures were processed using SPSS and GraphPad Prism v6.05 software.

In Studies II–IV, the data are expressed as medians and ranges. Differences in continuous variables between groups were tested by using the Kruskal–Wallis test and for post-hoc tests we used the Mann–Whitney U-test. Differences within the study groups were analysed by Wilcoxon's test. Spearman's correlation method was used to calculate correlation coefficients. Probabilities of less than 0.05 were

considered statistically significant. Statistical analyses were performed by using IBM-SPSS software, version 11.0. Figures were processed with SPSS (Studies II & III) and GraphPad Prism 8 software (Study IV).

4.7 Ethical aspects

This study was approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permits R11088 and R16184). This study conforms to the principles outlined in the Declaration of Helsinki. The use of human adipose stromal cells and use of human umbilical endothelial cells were separately approved by the Ethics Committee of Pirkanmaa Hospital District (Tampere, Finland); permit numbers of R15161 and R15033, respectively. Written informed consent was obtained from all participants.

5 RESULTS

5.1 Study I

The median gestational age at delivery, birth weight and birth weight standard deviation were significantly lower in the pre-eclampsia group compared with the control group. Eight women had severe pre-eclampsia; two of them had HELLP syndrome. Three pre-eclamptic women delivered before 32 weeks of gestation. In the control group one woman smoked, and one delivered before 37 weeks of gestation. Other data concerning clinical characteristics and neonatal outcome is presented in Table 8.

There was a strong inhibitory effect on tubule formation with sera from pre-eclamptic women, while sera from healthy women did not have such an effect. The difference between the groups was seen in both fibroblast-HUVEC ($p=0.010$) and hASC-HUVEC ($p=0.002$) tests. Umbilical serum was inhibitory in both groups. Umbilical samples from women with pre-eclampsia were more inhibitory, but the difference between the groups reached statistical significance only in the hASC-HUVEC test ($p=0.020$).

None of the serum samples caused cell death (decreased viability). On the contrary, the umbilical serum samples induced increased cell viability (i.e. stimulated proliferation of cells) in fibroblast angiogenesis tests. In the hASC test system all samples showed cell viability slightly above the control level.

There was no correlation between the inhibitory effects of maternal serum and corresponding umbilical serum in either of the groups. Neither was there a correlation between clinical features or laboratory findings and the inhibitory effects of the maternal sera. However, there was a correlation ($r=0.75$) between birth weight SD and the inhibitory effect of umbilical sera in the pre-eclampsia group ($p=0.020$).

Table 8. Clinical characteristics and neonatal outcome in Study I

	Pre-eclampsia (n=11)	Control (n=10)	*p*-value
Maternal age, years	29.8 (20–44)	27.0 (23–34)	NS
BMI, kg/m²	21.5 (19.7–32.1)	24.1 (17.9–38.2)	NS
Highest systolic BP, mmHg	166 (141–178)	133 (103–142)	0.000**
Highest diastolic BP, mmHg	100 (88–113)	75 (54–89)	0.000**
Proteinuria, grams/d	4.2 (1.3–9.4)		
Gestational age at delivery, wks	35.1 (28.1–39.6)	39.9 (35.4–42.3)	0.002**
Birth weight, grams	2171 (1020–3140)	3607 (2685–4535)	0.000**
Birth weight, SD	-2.2 (-0.4– -1.9)	-0.2 (-1–1.5)	0.002**
Apgar scores, at 5 min	8 (5–10)	9 (9)	NS

Data are gives as median (range). BMI, body mass index; BP, blood pressure; SD, standard deviation

**p-value <0.01, Mann–Whitney U-test.

5.2 Study II

There were two preterm births in the pre-eclampsia group and one in the control group. There was no difference in mean gestational age between the groups. Most women (5/6) with pre-eclampsia had severe disease. One woman in the control group smoked. Maternal characteristics and neonatal outcomes are presented in Table 9.

In the first trimester maternal sera were stimulatory, and tubule formation was equally high in both groups (Fig. 5). There were no differences in values of angiogenic biomarkers between the groups. The amounts of pro-angiogenic proteins (VEGF and PlGF) did not show any correlation with the stimulatory effect seen in the *in vitro* tests. Neither did any other biomarker or tubule-formation measure have a correlation with baseline demographic characteristics, severity of pre-eclampsia, gestational weeks at delivery, or birthweight.

In the third trimester, maternal sera from pre-eclamptic pregnancies exhibited an inhibitory effect on tubule formation. In comparison with healthy women, tubule

formation was significantly lower (p=0.026), and sFlt-1 levels were higher (p=0.004) in the pre-eclampsia group (Fig. 5, Table 10).

Compared with the first trimester, tubule formation was lower (p=0.043) in connection with women with pre-eclampsia in the third trimester, whereas in the healthy control group there was no change in tubule formation between the trimesters (Fig. 5). In the third trimester PlGF levels were lower (p=0.043) and those of sFlt-1 (p=0.028) higher in women with pre-eclampsia when compared with serum samples taken in the first trimester (Table 10).

Umbilical serum had an equally strong inhibitory effect on tubule formation in both groups (Fig. 5). There were no differences between the groups in the concentrations of angiogenic biomarkers. Only in the group of healthy women was the inhibitory effect on tubule formation stronger in umbilical-cord blood than in maternal serum (p=0.028). In women with pre-eclampsia sFlt-1 concentrations were significantly lower (p=0.028) in umbilical serum than in third-trimester maternal serum (Table 10).

In pre-eclamptic pregnancies both maternal serum in the third trimester and umbilical serum showed a correlation between the inhibitory effect on tubule formation and low birth weight SD (r=-0.754, p=0.050) and (r=0.841, p=0.036), respectively.

Table 9. Maternal characteristics and neonatal outcome in Study II

	Pre-eclampsia (n=6)	Control (n=6)	*p*-value
Maternal age, years	29.0 (25–33)	25.0 (24–34)	0.240
BMI, kg/m²	21.3 (19.7–22.3)	26.4 (17.9–38.2)	0.065
Highest systolic BP, mmHg	160 (141–171)	130 (103–138)	0.009**
Highest diastolic BP, mmHg	96 (88–110)	73 (54–88)	0.002**
Mode of delivery, vaginal/CS, n	4/2	3/3	
Gestational age at delivery, wks	37.5 (35.3–38.3)	40.5 (35.4–41.6)	0.065
Birth weight, grams	2620 (2040–3140)	3278 (2685–4535)	0.026*
Birth weight, SD	-1.2 [-3.1–(-0.4)]	-0.5 (-1.7–1.5)	0.093
Umbilical artery pH	7.23 (7.13–7.36)	7.33 (7.30–7.41)	0.052

Data are given as median (range). BMI, body mass index; BP, blood pressure; CS, Caesarean section; SD, standard deviation.
*p value < 0.05, **p-value <0.01, Mann–Whitney U-test.

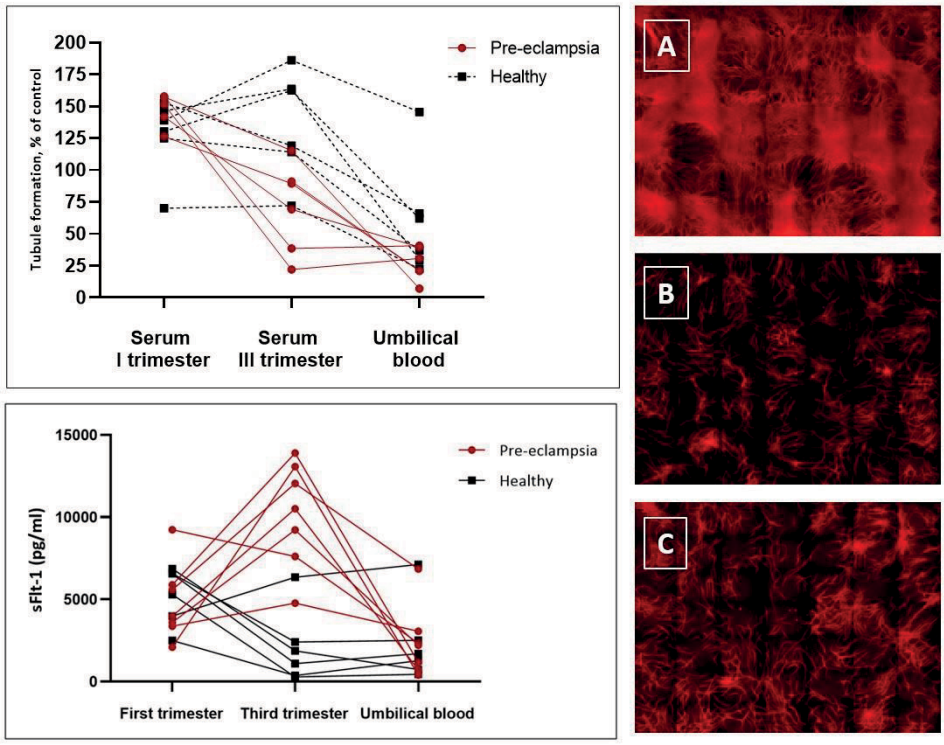

Figure 5. Longitudinal changes in tubule formation and in sFlt-1 concentrations in pre-eclamptic and healthy women. Immunofluorescence images of vascular structures show the angiogenic effect of maternal sera from women with pre-eclampsia. A) first trimester, B) third trimester and C) umbilical sera.

Table 10. Concentrations of angiogenic biomarkers in maternal and umbilical sera in the pre-eclamptic and control group

	First trimester			Third trimester			Umbilical cord		
	PE	CONTR		PE	CONTR		PE	CONTR	
			p-value			*p*-value			*p*-value
VEGF (pg/ml)	2572 (1980-25889)	2961 (1875-14003)	0.940	2196 (1417-9281)	1818 (856-17321)	0.240	6374 (5185-8619)	3893 (2238-16486)	0.180
PlGF (pg/ml)	144 (1-14155)	120 (1-1514)	0.700	23 (1-3535)	1 (1-1216)	0.240	28 (1-923)	1.6 (1-1450)	0.590
sFlt-1 (pg/ml)	3780 (2092-5878)	5914 (2489-6851)	0.180	11278 (4768-13899)	1480 (277-6339)	0.004**	1684 (397-6845)	1485 (445-7113)	0.940
sEng (pg/ml)	2550†	2550†	1.000	2550 (1009-2550)	1071 (595-2550)	0.093	1885 (846-2550)	1159 (701-2550)	0.700
sFlt-1/PlGF	35 (0.2-2092)	44 (4-6538)	0.590	614 (1.4-13064)	734 (2-6339)	0.940	52 (3-6845)	382 (1.7-7113)	0.390
sFlt-1/VEGF	1.3 (0.1-2.8)	1.6 (0.5-2.8)	0.700	5.6 (0.5-8.5)	0.5 (0.1-5.0)	0.041*	0.3 (0.1-1.3)	0.3 (0.1-2.1)	0.700

Data are gives as median (range). PE, pre-eclampsia; CONTR, control; VEGF, vascular endothelial growth factor; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; sEng, soluble endoglin. †All values were above the range of the standards.
*p-value < 0.05, Mann–Whitney U-test.

5.3 Studies III and IV

The clinical characteristics and perinatal outcomes of the study groups are presented in Table 11. One woman in the early-onset pre-eclampsia group and one in the IUGR group had chronic hypertension; one of them had used labetalol since the beginning of the pregnancy. None of the participants had used acetylsalicylic acid. Two women in the IUGR group smoked. There were five preterm deliveries (before 37 weeks) in the early-onset pre-eclampsia group and four in the IUGR group (two of which were before 32 weeks).

The results of the measurements of various biomarkers in maternal sera are presented in Table 12. There were significant differences in levels of Ang2, sEng and ET-1 between the pre-eclampsia, IUGR and control groups, but no significant differences in levels of anti-inflammatory markers (TNF-α, VCAM-1) were observed between the groups.

Maternal sera alone did not have stimulatory or inhibitory effects on tubule formation in any study group, and there was no difference between the groups (Figs. 6 and 7). Neither was there a correlation between tubule formation and the concentrations of angiogenic or inflammatory biomarkers in any group.

Table 11. Clinical characteristics and neonatal outcome in studies III & IV

	Early-onset PE (n=5)	Late-onset PE (n=5)	IUGR (n=5)	Healthy (n=5)
Maternal age, years	22 (19-40)	24 (20-33)	27 (25-33)	34 (33-36)
BMI, kg/m²	25.3 (18.0-30.0)	25.3 (21.8-34.8)	25.3 (18.3-38.4)	25.5 (21.9-26.1)
Highest systolic BP, mmHg	165 (152-186)	160 (142-202)	136 (118-166)	122 (112-132)
Highest diastolic BP, mmHg	107 (92-115)	106 (93-118)	84 (66-104)	83 (68-94)
Umbilical artery, PI	1.14 (1.02-1.36)	0.86 (0.74-1.08)	1.3 (1.1-1.5)	0.72 (0.57-0.94)
Proteinuria, grams/d	8.0 (1.5-13.5)	2.8 (1.0-4.1)		
Gestational age at delivery, wks	33.6 (32.3-35.3)	40.5 (39.1-41.1)	34.0 (26.3-39.9)	39.7 (39-41.3)
Birth weight, grams	1880 (1645-2040)	3860 (3300-4230)	1735 (600-2420)	3920 (3400-4160)
Birth weight, SD	-1.2 (-1.5-0)	0.9 (-0.4-1.6)	-2.9 [-1.9-(-3.8)]	0.5 (-0.9-1.3)
Umbilical arterial pH	7.35 (7.18-7.40)	7.22 (7.16-7.34)	7.35 (7.31-7.39)	7.31 (7.26-7.35)

Data are given as median (range). BMI, body mass index; BP, blood pressure; PI, pulsatility index; SD, standard deviation

5.3.1 Effect of metformin on angiogenesis (Study III)

When the direct effect of metformin was studied (without maternal serum) we found that the two therapeutic concentrations (5 and 50 μg/ml) did not affect tubule formation (angiogenesis), but at a concentration of 600 μg/ml metformin inhibited tubule formation ($p=0.002$) when compared with tubule growth without the drug. There were also significant differences in tubule formation between the three doses of metformin. Tubule formation (direct effect) was highest at a concentration of 5 μg/ml and lowest at a concentration of 600 μg/ml of metformin.

Compared with tubule growth in the presence of maternal sera alone, metformin at concentrations of 5 and 50 μg/ml did not significantly change tubule formation in

any group. However, metformin at 600 μg/ml resulted in a significant decrease in tubule formation in all groups (p=0.043).

When we investigated differences in tubule formation within study groups, there was a significant decrease in tubule formation with increasing doses of metformin in the two pre-eclampsia groups and the IUGR group (p=0.043). In the healthy group, this decrease in tubule formation followed the same pattern as in the other groups, but the difference was significant only at concentrations of 50 and 600 μg/ml (p=0.043) (Fig. 6).

Serum samples alone, or with 5, 50 or 600 μg/ml concentrations of metformin, were not cytotoxic as determined by WST-1 measurement (using 80% viability). In fact, metformin at a concentration of 600 μg/ml even increased cell viability/mitochondrial activity.

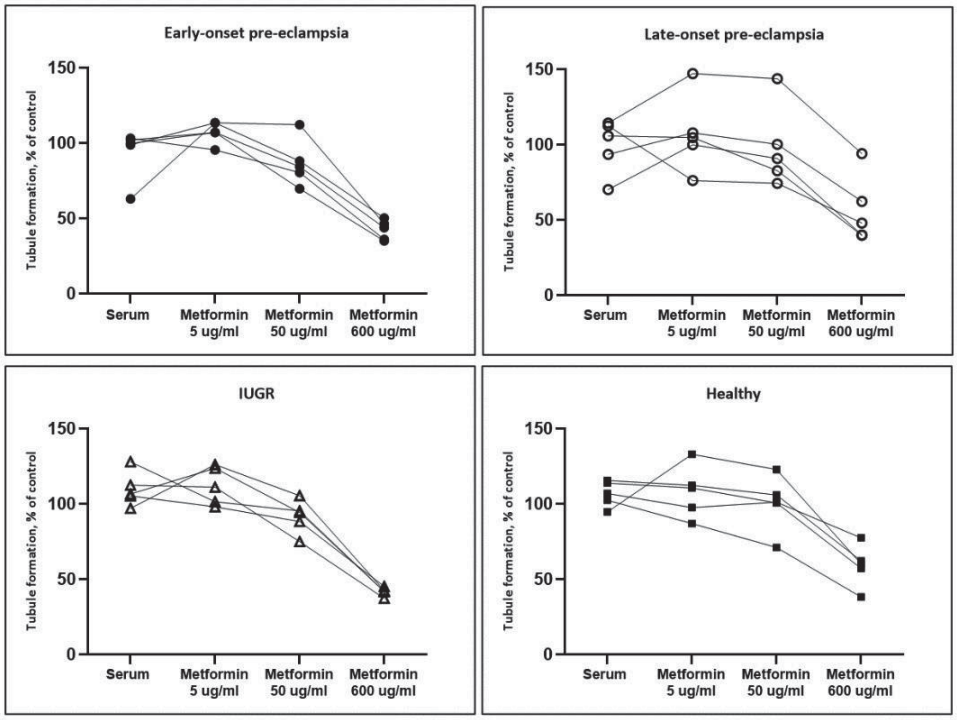

Figure 6. Effect of metformin on tubule formation at three concentrations in each study group.

5.3.2 Effect of pravastatin on angiogenesis (Study IV)

When the direct effects of pravastatin (without maternal sera) were evaluated, a therapeutic concentration (20 ng/ml) did not have an effect on angiogenesis, but at concentrations of 1000 and 8000 ng/ml pravastatin inhibited tubule formation ($p=0.028$).

Compared with tubule growth caused by maternal sera alone, angiogenesis was not significantly enhanced with any dose of pravastatin. However, there seemed to be enhancement of angiogenesis in some individual cases, particularly in the early-onset pre-eclampsia group. In the healthy group there was significant inhibition of angiogenesis at a concentration of 1000 ng/ml ($p=0.043$) when compared with tubule formation caused by maternal sera alone. Within-group differences existed in the IUGR and early-onset pre-eclampsia groups. There was a significant decrease in tubule formation between 20 ng/ml and 8000 ng/ml doses of pravastatin in the IUGR group ($p=0.043$) and between 1000 ng/ml and 8000 ng/ml in the early-onset pre-eclampsia group ($p=0.043$) (Fig. 7).

When the study groups were compared with healthy pregnancies, there was significantly more tubule formation at a pravastatin concentration of 20 ng/ml ($p=0.047$) in the IUGR group. There were no differences in tubule formation between the two pre-eclampsia groups and healthy pregnancies at different doses of pravastatin. However, when the late-onset pre-eclampsia group was compared with the IUGR group, there was more tubule formation in the latter group with every dose of pravastatin (from the lowest to the highest dose, $p=0.009$, $p=0.009$, $p=0.028$). Otherwise, there were no differences in tubule formation between the groups (Fig. 7).

Serum samples alone, or with three concentrations of pravastatin, were not cytotoxic, as viability was above 80% in all tested samples.

Concentrations of sFlt-1, PlGF, VEGF, sEng, VCAM-1, TNF-α and IL-6 in test media exposed to maternal sera did not change after pravastatin (1000 ng/ml) supplementation.

Table 12. Concentrations of angiogenic and inflammatory biomarkers in maternal sera in Studies III and IV.

	Early-onset PE		Late-onset PE		IUGR		Healthy
		p-value		*p*-value		*p*-value	
PlGF (pg/ml)	270 (16.8-356)	0.564	284 (120-442)	0.355	127 (65-302)	0.564	172 (171-173)
sFlt-1 (pg/ml)	3375 (2584-9906)	0.117	6244 (1801-9781)	0.028*	6036 (459-8623)	0.117	1335 (717-3434)
Ang2 (pg/ml)	8242 (4750-10332)	0.014*	7552 (514-10927)	0.086	6236 (3645-7619)	0.014*	1789 (471-2530)
ET-1 (pg/ml)	1.8 (0.7-5.0)	0.050	3.5 (0.9-8.3)	0.027*	0.6 (0.2-3.9)	0.462	0.4 (0.1-1.0)
sEng (pg/ml)	2995 (2375-3418)	0.028*	3552 (1469-3787)	0.117	2827 (1767-3674)	0.117	2091 (1661-2548)
HO-1 (ng/ml)	0.2 (0.2-0.8)	0.806	0.2 (0.1-0.5)	0.142	0.5 (0.2-0.9)	0.806	0.5 (0.2-0.7)
TNF-α (pg/ml)	18.0 (16.6-18.0)	1.000	7.0 (2.1-23.6)	0.643	8.9 (6.1-31.2)	0.245	15.0 (9.8-20.2)
sFlt-1/PlGF	14.2 (9.6-591.1)	0.564	30.7 (17.5-47.0)	0.355	67.8 (1.5-92.4)	0.564	11.6 (4.2-19.0)
VCAM-1 (pg/ml)	79471 (23157-135785)	0.439	23246 (4287-27724)	0.439	20586 (9792-25728)	1.000	17704 (12191-23216)

Data are gives as median (range). PE, pre-eclampsia; IUGR, intrauterine growth restriction; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; Ang2, angiopoietin 2; ET-1, endothelin 1; sEng, soluble endoglin; HO-1, heme oxygenase 1; TNF-α, tumour necrosis factor α; VCAM-1, vascular cell adhesion protein 1. Values of p refer to the differences between the study groups and healthy pregnancies.
**p*-value < 0.05, Mann–Whitney U-test.

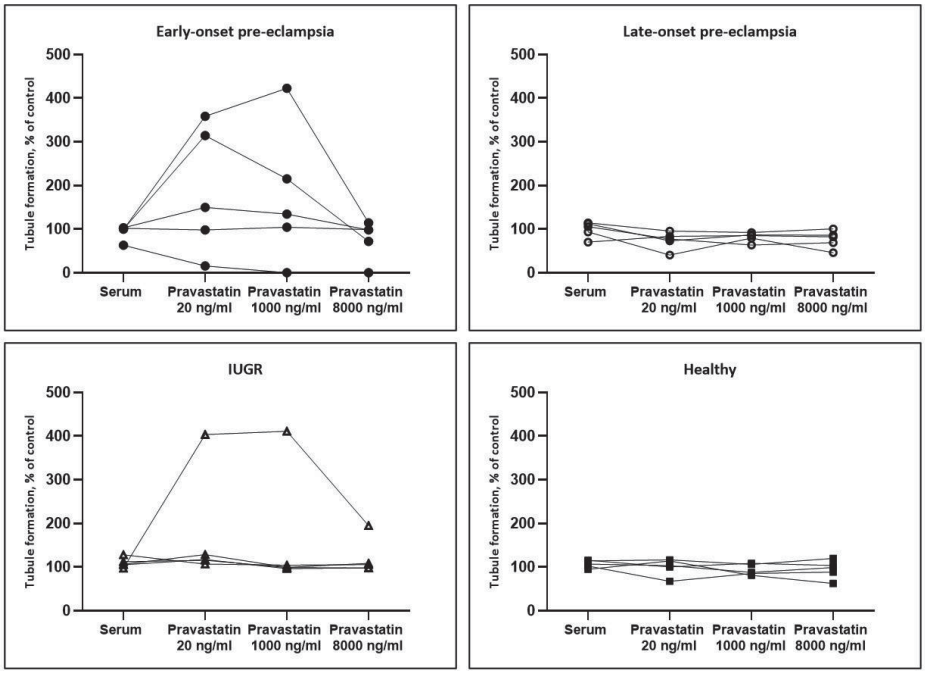

Figure 7. Effect of pravastatin on tubule formation at three concentrations in each study group.

6 DISCUSSION

6.1 Main findings

This study showed that a human cell-based vasculogenesis/angiogenesis model can be utilized to study angiogenic properties of sera from healthy and complicated pregnancies. Since maternal and umbilical sera contain several pro- and anti-angiogenic factors, vasculogenesis/angiogenesis assays may reflect the functional angiogenic capacity of the sera better than assay of separate angiogenic factors. Additionally, the *in vitro* method used is suitable for evaluation of the direct effects of drugs on angiogenesis as well as for the study of interactions between maternal sera and pharmaceutical agents. In the first trimester, maternal sera from healthy and pre-eclamptic pregnancies stimulated angiogenesis, whereas in the third trimester only sera from women with pre-eclampsia exhibited anti-angiogenic properties. Umbilical-cord blood was inhibitory after both pre-eclamptic and healthy pregnancies, and there was no difference between the groups. When the direct effects of metformin or pravastatin on angiogenesis were evaluated, neither of the drugs enhanced angiogenesis at therapeutic concentrations. However, maternal sera along with pravastatin had stimulatory effect on angiogenesis in some women with early-onset pre-eclampsia. Metformin, in contrast, had a strong inhibitory effect on angiogenesis at high doses.

6.1.1 *In vitro* hASC-HUVEC assay as an angiogenesis research tool

In vitro methods have been traditionally employed in efficacy and toxicity testing of chemicals and drugs, but currently they are additionally being utilized to study underlying mechanisms of diseases and in the development of new therapeutic strategies (Rouwkema et al., 2011). Despite significant advances in *in vitro* technology in recent years, a great deal of medical research is still carried out with animal testing (Hajar, 2011). Various animal models of pre-eclampsia have been established to study angiogenesis and knowledge of pathophysiological mechanisms behind pre-eclampsia has increased via studies in which such models have been utilized.

Nevertheless, there are disadvantages both in animal experiments as well as in *in vitro* models. Animal testing is costly and requires time-consuming protocols (Staton et al., 2009), and most of all, there is major concern regarding repeatability in humans (Anderesen, 2018). In *in vitro* tests, the microenvironment of the cells is different from their *in vivo* environment, and the concentrations of pharmaceutical agents in *in vitro* conditions rarely correlate directly to *in vivo* concentrations (Huntjens et al., 2006) (Checkley et al., 2016). However, it is possible to calculate exact in vitro-in vivo correlations (IVIVCs), but the predictive mathematical models are complex, requiring data on several parameters. Among other limitations of *in vitro* tests, of particular importance in obstetric research is their inability to replicate placental transfer.

We utilized human cell-based vasculogenesis/angiogenesis *in vitro* tests to evaluate the performance of this model in pre-eclampsia research. Our aim was to study the angiogenic capacity of maternal and umbilical sera in healthy and pre-eclamptic pregnancies, and to investigate the effects of two pharmaceutical agents on angiogenesis with and without maternal sera. In the first study we used both fibroblast-HUVEC and hASC-HUVEC assays, but in Studies II–IV only the latter was used. The hASC-HUVEC model is considered to be a more advanced tool to study angiogenesis than the fibroblast-HUVEC model since the vascular network formed is three-dimensional, and additionally there are pericytes lining the endothelial cells resembling better in vivo vascular structures (Huttala et al., 2015). The hASC-HUVEC model is considered to reveal also changes in vasculogenesis. The existence of angioblasts has not been directly confirmed, but there are findings that supports the presence of an endothelial progenitor cell population capable of vasculogenesis in hASC (Huttala et al., 2015). The hASC-HUVEC model has been developed and intra-laboratory-validated at FICAM (The Finnish Centre for Alternative Methods) (Toimela et al., 2017), which is a centre of expertise as regards alternatives to animal experimentation. It is specialized in development and validation of human cell-based tissue/organ models that mimic normal functions of human tissues. In addition, FICAM acts as the Finnish reference laboratory for EURL-ECVAM (the European Union Reference Laboratory for Alternatives to Animal testing). The hASC-HUVEC model has been shown to be reliable, with high repeatability and good concordance with blood vessels *in vivo* (Toimela et al., 2017). It reveals mostly branching angiogenesis, but additionally intussusceptive angiogenesis is suggested to happen. It has been considered as a 'pathbreaker', since it is the first *in vitro* model that has not employed animal-derived components such as Matrigel or foetal bovine serum (Andree et al., 2019). It is mostly utilized for

testing angiogenic properties of pharmaceuticals and other chemicals. Additionally, however, it is employed in the development of tissue/organ models such as the human cellular cardiovascular 'mini heart' and the human adipose cell model (Huttala et al., 2020) (Vuorenpää et al., 2014) (Huttala et al., 2018). Since pre-eclampsia is strongly associated with vascular dysfunction, we considered that an advanced *in vitro* model would provide an alternative method to study modulators of angiogenesis in healthy and complicated pregnancies. Angiogenesis involved in pre-eclampsia has been commonly studied by measuring levels of pro- and anti-angiogenic factors in the maternal circulation, whereas the *in vitro* test utilized in this study reveals functional capabilities of sera to promote, maintain or inhibit angiogenesis.

6.1.2 Angiogenesis in early gestation in pregnancies with subsequent pre-eclampsia

Controlled angiogenesis is essential for successful pregnancy outcome, and the balance between pro- and anti-angiogenic factors varies depending on gestational stage (Kaufmann et al., 2004). In early gestation, during formation of the vascular foetoplacental system, a predominance of pro-angiogenesis exists (Charnock-Jones et al., 2004), whereas towards the end of pregnancy, the balance shifts in favour of anti-angiogenesis (Levine et al., 2004). Angiogenic biomarkers have been widely studied in the first trimester. The earliest measurements have been made at 6–9 weeks of pregnancy, but those studies have mainly been concentrated on miscarriages rather than identification of subsequent pre-eclampsia (Vuorela et al., 2000) (Pang et al., 2013). In pre-eclampsia studies, measurements of serum biomarkers has been mostly scheduled between 8–14 weeks of gestation (Kuc et al., 2011). In our study, serum samples were taken at 9–11 weeks. Congruent with previous data, we noticed that maternal sera were pro-angiogenic in the first trimester, and there were no differences either in tubule formation or in concentrations of angiogenic biomarkers between women with subsequent pre-eclampsia and those with healthy pregnancies. The concentrations of pro-angiogenic proteins (VEGF and PlGF) did not show correlation with the stimulatory effect seen in the *in vitro* test, and there was no inhibition of tubule formation even though the levels of sFlt-1 were already relatively high. In early gestation the association of angiogenic biomarkers with pre-eclampsia is unclear. Several investigators have reported some differences in trophoblast and angiogenic biomarkers between women with healthy pregnancies and those with subsequent pre-eclampsia

(Townsend et al., 2019), but there are also opposite findings (Wu et al., 2015). In the first trimester, concentrations of angiogenic biomarkers have mostly been studied with the intention of identifying women destined to have pre-eclampsia. It is commonly recognized that a combination of multiple biomarkers yields a higher detection rate than a single biomarker in predicting pre-eclampsia, and biomarkers overall show better performance in identifying early-onset rather than late-onset pre-eclampsia (Chaiworapongsa et al., 2014). Nevertheless, for predictive purposes the overall significance of biomarkers is questionable (Myatt et al., 2012). The general incidence of pre-eclampsia is so low that positive predictive values for subsequent pre-eclampsia have been reported to be only 13% when using biomarkers (Roberts & Bell, 2013). The focus of our study was not on prediction, and altogether the significance of measuring angiogenic biomarkers was limited. Maternal serum samples were obtained relatively early in the first trimester, sample sizes were small and most women in the pre-eclamptic group whom first-trimester serum samples were available had late-onset pre-eclampsia. Overall, we think that the results from the *in vitro* tests better reflect the pro-angiogenic state in early gestation than levels of individual angiogenic proteins.

6.1.3 Angiogenesis and clinical manifestation of pre-eclampsia

Since the discovery of angiogenic disturbance in pre-eclampsia, several studies have been focused on levels of angiogenic biomarkers longitudinally or at a certain point of pregnancy. We also measured levels of individual angiogenic proteins, but the main goal was to evaluate the overall angiogenic capacity of maternal and umbilical sera. We hypothesized that angiogenic properties determined by human cell-based vasculogenesis/angiogenesis assays offer a wider approach to angiogenesis, as serum contains various factors other than known angiogenic biomarkers that are involved in disease pathophysiology. There is increased knowledge of the pathophysiological mechanisms behind pre-eclampsia, but the exact aetiology is still unknown (Steegers et al., 2010). Currently, it is thought that the endpoint of pre-eclampsia exhibits oxidative stress, excess inflammation and endothelial dysfunction, but the mechanisms that lead there are different in early- and late-onset pre-eclampsia (Chaiworapongsa et al., 2014). The "two-stage" theory of pre-eclampsia is commonly known, but it mainly concerns the angiogenic early-onset disease. Non-angiogenic type of pre-eclampsia also exists, and in that disease the angiogenic balance in the

maternal circulation is not altered (Rana et al., 2013). It is characterized by mild disease presentation occurring in late gestation.

In Studies I and II sera from women with pre-eclampsia showed anti-angiogenic properties. There was a decrease in tubule formation, and a significant change in the concentrations of angiogenic proteins toward an anti-angiogenic state between the first and third trimesters in women with pre-eclampsia. Additionally, levels of sFlt-1 and sEng were higher in the pre-eclampsia group than in the control group in the third trimester, but levels of individual pro- and anti-angiogenic proteins showed no correlation with tubule formation. In Study I, almost half of the pregnancies proceeded to full term, but most women had findings of severe pre-eclampsia, which explained the anti-angiogenic findings. In Studies III and IV the angiogenic effect of maternal sera was neutral, and there were no differences in tubule formation between the healthy, IUGR and pre-eclampsia groups. We expected that there would have been some differences in angiogenesis between the healthy and pre-eclampsia groups, since our previous findings supported that. Additionally, we hypothesized that IUGR pregnancies would have resembled early-onset pre-eclamptic pregnancies in angiogenic profile, as the two disorders share similarities in placental structure. We suggested that the results may have been different with a larger sample size.

There are previous studies concerning *in vitro* models of pre-eclampsia (McNally et al., 2017), and in several studies human umbilical endothelial cells have been isolated from umbilical cords from pre-eclamptic and uncomplicated pregnancies (Brodowski et al., 2017). However, maternal sera from women with pre-eclampsia have been less well investigated in *in vitro* assays (Maynard et al., 2003). In the study by Maynard and colleagues, tubule formation was determined in their HUVEC model (Maynard et al., 2003). HUVECs are the most commonly used cell type for studying angiogenesis (Stryker et al., 2019). However, HUVECs have been commonly combined with other cell types such as fibroblasts, smooth-muscle cells/pericytes and other stromal cells to improve test performance, reliability and to add more aspects of *in vivo* biology (Toimela et al., 2017) (Staton et al., 2004).

6.1.4 Disturbed angiogenesis affecting children after pre-eclampsia

Pre-eclampsia is increasingly recognized as a disease that has long-term impacts on maternal and foetal health. Epidemiological studies have shown that the intrauterine environment programs permanent changes in foetal vascular, lipid and hormone responses (Barker, 1992). There is also growing evidence that foetal exposure to

hypertensive disorders in utero can result in an increased risk of adult cardiovascular diseases (Øglaend et al., 2009) (Davis et al., 2012). There are several studies on angiogenic biomarkers in maternal sera after pre-eclamptic pregnancy, but data concerning angiogenic biomarkers in infants born to mothers with pre-eclampsia is limited. Angiogenic biomarkers in umbilical blood are considered to reflect the angiogenic profile in the foetal circulation. In our study umbilical serum was anti-angiogenic after childbirth. There were no differences in the levels of angiogenic proteins between maternal and umbilical sera, with the exception of sFlt-1 concentrations, which were higher in sera from women with pre-eclampsia. Increased concentrations of sFlt-1 in umbilical blood, and even structural changes in the umbilical cord have been reported after pre-eclamptic pregnancy (Staff et al., 2005) (Blanco et al., 2011). Findings concerning levels of sFlt-1 in umbilical blood are not consistent (Sezer et al., 2012), and most investigators reporting increased concentrations of sFlt-1 in umbilical blood have also detected significantly elevated levels of sFlt-1 in maternal sera. In our study, umbilical blood samples included both venous and arterial blood. This could explain the minor differences between maternal and umbilical sera, as umbilical arterial blood better represents the status of the foetus, and umbilical venous blood that of the uteroplacental circulation. In our study, levels of PlGF were below the lowest concentration standard of the immunoassay kit. The same finding has also been reported previously (Staff et al., 2005). In umbilical serum samples, there was a correlation between the anti-angiogenic effect noticed in *in vitro* assays and low birth-weight in the pre-eclampsia group, presumably reflecting the predominance of anti-angiogenesis in pregnancies complicated by pre-eclampsia.

Altogether, it seems that pre-eclampsia affects the foetal endothelium. Gilabert and colleagues reported already two decades ago that umbilical endothelial cells from pre-eclamptic pregnancies exhibit morphofunctional alterations (Gilabert et al., 1999). Later it was confirmed that HUVECs isolated from umbilical cords obtained from pre-eclamptic pregnancies versus healthy pregnancies exhibit reduced migration and tubule formation (Brodowski et al., 2017). It has also been demonstrated that the level of cord-blood endothelial colony-forming cells is decreased after pre-eclamptic pregnancy (Robb et al., 2007). These cells are postulated to contribute to the formation of the foetal vasculature and to the maintenance of vascular integrity. Recently, Lin and colleagues observed that neonates of women with pre-eclampsia had larger coronary arteries at birth than neonates from normotensive pregnancies. The finding was found to correlate with

increased expression of vascular cell adhesion molecule 1 (an inflammatory marker synthesized by endothelial cells) in the umbilical artery (Lin et al., 2021).

6.1.5 Effects of metformin and pravastatin on angiogenesis

We chose to study metformin and pravastatin since both drugs have been hypothesized to reverse the angiogenic imbalance in pre-eclampsia. Additionally, they have beneficial qualities such as oral administration, cheap price, easy availability and good safety profiles. Neither of the drugs have therapeutic indications for use during gestation, but there has not been evidence of teratogenicity.

There is wide experience of metformin use during gestation as it has been commonly prescribed for the treatment of type 2 diabetes and PCOS. A possible association between metformin use and a decreased incidence of hypertensive disorders of pregnancy has been suggested in studies concerning these disorders (Tan et al., 2016) (Feig et al., 2020). The effect of metformin on angiogenesis during gestation has been investigated only in few studies. In a study of Brownfoot and colleagues, metformin improved endothelial dysfunction in a HUVEC model and induced angiogenesis in omental vessels obtained from women with pre-eclampsia (Brownfoot et al., 2016). In an animal study by Wang and colleagues metformin promoted the expression of VEGF and matrix metalloproteinase-2 (MMP-2) proteins, and further improved shallow placental implantation in pre-eclamptic mice (Wang et al., 2019). In our study, metformin at therapeutic concentrations along with maternal sera did not affect tubule formation, but at a high non-therapeutic concentration it inhibited angiogenesis. Within study groups, increasing doses of metformin caused a significant increase in its anti-angiogenic effect on tubule formation. The finding was not due to cell death, as no cytotoxicity was seen in the cell-viability assay, and it seems that an anti-angiogenic state resulted from a specific mechanism of metformin. In addition to findings of a stimulatory angiogenic effect of metformin, there are also contrary reports. In cardiovascular-disease model metformin treatment was associated with VEGF upregulation (Sena et al., 2011), whereas in a HUVEC model metformin inhibited tubule formation in a dose-dependent manner (Dallaglio et al., 2014). Altogether, data concerning the association between metformin and angiogenesis in pre-eclampsia is scarce. It has been hypothesized that since metformin improves insulin sensitivity, decreases weight gain and might have an impact on nitric oxide synthase, the observed decrease in hypertensive disorders is associated with those pathways rather than improved

angiogenesis. There is now an on-going double-bind, randomized, placebo-controlled trial concerning the efficacy of metformin in the treatment of preterm pre-eclampsia, but results are not yet available (Cluver et al., 2019).

A history of pre-eclampsia has been recognized as an independent cardiovascular risk factor (Mosca et al., 2011), but it is not known whether the increased risk is attributed to pre-eclampsia-associated changes in maternal vasculature or to pre-pregnancy predisposing factors that connect pre-eclampsia and cardiovascular disease. It has been observed that pre-eclampsia has a long-term effect on the maternal vasculature. Women with prior pre-eclampsia have been demonstrated to have impairment in endothelium-dependent vasodilatation for up to three years after an affected pregnancy (Chambers et al., 2001), and markers of endothelial activation have been seen to be high even much longer (Sattar et al., 2003). Statins have been considered as a potential therapeutic option for the treatment of pre-eclampsia, since there are similarities in the pathogenic mechanism of pre-eclampsia and cardiovascular disease, and additionally, statins are known to have protective effects on vascular endothelial cells. In several animal, human pre-clinical and *in vitro* studies, pravastatin has been associated with a more favourable angiogenic profile. However, not all studies support that finding, and in the only randomized trial, pravastatin therapy did not lower maternal plasma sFlt-1 levels once early-onset pre-eclampsia had developed (Ahmed et al., 2019). In our study, pravastatin at a therapeutic concentration did not have a significant effect on angiogenesis, but at a high concentration it inhibited angiogenesis. Pravastatin did not change the levels of biomarkers in test media, but the test was performed at only one concentration. When a therapeutic dose of pravastatin along with maternal sera was studied, there were no changes in angiogenesis. However, sera from a few women with early-onset pre-eclampsia had strong stimulatory effect on tubule formation.

Our results did not support previous findings of improved angiogenesis associated with metformin and pravastatin, but the study setting differed from those in previous research. We studied maternal sera, and the cells for the *in vitro* model were obtained from healthy donors, whereas in previous studies placental tissue was obtained from women with pre-eclampsia, or the study was conducted with an animal model of pre-eclampsia. It is possible that our results would have been different if we had used cells from women with pre-eclampsia, and the endothelium of the vessels had been dysfunctional. Also, the angiogenic properties of maternal sera did not differ between the groups. Had the sera been anti-angiogenic, the results may have been different. In the pravastatin study in particular, there was wide variation in the results of the *in vitro* test. A large part of the variation is probably a

result of person-to-person variation, but the confidence level of this study would have benefited from a larger sample size per group. In both studies we also tested high non-therapeutic concentrations of the drugs, because in previous studies anti-angiogenic effects have been reported with high doses (Weis et al., 2002) (Dallaglio et al., 2014). The angiogenic effects observed with high doses were considered real, since the drugs did not cause cytotoxicity.

6.2 Strengths and limitations of the study

The strength of this study is that we used an entirely human cell-based *in vitro* model to assess angiogenesis. Use of advanced *in vitro* technology to study disease pathophysiology is topical, since there is an increasing need for non-animal approaches in medical research. The hASC/HUVEC model has been previously employed for studying anti-angiogenic effect of chemicals and as *in vitro* vasculature in tissue models but it has not been utilized in vascular-system related disease mechanism studies nor in pre-eclampsia study. We suggest that results obtained from the *in vitro* model offer a wider and more overall approach to angiogenic capacity of maternal sera than determining levels of single angiogenic biomarkers in the maternal circulation. Additionally, we studied the angiogenic effects of two pharmaceutical agents, metformin and pravastatin. Both drugs have been recently proposed as promising agents to treat and prevent pre-eclampsia, since their use has been associated with restored angiogenic balance. The results of our study do not support previous findings, but we obtained valuable new information on the angiogenic actions of these drugs. Additionally, the effects of metformin and pravastatin on angiogenesis have not been studied before in the setting of pre-eclamptic, IUGR and healthy pregnancies. Our results highlight the importance of further research in this area, and the need for novel approaches in the development of therapeutics in the treatment of pre-eclampsia.

One limitation of the study was the small sample-size per group, and therefore some true associations may not have reached statistical significance. There was also some heterogeneity in the study population. Although most women in Study I had severe pre-eclampsia, there was variation in the time of onset of the disease, which might have had an effect on the angiogenic properties of the sera. In Study III/IV the women with pre-eclampsia were primiparous, but in the control group multiparous women were also recruited. Gestational age affects angiogenesis. The controls were supposed to be gestational-age-matched, but at the end there was a

significant difference in gestational age between the groups (Study I/II). There were two smokers in the IUGR group (Study III/IV). They should not have been included in the study, since smoking has been observed to have an effect on angiogenesis by decreasing sFlt-1 concentration. There were also weaknesses in the methods and in the description of detailed cellular mechanisms behind the used method. Some protein concentrations were below or above the standards in the immunoassay kits, which creates difficulties in comparing the study groups. And, although there have been advances in *in vitro* methods, there are still differences in effective drug concentrations between *in vivo* and *in vitro* test settings.

6.3 Future perspectives

There have been great advances in understanding the molecular basis of pre-eclampsia during the last two decades. Pre-eclampsia is known to be associated with disturbed angiogenesis, an excessive inflammatory response and production of vasoconstrictors, but there are still several unsolved questions in disease pathophysiology. In recent literature the two-stage theory of pre-eclampsia has been expanded to a four-stage model. In the first two stages environmental and genetic factors are critical components, impaired placentation occurs at the third stage as a consequence of immunological maladaptation, and the fourth stage represents the clinical disease. Since the discovery of angiogenic imbalance in pre-eclampsia, therapeutic approaches have been mainly concentrated on treatments that restore angiogenic balance in the vasculature of pre-eclamptic women. Animal experiments are commonly used in medical research, including pre-eclampsia studies, and it is probable that not even in the future will they be entirely replaced by *in vitro* methods. However, there have been great advances in *in vitro* technology, and current methods offer the possibility to generate tissue models such as the "mini-heart" and liver models. The human cell-based *in vitro* model that has been utilized in this study is suitable for studying the effects of pharmaceutical agents on angiogenesis, but in future it would be interesting to study the effects of other drugs in an *in vitro* model that better resembles the true vascular condition in pre-eclamptic pregnancies. In such model the vessels should be excessively responsive to vasoconstrictive agents and the endothelium should exhibit dysfunctional properties.

7 CONCLUSIONS

The main findings and conclusions of the study are:

1. The human cell-based vasculogenesis/angiogenesis *in vitro* model can be utilized to evaluate the angiogenic properties of maternal and umbilical-cord blood in pre-eclamptic and normotensive pregnancies. The hASC/HUVEC assay provides a wider functional perspective in assessment of the angiogenic capacity of sera than assay of the concentrations of individual angiogenic biomarkers in maternal and/or umbilical-cord sera.

2. In the third trimester, sera from women with clinical pre-eclampsia is more anti-angiogenic than sera from healthy women. Umbilical-cord sera is anti-angiogenic after both pre-eclamptic and healthy pregnancies.

3. In the first trimester, maternal sera have a stimulatory effect on angiogenesis, and there are no differences between women with healthy or subsequently pre-eclamptic pregnancies.

4. The hASC/HUVEC assay can be utilized to study the direct effects of metformin and pravastatin on angiogenesis, and to evaluate angiogenic interactions between maternal sera and these drugs.

5. Therapeutic concentrations of metformin alone or with maternal sera do not have an impact on angiogenic properties, but at a concentration above the therapeutic level there is a significant anti-angiogenic effect on tubule formation.

6. A therapeutic concentration of pravastatin alone or with maternal sera does not significantly change angiogenic capacity, although interaction with maternal sera from early-onset pre-eclamptic pregnancies results in stimulation of angiogenesis in some women. At high doses, above the therapeutic level, pravastatin has an anti-angiogenic effect.

8 REFERENCES

Adair, T. H., & Montani, J.-P. (2010). *Angiogenesis*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK53238/

Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Benthem, J., Zuang, V., … Zaldivar, J.-M. (2011). Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. *Archives of Toxicology*, *85*(5), 367–485. https://doi.org/10.1007/s00204-011-0693-2

Adu-Gyamfi EA, Lamptey J, Duan F, Wang YX, Ding YB. The transforming growth factor β superfamily as possible biomarkers of preeclampsia: a comprehensive review. Biomark Med. 2019 Oct;13(15):1321-1330. doi: 10.2217/bmm-2019-0208.

Ahmed, A., Williams, D. J., Cheed, V., Middleton, L. J., Ahmad, S., Wang, K., … Daniels, J. P. (2019). Pravastatin for early-onset preeclampsia: a randomized, blinded, placebo-controlled trial. *BJOG : An International Journal of Obstetrics and Gynaecology*. https://doi.org/10.1111/1471-0528.16013

Ahn, H., Park, J., Gilman-Sachs, A., & Kwak-Kim, J. (2011). Immunologic characteristics of preeclampsia, a comprehensive review. *American Journal of Reproductive Immunology*, *65*(4), 377–394. https://doi.org/10.1111/j.1600-0897.2010.00913.x

Akolekar, R., Syngelaki, A., Poon, L., Wright, D., & Nicolaides, K. H. (2013). Competing risks model in early screening for preeclampsia by biophysical and biochemical markers. *Fetal Diagnosis and Therapy*, *33*(1), 8–15. https://doi.org/10.1159/000341264

Alqudah, A., McKinley, M. C., McNally, R., Graham, U., Watson, C. J., Lyons, T. J., & McClements, L. (2018). Risk of pre-eclampsia in women taking metformin: a systematic review and meta-analysis. *Diabetic Medicine : A Journal of the British Diabetic Association*, *35*(2), 160–172. https://doi.org/10.1111/dme.13523

Ambati, B. K., Nozaki, M., Singh, N., Takeda, A., Jani, P. D., Suthar, T., … Ambati, J. (2006). Corneal avascularity is due to soluble VEGF receptor-1.

Nature, *443*(7114), 993–997. https://doi.org/10.1038/nature05249

Anderesen, M. (2018). No Title. In *Experimental Animal Models of Human Diseases*. Retrieved from https://www.intechopen.com/books/experimental-animal-models-of-human-diseases-an-effective-therapeutic-strategy/animal-models-of-fetal-medicine-and-obstetrics

Andree, B., Ichanti, H., Kalies, S., Heisterkamp, A., Strauss, S., Vogt, P.-M., … Hilfiker, A. (2019). Formation of three-dimensional tubular endothelial cell networks under defined serum-free cell culture conditions in human collagen hydrogels. *Scientific Reports*, *9*(1), 5437. https://doi.org/10.1038/s41598-019-41985-6

Armaly, Z., Jadaon, J. E., Jabbour, A., & Abassi, Z. A. (2018). Preeclampsia: Novel Mechanisms and Potential Therapeutic Approaches. *Frontiers in Physiology*, *9*, 973. https://doi.org/10.3389/fphys.2018.00973

Aske, K. C., & Waugh, C. A. (2017). Expanding the 3R principles: More rigour and transparency in research using animals. *EMBO Reports*, *18*(9), 1490–1492. https://doi.org/10.15252/embr.201744428

Aykas, F., Solak, Y., Erden, A., Bulut, K., Dogan, S., Sarli, B., … Kanbay, M. (2015). Persistence of cardiovascular risk factors in women with previous preeclampsia: a long-term follow-up study. *Journal of Investigative Medicine*, *63*(4), 641–645. https://doi.org/10.1097/JIM.0000000000000189

Barker, D. J. (1992). The fetal origins of diseases of old age. *European Journal of Clinical Nutrition*, *46 Suppl 3*, S3-9.

Barker, D. J., Gluckman, P. D., Godfrey, K. M., Harding, J. E., Owens, J. A., & Robinson, J. S. (1993). Fetal nutrition and cardiovascular disease in adult life. *Lancet*, *341*(8850), 938–941. https://doi.org/10.1016/0140-6736(93)91224-a

Bateman, B. T., Hernandez-Diaz, S., Fischer, M. A., Seely, E. W., Ecker, J. L., Franklin, J. M., … Huybrechts, K. F. (2015). Statins and congenital malformations: cohort study. *BMJ*, *350*, h1035. https://doi.org/10.1136/bmj.h1035

Bauer, A. J., Banek, C. T., Needham, K., Gillham, H., Capoccia, S., Regal, J. F., & Gilbert, J. S. (2013). Pravastatin attenuates hypertension, oxidative stress, and angiogenic imbalance in rat model of placental ischemia-induced hypertension. *Hypertension*, *61*(5), 1103–1110. https://doi.org/10.1161/HYPERTENSIONAHA.111.00226

Bell, E. (2004). A bad combination. *Nature Reviews Immunology*, *4*, 927. Retrieved from https://doi.org/10.1038/nri1514

Blaauw, J., Graaff, R., van Pampus, M. G., van Doormaal, J. J., Smit, A. J., Rakhorst, G., & Aarnoudse, J. G. (2005). Abnormal endothelium-dependent microvascular reactivity in recently preeclamptic women. *Obstetrics and Gynecology*, *105*(3), 626–632. https://doi.org/10.1097/01.AOG.0000153490.41973.e0

Blanco, M. V, Vega, H. R., Giuliano, R., Grana, D. R., Azzato, F., Lerman, J., & Milei, J. (2011). Histomorphometry of umbilical cord blood vessels in preeclampsia. *Journal of Clinical Hypertension*, *13*(1), 30–34. https://doi.org/10.1111/j.1751-7176.2010.00384.x

Bondareva, O., & Sheikh, B. N. (2020). Vascular Homeostasis and Inflammation in Health and Disease-Lessons from Single Cell Technologies. *International Journal of Molecular Sciences*, *21*(13). https://doi.org/10.3390/ijms21134688

Boutsikou, T., Malamitsi-Puchner, A., Economou, E., Boutsikou, M., Puchner, K.-P., & Hassiakos, D. (2006). Soluble vascular endothelial growth factor receptor-1 in intrauterine growth restricted fetuses and neonates. *Early Human Development*, *82*(4), 235–239. https://doi.org/10.1016/j.earlhumdev.2005.09.010

Bracken, M. B. (2009). Why animal studies are often poor predictors of human reactions to exposure. *Journal of the Royal Society of Medicine*, *102*(3), 120–122. https://doi.org/10.1258/jrsm.2008.08k033

Brodowski, L., Burlakov, J., Hass, S., von Kaisenberg, C., & von Versen-Höynck, F. (2017). Impaired functional capacity of fetal endothelial cells in preeclampsia. *PloS One*, *12*(5), e0178340. https://doi.org/10.1371/journal.pone.0178340

Brosens, I., Pijnenborg, R., Vercruysse, L., & Romero, R. (2011). The "Great Obstetrical Syndromes" are associated with disorders of deep placentation. *American Journal of Obstetrics and Gynecology*, *204*(3), 193–201. https://doi.org/10.1016/j.ajog.2010.08.009

Brown, M. A., Lindheimer, M. D., de Swiet, M., Van Assche, A., & Moutquin, J. M. (2001). The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertension in Pregnancy*. England. https://doi.org/10.1081/PRG-100104165

Brownfoot, F. C., Hastie, R., Hannan, N. J., Cannon, P., Tuohey, L., Parry, L. J., … Tong, S. (2016). Metformin as a prevention and treatment for preeclampsia: effects on soluble fms-like tyrosine kinase 1 and soluble endoglin secretion and endothelial dysfunction. *American Journal of Obstetrics and Gynecology*, *214*(3), 356.e1-356.e15. https://doi.org/10.1016/j.ajog.2015.12.019

Burton, G. J., Redman, C. W., Roberts, J. M., & Moffett, A. (2019). Pre-eclampsia: pathophysiology and clinical implications. *BMJ*, *366*, l2381. https://doi.org/10.1136/bmj.l2381

Caillon, H., Tardif, C., Dumontet, E., Winer, N., & Masson, D. (2018). Evaluation of sFlt-1/PlGF Ratio for Predicting and Improving Clinical Management of Pre-eclampsia: Experience in a Specialized Perinatal Care Center. *Annals of Laboratory Medicine*, *38*(2), 95–101. https://doi.org/10.3343/alm.2018.38.2.95

Calabro, P., & Yeh, E. T. H. (2005). The pleiotropic effects of statins. *Current Opinion in Cardiology*, *20*(6), 541–546. https://doi.org/10.1097/01.hco.0000181482.99067.bf

Carmeliet, P. (2003). Angiogenesis in health and disease. *Nature Medicine*, *9*(6), 653–660. https://doi.org/10.1038/nm0603-653

Carmeliet, P., & Collen, D. (2000). Transgenic mouse models in angiogenesis and cardiovascular disease. *The Journal of Pathology*, *190*(3), 387–405. https://doi.org/10.1002/(SICI)1096-9896(200002)190:3<387::AID-PATH595>3.0.CO;2-R

Cassina, M., Dona, M., Di Gianantonio, E., Litta, P., & Clementi, M. (2014). First-trimester exposure to metformin and risk of birth defects: a systematic review and meta-analysis. *Human Reproduction Update*, *20*(5), 656–669. https://doi.org/10.1093/humupd/dmu022

Chaiworapongsa, T., Chaemsaithong, P., Korzeniewski, S. J., Yeo, L., & Romero, R. (2014). Pre-eclampsia part 2: prediction, prevention and management. *Nature Reviews. Nephrology*, *10*(9), 531–540. https://doi.org/10.1038/nrneph.2014.103

Chaiworapongsa, T., Chaemsaithong, P., Yeo, L., & Romero, R. (2014). Pre-eclampsia part 1: current understanding of its pathophysiology. *Nature Reviews. Nephrology*, *10*(8), 466–480. https://doi.org/10.1038/nrneph.2014.102

Chambers, J. C., Fusi, L., Malik, I. S., Haskard, D. O., De Swiet, M., & Kooner, J. S. (2001). Association of maternal endothelial dysfunction with preeclampsia.

JAMA, *285*(12), 1607–1612. https://doi.org/10.1001/jama.285.12.1607

Charnock-Jones, D. S., Kaufmann, P., & Mayhew, T. M. (2004). Aspects of human fetoplacental vasculogenesis and angiogenesis. I. Molecular regulation. *Placenta*, *25*(2–3), 103–113. https://doi.org/10.1016/j.placenta.2003.10.004

Chau, K., Hennessy, A., & Makris, A. (2017). Placental growth factor and pre-eclampsia. *Journal of Human Hypertension*, *31*(12), 782–786. https://doi.org/10.1038/jhh.2017.61

Checkley, S., MacCallum, L., Yates, J., Jasper, P., Luo, H., Tolsma, J., & Bendtsen, C. (2016). Corrigendum: Bridging the gap between in vitro and in vivo: Dose and schedule predictions for the ATR inhibitor AZD6738. *Scientific Reports*. https://doi.org/10.1038/srep16545

Chen, C. W., Jaffe, I. Z., & Karumanchi, S. A. (2014). Pre-eclampsia and cardiovascular disease. *Cardiovascular Research*, *101*(4), 579–586. https://doi.org/10.1093/cvr/cvu018

Chen, D.-B., & Zheng, J. (2014). Regulation of placental angiogenesis. *Microcirculation*, *21*(1), 15–25. https://doi.org/10.1111/micc.12093

Cheung, B. M. Y., Lauder, I. J., Lau, C.-P., & Kumana, C. R. (2004). Meta-analysis of large randomized controlled trials to evaluate the impact of statins on cardiovascular outcomes. *British Journal of Clinical Pharmacology*, *57*(5), 640–651. https://doi.org/10.1111/j.1365-2125.2003.02060.x

Cindrova-Davies, T. (2014). The therapeutic potential of antioxidants, ER chaperones, NO and H2S donors, and statins for treatment of preeclampsia. *Frontiers in Pharmacology*, *5*, 119. https://doi.org/10.3389/fphar.2014.00119

Cluver, C., Walker, S. P., Mol, B. W., Hall, D., Hiscock, R., Brownfoot, F. C., … Tong, S. (2019). A double blind, randomised, placebo-controlled trial to evaluate the efficacy of metformin to treat preterm pre-eclampsia (PI2 Trial): study protocol. *BMJ Open*, *9*(4), e025809. https://doi.org/10.1136/bmjopen-2018-025809

Costantine, M. M., Cleary, K., Hebert, M. F., Ahmed, M. S., Brown, L. M., Ren, Z., … Hankins, G. (2016). Safety and pharmacokinetics of pravastatin used for the prevention of preeclampsia in high-risk pregnant women: a pilot randomized controlled trial. *American Journal of Obstetrics and Gynecology*, *214*(6), 720.e1-720.e17. https://doi.org/10.1016/j.ajog.2015.12.038

Coyle, C., Cafferty, F. H., Vale, C., & Langley, R. E. (2016). Metformin as an adjuvant treatment for cancer: a systematic review and meta-analysis. *Annals of Oncology : Official Journal of the European Society for Medical Oncology*, *27*(12), 2184–2195. https://doi.org/10.1093/annonc/mdw410

Creanga, A. A., Bradley, H. M., McCormick, C., & Witkop, C. T. (2008). Use of metformin in polycystic ovary syndrome: a meta-analysis. *Obstetrics and Gynecology*, *111*(4), 959–968. https://doi.org/10.1097/AOG.0b013e31816a4ed4

Crispi, F., Dominguez, C., Llurba, E., Martin-Gallan, P., Cabero, L., & Gratacos, E. (2006). Placental angiogenic growth factors and uterine artery Doppler findings for characterization of different subsets in preeclampsia and in isolated intrauterine growth restriction. *American Journal of Obstetrics and Gynecology*, *195*(1), 201–207. https://doi.org/10.1016/j.ajog.2006.01.014

D'Alessio, A., Moccia, F., Li, J.-H., Micera, A., & Kyriakides, T. R. (2015). Angiogenesis and Vasculogenesis in Health and Disease. *BioMed Research International*. United States. https://doi.org/10.1155/2015/126582

Dallaglio, K., Bruno, A., Cantelmo, A. R., Esposito, A. I., Ruggiero, L., Orecchioni, S., … Albini, A. (2014). Paradoxic effects of metformin on endothelial cells and angiogenesis. *Carcinogenesis*, *35*(5), 1055–1066. https://doi.org/10.1093/carcin/bgu001

Davis, E. F., Lazdam, M., Lewandowski, A. J., Worton, S. A., Kelly, B., Kenworthy, Y., … Leeson, P. (2012). Cardiovascular risk factors in children and young adults born to preeclamptic pregnancies: a systematic review. *Pediatrics*, *129*(6), e1552-61. https://doi.org/10.1542/peds.2011-3093

De Falco, S. (2012). The discovery of placenta growth factor and its biological activity. *Experimental & Molecular Medicine*, *44*(1), 1–9. https://doi.org/10.3858/emm.2012.44.1.025

De Leo, V., Musacchio, M. C., Piomboni, P., Di Sabatino, A., & Morgante, G. (2011). The administration of metformin during pregnancy reduces polycystic ovary syndrome related gestational complications. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, *157*(1), 63–66. https://doi.org/10.1016/j.ejogrb.2011.03.024

Deanfield, J. E., Halcox, J. P., & Rabelink, T. J. (2007). Endothelial function and dysfunction: testing and clinical relevance. *Circulation*, *115*(10), 1285–1295. https://doi.org/10.1161/CIRCULATIONAHA.106.652859

Duley, L. (2009). The global impact of pre-eclampsia and eclampsia. *Seminars in Perinatology*, *33*(3), 130–137. https://doi.org/10.1053/j.semperi.2009.02.010

Easterling, T. R. (2016). Apheresis to Treat Preeclampsia: Insights, Opportunities and Challenges. *Journal of the American Society of Nephrology : JASN*. https://doi.org/10.1681/ASN.2015070794

Edison, R. J., & Muenke, M. (2004). Mechanistic and epidemiologic considerations in the evaluation of adverse birth outcomes following gestational exposure to statins. *American Journal of Medical Genetics. Part A*, *131*(3), 287–298. https://doi.org/10.1002/ajmg.a.30386

Egom, E. E. A., & Hafeez, H. (2016). Biochemistry of Statins. *Advances in Clinical Chemistry*, *73*, 127–168. https://doi.org/10.1016/bs.acc.2015.10.005

Eilken, H. M., & Adams, R. H. (2010). Dynamics of endothelial cell behavior in sprouting angiogenesis. *Current Opinion in Cell Biology*, *22*(5), 617–625. https://doi.org/10.1016/j.ceb.2010.08.010

El-Sayed, A. A. F. (2017). Preeclampsia: A review of the pathogenesis and possible management strategies based on its pathophysiological derangements. *Taiwanese Journal of Obstetrics & Gynecology*, *56*(5), 593–598. https://doi.org/10.1016/j.tjog.2017.08.004

Eppler, S. M., Combs, D. L., Henry, T. D., Lopez, J. J., Ellis, S. G., Yi, J.-H., … Zioncheck, T. F. (2002). A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. *Clinical Pharmacology and Therapeutics*, *72*(1), 20–32. https://doi.org/10.1067/mcp.2002.126179

Evans, J. M. M., Donnelly, L. A., Emslie-Smith, A. M., Alessi, D. R., & Morris, A. D. (2005). Metformin and reduced risk of cancer in diabetic patients. *BMJ*, *330*(7503), 1304–1305. https://doi.org/10.1136/bmj.38415.708634.F7

Eyal, S., Easterling, T. R., Carr, D., Umans, J. G., Miodovnik, M., Hankins, G. D. V, … Hebert, M. F. (2010). Pharmacokinetics of metformin during pregnancy. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *38*(5), 833–840. https://doi.org/10.1124/dmd.109.031245

Farrar, D., Simmonds, M., Bryant, M., Sheldon, T. A., Tuffnell, D., Golder, S., & Lawlor, D. A. (2017). Treatments for gestational diabetes: a systematic review and meta-analysis. *BMJ Open*, *7*(6), e015557. https://doi.org/10.1136/bmjopen-2016-015557

FDA. (n.d.). *Pravachol Label, Highlights of prescribing information*. Retrieved from https://www.accessdata.fda.gov/drugsatfda_docs/label/2012/019898s062lbl.pdf

Feig, D. S., Donovan, L. E., Zinman, B., Sanchez, J. J., Asztalos, E., Ryan, E. A., … Murphy, K. E. (2020). Metformin in women with type 2 diabetes in pregnancy (MiTy): a multicentre, international, randomised, placebo-controlled trial. *The Lancet. Diabetes & Endocrinology*, *8*(10), 834–844. https://doi.org/10.1016/S2213-8587(20)30310-7

Flamme, I., Frolich, T., & Risau, W. (1997). Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *Journal of Cellular Physiology*, *173*(2), 206–210. https://doi.org/10.1002/(SICI)1097-4652(199711)173:2<206::AID-JCP22>3.0.CO;2-C

Fox, K. A., Longo, M., Tamayo, E., Kechichian, T., Bytautiene, E., Hankins, G. D. V, … Costantine, M. M. (2011). Effects of pravastatin on mediators of vascular function in a mouse model of soluble Fms-like tyrosine kinase-1-induced preeclampsia. *American Journal of Obstetrics and Gynecology*, *205*(4), 366.e1-5. https://doi.org/10.1016/j.ajog.2011.06.083

Franks, M. E., Macpherson, G. R., & Figg, W. D. (2004). Thalidomide. *Lancet*, *363*(9423), 1802–1811. https://doi.org/10.1016/S0140-6736(04)16308-3

Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. (2020). *Obstetrics & Gynecology*, *135*(6). Retrieved from https://journals.lww.com/greenjournal/Fulltext/2020/06000/Gestational_Hypertension_and_Preeclampsia__ACOG.46.aspx

Ghallab, A. (2013). In vitro test systems and their limitations. *EXCLI Journal*, *12*, 1024–1026.

Gilabert, R., Bellart, J., Jové, M., Miralles, R. M., & Piera, V. (1999). Endothelial cell lesion in preeclampsia. Morphofunctional study using umbilical endothelial cells. *Gynecologic and Obstetric Investigation*, *47*(2), 95–101. https://doi.org/10.1159/000010070

Gilbert, J. S., Verzwyvelt, J., Colson, D., Arany, M., Karumanchi, S. A., & Granger, J. P. (2010). Recombinant vascular endothelial growth factor 121 infusion lowers blood pressure and improves renal function in rats with placentalischemia-induced hypertension. *Hypertension*, *55*(2), 380–385. https://doi.org/10.1161/HYPERTENSIONAHA.109.141937

Gluckman, P. D., Hanson, M. A., Cooper, C., & Thornburg, K. L. (2008). Effect of in utero and early-life conditions on adult health and disease. *The New England Journal of Medicine*, *359*(1), 61–73. https://doi.org/10.1056/NEJMra0708473

Goel, S., Duda, D. G., Xu, L., Munn, L. L., Boucher, Y., Fukumura, D., & Jain, R. K. (2011). Normalization of the vasculature for treatment of cancer and other diseases. *Physiological Reviews*, *91*(3), 1071–1121. https://doi.org/10.1152/physrev.00038.2010

Gong, L., Goswami, S., Giacomini, K. M., Altman, R. B., & Klein, T. E. (2012). Metformin pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenetics and Genomics*, *22*(11), 820–827. https://doi.org/10.1097/FPC.0b013e3283559b22

Graham, G. G., Punt, J., Arora, M., Day, R. O., Doogue, M. P., Duong, J. K., … Williams, K. M. (2011). Clinical pharmacokinetics of metformin. *Clinical Pharmacokinetics*, *50*(2), 81–98. https://doi.org/10.2165/11534750-000000000-00000

Gui, J., Liu, Q., & Feng, L. (2013). Metformin vs insulin in the management of gestational diabetes: a meta-analysis. *PloS One*, *8*(5), e64585. https://doi.org/10.1371/journal.pone.0064585

Hajar, R. (2011). Animal testing and medicine. *Heart Views : The Official Journal of the Gulf Heart Association*, *12*(1), 42. https://doi.org/10.4103/1995-705X.81548

Hammad, S. (2013). Advances in 2D and 3D in vitro systems for hepatotoxicity testing. *EXCLI Journal*, *12*, 993–996.

Henriques, A. C. P. T., Carvalho, F. H. C., Feitosa, H. N., Macena, R. H. M., Mota, R. M. S., & Alencar, J. C. G. (2014). Endothelial dysfunction after pregnancy-induced hypertension. *International Journal of Gynaecology and Obstetrics: The Official Organ of the International Federation of Gynaecology and Obstetrics*, *124*(3), 230–234. https://doi.org/10.1016/j.ijgo.2013.08.016

Hentges, C. R., Silveira, R. C., & Procianoy, R. S. (2015). Angiogenic and Antiangiogenic Factors in Preterm Neonates Born to Mothers with and without Preeclampsia. *American Journal of Perinatology*, *32*(12), 1185–1190. https://doi.org/10.1055/s-0035-1552932

Hofmeyr, G. J., Lawrie, T. A., Atallah, Á. N., & Torloni, M. R. (2018). Calcium supplementation during pregnancy for preventing hypertensive disorders and

related problems. *The Cochrane Database of Systematic Reviews*, *10*(10), CD001059. https://doi.org/10.1002/14651858.CD001059.pub5

Holmes, D. I. R., & Zachary, I. (2005). The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease. *Genome Biology*, *6*(2), 209. https://doi.org/10.1186/gb-2005-6-2-209

Huntjens, D. R. H., Spalding, D. J. M., Danhof, M., & Della Pasqua, O. E. (2006). Correlation between in vitro and in vivo concentration-effect relationships of naproxen in rats and healthy volunteers. *British Journal of Pharmacology*, *148*(4), 396–404. https://doi.org/10.1038/sj.bjp.0706737

Hutcheon, J. A., Lisonkova, S., & Joseph, K. S. (2011). Epidemiology of pre-eclampsia and the other hypertensive disorders of pregnancy. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, *25*(4), 391–403. https://doi.org/10.1016/j.bpobgyn.2011.01.006

Huttala, O., Palmroth, M., Hemminki, P., Toimela, T., Heinonen, T., Ylikomi, T., & Sarkanen, J.-R. (2018). Development of Versatile Human In Vitro Vascularized Adipose Tissue Model with Serum-Free Angiogenesis and Natural Adipogenesis Induction. *Basic & Clinical Pharmacology & Toxicology*, *123 Suppl 5*, 62–71. https://doi.org/10.1111/bcpt.12987

Huttala, O., Staff, S., Heinonen, T., Mäenpää, J., Tanner, M., & Ylikomi, T. (2020). In Vitro Vascular Network Modified to Function as Culture Platform and Angiogenic Induction Potential Test for Cancer Cells. *International Journal of Molecular Sciences*, *21*(5). https://doi.org/10.3390/ijms21051833

Huttala, O., Vuorenpaa, H., Toimela, T., Uotila, J., Kuokkanen, H., Ylikomi, T., … Heinonen, T. (2015). Human vascular model with defined stimulation medium - a characterization study. *ALTEX*, *32*(2), 125–136. https://doi.org/10.14573/altex.1411271

Ijas, H., Vaarasmaki, M., Saarela, T., Keravuo, R., & Raudaskoski, T. (2015). A follow-up of a randomised study of metformin and insulin in gestational diabetes mellitus: growth and development of the children at the age of 18 months. *BJOG : An International Journal of Obstetrics and Gynaecology*, *122*(7), 994–1000. https://doi.org/10.1111/1471-0528.12964

Inzucchi, S. E., Bergenstal, R. M., Buse, J. B., Diamant, M., Ferrannini, E., Nauck, M., … Matthews, D. R. (2012). Management of hyperglycaemia in type 2 diabetes: a patient-centered approach. Position statement of the American Diabetes Association (ADA) and the European Association for the Study of

Diabetes (EASD). *Diabetologia*, *55*(6), 1577–1596. https://doi.org/10.1007/s00125-012-2534-0

Irgens, H. U., Reisaeter, L., Irgens, L. M., & Lie, R. T. (2001). Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study. *BMJ*, *323*(7323), 1213–1217. https://doi.org/10.1136/bmj.323.7323.1213

Jansen, M. A., Pluymen, L. P., Dalmeijer, G. W., Groenhof, T. K. J., Uiterwaal, C. S., Smit, H. A., & van Rossem, L. (2019). Hypertensive disorders of pregnancy and cardiometabolic outcomes in childhood: A systematic review. *European Journal of Preventive Cardiology*, *26*(16), 1718–1747. https://doi.org/10.1177/2047487319852716

Jayet, P.-Y., Rimoldi, S. F., Stuber, T., Salmon, C. S., Hutter, D., Rexhaj, E., … Sartori, C. (2010). Pulmonary and systemic vascular dysfunction in young offspring of mothers with preeclampsia. *Circulation*, *122*(5), 488–494. https://doi.org/10.1161/CIRCULATIONAHA.110.941203

Jayson, G. C., Kerbel, R., Ellis, L. M., & Harris, A. L. (2016). Antiangiogenic therapy in oncology: current status and future directions. *Lancet*, *388*(10043), 518–529. https://doi.org/10.1016/S0140-6736(15)01088-0

Jeyabalan, A., McGonigal, S., Gilmour, C., Hubel, C. A., & Rajakumar, A. (2008). Circulating and placental endoglin concentrations in pregnancies complicated by intrauterine growth restriction and preeclampsia. *Placenta*, *29*(6), 555–563. https://doi.org/10.1016/j.placenta.2008.03.006

Jiang L, Zhou Y, Huang Q. Serum fibroblast growth factor 21 level is increased in pre-eclampsia patients: Association with blood pressure and lipid profile. J Obstet Gynaecol Res. 2021 Jan;47(1):375-381. doi: 10.1111/jog.14534.

Kajantie, E., Eriksson, J. G., Osmond, C., Thornburg, K., & Barker, D. J. P. (2009). Pre-eclampsia is associated with increased risk of stroke in the adult offspring: the Helsinki birth cohort study. *Stroke*, *40*(4), 1176–1180. https://doi.org/10.1161/STROKEAHA.108.538025

Kaufmann, P., Mayhew, T. M., & Charnock-Jones, D. S. (2004). Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy. *Placenta*, *25*(2–3), 114–126. https://doi.org/10.1016/j.placenta.2003.10.009

Karakus S, Bozoklu Akkar O, Yildiz C, Sancakdar E, Cetin M, Cetin A. Serum levels of ET-1, M30, and angiopoietins-1 and -2 in HELLP syndrome and

preeclampsia compared to controls. Arch Gynecol Obstet. 2016 Feb;293(2):351-9. doi: 10.1007/s00404-015-3803-1.

Kenny, L. C., Black, M. A., Poston, L., Taylor, R., Myers, J. E., Baker, P. N., … North, R. A. (2014). Early pregnancy prediction of preeclampsia in nulliparous women, combining clinical risk and biomarkers: the Screening for Pregnancy Endpoints (SCOPE) international cohort study. *Hypertension*, *64*(3), 644–652. https://doi.org/10.1161/HYPERTENSIONAHA.114.03578

Kerbel, R. S. (2008). Tumor angiogenesis. *The New England Journal of Medicine*, *358*(19), 2039–2049. https://doi.org/10.1056/NEJMra0706596

Khan, K. S., Wojdyla, D., Say, L., Gulmezoglu, A. M., & Van Look, P. F. (2006). WHO analysis of causes of maternal death: a systematic review. *Lancet*, *367*(9516), 1066–1074. https://doi.org/10.1016/S0140-6736(06)68397-9

Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., … Strieter, R. M. (1992). Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science*, *258*(5089), 1798–1801. https://doi.org/10.1126/science.1281554

Kuc, S., Wortelboer, E. J., van Rijn, B. B., Franx, A., Visser, G. H. A., & Schielen, P. C. J. I. (2011). Evaluation of 7 serum biomarkers and uterine artery Doppler ultrasound for first-trimester prediction of preeclampsia: a systematic review. *Obstetrical & Gynecological Survey*, *66*(4), 225–239. https://doi.org/10.1097/OGX.0b013e3182227027

Kumasawa, K., Ikawa, M., Kidoya, H., Hasuwa, H., Saito-Fujita, T., Morioka, Y., … Okabe, M. (2011). Pravastatin induces placental growth factor (PGF) and ameliorates preeclampsia in a mouse model. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(4), 1451–1455. https://doi.org/10.1073/pnas.1011293108

Kusanovic, J. P., Romero, R., Chaiworapongsa, T., Erez, O., Mittal, P., Vaisbuch, E., … Hassan, S. S. (2009). A prospective cohort study of the value of maternal plasma concentrations of angiogenic and anti-angiogenic factors in early pregnancy and midtrimester in the identification of patients destined to develop preeclampsia. *The Journal of Maternal-Fetal & Neonatal Medicine*, *22*(11), 1021–1038. https://doi.org/10.3109/14767050902994754

Kvehaugen, A. S., Dechend, R., Ramstad, H. B., Troisi, R., Fugelseth, D., & Staff, A. C. (2011). Endothelial function and circulating biomarkers are disturbed in women and children after preeclampsia. *Hypertension*, *58*(1), 63–69.

https://doi.org/10.1161/HYPERTENSIONAHA.111.172387

Laskowska, M., Laskowska, K., & Oleszczuk, J. (2012). Endoglin in pregnancy complicated by fetal intrauterine growth restriction in normotensive and preeclamptic pregnant women: a comparison between preeclamptic patients with appropriate-for-gestational-age weight infants and healthy pregnant women. *The Journal of Maternal-Fetal & Neonatal Medicine*, *25*(6), 806–811. https://doi.org/10.3109/14767058.2011.595852

Leanos-Miranda, A., Navarro-Romero, C. S., Sillas-Pardo, L. J., Ramirez-Valenzuela, K. L., Isordia-Salas, I., & Jimenez-Trejo, L. M. (2019). Soluble Endoglin As a Marker for Preeclampsia, Its Severity, and the Occurrence of Adverse Outcomes. *Hypertension*, *74*(4), 991–997. https://doi.org/10.1161/HYPERTENSIONAHA.119.13348

Lee, W. L., & Slutsky, A. S. (2010). Sepsis and endothelial permeability. *The New England Journal of Medicine*, *363*(7), 689–691. https://doi.org/10.1056/NEJMcibr1007320

Levine, R. J., & Karumanchi, S. A. (2005). Circulating angiogenic factors in preeclampsia. *Clinical Obstetrics and Gynecology*, *48*(2), 372–386. https://doi.org/10.1097/01.grf.0000160313.82606.d7

Levine, R. J., Lam, C., Qian, C., Yu, K. F., Maynard, S. E., Sachs, B. P., … Karumanchi, S. A. (2006). Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *The New England Journal of Medicine*, *355*(10), 992–1005. https://doi.org/10.1056/NEJMoa055352

Levine, R. J., Maynard, S. E., Qian, C., Lim, K.-H., England, L. J., Yu, K. F., … Karumanchi, S. A. (2004). Circulating angiogenic factors and the risk of preeclampsia. *The New England Journal of Medicine*, *350*(7), 672–683. https://doi.org/10.1056/NEJMoa031884

Li, Z., Zhang, Y., Ying Ma, J., Kapoun, A. M., Shao, Q., Kerr, I., … Pollitt, N. S. (2007). Recombinant vascular endothelial growth factor 121 attenuates hypertension and improves kidney damage in a rat model of preeclampsia. *Hypertension*, *50*(4), 686–692. https://doi.org/10.1161/HYPERTENSIONAHA.107.092098

Lin Chun. (2021). Coronary dilatation and endothelial inflammation. *The Journal of Pediatrics*, *228*, 58–65.

Lindsay, R. S., & Loeken, M. R. (2017). Metformin use in pregnancy: promises and

uncertainties. *Diabetologia*, *60*(9), 1612–1619. https://doi.org/10.1007/s00125-017-4351-y

Lisonkova, S., & Joseph, K. S. (2013). Incidence of preeclampsia: risk factors and outcomes associated with early- versus late-onset disease. *American Journal of Obstetrics and Gynecology*, *209*(6), 544.e1-544.e12. https://doi.org/10.1016/j.ajog.2013.08.019

Lisowska, M., Pietrucha, T., & Sakowicz, A. (2018). Preeclampsia and Related Cardiovascular Risk: Common Genetic Background. *Current Hypertension Reports*, *20*(8), 71. https://doi.org/10.1007/s11906-018-0869-8

Lokki, A. I., Daly, E., Triebwasser, M., Kurki, M. I., Roberson, E. D. O., Happola, P., … Laivuori, H. (2017). Protective Low-Frequency Variants for Preeclampsia in the Fms Related Tyrosine Kinase 1 Gene in the Finnish Population. *Hypertension*, *70*(2), 365–371. https://doi.org/10.1161/HYPERTENSIONAHA.117.09406

López, J. F., Sarkanen, J.-R., Huttala, O., Kaartinen, I. S., Kuokkanen, H. O., & Ylikomi, T. (2018). Adipose tissue extract shows potential for wound healing: in vitro proliferation and migration of cell types contributing to wound healing in the presence of adipose tissue preparation and platelet rich plasma. *Cytotechnology*, *70*(4), 1193–1204. https://doi.org/10.1007/s10616-018-0211-y

Ludman, A., Venugopal, V., Yellon, D. M., & Hausenloy, D. J. (2009). Statins and cardioprotection--more than just lipid lowering? *Pharmacology & Therapeutics*, *122*(1), 30–43. https://doi.org/10.1016/j.pharmthera.2009.01.002

Lyall, F. (2005). Priming and remodelling of human placental bed spiral arteries during pregnancy--a review. *Placenta*, *26 Suppl A*, S31-6. https://doi.org/10.1016/j.placenta.2005.02.010

Lykke, J. A., Langhoff-Roos, J., Sibai, B. M., Funai, E. F., Triche, E. W., & Paidas, M. J. (2009). Hypertensive pregnancy disorders and subsequent cardiovascular morbidity and type 2 diabetes mellitus in the mother. *Hypertension*, *53*(6), 944–951. https://doi.org/10.1161/HYPERTENSIONAHA.109.130765

Makris, A., Yeung, K. R., Lim, S. M., Sunderland, N., Heffernan, S., Thompson, J. F., … Hennessy, A. (2016). Placental Growth Factor Reduces Blood Pressure in a Uteroplacental Ischemia Model of Preeclampsia in Nonhuman Primates. *Hypertension*, *67*(6), 1263–1272. https://doi.org/10.1161/HYPERTENSIONAHA.116.07286

Matsumoto, T., & Claesson-Welsh, L. (2001). VEGF receptor signal transduction. *Science's STKE : Signal Transduction Knowledge Environment*, (112), re21. https://doi.org/10.1126/stke.2001.112.re21

Matthews, R. A. J. (2008). Medical progress depends on animal models - doesn't it? *Journal of the Royal Society of Medicine*, *101*(2), 95–98. https://doi.org/10.1258/jrsm.2007.070164

Maynard, S. E., & Karumanchi, S. A. (2011). Angiogenic factors and preeclampsia. *Seminars in Nephrology*, *31*(1), 33–46. https://doi.org/10.1016/j.semnephrol.2010.10.004

Maynard, S. E., Min, J.-Y., Merchan, J., Lim, K.-H., Li, J., Mondal, S., … Karumanchi, S. A. (2003). Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *The Journal of Clinical Investigation*, *111*(5), 649–658. https://doi.org/10.1172/JCI17189

McElrath, T. F., Lim, K.-H., Pare, E., Rich-Edwards, J., Pucci, D., Troisi, R., & Parry, S. (2012). Longitudinal evaluation of predictive value for preeclampsia of circulating angiogenic factors through pregnancy. *American Journal of Obstetrics and Gynecology*, *207*(5), 407.e1-7. https://doi.org/10.1016/j.ajog.2012.08.010

McNally, R., Alqudah, A., Obradovic, D., & McClements, L. (2017). Elucidating the Pathogenesis of Pre-eclampsia Using In Vitro Models of Spiral Uterine Artery Remodelling. *Current Hypertension Reports*, *19*(11), 93. https://doi.org/10.1007/s11906-017-0786-2

Mentzer, S. J., & Konerding, M. A. (2014). Intussusceptive angiogenesis: expansion and remodeling of microvascular networks. *Angiogenesis*, *17*(3), 499–509. https://doi.org/10.1007/s10456-014-9428-3

Miquerol, L., Langille, B. L., & Nagy, A. (2000). Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development*, *127*(18), 3941–3946.

Moran, C. J., Ramesh, A., Brama, P. A. J., O'Byrne, J. M., O'Brien, F. J., & Levingstone, T. J. (2016). The benefits and limitations of animal models for translational research in cartilage repair. *Journal of Experimental Orthopaedics*, *3*(1), 1. https://doi.org/10.1186/s40634-015-0037-x

Mosca, L., Benjamin, E. J., Berra, K., Bezanson, J. L., Dolor, R. J., Lloyd-Jones, D.

M., … Wenger, N. K. (2011). Effectiveness-based guidelines for the prevention of cardiovascular disease in women--2011 update: a guideline from the american heart association. *Circulation*, *123*(11), 1243–1262. https://doi.org/10.1161/CIR.0b013e31820faaf8

Myatt, L., Clifton, R. G., Roberts, J. M., Spong, C. Y., Hauth, J. C., Varner, M. W., … Anderson, G. D. (2012). First-trimester prediction of preeclampsia in nulliparous women at low risk. *Obstetrics and Gynecology*, *119*(6), 1234–1242. https://doi.org/10.1097/AOG.0b013e3182571669

Myers, J. E., Kenny, L. C., McCowan, L. M. E., Chan, E. H. Y., Dekker, G. A., Poston, L., … North, R. A. (2013). Angiogenic factors combined with clinical risk factors to predict preterm pre-eclampsia in nulliparous women: a predictive test accuracy study. *BJOG : An International Journal of Obstetrics and Gynaecology*, *120*(10), 1215–1223. https://doi.org/10.1111/1471-0528.12195

Nanovskaya, T. N., Patrikeeva, S. L., Paul, J., Costantine, M. M., Hankins, G. D. V, & Ahmed, M. S. (2013). Transplacental transfer and distribution of pravastatin. *American Journal of Obstetrics and Gynecology*, *209*(4), 373.e1-5. https://doi.org/10.1016/j.ajog.2013.05.038

Nowak-Sliwinska, P., Alitalo, K., Allen, E., Anisimov, A., Aplin, A. C., Auerbach, R., … Griffioen, A. W. (2018). Consensus guidelines for the use and interpretation of angiogenesis assays. *Angiogenesis*, *21*(3), 425–532. https://doi.org/10.1007/s10456-018-9613-x

Ogge, G., Chaiworapongsa, T., Romero, R., Hussein, Y., Kusanovic, J. P., Yeo, L., … Hassan, S. S. (2011). Placental lesions associated with maternal underperfusion are more frequent in early-onset than in late-onset preeclampsia. *Journal of Perinatal Medicine*, *39*(6), 641–652. https://doi.org/10.1515/jpm.2011.098

Øglaend, B., Forman, M. R., Romundstad, P. R., Nilsen, S. T., & Vatten, L. J. (2009). Blood pressure in early adolescence in the offspring of preeclamptic and normotensive pregnancies. *Journal of Hypertension*, *27*(10), 2051–2054. https://doi.org/10.1097/HJH.0b013e328330052a

Pang, L., Wei, Z., Li, O., Huang, R., Qin, J., Chen, H., … Chen, Z.-J. (2013). An increase in vascular endothelial growth factor (VEGF) and VEGF soluble receptor-1 (sFlt-1) are associated with early recurrent spontaneous abortion. *PloS One*, *8*(9), e75759. https://doi.org/10.1371/journal.pone.0075759

Paredes, V., Espinoza-Caicedo, J. A., Salazar-Pousada, D., Escobar, G. S., Perez-

Lopez, F. R., & Chedraui, P. (2017). Lower placental growth factor and higher free beta-hCG and PAPP-A levels in the fetal circulation of near-term pregnancies complicated with severe preeclampsia. *Gynecological Endocrinology : The Official Journal of the International Society of Gynecological Endocrinology*, *33*(1), 79–81. https://doi.org/10.1080/09513590.2016.1241228

Pearson, J. D. (2000). Normal endothelial cell function. *Lupus*, *9*(3), 183–188. https://doi.org/10.1191/096120300678828299

Phipps, E. A., Thadhani, R., Benzing, T., & Karumanchi, S. A. (2019). Pre-eclampsia: pathogenesis, novel diagnostics and therapies. *Nature Reviews. Nephrology*, *15*(5), 275–289. https://doi.org/10.1038/s41581-019-0119-6

Porter, F. D. (2003). Human malformation syndromes due to inborn errors of cholesterol synthesis. *Current Opinion in Pediatrics*, *15*(6), 607–613. https://doi.org/10.1097/00008480-200312000-00011

Potente, M., Gerhardt, H., & Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell*, *146*(6), 873–887. https://doi.org/10.1016/j.cell.2011.08.039

Powers, R. W., Roberts, J. M., Cooper, K. M., Gallaher, M. J., Frank, M. P., Harger, G. F., & Ness, R. B. (2005). Maternal serum soluble fms-like tyrosine kinase 1 concentrations are not increased in early pregnancy and decrease more slowly postpartum in women who develop preeclampsia. *American Journal of Obstetrics and Gynecology*, *193*(1), 185–191. https://doi.org/10.1016/j.ajog.2004.11.038

Pugsley, M. K., & Tabrizchi, R. (2000). The vascular system. An overview of structure and function. *Journal of Pharmacological and Toxicological Methods*, *44*(2), 333–340. https://doi.org/10.1016/s1056-8719(00)00125-8

Quinton, A. E., Cook, C.-M., & Peek, M. J. (2007). A longitudinal study using ultrasound to assess flow-mediated dilatation in normal human pregnancy. *Hypertension in Pregnancy*, *26*(3), 273–281. https://doi.org/10.1080/10641950701366841

Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G., & Nishigaki, I. (2013). The vascular endothelium and human diseases. *International Journal of Biological Sciences*, *9*(10), 1057–1069. https://doi.org/10.7150/ijbs.7502

Rana, S., Powe, C. E., Salahuddin, S., Verlohren, S., Perschel, F. H., Levine, R. J., … Karumanchi, S. A. (2012). Angiogenic factors and the risk of adverse

outcomes in women with suspected preeclampsia. *Circulation*, *125*(7), 911–919. https://doi.org/10.1161/CIRCULATIONAHA.111.054361

Rana, S., Schnettler, W. T., Powe, C., Wenger, J., Salahuddin, S., Cerdeira, A. S., … Karumanchi, S. A. (2013). Clinical characterization and outcomes of preeclampsia with normal angiogenic profile. *Hypertension in Pregnancy*, *32*(2), 189–201. https://doi.org/10.3109/10641955.2013.784788

Redman, C. W. G., & Staff, A. C. (2015). Preeclampsia, biomarkers, syncytiotrophoblast stress, and placental capacity. *American Journal of Obstetrics and Gynecology*, *213*(4 Suppl), S9.e1, S9-11. https://doi.org/10.1016/j.ajog.2015.08.003

Reynolds, L. P., Borowicz, P. P., Caton, J. S., Vonnahme, K. A., Luther, J. S., Buchanan, D. S., … Redmer, D. A. (2010). Uteroplacental vascular development and placental function: an update. *The International Journal of Developmental Biology*, *54*(2–3), 355–366. https://doi.org/10.1387/ijdb.082799lr

Risau, W. (1997). Mechanisms of angiogenesis. *Nature*, *386*(6626), 671–674. https://doi.org/10.1038/386671a0

Robb, A. O., Mills, N. L., Newby, D. E., & Denison, F. C. (2007). Endothelial progenitor cells in pregnancy. *Reproduction*, *133*(1), 1–9. https://doi.org/10.1530/REP-06-0219

Roberge, S., Nicolaides, K. H., Demers, S., Villa, P., & Bujold, E. (2013). Prevention of perinatal death and adverse perinatal outcome using low-dose aspirin: a meta-analysis. *Ultrasound in Obstetrics & Gynecology : The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology*, *41*(5), 491–499. https://doi.org/10.1002/uog.12421

Roberts, J. M., & Bell, M. J. (2013). If we know so much about preeclampsia, why haven't we cured the disease? *Journal of Reproductive Immunology*, *99*(1–2), 1–9. https://doi.org/10.1016/j.jri.2013.05.003

Roberts, J. M., Taylor, R. N., Musci, T. J., Rodgers, G. M., Hubel, C. A., & McLaughlin, M. K. (1989). Preeclampsia: an endothelial cell disorder. *American Journal of Obstetrics and Gynecology*, *161*(5), 1200–1204. https://doi.org/10.1016/0002-9378(89)90665-0

Roggen, E. L. (2011). In vitro Toxicity Testing in the Twenty-First Century. *Frontiers in Pharmacology*, *2*, 3. https://doi.org/10.3389/fphar.2011.00003

Romero, R., Nien, J. K., Espinoza, J., Todem, D., Fu, W., Chung, H., … Karumanchi, S. A. (2008). A longitudinal study of angiogenic (placental growth factor) and anti-angiogenic (soluble endoglin and soluble vascular endothelial growth factor receptor-1) factors in normal pregnancy and patients destined to develop preeclampsia and deliver a small for gestational age neonate. *The Journal of Maternal-Fetal & Neonatal Medicine*, *21*(1), 9–23. https://doi.org/10.1080/14767050701830480

Rouwkema, J., Gibbs, S., Lutolf, M. P., Martin, I., Vunjak-Novakovic, G., & Malda, J. (2011). In vitro platforms for tissue engineering: implications for basic research and clinical translation. *Journal of Tissue Engineering and Regenerative Medicine*, *5*(8), e164-7. https://doi.org/10.1002/term.414

Rowan, J. A., Hague, W. M., Gao, W., Battin, M. R., & Moore, M. P. (2008). Metformin versus insulin for the treatment of gestational diabetes. *The New England Journal of Medicine*, *358*(19), 2003–2015. https://doi.org/10.1056/NEJMoa0707193

Russell, W. M. S. (1995). The development of the three Rs concept. *Alternatives to Laboratory Animals : ATLA*, *23*(3), 298–304.

Saad, A. F., Kechichian, T., Yin, H., Sbrana, E., Longo, M., Wen, M., … Costantine, M. M. (2014). Effects of pravastatin on angiogenic and placental hypoxic imbalance in a mouse model of preeclampsia. *Reproductive Sciences*, *21*(1), 138–145. https://doi.org/10.1177/1933719113492207

Salajegheh A. (2016). *Introduction to Angiogenesis in Normal Physiology, Disease and Malignancy. In: Angiogenesis in Health, Disease and Malignancy*. Springer, Cham. Retrieved from https://doi.org/10.1007/978-3-319-28140-7_1

Sattar, N., Ramsay, J., Crawford, L., Cheyne, H., & Greer, I. A. (2003). Classic and novel risk factor parameters in women with a history of preeclampsia. *Hypertension*, *42*(1), 39–42. https://doi.org/10.1161/01.HYP.0000074428.11168.EE

Scandinavian Simvastatin Survival Study Group. (1994). Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet*, *344*(8934), 1383–1389.

Schmidt, A., Brixius, K., & Bloch, W. (2007). Endothelial precursor cell migration during vasculogenesis. *Circulation Research*, *101*(2), 125–136. https://doi.org/10.1161/CIRCRESAHA.107.148932

Schmidt, M., Raghavan, B., Muller, V., Vogl, T., Fejer, G., Tchaptchet, S., … Goebeler, M. (2010). Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nature Immunology*, *11*(9), 814–819. https://doi.org/10.1038/ni.1919

Sena, C. M., Matafome, P., Louro, T., Nunes, E., Fernandes, R., & Seiça, R. M. (2011). Metformin restores endothelial function in aorta of diabetic rats. *British Journal of Pharmacology*, *163*(2), 424–437. https://doi.org/10.1111/j.1476-5381.2011.01230.x

Sezer, S. D., Kucuk, M., Yenisey, C., Yuksel, H., Odabasi, A. R., Turkmen, M. K., … Omurlu, I. K. (2012). Comparison of angiogenic and anti-angiogenic factors in maternal and umbilical cord blood in early- and late-onset pre-eclampsia. *Gynecological Endocrinology : The Official Journal of the International Society of Gynecological Endocrinology*, *28*(8), 628–632. https://doi.org/10.3109/09513590.2011.650759

Shore, V. H., Wang, T. H., Wang, C. L., Torry, R. J., Caudle, M. R., & Torry, D. S. (1997). Vascular endothelial growth factor, placenta growth factor and their receptors in isolated human trophoblast. *Placenta*, *18*(8), 657–665. https://doi.org/10.1016/s0143-4004(97)90007-2

Spradley, F. T., Tan, A. Y., Joo, W. S., Daniels, G., Kussie, P., Karumanchi, S. A., & Granger, J. P. (2016). Placental Growth Factor Administration Abolishes Placental Ischemia-Induced Hypertension. *Hypertension*, *67*(4), 740–747. https://doi.org/10.1161/HYPERTENSIONAHA.115.06783

Staff, A. C., Braekke, K., Harsem, N. K., Lyberg, T., & Holthe, M. R. (2005). Circulating concentrations of sFlt1 (soluble fms-like tyrosine kinase 1) in fetal and maternal serum during pre-eclampsia. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, *122*(1), 33–39. https://doi.org/10.1016/j.ejogrb.2004.11.015

Staff, A. C., Redman, C. W. G., Williams, D., Leeson, P., Moe, K., Thilaganathan, B., … Roberts, J. M. (2016). Pregnancy and Long-Term Maternal Cardiovascular Health: Progress Through Harmonization of Research Cohorts and Biobanks. *Hypertension*, *67*(2), 251–260. https://doi.org/10.1161/HYPERTENSIONAHA.115.06357

Staton, C. A., Reed, M. W. R., & Brown, N. J. (2009). A critical analysis of current in vitro and in vivo angiogenesis assays. *International Journal of Experimental Pathology*, *90*(3), 195–221. https://doi.org/10.1111/j.1365-2613.2008.00633.x

Staton, C. A., Stribbling, S. M., Tazzyman, S., Hughes, R., Brown, N. J., & Lewis, C. E. (2004). Current methods for assaying angiogenesis in vitro and in vivo. *International Journal of Experimental Pathology*, *85*(5), 233–248. https://doi.org/10.1111/j.0959-9673.2004.00396.x

Steegers, E. A. P., von Dadelszen, P., Duvekot, J. J., & Pijnenborg, R. (2010). Pre-eclampsia. *Lancet*, *376*(9741), 631–644. https://doi.org/10.1016/S0140-6736(10)60279-6

Stepan, H., Geide, A., & Faber, R. (2004, November). Soluble fms-like tyrosine kinase 1. *The New England Journal of Medicine*. United States. https://doi.org/10.1056/NEJM200411183512123

Stepan, H., Herraiz, I., Schlembach, D., Verlohren, S., Brennecke, S., Chantraine, F., … Galindo, A. (2015). Implementation of the sFlt-1/PlGF ratio for prediction and diagnosis of pre-eclampsia in singleton pregnancy: implications for clinical practice. *Ultrasound in Obstetrics & Gynecology : The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology*, *45*(3), 241–246. https://doi.org/10.1002/uog.14799

Stryker, Z. I., Rajabi, M., Davis, P. J., & Mousa, S. A. (2019). Evaluation of Angiogenesis Assays. *Biomedicines*, *7*(2). https://doi.org/10.3390/biomedicines7020037

Sunderland, N., Hennessy, A., & Makris, A. (2011). Animal models of pre-eclampsia. *American Journal of Reproductive Immunology*, *65*(6), 533–541. https://doi.org/10.1111/j.1600-0897.2010.00929.x

Syngelaki, A., Nicolaides, K. H., Balani, J., Hyer, S., Akolekar, R., Kotecha, R., … Shehata, H. (2016). Metformin versus Placebo in Obese Pregnant Women without Diabetes Mellitus. *The New England Journal of Medicine*, *374*(5), 434–443. https://doi.org/10.1056/NEJMoa1509819

Szekanecz, Z., & Koch, A. E. (2007). Mechanisms of Disease: angiogenesis in inflammatory diseases. *Nature Clinical Practice. Rheumatology*, *3*(11), 635–643. https://doi.org/10.1038/ncprheum0647

Tabit, C. E., Chung, W. B., Hamburg, N. M., & Vita, J. A. (2010). Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. *Reviews in Endocrine & Metabolic Disorders*, *11*(1), 61–74. https://doi.org/10.1007/s11154-010-9134-4

Taguchi, N., Rubin, E. T., Hosokawa, A., Choi, J., Ying, A. Y., Moretti, M. E., …

Ito, S. (2008). Prenatal exposure to HMG-CoA reductase inhibitors: effects on fetal and neonatal outcomes. *Reproductive Toxicology*, *26*(2), 175–177. https://doi.org/10.1016/j.reprotox.2008.06.009

Tan, X., Li, S., Chang, Y., Fang, C., Liu, H., Zhang, X., & Wang, Y. (2016). Effect of metformin treatment during pregnancy on women with PCOS: a systematic review and meta-analysis. *Clinical and Investigative Medicine. Medecine Clinique et Experimentale*, *39*(4), E120-31. https://doi.org/10.25011/cim.v39i4.27091

Tepper, O. M., Capla, J. M., Galiano, R. D., Ceradini, D. J., Callaghan, M. J., Kleinman, M. E., & Gurtner, G. C. (2005). Adult vasculogenesis occurs through in situ recruitment, proliferation, and tubulization of circulating bone marrow-derived cells. *Blood*, *105*(3), 1068–1077. https://doi.org/10.1182/blood-2004-03-1051

Thadhani, R., Hagmann, H., Schaarschmidt, W., Roth, B., Cingoez, T., Karumanchi, S. A., … Benzing, T. (2016). Removal of Soluble Fms-Like Tyrosine Kinase-1 by Dextran Sulfate Apheresis in Preeclampsia. *Journal of the American Society of Nephrology : JASN*, *27*(3), 903–913. https://doi.org/10.1681/ASN.2015020157

Thissier-Levy S, Boucoiran I, Luo ZC, Nuyt AM, Julien P, Fraser WD, Audibert F. Endostatin levels and the risk of subsequent preeclampsia. Eur J Obstet Gynecol Reprod Biol. 2013 Oct;170(2):396-400. doi: 10.1016/j.ejogrb.2013.07.039.

Toimela, T., Huttala, O., Sabell, E., Mannerstrom, M., Sarkanen, J. R., Ylikomi, T., & Heinonen, T. (2017). Intra-laboratory validated human cell-based in vitro vasculogenesis/angiogenesis test with serum-free medium. *Reproductive Toxicology*, *70*, 116–125. https://doi.org/10.1016/j.reprotox.2016.11.015

Torry, D. S., Leavenworth, J., Chang, M., Maheshwari, V., Groesch, K., Ball, E. R., & Torry, R. J. (2007). Angiogenesis in implantation. *Journal of Assisted Reproduction and Genetics*, *24*(7), 303–315. https://doi.org/10.1007/s10815-007-9152-7

Townsend, R., Khalil, A., Premakumar, Y., Allotey, J., Snell, K. I. E., Chan, C., … Thangaratinam, S. (2019). Prediction of pre-eclampsia: review of reviews. *Ultrasound in Obstetrics & Gynecology : The Official Journal of the International Society of Ultrasound in Obstetrics and Gynecology*, *54*(1), 16–27. https://doi.org/10.1002/uog.20117

Tranquilli, A. L., Dekker, G., Magee, L., Roberts, J., Sibai, B. M., Steyn, W., … Brown, M. A. (2014, April). The classification, diagnosis and management of the hypertensive disorders of pregnancy: A revised statement from the ISSHP. *Pregnancy Hypertension*. Netherlands. https://doi.org/10.1016/j.preghy.2014.02.001

Tsao, P.-N., Wei, S.-C., Su, Y.-N., Chou, H.-C., Chen, C.-Y., & Hsieh, W.-S. (2005). Excess soluble fms-like tyrosine kinase 1 and low platelet counts in premature neonates of preeclamptic mothers. *Pediatrics*, *116*(2), 468–472. https://doi.org/10.1542/peds.2004-2240

Ucuzian, A. A., Gassman, A. A., East, A. T., & Greisler, H. P. (2010). Molecular mediators of angiogenesis. *Journal of Burn Care & Research : Official Publication of the American Burn Association*, *31*(1), 158–175. https://doi.org/10.1097/BCR.0b013e3181c7ed82

Urbich, C., Dernbach, E., Zeiher, A. M., & Dimmeler, S. (2002). Double-edged role of statins in angiogenesis signaling. *Circulation Research*, *90*(6), 737–744.

van der Merwe, J. L., Hall, D. R., Wright, C., Schubert, P., & Grove, D. (2010). Are early and late preeclampsia distinct subclasses of the disease--what does the placenta reveal? *Hypertension in Pregnancy*, *29*(4), 457–467. https://doi.org/10.3109/10641950903572282

van Weelden, W., Wekker, V., de Wit, L., Limpens, J., Ijas, H., van Wassenaer-Leemhuis, A. G., … Painter, R. C. (2018). Long-Term Effects of Oral Antidiabetic Drugs During Pregnancy on Offspring: A Systematic Review and Meta-analysis of Follow-up Studies of RCTs. *Diabetes Therapy : Research, Treatment and Education of Diabetes and Related Disorders*, *9*(5), 1811–1829. https://doi.org/10.1007/s13300-018-0479-0

Vargesson, N. (2015). Thalidomide-induced teratogenesis: history and mechanisms. *Birth Defects Research. Part C, Embryo Today : Reviews*, *105*(2), 140–156. https://doi.org/10.1002/bdrc.21096

Venkatesha, S., Toporsian, M., Lam, C., Hanai, J., Mammoto, T., Kim, Y. M., … Karumanchi, S. A. (2006). Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nature Medicine*, *12*(6), 642–649. https://doi.org/10.1038/nm1429

Voller, S. B., Chock, S., Ernst, L. M., Su, E., Liu, X., Farrow, K. N., & Mestan, K. K. (2014). Cord blood biomarkers of vascular endothelial growth (VEGF and

sFlt-1) and postnatal growth: a preterm birth cohort study. *Early Human Development*, *90*(4), 195–200. https://doi.org/10.1016/j.earlhumdev.2014.01.003

von Dadelszen, P., Magee, L. A., & Roberts, J. M. (2003). Subclassification of preeclampsia. *Hypertension in Pregnancy*, *22*(2), 143–148. https://doi.org/10.1081/PRG-120021060

Vuorela, P., Carpén, O., Tulppala, M., & Halmesmäki, E. (2000). VEGF, its receptors and the tie receptors in recurrent miscarriage. *Molecular Human Reproduction*, *6*(3), 276–282. https://doi.org/10.1093/molehr/6.3.276

Vuorenpää, H., Ikonen, L., Kujala, K., Huttala, O., Sarkanen, J.-R., Ylikomi, T., … Heinonen, T. (2014). Novel in vitro cardiovascular constructs composed of vascular-like networks and cardiomyocytes. *In Vitro Cellular & Developmental Biology. Animal*, *50*(4), 275–286. https://doi.org/10.1007/s11626-013-9703-4

Wang, F., Cao, G., Yi, W., Li, L., & Cao, X. (2019). Effect of Metformin on a Preeclampsia-Like Mouse Model Induced by High-Fat Diet. *BioMed Research International*, *2019*, 6547019. https://doi.org/10.1155/2019/6547019

Weis, M., Heeschen, C., Glassford, A. J., & Cooke, J. P. (2002). Statins have biphasic effects on angiogenesis. *Circulation*, *105*(6), 739–745.

Weissgerber, T. L., Milic, N. M., Milin-Lazovic, J. S., & Garovic, V. D. (2016). Impaired Flow-Mediated Dilation Before, During, and After Preeclampsia: A Systematic Review and Meta-Analysis. *Hypertension*, *67*(2), 415–423. https://doi.org/10.1161/HYPERTENSIONAHA.115.06554

WHO. (n.d.). International Statistical Classification of Diseases and Related Health Problems. Retrieved from https://www.who.int/standards/classifications/classification-of-diseases

Wu, P., van den Berg, C., Alfirevic, Z., O'Brien, S., Röthlisberger, M., Baker, P. N., … Duvekot, J. J. (2015). Early Pregnancy Biomarkers in Pre-Eclampsia: A Systematic Review and Meta-Analysis. *International Journal of Molecular Sciences*, *16*(9), 23035–23056. https://doi.org/10.3390/ijms160923035

Wu, Q., Liu, J., Wang, X., Feng, L., Wu, J., Zhu, X., … Gong, X. (2020). Organ-on-a-chip: recent breakthroughs and future prospects. *Biomedical Engineering Online*, *19*(1), 9. https://doi.org/10.1186/s12938-020-0752-0

Yoo, S. Y., & Kwon, S. M. (2013). Angiogenesis and its therapeutic opportunities.

Mediators of Inflammation, *2013*, 127170. https://doi.org/10.1155/2013/127170

Zarek, J., DeGorter, M. K., Lubetsky, A., Kim, R. B., Laskin, C. A., Berger, H., & Koren, G. (2013). The transfer of pravastatin in the dually perfused human placenta. *Placenta*, *34*(8), 719–721. https://doi.org/10.1016/j.placenta.2013.05.002

9 ORIGINAL PUBLICATIONS

PUBLICATION I

Strong inhibitory effect of pre-eclampsia serum on angiogenesis detected in vitro by human cell-based angiogenesis tests

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Strong inhibitory effect of pre-eclampsia serum on angiogenesis detected *in vitro* by human cell-based angiogenesis tests

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ABSTRACT

Objective: To explore *in vitro* angiogenic properties of maternal and umbilical cord blood sera from women with symptomatic pre-eclampsia in comparison with sera from women with normotensive pregnancies.

Study design: Maternal and umbilical blood serum samples were collected from eleven primiparous women with pre-eclampsia and ten healthy gestational-age-matched primiparous controls. The samples were tested for tubule formation in two different types of *in vitro* angiogenesis tests. The first test (fibroblast-HUVEC) showed effects on angiogenesis and the second test (hASC-HUVEC), in addition to angiogenesis, also showed effects on vasculogenesis. The pro-angiogenic and inhibitory properties of the samples were microscopically quantified after immunostaining tubular structures, using markers for von Willebrand factor (vWf) and collagen IV.

Results: Serum samples from pre-eclamptic women inhibited tubule formation in both models, while those from normal pregnancy didn't. Umbilical blood samples were inhibitory both after pre-eclampsia and normal pregnancy. In the fibroblast-HUVEC model the inhibition was stronger after preeclampsia pregnancy, and the difference between groups was statistically significant. In the pre-eclampsia group a correlation between the inhibitory effect of umbilical blood and birth weight adjusted to gestational age was found. No clear correlation between sera from pregnant women and corresponding umbilical sera was found.

Conclusion: The strong inhibitory effect of maternal serum samples on tubule formation reflects the anti-angiogenic state that is present in pre-eclampsia.

1. Introduction

Although the exact pathogenesis of pre-eclampsia remains unclear, there is incremental data indicating that angiogenic factors play a major role in the development of this disease [1–6]. An imbalance between pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), and anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) has been detected in numerous studies [1–4,7]. Changes in the concentrations of circulating angiogenic and anti-angiogenic proteins may occur weeks before the clinical recognition of pre-eclampsia [8,9].

The angiogenic properties of umbilical blood after pre-eclamptic pregnancy have been studied to a lesser extent. It is known that sFlt-1 levels are high in umbilical blood after pre-eclampsia [5,10]. According to the results of recent studies there are also other factors that affect the vasculogenic properties of umbilical blood. In a study by Xia et al. (2007) venous cord blood samples were collected during labour from pre-eclamptic mothers and normotensive controls in order to investigate endothelial progenitor cells (EPCs). It was noticed that the level of EPCs was significantly lower after pre-eclamptic pregnancies when compared with normal ones. Endothelial progenitor cells mediate neovascularization in uterine

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endometrium and it has been hypothesized that they might be involved in neovascularization of the placental vasculature [9].

Vasculogenesis (formation of blood vessels from EPCs) and angiogenesis (formation of new blood vessels from existing ones) are crucial for placentation and embryonic development [11]. Later in human life angiogenesis is involved in physiological processes such as wound healing and the menstrual cycle as well as in pathological processes such as tumour development [12]. Angiogenesis is a multi-step process. The key stages of angiogenesis are endothelial cell proliferation, migration, differentiation and tubule formation. Each stage is regulated by different growth factors and inhibitors [13]. Evaluation of the factors that affect angiogenesis would optimally be studied *in vivo*, but animal models have several disadvantages such as variability and animal-specificity. However, a standardized *in vitro* angiogenesis assay is reliable for testing the modulators of angiogenesis [14]. The human primary cell-based *in vitro* assay, which was used in the present study, mimics the effects in humans well [14,15]. As yet only a few models of angiogenesis using cell-culture techniques are available to explore the effects of pre-eclampsia. Maynard et al. measured endothelial tubule formation in an *in vitro* model of angiogenesis using human umbilical vein endothelial cells (HUVECs) and proved that sera from women with pre-eclampsia inhibit tubule formation [16].

Our aim was to utilize two different *in vitro* angiogenesis models to study whether sera from women with pre-eclampsia exhibit different angiogenic effects compared with sera from healthy controls. Furthermore, we wanted to explore the angiogenic properties of umbilical blood sera from newborns after pre-eclamptic and normotensive pregnancies.

2. Material and methods

2.1. Ethics statement

This study was approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit number R11088). All patients gave written informed consent. The study conforms to the principles outlined in the Declaration of Helsinki. The use of human adipose stromal cells (hASCs) and human umbilical cord endothelial cells (HUVECs) was separately approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit numbers R03058 and R08028, respectively).

2.2. Study population

In this cross-sectional study, we collected maternal blood samples from eleven primiparous women with pre-eclampsia and ten primiparous controls. Corresponding umbilical cord blood samples were obtained from nine women in both groups. Each of the controls had the same gestational age as the women with pre-eclampsia at maternal blood sample collection. The inclusion criteria for healthy controls were: blood pressure <140/90 mmHg, urine dip stick test negative for proteinuria and previously uncomplicated singleton pregnancy. Hypertension later in pregnancy was an exclusion criterion.

The samples were collected during 2011–2014 at the Department of Obstetrics and Gynaecology, Tampere University Central Hospital. In the study group the samples were taken at a maximum of three days before delivery when the patients were already hospitalized because of clinical symptoms of pre-eclampsia. Cord blood samples were taken after delivery of the placenta in both groups. There was no separation between umbilical arterial and venous blood in the cord samples. The serum samples were frozen and conserved at −70 °C until assay. Blood tests for haemoglobin level, platelet count and alanine aminotransferase level were carried out at admission from the women with pre-eclampsia. Baseline demographic details and data on pregnancy outcome were collected from the hospital maternity records. Gestational age was calculated on the basis of the last menstruation and corrected if necessary at first-trimester screening ultrasonography.

Pre-eclampsia was defined according to the guidelines of the International Society for the Study of Hypertension in Pregnancy. Systolic blood pressure should be $\geqslant$140 mmHg or diastolic blood pressure $\geqslant$90 mmHg on at least two occasions 4 h apart after 20 weeks of gestation in previously normotensive women, with proteinuria of 300 mg or more in 24 h [17]. Pre-eclampsia was defined as severe if HELLP syndrome (haemolysis, elevated levels of liver enzymes and low platelet count), eclampsia or exceptionally high blood pressure (>160 mmHg systolic or >110 mmHg diastolic) appeared [18,19].

Deviation from normal growth (mean weight of newborns at the same gestational age) was determined for all newborns according to national weight curves [20]. Small-for gestational-age (SGA) was defined as birth weight more than two standard deviations below the mean.

Pre-eclampsia was divided into early- and late-onset depending on the gestational age when it was diagnosed. The cut-off point between these two groups was 34 weeks [21].

2.3. In vitro angiogenesis/vasculogenesis tests

The tests were performed at the Finnish Centre for Alternative Methods, School of Medicine, University of Tampere. Two different tests were utilized in the study:

1) The fibroblast-HUVEC test (angiogenesis test method), which involves co-culture of human BJ fibroblasts (20,000 cells/cm^2) and primary HUVECs (4000 cells/cm^2) and is used to show basic effects of angiogenesis [14].
2) The hASC-HUVEC test (angiogenesis/vasculogenesis test), based on co-culture of human adipose stem cells (hASCs, 20,000 cells/cm^2) and HUVECs (4000 cells/cm^2) was used to include vasculogenesis [15,22].

Human adipose tissue samples were obtained from waste material from surgical operations and human umbilical cords were received after Caesarean sections (with informed consent) at Tampere University Hospital, Tampere, Finland. The culture medium contained specific exogenous growth factors to induce formation of tubular structures and networks. Two different growth factor concentrations were used to induce either strong vascular formation (to reveal possible inhibitory effects of test samples on vascular formation) or to induce only moderate vascular formation, where stimulation of vascular formation may also take place. In inhibition set-ups the co-cultures were exposed to 1 ng fibroblast growth factor β (FGF-β)/ml and 10 ng VEGF/ml, whereas in stimulation tests the concentrations were 0.25 ng/ml and 2.5 ng/ml, respectively. Table 1 shows assay media components. Serum-free hASC assay medium was modified from our earlier-described medium [14].

During both *in vitro* tests, the co-cultures were exposed to patient serum samples at a dilution of 1:15 and cultured for a further six days (with one replenishment of the growth medium). After exposure, the number of living cells (viability) was evaluated. The purpose was to distinguish possible cytotoxicity (unspecific general toxicity causing cell death) from anti-angiogenetic effects induced by the samples, seen as a reduced amount of formed vascular structures. Viability/cytotoxicity of the samples was evaluated by using a WST-1 assay (Cell Proliferation Reagant, Roche, Basel, Switzerland) which measures mitochondrial activity present only in living cells. After WST-1 assay, the cells were fixed with 70% ethanol and immunostained for vWf (Sigma Aldrich, Manassas, VA, USA) and

Table 1
Assay media components.

Medium name	Content	Manufacturer
Fibroblast inhibition test medium	EBM-2 Basal medium	Lonza
	2% Foetal bovine serum	Gibco Invitrogen
	2 mM L-glutamine	Gibco Invitrogen
	10 ng VEGF/ml	R&D Systems
	1 ng FGF-β/ml	R&D Systems
Fibroblast stimulation test medium ("moderate vascular formation")	EBM-2 Basal medium	Lonza
	1% Foetal bovine serum	Gibco Invitrogen
	2 mM L-glutamine	Gibco Invitrogen
	2.5 ng VEGF/ml	R&D Systems
	0.25 ng FGF-β/ml	R&D Systems
hASC inhibition test medium	DMEM/F12	Gibco Invitrogen
	2.56 mM L-glutamine	Gibco Invitrogen
	0.1 nM 3,3′,5-triiodo-L-thyronine sodium salt	BD Biosciences
	ITS™ Premix:	BD Biosciences
	1.15 μM: 6.65 μg insulin/ml	
	6.65 μg transferrin/ml	
	6.65 ng selenious acid/ml	
	1% Bovine serum albumin	PAA
	2.8 mM Sodium pyruvate	Gibco Invitrogen
	200 μg Ascorbic acid/ml	Sigma Aldrich
	0.5 μg Heparin/ml	Stemcell Technologies
	2 μg Hydrocortisone/ml	Sigma Aldrich
	10 ng VEGF/ml	R&D Systems
	1 ng FGF-β/ml	R&D Systems

collagen IV (Sigma Aldrich). For visualization, an avidin-biotin system (Vectastain Elite ABC Kit, Vector laboratories Inc., Burlingame, CA, USA) was applied with DAB (Diaminobenzidine, Zymed Laboratories Inc., Invitrogen, Carlsbad, CA, USA) as a substrate in the fibroblast angiogenesis assay. The stained tubular structures were then evaluated (graded) microscopically. Automated analysis was applied in the vasculogenesis/angiogenesis test. For the analysis, fluorescent secondary antibodies against the primary antibodies, anti-rabbit TRITC (Tetramethylrhodamine isothiocyanante, Sigma Aldrich) for vWf and anti-mouse FITC (Fluorescein 5-Isothiocyanate, Sigma Aldrich) for collagen IV were applied for approx. 45 min for visualization of the tubules (resulting in fluorescent tubules suitable for image capture and analysis by using an automated image analysis platform – Cell-IQ, CM-Technologies, Tampere, Finland). On the grading scale, the intensity of the formed tubular network (tubule length and branching) was the scoring criterion. The angiogenic effect score was compared with the positive tubule formation control score (highest level of tubule formation induced with stimulatory factors) to obtain a percentage of control value for the tubule effect. This is depicted as mean% ± SD.

2.4. Statistical analysis

The results concerning angiogenesis are presented as mean values with SD. Angiogenesis assay results were analysed by independent-samples *t*-test, using GraphPad Prism v6.05 software. Differences were considered significant at $p < 0.05$.

The rest of the data are expressed as medians and range. Differences in continuous variables between groups were tested by using (non-parametric) Mann–Whitney *U*-tests. Pearson's correlation was used to calculate correlation coefficients. Probabilities of less than 0.05 were considered statistically significant. Statistical analyses were performed by using the Statistical Package for the Social Sciences (SPSS), version 11.0.

3. Results

Table 2 presents demographic data and clinical characteristics of the deliveries in the pre-eclamptic and control pregnancy groups. None of the women had essential hypertension, diabetes mellitus or a thrombophilic disorder. One woman in the control group smoked. Eight women had severe pre-eclampsia. Seven women had an early-onset pre-eclampsia, and of these, three delivered before 32 weeks of gestation. In the control group one woman delivered before 37 weeks of gestation. Two women had HELLP syndrome.

The median gestational age at delivery, birth weight and birth weight standard deviation were significantly lower in the pre-eclampsia group compared with the control group. Two women in the pre-eclampsia group delivered a small-for-gestational age infant and their birth-weight standard deviations were −2.6 and −3.1. Data concerning neonatal outcome is also described in Table 2.

To rule out possible basal cytotoxicity of the serum samples, the amount of living cells (viability) after exposure of the test system to serum samples was evaluated. None of the serum samples caused cell death (decreased viability) at the selected dilution of 1:15 (Fig. 1). On the contrary, the umbilical serum samples induced increased cell viability (i.e. stimulated proliferation of cells) compared with positive tubule formation controls and maternal serum in fibroblast angiogenesis tests ($p \leqslant 0.024$). In the hASC test system all samples showed cell viability slightly above the control level.

Table 2
Demographic characteristics, delivery details and neonatal outcome.

	PE (n = 11)	Control (n = 10)	P-value
Maternal age (years)	29.8 (20–44)	27 (23–34)	NS
BMI	21.5 (19.7–32.1)	24.1 (17.9–38.2)	NS
GA at delivery (weeks)	35.1 (28.1–39.6)	39.9 (35.4–42.3)	0.002
Mode of delivery			
Vaginal (%)	5 (45)	6 (60)	NS
Cesarean section (%)	6 (55)	4 (40)	NS
Birth weight (grams)	2171 (1020–3140)	3607 (2685–4535)	0.000
Birth weight SD	−2.2 (−0.4–(−1.9))	−0.2 (−1–1.5)	0.002
Apgar 1 min	9 (7–9)	9 (8–9)	NS
Apgar 5 min	8 (5–10)	9 (9)	NS

Values expressed as median (range) or n (%).
PE, pre-eclampsia; NS, non-significant; BMI, body mass index (kg/m^2); GA, gestational age.

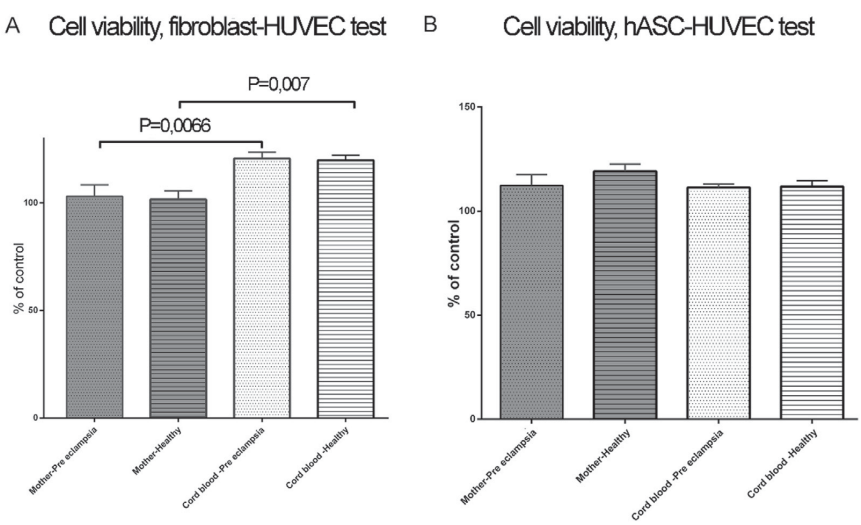

Fig. 1. The effect of serum samples on viability of cells in the pre-eclampsia and control groups as evaluated by using the mitochondrial viability test WST-1. A) In the fibroblast-HUVEC test, maternal samples did not change the viability of the cells. In both groups, cord blood serum samples induced a significant increase in cell viability/cell number when compared with serum samples from corresponding pregnant women. B) In the hASC-HUVEC test all samples showed cell viability slightly above the control level.

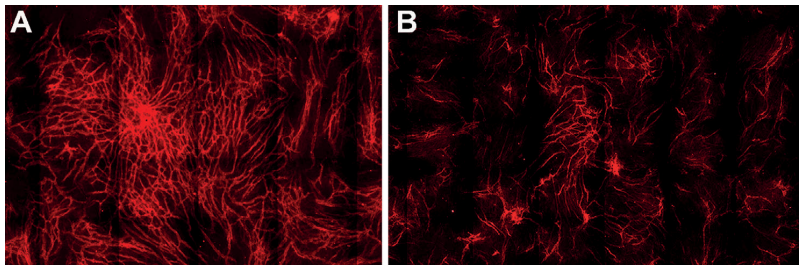

Fig. 2. Fluorescent images of the vascular structures. A) Image of vWf-TRITC-stained tubules exposed to healthy maternal serum. No inhibition is seen. B) Tubules exposed to serum from pre-eclamptic mothers. Tubular structures and branching are seen to a lesser extent, as a reflection of stronger inhibition of tubule formation.

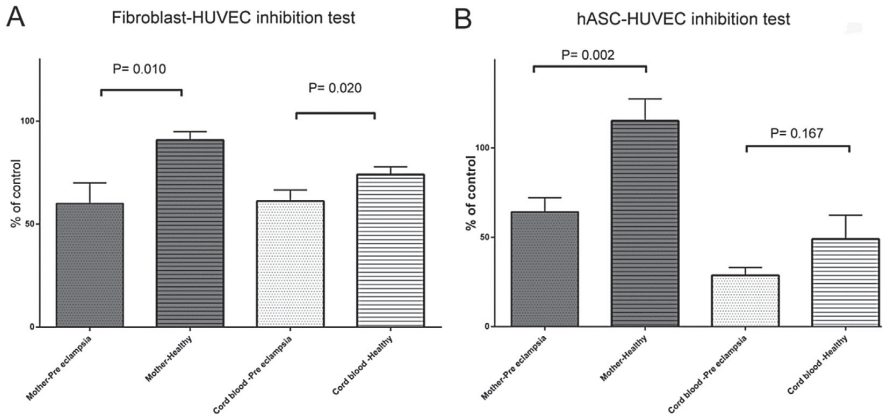

Fig. 3. Inhibition set-up of both angiogenesis tests. A, B) Significant inhibition was seen with maternal serum samples from pre-eclamptic patients when compared with healthy controls (and positive tubule formation control). B) In the vasculogenesis hASC test cord serum samples were inhibitory compared with positive controls whereas healthy maternal samples were not inhibitory as regards tubule formation.

There were no stimulatory effects as regards tubule formation with any of the samples (data not shown), but there was a strong inhibitory effect on tubule formation with sera from pre-eclamptic mothers (Figs. 2B and 3), while sera from healthy mothers showed no inhibition. Umbilical blood serum was inhibitory in both groups; a stronger inhibition was seen after preeclamptic than normal pregnancy. In fibroblast-HUVEC test the difference between the groups was significant ($p = 0.020$). There was no correlation between the inhibitory effects of maternal serum and the corresponding umbilical serum in either of the groups.

In the pre-eclampsia group no significant correlations were found between clinical features or laboratory findings *vs.* the inhibitory effects of the sera (Table 3). There was a significant correlation ($r = 0.75$) between birth weight SD and the inhibitory effect of umbilical blood in pre-eclampsia group (Fig. 4) ($p = 0.02$).

4. Discussion

Although there is ample documentation of an imbalance between various angiogenic and anti-angiogenic factors in pre-eclampsia, this has been the first study in which two advanced *in vitro* models to assess the functional effects of pre-eclamptic serum on vasculogenesis and angiogenesis have been utilized. In addition, for the first time, we also included corresponding umbilical blood serum samples in the tests.

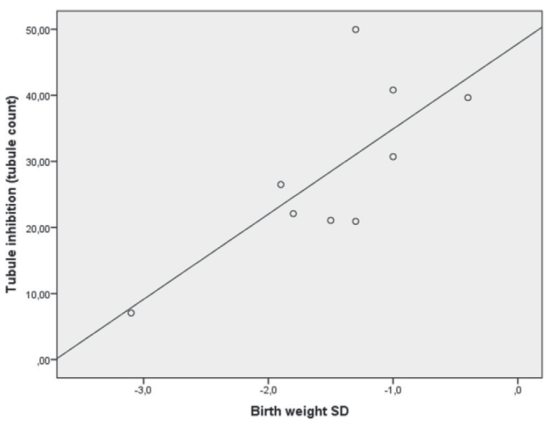

Fig. 4. Correlation between birth weight standard deviation and tubule inhibition in umbilical blood after pre-eclampsia pregnancy. The lower the tubule count, the stronger the inhibition. ($r = 0.75$, $p = 0.020$).

Both fibroblast-HUVEC and hASC-HUVEC models, forming tubular structures and branching mimicking angiogenesis *in vivo*, can be used to test the effects of various drugs and chemicals on angiogenesis, but adding patient serum to the models also enables evaluation of clinical conditions. In this study we noticed that suitable dilution of the serum sample induced no direct cytotoxicity and thus enabled the testing of inhibition and stimulation of angiogenesis in the model. Higher serum concentrations inhibited tubule formation completely; a serum concentration of 1:15 (6.7%) allowed tubule formation at a level at which differences between samples could be quantified.

Angiogenic capacity in pre-eclampsia and in normotensive pregnancy has been earlier investigated in a HUVEC-based *in vitro* model [16]. These authors reported that sera from four patients with pre-eclampsia inhibited and sera from four patients with normotensive pregnancies induced tubule formation. The results of our study, with more advanced vasculogenesis and angiogenesis co-culture models support this finding.

Our main finding was that pre-eclamptic maternal serum showed strong inhibitory effects on *in vitro* vasculogenesis and angiogenesis. It is probable that the sample concentrations of VEGF and FGF-β were not the main causative reason for the angiogenic inhibition seen. Serum concentrations of these factors are normally at the level of picograms/ml, whereas in our test systems the growth factor concentrations were at the level of nanograms/ml. This suggests that the effects we found were independent of minor differences in the amounts of these growth factors and would actually be due to other factors resulting in powerful vasculogenesis inhibition. One plausible cause could be over-expression of sFlt-1 (VEGFR-1), which antagonizes VEGF. This is in line with the results of previous studies where high levels of sFlt-1 and low levels of free VEGF and PlGF in women with pre-eclampsia have been demonstrated [2,4,5]. In a study by [23] Tsatsaris et al. (2003) sFlt-1 was significantly up-regulated in placentas from pre-eclamptic pregnancies and plasma levels of sFlt-1 were significantly higher in cases of pre-eclampsia compared with women with normal pregnancies. Furthermore, plasma concentrations of sFlt-1 have been shown to be higher in cases of severe *vs.* mild pre-eclampsia and high concentrations of sFlt-1 are also associated with early onset of the disease [24].

In our study, although there was a strong inhibitory effect of pre-eclamptic sera on vasculogenesis and angiogenesis, we found

Table 3
Tubule inhibition caused by maternal serum and umbilical blood in fibroblast-HUVEC test and clinical characteristics of the pre-eclampsia group.

GA at delivery	Prot g/24 h	BP	Tubule inhibition maternal serum	Tubule inhibition umbilical blood	Characteristics
35 + 2	6.15	167/102	Strong	Strong	Low platelet count, severe preeclampsia symptoms
39 + 4	2.63	177/96	Low	Moderate	Low platelet count
37 + 2	1.27	165/95	Strong	Moderate	
28 + 1	7.14	167/100	Moderate	Low	Low platelet count, severe preeclampsia symptoms
31 + 1	2.68	154/90	Low		IUGR, block in the umbilical artery
34	1.6	178/108	Moderate	Moderate	HELLP, severe preeclampsia symptoms
37 + 4	4.2	155/103	Strong	Moderate	
35 + 6	2.61	147/88	Low	Moderate	
30 + 6	8.58	166/113	Strong		HELLP, severe preeclampsia symptoms
38	9.35	171/110	Low	Moderate	
38 + 2	6.07	141/92	Low	Moderate	IUGR, low platelet count

GA, gestational age; Prot, proteinuria; BP, blood pressure; IUGR, intrauterine growth restriction.

no correlation between maternal clinical characteristics, onset or severity of the disease and the strength of the inhibitory effects of the sera. One reason could be the small sample size and the relatively heterogeneous group of pre-eclamptic patients, but the observation may imply that pre-eclampsia represents an anti-angiogenic state, independently of the severity of the disease. Serum sFlt-1 concentrations have been observed to be high in pregnancies with foetal growth restriction but to a lesser extent than in pre-eclampsia [25]. It is not known if foetal growth restriction in pre-eclampsia changes the angiogenic balance toward a more anti-angiogenic state [26]. In our study there were two SGA foetuses in the pre-eclampsia group and there was not a stronger inhibitory effect on tubule formation compared with pregnancies where foetal growth was normal. On the other hand, there was a positive correlation between birth weight SD and inhibitory effect of the umbilical blood after pre-eclampsia pregnancy.

The inhibition was always stronger in the pre-eclampsia group than in the healthy control group. The effect was even more pronounced when the vasculogenesis test (utilizing hASC cells) was employed. The exact mechanism is not known, but it may be due to the involvement of endothelial progenitor cells (EPCs) which are a heterogeneous group of circulating cells, derived from the bone marrow as well as the vascular wall, that are thought to play a role in endothelial homeostasis and vascular remodeling [27]. It has been found that EPCs are reduced in maternal serum and in umbilical blood from pregnant women with pre-eclampsia [27,28]. The hASC vasculogenesis test showed a remarkable difference between umbilical serum and respective maternal serum, which was not so evident in the angiogenesis set-up. In a study by Staff et al. (2005) sFlt-1 concentrations were significantly higher in maternal serum, both in cases of pre-eclampsia and in a healthy group, compared with corresponding umbilical serum. Therefore, the strong inhibition of tubule formation caused by umbilical serum, seen in this study, may not be explained by sFlt-1 concentrations alone. In a study by Luppi et al. reduced endothelial progenitor cell count observed in pre-eclampsia pregnancies did not correlate with s-flt-1 or free PlGF values. In previous study significantly elevated sFlt-1 levels have been detected in umbilical cord blood after pre-eclamptic pregnancies compared with healthy ones [5]. It has been found that in newborns sFlt-1 levels remain much higher after pre-eclamptic pregnancies compared with uncomplicated ones days after delivery [27]. A positive association between foetal and maternal serum levels of sFlt-1 in pre-eclamptic pregnancy has been shown [5,28], and lower serum VEGF levels have been found in cord blood in cases of pre-eclampsia [16,29,30]. The results of our study suggest that at birth, cord blood is highly anti-angiogenic in newborns born to mothers with normotensive pregnancies also.

There was no stimulation of tubule formation induced by any of the test samples, with or without added VEGF and FGF-β. This suggests that the levels of these growth factors were not sufficiently high in the serum samples to induce angiogenesis.

This study has its strengths and limitations. A Pubmed search did not reveal any other study involving comparison of maternal serum and corresponding umbilical serum from patients with pre-eclampsia and women with uncomplicated pregnancies and concerning an *in vitro* model of angiogenesis. Recently, most studies concerning pre-eclampsia have been focused on measurement of specific circulating angiogenic or anti-angiogenic factors. In this study we wanted to approach the angiogenic balance via a functional test to measure the overall angiogenic capacity present in pre-eclamptic and uncomplicated pregnancies. The sample size was relatively small, but when compared with a previous *in vitro* model study [16], it was reasonably large. However, because of small sample size it was difficult to assess potentially important interactions in this study. Another limitation is that the pre-eclampsia group was not homogeneous as regards severity of the disease.

In conclusion, our findings demonstrate that there is imbalance toward an anti-angiogenic state in uncomplicated normotensive pregnancies as well in pregnancies complicated by pre-eclampsia. The anti-angiogenic state was also noticed in umbilical blood. The anti-angiogenic property was always stronger in the pre-eclampsia group. The strength of the current study is that we have evaluated the overall angiogenic capacity of sera from maternal blood samples and from corresponding umbilical blood samples from patients with pre-eclampsia and healthy controls. Recently, most studies have been focused on single biomarkers.

References

[1] T. Chaiworapongsa, P. Chaemsaithong, L. Yeo, R. Romero, et al., Pre-eclampsia part 1: current understanding of its pathophysiology, Nat. Rev. Nephrol. 10 (8) (2014) 466–480.
[2] R. Levine, S. Maynard, C. Qian, K.-H. Lim, L. England, Yu Kai, E. Schisterman, R. Thadhani, B. Sachs, F. Epstein, B. Sibai, V. Sukhatme, S. Karumanchi, Circulating angiogenic factors and the risk of preeclampsia, N. Engl. J. Med. 350 (7) (2004) 672–683.
[3] S. Maynard, A. Karumanchi, Angiogenic factors and preeclampsia, Semin. Nephrol. 31 (1) (2011) 33–46.
[4] A. Pratt, Costa.F. Da Silva, A. Borg, B. Kalionis, R. Keogh, P. Murthi, Placenta-derived angiogenic proteins and their contribution to the pathogenesis of preeclampsia, Angiogenesis 18 (2) (2015) 115–123.
[5] A.C. Staff, K. Braekke, N. Kittelsen, T. Lyberg, M. Holthe, Circulating concentrations of sFlt1 (soluble fms-like tyrosine kinase 1) in fetal and maternal serum during pre-eclampsia, Eur. J. Obstet. Gynecol. Reprod. Biol. 122 (1) (2005) 33–39.
[6] E. Llurba, F. Crispi, S. Verlohren, Update on the pathophysiological implications and clinical role of angiogenic factors in pregnancy, Fetal Diagn. Ther. 37 (2) (2015) 81–92.
[7] A. Vest, L. Cho, Hypertension in pregnancy, Curr. Atheroscler. Rep. 16 (3) (2014) 395.
[8] T. Chaiworapongsa, R. Romero, Y.M. Kim, G.J. Kim, M.R. Kim, J. Espinoza, et al., Plasma soluble vascular endothelial growth factor receptor-1 concentration is elevated prior to the clinical diagnosis of preeclampsia, J. Matern. Fetal Neonatal Med. 17 (1) (2005) 3–18.
[9] B.M. Polliotti, A.G. Fry, D.N. Saller, R.A. Mooney, C. Cox, R.K. Miller, Second-trimester maternal serum placental growth factor and vascular endothelial growth factor for predicting severe, early-onset preeclampsia, Obstet. Gynecol. 101 (6) (2003) 1266–1274.
[10] L. Xia, X.P. Zhou, J.H. Zhu, X.D. Xie, H. Zhang, X.X. Wang, et al., Decrease and dysfunction of endothelial progenitor cells in umbilical cord blood with maternal pre-eclampsia, J. Obstet. Gynaecol. Res. 33 (4) (2007) 465–474.
[11] P. Kaufmann, T.M. Mayhew, D.S. Charnock-Jones, Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy, Placenta 25 (2–3) (2004) 114–126.
[12] A. D'Alessio, F. Moccia, J.H. Li, A. Micera, T. Kyriakides, Angiogenesis and vasculogenesis in health and disease, Biomed. Res. Int. 2015 (2015) 126582.
[13] D.S. Charnock-Jones, P. Kaufmann, T.M. Mayhew, Aspects of human fetoplacental vasculogenesis and angiogenesis. I. Molecular regulation, Placenta 25 (2–3) (2004) 103–113.
[14] J.R. Sarkanen, M. Mannerström, H. Vuorenpää, J. Uotila, T. Ylikomi, T. Heinonen, Intra-laboratory pre-validation of a human cell based in vitro angiogenesis assay for testing angiogenesis modulators, Front. Pharmacol. 20 (1) (2011) 147.
[15] O. Huttala, H. Vuorenpaa, T. Toimela, J. Uotila, H. Kuokkanen, T. Ylikomi, et al., Human vascular model with defined stimulation medium – a characterization study, ALTEX 32 (2) (2015) 125–136.
[16] S. Maynard, J.-Y. Min, J. Merchan, K.-H. Lim, J. Li, S. Mondal, et al., Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia, J. Clin. Invest. 111 (5) (2003) 649–658.
[17] M.A. Brown, M.D. Lindheimer, M. de Swiet, A. Van Assche, J.M. Moutquin, The classification and diagnosis of the hypertension disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP), Hypertens Pregnancy 20 (1) (2001) IX–XIV.
[18] B. Sibai, G. Dekker, M. Kupferminc, Pre-eclampsia, Lancet 365 (9461) (2005) 785–799.
[19] J.A. Turner, Diagnosis and management of pre-eclampsia: an update, Int. J. Womens Health 30 (2) (2010) 327–337.
[20] U. Sankilampi, M.-L. Hannila, A. Saari, M. Gissler, L. Dunkel, New population-based references for birth weight, length, and head circumference in singletons and twins from 23 to 43 gestation weeks, Ann. Med. 45 (5–6) (2013) 446–454.
[21] A. Tranquilli, M. Brown, G. Zeeman, G. Dekker, B. Sibai, The definition of severe and early-onset preeclampsia. Statement from the international society for the

study of hypertension in pregnancy (ISSHP), Pregnancy Hypertens 3 (1) (2013) 44–47.
[22] J.R. Sarkanen, H. Vuorenpää, O. Huttala, B. Mannerström, H. Kuokkanen, S. Miettinen, et al., Adipose stromal cell tubule network model provides a versatile tool for vascular research and tissue engineering, Cells Tissues Organs 196 (5) (2012) 385–397.
[23] V. Tsatsaris, F. Goffin, C. Munaut, J.F. Brichant, M.R. Pignon, A. Noel, et al., Overexpression of the soluble vascular endothelial growth factor receptor in preeclamptic patients: pathophysiological consequences, J. Clin. Endocrinol. Metab. 88 (11) (2003) 5555–5563.
[24] T. Chaiworapongsa, R. Romero, J. Espinoza, E. Bujold, K.Y. Mee, L.F. Goncalves, et al., Evidence supporting a role for blockade of the vascular endothelial growth factor system in the pathophysiology of preeclampsia. Young Investigator Award, Am. J. Obstet. Gynecol. 190 (6) (2004) 1541–1547.
[25] D. Borras, A. Perales-Puchalt, N. Ruiz Sacedon, A. Perales, Angiogenic growth factors in maternal and fetal serum in pregnancies complicated with intrauterine growth restriction, J. Obstet. Gynaecol. 34 (3) (2014) 218–220.
[26] N. Uras, S.S. Oguz, S. Zergeroglu, A. Akdag, B. Polat, E.A. Dizdar, et al., CD31 and Factor VIII in angiogenesis of normal and pre-eclamptic human placentas, J. Obstet. Gynaecol. 32 (6) (2012) 533–536.
[27] P. Luppi, R. Powers, V. Verma, L. Edmunds, D. Plymire, C. Hubel, Maternal Circulating CD34+, VEGFR-2+ and CD133+, VEGFR-2+ progenitor cells increase during normal pregnancy but are reduced in women with preeclampsia, Reprod. Sci. 17 (7) (2010) 643–652.
[28] C.R. Hentges, R.C. Silveira, R.S. Procianoy, Angiogenic and antiangiogenic factors in preterm neonates born to mothers with and without preeclampsia, Am. J. Perinatol. 32 (12) (2015) 1185–1190.
[29] W. Wallner, R. Sengenberger, R. Strick, P.L. Strissel, M.W. Beckmann, D. Schlembach, Angiogenic growth factors in maternal and fetal serum in pregnancies complicated by intrauterine growth restriction, Clin. Sci. 112 (1) (2007) 51–57.
[30] C. Catarino, I. Rebelo, L. Belo, S. Rocha, E.B. Castro, B. Patrício, et al., Fetal and maternal angiogenic/anti-angiogenic factors in normal and preeclamptic pregnancy, Growth Factors 27 (6) (2009) 345–351.

PUBLICATION II

Angiogenic capacity in pre-eclampsia and uncomplicated pregnancy estimated by assay of angiogenic proteins and in vitro vasculogenesis/ angiogenesis test

Virtanen A., Huttala O., Tihtonen K., Toimela T., Heinonen T., Uotila J.

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ORIGINAL PAPER

Angiogenic capacity in pre-eclampsia and uncomplicated pregnancy estimated by assay of angiogenic proteins and an in vitro vasculogenesis/angiogenesis test

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Abstract

Objective The purpose of the study was to determine the angiogenic capacity of sera in early and late pregnancy and in umbilical blood serum after childbirth, and to define how angiogenic properties assessed in a functional in vitro test are related to individual angiogenic proteins in six women with pre-eclampsia and in six healthy pregnant controls.

Methods Maternal first and third trimester serum samples, and umbilical blood samples after childbirth, were tested in an in vitro human adipose stromal cell—human umbilical vein endothelial cell (hASC-HUVEC) vasculogenesis/angiogenesis assay. The angiogenic properties of the samples were measured by quantifying tubule formation. Concentrations of total placental growth factor (PlGF), total vascular endothelial growth factor (VEGF), soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) were determined by immunoassay.

Results First-trimester maternal sera of both groups had a stimulatory effect on angiogenesis in vitro and levels of angiogenic proteins did not differ between the groups. Third-trimester maternal sera in the pre-eclampsia group had an inhibitory effect on tubule formation, while those from normal pregnancies remained stimulatory. Compared with the first trimester there was a significant change in the concentrations of angiogenic proteins toward an anti-angiogenic state in pre-eclampsia. Umbilical blood serum exhibited strong anti-angiogenic effects without a significant difference between groups.

Conclusions Third-trimester serum of pre-eclamptic patients is anti-angiogenic. This phenomenon is not yet present in the first trimester. Umbilical blood serum shows inhibitory effects on angiogenesis after normal as well as pre-eclamptic pregnancy.

Keywords In vitro vasculogenesis/angiogenesis · Angiogenic proteins · Inhibition of angiogenesis · Pre-eclampsia · VEGF · sFlt-1 · PlGF · sEng

Introduction

There have been several studies on pro- and anti-angiogenic factors and their differences in normal pregnancies and in women with pre-eclampsia [1, 2]. The most studied proteins are pro-angiogenic factors such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) and anti-angiogenic factors such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) [1, 3]. Even in normal pregnancies changes in favour of anti-angiogenic proteins in the third trimester have been observed, but in pre-eclampsia the imbalance between pro- and anti-angiogenic proteins is stronger and a change in concentrations of these proteins toward an anti-angiogenic state has been seen to exist several weeks before the onset of clinical disease [4, 5]. There are fewer studies on angiogenesis in connection with umbilical blood after pre-eclamptic pregnancy. Pre-eclampsia seems to have an effect on the umbilical cord itself and on cord blood, as structural changes have been found in endothelia of umbilical vessels and increased concentrations of sFlt-1 in umbilical blood after childbirth [6, 7].

The net angiogenic capacity of serum can be measured in in vitro tests. Human primary cell-based in vitro assays mimic the effects in humans reliably [8, 9]. In in vitro

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assays, the endothelial cells form capillary-like structures (tubule formation) in response to angiogenic signals from added maternal serum [10]. In contrast to quantitative tests involving measurement of concentrations of single specific pro- and anti-angiogenic factors, in vitro angiogenesis tests reveal functional capabilities of tested samples to promote, maintain or inhibit tubule formation [8, 10]. In addition, they provide quantitative information on the extent of tubule formation [8, 11]. The in vitro vasculogenesis/angiogenesis human adipose stromal cell—human umbilical cord vein endothelial cell (hASC-HUVEC) test offers a possibility to study the effects of serum on angiogenesis (formation of new blood vessels from existing ones) and vasculogenesis (de novo blood vessel generation from vascular progenitor cells) [8, 11].

In the present study, we wanted to assess inter-group differences and longitudinal changes in angiogenic capacity of maternal serum during pregnancy in healthy women and in women with pre-eclampsia, utilising the hASC-HUVEC test. Another aim was to investigate how the angiogenic capacity of maternal serum is associated with known pro- and anti-angiogenic factors. Furthermore, we wanted to evaluate the angiogenic capacity of umbilical blood samples after pre-eclampsia and healthy pregnancy.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit number R11088). Written informed consent was obtained from the women agreeing to participate in the study. The use of human adipose stromal cells (hASCs) and human umbilical cord vein endothelial cells (HUVECs) was separately approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit numbers R03058 and R08028, respectively).

Study population

This was a cross-sectional research project where the study population consisted of six primiparous women with pre-eclampsia and six controls. All women gave two blood samples during pregnancy and an umbilical blood sample after childbirth in 2011–2014. The women were recruited to the study during the third trimester of pregnancy. The inclusion criteria for healthy controls were blood pressure < 140/90 mmHg, urine dip stick test negative for proteinuria and previously uncomplicated singleton pregnancy. Hypertension later in pregnancy was an exclusion criterion. Early-gestation serum samples from both groups were obtained from the National Institute for Health and Welfare, where maternal sera from first-trimester screening was stored. The samples had been taken between the ninth and eleventh weeks of gestation. Gestational age was calculated on the basis of the last menstruation and corrected if necessary at first-trimester screening ultrasonography.

The definition of pre-eclampsia was that of the International Society for the Study of Hypertension in Pregnancy (ISSHP) in 2000. Systolic blood pressure should be ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on at least two occasions 4 h apart after 20 weeks of gestation in previously normotensive women, with proteinuria of 300 mg or more in 24 h [12]. Pre-eclampsia was defined as severe if HELLP syndrome (haemolysis, elevated levels of liver enzymes and low platelet count), eclampsia or exceptionally high blood pressure (> 160 mmHg systolic or > 110 mmHg diastolic) appeared [13, 14]. In the study group, maternal blood samples were taken at a maximum of 3 days before delivery when the patient was already hospitalised because of clinical symptoms of pre-eclampsia. Cord blood samples were taken after delivery of the placenta in both groups. There was no separation between umbilical arterial and venous blood in the cord samples. The serum samples were frozen and conserved at − 70 °C until assay. Blood tests for haemoglobin level, platelet count and alanine aminotransferase level were carried out at admission for the women with pre-eclampsia. Baseline demographic details and data on pregnancy outcome were collected from the hospital maternity records.

Deviation from normal growth (mean weight of newborns at the same gestational age) was determined for all newborns according to national weight curves [15]. Small-for gestational-age (SGA) was defined as birth weight more than two standard deviations below the mean.

Immunoassays

The concentrations of sFlt-1, PlGF, VEGF and sEng were determined in all samples by using ProcartaPlex assays (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, samples were centrifuged at 1000×*g* for 10 min before use in the assay. The samples were not diluted. Antigen standards and magnetic beads were prepared according to the manufacturer's instructions. Samples and standards were then added to the beads and incubated with shaking for 2 h at room temperature. The detection antibodies were added and incubated for 30 min at room temperature. To stain the proteins, streptavidin-PE was added and incubated for 30 min at room temperature, after which the beads were analysed in reading buffer. The results were analysed with Bio-Plex 200 (Bio-Rad, California, USA) and Bio-Plex Manager™ 6.0 software (Bio-Rad). Concentrations were obtained in pg/ml. Standard concentrations for VEGF

were 5.74–23,500 pg/ml, for sFlt-1 49–200,000 pg/ml, for sEng 0.61–2500 pg/ml and for PlGF 1.66–6800 pg/ml. If the result obtained was lower or higher than the range of the standards, a fixed concentration above or below the standards was used in analysis of the results. The concentration used for sEng was 2550 pg/ml when the result was over the standard range, and for PlGF 1 pg/ml when the result was lower than the lowest standard. The result of the VEGF and PlGF assays can include also the bound version of these growth factors and hence the concentration result should be considered as total concentration.

Cells

Human adipose tissue samples were obtained from waste material from surgical operations, and human umbilical cords from uncomplicated pregnancies were received after Caesarean sections. Isolation of HUVECs from umbilical cord veins was performed as described earlier (Sarkanen et al. [16]) using 0.05% collagenase I (Gibco, Thermo Fisher scientific, Waltham, USA) inserted into the umbilical vein. Isolation of hASCs was performed as described earlier (Sarkanen et al. [16]) using 0.15% Collagenase type I (Gibco). HUVECs were cultured in EGM-2 medium (Lonza, Basel, Switzerland) and hASCs in hASC medium (Table 1). Cells were negative for mycoplasma contamination, tested by using MycoAlert™ kits (Lonza).

Table 1 Media used in the in vitro test

Medium name	Content	Manufacturer
hASC medium	DMEM/F12	Gibco
	10% human serum	PAA laboratories
	2 mM L-glutamine	Gibco
Vasculogenesis/angiogenesis test medium	DMEM/F12	Gibco Invitrogen
	2.56 mM L-glutamine	Gibco Invitrogen
	0.1 nM 3,3′,5-triiodo-L-thyronine sodium salt	BD Biosciences
	ITS™ Premix	BD Biosciences
	6.65 µg insulin/ml	
	6.65 µg transferrin/ml	
	6.65 ng selenious acid/ml	
	1% bovine serum albumin	PAA
	2.8 mM sodium pyruvate	Gibco Invitrogen
	200 µg/ml ascorbic acid	Sigma Aldrich
	0.5 µg/ml heparin	Stemcell Technologies
	2 µg/ml hydrocortisone	Sigma Aldrich
	2.5 ng/ml VEGF	R&D Systems
	0.25 ng/ml FGF-β	R&D Systems

In vitro vasculogenesis/angiogenesis test

To study the angiogenic potency, i.e. bioactive growth factor content, of the serum samples, all three sample groups (first trimester, third trimester and umbilical blood serum samples) were studied by using the in vitro vasculogenesis/angiogenesis test, with a co-culture of hASCs (20,000 cells/cm^2) and HUVECs (4000 cells/cm^2). The test was performed as described earlier (Virtanen et al. [10]). Briefly, vasculogenesis/angiogenesis was induced using vasculogenesis/angiogenesis test medium (Table 1) and the co-cultures were exposed to patient serum samples at a dilution of 1:15 and cultured for 6 days in total with one replenishment of the growth medium. After exposure, the number of living cells was evaluated by using a WST-1 Cell Proliferation Reagent (Roche, Basel, Switzerland). Following the WST-1, the cells were fixed with ethanol and immunostained for vWf (Sigma Aldrich) to detect the endothelial cells and collagen IV (Sigma Aldrich) to detect the basement membrane of the tubules. For visualisation of the tubules, fluorescent secondary antibodies against the primary antibodies were applied: anti-rabbit tetramethylrhodamine isothiocyanate (TRITC, Sigma Aldrich) for vWf and anti-mouse fluorescein 5-isothiocyanate (FITC, Sigma Aldrich) for collagen IV. The resulting fluorescent tubules were imaged and analysed using an automated image analysis platform (Cell-IQ, CM-Technologies, Tampere, Finland). Tubule formation was quantified on the basis of the intensity of the tubular network formed (tubule length and branching). The obtained result was compared with the positive tubule formation control (highest level of tubule formation induced with stimulatory factors). Tubule formation was defined as stimulatory when there was more tubule formation after exposure of the serum sample compared with the positive tubule formation control and inhibitory when there was less tubule formation after exposure to serum. Values were expressed as percentage of positive tubule formation.

Statistical analysis

The data are expressed as medians and range. Differences in continuous variables between groups were tested by using Mann–Whitney *U* tests and differences within the study group by using Wilcoxon's test. Spearman's correlation method was used to calculate correlation coefficients. Probabilities of less than 0.05 were considered statistically significant. Statistical analyses were performed by using the Statistical Package for the Social Sciences (IBM-SPSS), version 11.0.

Results

The characteristics of the study population are summarised in Table 2. There were two preterm births in the pre-eclampsia group, but none of the women had early-onset pre-eclampsia. Most women with pre-eclampsia had severe disease.

In the first trimester, maternal sera were stimulatory, and tubule formation was equally high in the in vitro test in both groups (Fig. 2). Neither were there differences between groups in concentrations of the individual pro- and anti-angiogenic proteins or the sFlt-1:PlGF ratio (Table 3). In both groups, the median concentrations of PlGF were higher in maternal sera in the first trimester when compared with serum samples taken in the third trimester ($p = 0.043$) or umbilical blood serum ($p = 0.028$) (Table 3).

In contrast to the first trimester, sera from women with pre-eclampsia in the third trimester exhibited an inhibitory effect on tubule formation. In comparison with healthy women, tubule formation was significantly lower in the pre-eclampsia group ($p = 0.026$) (Figs. 2, 3). Compared with the first trimester, there was remarkably lower ($p = 0.043$) tubule formation in the third trimester in women with pre-eclampsia, whereas in the healthy control group there was no change in the capacity of tubule formation (Figs. 2, 3). In the third trimester, sFlt-1 levels were higher in the pre-eclampsia group than in the healthy control group ($p = 0.004$) (Table 3) and compared with the first trimester, sFlt-1 levels had increased ($p = 0.028$) and total PlGF concentrations had decreased ($p = 0.043$) in women with pre-eclampsia. In addition, the sFlt-1:PlGF and sFlt-1/VEGF ratios increased between the first and third trimester in the pre-eclampsia group (Fig. 1). In normal pregnancies, sFlt-1 concentrations and the sFlt-1/VEGF ratio were similar in the first and third trimesters.

Umbilical blood serum had an equally strong inhibitory effect on tubule formation in both groups. The inhibitory effect seen in the in vitro test was stronger in umbilical serum than in third-trimester maternal serum when the whole study population (i.e. including controls) was analysed as one group ($p = 0.005$). The sFlt-1/VEGF ratio was also significantly higher in umbilical than in maternal sera ($p = 0.015$). When the data were studied in separate groups, only in healthy women was the inhibitory effect on tubule formation stronger in umbilical than in maternal serum ($p = 0.028$). There were no differences between the groups in the concentrations of individual pro- and anti-angiogenic proteins, or in the sFlt-1:VEGF ratio in cord sera. In women with pre-eclampsia, sFlt-1 concentrations were significantly lower ($p = 0.028$) in umbilical serum than in third-trimester maternal serum.

In the first trimester, total amount of pro-angiogenic proteins (VEGF and PlGF) did not show any correlation with the stimulatory effect seen in the in vitro test. Neither did any pro- or anti-angiogenic protein or tubule formation measure in the first trimester have a correlation with baseline demographic characteristics, severity of pre-eclampsia, gestational weeks at birth or weight of the newborn.

In the third trimester, when the whole study population was analysed as one group, there were significant negative correlations between tubule formation and sFlt-1 ($r = -0.902$, $p < 0.001$), sEng ($r = -0.595$, $p = 0.041$) and the sFlt-1/VEGF ratio ($r = -0.748$, $p = 0.005$). Furthermore, positive correlations between the concentration of sFlt-1 and both systolic ($r = 0.741$, $p = 0.006$) and diastolic blood pressure ($r = 0.849$, $p < 0.001$) were found. A correlation between a greater inhibitory effect on tubule formation and a lower birth weight SD score reached statistical

Table 2 Maternal characteristics and neonatal outcome

	PE group ($n = 6$)	Controls ($n = 6$)	p value
Maternal age, years[a]	29.0 (25–33)	25 (24–34)	0.240
BMI, kg/m^2[a]	21.3 (19.7–22.3)	26.4 (17.9–38.2)	0.065
Highest systolic BP, mmHg[a]	160 (141–171)	130 (103–138)	0.009**
Highest diastolic BP, mmHg[a]	96 (88–110)	73 (54–88)	0.002**
Severe pre-eclampsia (%)	5/6 (83.3)		
Gestational age, weeks[a]	37.5 (35.3–38.3)	40.5 (35.4–41.6)	0.065
Mode of delivery			
Vaginal/CS, *n* (%)	4/2 (66.7/33.3)	3/3 (50/50)	
Birth weight, grams[a]	2620 (2040–3140)	3278 (2685–4535)	0.026*
Birth weight, SD score[a]	−1.2 [−3.1 to (−0.4)]	−0.5 (−1.7 to 1.5)	0.093
Umbilical arterial pH[a]	7.23 (7.13–7.36)	7.33 (7.30–7.41)	0.052

PE pre-eclampsia, *BMI* body mass index, *BP* blood pressure, *CS* Caesarean section, *SD* standard deviation

*$p < 0.05$; **$p < 0.01$, Mann–Whitney *U*-test

[a]Data are given as median (range)

Table 3 Concentrations of angiogenic proteins in maternal serum and in umbilical blood serum in the pre-eclampsia and control groups

	First trimester			Third trimester			Umbilical cord		
	PE	CONTR	*p* value	PE	CONTR	*p* value	PE	CONTR	*p* value
VEGF (pg/ml)	2572 (1980–25,889)	2961 (1875–14,003)	0.94	2196 (1417–9281)	1818 (856–17321)	0.24	6374 (5185–8619)	3893 (2238–16,486)	0.18
PlGF (pg/ml)	144 (1–14155)	120 (1–1514)	0.70	23 (1–3535)	1 (1–1216)	0.24	28 (1–923)	1.6 (1–1450)	0.59
sFlt-1 (pg/ml)	3780 (2092–5878)	5914 (2489–6851)	0.18	11,278 (4768–13,899)	1480 (277–6339)	0.004**	1684 (397–6845)	1485 (445–7113)	0.94
sEng (pg/ml)	2550[b]	2550[b]	1.00	2550 (1009–2550)	1071 (595–2550)	0.093	1885 (846–2550)	1159 (701–2550)	0.70
sFlt-1/PlGF	35 (0.2–2092)	44 (4–6538)	0.59	614 (1.4–13064)	734 (2–6339)	0.94	52 (3–6845)	382 (1.7–7113)	0.39
sFlt-1/VEGF	1.3 (0.1–2.8)	1.6 (0.5–2.8)	0.70	5.6 (0.5–8.5)	0.5 (0.1–5.0)	0.041*	0.3 (0.1–1.3)	0.3 (0.1–2.1)	0.70
Tubule formation (%)[a]	152 (126–158)	135 (70–153)	0.18	79 (22–115)	140 (72–186)	0.026*	26 (7–41)	50 (23–146)	0.13

Data are given as median (range)

The concentrations of VEGF and PlGF should be considered as total concentration

PE pre-eclampsia, *CONTR* control, *VEGF* vascular endothelial growth factor, *PlGF* placental growth factor, *sFlt*-1 soluble fms-like tyrosine kinase-1, *sEng* soluble endoglin

[a]Tubule intensity compared with the positive tubule control

[b]All values were above the range of the standards

*$p < 0.05$; **$p < 0.01$, Mann–Whitney *U*-test

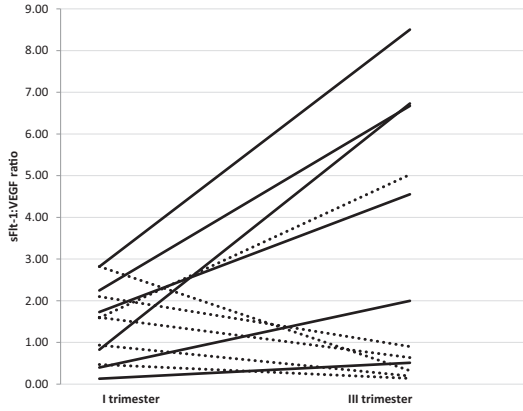

Fig. 1 The sFlt-1:VEGF ratio in maternal sera in the first and third trimesters. The ratio was significantly higher in the pre-eclampsia group in the third trimester when compared with the healthy control group ($p = 0.041$). There was also a significant increase in the sFlt-1:VEGF ratio between the first and third trimester in the pre-eclampsia group ($p = 0.028$). Solid lines = pre-eclampsia, dotted lines = healthy

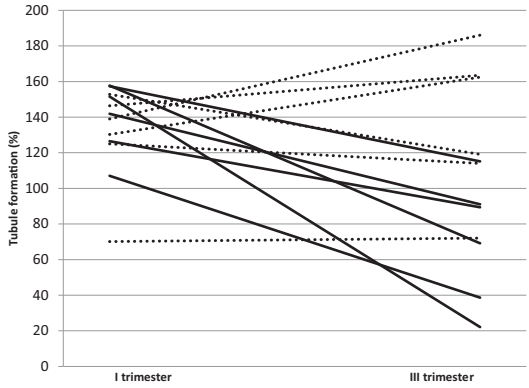

Fig. 2 Tubule formation (%) in the first and third trimester of pregnancy. The decrease in tubule formation was significant between the first and third trimester in women with pre-eclampsia ($p = 0.043$). Solid lines = pre-eclampsia, dotted lines = healthy

significance ($r = -0.754$, $p = 0.050$) in the pre-eclampsia group.

In umbilical blood serum samples, when the whole study population was analysed as one group, there were no correlations between the concentrations of pro- and anti-angiogenic proteins versus tubule formation, but there were correlations between a strong inhibitory effect in in vitro tests and low birth weight ($r = 0.790$, $p = 0.002$), low birth weight SD score ($r = 0.582$, $p = 0.047$) and earlier gestational weeks at birth ($r = 0.622$, $p = 0.031$). When

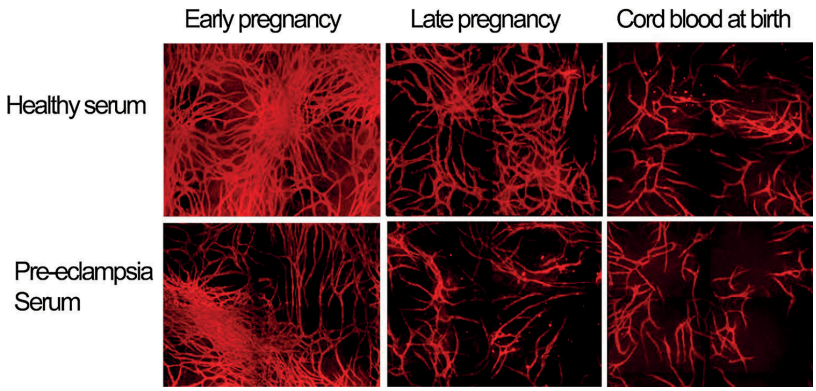

Fig. 3 Fluorescence images of vWf-TRITC-stained tubules exposed to healthy and pre-eclamptic maternal serum and cord blood serum. Top row: healthy serum in early pregnancy, late pregnancy and cord blood. Bottom row: pre-eclamptic serum collected early in pregnancy, late pregnancy and from cord blood of the infant. Both early pregnancy samples induced strong tubule formation, in places forming dense tubule clumps in which the tubules are not distinguishable enough to be analysed. Late pregnancy samples show that healthy serum induced more tubule formation. The two cord blood groups did not show a significant difference

the same correlations were studied in the two separate groups, the correlations with low birth weight ($r = 0.829$, $p = 0.042$) and low birth weight SD score ($r = 0.841$, $p = 0.036$) remained significant in the pre-eclampsia group.

Discussion

In the present study, we demonstrated that assay of pro- and anti-angiogenic proteins is not the only method to measure angiogenic capacity of maternal or umbilical blood serum, but that advanced in vitro models provide an alternative method to study angiogenesis. We longitudinally measured concentrations of pro- and anti-angiogenic proteins by immunoassay and at the same time evaluated functional angiogenic capacity in in vitro tests in women with pre-eclampsia and in their controls with uncomplicated pregnancies. We assessed the overall angiogenic profile not only in women with symptomatic pre-eclampsia but also in the first trimester and after childbirth.

Angiogenesis and vasculogenesis are complex processes which are regulated not only by pro- and anti-angiogenic proteins but by other factors in addition [17, 18]. Immunoassays give us quantitative information on specific proteins in maternal sera, but in order to assess overall angiogenic capacity, we used an additional functional approach to angiogenesis. The human cell-based in vitro vasculogenesis/angiogenesis test can be described as a combination of a functional and a quantitative test. In the in vitro assay, the endothelial cells form capillary-like structures in response to angiogenic signals from the added maternal serum and the effect of maternal serum on tubule formation can be stimulatory, neutral or inhibitory. The strength of an effect can be measured as tubule count. What we have gathered from the literature is that there is only one earlier study concerning an in vitro model of angiogenesis and pro- and anti-angiogenic proteins. In the study, in vitro tubule formation and sFlt-1 concentrations in maternal sera were assessed in women with pre-eclampsia [19]. In the present study, we used an in vitro model that in addition to giving a measure of the effects on angiogenesis, also shows effects on vasculogenesis.

The pro-angiogenic factors VEGF and PlGF are known to play important roles in placental development [18]. Angiogenic proteins have been widely studied in the first trimester in order to identify women destined to develop pre-eclampsia later in pregnancy, but levels of single angiogenic proteins have been observed to be of poor predictive value [2, 20, 21]. Our results are in concordance with these findings, since in early gestation we found no significant differences in functional test results or in the concentrations of pro- and anti-angiogenic proteins between the study groups. In our in vitro model of angiogenesis, an equally strong stimulatory effect on tubule formation was seen in both groups in the first trimester, which reflects the pro-angiogenic state in early weeks of pregnancy. Serum levels of PlGF were higher in the first trimester than later in pregnancy in both groups, but there was wide variation in the concentrations and we found no significant correlation between stimulatory effects on tubule formation and levels of pro-angiogenic proteins. There was no inhibition of tubule formation in the first trimester even though the levels of sFlt-1 were already relatively high. The results of earlier studies concerning sFlt-1 concentrations in the first trimester are variable, but there

are more consistent findings that concentrations of sFlt-1 in women who subsequently develop pre-eclampsia are not significantly different from those in unaffected controls [22]. With a larger study population the results could be different, and it is also known that there are factors other than those we have measured involved in angiogenesis during the first trimester of pregnancy [18]. Concentrations of soluble endoglin were at the same level in both groups in the first trimester, as has been published earlier [23]. In summary, results concerning the first trimester in the present study indicate that the strong stimulatory effect seen in the in vitro test may better reflect the pro-angiogenic state in early gestation than levels of individual angiogenic proteins.

We have earlier shown that sera in women with symptomatic pre-eclampsia are strongly inhibitory in our in vitro test [10]. Our finding supported the results of the study by Maynard et al., where sera from women with pre-eclampsia inhibited and sera from women with normotensive pregnancies induced tubule formation in a HUVEC-based in vitro model [19]. In the present study, the decrease in tubule formation was remarkable between the first and third trimester in women with pre-eclampsia. Simultaneously there were significant changes in concentrations of sFlt-1 and PlGF toward a clearly anti-angiogenic balance. However, despite prevailing anti-angiogenesis, levels of individual pro- and anti-angiogenic proteins showed no correlation with tubule formation in the pre-eclampsia group. This could indicate that despite a steep increase in sFlt-1 concentrations there are also other factors related to anti-angiogenesis in pre-eclampsia. In the present work, the small study population and wide variation in the concentrations of pro- and anti-angiogenic proteins limit the finding of true associations. As in previous studies, in our study also sFlt-1 and sEng levels were higher in the pre-eclampsia group than in the healthy control group [2, 4]. Decreases in PlGF concentrations and increases in sFlt-1:PlGF ratios were significant only in the pre-eclampsia group.

The angiogenic properties of umbilical cord blood after pre-eclampsia and uncomplicated pregnancy have not been studied to the same extent as in maternal sera, and to our knowledge there are no other studies concerning the angiogenic quality of umbilical blood together with immunoassay measurements and in vitro test data. After childbirth, umbilical blood serum tended to be anti-angiogenic in the in vitro test, and it was even stronger that in maternal serum. This was surprising since there were no significant differences in the concentrations of pro- and anti-angiogenic proteins (excluding higher levels of maternal sFlt-1 in the pre-eclampsia group) between mother and foetus. The result might be affected by the structural variety of umbilical vessels when compared with vessels of the same calibre in the mother. Umbilical vessels are thought to be more susceptible to changes in haemodynamic conditions and that might have an influence on the consistency of umbilical blood [7, 24]. The results of studies on pro-angiogenic proteins in umbilical blood after pre-eclampsia and unaffected pregnancies are inconsistent [6, 25]. In a recent study, it was observed that median PlGF levels were lower in umbilical blood after pre-eclamptic pregnancy when compared with gestational age-matched controls [26], whereas Staff et al. reported the same finding as ourselves, that in the majority of samples levels of PlGF were below the lowest concentration standard of the immunoassay kit [6]. In the study by Sezer et al., it was found that there were no differences in sFlt-1 levels between a pre-eclampsia group and a control group as regards umbilical blood [26], as we reported, but there are also studies indicating that sFlt-1 concentrations are higher in umbilical blood after pre-eclampsia than after unaffected pregnancies [6]. In the present study, concentrations of pro- and anti-angiogenic proteins in umbilical blood sera were similar to maternal levels, with the exception of lower sFlt-1 levels after pre-eclamptic pregnancy. A correlation between high sFlt-1 concentrations and low-level tubule formation was found only in the healthy control group, as was also the case in maternal samples in the third trimester. There are probably fewer confounding factors that have an influence on angiogenesis in healthy pregnant women than in those with pre-eclampsia. In umbilical samples, there was also a correlation between a pronounced inhibitory effect in the in vitro test and low birth weight in the pre-eclampsia group, presumably reflecting the predominance of anti-angiogenesis present in foetal growth in pregnancies complicated by hypertensive disorders.

Strengths and limitations

To our knowledge, this is first study carried out to investigate an association between the results of an in vitro angiogenesis test and quantitative measurements of pro- and anti-angiogenic proteins at three different stages of pregnancy. As the proteins concerned have been extensively studied recently, we intended to achieve a new approach to the study of angiogenic changes during pregnancy in women with pre-eclampsia as well in healthy pregnant women, by using two distinct methods. In particular, the results of an advanced in vitro vasculogenesis/angiogenesis test give us new information about the overall balance of angiogenesis in complicated and healthy pregnancies. The study was carried out with a small number of cases, and therefore some true associations may not have reached statistical significance. Another limitation of this study was that some protein concentrations were below or above the standards in the immunoassay kits, which creates difficulties in comparing the study groups. The high level of sFlt-1 present in the studied serum samples may cross-react or interfere in the immunoassay for VEGF and PlGF.

In conclusion, in the first trimester, maternal sera had a stimulatory effect on tubule formation reflecting a pro-angiogenic state in early pregnancy. In the third trimester, maternal sera from women with pre-eclampsia exhibited inhibitory properties on angiogenesis, and simultaneously there was change toward a more anti-angiogenic state as regards individual proteins. The angiogenic balance in umbilical blood sera did not differ between the study groups.

References

1. Maynard S, Karumanchi A (2011) Angiogenic factors and preeclampsia. Semin Nephrol 31:33–46
2. McElrath T, Lim KH, Pare E, Rich-Edwards J, Pucci D, Troisi R, Parry S (2012) Longitudinal evaluation of predictive value for preeclampsia of circulating angiogenic factors through pregnancy. Am J Obstet Gynecol 207:407.e1–407.e7
3. Rana S, Karumanchi SA, Lindheimer MD (2014) Angiogenic factors in diagnosis, management, and research in preeclampsia. Hypertension 63:198–202
4. Levine R, Maynard S, Qian C, Lim K-H, England L, Yu KF, Schisterman EF, Thadhani R, Sachs B, Epstein F, Sibai B, Sukhatme V, Karumanchi S (2004) Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 350:672–683
5. Schaarschmidt W, Rana S (2013) The course of angiogenic factors in early- vs. late-onset preeclampsia and HELLP syndrome. J Perinat Med 41:511–516
6. Staff AC, Braekke K, Kittelsen N, Lyberg T, Holthe M (2005) Circulating concentrations of sFlt1 (soluble fms-like tyrosine kinase 1) in fetal and maternal serum during pre-eclampsia. Eur J Obstet Gynecol Reprod Biol 122:33–39
7. Blanco MV, Vega HR, Giuliano R, Grana DR, Azzato F, Lerman J, Milei J (2011) Histomorphometry of umbilical cord blood vessels in preeclampsia. J Clin Hypertens 13:30–34
8. Toimela T, Huttala O, Sabell E, Mannerström M, Sarkanen JR, Ylikomi T, Heinonen T (2017) Intra-laboratory validated human cell-based in vitro vasculogenesis/angiogenesis test with serum-free medium. Reprod Toxicol 70:116–125
9. Sarkanen JR, Mannerström M, Vuorenpää H, Uotila J, Ylikomi T, Heinonen T (2011) Intra-laboratory pre-validation of a human cell based in vitro angiogenesis assay for testing angiogenesis modulators. Front Pharmacol 1:147
10. Virtanen A, Toimela T, Tihtonen K, Sarkanen JR, Huttala O, Heinonen T, Uotila J (2016) Strong inhibitory effect of pre-eclampsia serum on angiogenesis detected in vitro by human cell-based angiogenesis tests. Pregnancy Hypertens 6:367–373
11. Huttala O, Vuorenpaa H, Toimela T, Uotila J, Kuokkanen H, Ylikomi T, Sarkanen JR, Heinonen T (2015) Human vascular model with defined stimulation medium—a characterization study. ALTEX 32:125–136
12. Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM (2001) The classification and diagnosis of the hypertension disorders of pregnancy: statement from the International Society for the Study of Hypertension in Preganancy (ISSHP). Hypertens Pregnancy 20:IX–XIV
13. Sibai B, Dekker G, Kupferminc M (2005) Pre-eclampsia. Lancet 365:785–799
14. Turner J (2010) Diagnosis and management of pre-eclampsia: an update. Int J Womens Health 2:327–337
15. Sankilampi U, Hannila M-L, Saari A, Gissler M, Dunkel L (2013) New population-based references for birth weight, length, and head circumference in singletons and twins from 23 to 43 gestation weeks. Ann Med 45:446–454
16. Sarkanen JR, Vuorenpää H, Huttala O, Mannerström B, Kuokkanen H, Miettinen S, Heinonen T, Ylikomi T (2012) Adipose stromal cell tubule network model provides a versatile tool for vascular research and tissue engineering. Cells Tissues Organs 196:385–397
17. Chaiworapongsa T, Chaemsaithong P, Yeo L, Romero R (2014) Pre-eclampsia part 1: current understanding of its pathophysiology. Nat Rev Nephrol 10:466–480
18. Kaufmann P, Mayhew TM, Charnock-Jones DS (2004) Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy. Placenta 25:114–126
19. Maynard S, Min J, Merchan J, Lim K, Li J, Mondal S, Libermann T, Morgan J, Sellke F, Stillman I, Epstein F, Sukhatme V, Karumanchi S (2003) Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 111:649–658
20. Kleinrouweler CE, Wiegerinck MM, Ris-Stalpers C, Bossuyt PM, van der Post JA, von Dadelszen P, Mol BW, Pajkrt E, EBM CONNECT Collaboration (2012) Accuracy of circulating placental growth factor, vascular endothelial growth factor, soluble fms-like tyrosine kinase 1 and soluble endoglin in the prediction of pre-eclampsia: a systematic review and meta-analysis. BJOG 119:778–787
21. Kusanovic J, Romero R, Chaiworapongsa T, Erez O, Mittal P, Vaisbuch E, Mazaki-Tovi S, Gotsch F, Edwin S, Gomez R, Yeo L, Conde-Agudelo A, Hassan S (2009) A prospective cohort study of the value of maternal plasma concentrations of angiogenic and anti-angiogenic factors in early pregnancy and midtrimester in the identification of patients destined to develop preeclampsia. J Matern Fetal Neonatal Med 22:1021–1038
22. Akolekar R, de Cruz J, Foidart JM, Munaut C, Nicolaides KH (2010) Maternal plasma soluble fms-like tyrosine kinase-1 and free vascular endothelial growth factor at 11 to 13 weeks of gestation in preeclampsia. Prenat Diagn 30:191–197
23. Erez O, Romero R, Espinoza J, Fu W, Todem D, Kusanovic JP, Gotsch F, Edwin S, Nien JK, Chaiworapongsa T, Mittal P, Mazaki-Tovi S, Than NG, Gomez R, Hassan SS (2008) The change in concentrations of angiogenic and anti-angiogenic factors in maternal plasma between the first and second trimesters in risk assessment for the subsequent development of preeclampsia and small-for-gestational age. J Matern Fetal Neonatal Med 21:279–287
24. Spurway J, Logan P, Pak S (2012) The development, structure and blood flow within the umbilical cord with particular reference to the venous system. Australas J Ultrasound Med 15:97–102
25. Sezer SD, Küçük M, Yenisey C, Yüksel H, Odabaşı AR, Türkmen MK, Çetinkaya Çakmak B, Ömürlü IK (2012) Comparison of angiogenic and anti-angiogenic factors in maternal and umbilical cord blood in early- and late-onset pre-eclampsia. Gynecol Endocrinol 28:628–632
26. Paredes V, Espinoza-Caicedo J, Salazar-Pousada D, Escobar G, Perez-Lopez F, Chedraui P (2017) Lower placental growth factor and higher free β-hCG and PAPP-A levels in the circulation of near-term pregnancies complicated with severe preeclampsia. J Gynecol Endocrinol 33:79–81

PUBLICATION III

Therapeutic doses of metformin do not have impact on angiogenesis in presence of sera from pre-eclamptic, IUGR and healthy pregnancies

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Therapeutic doses of metformin do not have impact on angiogenesis in presence of sera from pre-eclamptic, IUGR and healthy pregnancies

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ABSTRACT

Recent evidence suggests that metformin may prevent pre-eclampsia by reverting the angiogenic imbalance in maternal sera. In this study, we investigated effect of metformin on angiogenesis by quantifying tubule formation in a human-based *in vitro* test with co-culture of human adipose stromal cell (hASC) and human umbilical vein endothelial cell (HUVEC). A total of 20 pregnant women were recruited in the study. Serum samples were obtained from women with early- and late-onset pre-eclampsia and from women with pregnancies complicated by intrauterine growth restriction (IUGR) without pre-eclampsia (N = 5 in each of the three groups). Serum samples from women with healthy pregnancies served as controls (N = 5). The direct effect of metformin on angiogenesis was first assessed without maternal sera. Secondly, we investigated the impact of metformin on angiogenesis in the present of maternal sera. Metformin was used at 5, 50 and 600 μg/ml concentrations. Angiogenic and inflammatory biomarkers in maternal sera were analyzed by immunoassays. When the direct effect of metformin was studied, the two lowest concentrations of metformin did not affect tubule formation (angiogenesis), but the highest concentration inhibited angiogenesis. When metformin was supplemented at therapeutic concentrations of 5 and 50 μg/ml along with serum samples, there was no change in tubule formation in comparison to maternal sera alone. However, strong inhibitory effect on tubule formation was observed in all groups with the highest, non-therapeutic (600 μg/ml), concentration of metformin.

1. Introduction

Metformin has beneficial effects in various diseases apart from its original use in type 2 diabetes [1]. Its impact on angiogenesis has been investigated extensively in oncological studies since the observation of reduced risk and improved survival of cancers in patients using metformin [2]. Suggested anti-cancer potential of metformin has been linked to anti-angiogenic activity of the drug as it has been observed to inhibit the formation of capillary-like networks by endothelial cells. Paradoxically, metformin has also been observed to up-regulate vascular endothelial growth factor A (VEGF-A) expression, enhancing angiogenesis [2]. The association between pre-eclampsia and disturbed angiogenesis is widely accepted [3,4]. The focus of pre-eclampsia studies has shifted towards research concerning pathophysiological changes that improve endothelial dysfunction and restore angiogenic balance [5,6]. Recently, metformin has been proposed as an alternate drug for the treatment of hypertensive pregnancy disorders [7]. The incidence of gestational hypertension has been shown to be lower in women with polycystic ovary syndrome using metformin throughout pregnancy [8]. Interestingly, metformin has been shown to improve endothelial dysfunction in an *in vitro* human umbilical vein endothelial cell (HUVEC) test and to induce angiogenesis in omental vessels obtained from women with pre-eclampsia [6]. Nevertheless, the association between metformin use and a lower risk of pre-eclampsia is still unclear [9,10].

The aim of our study was to elucidate the direct effect of metformin on angiogenesis, and to evaluate the impact of maternal sera from healthy and complicated pregnancies along with metformin on angiogenesis by using an *in vitro* hASC-HUVEC vasculogenesis/angiogenesis assay.

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2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit R16184). The isolation and use of hASC and HUVEC was approved (permits R15161 and R15033, respectively). Written informed consent was obtained from all participants.

2.2. Study population

In this *in vitro* trial we studied serum samples from women with early- and late-onset pre-eclampsia and from women with pregnancies complicated by intrauterine growth restriction (IUGR) without pre-eclampsia (N = 5 in each of the three groups). Serum samples from women with healthy pregnancies served as controls (N = 5). Recruitment was conducted prospectively at the Department of Obstetrics and Gynaecology, Tampere University Hospital, from April 2017 until March 2018.

Blood samples were obtained from patients at the time of admission to the hospital. The serum samples were stored at −70 °C until assay. Baseline demographic details and data on pregnancy outcomes were collected from the medical records. Gestational age was calculated according to last menstrual period, or on the basis of first-trimester ultrasonographic screening results.

Pre-eclampsia was diagnosed as hypertension and proteinuria occurring after 20 weeks of gestation. Hypertension was diagnosed as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and proteinuria as urinary excretion of ≥300 mg protein in a 24-hour specimen. Pre-eclampsia was defined as early-onset when diagnosed before 34 0/7 weeks of gestation and late-onset when diagnosed at 34 0/7 weeks of gestation or later. IUGR was defined as a fetal abdominal circumference below the 10th percentile or estimated fetal weight below the 10th percentile in ultrasonographic examination.

2.3. Immunoassays

The serum samples were analyzed for the concentrations of several angiogenic and inflammatory key proteins. The concentrations of heme oxygenase 1 (HO-1), endothelin-1 (ET-1) and angiopoietin 2 (Ang2) were determined by using ELISA kits. HO-1 (sensitivity 0.78 ng/ml) and ET-1 (sensitivity 0.41 pg/ml) assays (ADI-EKS-800 and ADI-900-020A, Enzo life sciences, Farmingdale, NY, USA) and Ang2 assays (sensitivity 10 pg/ml; ELH-angiopoietin 2, Raybiotech, Norcross, GA, USA) were performed according to the manufactureŕs instructions as follows: In the HO-1 and Ang2 ELISAs standards and samples were incubated at room temperature for 30 min and 2.5 h, respectively, and in the ET-1 ELISA they were incubated overnight at +4 °C followed by removal of the liquids from the plates. The antibody was added and incubation carried out for 1 h for HO-1 and Ang2 and for 30 min for ET-1, at RT. HRP conjugate was added to the HO-1 and Ang2 plates (ET-1 antibody was readily conjugated) and incubation carried out for 30 min and 45 min, respectively, at room temperature. Next, TMB substrate was added and incubation carried out with slow shaking in the dark at room temperature for 15–30 min, followed by addition of stop solution. Measurements were performed at 450 nm with a Varioskan Flash Multimode Reader (Thermo Fischer Scientific, Waltham, MA, USA).

The concentrations of tumor necrosis factor α (TNF-α), soluble fms-like tyrosine kinase-1 (sFlt-1), placental growth factor (PlGF), vascular endothelial growth factor A (VEGF-A), vascular cell adhesion molecule 1 (VCAM-1) and soluble endoglin (sEng) were determined in all samples using ProcartaPlex assays (Thermo Fisher Scientific) according to the manufacturer's instructions and as described earlier [11]. Concentrations were obtained in pg/ml. Standard concentrations were: VEGF-A 5.76–23600 pg/ml, sFlt-1 47.53–194700 pg/ml, sEng 0.61–2500 pg/ml, VCAM-1 9.45–38700 pg/ml, TNF-α 8.47–34700 pg/ml and PlGF 1.81–7400 pg/ml.

Table 1
Cell culture media compositions.

hASC culture medium	DMEM/F12	Gibco
	10% human serum	PAA laboratories
	1% L-Glutamine	Gibco
hASC-HUVEC test medium	DMEM/F12	Gibco
	2.56 mM L-glutamine	Gibco
	0.1 nM 3,3′,5-triiodo-L-thyronine sodium salt	BD Biosciences
		BD Biosciences
	ITS™ Premix: insulin (6.65 μg/ml) transferrin (6.65 μg/ml) selenious acid (6.65 ng/ml)	
	1% Bovine serum albumin	PAA
	2.8 mM Sodium pyruvate	Gibco Invitrogen
	70 μg/ml Ascorbic acid	Sigma Aldrich
	175 ng/ml Heparin	Stemcell Technologies
	0.7 μg/ml Hydrocortisone	Sigma Aldrich
	3,5 ng/ml VEGF	R&D Systems
	0,35 ng/ml FGF-β	R&D Systems

2.4. Cells

Human adipose-tissue samples were obtained from excess material of surgical operations. Human umbilical cords from uncomplicated pregnancies were obtained after Caesarean deliveries. Isolation of HUVECs from umbilical cord veins was performed as described earlier [12] using 0.05% collagenase I (Gibco, Thermo Fisher scientific, Waltham, USA) injected into the umbilical vein. Isolated HUVECs were cultured in EGM-2 medium (Lonza, Basel, Switzerland) (Table 1).

Isolation of hASCs was performed as described earlier [12] using 0.15% collagenase type I (Gibco). Isolated hASCs were cultured in hASC medium (Table 1). Both HUVECs and hASCs were negative for mycoplasma contamination, as tested by using MycoAlert™ kits (Lonza).

2.5. In vitro vasculogenesis/angiogenesis test

To study angiogenesis, an *in vitro* vasculogenesis/angiogenesis test, a co-culture of hASCs and HUVECs, was utilized. Direct effect of metformin (without maternal sera) on tubule formation was first assessed. After that, the angiogenic capacity of serum samples with and without metformin were studied by using the same study protocol. The test was performed as described earlier [13,11] with modification of the concentrations of supplements (Table 1). Briefly, a co-culture of hASCs (20,000 cells/cm^2) and HUVECs (4000 cells/cm^2) was plated on 48-well plates on day 0. On day 1, vasculogenesis/angiogenesis was induced using vasculogenesis/angiogenesis test medium (Table 1) and patient serum samples at a dilution of 1:15. The cells were cultured for six days in total with one replenishment of growth medium. To evaluate the effect of metformin on tubule formation, metformin was added to the test medium along with patient serum samples on day 1 and replenished once during the six days of culture. The studied concentrations of metformin were 5 μg/ml, 50 μg/ml and 600 μg/ml. The two lowest concentrations were chosen to correspond reported therapeutic concentrations of metformin [14] and the highest to ensure a sufficiently high concentration of free metformin for the detection of angiogenic effects in *in vitro* model. The highest concentration of metformin (600 μg/ml) was markedly over the therapeutic concentration of metformin.

After exposure, the number of living cells was evaluated by using a WST-1 Cell Proliferation Reagent (Roche, Basel, Switzerland). The WST-1 reagent measures the activity of mitochondria of cells, as a

surrogate marker of the number of living cells, i.e. cell viability. Following this, the cells were fixed with 70% ethanol and immunostained for von Willebrand factor (vWf) (Sigma Aldrich) to detect endothelial cells, and collagen IV (Sigma Aldrich) to detect the basement membrane of tubules. For visualisation of the tubules, fluorescent secondary antibodies against the primary antibodies were applied: antirabbit tetramethylrhodamine isothiocyanate (TRITC, Sigma Aldrich) for vWf and anti-mouse fluorescein 5-isothiocyanate (FITC, Sigma Aldrich) for collagen IV.

The resulting fluorescent tubules were imaged and analyzed using an automated image analysis platform (Cell-IQ, CM-Technologies, Tampere, Finland). Tubule formation was quantified on the basis of the intensity of the tubular network formed (tubule length and branching). The obtained result was compared with the positive tubule formation control (highest level of tubule formation induced with stimulatory factors). Values were first normalized to the in-plate control to remove variation between the plates. To see the effect of serum between the groups, the resulting values were compared with the mean value of the healthy group. To determine the effect of metformin on the tubules, the normalized values were compared with the unexposed tubules containing the serum of the corresponding patient. These results were expressed as percentages of positive tubule formation.

2.6. Statistical analysis

The data of clinical characteristics and bioactive markers are expressed as medians and range. Differences in continuous variables between the groups were tested by using Mann–Whitney *U*-tests and differences within the study group by using Wilcoxon's test. Spearman's correlation method was used to calculate correlation coefficients. Probabilities of less than 0.05 were considered statistically significant. Statistical analyses were performed by using the Statistical Package for the Social Sciences (IBM-SPSS), version 11.0. The graphs were processed with GraphPadPrism 8 (GraphPad Software, San Diego, CA, USA), and the results of tubule formation at the graphs were presented as mean ± SD.

3. Results

The clinical characteristics and neonatal outcomes of the study groups are presented in Table 2. One woman with early-onset pre-eclampsia and one with IUGR had chronic hypertension; one of them was on labetalol from the beginning of the pregnancy. None of the participants used low-dose aspirin, nor had pregestational diabetes or a thrombophilic disorder. There were five preterm deliveries (before 37 0/7 weeks of gestation) in the early-onset pre-eclampsia group and four in the IUGR group (two of which before 32 0/7 weeks of gestation).

The median gestational age at delivery, birth weight and birth weight standard deviation were significantly lower in pregnancies complicated by early-onset pre-eclampsia and IUGR compared with pregnancies complicated by late-onset pre-eclampsia and healthy pregnancies (Table 2). The blood samples were obtained within four days before delivery, and there was no difference in the time interval between blood sampling and the delivery in the control and study groups.

The results of the measurements of various bioactive markers in maternal sera are presented in Table 3. There were significant differences in levels of sFlt-1, Ang2, sEng and ET-1 between the pre-eclampsia, IUGR and control group, whereas no significant differences in sFlt-1/PlGF ratio nor in levels of pro-inflammatory markers (TNF-α, VCAM-1) were observed between the groups.

Maternal sera alone did not have stimulatory or inhibitory effects on tubule formation in any study group, and there was no difference between the control and study groups (Figs. 2 and 3). Neither was there a correlation between tubule formation and the concentrations of angiogenic or inflammatory biomarkers in any group.

When the direct effect of metformin was studied (without maternal serum) we found that the two lowest tested concentrations (5 and 50 μg/ml) did not affect tubule formation (angiogenesis), but at concentration of 600 μg/ml metformin inhibited tubule formation ($p = 0.002$) when compared to tubule growth without drug. There were also significant differences in tubule formation between the three doses of metformin (Fig. 1).

Metformin at concentration of 5 and 50 μg/ml did not significantly change tubule formation in any group when compared to tubule growth affected by maternal sera alone (Fig. 2). Either, there were no differences between the control and study groups. However, metformin at 600 μg/ml resulted in an equally strong inhibition on tubule formation in each study group ($p = 0.005$, $p = 0.011$, $p = 0.000$, $p = 0.002$) (Figs. 2 and 3). When tubule formation with and without maternal sera were compared, there was a significant difference only with the highest concentration of metformin; the presence of maternal sera increased tubule growth in each study group (early-onset pre-eclampsia; $p = 0.037$, late-onset pre-eclampsia; $p = 0.018$, IUGR; $p = 0.035$, healthy; $p = 0.004$).

When changes in tubule formation were investigated within each study group, there seems to be decrease in tubule formation with increasing doses of metformin (Fig. 2). The only difference between the groups were observed between the two lowest concentrations of metformin since only the sera from early-onset pre-eclampsia or IUGR group, caused a significant inhibition of angiogenesis with increasing dose (Fig. 2).

Serum samples alone, or with 5, 50 or 600 μg/ml concentrations of metformin, were not cytotoxic, as determined by WST-1 measurement, using 80% viability compared with the unexposed controls as the non-cytotoxicity limit. In contrast, metformin at a concentration of 600 μg/ml even increased cell viability/mitochondrial activity.

Table 2
Maternal characteristics and neonatal outcome.

	Early-onset PE (n = 5)	Late-onset PE (n = 5)	IUGR (n = 5)	Healthy (n = 5)
Maternal age, years	22 (19–40)	22.5 (20–33)	27 (25–33)	34 (33–36)
BMI, kg/m^2	25.3 (18–30)	24.5 (21.8–34.8)	25.3 (18.3–38.4)	25.5 (21.9–26.1)
Non-smoker (%)	100	100	60	100
Highest systolic BP, mmHg	165 (152–186)	160 (142–202)	136 (118–166)	122 (112–132)
Highest diastolic BP, mmHg	107 (92–115)	106 (93–118)	84 (66–104)	83 (68–94)
Proteinuria, grams	8.0 (1.5–13.5)	2.8 (1.0–4.1)		
Umb(A) PI	1.14 (1.02–1.36)	0.83 (0.74–1.08)	1.3 (1.1–1.5)	0.72 (0.57–0.94)
Gestational age at delivery, wks	33.6 (32.3–35.3)	40.5 (39.1–41.1)	34 (26.3–39.9)	39.7 (39–41.3)
Birth weight, grams	1880 (1645–2040)	3655 (3300–4230)	1735 (600–2420)	3920 (3400–4160)
Birth weight, SD score	−1.2 (−1.5–0)	0.4 (−0.4–1.6)	−2.9 [−1.9 − (−3.8)]	0.5 (−0.9–1.3)
Umbilical arterial pH	7.35 (7.18–7.40)	7.23 (7.16–7.34)	7.35 (7.31–7.39)	7.31 (7.26–7.35)

Data are given as median (range). PE, pre-eclampsia; IUGR, intrauterine growth restriction; BMI, body mass index; BP, blood pressure; Umb(A) PI, umbilical artery pulsatility index; SD, standard deviation.

Table 3
Concentrations of angiogenic and inflammatory proteins in maternal serum in early- and late-onset pre-eclampsia, IUGR and healthy group. Values of *p* refer to the differences between pre-eclampsia or IUGR and healthy pregnancies.

	Early-onset PE		Late-onset PE		IUGR		Healthy
		p-value		*p*-value		*p*-value	
PlGF (pg/ml)	270 (16.8–356)	0.564	284 (120–442)	0.355	127 (65–302)	0.564	172 (171–173)
sFlt-1 (pg/ml)	3375 (2584–9906)	0.117	6244 (1801–9781)	0.028*	6036 (459–8623)	0.117	1335 (717–3434)
Ang2 (pg/ml)	8242 (4750–10332)	0.014*	7552 (514–10927)	0.086	6236 (3645–7619)	0.014*	1789 (471–2530)
ET-1 (pg/ml)	1.77 (0.69–4.97)	0.050	3.52 (0.86–8.29)	0.027*	0.62 (0.16–3.86)	0.462	0.44 (0.11–1.01)
sEng (pg/ml)	2995 (2375–3418)	0.028*	3552 (1469–3787)	0.117	2827 (1767–3674)	0.117	2091 (1661–2548)
HO-1 (ng/ml)	0.24 (0.18–0.82)	0.806	0.21 (0.12–0.52)	0.142	0.48 (0.18–0.94)	0.806	0.46 (0.15–0.70)
TNF-α (pg/ml)	18.04 (16.58–18.04)	1.000	7.01 (2.06–23.58)	0.643	8.92 (6.08–31.22)	0.245	14.99 (9.81–20.17)
VCAM-1 (pg/ml)	79,471 (23157–135785)	0.439	23,246 (4287–27724)	0.439	20,586 (9792–25728)	1.000	17,704 (12191–23216)
sFlt-1/PlGF	14.17 (9.56–591.08)	0.564	30.67 (17.48–46.98)	0.355	67.77 (1.52–92.42)	0.564	11.60 (4.19–19.00)

Data are given as median (range). PE, pre-eclampsia; IUGR, intrauterine growth restriction; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; Ang2, angiopoietin 2; ET-1, endothelin 1; sEng, soluble endoglin, HO-1; heme oxygenase 1, TNF-α; tumor necrosis factor alpha, VCAM-1; vascular cell adhesion molecule 1.

* p-value less than 0.05, Mann–Whitney *U* test.

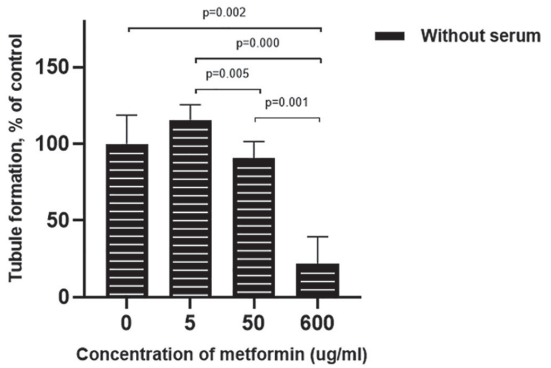

Fig. 1. When the direct effect of metformin was studied (without maternal serum) we found that the two lowest tested concentrations (5 and 50 μg/ml) did not affect tubule formation (angiogenesis) when compared to tubule formation without drug, but at concentration of 600 μg/ml metformin inhibited tubule formation (p = 0.002).

4. Discussion

4.1. Main findings

The therapeutic concentrations of 5 and 50 μg/ml of metformin did not affect tubule formation (angiogenesis), but the highest concentration inhibited angiogenesis when the direct effect of metformin was studied. When metformin was supplemented at the two lowest concentrations along with maternal serum samples, there was no change in tubule formation in comparison to maternal sera alone. However, strong inhibitory effect on tubule formation was observed in all groups with the highest, non-therapeutic (600 μg/ml), concentration of metformin.

The vasculogenesis/angiogenesis model utilized in this study has gone through intra-laboratory validation (Environment Directorate OECD), and it has been found to show good concordance in comparison with blood vessel formation in vivo [15]. The model has been confirmed to be suitable for testing the angiogenic properties of drugs and other chemicals [15,16]. We have previously utilized the model to study maternal and umbilical sera in order to assess the overall angiogenic capacity in pre-eclamptic and healthy pregnancies [13]. According to Kajbaf *et al.* therapeutic human plasma levels of metformin range from 0.129 to 90 mg/l (=μg/ml)[14]. Hence, the two lowest concentrations used in our study correspond to measured metformin plasma concentrations, but the highest concentration is markedly over the therapeutic level. Calculation of *in vitro*-in vivo correlation (IVIVC) requires complicated mathematical modelling which takes into account various characteristics of the substance, such as pharmacokinetics and bioavailability [17]. Free concentrations of the drug may be drastically different compared to nominal concentrations, and it is common that effective *in vitro* concentrations are higher than those measured from plasma. We have previously shown that higher than usual plasma concentrations of a studied compound might be needed to demonstrate known effects in hASC-HUVEC vasculogenesis/angiogenesis model [15]. The concentrations in this study were chosen to span a wide range in order to ensure a sufficiently high concentration of free metformin for the detection of possible angiogenic effects.

So far, only one other research group has evaluated the angiogenic capacity of metformin in obstetric patients. Brownfoot et al. found that metformin ameliorates endothelial dysfunction in an *in vitro* HUVEC test and induces angiogenesis in an omental artery explant assay [6]. However, their study design differed from ours. In their study maternal sera were not tested, but placental villous explants and omental tissues were collected from pre-eclamptic women. Explants of omental vessels were cultured with and without sFlt-1, and the reduction in angiogenic sprouting caused by sFlt-1, was rescued by the addition of metformin at 1 mmol/l[6]. In our study the lowest concentration of metformin was 5 μg/ml, which corresponds to twice the concentration used in the study by Brownfoot et al. Our results were not in accordance with Brownfoot et al, as we did not observe pro-angiogenic effects of metformin at all. At therapeutic concentrations, metformin along with maternal sera did not affect angiogenesis when compared to angiogenic capacity of maternal sera alone.

When the basic tubule formation was measured (without metformin) we found no differences in effects of maternal sera between the control and study groups. Either there were no correlations between the effect of maternal sera on tubule formation and measured angiogenic biomarkers. We expected that the sera from pre-eclampsia and IUGR pregnancies, would have been more anti-angiogenic than the sera from women with healthy pregnancies. The result is possible affected by a small sample size. The lack of correlations was understandable as we have already earlier suggested that there might be interacting factors other than known angiogenic proteins that have an influence on angiogenesis [11]. Although, the basic tubule formation of maternal sera did not differ between study and control groups, there were difference in angiogenic capacity in the present of maternal sera along with metformin. When the effect of metformin and maternal sera on angiogenesis was investigated within each group, only in the early-onset pre-eclampsia and IUGR groups the inhibition of angiogenesis was stronger in every dosage increase. The highest, non-therapeutic concentration of

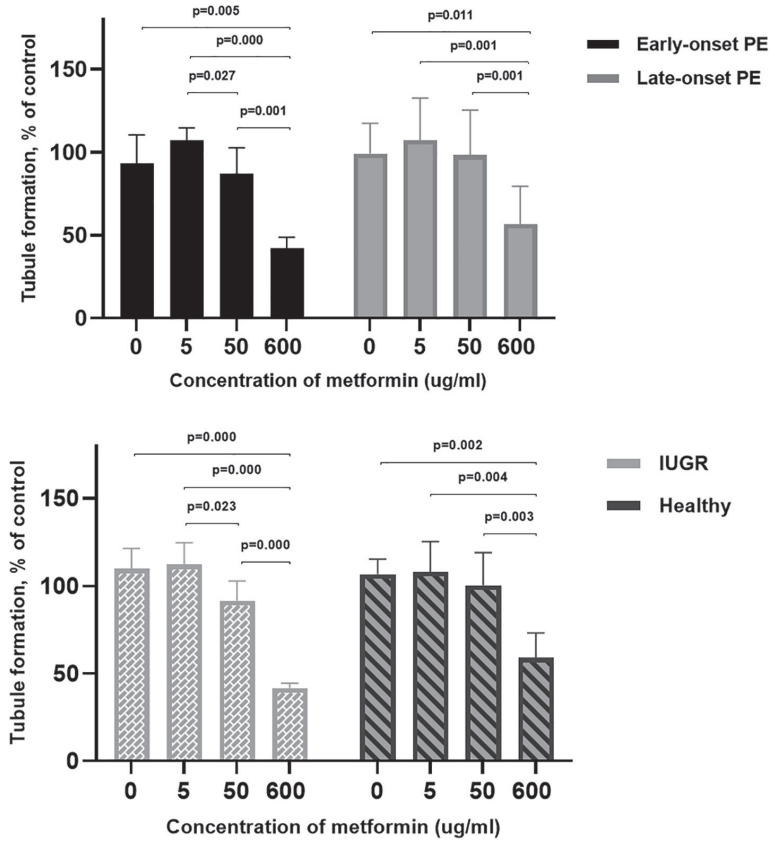

Fig. 2. Tubule formation (mean + SD) with and without metformin in the presence of maternal sera from each study group. In comparison to maternal sera alone, there was a strong inhibitory effect on angiogenesis with the highest concentration (600 μg/ml) of metformin. In addition, within groups there were decrease in tubule formation with increasing dose of metformin.

metformin caused a strong inhibitory effect on angiogenesis with and without maternal sera. Interestingly, maternal sera from each group increased tubule formation in comparison to effects of the drug without maternal sera when metformin was used at 600 ug/ml concentration. The highest concentration of metformin used in this study is higher than therapeutic concentrations of metformin. The inhibition of angiogenesis observed at high metformin concentration was not due to cell death, as there was no cytotoxicity in the cell viability assay. Thus, our findings suggest that using metformin at high concentrations results in an anti-angiogenic state by specific mechanisms of this drug.

Metformin use in pregnancy has increased, as it has been shown to be an efficient treatment for gestational diabetes [18]. Recently, metformin has been associated with a reduced risk of pre-eclampsia and/or hypertensive pregnancy disorders [19]. One of the suggested beneficial mechanisms is a reduction in levels of sFlt-1 [20]. Our finding of metformin having no pro-angiogenic effects may suggest that its beneficial effects in diabetic and non-diabetic pregnancies may be based on other effects, such as improvement of cardiovascular function and insulin sensitivity. Moreover, metformin may prevent pre-eclampsia by limiting gestational weight gain [19]. However, little is known about the direct effects of metformin on angiogenesis during pregnancy and information is controversial in non-pregnant state [21]. Currently, metformin is commonly used in early gestation when angiogenesis plays a crucial role in normal placental development. Our finding of metformin causing inhibition of angiogenesis at high concentrations, encourages to continue research to clarify underlying mechanisms.

4.2. Strengths and limitations

The strength of this study is that we assessed both direct effect of metformin on angiogenesis and the impact of maternal sera along with metformin on angiogenic capacity in a human based *in vitro* assay. The angiogenic effects of metformin have not been studied before in the setting of pre-eclamptic, IUGR and healthy pregnancies. The results presented here are limited by a small number of samples, and the effects of metformin on healthy and dysfunctional endothelium may differ.

5. Conclusion

In comparison with tubule growth in the presence of maternal sera alone, therapeutic doses of metformin did not have an impact of angiogenetic capacity of maternal sera. Metformin at concentration remarkably above therapeutic level resulted in a significant anti-angiogenic effect on tubule formation.

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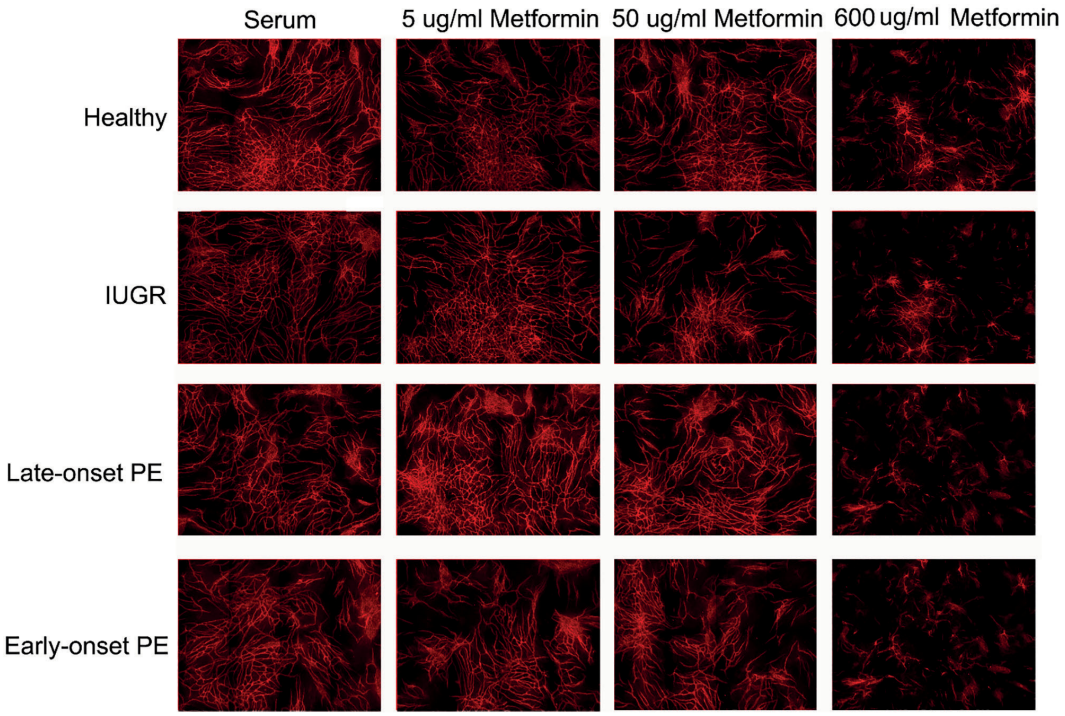

Fig. 3. Fluorescence images of vWf-TRITC-stained tubules exposed to healthy, IUGR, late- and early-onset pre-eclamptic (PE) maternal serum alone and with metformin at 5, 50 and 600 μg/ml. In comparison with tubule growth in the presence of maternal sera alone, metformin supplementation at 600 μg/ml concentration resulted in a significant anti-angiogenic effect on tubule formation in each group.

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Disclosure of interests

The authors report no conflicts of interest.

Details of ethics approval

This study was approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit R16184, date of approval 17.3.2017).

References

[1] M.J. Crowley, C.J. Diamantidis, J.R. McDuffie, et al., Clinical outcomes of metformin use in populations with chronic kidney disease, congestive heart failure, or chronic liver disease: a systematic review, Ann. Internal Med. 166 (3) (2017) 191–200, https://doi.org/10.7326/M16-1901.

[2] J.M.M. Evans, L.A. Donnelly, A.M. Emslie-Smith, D.R. Alessi, A.D. Morris, Metformin and reduced risk of cancer in diabetic patients, BMJ 330 (7503) (2005) 1304–1305, https://doi.org/10.1136/bmj.38415.708634.F7.

[3] E.A.P. Steegers, P. von Dadelszen, J.J. Duvekot, R. Pijnenborg, Pre-eclampsia, Lancet (London, England). 376 (9741) (2010) 631–644, https://doi.org/10.1016/S0140-6736(10)60279-6.

[4] S.A. Karumanchi, Angiogenic factors in preeclampsia: from diagnosis to therapy. Hypertens. (Dallas, Tex 1979). 67(6) (2016) 1072–1079. doi:10.1161/HYPERTENSIONAHA.116.06421.

[5] S. Ornaghi, M.J. Paidas, Upcoming drugs for the treatment of preeclampsia in pregnant women, Expert Rev. Clin. Pharmacol. 7 (5) (2014) 599–603, https://doi.org/10.1586/17512433.2014.944501.

[6] F.C. Brownfoot, R. Hastie, N.J. Hannan, et al., Metformin as a prevention and treatment for preeclampsia: effects on soluble fms-like tyrosine kinase 1 and soluble endoglin secretion and endothelial dysfunction, Am. J. Obstet. Gynecol. 214 (3) (2016) 356.e1–356.e15, https://doi.org/10.1016/j.ajog.2015.12.019.

[7] N. Soobryan, S. Murugesan, A. Pandiyan, J. Moodley, I. Mackraj, angiogenic dysregulation in pregnancy-related hypertension-a role for metformin, Reprod. Sci. 25 (11) (2018) 1531–1539, https://doi.org/10.1177/1933719118773484.

[8] V. De Leo, M.C. Musacchio, P. Piomboni, A. Di Sabatino, G. Morgante, The administration of metformin during pregnancy reduces polycystic ovary syndrome related gestational complications, Eur. J. Obstet. Gynecol. Reprod. Biol. 157 (1) (2011) 63–66, https://doi.org/10.1016/j.ejogrb.2011.03.024.

[9] J. Gui, Q. Liu, L. Feng, Metformin vs insulin in the management of gestational diabetes: a meta-analysis, PLoS One 8 (5) (2013) e64585, , https://doi.org/10.1371/journal.pone.0064585.

[10] A. Alqudah, M.C. McKinley, R. McNally, et al., Risk of pre-eclampsia in women taking metformin: a systematic review and meta-analysis, Diabet. Med. 35 (2) (2018) 160–172, https://doi.org/10.1111/dme.13523.

[11] A. Virtanen, O. Huttala, K. Tihtonen, T. Toimela, T. Heinonen, J. Uotila, Angiogenic capacity in pre-eclampsia and uncomplicated pregnancy estimated by assay of angiogenic proteins and an in vitro vasculogenesis/angiogenesis test, Angiogenesis (July 2018), https://doi.org/10.1007/s10456-018-9637-2.

[12] J.-R. Sarkanen, H. Vuorenpaa, O. Huttala, et al., Adipose stromal cell tubule network model provides a versatile tool for vascular research and tissue engineering, Cells Tissues Organs 196 (5) (2012) 385–397, https://doi.org/10.1159/000336679.

[13] A. Virtanen, T. Toimela, K. Tihtonen, et al., Strong inhibitory effect of pre-eclampsia serum on angiogenesis detected in vitro by human cell-based angiogenesis tests, Pregnancy Hypertens. 6 (4) (2016) 367–373, https://doi.org/10.1016/j.preghy.2016.08.239.

[14] F. Kajbaf, M.E. De Broe, J.-D. Lalau, Therapeutic concentrations of metformin: a systematic review, Clin. Pharmacokinet. 55 (4) (2016) 439–459, https://doi.org/10.1007/s40262-015-0323-x.

[15] T. Toimela, O. Huttala, E. Sabell, et al., Intra-laboratory validated human cell-based in vitro vasculogenesis/angiogenesis test with serum-free medium, Reprod Toxicol. 70 (2017) 116–125, https://doi.org/10.1016/j.reprotox.2016.11.015.

[16] O. Huttala, H. Vuorenpaa, T. Toimela, et al., Human vascular model with defined stimulation medium – a characterization study, ALTEX 32 (2) (2015) 125–136, https://doi.org/10.14573/altex.1411271.

[17] M. Kakhi, P. Marroum, J. Chittenden, Analysis of level A in vitro-in vivo correlations for an extended-release formulation with limited bioavailability, Biopharm. Drug Dispos. 34 (5) (2013) 262–277, https://doi.org/10.1002/bdd.1820.

[18] R.S. Lindsay, M.R. Loeken, Metformin use in pregnancy: promises and uncertainties, Diabetologia 60 (9) (2017) 1612–1619, https://doi.org/10.1007/s00125-017-4351-y.

[19] E. Kalafat, Y.E. Sukur, A. Abdi, B. Thilaganathan, A. Khalil, Metformin for

prevention of hypertensive disorders of pregnancy in women with gestational diabetes or obesity: systematic review and meta-analysis of randomized trials, Ultrasound Obstet. Gynecol. (May 2018), https://doi.org/10.1002/uog.19084.
[20] R. Romero, O. Erez, M. Huttemann, et al., Metformin, the aspirin of the 21st century: its role in gestational diabetes mellitus, prevention of preeclampsia and cancer, and the promotion of longevity, Am. J. Obstet. Gynecol. 217 (3) (2017) 282–302, https://doi.org/10.1016/j.ajog.2017.06.003.
[21] K. Dallaglio, A. Bruno, A.R. Cantelmo, et al., Paradoxic effects of metformin on endothelial cells and angiogenesis, Carcinogenesis 35 (5) (2014) 1055–1066, https://doi.org/10.1093/carcin/bgu001.

PUBLICATION IV

Angiogenic effect of pravastatin alone and with sera from healthy and complicated pregnancies studied by in vitro vasculogenesis/angiogenesis assay

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Angiogenic Effect of Pravastatin Alone and with Sera from Healthy and Complicated Pregnancies Studied by in vitro Vasculogenesis/Angiogenesis Assay

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Keywords
Pravastatin · In vitro angiogenesis · Pre-eclampsia · Intrauterine growth restriction · Angiogenic biomarkers

Abstract
Objective: To determine the direct effect of pravastatin on angiogenesis and to study the interaction between pravastatin and maternal sera from women with early- or late-onset pre-eclampsia (PE), intrauterine growth restriction, or healthy pregnancy. ***Methods:*** We collected 5 maternal serum samples from each group. The effect of pravastatin on angiogenesis was assessed with and without maternal sera by quantifying tubule formation in a human-based in vitro assay. Pravastatin was added at 20, 1,000, and 8,000 ng/mL concentrations. Concentrations of angiogenic and inflammatory biomarkers in serum and in test medium after supplementation of serum alone and with pravastatin (1,000 ng/mL) were measured. ***Results:*** Therapeutic concentration of pravastatin (20 ng/mL) did not have significant direct effect on angiogenesis, but the highest concentrations inhibited angiogenesis. Pravastatin did not change the levels of biomarkers in the test media. There were no changes in angiogenesis when therapeutic dose of pravastatin was added with maternal sera, but there was a trend to wide individual variation towards enhanced angiogenesis, particularly in the early-onset PE group. ***Conclusions:*** At therapeutic concentration, pravastatin alone or with maternal sera has no significant effect on angiogenesis, but at high concentrations the effect seems to be anti-angiogenic estimated by in vitro assay.

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in women worldwide. Since 2011, a history of pre-eclampsia (PE) has been recognized as an independent cardiovascular risk factor in American Heart Association guidelines [1]. PE shares several risk factors and pathophysiological mechanisms with cardiovascular diseases, such as angiogenic imbalance, endothelial injury, inflammation, and oxidative stress [2, 3]. It has been hypothesized that an increased risk of future cardiovascular disease is either because of a shared aetiology of the diseases or because of vascular damage during PE [4].

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Table 1. Cell culture media compositions

hASC culture medium	DMEM/F12 10% human serum 1% L-glutamine	Gibco PAA laboratories Gibco
Vasculogenesis/angiogenesis assay medium	DMEM/F12 2.56 mM L-glutamine 0.1 nM 3,3′,5-triiodo-L-thyronine sodium salt ITS™ Premix Insulin (6.65 μg/mL) Transferrin (6.65 μg/mL) Selenious acid (6.65 ng/mL) 1% bovine serum albumin 2.8 mM sodium pyruvate 70 μg/mL ascorbic acid 175 ng/mL heparin 0.7 μg/mL hydrocortisone 3,5 ng/mL VEGF 0,35 ng/mL FGF-β	Gibco Gibco BD Biosciences BD Biosciences PAA Gibco Invitrogen Sigma Aldrich Stemcell Technologies Sigma Aldrich R&D Systems R&D Systems

hASC, human adipose stromal cell; VEGF, vascular endothelial growth factor.

Statin use for the prevention of cardiovascular disease is widely accepted and its benefit well documented [5]. The beneficial effects of statins are associated not only with their lipid-lowering action but also with other pleiotropic effects [6]. As a result of similarities in pathogenic mechanisms, statins have been proposed as potential preventive therapy for PE. Pravastatin has favourable characteristics for use during pregnancy. It is one of the least potent inhibitors of HMG-CoA reductase, and its transplacental transfer is limited because of its hydrophilicity [7]. However, there are conflicting findings regarding the association between pravastatin and PE, and the underlying mechanism behind the proposed beneficial effect of pravastatin is unclear. In animal and human pilot studies, pravastatin treatment has been associated with reverse angiogenic imbalance [8, 9]. In contrast, in a recently published randomized trial, pravastatin given to women with early-onset PE did not reduce maternal plasma soluble fms-like tyrosine kinase-1 (sFlt-1) levels [10], which would suggest lack of an angiogenic effect.

In this study, we evaluated the direct effects of pravastatin in terms of promoting, maintaining, or inhibiting angiogenesis. We also investigated how maternal sera from healthy and complicated pregnancies change the angiogenic properties of pravastatin. Angiogenic effect is estimated by in vitro human-based vasculogenesis/angiogenesis assay that has been validated according to OECD guidance for studying effects of chemicals [11]. The cells utilized in this assay, human adipose stromal cells (hASCs) and human umbilical vein endothelial cells (HUVECs) have been shown to produce in vivo like vascular structures without addition of biomaterial [12, 13].

Materials and Methods

Study Population

In this in vitro trial, we studied serum samples from women with early-onset PE (n=5), late-onset PE (n=5), or intrauterine growth restriction (IUGR, n=5). Serum samples from women with healthy pregnancies served as controls (n=5). Recruitment was conducted prospectively at the Department of Obstetrics and Gynaecology, Tampere University Hospital, from April 2017 to March 2018.

Blood samples were drawn within 4 days prior to delivery. The serum samples were stored at −70°C until assay. Baseline demographic details and data on pregnancy outcomes were collected from hospital maternity records.

PE was diagnosed as hypertension and proteinuria occurring after 20 weeks of gestation. Hypertension was diagnosed as systolic blood pressure (BP) ≥140 mm Hg and/or diastolic BP ≥90 mm Hg and proteinuria as urinary excretion of ≥300 mg protein in a 24-h specimen. PE was defined as early onset when diagnosed before 34 0/7 weeks of gestation and late onset when diagnosed at 34 0/7 weeks of gestation or later. IUGR was defined as foetal abdominal circumference below the 10th percentile or estimated foetal weight below the 10th percentile in ultrasonographic examination.

Immunoassays

The serum samples were analysed for their protein concentrations. Concentrations of haem oxygenase 1, endothelin-1, and angiopoietin-2 were determined by using ELISA kits according to

Table 2. Background data and neonatal outcome

	Early-onset PE ($n = 5$)	Late-onset PE ($n = 5$)	IUGR ($n = 5$)	Healthy ($n = 5$)	p value
Maternal age, years	22 (19–40)	24.0 (20–33)	27 (25–33)	34 (33–36)	0.053
BMI, kg/m^2	25.3 (18–30)	25.3 (21.8–34.8)	25.3 (18.3–38.4)	25.5 (21.9–26.1)	0.992
Non-smoker, %	100	100	60	100	0.057
Highest syst BP, mm Hg	165 (152–186)$^{\#,\approx}$	160 (142–202)$^{\approx}$	136 (118–166)	122 (112–132)	0.008*
Highest diast BP, mm Hg	107 (92–115)$^{\#,\approx}$	101 (93–118)$^{\approx}$	84 (66–104)	83 (68–94)	0.017*
Proteinuria, g	8.0 (1.5–13.5)	2.8 (1.0–4.1)			0.131
Umb(A) PI	1.14 (1.02–1.36)$^{\dagger,\approx}$	0.86 (0.74–1.08)	1.3 (1.1–1.5)	0.72 (0.57–0.94)	0.009*
Gestational age at delivery, weeks	33.6 (32.3–35.3)$^{\dagger,\approx}$	40.5 (39.1–41.1)$^{\#}$	34 (26.3–39.9)$^{\approx}$	39.7 (39–41.3)	0.007*
Birth weight, g	1,880 (1,654–2,040)$^{\dagger,\approx}$	3,860 (3,300–4,230)$^{\#}$	1,735 (600–2,420)$^{\approx}$	3,920 (3,400–4,160)	0.002*
Birth weight, SD score	−1.2 (−1.5 to 0)$^{\dagger,\#,\approx}$	0.9 (−0.4 to 1.6)$^{\#}$	−2.9 [−1.9 to −3.8)]$^{\approx}$	0.5 (−0.9 to 1.3)	0.002*
Umbilical arterial pH	7.35 (7.18–7.40)	7.22 (7.16–7.34)	7.35 (7.31–7.39)	7.31 (7.26–7.35)	0.130

Data are given as median (range). Analyses of differences between medians were performed by the Kruskal-Wallis test. PE, pre-eclampsia; IUGR, intrauterine growth restriction; BP, blood pressure; Umb(A) PI, umbilical artery pulsatility index; SD, standard deviation. * $p < 0.05$, and post hoc with the Mann-Whitney test. † Compared with the late-onset pre-eclampsia group. $^{\#}$ Compared with the IUGR group. $^{\approx}$ Compared with the healthy group.

the manufacturer's instructions. The concentrations of tumour necrosis factor α (TNF-α), sFlt-1, placental growth factor (PlGF), vascular endothelial growth factor A, vascular cell adhesion molecule 1 (VCAM-1), interleukin-6 (IL-6), and soluble endoglin (sEng) were determined in all samples by using ProcartaPlex assays (Thermo Fisher Scientific), following the manufacturer's protocols and as described earlier [14]. In addition, medium samples collected from the cell cultures on day 6 were analysed to determine concentrations of TNF-α, sFlt-1, PlGF, vascular endothelial growth factor A, VCAM-1, IL-6, and sEng. The samples were collected from cultures containing (1) maternal sera from each group ($n = 9$) and (2) maternal sera from the same individuals with pravastatin at a concentration of 1,000 ng/mL. Samples were analysed in 2 parallels, and no repetitions of the immunoassays were performed.

Cells

Human adipose-tissue samples were obtained from excess material from surgical operations, and human umbilical cords were obtained after caesarean deliveries from uncomplicated pregnancies. Isolation of HUVECs from umbilical cord veins was performed as described earlier [12] using 0.05% collagenase type I (Gibco, Thermo Fisher scientific, Waltham, MA, USA) injected into the umbilical vein. Isolation of hASCs was performed as described earlier [12] using 0.15% collagenase type I (Gibco). Isolated HUVECs and hASCs were cultured in EGM-2 medium (Lonza, Basel, Switzerland) and in hASC medium, respectively (Table 1). HUVECs and hASCs were negative for mycoplasma contamination, tested by using MycoAlert™ kits (Lonza). The hASC utilized in this study were obtained from 1 healthy donor and HUVEC from 1 healthy donor, that is, the cells utilized in all of the presented experiments were same in order to remove the person-to-person variation in the model. Cell donors were different from the serum donors.

In vitro Vasculogenesis/Angiogenesis Test

The in vitro vasculogenesis/angiogenesis test, a co-culture of hASC and HUVEC, was utilized (1) to study direct effect of pravastatin on angiogenesis, (2) to determine angiogenic properties of maternal sera from each group, and (3) to evaluate an interaction between pravastatin and maternal sera. In this assay, vascular structures containing lumen inside the endothelial tubule, basement membrane, and pericytes surrounding the tubule are self-organized from hASCs and HUVECs in the stimulation medium [13]. The angiogenic capacity is measured from the properties of vascular structures (the length of the tubules and number of branches, i.e., tubule formation). Different stages of angiogenesis (sprouting, de novo tubule formation, and stabilization of the new vessels) cannot be separated from each other in this assay. To assess the direct effects of pravastatin on angiogenesis, tubule formation was first studied without maternal serum. The tests were performed as described earlier [14, 15] with modification of the concentrations of supplements. Briefly, a co-culture of hASCs (20,000 cells/cm^2) and HUVECs (4,000 cells/cm^2) was plated on 48-well plates on day 0. Cells were plated directly on cell culture plastic without pre-coating the plates and without addition of biomaterial. On day 1, vasculogenesis/angiogenesis was induced using vasculogenesis/angiogenesis test medium and patient serum samples at a dilution of 1:15. The cells were cultured for 6 days in total, with one replenishment of the growth medium. To evaluate the effect of pravastatin on tubule formation, pravastatin was added to the test media along with patient serum samples on day 1 and replenished once during the 6 days of culture at day 3. The studied concentrations of pravastatin were 20, 1,000, and 8,000 ng/mL. When a 20 mg dose of pravastatin has been administered, the highest concentration of pravastatin in plasma is 26.5 ng/mL [16]. Thus, the lowest dose chosen for this study corresponds to the reported therapeutic plasma concentration. The other 2 concentrations were higher in order to correspond to higher doses of the drug and also in order to compare the results with those in previous in vitro studies [17].

Table 3. Concentrations of angiogenic and inflammatory biomarkers in maternal sera

	Early-onset PE	*p* value	Late-onset PE	*p* value	IUGR	*p* value	Healthy
PlGF, pg/mL	270 (17–356)	0.564	284 (120–442)	0.355	127 (65–302)	0.564	172 (171–173)
sFlt-1, pg/mL	3,375 (2,584–9,906)	0.221	6,244 (1,801–9,781)	0.028*	6,036 (459–8,623)	0.117	1,335 (717–3,434)
sFlt-1/PlGF	14.17 (9.56–591.08)	0.564	30.67 (17.48–46.98)	0.355	67.77 (1.52–92.42)	0.564	11.60 (4.19–19.00)
Ang2, pg/mL	8,242 (4,750–10,332)	0.014*	7,552 (514–10,927)	0.086	6,236 (3,645–7,619)	0.014*	1,789 (471–2,530)
ET-1, pg/mL	1.77 (0.69–4.97)	0.050	3.52 (0.86–8.29)	0.027*	0.62 (0.16–3.86)	0.462	0.44 (0.11–1.01)
sEng, pg/mL	2,995 (2,375–3,418)	0.028*	3,552 (1,469–3,787)	0.117	2,827 (1,767–3,674)	0.117	2,091 (1,661–2,548)
HO-1, ng/mL	0.24 (0.18–0.82)	0.806	0.21 (0.12–0.52)	0.142	0.48 (0.18–0.94)	0.806	0.46 (0.15–0.70)
TNF-α, pg/mL	18.04 (16.58–18.04)	1.000	7.01 (2.06–23.58)	0.643	8.92 (6.08–31.22)	0.245	14.99 (9.81–20.17)
VCAM-1, pg/mL	79,471 (23,157–135785)	0.439	23,246 (4,287–27,724)	0.439	20,586 (9,792–25,728)	1.000	17,704 (12,191–23,216)

Data are given as median (range). Values of *p* refer to the differences between the study group and healthy pregnancies. PE, preeclampsia; IUGR, intrauterine growth restriction; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; Ang2, angiopoietin-2; ET1, endothelin-1; sEng, soluble endoglin; HO-1, haem oxygenase 1; TNF-α, tumour necrosis factor alpha, VCAM-1; vascular cell adhesion molecule 1. * *p* value <0.05, Mann-Whitney U-test.

After exposure, the number of living cells was evaluated by using WST-1 Cell Proliferation Reagent (Roche, Basel, Switzerland). Following this, the cells were fixed with 70% ethanol and immunostained for vWf (Sigma Aldrich) to detect endothelial cells, and collagen IV (Sigma Aldrich) to detect basement membranes of tubules. For visualization of the tubules, fluorescent secondary antibodies against the primary antibodies were applied: anti-rabbit tetramethylrhodamine isothiocyanate (TRITC, Sigma Aldrich) for vWf and anti-mouse fluorescein 5-isothiocyanate (FITC, Sigma Aldrich) for collagen IV.

The resulting fluorescent tubules were imaged and analysed using an automated image analysis platform (Cell-IQ, CM-Technologies, Tampere, Finland). Tubule formation was quantified on the basis of the intensity of the tubular network formed (tubule length and branching). The obtained result was compared with that of a positive tubule formation control (highest level of tubule formation induced with stimulatory factors). Values were first normalized against the in-plate control to remove variation between the plates. To see the effect of serum between the groups, the resulting values were compared with the mean value of the healthy group. To determine the effect of pravastatin on the tubules, the normalized values were compared with that of unexposed tubules containing the serum of the corresponding patient. These results were expressed as percentages of positive tubule formation.

Statistical Analysis

The data are expressed as medians and range. Differences in continuous variables between groups were tested by using Kruskal-Wallis test and Mann-Whitney U-test. Differences within groups were studied by using Wilcoxon's test. Spearman's correlation method was used to calculate correlation coefficients. Probabilities of less than 0.05 were considered statistically significant. Statistical analyses were performed by using the Statistical Package for the Social Sciences (IBM-SPSS), version 11.0. Graphs were processed with GraphPadPrism 8 (GraphPad Software, San Diego, CA, USA).

Results

Demographic data and neonatal outcomes of the study groups are summarized in Table 2. One woman with early-onset PE and one with IUGR had a chronic hypertension. One of them had been on labetalol since the beginning of pregnancy. The remaining participants were healthy. Low-dose aspirin prophylaxis was not used. There were 9 preterm deliveries; 5 among pregnancies with early-onset PE

Table 4. Concentrations of angiogenic and inflammatory biomarkers in test medium after supplementation of maternal sera alone and with pravastatin at 1,000 ng/mL

	Maternal sera + test medium	Maternal sera + test medium + pravastatin	*p* value
sFlt-1, pg/mL	5,436 (4,278–7,951)	5,246 (4,361–6,563)	0.314
PlGF, pg/mL	277 (173–320)	275 (154–466)	0.859
VEGF, pg/mL	25,533 (19,398–35,694)	27,733 (20,594–31,421)	0.327
sEng, pg/mL	1,844 (1,547–2,055)	1,868 (1,347–2,070)	0.515
VCAM-1, pg/mL	8,524 (1,107–34,869)	10,164 (6,156–201,606)	0.917
TNF-α, pg/mL	8.8 (1.0–24.0)	6.2 (3.8–10.0)	0.753
IL6, pg/mL	886 (572–1,296)	774 (541–1,078)	0.066

Data are given as median (range). sFlt-1, soluble fms-like tyrosine kinase-1; PlGF, placental growth factor; VEGF, vascular endothelial growth factor; sEng, soluble endoglin, VCAM-1, vascular cell adhesion molecule 1; TNF-α, tumour necrosis factor alpha; IL-6, interleukin-6. Wilcoxon signed-rank test.

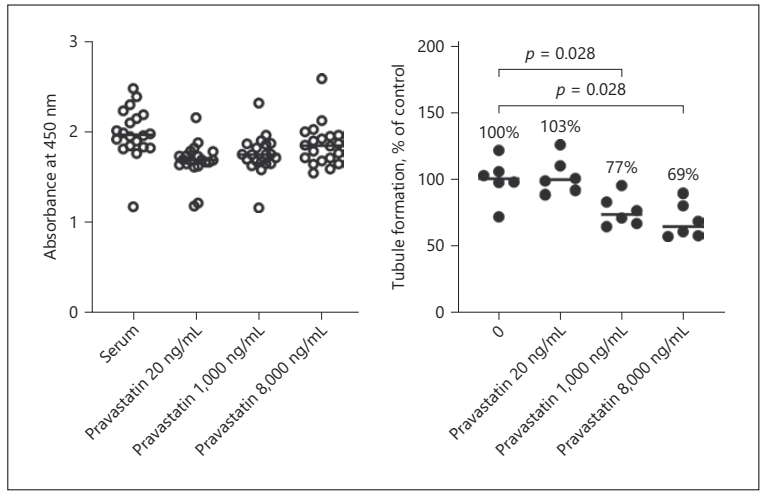

Fig. 1. None of the tested concentrations of pravastatin showed cytotoxicity (WST assay) (left). Direct effect of pravastatin on tubule formation (right). Pravastatin inhibited tubule formation (angiogenesis) at concentrations of 1,000 and 8,000 ng/mL. Percentages correspond to values in *y*-axis. Wilcoxon signed-rank test, *p* value <0.05.

and 4 among cases of IUGR (2 of which took place before 32 gestational weeks). Two women in the IUGR group had smoked during gestation (Table 2).

Concentrations of angiogenic and inflammatory biomarkers in maternal sera are presented in Table 3. In short, there were no significant differences in sFlt-1/PlGF ratios or in levels of inflammatory markers (TNF-α and VCAM-1) between the groups. However, there were differences in levels of Ang2, endothelin-1, sEng, and sFlt-1 between the study groups and healthy women (Table 3). Concentrations of sFlt-1, PlGF, VEGF, sEng, VCAM-1, TNF-α, and IL-6 in test media exposed to maternal sera from 9 women did not change after pravastatin (1,000 ng/mL) supplementation (Table 4).

When the direct effects of pravastatin were studied (without maternal serum) we found that the therapeutic tested concentration (20 ng/mL) did not affect tubule formation (angiogenesis), but at high concentrations (1,000 and 8,000 ng/mL), pravastatin inhibited tubule formation significantly (p=0.028). Viability was above 80% (the cut-off value for toxic concentrations) with all tested concentrations; hence the concentrations were non-toxic and the effects seen were due to the specific anti-angiogenic property of pravastatin (Fig. 1).

Maternal sera did not have stimulatory or inhibitory effects on tubule formation in any study group or in healthy pregnancies. Furthermore, there were no significant differences in tubule formation between the

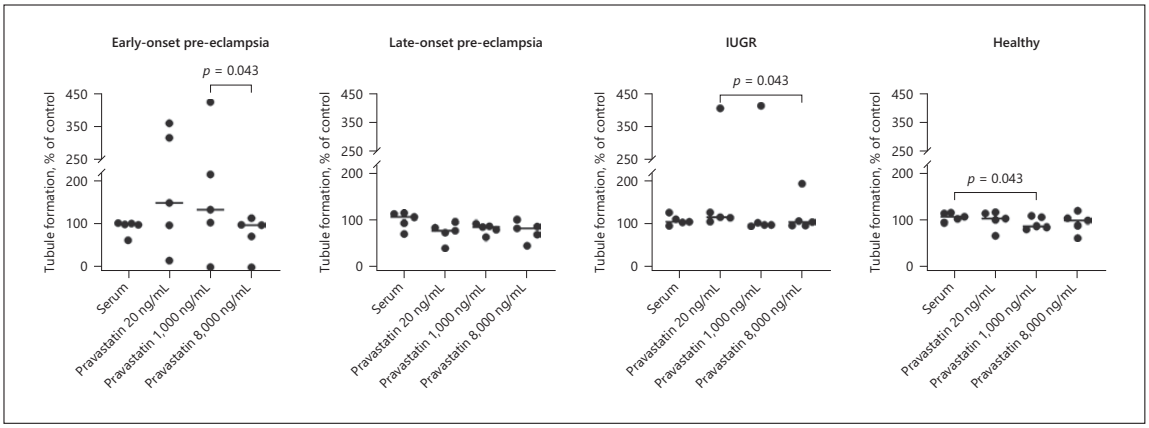

Fig. 2. The graphs represent individual values of tubule formation with maternal sera alone and with 3 doses of pravastatin in each group. In healthy pregnancies, pravastatin at a concentration of 1,000 ng/mL inhibited tubule formation (angiogenesis) when compared with tubule growth associated with maternal sera alone. In early-onset PE and IUGR groups, there was a decrease in tubule formation with increasing dose of pravastatin from 1,000 to 8,000 ng/mL and from 20 to 8,000 ng/mL, respectively. Wilcoxon signed-rank test, p value <0.05. PE, pre-eclampsia; IUGR, intrauterine growth restriction.

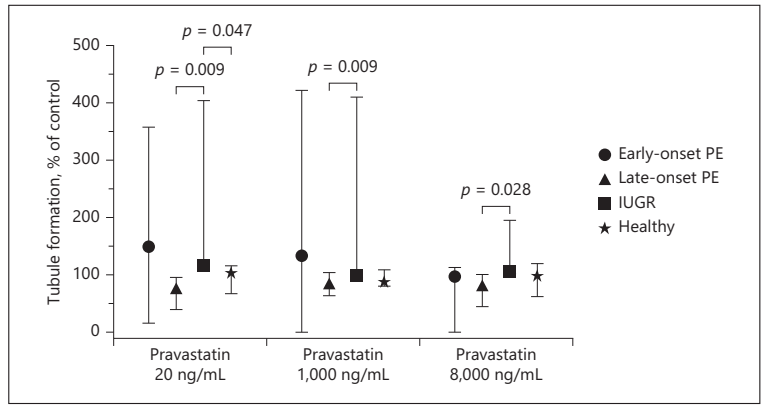

Fig. 3. Intergroup differences in tubule formation. Plots represent median values with range. There was more tubule formation (angiogenesis) in the IUGR group compared to the late-onset PE and the healthy groups with therapeutic concentration of pravastatin (20 ng/mL). Pravastatin at 1,000 and 8,000 ng/mL concentrations caused more tubule formation in IUGR than in late-onset PE group. Mann-Whitney U-test, p value <0.05. PE, pre-eclampsia; IUGR, intrauterine growth restriction.

study groups or compared to healthy pregnancies (Fig. 2).

There were no significant changes in angiogenesis with therapeutic dose of pravastatin when compared with tubule growth caused by maternal sera alone in any of the study groups or in healthy pregnancies. Although in some individual cases, particularly within the early-onset PE group, there seemed to enhancement of tubular formation. On the contrary, there was significant inhibition of angiogenesis at a concentration above therapeutic dose of pravastatin (1,000 ng/mL) in the healthy group (p=0.043). Additionally, in the IUGR group, there was a significant decrease in tubule formation between cultures with the lowest and the highest dose of pravastatin (p=0.043), and in the early-onset PE group between concentrations of 1,000 and 8,000 ng/mL of pravastatin (p=0.043) (Fig. 2).

When the study groups were compared with healthy pregnancies, there was significantly more tubule formation at concentrations of 20 ng/mL (p=0.047) of pravastatin in a comparison with the IUGR group. There were no differences in tubule formation between the 2 PE groups and healthy pregnancies at any dose of pravas-

tatin. However, when the late-onset PE group was compared with the IUGR group, there was more tubule formation in the IUGR group with every dose of pravastatin (from the lowest to the highest dose; p=0.009, p=0.009, and p=0.028). Otherwise, the were no differences in tubule formation between the groups (Fig. 3).

Discussion

In the in vitro hASC-HUVEC test, the therapeutic concentration of pravastatin did not affect tubule formation. At high concentrations, above therapeutic doses, pravastatin had an anti-angiogenic effect. In the presence of maternal sera from healthy or complicated pregnancies, angiogenesis was not enhanced by therapeutic concentration of pravastatin although maternal sera seem to modulate the effect of pravastatin on angiogenesis.

The vasculogenesis/angiogenesis test utilized in this study offers the possibility to study the effects of drugs on angiogenesis [11, 13]. Intra-laboratory validation has been performed for the hASC-HUVEC test (Environment Directorate, OECD), and good concordance in comparison with blood vessels in vivo has been observed [11]. We have previously utilized the present in vitro model to study maternal and umbilical sera in order to assess overall angiogenic capacity in PE and healthy pregnancies and to evaluate the effect of metformin on angiogenesis [14, 15, 18]. Since statins have reported to have anti-angiogenic effects in high doses, the pravastatin concentrations chosen for this study correspond to therapeutic and supratherapeutic levels in human. In pre-clinical and clinical studies, daily dose of pravastatin has varied from 10 to 40 mg [8, 10, 17]. When a 20 mg dose of pravastatin has been administered, the highest concentration of pravastatin in plasma has been 26.5 ng/mL [16]. Thus, in the present study the lowest dose of pravastatin (20 ng/mL) corresponds to the therapeutic dose. Although the other tested concentrations (1,000 and 8,000 ng/mL, respectively) are high compared to the therapeutic concentration, these are in line with other in vitro studies [17]. Despite the emergence of more and more in vivo resembling in vitro assays, there is still differences in effective drug concentrations between in vivo and in vitro test settings [19, 20]. Hence, the concentration results of in vitro assays need extrapolation to human in vivo effective concentrations. Since the data concerning the effect of pravastatin on angiogenesis are inconsistent, we wanted to analyse the effects of pravastatin with larger scale of concentrations and to limit our studies to concentrations which are not cytotoxic.

Pravastatin treatment has been shown to ameliorate PE symptoms in animal models [21, 22] and to improve pregnancy outcomes in human pilot studies [8, 23]. The pro-angiogenic effect of pravastatin has been suggested to be one of the pharmacodynamic mechanisms. In sFlt-1-induced animal models of PE, pravastatin treatment has resulted in increased PlGF concentrations [21] and reduced sFlt-1 and sEng concentrations [24]. In a human pilot study of Constantine et al. [8], it was reported that daily administration of pravastatin from 12 to 16 weeks onwards resulted in lower sFlt-1 and sEng concentrations and higher PlGF levels in the third trimester, versus placebo. Although the results were not statistically significant, pravastatin was thought to have contributed to the improved angiogenic profile [8]. In our study, the therapeutic concentration of pravastatin did not result in pro-angiogenesis. The presence of maternal sera from any of the groups did not change the result although there was a wide individual variation towards enhanced angiogenesis in the early-onset PE and IUGR groups. The concentrations of VEGF, PlGF, and sFlt-1 in test media were unaltered after pravastatin supplementation but the levels of the biomarkers were not detected from all. It is known that plasma sFlt-1 increases and PlGF decreases with advancing gestational age [25]. In our study, despite significant difference in gestational age between the groups, there were no difference in overall angiogenic properties of maternal sera between the groups. Hence, we cannot exclude beneficial effects of pravastatin on angiogenic capacity if maternal sera had been more anti-angiogenic.

Although many investigators have reported pro-angiogenic effects of pravastatin, some have found it to inhibit capillary-like tubular formation [22, 26]. Bauer et al. [22] demonstrated decreased endothelial tube formation in pravastatin-treated healthy rats. We also found an anti-angiogenic effect of high-dose pravastatin. This finding persisted when the effect of pravastatin (1,000 ng/mL) was studied in the presence of maternal sera from healthy pregnancies. The inhibitory effect on angiogenesis was not a result of cell death as there was no sign of cytotoxicity in WST-1 assay. Also other groups have previously observed that low concentrations of statins enhance angiogenesis and may promote vasculogenesis, whereas high statin concentrations inhibit angiogenesis [27, 28]. An anti-angiogenic effect at high concentrations has been associated with decreased endothelial release of VEGF and increased endothelial apoptosis [27]. In our study, there was a significant decrease in tubule formation in

connection with the lowest versus the highest dose of pravastatin in the IUGR group. Otherwise, such an effect was not observed. However, it seems that maternal sera modulate the effect of pravastatin on angiogenesis since the anti-angiogenic effect of high-dose of pravastatin was less strong or did not exist in the presence of maternal sera.

PE and cardiovascular diseases share several pathophysiological pathways as well as many risk factors. Currently it is known that women diagnosed with PE have an increased risk of developing chronic hypertension and cardiovascular disease when compared with women with a history of normotensive pregnancy [29]. Beyond their lipid-lowering effect, statins have several beneficial pleiotropic effects such as protection of vascular endothelium and inhibition of inflammatory responses [30]. Current data suggesting pravastatin as a promising drug for the prevention and treatment of PE is mainly limited to animal and pilot human studies. In the only randomized trial published, no difference in sFlt-1 levels after pravastatin treatment was seen in women with early-onset PE [10]. Hence, beneficial effects of pravastatin on BP and placental vasculature [31] may be the consequence of several pleiotropic effects rather than restored angiogenic balance. Above all, the potential contribution of pravastatin to angiogenesis is controversial as it has been shown to have both anti- and pro-angiogenic effects.

The angiogenic effect of pravastatin has not been studied before in the current setting. The strength of this study is that we measured not only direct effects of pravastatin on angiogenesis but also the interaction between pravastatin and maternal sera from complicated and healthy pregnancies. The results presented here are based on small numbers of serum samples, which is a limitation when interpreting the findings. In addition, 2 women were smoking which can have an influence on angiogenic biomarkers. Although larger sample number would have decreased the variation in the results, the information obtained in this study gives indication on where to focus the study of pravastatin in the treatment of PE.

Conclusions

Therapeutic dose of pravastatin alone or with sera from complicated pregnancies do not significantly change angiogenic capacity estimated by hASC/HUVEC in vitro assay. At high concentrations, pravastatin alone has a significant inhibitory effect on tubule formation. Interaction between pravastatin and maternal sera changes the direct effect on angiogenesis caused by pravastatin alone.

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Statement of Ethics

This study was approved by the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (permit R16184). The isolation and use of human adipose stromal cells (hASCs) and human umbilical vein endothelial cells (HUVECs) were approved (permits R15161 and R15033, respectively). Written informed consent was obtained from all participants.

Conflict of Interest Statement

The authors report no conflicts of interest.

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Author Contributions

A.V., O.H., K.T., T.T., T.H., and J.U. designed the study. O.H. and T.T. conducted the assays. A.V. ran statistical analyses and wrote the manuscript, except the methods section. Graphs and figures were prepared by A.V. and O.H. K.T., T.T., T.H., H.L., and J.U. have critically revised the manuscript and approved the final version for publication.

References

1 Mosca L, Benjamin EJ, Berra K, Bezanson JL, Dolor RJ, Lloyd-Jones DM, et al. Effectiveness-based guidelines for the prevention of cardiovascular disease in women-2011 update: a guideline from the american heart association. Circulation. 2011 Mar;123(11):1243–62.

2 Chen CW, Jaffe IZ, Karumanchi SA. Pre-eclampsia and cardiovascular disease. Cardiovasc Res. 2014 Mar;101(4):579–86.

3 Costantine MM, Cleary K. Pravastatin for the prevention of preeclampsia in high-risk pregnant women. Obstet Gynecol. 2013;121(2 Pt 1):349–53.

4 Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. BMJ. 2007 Nov; 335(7627):974.

5 Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet. 1994 Nov;344(8934): 1383–9.

6 Zhou Q, Liao JK. Statins and cardiovascular diseases: from cholesterol lowering to pleiotropy. Curr Pharm Des. 2009;15(5):467–78.

7 Nanovskaya TN, Patrikeeva SL, Paul J, Costantine MM, Hankins GD, Ahmed MS. Transplacental transfer and distribution of pravastatin. Am J Obstet Gynecol. 2013 Oct; 209(4):373–5.
8 Costantine MM, Cleary K, Hebert MF, Ahmed MS, Brown LM, Ren Z, et al. Safety and pharmacokinetics of pravastatin used for the prevention of preeclampsia in high-risk pregnant women: a pilot randomized controlled trial. Am J Obstet Gynecol. 2016 Jun; 214(6):720–e17.
9 Chaiworapongsa T, Romero R, Korzeniewski SJ, Chaemsaithong P, Hernandez-Andrade E, Segars JH, et al. Pravastatin to prevent recurrent fetal death in massive perivillous fibrin deposition of the placenta (MPFD). J Matern Fetal Neonatal Med. 2016 Mar;29(6):855–62.
10 Ahmed A, Williams DJ, Cheed V, Middleton LJ, Ahmad S, Wang K, et al. Pravastatin for early-onset preeclampsia: a randomized, blinded, placebo-controlled trial. BJOG. 2020 Mar;127(4):478–88.
11 Toimela T, Huttala O, Sabell E, Mannerström M, Sarkanen JR, Ylikomi T, et al. Intra-laboratory validated human cell-based in vitro vasculogenesis/angiogenesis test with serum-free medium. Reprod Toxicol. 2017 Jun;70: 116–25.
12 Sarkanen JR, Vuorenpää H, Huttala O, Mannerström B, Kuokkanen H, Miettinen S, et al. Adipose stromal cell tubule network model provides a versatile tool for vascular research and tissue engineering. Cells Tissues Organs. 2012;196(5):385–97.
13 Huttala O, Vuorenpää H, Toimela T, Uotila J, Kuokkanen H, Ylikomi T, et al. Human vascular model with defined stimulation medium: a characterization study. ALTEX. 2015; 32(2):125–36.
14 Virtanen A, Huttala O, Tihtonen K, Toimela T, Heinonen T, Uotila J. Angiogenic capacity in pre-eclampsia and uncomplicated pregnancy estimated by assay of angiogenic proteins and an in vitro vasculogenesis/angiogenesis test. Angiogenesis. 2019 Feb;22(1):67–74.
15 Virtanen A, Toimela T, Tihtonen K, Sarkanen JR, Huttala O, Heinonen T, et al. Strong inhibitory effect of pre-eclampsia serum on angiogenesis detected in vitro by human cell-based angiogenesis tests. Pregnancy Hypertens. 2016 Oct;6(4):367–73.
16 Food and Drug Administration 2011 Pravachol labeling [Internet]. Available from: https: //www.accessdata.fda.gov/drugsatfda_docs/nda/2011/019898Orig1s061.pdf.
17 Brownfoot FC, Tong S, Hannan NJ, Binder NK, Walker SP, Cannon P, et al. Effects of pravastatin on human placenta, endothelium, and women with severe preeclampsia. Hypertension. 2015 Sep;66(3):687–445; .
18 Virtanen A, Huttala O, Tihtonen K, Toimela T, Heinonen T, Laivuori H, et al. Therapeutic doses of metformin do not have impact on angiogenesis in presence of sera from pre-eclamptic, IUGR and healthy pregnancies. Pregnancy Hypertens. 2020 Jul;22:7–13.
19 Huntjens DR, Spalding DJ, Danhof M, Della Pasqua OE. Correlation between in vitro and in vivo concentration-effect relationships of naproxen in rats and healthy volunteers. Br J Pharmacol. 2006 Jun;148(4):396–404.
20 Checkley S, MacCallum L, Yates J, Jasper P, Luo H, Tolsma J, et al. Corrigendum: bridging the gap between in vitro and in vivo: Dose and schedule predictions for the ATR inhibitor AZD6738. Sci Rep. 2016;6:16545.
21 Kumasawa K, Ikawa M, Kidoya H, Hasuwa H, Saito-Fujita T, Morioka Y, et al. Pravastatin induces placental growth factor (PGF) and ameliorates preeclampsia in a mouse model. Proc Natl Acad Sci U S A. 2011 Jan;108(4):1451–5.
22 Bauer AJ, Banek CT, Needham K, Gillham H, Capoccia S, Regal JF, et al. Pravastatin attenuates hypertension, oxidative stress, and angiogenic imbalance in rat model of placental ischemia-induced hypertension. Hypertension. 2013 May;61(5):1103–10.
23 Lefkou E, Mamopoulos A, Dagklis T, Vosnakis C, Rousso D, Girardi G. Pravastatin improves pregnancy outcomes in obstetric antiphospholipid syndrome refractory to antithrombotic therapy. J Clin Invest. 2016 Aug; 126(8):2933–40.
24 Saad AF, Kechichian T, Yin H, Sbrana E, Longo M, Wen M, et al. Effects of pravastatin on angiogenic and placental hypoxic imbalance in a mouse model of preeclampsia. Reprod Sci. 2014 Jan;21(1):138–45.
25 Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med. 2004 Feb;350(7):672–83.
26 Asakage M, Tsuno NH, Kitayama J, Kawai K, Okaji Y, Yazawa K, et al. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor (pravastatin) inhibits endothelial cell proliferation dependent on G1 cell cycle arrest. Anticancer Drugs. 2004 Jul;15(6): 625–32.
27 Weis M, Heeschen C, Glassford AJ, Cooke JP. Statins have biphasic effects on angiogenesis. Circulation. 2002 Feb;105(6):739–45.
28 Urbich C, Dernbach E, Zeiher AM, Dimmeler S. Double-edged role of statins in angiogenesis signaling. Circ Res. 2002 Apr;90(6):737–44.
29 Lykke JA, Langhoff-Roos J, Sibai BM, Funai EF, Triche EW, Paidas MJ. Hypertensive pregnancy disorders and subsequent cardiovascular morbidity and type 2 diabetes mellitus in the mother. Hypertension. 2009 Jun; 53(6):944–51.
30 Davignon J. Beneficial cardiovascular pleiotropic effects of statins. Circulation. 2004 Jun; 109(23 Suppl 1):III39–43.
31 Singh J, Ahmed A, Girardi G. Role of complement component C1q in the onset of preeclampsia in mice. Hypertension. 2011 Oct; 58(4):716–24.