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Heli Saarelainen

Endothelial Function and Regulation of Vascular Tone in Normal and Complicated Pregnancies

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Author's address: Department of Obstetrics and Gynecology
Kuopio University Hospital
P.O. Box 1777, FI-70211 KUOPIO
FINLAND
e-mail: heli.saarelainen@kuh.fi

Supervisors: Professor Seppo Heinonen, M.D.,Ph.D.
Department of Obstetrics and Gynecology
Kuopio University Hospital
P.O. Box 1777, FI-70211 KUOPIO
FINLAND

Professor Tomi Laitinen, M.D., Ph.D.
Department of Clinical Physiology and Nuclear Medicine
Kuopio University Hospital
P.O. Box 1777, FI-70211 KUOPIO
FINLAND

Reviewers: Professor Ganesh Acharya, M.D., Ph.D
Department of Obstetrics and Gynecology
University Hospital of North Norway
P.O. Box 24, N-9038 TROMSØ
NORWAY

Docent Eeva Ekholm, M.D., Ph.D
Department of Obstetrics and Gynecology
University Hospital of Turku
P.O. Box 52, FI-20521 TURKU
FINLAND

Opponent: Professor Risto Kaaja, M.D., Ph.D.
Satakunta Central Hospital and University Hospital of Turku
P.O. Box 52, FI-20521 TURKU
FINLAND

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ABSTRACT

Preeclampsia and cardiovascular disease, and also gestational diabetes and type 2 diabetes share the same risk factors (genetic predisposition, obesity, metabolic syndrome, hyperlipidemia, high blood pressure) and probably at least partly the same pathophysiology. Knowledge of how normal or complicated pregnancy affects the endothelium can help in evaluating the risks for future cardiovascular disease and being able to provide advice on how to prevent or postpone the disease.

This study was undertaken to explore endothelial function in normal pregnancy as well as in pregnancy related complications that are related to endothelial damage. During pregnancy the increase in nitric oxide bioavailability enhances endothelial function despite the presence of marked hyperlipidemia and inflammation. Even in pregnancy related hypertensive and metabolic disorders, i.e. mild to moderate preeclampsia and gestational diabetes, endothelial function was not impaired when measured noninvasively from the brachial artery. Thus brachial artery flow mediated dilation does not seem to be suitable for clinical use for example in predicting preeclampsia or detecting the endothelial damage in current disease according to our results. Pregnancy related complications are known risk factors for future diseases. Hypertension in pregnancy and preeclampsia carry the risk for hypertension and cardiovascular diseases later in life. Approximately 30 % of the women with gestational diabetes but almost none of those with normal glucose tolerance during pregnancy will develop type 2 diabetes in 15 years surveillance. We found that soon after parturition, women with previous gestational diabetes exhibit marked cardiovascular risk factors, such as obesity and elevated serum lipid and glucose concentrations. Although these risk factors were not associated with endothelial damage, these women have an increased risk of developing diabetes mellitus type 2 and cardiovascular disease. An intervention at this stage may be highly advantageous when arteriosclerosis is still preventable with lifestyle changes and, if required, other interventions.

National Library of Medicine Classification: WQ200; WQ 500; WQ 240; WQ 248; WQ 215
Medical Subject Headings: Pregnancy; endothelium; vasodilation; preeclampsia; diabetes; gestational.

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TIIVISTELMÄ

Tutkimuksen tavoitteena oli tarkastella verisuonten sisäkerroksen eli endoteelin toimintaa normaaliraskaudessa ja sellaisissa raskauksissa, joissa synnyttäjällä oli raskausmyrkytys, raskauden aikainen verenpainetauti tai raskausajan diabetes. Verisuonten sisäkerroksen eli endoteelin toimintaa arvioitiin olkavarsivaltimosta mittaamalla ultraäänellä verisuonen laajenemista puristuksen jälkeen. Verisuonen endoteelin toiminta parani normaalin raskauden aikana. Verisuonet laajenivat lisääntyneen typpioksidipitoisuuden ansiosta parantaen näin kohdun verenkiertoa sikiötä varten. Raskaudenaikainen veren rasva-arvojen voimakas nousu tai tulehdusta välittävien aineiden pitoisuuden lisääntyminen eivät huonontaneet verisuonten toimintaa raskauden aikana, vaikka ilman raskautta niiden tiedetään aiheuttavan verisuonen sisäkerroksen toimintahäiriöitä. Raskaudenaikaisen verenpainetaudin tai raskausmyrkytyksen ei todettu huonontavan olkavaltimon endoteelin toimintaa. Aiemmin on osoitettu, että naisen raskausajan verenpainetauti ja raskausmyrkytys lisäävät merkittävästi myöhempää verenpainetauti- ja sydän- ja verisuonisairausriskiä. Hyvässä hoitotasapainossa oleva raskausdiabetes ei heikentänyt verisuonten toimintaa. Raskausajan diabeteksen ajatellaan paranevan synnytyksen jälkeen, mutta näillä naisilla on todettu suurentunut todennäköisyys sairastua 1 tai 2 tyypin diabetekseen. Tutkimuksessamme raskauden ajan diabetesta sairastaneilla naisilla veren rasva- ja sokeriarvot sekä paino olivat merkittävästi koholla kolmen kuukauden kuluttua synnytyksestä verrattuina normaaliraskauden jälkeen tutkittuihin naisiin. Jo raskauden aikana tulisi antaa tietoa raskausajan komplikaatioiden pitkäaikaisvaikutuksista ja elämäntapaohjausta diabeteksen ja sydän- ja verisuonisairauksien ehkäisystä.

Luokitus:
Yleinen Suomalainen asiasanasto: Raskaus; verisuonet; endoteeli, pre-eklampsia, raskausdiabetes.

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List of original publications

This thesis is based on the following original publications, which are referred in the text by their Roman numerals I-V:

I Saarelainen H, Laitinen T, Raitakari OT, Juonala M, Heiskanen N, Lyyra-Laitinen T, Viikari JS, Vanninen E, Heinonen S. Pregnancy-related hyperlipidemia and endothelial function in healthy women. *Circ J 2006;70(6):768-72*.

II Saarelainen H, Valtonen P, Punnonen K, Laitinen T, Raitakari OT, Juonala M, Heiskanen N, Lyyra-Laitinen T, Viikari JS, Vanninen E, Heinonen S. Subtle changes in ADMA and l-arginine concentrations in normal pregnancies are unlikely to account for pregnancy-related increased flow-mediated dilatation. *Clin Physiol Funct Imaging 2008;28(2):120-4.*

III Saarelainen H, Valtonen P, Punnonen K, Laitinen T, Raitakari OT, Juonala M, Heiskanen N, Lyyra-Laitinen T, Viikari JS, Heinonen S. Flow mediated vasodilation and circulating concentrations of high sensitive C-reactive protein, interleukin-6 and tumor necrosis factor-alpha in normal pregnancy – The Cardiovascular Risk in Young Finns Study. *Clin Physiol Funct Imaging 2009;29(5):347-52.*

IV Heli Saarelainen, Henna Kärkkäinen, Pirjo Valtonen, Kari Punnonen, Tomi Laitinen, Nonna Heiskanen, Tiina Lyyra-Laitinen, Esko Vanninen, and Seppo Heinonen. Flow-Mediated Vasodilation Is Not Attenuated in Hypertensive Pregnancies Despite Biochemical Signs of Inflammation. *ISRN Obstetrics and Gynecology 2012;2012: Article ID 709464*. Epub 2012 Jan 17.

V Heli Saarelainen, Henna Kärkkäinen, Pirjo Valtonen, Tomi Laitinen, Nonna Heiskanen and Seppo Heinonen. Adequately managed patients with gestational diabetes have normal endothelial function during pregnancy. Submitted.

Contents

Abbreviations

ACh	acetylcholine
ACEI	angiotensin converting enzyme inhibitor
ADMA	asymmetric dimethylarginine
AF-1	activation function 1
AGA	appropriate for gestational age
AGE	advanced glycation end product
ANOVA	analysis of variance
AUC	area under curve
BMI	body mass index
BP	blood pressure
cAMP	cyclic adenosinemono phosphate
cGMP	cyclic guanosinemono phosphate
CKD	chronic kidney disease
CMV	cytomegalovirus
CO	cardiac output
CRP	C-reactive protein
CT	computerized tomography
CVD	cardiovascular disease
DM	diabetes mellitus
DDAH	dimethylarginine dimethylaminohydrolase
E2	17-beta-estradiol
EC	endothelial cell
ECG	electrocardiography
EDHF	endothelium-derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
EMP	endothelial micro particles
EPC	endothelial progenitor cells
EPO	erytropoietin
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
FMD	flow mediated dilation

GDM	gestational diabetes mellitus
GFR	glomerular filtration rate
GTN	glyceryl trinitrate
HDL	high density lipoprotein
ICAM	intercellular adhesion molecule
IGT	impaired glucose tolerance
INF	interferon
IL	interleukin
IUGR	intra uterine growth restriction
Kc	calcium activated potassium channel
LDL	low density lipoprotein
LPC	lysophosphatidylcholine
MCP-1	monocyte chemotactic protein-1
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NF-κB	nuclear factor κ B
NO	nitric oxide
NS	non-significant
NTG	nitroglycerine
OC	oral contraceptive
OGTT	oral glucose intolerance test
OPA	ortho-phthaldialdehyde
PAT	finger pletysmography
PCA	pulse wave contour analysis
PDE	cyclic nucleotide phosphodiesterase
PET	positron emission tomography
PG12	prostacycline
PRMT	protein arginine N-methyltransferase
PWV	pulse wave velocity
RCT	reverse cholesterol transport
ROS	reactive oxygen species
RR	relative risk, risk ratio
SDMA	symmetric dimethylarginine

SERM	selective estrogen receptor modulator
TC	total cholesterol
TNF	tumour necrosis factor
TLR	toll-like receptor
trigly	triglycerides
VCAM-1	vascular cell adhesion molecule-1

1 Introduction

Sir Thomas Lauder Brunton first used amyl nitrite in the treatment of angina pectoris in 1867(Brunton. 1867). As a medical student, Brunton had become aware of the prior clinical findings of Benjamin Ward Richardson that inhaled amyl nitrite rapidly increased the activity of the heart(Richardson. 1864), and also the unpublished observations of Arthur Gamgee demonstrating that amyl nitrite greatly lessened 'arterial tension' in both animals and man. During the same period in which Brunton used amyl nitrite, another British physician, William Murrell, began using the organic nitrate, GTN, in the treatment of angina pectoris. With nitroglycerine therapy, patients would obtain relief from angina with some patients also reporting that their angina attack could be aborted by taking the drug at the very onset of symptoms(Murrel. 1897). Murrell's discovery of NTG, the world's first synthesized drug, for the treatment of angina pectoris is still in therapeutic use 140 years later(Nossaman, et al. 2010).

In pregnancy the endothelial production of nitric oxide (NO) is increased in the systemic and in the uterine vasculature in order to maintain adequate oxygen and nutrient supply to the growing fetus(Boeldt, et al. 2011). Several physiological changes take place during pregnancy: cardiac output increases, plasma volume increases, peripheral resistance falls, blood pressure first falls reaching its nadir in the second trimester, and then rises again with the approach of term. The metabolic changes include alterations in insulin sensitivity, requirements for insulin secretion increase, and there are elevated serum concentrations of lipids(An-Na, et al. 1995). The parasympathetic nervous system deactivates(Ekholm, et al. 1994) and a complex interplay of inflammatory events is regulated by both the innate and acquired immune systems(MacIntyre, et al. 2012). Sometimes these physiologic demands are excessive, and complications such as hypertension in pregnancy, preeclampsia and gestational diabetes appear. These complications are relatively common and usually reversible and forgotten after pregnancy. However these complications can be viewed as a canary in a coal mine, i.e. they are the first warning signs of looming chronic diseases. If a woman has suffered preeclampsia then the Framingham score based, calculated 10-year cardiovascular disease risk odds ratio is 1.31 (95% confidence interval 1.11 – 1.53) when compared to those with normal pregnancies(Fraser, et al. 2012). There is much evidence that pregnancy seems to expose those women who have reduced capacity of insulin secretion or who have insulin resistance and are at risk of developing diabetes later in life. Women with earlier gestational diabetes display an increased risk of developing type 2 diabetes compared with those who had a normoglycemic pregnancy (RR 7.43, 95% CI 4.79-11.51)(Bellamy, et al. 2009) and in another study, the diabetes risk was 40% in those with GDM in pregnancy over the ten subsequent years (Lauenborg, et al. 2004). In contrast, none of the women with normal glucose metabolism developed diabetes in six years follow-up(Järvelä, et al. 2006). Preeclampsia is associated with insulin resistance(Kaaja, et al. 1999) and may also increase the subsequent risk for diabetes(McDonald, et al. 2008). If it were possible to predict, diagnose and treat these pregnancy-related complications, this would reduce the morbidity of the

mothers and fetuses not only during pregnancy and shortly after birth, but could also have beneficial effects on their health far into the future.

The aim of this thesis was to clarify the factors contributing to the vascular health of pregnant women. Normal pregnant women and women with pregnancy related complications such as hypertension, preeclampsia and gestational diabetes were examined during and after pregnancy. We assessed the endothelial function using flow mediated vasodilation (FMD) from brachial artery. We further evaluated the concentrations of glucose, lipids, inflammatory markers and asymmetric dimethylarginine (ADMA) in these women and we estimated whether these markers were related to endothelial function.

2 *Review of the literature*

2.1 THE VASCULAR ENDOTHELIUM AND ITS FUNCTIONS

The arterial wall has 3 layers: the intima, including the endothelium, the media, and the adventitia (Figure 1). Each of these layers has individual roles in the systemic circulation. The endothelium is a monolayer of cells on blood and lymphatic vessels. The thin squamous type of epithelium was virtually invisible in light microscopy and initially considered as a nonessential cellophane-like sheet. Now the endothelial cells (ECs), have earned the profound respect of biologists and pathologists in the relatively short time of 50 years (Simionescu. 2007).

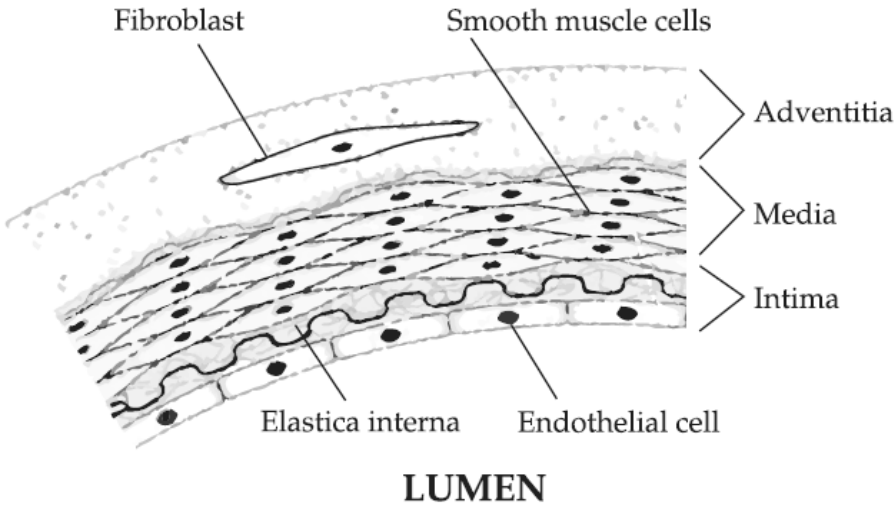


Figure 1. The arterial wall.

The endothelium is an active organ, and its functions are listed below and presented in figure 2.

The functions of endothelium.

1. Regulation of vascular tone
2. Regulation of vascular permeability
3. Pro- and anticoagulant activity
4. Contribution to the balance of pro- and anti-inflammatory mediators
5. Role in generation of new blood vessels
6. Interaction with circulating blood cells

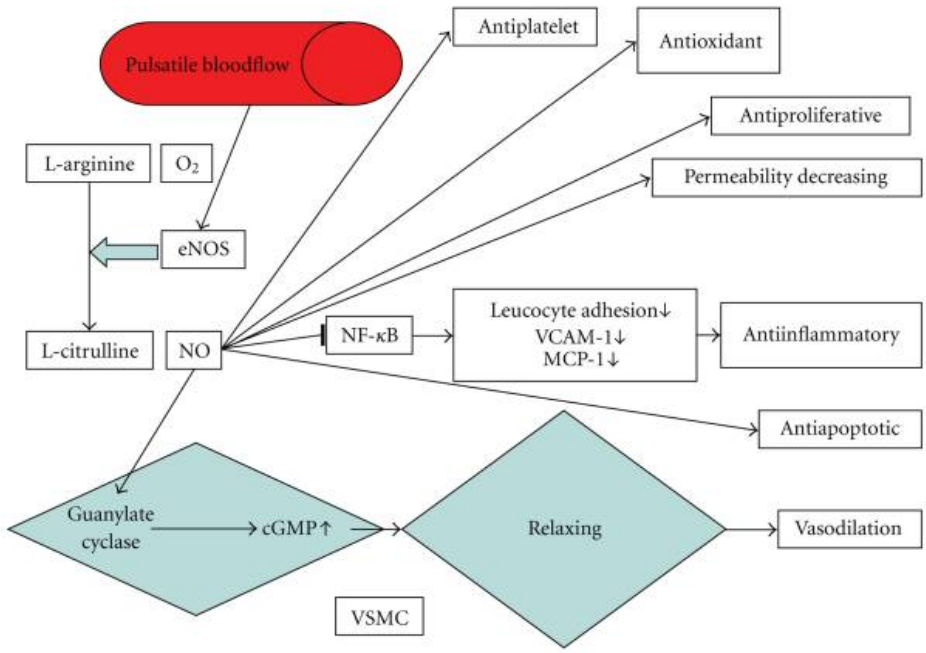


Figure 2. Functions of endothelium. eNOS endothelial nitric oxide synthase, NO nitric oxide, cGMP cyclic guanisine monophosphate, NF-κβ nuclear factor κβ, VCAM-1 vascular cell adhesion molecule -1, MCP-1 monocyte chemotactic protein-1. With permission from(Van der Oever, et al. 2010).

Endothelial cells are heterogenic; the phenotype varies according to the requirements of the individual organ(Deanfield, et al. 2007). Endothelial cells receive and respond to signals from both surrounding cells and tissues and flowing blood. The response to a given stimulus may vary dramatically from one vascular bed to another. Quiescent endothelial cells display the thromboresistant, anti-adhesive and vasodilatory phenotype, whereas activated endothelial cells have procoagulant, pro-adhesive, and vasoconstricting properties. The normal relatively dilated state of the vascular wall is maintained mainly by nitric oxide(Aird. 2008). The principal

physiologic stimulus for endothelial NO synthesis is blood flow-induced shear stress. This process is called flow mediated vasodilation.

2.2 ASSESSMENT OF ENDOTHELIAL FUNCTION

Endothelial function can be evaluated via the following different approaches: (1) measurement of morphological and mechanical characteristics of the vascular wall (eg, intima media thickness, compliance, distensibility, and remodelling indexes); (2) determination of soluble endothelial markers (eg,von Willebrandt factor, thrombomodulin, adhesion molecules, plasminogen activator inhibitor complex and N-oxides); and (3) measurement of the endothelium-dependent regulation of vascular tone at focal sites of the circulation. Endothelial function can be measured in coronary arteries and in the peripheral vessels by measuring vasomotor function after intra-arterial infusion of vasoactive substances e.g. acetylcholine (ACh) which enhances the release of endothelial NO (Table 1.). The disadvantage of these methods is their invasive nature involving arterial cannulation. These tests are generally unsuitable for subjects who are at risk for atherosclerosis but who have no clinical symptoms or signs of disease(Raitakari, et al. 2000). For this reason, non-invasive tests of endothelial function have been developed. Currently the main noninvasive techniques to assess endothelial functions are flow-mediated vasodilation (FMD) as measured by ultrasound of the brachial artery, pulse wave contour analysis (PCA), and finger plethysmography during postischemic hyperemia(PAT) (Table 2.). Other invasive and noninvasive methods for measuring coronary microvascular function have been recently reviewed, such as magnetic resonance imaging (MRI), positron emission tomography scanning (PET), CT scanning, single photon emission CT, Doppler echocardiography, Doppler flow wire, temperature and pressure sensor tripped coronary wire, or thrombolysis in myocardial infarction framecount and myocardial blush score(Arrebola-Moreno, et al. 2011).

Table 1. Pharmacological stimuli that affect endothelial function

Substance	Result
ACh infusion	vasodilation
	paradoxical vasoconstriction in atherosclerosis based on smooth muscle cell contraction in the absence of NO release from endothelium
Nitroglycerine	vasodilation
Substance P	vasoconstriction
Adenosine	vasodilation
Bradykinine	vasoconstriction

Table 2. Noninvasive techniques to assess endothelial function

Technique	Site	Method	Description	Interpretation
FMD	Brachial artery	Ultrasound	A pneumatic cuff is placed distally to the arm, inflated and then released causing increase in flow "shear stress", then maximum vasodilation is measured with ultrasound.	Measures the capacity of endothelial cells to produce NO, and smooth muscle cell capacity to relax, percentage of maximum change from the baseline diameter.
PCA	Radial artery	Photo-plethysmo-graphy/ tonometry	Radial artery pulse waves are recorded.	Oscillatory compliance is reduced in disease states.
PAT	Finger	Finger pneumatic plethysmo-graphic cuff	Beat to beat blood flow volume is assessed.	Post-ischemic blood flow is compared to baseline blood flow to assess the endothelial function.

List of commonly used markers of endothelial dysfunction.

Endothelin-1

Tissue plasminogen activator (TPA)

Plasminogen activator inhibitor-1 (PAI-1)

Soluble adhesion molecules

Von Willebrandt factor (vWF)

Asymmetric dimethylarginine (ADMA)

Vascular endothelial growth factor (VEGF)

Endothelial microparticles (EMPs)

Endothelial progenitor cells (EPCs)

The concentrations of these peptides increase when the endothelium is activated or damaged, and they are predictive at least to a certain extent of the risk, presence and the severity of vascular disease. Endothelial dysfunction may be assessed indirectly by plasma biomarkers that, although generally nonspecific, can provide an indication of cellular malfunction. Currently, in hypercholesterolemic or diabetic patients, the best indicators of EC dysfunction are showed to be the plasma NO metabolites (nitrites, nitrates), the increased concentration of vasoconstrictors (i.e. endothelin-1), von Willebrandt factor, and others(Simionescu. 2007). Various cell types release small membrane vesicles called microparticles (MP) on their activation, as well as during the process of apoptosis. The properties and roles of MP generated in different contexts are both diverse and are determined by their parent cell and the pathway of their generation which affects their content. MPs have been reported to be involved in multiple cellular functions, including immunomodulation, inflammation, coagulation, and intercellular communication(Shai and Varon. 2011). Investigations of MP could elucidate new cellular communication pathways and may lead to a better understanding of the underlying pathophysiological processes. From a clinical point of view, MP may serve as biomarkers for disease and may be found useful for developing novel therapeutic strategies targeting angiogenesis-related conditions(Shai and Varon. 2011).

2.2.1 High sensitive C-reactive protein

Elevated high sensitive C-reactive protein (hsCRP) serum levels are indicative of a systemic inflammatory response and have been associated with a blunted systemic endothelial vasodilator function(Fichtlscherer, et al. 2000). In Framingham study, CRP correlated significantly to reactive hyperemia even after adjustment of traditional cardiovascular risk factors, but not to flow mediated vasodilation. It was concluded that inflammation had no attributable effects on endothelial function beyond traditional risk factors(Vita, et al. 2004).

Järvisalo et al. showed that children with higher levels of serum ultrasensitive C-reactive protein (≥0.1 to ≤0.7 and >0.7 mg/liter) demonstrated lower flow-mediated vasodilation than children with ultrasensitive CRP levels that were under the detection limit (less than 0.1 mg/l) ($P = 0.015$ for trend). The level of CRP remained a significant independent predictor for brachial artery flow-mediated vasodilation in multivariate analysis(Järvisalo, et al. 2002).

2.2.2 Proinflammatory cytokines tumor necrosis factor-α and interleukin-6

Tumor necrosis factor-α (TNF-α) is a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor superfamily. This cytokine is mainly secreted by macrophages. It can bind to and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. It has been implicated in a variety of diseases, including autoimmune diseases, insulin resistance, and cancer. Knockout studies in mice also indicated that TNF-α has a neuroprotective function(Chudek and Wiecek. 2006, Gu, et al.).

Interleukin-6 (IL-6) is a cytokine that functions in inflammation and the maturation of B cells. In addition, it has been shown to be an endogenous pyrogen capable of inducing fever in subjects with autoimmune diseases or infections. The protein is primarily produced at sites of acute and chronic inflammation, where it is

secreted into the serum and induces a transcriptional inflammatory response through the interleukin-6 receptor. IL-6 is implicated in a wide variety of inflammation-associated disease states, including susceptibility to diabetes mellitus and systemic juvenile rheumatoid arthritis(Gu, et al.).

Pro-inflammatory cytokines (TNF-α and IL-1β alone but not IL-6) induce transient and reversible endothelial dysfunction and cyclooxygenase activity may contribute to the genesis of the effect(Bhagat, et al. 1997).

2.2.3 Asymmetric dimethylarginine

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase (NOS) since it can compete with the binding of the natural substrate L-arginine(Leone, et al. 1992). The pathway of the synthesis and elimination of ADMA is presented in the figure 3.

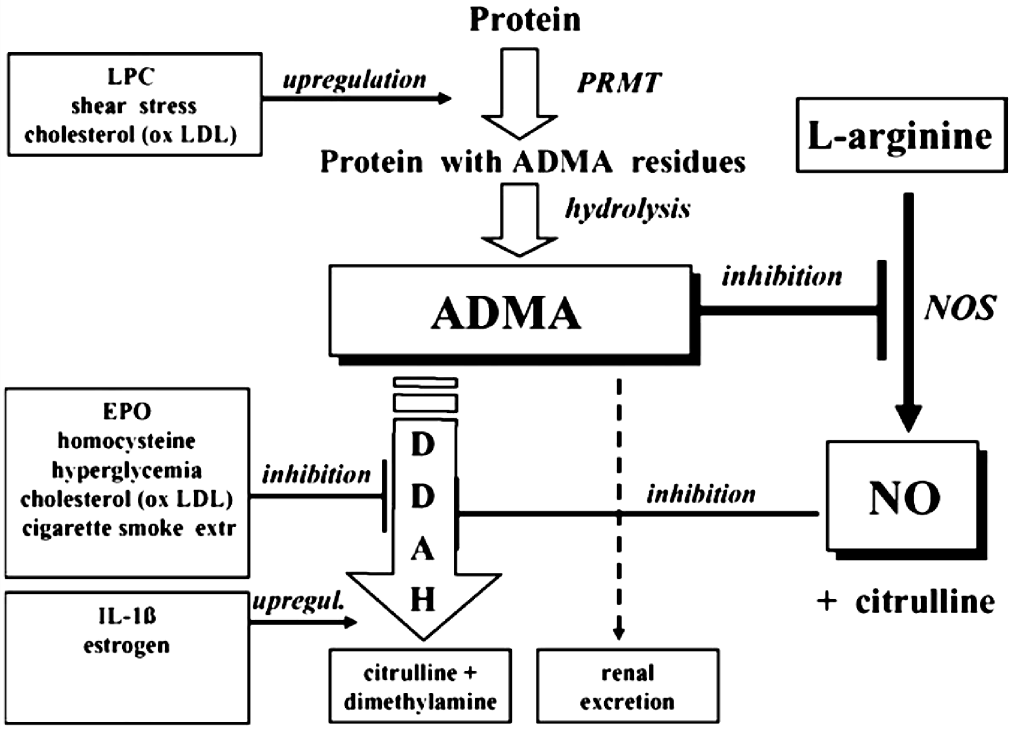


Figure 3. Biochemical pathways for the generation, elimination and degradation of ADMA. ADMA derives from methylation of arginine residues in proteins. The reaction is catalyzed by protein arginine N-methyltransferases (PRMT). Hydrolysis of the methylated proteins releases ADMA, which competitively inhibits NOS. Renal excretion accounts for only 20% of ADMA elimination. The primary route of elimination (80%) is ADMA metabolism through the enzyme dimethylargininedimethylaminohydrolase (DDAH). DDAH hydrolyzes ADMA to form dimethylamine and L-citrulline. Generation of ADMA can be increased by shear stress, oxidized LDL and lysophosphatidylcholine (LPC). Many cardiovascular risk factor like cholesterol (oxLDL), homocysteine, hyperglycemia, cytomegalovirus (CMV) infection and cigarette smoke decrease DDAH activity and thereby increase ADMA. IL-1β and estrogen have been shown to increase DDAH activity and to lower plasma ADMA. With permission from(Kielstein et al. 2007).

ADMA increases vascular resistance and blood pressure in humans. ADMA is a strong and independent risk factor for mortality and cardiovascular events, both in the general population and in patients at different stages of chronic kidney disease(Jourde-Chiche, et al. 2011). Elevated plasma ADMA concentrations are known to be associated with decreased brachial FMD responses in healthy adults(Juonala, et al. 2007). However, in a recent study among 289 patients with coronary artery disease, no correlation was detected between plasma ADMA concentration and coronary response to acetylcholine, adenosine, or nitroglycerin(Maas, et al. 2007) The associations between FMD and methylarginines have been rather weak and this may explain why the relation has not been detected in all studies. Pregnancy related changes in ADMA concentrations are discussed in the chapter "Endothelium and regulation of vascular tone in preeclampsia".

2.3 FLOW MEDIATED DILATION

Flow mediated dilation (FMD) is a term used to describe any vasodilation of an artery following an increase in luminal blood flow and internal-wall shear stress (Fig. 4). However, the term has conventionally come to describe subtle variations to the technique introduced by Celermajer, Deanfield, and colleagues in the Lancet in 1992(Celermajer, et al. 1992). Today FMD is now the most commonly used noninvasive assessment of vascular endothelial function in humans.

FMD refers to the assessment of peripheral conduit artery diameter following a period of distal limb ischemia by ultrasound. Nobel prize-winning experiments by Furchgott established that the endothelium produces a labile vasodilator substance(Furchgott and Zawadzki. 1980). Subsequently it was discovered that in response to flow, the endothelium had released a substance that possessed all of the characteristics of Furchgott's endothelium-derived relaxing factor; it was later identified as nitric oxide (NO)(Moncada, et al. 1988). The FMD technique has increasingly been applied in physiological studies to examine the mechanisms that alter vascular function and CVD risk (e.g., exercise training, smoking, hypercholesterolemia, hypertension)(Thijssen, et al. 2011). The FMD test represents an important way to improve our physiological insights and understanding of mechanisms that alter endothelial and vascular function. However, it is clear that minor changes in the methodological approach can critically impact on the nature and magnitude of the FMD response(Thijssen, et al. 2011). Since it is non-invasive, this method has been applied widely in asymptomatic subject groups, including children and young adults(Järvisalo, et al. 2006) and it also is suitable for pregnant women. The method has even been used in large population-based studies involving thousands of study subjects(Juonala, et al. 2004).

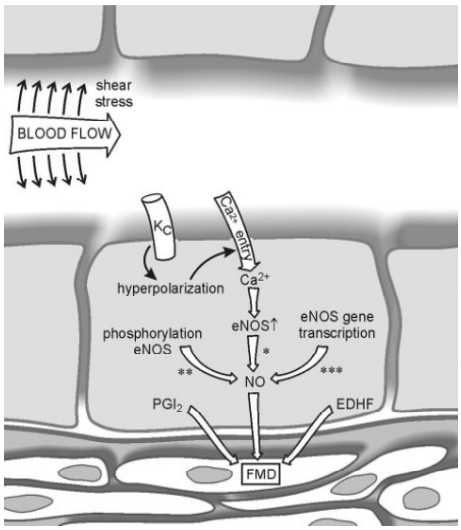


Figure 4. The genesis of FMD, in response to different changes in shear stress. * Very short-term changes; ** changes taking place over slightly longer periods (minutes); *** changes taking place over a longer time (many minutes or hours). PGI2 prostacyclin; EDHF endothelium-derived hyperpolarizing factor; Kc calcium-activated potassium channel. With permission from(Moens, et al. 2005).

2.3.1 Limitations and weaknesses of flow mediated vasodilation

The technical and methodological limitations of FMD are listed below(Inaba, et al. 2010). In addition, there is extensive variability between studies with respect to the protocols applied, methods of analysis, and interpretation of results. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery have been established(Corretti, et al. 2002) This technique is considered to be particularly well suited for study of the earliest stages of atherosclerosis in children and young adults, thus providing maximal opportunities for implementing prevention measures(Corretti, et al. 2002).

Limitations of FMD

1. Methodologies used for the measurement of FMD are not standardized between various vascular laboratories and thus there are no universal cut-off values for FMD, making it difficult to compare the results
2. Due to the physiological fluctuations, there is a large intraindividual variation in intersession FMD
3. A high-quality ultrasound device is necessary, those devices are expensive and demanding to use

There is a wide interindividual variation in mean FMD so the accurate comparison of individual FMD data is somewhat troublesome. Figure 5 presents the FMD% values of 157 women in pregnancy by gestational weeks.

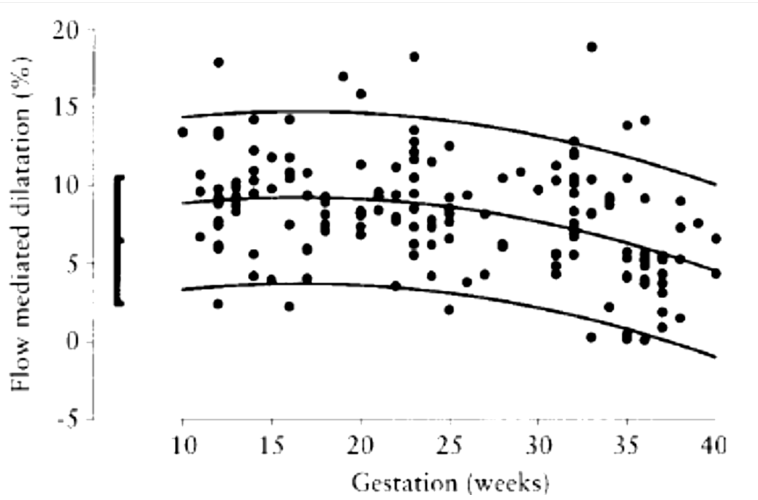


Figure 5. Flow mediated dilation of the brachial artery with gestation. Individual values and regression lines of the mean, 95th and 5 th centiles. Vertical line stands for 95th, mean and 5th percentiles for 19 nonpregnant controls. With permission from (Savvidou, et al. 2000).

2.3.2 Clinical use of flow mediated vasodilation

Brachial artery flow mediated vasodilation is mainly used for studying populations, but FMD is not merely a laboratory curiosity, it also has clinical applications. The most common finding is that reduced FMD is associated with various cardiovascular risk factors. It has been claimed that FMD strongly predicts cardiovascular events in patients with established cardiovascular disease(Thijssen, et al. 2011). Over a 6-year follow-up, FMD was reported to correlate with the progression of preclinical carotid artery disease, showing a closer relationship with disease progression than conventional risk factors(Halcox, et al. 2009). Impaired FMD has been reported to predict short and long term cardiovascular events(Gokce, et al. 2003)(Karatzis, et al. 2006)(Schachinger, et al. 2000). Impaired flow mediated vasodilation in the brachial artery (5.8 % ± 3.4 vs. 9.0 % ± 4.8, p = 0.005) four weeks after percutaneous coronary intervention independently predicted the risk of clinical restenosis(Munk, et al. 2011).

2.4 THE FACTORS THAT ALTER ENDOTHELIAL FUNCTION

In addition to specific medical illnesses FMD can be influenced by recent aerobic or resistance exercise(Black, et al. September 2009)(Dawson, et al. 2008) (Harris, et al. 2008), dietary intake, caffeine(Papamichael, et al. 2005) and alcohol ingestion(Hijmering, et al. 2007), and use of supplements or medications(Magen, et al. 2005)(Harris, et al. 2009), smoking, menstrual phase and repeated measurements(Harris, et al. 2010). Endothelial dysfunction referred initially to structural changes in endothelium, such as those seen in atherosclerosis, but nowadays this term is used to describe the loss of the endothelium's ability to regulate vascular resistance.

2.4.1 Inflammation

Almost every stimulus evoking to a systemic inflammatory response i.e. severe infection, trauma, excessive tissue breakdown, solid tumours, leukaemia, pregnancy associated complications such as hypertensive disorders and GDM, liver failure, and toxicological or immunological responses and activation of the coagulation system can be associated with endothelial damage(Paulus, et al. 2011). Inflammatory processes associated with endothelial damage, with a special focus on arteriosclerosis, are presented in figure 6.

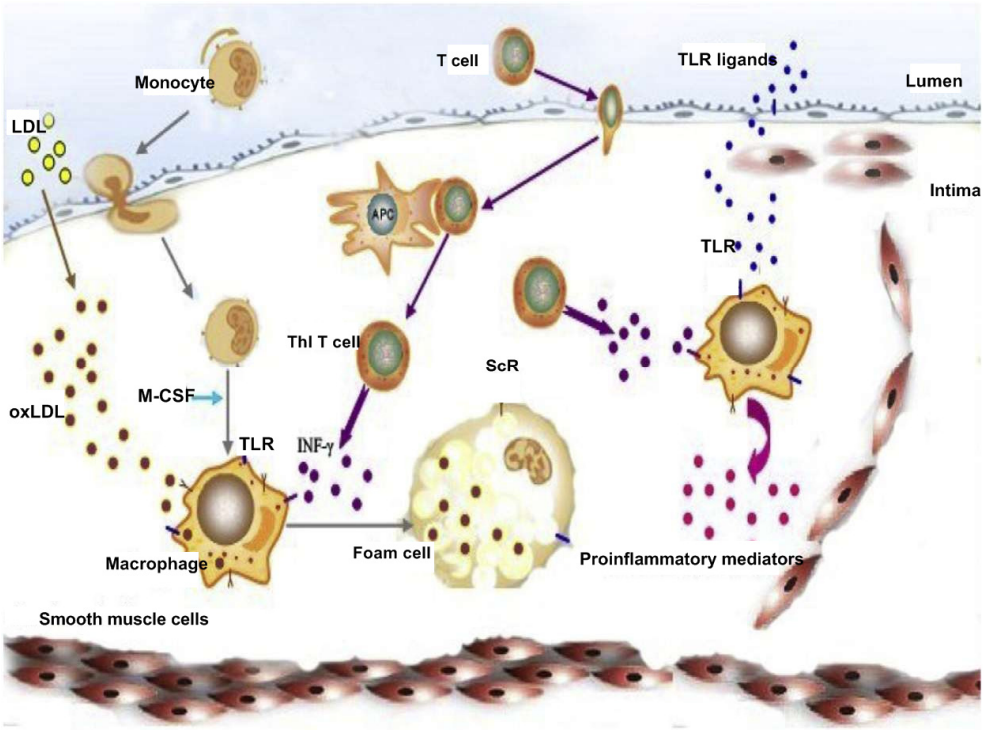


Figure 6. Inflammatory processes that are involved in arteriosclerosis. Low-density lipoprotein (LDL) infiltrates the artery wall and undergoes modification by oxidation. The modified LDL particles then induce the expression of adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Monocytes migrate into the vessel wall and differentiate into macrophages. After migration through the endothelial layer, the monocytes differentiate into macrophages and scavenge the oxidized low density lipoprotein (oxLDL) from the vessel wall, resulting in foam cell formation. Stimulation of toll-like receptors (TLRs) of macrophages results in the release of several proinflammatory cytokines, such as TNF-α, IL-1β and IL-6. T cells become activated during this time and produce other mediators such as interferon (INF-γ), which further amplifies the inflammatory response and contributes to atherogenesis. The activation of TLRs also induces expression of matrix-degrading matrix metalloproteinases (MMPs), which probably play a role in weakening the fibrous cap and promoting plaque vulnerability. With permission from(Gu, et al. 2012).

Cardiovascular risk factors, such as smoking, hypercholesterolemia, and elevated blood pressure give rise to a variety of noxious stimuli that elicit secretion of both

leukocyte soluble adhesion molecules, which facilitate the attachment of monocytes to endothelial cells, and chemotactic factors, which encourage the migration of monocytes into the subintimal space. The transformation of monocytes into macrophages and the uptake of cholesterol lipoproteins are thought to initiate the fatty streak. Further injurious stimuli may continue the attraction and accumulation of macrophages, mast cells, and activated T cells within the growing atherosclerotic lesion, and secretion of metalloproteinases and other connective tissue enzymes by activated macrophages may break down collagen, weakening the cap and making it prone to rupture. Thus, virtually every step in atherogenesis is believed to involve cytokines, other bioactive molecules, and cells that are characteristically associated with inflammation(Pearson, et al. 2003).

2.4.2 Hypertension

An association between endothelial dysfunction and hypertension is well established. Data from the Framingham offspring cohort suggest that the severity of hypertension is positively associated with the degree of impairment of endothelial function(Benjamin, et al 2004).

Endothelium mediated vascular relaxation following acetylcholine infusion is reduced in essential hypertension compared to normotensive controls(Panza, et al. 1990). The pathophysiology of hypertension involves a complex interaction of multiple vascular effectors including the activation of the sympathetic nervous system, of the renin–angiotensin–aldosterone system and of the inflammatory mediators. Subsequently vasoconstriction and inflammation ensue, leading to vessel wall remodeling and finally, to the formation of atherosclerotic lesions as the hallmark of advanced disease. Oxidative stress and endothelial dysfunction are consistently observed in hypertensive subjects, but emerging evidence suggests that they also have a causal role in the molecular processes leading to hypertension(Schulz, et al. 2011).

The discovery of vascular receptors that control vessel tone and neurohumoral mediators in hypertension has led to the development of modern antihypertensive drugs such as beta-blockers, angiotensin converting enzyme inhibitors, AT-1 receptor blockers or calcium channel blockers. Although the pathophysiology of hypertension is extremely complex and multifactorial, and the role of factors such as endothelin, cyclooxygenase-dependent vasoconstrictors and endothelium-derived hyperpolarizing factor needs to be acknowledged, numerous experimental animal studies indicate that this condition is associated with an increased formation of reactive oxygen species (ROS) from all layers of the vascular wall(Schulz, et al. 2011).

2.4.3 Renal disease

Endothelial dysfunction is a hallmark of chronic kidney disease (CKD). Patients with CKD show endothelial dysfunction resulting from increased endothelial injury and decreased endothelial repair. Patients with CKD are known to display impaired endothelium-dependent vasodilation, elevated soluble biomarkers of endothelial dysfunction, and increased oxidative stress(Jourde-Chiche, et al. 2011). Several uremic toxins, mostly protein-bound, have been associated with specific endothelial toxicity: ADMA, homocysteine, advanced glycation end products (AGEs), and more recently, p-cresyl sulfate and indoxyl sulfate. These toxins, all poorly removed by hemodialysis therapies, share mechanisms of endothelial toxicity: they promote pro-

oxidant and pro-inflammatory responses and inhibit endothelial repair(Jourde-Chiche, et al. 2011).

2.4.4 Hyperlipidemia

Increased plasma levels of low density lipoprotein (LDL) particles damage the endothelium favouring LDL entry and accumulation within the arterial wall. Oxidized low-density lipoproteins may be one of several factors that contribute to loss of smooth muscle cells through apoptosis in the atherosclerotic plaque cap(Pearson, et al. 2003). Elevated high density lipoprotein (HDL) levels reduce the risk of coronary events regardless of LDL levels. It is currently believed that most of the atheroprotective effects of HDLs stem from their capacity to remove cholesterol from the vasculature, and to deliver it to the liver for disposal in a process commonly referred to as reverse cholesterol transport (RCT)(Badimon, et al. 2011). There is no consensus as to whether flow-mediated vasodilation is correlated with LDL cholesterol.

2.4.5 Obesity

Adipose tissue releases a large number of bioactive mediators that influence not only body weight homeostasis but also insulin resistance, which is the core feature of type 2 diabetes, as well as alterations in lipids, blood pressure, coagulation, fibrinolysis and inflammation, leading to endothelial dysfunction and atherosclerosis(Van Gaal, et al. 2006). Obesity increases the risk of cardiovascular disease and premature death(Yusuf, et al. 2004). The accumulation of abdominal fat independently increases cardiovascular risk. In The Nurses' Health Study the waist-to-hip ratio reflects abdominal fat in predicting type 2 diabetes, stroke, myocardial infarction and cardiovascular mortality in middle-aged individuals(Rexrode, et al. 1998). Obesity has been linked to impaired coronary and peripheral endothelial function(Benjamin, et al. 2004). In The Cardiovascular Risk in Young Finns Study, an opposite finding was done - increased BMI was found to correlate positively to FMD(Juonala, et al. 2004). An increase in body size within the nonobese range in a population of healthy young adults seems to be associated with physiological changes that lead to enhanced FMD responses and overcomes the opposing influences of the larger vessel size and increased oxidative stress associated with higher BMIs(Juonala, et al. 2004).

2.4.6 Metabolic syndrome

Metabolic syndrome is characterized by atherogenic dyslipidemia (low HDL and high triglycerides levels), elevated blood pressure, elevated plasma glucose levels, a prothrombotic state, and a proinflammatory state(Cho. 2011). The proinflammatory state is a result of an increase in adipocyte mass and macrophage infiltration in fat tissue and other organs. The presence of elevated blood pressure, elevated blood glucose and serum free fatty acid levels, hyperinsulinemia, and insulin resistance lead to endothelial dysfunction in skeletal muscle, liver, and impairments in microcirculations(Vykoukal, et al. 2011).

2.4.7 Diabetes

Both type 1 and type 2 DM patients suffer an increased risk for cardiovascular morbidity and mortality. Patients with diabetes have twice the risk of incident myocardial infarction and stroke as that of the general population(Buse et al., 2007).

As many as 80% of patients with type 2 DM will develop and possibly die of macrovascular disease(Buse et al., 2007). The relation between diabetic micro- and macroangiopathy and endothelial dysfunction is complex and is still a subject of extensive research. In addition to the metabolic actions of insulin in coordinating glucose homeostasis, a complete biochemical signaling pathway linking the insulin receptor to activation of eNOS in vascular endothelium has recently been elucidated(Yu, et al. 2011). The increased and accelerated rate of apoptosis of endothelial cells is probably a crucial factor in diabetic co-morbidity. There are many pathways involved in activating endothelial cell apoptosis and all of these pathways can be activated in multiple ways. A common mechanism triggering endothelial dysfunction and endothelial cell apoptosis is oxidative stress(Giugliano, et al. 1996)(Kuroki, et al. 2003). Hyperglycemia alone accelerates the development and progression of atherosclerotic lesions and the rapid formation of EC-derived foam cells(Simionescu, et al. 1996). In pregnancy type 1 diabetes has been found to associate with impaired endothelial function(Savvidou, et al. 2002).

2.4.8 Alcohol

Low concentrations of alcohol induce increased release of NO from the endothelium due to activation and expression of NO synthase (NOS). In contrast, administration of high concentrations of alcohol or its chronic ingestion impairs endothelial function in association with reduced NO bioavailability. The endogenous NOS inhibitor asymmetric dimethylarginine may be involved in the decreased synthesis of NO(Toda and Ayajiki. 2010).

2.4.9 Oxidative stress

Reactive oxygen species (ROS) are a family of molecules including molecular oxygen and its derivatives produced in all aerobic cells(Cai and Harrison. 2000). Excessive production of ROS, outstripping endogenous antioxidant defense mechanisms, has been implicated in processes where these reactive radicals oxidize biological macromolecules, such as DNA, protein, carbohydrates, and lipids. This condition has commonly been referred to as oxidative stress. There is evidence that ROS induce endothelial dysfunction by affecting eNOS expression or by inactivation of NO through the formation of lipid peroxidation products and peroxynitrite radicals that disturb the EC membrane directly(Cai and Harrison. 2000)

2.4.10 Estrogens

Estrogens, and in particular 17beta-estradiol (E2), play a pivotal role in sexual development and reproduction. Both acetylcholine-induced and flow-dependent vasodilation are preserved or potentiated by estrogen treatment in both animal models and humans(Miller et al. 2008). E2 increases the endothelial production of nitric oxide and prostacyclin and prevents early atheroma through endothelial-mediated mechanisms. Furthermore E2 potentiates the ability of several subpopulations of the circulating or resident immune cells to produce proinflammatory cytokines. E2 also promotes endothelial healing, and it is involved in angiogenesis(Arnal, et al. 2010). The actions of estrogens are essentially mediated by two molecular targets: estrogen receptor-alpha (ERα) and ERbeta. ERα appears to mediate most of the actions of E2 on the endothelium and the immune system, Arnal, et al. 2009). ERα activation function-1 (AF-1) is not required for the vasculoprotective actions of E2, whereas it is necessary for the effects on its

reproductive targets. Selective estrogen receptor modulators (SERMs) stimulating ERα, with minimal activation of ERα AF-1, are used in order to achieve beneficial vascular actions, while minimizing the sexual effects(Arnal, et al. 2009). The types of receptor isoforms vary from tissue to tissue and from species to species. This may account for considerable functional diversity, but this emerging field has not yet matured enough to give clear insights into implications for the actions of estrogen on a particular organ system, such as the vasculature(Miller and Duckles. 2008). Thus the clinical use of estrogen in preventing or treating cardiovascular disease is controversial.

2.5 ENDOTHELIUM IN PREGNANCY

2.5.1 Endothelium and regulation of vascular tone in normal pregnancy

Normal pregnancy is characterized by vasodilation resulting in reduction of peripheral vascular resistance. Blood pressure begins to decrease early in the first trimester and reaches its nadir by 20 to 24 weeks' gestation. The physiological changes are not completely understood, but the human chorionic gonadotropin–induced increased production of relaxin by the corpus luteum may facilitate vasodilation in normal pregnancy. Relaxin up-regulates vascular gelatinase activity thereby contributing to vasodilation and reduced myogenic reactivity of small arteries through activation of the endothelial endothelin B receptor–nitric oxide pathway(Boeldt, et al. 2011). The amount of NO produced by endothelial NO synthase (eNOS) is determined by the maximum capacity of the cell (eNOS expression levels), the eNOS phosphorylation state, and the intracellular $[Ca^{2+}]_i$ concentration in response to circulating hormones or physical forces(Boeldt, et al. 2011). In early pregnancy, the magnitude of the endothelium-dependent FMD of the brachial artery is determined in part by carriage of the endothelial nitric oxide synthase gene polymorphism Asp298 variant(Savvidou, et al. 2001). Angiogenic factors such as vascular endothelial growth factor (VEGF) also may have an important function in the increased production of NO and prostacyclin in pregnancy via pathways involving phospholipase C, mitogen-activated protein kinase, and protein kinase C. The balance between vasodilatory (NO, prostacyclin) and vasoconstrictive (thromboxane A_2, endothelin) substances, and in parallel the balance between angiogenic and anti-angiogenic factors, are speculated to be important determinants of blood pressure in pregnancy(Cornelis, et al. 2011).

In pregnancy, vascular nitric oxide (NO) production is increased in the systemic and more so in the uterine vasculature, thereby supporting maximal perfusion of the uterus(Boeldt, et al. 2011). Increased activity of the NO vasodilatory mechanism occurs in the maternal systemic vasculature in general and this is even more pronounced in the uterine vasculature. Several studies have described improvements in endothelial function in terms of brachial artery FMD during normal pregnancy with some alterations between the three trimesters(Dorup, et al. 1999) (Savvidou, et al. 2000) (Faber-Swensson, et al. 2004) (Seeliger, et al. 2011). In the first study 71 normal pregnant women were compared to 37 controls and FMD% was increased in all three trimesters (9.1 and 9.1. and 10.6%) compared to nonpregnant controls who had mean FMD% of 7.2%(Dorup, et al. 1999). The largest of these studies consisted of a sample of 157 pregnant women who were investigated at gestational weeks 10-40 and compared to 19 non-pregnant controls. In final

analysis they took pregnancies from 10 to 30 weeks, because FMD% decreased later in pregnancy. Mean FMD% in pregnancy was 8.84 ± 3.18% and in nonpregnant controls 6.42 ± 2.45% (P=0.002) (Savvidou, et al. 2000). In 74 twin pregnancies the flow mediated vasodilation was comparable to 98 singleton pregnancies (9.61 ± 4.36 vs. 8.84 ± 3.18%) during the weeks 11-30 (Savvidou, et al. 2001). In a longitudinal study increase of FMD% did not reach significance in pregnancy up to 32 weeks, but decreased after 36 weeks significantly(Quinton AE, et al. 2007). Another longitudinal study completed in all the three trimesters and postpartum showed that FMD% increased from the first trimester to the second trimester (8.0 ± 5.58 vs. 15.2 ± 5.19, P=0.003) and attenuated again to the third trimester (9.15 ± 3.61%, P=0.004 between the second and third trimesters) and postpartum values were comparable to first trimester(Seeliger, et al. 2011).

2.5.2 Endothelium and regulation of vascular tone in preeclampsia

Although preeclampsia appears to originate in the placenta, the tissue affected most is the maternal endothelium. The clinical manifestations of preeclampsia reflect widespread endothelial dysfunction, with vasoconstriction and end-organ ischemia. The hypertension is characterized by peripheral vasoconstriction and decreased arterial compliance(Powe, et al. 2011). The proteinuria of preeclampsia is associated with a pathognomonic renal lesion known as glomerular endotheliosis, in which the endothelial cells of the glomerulus become swollen and endothelial fenestrations are lost(Powe, et al. 2011).

Exposure of endothelial cells to serum from women with preeclampsia results in endothelial dysfunction. It has been hypothesized that circulating factors, probably originating in the placenta, are responsible for the manifestations of the disease(Boeldt, et al. 2011). Dozens of serum markers of endothelial activation and endothelial dysfunction are known to be deranged in women with preeclampsia, including von Willebrand antigen, cellular fibronectin, soluble tissue factor, soluble E-selectin, platelet-derived growth factor, and endothelin(Maynard, et al. 2008).

Women who developed pre-eclampsia or intra uterine growth restriction (IUGR) later in pregnancy had significantly lower FMD at gestational weeks 23-25 than those women who had normal outcome (3.6 vs. 6.17 vs. 8.6%, $p<0.0001$)(Savvidou, et al. 2003). Irrespective of pregnancy outcome, women with evidence of impaired placental perfusion had significantly higher levels of ADMA than women with normal Doppler waveforms (2.4 μmol/L [IQR 1.97–3.14] vs. 0.81 μmol/L [0.49–1.08]; $p<0.0001$). There was a strong inverse correlation between ADMA levels and flow-mediated dilation but only in the group of women who eventually developed pre-eclampsia ($r=-0.8$, $p=0.005$)(Savvidou, et al. 2003). In the placenta it has been reported that DDAH expression and activity in pre-eclampsia were almost undetectable and ADMA plasma levels were higher in women with pre-eclampsia compared to normal pregnant women(Anderssohn, et al. 2012)

Endothelial dysfunction in preeclampsia may be more pronounced in uteroplacental vasculature than in general vasculature and failure in shear stress mediated vasodilation in myometrial arteries might contribute to impaired uteroplacental blood flow(Kublickiene, et al. 2000)(Nisell, et al. 1991)(Acharya et al. 2009).

2.5.3 Endothelial function in women with previous preeclampsia

Flow mediated vasodilation was found to be significantly reduced in women with previous early-onset preeclampsia and IUGR compared with women with previous late-onset preeclampsia and control subjects (3.2±2.7% and 2.1±1.2% versus 7.9±3.8% and 9.1±3.5%, respectively; P<0.0001) 6 to 24 months postpartum(Yinon, et al. 2010). Endothelium independent vasodilation was similar among all groups. In contrast, women with a history of late-onset preeclampsia exhibited normal FMD i.e. similar to control subjects. Using pulse-wave analysis, it was demonstrated that arterial stiffness was increased in both women with a history of early preeclampsia and women with previous normotensive IUGR relative to women with previous late-onset preeclampsia and healthy control subjects(Yinon, et al. 2010) In a recently published study no evidence of endothelial dysfunction (measured with strain gauge plethysmography) nor sympathetic overactivity was detected in the postpartum state in women with a history of preeclampsia or gestational hypertension(Mangos, et al. 2012).

In a Swedish study with small population (N=18) the mean value of FMD measured one year after pregnancy was remarkably lower in women with history of preeclampsia than in control women (2.5 ± 2.9% vs. 10.3 ± 2.0%)(Hamad, et al. 2007). Lampinen et al. described impairement in endothelium independent and -dependent vascular dilatory capacity in women with a history of moderate/severe preeclampsia after 5-6 years of pregnancy(Lampinen, et al. 2006). In their study vasodilatory function was assessed with venous occlusion plethysmography with the change in flow being measured in resistance vessels of the forearm resulting from vasodilation after intra-arterial infusions of vasodilatory substances(Lampinen, et al. 2006).

2.5.4 Preeclampsia and subsequent risk for hypertension and cardiovascular disease

Women with two episodes of preeclampsia were approximately 10 times more likely to be using antihypertensive medication at follow-up (adjusted odds ratio, 11.6, 95% CI 7.1-26.3), and in women with gestational hypertension in three consecutive pregnancies, systolic pressure was on average 27 mmHg (95% CI 18-37 mm Hg) higher, and diastolic pressure was 12 mmHg (95% CI 5-19 mmHg) higher, compared with women without a history of gestational hypertensive disorders(Magnussen, et al. 2009) According to another study women with a history of preeclampsia had double the risk of hypertension and coronary artery disease compared with control subjects(Andersgaard, et al. 2012). They had carotid plaques more often, had larger total carotid plaque area and thicker intima-media layer compared with control subjects. A family history of CVD was more common among these women, which suggested that the familial risk may be associated with underlying genetic predisposition towards vascular dysfunction or other related factors (such as familial food habits, life style) (Andersgaard, et al. 2012). In a large meta-analysis, it was found that after preeclampsia women displayed an increased risk of vascular disease. The relative risks (95% confidence intervals) for hypertension were 3.70 (2.70 to 5.05) after 14.1 years weighted mean follow-up, for ischemic heart disease 2.16 (1.86 to 2.52) after 11.7 years, for stroke 1.81 (1.45 to 2.27) after 10.4 years, and for venous thromboembolism 1.79 (1.37 to 2.33) after 4.7 years. No increase in risk of any cancer was found (0.96, 0.73 to 1.27), including breast cancer (1.04, 0.78 to 1.39) 17 years after pre-eclampsia. Overall mortality after preeclampsia was increased: 1.49 (1.05 to 2.14) after 14.5 years(Bellamy, et al. 2007). In a Finnish study, it was found

that a history of preeclampsia was an independent risk factor for subsequent coronary artery disease among other well known risk factors (hypertension, diabetes, hypercholesterolemia, advanced age, smoking), and even after adjusting for other risk factors (OR 4.8 (1.2–19)) (Haukkamaa, et al. 2004).

Although the increased risk for hypertension and cardiovascular disease seems evident, the mechanisms behind this phenomenon are somewhat unclear. A permanent endothelial dysfunction and metabolic risk factors have been suggested to provoke the risk. In the Avon Longitudinal Study of Parents and Children (ALSPAC), which is a prospective population-based birth cohort study with 14,541 pregnancies resident in Avon, UK both gestational hypertension and preeclampsia were associated with a greater number of cardiovascular risk factors: BMI, waist circumference, systolic and diastolic blood pressure values, insulin, proinsulin, triglycerides, and HDL cholesterol. Mothers of small for gestational age babies had higher systolic and diastolic blood pressure compared to mothers with appropriate for gestational age babies, as did mothers who delivered before term(Fraser, et al. 2012). In other studies, women with previous preeclampsia have exhibited features of metabolic syndrome which were presumably present already before pregnancy, predisposing them to hypertensive disorders of pregnancy and a later cardiovascular risk.(Mangos, et al. 2012) No difference in insulin sensitivity in women with previous preeclampsia as compared with the control women was found(Mangos, et al. 2012) and similar results were reported in the study by Lampinen et al. Insulin sensitivity was found to correlate with vascular dilatory function in women with previous preeclampsia, suggesting that there was a relationship between the metabolic and vascular functions(Lampinen, et al. 2008).

2.5.5 Gestational diabetes and endothelial function

Abnormal carbohydrate metabolism associated with endothelial dysfunction during pregnancy. In the last trimester of pregnancy, FMD% was 4.1 % in GDM group vs. 10.9 % in control group and a strong negative relation was found between FMD and glucose area under curve (AUC)(Paradisi, et al. 2002). In addition impaired endothelium dependent vasodilation has been found in euglycemic women with previous GDM(Anastasiou, et al. 1998). Endothelial dysfunction in diabetes is thought to be mainly due to hyperglycemia(Williams, et al. 1998) and its adverse effects on endothelium have been discussed earlier.

In the ALSPAC study, it was found that pre-gestational diabetes, GDM and glucosuria were all associated with higher glucose concentrations eighteen years after pregnancy even when controlling for potential confounders including pre-pregnancy BMI(Fraser, et al. 2012). Pregnancy diabetes was associated with higher glucose levels, GDM and glucosuria were also associated with higher insulin and proinsulin, and furthermore glucosuria was associated with higher triglyceride levels(Fraser, et al. 2012). Similarly, mothers to large for gestational age (LGA) babies had higher glucose levels than mothers of AGA babies - in line with the established linear association between maternal glycemic status and the risk of delivering an LGA infant(Fraser, et al. 2012).

2.5.6 Gestational diabetes and hypertensive disorder in pregnancy and future cardiovascular risk

There are many women that experience more than one pregnancy complication. There are few studies about how the risks accumulate in these women. Hypertension

in pregnancy and pregnancy diabetes are independently associated with an increased calculated 10 year CVD risk(Fraser, et al. 2012). Preeclampsia may be a better predictor of future CVD since it was associated with a wider range of cardiovascular risk factors(Fraser, et al. 2012). Pregnant mothers with chronic hypertension and GDM are more insulin resistant than those with GDM alone(Caruso, et al. 1999). Blood pressure is a stronger predictor of insulin resistance than adiposity in a population of pregnant women with normal and abnormal carbohydrate metabolism(Caruso, et al. 1999). After delivery, women with GDM often have an increased risk for developing metabolic syndrome and they present with early markers of vascular disease such as endothelial dysfunction and increased intima-media thickness of carotid arteries(Bo, et al. 2007, Kaaja and Ronnemaa. 2008) Thus, pregnancy may act as a "stress test", revealing a woman's predisposition to type 2 diabetes and it can be viewed as an opportunity for providing focused prevention of important chronic diseases.(Kaaja and Ronnemaa. 2008).

2.5.7 Complications in pregnancy and vascular health of the offspring

Coronary heart disease is believed to result from the unhealthy lifestyles of westernized adults in conjunction with a contribution from genetic inheritance. Geographical studies provided the first clue that the disease originates during intra-uterine development. In animal studies it has been found that if adult rat offspring were exposed to prenatal hypoxia then their flow-mediated vasodilation was impaired (Morton, et al. 2011). According to Barker's studies, small birth weight correlated to cardiovascular disease(Barker and Osmond. 1986). Impaired fetal growth and prematurity were associated with impaired endothelial function and elevated preclinical atherosclerosis in young adults, partly mediated by inflammation, blood pressure, and triglyceride levels(Skilton, et al. 2011). If the fetus is exposed to GDM then this will add an intrauterine environmental risk factor to an already increased genetic risk for the development of obesity and/or diabetes. Maternal nutritional status (under- or overnutrition) can also affect the vascular function in the offspring(Poston, 2011).

3 *Aims of the study*

The overall aim of this study was to assess the endothelial function in normal and complicated pregnancies. Firstly we wanted to evaluate the normal range of FMD in women with uncomplicated pregnancies. Secondly, we hypothesized that in gestational diabetes and preeclampsia we would find endothelial dysfunction compared to normal pregnant women. Thridly, we wanted to investigate factors that alter the vascular tone during normal and complicated pregnancy. The individual aims were to determine

1. FMD and lipid levels in pregnant vs. non-pregnant healthy women.

2. FMD and the concentrations of asymmetric dimethylarginine (ADMA) in healthy pregnant vs. non-pregnant women.

3. FMD and the levels of proinflammatory cytokines and hsCRP in normal pregnancy vs. non-pregnant healthy women and their association with FMD during pregnancy.

4. the endothelial function and the concentrations of proinflammatory cytokines and hsCRP in normal vs. pre-eclamptic pregnancies and postpartum.

5. FMD and levels of proinflammatory cytokines in gestational diabetes vs. normal pregnancy and postpartum period.

4 Material and methods

4.1 PATIENTS IN STUDIES I-III

The Cardiovascular Risk in Young Finns Study is an ongoing population-based 5-centre follow-up study of atherosclerosis risk factors in Finnish children and adolescents. The first cross-sectional survey was conducted in 1980. The original sample size was 4,320 children and adolescents aged 3, 6, 9, 12, 15 and 18 years. The individuals were randomly chosen from the national register. There were 3,596 participants who participated in 1980. We re-examined 2,283 of these individuals in 2001 when they were aged 24–39 years. The loss of participants was 34%. Out of this sample, 62 of the participants were pregnant and in 57 cases there was complete data about endothelial function. There were 13 women (23%) in the first trimester (≤14 weeks), 20 women (35%) in the second trimester (15–27 weeks) and 22 women (39%) in the third trimester (≥28 weeks); the gestational age data were not available for 2 patients (3%). The mean gestational age was 23±9 weeks. For the present analysis we chose these 57 pregnant women and 62 non-pregnant women matched for age and smoking status. There were 25/62 oral contraceptive (OC) users in control group.

The parameters used in this thesis from the Cardiovascular Risk in Young Finns Study

Weight, height, BMI

Smoking

Blood pressure

Total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides

CRP, ADMA, TNF-α, IL-6

Brachial artery flow-mediated dilation

4.2 PATIENTS IN STUDIES IV-V

A total of 110 women were recruited from Kuopio University Hospital maternity clinic for the Complicated Pregnancy study Group in the years 2003-2008. The subgroups and the number of women are presented in Table 7.

Table 3. Subgroups and number of women recruited in Kuopio University Hospital Maternity Clinic for The Complicated Pregnancy Study.

Study group	Pregnancy(N)	After pregnancy (N)
Normal	32	27
Hypertensive	14	9
Preeclampsia	12	7
GDM (diet)	42	26
GDM (insulin)	10	7

Hypertension was defined as blood pressure ≥140/90 mmHg in two separate measurements 6 hours apart or 30/15 mmHg increase in blood pressure during pregnancy. Preeclampsia was defined as hypertension and proteinuria ≥ 300 mg over 24 hours. If blood pressure was over 160/100 mmHg in two separate measurements or the patient had symptoms such as headache, labetalol medication was started. At the time of FMD measurement 16/26 hypertensive patients had antihypertensive medication.

Gestational diabetes was defined as an abnormal 2-h 75 g glucose test. In Finland screening for GDM is a selective risk-related procedure (only pregnant women with one or more risk factors for GDM are screened. Plasma venous values lower than 4.8–11.2–9.9 mmol/l were considered normal (fasting–1 h–2 h) in 2003-2008. Of the GDM women, 91.7% had fasting glucose value higher than reference value, 27.7% had 1-h value and 4.3% had 2-h value higher than the reference value. If the mother had at least one value over the reference range, she was included to the GDM group and the diagnosis was confirmed with a blood glucose profile, measuring blood glucose every 4 h over 24 h. Women with GDM received dietary advice, regular blood glucose controls (at home, capillary plasma glucose levels 3 days/week), and insulin when necessary (fasting capillary plasma glucose levels repeatedly over 5.5 mmol/l or postprandial over 7.8 mmol/l).

4.3 METHODS

4.3.1 Anthropometry and Physiology

Height and weight were measured. Blood pressure was measured with a random zerosphygmomanometer (Hawksley & Sons Ltd, Lancin, UK) while the patient was seated after 5 min rest. Korotkoff's fifth phase was used as the sign of diastolic blood pressure and first phase as the sign of systolic blood pressure. Readings to the nearest even number of millimetres of mercury were performed at least 3 times for each subject and the average of these measurements was used in the analysis in The Cardiovascular Risk in Young Finns Study and The Complicated Pregnancy Study.

For the assessment of cardiac output, the maternal electrocardiography (ECG) (Rigel Multicare 302, Morden, Surrey, England) and non-invasive beat-to-beat arterial blood pressure (Finapres digital plethysmograph, Ohmeda Inc., Englewood, CO, USA) were recorded for 5 minutes at rest in the supine position during and after pregnancy. Continuous blood pressure recording was performed from the middle finger of the right hand. Stroke volume was assessed from the non-invasive blood pressure signal by using the arterial pulse contour method, which is modified from the model flow method. Cardiac output was calculated from the equation: cardiac output = heart rate x stroke volume. All recordings and data analyses were performed with an IBM PC-compatible microcomputer with commercial

WINACQ/WINCPRS acquisition electronics and software (Absolute Aliens Oy, Turku, Finland), which includes a sophisticated analysis program designed for research in cardiovascular physiology.

In addition, in The Complicated Pregnancy Study, all subjects went through twenty-four-hour ambulatory ECG, ambulatory blood pressure recordings, autonomic nervous system assessment and body composition analysis by bioimpedance during and after pregnancy but the data were not used in this analysis.

4.3.2 Blood samples

In the Cardiovascular Risk in Young Finns Study venous blood samples were drawn from the right antecubital vein of recumbent subjects after an overnight fast. Fasting blood samples were drawn from each subject during pregnancy and three months postpartum in the Complicated Pregnancy Study. Venous blood samples were drawn from the right antecubital vein of recumbent subjects after a 12-hour overnight fast for the determination of serum hsCRP, TNF-α and IL-6 and serum lipids and lipoprotein concentrations. Serum was separated by centrifugation and samples were stored frozen (-70 °C) until analysis.

4.3.2.1 Glucose

In the Cardiovascular Risk in Young Finns Study glucose concentrations were analyzed enzymatically (Olympus Diagnostica GmbH, Hamburg, Germany). In the Complicated Pregnancy Study plasma glucose was determined by the hexokinase method (Konelab 60i Clinical Chemistry Analyzer, Thermo Electron Co., Finland).

4.3.2.2 Lipid determinations

Lipid determinations in the Cardiovascular Risk in Young Finns study were determined enzymatically (Olympus System Reagent; Olympus Diagnostica GmbH, Hamburg, Germany) in a clinical chemistry analyser (AU400; Olympus Optical Ltd, Mishima, Japan). HDL cholesterol was analysed after precipitation of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) with dextrane sulphate 500 000. The concentration of LDL cholesterol was calculated using the Friedewald formula(Friedewald, et al. 1972). Subjects with triglycerides above 4 mmol L^{-1} (n = 32) were excluded from this analysis(Juonala, et al. 2004). In the Complicated Pregnancy Study cholesterol and triglyceride levels were assayed by standard enzymatic photometric methods with Konelab 60i Clinical Chemistry Analyzer (Thermo Electron Co, Finland).

4.3.2.3 L-Arginine and asymmetric dimethylarginine analysis

Serum L-arginine, ADMA and symmetric dimethylarginine (SDMA) levels were determined by a high performance liquid chromatography (HPLC) method with the precolumn ortho-phthaldialdehyde (OPA) derivatization described earlier (Valtonen, et al. 2005). Prior to analysis, the standards, controls and samples were extracted on Waters Oasis MCX solid phase extraction cartridges. HPLC analysis was carried out on a Merck Hitachi liquid chromatography system consisting of a gradient pump (D-6200), an autosampler (AS-4000), a fluorescence detector (F1000). Standards controls and samples were incubated for 2 min with the OPA reagent (1 mg/ml OPA in borate buffer, pH 9.5, containing 0.1% 3-mercaptopropionic acid)

before automatic injection in the HPLC. The OPA-derivatives were separated on Waters Symmetry C18 column (4.6 x 150 mm, 5 m) with fluorescence monitor set at λ^{ex} = 340 nm and λ^{em} = 455 nm. The column temperature was kept at +30 °C. Samples were eluted from the column with 50 mM K-phosphate buffer, pH 6.5 and 8.7% acetonitrile (v/v), at a flow rate of 1.1 ml/min.

4.3.2.4 High sensitive C-reactive protein analysis

Serum samples were analyzed by Immage automated analyzer using Beckman Coulter High Sensitivity C-Reactive Protein (CRPH) reagents (Beckman-Coulter, Fullerton, CA, USA) with working range of 0.2 to 1440 mg/l. The serum samples were analyzed according to the instructions of the manufacturer.

4.3.2.5 Interleukin-6 analysis

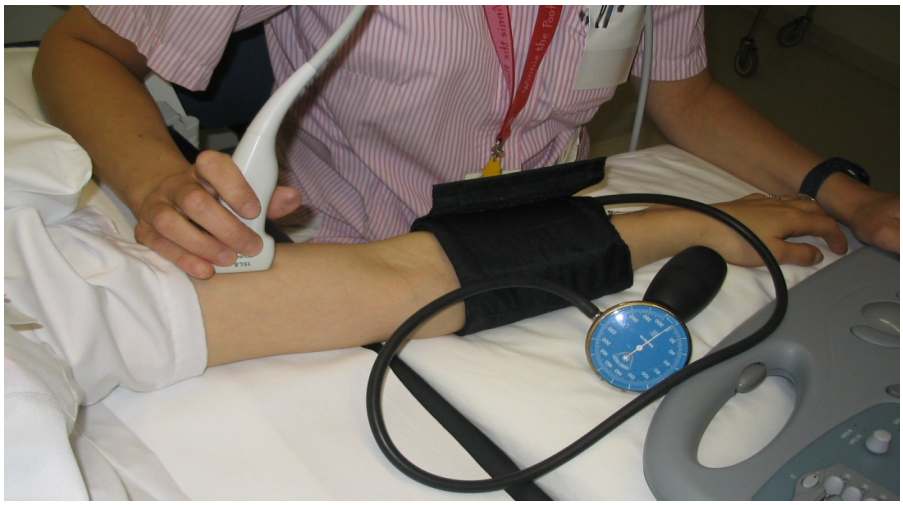
IL-6 was analyzed by R&D Systems Quantikine HS Human IL-6 Immunossay Kit (Minneapolis, USA) with working range of 0.156 to 10 pg/ml. Serum samples were analyzed according to the instructions of the manufacturer. Calibrators (supplied by the manufacturer) were analyzed in duplicate and samples as a single measurements. The absorbance at 490 nm was measured using a microplate reader (Tecan SPECTRAFluor, Tecan Group Ltd., Maennedorf, Switzerland).

4.3.2.6 Tumour necrosis factor-α analysis

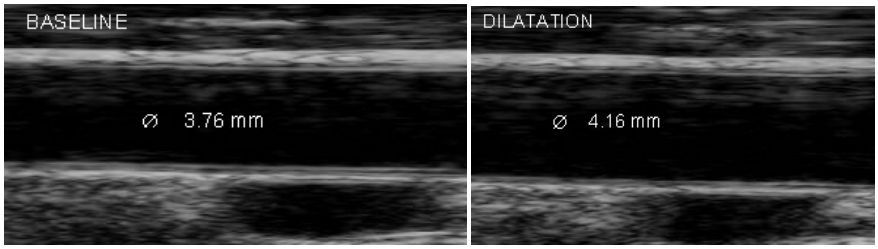
Serum samples were analyzed with the R&D Systems Quantikine HS Human TNF-α/TNFSF1A Immunossay Kit (Minneapolis, USA) with working range of 0.5 to 32 pg/ml. The serum samples were analyzed according to the instructions of the manufacturer. Calibrators (supplied by the manufacturer) were analyzed in duplicate and samples as a single measurements. The absorbance at 490 nm was measured using a microplate reader (Tecan SPECTRAFluor, Tecan Group Ltd., Maennedorf, Switzerland).

4.3.3 Ultrasound Imaging

Ultrasound studies were performed using Sequoia 512 ultrasound mainframes (Acuson) (Figure 7) with the same method in both the Cardiovascular Young Finns Study and the study conducted in Kuopio University Hospital. In the assessment of brachial artery FMD, the left brachial artery diameter was measured both at rest and after reactive hyperemia. Increased flow was induced by inflation of a pneumatic tourniquet placed around the forearm to a pressure of 250 mmHg for 4.5 min, followed by release. Three measurements of arterial diameter were performed at end-diastole at a fixed distance from an anatomic marker at rest and at 40, 60, and 80 s after cuff release. The vessel diameter in scans after reactive hyperemia was expressed both as the change in absolute diameter (FMD) and as the percentage relative to the resting scan (FMD%).



(a)



(b) (c)

Figure 7. Assessment of brachial artery flow mediated vasodilation (a). Brachial artery at baseline (b). Brachial artery after shear stress (c). FMD%= ((4.16-3.76)/3.76) x100=10.6 %≈11%

4.3.4 Statistical Analysis

The results are presented as means ± SD or when the distribution is not normal or when the variation was large, then the median values and minumums and maximums are used. The Kolmogorov-Smirnov test was used to evaluate data normality. The significance of differences was assessed by two independent samples t-test for observations that were normally distributed and when appropriate by nonparametric tests (Mann-Whitney U-test). When more than 2 groups were compared, the nonparametric Kruskall-Wallis test was used with Bonferroni correction. Univariate correlations were performed using the Pearson correlation. Multivariate analysis with linear regression was used to determine independent predictors of FMD. A p-value <0.05 was considered statistically significant. The sample size calculation revealed that 380 pregnant women would have been needed

in each ADMA tertile group to detect significant differences in FMD%. The SPSS version 19 (Chicago, IL, USA) was used in the statistical analysis.

5 Results

The results of flow mediated brachial artery vasodilation in studies I-V are presented in table 4. The main results are presented in the table 5.

Table 4. FMD% in studies I-V

Study	Study group	FMD % in pregnancy	FMD% nonpregnant/ postpartum
Cardiovascular Risk In Young Finns Study	Normal controls		9.5
	Normal pregnant		
	First trimester	7.4	
	Second trimester	10.9	
	Third trimester	11.1	
The Complicated Pregnancy Study	Normal	8.8	7.9
	Hypertension	10.0	8.5
	Preeclampsia	11.0	7.2
	GDM (diet)	7.7	8.8
	GDM (insulin)	8.7	6.1

Table 5. The main results in studies I-V.

Study	Main finding	Results	Other findings
I	FMD was enhanced in pregnant women compared to nonpregnant	1st trimester 0.22 mm (7.4%) 2nd trimester 0.34 mm (10.9%) 3rd trimester 0.36 mm (11.1%) nonpregnant 0.29 mm (9.5%) P=0.008 (0.067)	Pregnancy related marked hyperlipidemia FMD correlated with gestational age (r=0.345, P=0.010)
II	ADMA-concentrations were lower in pregnant women than in nonpregnant controls	0.513 vs. 0.577 µmol/l (P<0.001)	ADMA levels did not explain the improved FMD during pregnancy
III	HsCRP concentration was greater during pregnancy than in nonpregnant controls	2.52 mg/l vs. 1.21 mg/l (P<0.001)	Levels of IL-6 increased and TNF-α decreased (NS) in pregnant women Association between IL-6 and FMD in pregnancy
IV	FMD% was not attenuated in hypertensive pregnancies compared to normal pregnancies during or after pregnancy	11 % vs. 8.8 % (P=0.194) and postpartum 8.0 % vs. 7.9 % (P=0.978)	Markers of inflammation were high during pregnancy especially in hypertensive pregnancies vs. postpartum (medians): hs-CRP 4.5 vs. 0.80 mg/l (P=0.023) IL-6 2.1 vs. 1.2 pg/ml (P0.006) TNF-α 1.9 vs. 1.5 pg/ml (P=0.030)
V	In both diet and insulin treated gestational diabetes FMD remained good	diet 7.7 % vs. insulin 8.7 % vs. control 8.8% (P=0.597)	During pregnancy, lipids were similar in GDM and control groups irrespective of significantly higher BMI in GDM group but postpartum lipids and glucose levels were significantly higher and weight loss was diminished in GDM group

5.1 NORMAL PREGNANCY

5.1.1 Flow mediated vasodilation in normal pregnancy

Endothelium-dependent FMD during reactive hyperemia increased towards the end of pregnancy despite increasing serum lipid concentrations in the cross-sectional Cardiovascular Risk in Young Finns Study. The increase in the absolute value of FMD was statistically significant in pregnant women compared to nonpregnant controls (P=0.008). The value of FMD in the first trimester was diminished as compared with the non-pregnant controls and the increase in FMD% was statistically significant over the 3 trimesters (P=0.04). In pregnancy, FMD% correlated directly with gestational age, vessel size, total cholesterol (TC) and triglycerides (TG), but not with LDL or HDL. In the nonpregnant women, FMD% did not correlate with any of the serum lipid values. Overall, the univariate correlation analysis between FMD% and the other parameters during pregnancy revealed a significant correlation for maternal serum TC, TG and vessel size, whereas in non-pregnant women, the vessel size was the only significant correlate of

FMD%. A multiple regression analysis was used to identify the independent variables best predicting FMD% during pregnancy: these were TG and vessel size.

In the Complicated Pregnancy Study endothelial function was longitudinally determined in 32 normal pregnant women at gestational age of 24-38 weeks. The FMD% in normal pregnant women was 8.8. % during pregnancy and 7.9 % after pregnancy, the difference was not statistically different (P=0.439).

5.1.2 Lipid concentrations in normal pregnancy

Lipid profiles showed that in the third trimester of pregnancy, the mean concentrations of TC, LDL-C, HDL-C, TG and VLDL-C increased by 36, 35, 26, 116 and 115% respectively, when compared with the values of non-pregnant women. The presence of marked hypercholesterolemia associated with the increased HDL-C concentrations is common during pregnancy and 38% (21/56) of the pregnant women had a serum TC concentration exceeding 7.0 mmol/L.

5.1.3 Asymmetric dimethylarginine concentration in normal pregnancy

The mean ADMA concentration was significantly lower in pregnant women compared with the non-pregnant controls (0.513 ± 0.0593 versus 0.577 ± 0.0710, P < 0.001). The L-arginine concentration was also significantly lower in pregnant women than in their nonpregnant counterparts. To evaluate the effects of serum ADMA concentrations in normal pregnancy to endothelial function, ADMAMoM values were calculated based on the median of each of the three trimesters. These MoMvalues were then divided into tertiles and correlated to FMD. The three groups were: low ADMA [1 MoM (lowest – 0.967 MoM)], intermediate ADMA [2 MoM (0.968 – 1.0427] and high ADMA [3 MoM (1.0428 – highest MoM)]. We found no statistically significant relationship between maternal serum ADMA concentrations and endothelial function.

5.1.4 High sensitive C-reactive protein and proinflammatory cytokines in normal pregnancy

The median IL-6 concentration was 1.66 in pregnancy when all three trimesters were pooled vs. 1.32 pg/ml when compared to the non-pregnant (NS). In pregnancy the median TNF-α concentration was 2.11 vs. 2.38 pg/ml in the non-pregnant group (NS). During pregnancy, the median hsCRP concentration was 2.52 mg /l whereas the non-pregnant women had a median hsCRP concentration of 1.21 mg/l (P<0.001). Women using oral contraceptives (OCs) (25⁄62) in the control group displayed increased hsCRP concentrations compared to those not using OCs (median 0.71 mg/l for controls versus 1.92 mg/l for OC users, P = 0.002). In smoking women the median hsCRP value was 3.19 mg /l in pregnancy (n = 6) versus 2.63 mg/l in the non-pregnant group (n = 6) (NS). Exclusion of smokers from the analysis did not change the results: hsCRP was increased in pregnant women (P = 0.002) compared to non-pregnant women (median 1.17 (0.10–23.9) in non-pregnant versus 2.52 (0.44–23.6) mg/l in pregnant women). IL-6 showed a positive correlation to the FMD in pregnant women, R = 0.288, P = 0.031. In non-pregnant women, IL-6 correlated to baseline diameter (R = 0.336, P = 0.008). The results of flow mediated dilation, ADMA and hs-CRP and the cytokines are presented in the table 6. In addition FMD%, the concentrations of hs-CRP and cytokines in The Cardiovascular Risk in Young Finns Study are shown in figure 8.

Table 6. Flow mediated vasodilation and the concentrations of asymmetric dimethylarginine, high sensitive C-reactive protein, tumor necrosis factor-α and interleukin-6 in normal pregnant patients.

Normal pregnancy Study	**The Cardiovascular Risk in Young Finns Study**				**The Complicated Pregnancy Study**
Gestational age	1st trimester	2nd trimester	3rd trimester	P	weeks 24-38
FMD% (mean ± SD)	7.4 ± 3.5	10.9 ± 4.6	11.1 ± 5.3	NS	8.8 ± 5.1
FMD (mm) (mean ± SD)	0.29 ± 0.12	0.22 ± 0.10	0.34 ± 0.13	0.008	0.36 ± 0.036
ADMA (µmol/l) (mean ± SD)	0.521 ± 0.0745	0.505 ± 0.0459	0.513 ± 0.0638	NS	
hs-CRP (mg/l) (median, min., max.)	2.43 (0.52 – 15.5)	2.92 (0.78 – 16.9)	2.01 (0.62 – 6.38)	NS	3.0 (0.54 – 13.4)
IL-6 (pg/ml) (median, min., max.)	1.26 (0.48 – 3.1)	1.28 (0.62 – 6.38)	1.80 (0.56 – 5.6)	NS	1.8 (0.64 – 5.4)
TNF-α (pg/ml) (median, min., max.)	2.11 (1.45 – 4.1)	2.09 (0.69 – 34.7)	2.28 (1.28 – 5.4)	NS	2.0 (1.2 – 4.1)

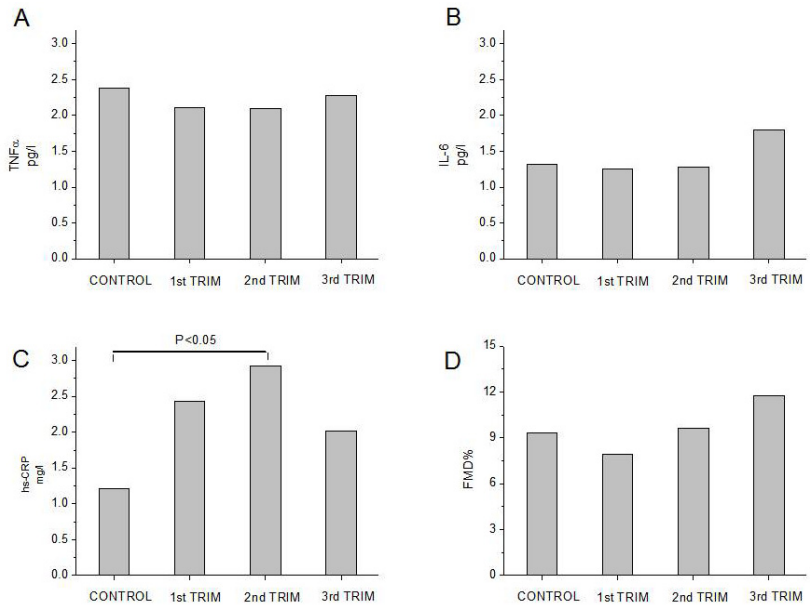


Figure 8. Medians of TNF-α, IL-6 and hs-CRP and means of FMD% in non-pregnant and pregnant women in the Cardiovascular Risk in Young Finns Study.

5.2 COMPLICATED PREGNANCY

5.2.1 Hypertensive pregnancies

Endothelial function during pregnancy was similar in pregnancy-related complications such as hypertension and preeclampsia and GDM when compared to normal pregnant women. The mean FMD% was 11.0% in the pooled group of hypertensive women and 8.8% in normal pregnant women during pregnancy (P=0.194) and 8.0% versus 7.9% postpartum (P=0.978). Concentrations of markers of inflammation were markedly increased in pregnant hypertensive group compared to those after delivery (hsCRP 4.5 versus 0.80 mg/l, P=0.023, IL-6 2.1 versus 1.2 pg/ml, P=0.006; TNF-α 1.9 versus 1.5 pg/ml, P=0.030). When compared the hypertensive pregnancies to the normal pregnancies the markers of inflammation were similar during and after pregnancy. FMD% in hypertensive pregnancies is presented in table 7 and markers of inflammation in table 8.

5.2.2 Gestational diabetes

FMD% among adequately managed GDM patients in either the dietary or the insulin treatment group compared to controls during pregnancy was not attenuated (7.7% and 8.7 % vs. 8.8 % concomitantly, NS) After the pregnancy FMD% in the former dietary GDM group was 8.8% and in the insulin treated group it was 6.1 % (only one person attended postpartum) vs. 7.9 %, (NS). Table 7 shows the results of the flow mediated dilation in the GDM pregnancies.

In a similar way, but in lesser extent GDM patients showed higher concentrations of markers of inflammation during pregnancy, see table 8. There were no statistically significant associations between the markers of inflammation and FMD.

During pregnancy, lipid values in the GDM group did not differ from the controls despite the glucose intolerance. Three months postpartum, BMI, fasting glucose and all lipids were significantly elevated in the GDM group, but this was not associated with endothelial dysfunction. The BMIs, glucose and lipis values for GDM groups are presented in table 9.

Table 7. FMD% in pregnancy complications.

Pregnancy complication	Hypertensive	GDM (diet)	GDM (insulin)	Controls
FMD% mean ± SD in pregnancy weeks 24-38	11 ± 6.3	7.7 ± 6.0	8.7 ± 5.2	8.8 ± 5.1
Postpartum	8.0 ± 4.2	8.8 ± 5.2	6.1	7.9 ± 3.5

Table 8. Hs-CRP and proinflammatory cytokine levels in women with hypertensive disorder during pregnancy or GDM (median, minimum, maximum).

Parameter	Hypertensive	GDM
hs-CRP (mg/l)	4.5 (0.89 – 38)	2.27 (0.199 – 8.3)
Postpartum	0.80 (0.27 – 5.9)	1.69 (0.199–7.04)
IL-6 (pg/ml)	2.1 (0.70 – 11)	2.32 (0.795–8.09)
Postpartum	1.2 (0.73 – 3.0)	1.83 (0.293–11.477)
TNF-α (pg/ml)	1.9 (0.88 – 3.2)	1.51 (0.778 – 2.91)
Postpartum	1.5 (0.71 – 2.1)	1.29 (0.673 – 4.01)

Table 9. BMIs, fasting glucose and lipids in GDM women during and after pregnancy.

Group	GDM diet		GDM insulin	
	Pregnancy	Postpartum	Pregnancy	Postpartum
BMI	28 ± 5.5	29 ± 5.42	31 ± 8.3	29 ± 2.70
fB-Gluc (mmol/l)	4.9 ± 0.36	5.4 ± 0.53	5.6 ± 1.5	6.7 ± 3.8
TC (mmol/l)	6.27 ± 1.07	5.01 ± 0.670	6.02 ± 1.42	5.49 ± 0.863
LDL (mmo/l)	3.74 ± 0.937	3.15 ± 0.680	3.63 ± 1.14	3.61 ± 0.953
HDL (mmol/l)	1.84 ± 0.492	1.38 ± 0.388	1.75 ± 0.237	1.23 ± 0.243
Trigly (mmol/l)	2.60 ± 0.901	1.21 ± 0.841	2.98 ± 1.24	1.59 ± 1.57

6 Discussion

The purpose of this study was to examine the vascular changes occurring during pregnancy, especially in preeclampsia. It was hypothesized that when compared to normal pregnant women the endothelium in preeclampsia would be injured. We have collaborated with Cardiovascular Risks in Young Finns Study-group to determine the FMD in normal pregnancies and had access to a considerable amount of data with regard to classical cardiovascular risk factors. At the same time material was collected from Kuopio University Maternity Clinic, although patients with pregnancy complications were harder to collect than expected.

Why was FMD chosen? This technique provides a noninvasive and direct measure of artery function and health in vivo and it is suitable for investigating large populations (as in Cardiovascular Risk in Young Finns Study). Further this technique can easily be conducted during pregnancy. The selected method is well validated and its reproducibility has been evaluated. There is increasing evidence that FMD provides valuable and independent prognostic information in humans. However, different methodological approaches limit its validity, comparability, and its potential use as a clinical and physiological research tool. Improving understanding of the physiological and technical principles on which the FMD technique is based will improve its application and interpretation of (patho)physiological changes that may occur between groups or after interventions(Thijssen, et al. 2011).

The main strength of the Young Finns Study is that it is a prospective cohort study, i.e. it represents the total population. These results can be generalized to white Caucasian pregnant women. OC users were not excluded from the control group, as there is data that FMD% was similar in OC users and non-OC users in the Cardiovascular Risk in Young Finns Study(Valtonen, et al 2010). In the first three studies we compared the vascular health of pregnant women to that of nonpregnant controls; this can cause an error due to the fact that pregnant women usually try to adopt a healthier lifestyle than their nonpregnant counterparts. In studies IV-V the same individuals could be compared during and after pregnancy. It has to be taken into account that there is marked individual and even interindividual variability in FMD measurements. In all the presented studies sample sizes are small and a type II error is possible. If one wanted to detect potential weak associations between FMD and investigated variables, much larger studies involving several thousands of subjects should be carried out. There was some loss of participants in The Complicated Pregnancy Study which reduced the sample size postpartum, many of the patients were hesitant to participate with the newborn baby. Dropouts may have affected the statistical analysis e.g. the nonsignificant result when comparing FMD% during and after pregnancy.

Endothelial function improved towards the end of normal pregnancy, despite the presence of marked hyperlipidemia. These results show that the elevated serum concentrations of TC, TG, LDL-C, and VLDL-C did not evoke endothelial dysfunction during pregnancy. On the contrary, endothelial function improved in the second and third trimesters of the pregnancy, probably partly because of the increased concentration of HDL-C, which may have inhibited the oxidation of low-

density lipoprotein and thus protected the endothelium. Interestingly the CVD risk in multiparous women later in life has been reported to be increased(Beral. 1985) or reduced in other studies(Jacobs, et al. 2012). The physiological short-term increase in the levels of serum lipids and the improvement of endothelium-dependent vasodilation responses are associated with gestation rather than with each other. The improvement of endothelial function in pregnancy in the Cardiovascular Risk in Young Finns Study was significant in terms of FMD but not as great as reported in previous studies(Dorup, et al. 1999) (Savvidou, et al. 2000) (Faber-Swensson, et al. 2004) (Seeliger, et al. 2011). In our longitudinal Complicated Pregnancy Study, FMD% was enhanced, although not significantly, in pregnancy vs. postpartum, which is in line with one previous study(Quinton, et al. 2007). Previously, in normal pregnancy mean FMD% has been reported to be 8.84 ± 3.18% between 10-30 weeks, which was significantly higher than in non-pregnant controls (6.42 ± 2.45%)(Savvidou, et al. 2000). In the Cardiovascular Risk In Young Finns Study the cross-sectional control group of 62 women had better FMD% (9.48%) than in Savvidou's study with 19 controls. In the first trimester FMD% has previously been reported to be smaller than in the second trimester, which is in line with our results. After 30 weeks FMD% has been found to decrease to prepregnancy levels (Savvidou, et al 2000) but this is in contrast to our results from the Cardiovascular Risk in Young Finns Study. On the other hand this might explain the insignificant increase in FMD% in the Complicated Pregnancy Study as the FMD was investigated during gestational weeks 24-38.

It seemed that lowered maternal serum ADMA and L-arginine concentrations did not modulate endothelial function during normal pregnancy in our study. The possibility of a type II error has to be taken into consideration when interpreting these results. However, according to the estimation of the sample size in retrospect, it was found that more than 1140 pregnant subjects would have needed to be studied in order to make statistically significant the weak association between ADMA and FMD. This is in line with a result based on the evaluation of an entire population in the Young Finns Study with respect to ADMA and FMD%(Juonala, et al. 2007). Rixos, et al. measured ADMA concentrations in the three trimesters of 41 uncomplicated pregnancies, and the result s are similar to ours: 0.51 ± 0.14, 0.52 ± 0.13, 0.58 ± 0.16 μmol/l(Rizos, et al. 2011). They also assessed ADMA concentrations in 10 pregnant women with preeclampsia and found a significant increase in the second trimester concentrations of ADMA when compared to the normal pregnant women (0.58 ± 0.10, 0.63 ± 0.14 (P=0.02), 0.68 ± 0.11 μmol/l). In 14 pregnancies with SGA infant the ADMA concentrations were significantly lower in each trimester (0.40 ± 0.10 (P=0.005), 0.42 ± 0.10 (P=0.0077), 0.45 ± 0.10 (P=0.007) μmol/l(Rizos, et al. 2011).

The concentrations of IL-6 and TNF-α did not change significantly from the non-pregnant state to the pregnant condition or during the three trimesters of pregnancy and thus these changes are unlikely to account to the pregnancy-related increased flow mediated dilation. The median concentration of TNF-α and IL-6 were 2.1 and 1.3 pg/ml in first trimester according to our results and 1.1 and 3.7 pg/ml according to a study of 100 normal pregnant women under 20 weeks(Fiorini, et al. 2012). Substatially higher concentrations have also been reported for IL-6 (8.1 pg/ml) and similar to TNF-α (1.3 pg/ml)(Coussons-Read, et al. 2012). We found a significant increase in the serum hsCRP concentration of normal pregnant women, and this is in line with the literature(Coussons-Read, et al. 2012). Incerased hs-CRP did not cause

endothelial dysfunction. The enhancement of endothelial function appears to occur despite the proinflammatory state, most probably due to an exaggerated NO response, which is specific to pregnancy itself.

Based on earlier studies one would have expected to find endothelial dysfunction in complicated pregnancies. Endothelial function has been found to be attenuated in severe preeclampsia before the signs of clinical disease in early pregnancy (Savvidou, et al. 2003) and even 5-6 years after moderate/severe preeclampsia(Lampinen, et al. 2006). On the contrary this present study showed that endothelial function in conduit arteries was similar in normal and in hypertensive pregnancies. In both groups, endothelial function was better during pregnancy than postpartum, and in hypertensive pregnancies endothelial vasodilation after a stimulus was even better than in normal pregnancies. There were no detectable signs of endothelial dysfunction in brachial artery in hypertensive pregnancies and in mild-to-moderate preeclampsia. The effect of medication on endothelial function among hypertensive women was not analysed, 16/26 women had a medication for hypertension. It is possible that the increased blood flow due to sympathetic overactivation and increased blood pressure in hypertensive pregnancies results in increased shear stress and thus an even greater increase in shear-stress mediated increased vasodilation. In a previous study, pulsed Doppler findings of maternal uterine artery, maternal ophthalmic artery, and brachial artery flow-mediated vasodilation varied among women with preeclampsia showing that there were vascular changes concomitantly or separately in uterus, ophthalmic vessels and conduit arteries(Takata, et al. 2002). The reason for this discrepant result might be the different vascular beds: In the microvascular bed, endothelial dysfunction is readily detectable and the oedema caused by leaking endothelium could interfere with the measurements.

The compromised nitric oxide production causing vasoconstriction is not the only mechanism leading to pregnancy-induced hypertension or preeclampsia(Powe, et al. 2011), and increased FMD was found also in late hypertensive pregnancies. This can also be explained by the selection of the material, as it is almost impossible to examine patients with severe preeclampsia and in our study the hypertension and preeclampsia appeared in the late pregnancy. From the clinical point of view early-onset (under 30 weeks) and late-onset preeclampsia are distinct syndromes(Valensise, et al. 2008). Similar findings were recently published in a retrospective study where only women with severe preeclampsia and IUGR had impaired vasodilatory function 6-24 months after pregnancy, and patients with late-onset preeclampsia had a comparable FMD to their control counterparts (3.2 ± 2.7% and 2.1 ± 1.2% versus 7.9 ± 3.8% and 9.1 ± 3.5%, respectively; $P<0.0001$)(Yinon, et al. 2010).

We found that even in normal pregnancies, the third trimester concentrations of hsCRP, IL-6, and TNF-α were increased as compared to the postpartum values. The difference during pregnancy was not statistically significant between normal and hypertensive pregnancies though increases in hsCRP and IL-6 concentrations were observed in hypertensive pregnancies. This negative result may have occurred due to a type 2 error, i.e. the sample size was too small to confirm the difference. From pregnancy to 3-month postpartum, all the investigated markers of inflammation declined significantly in the hypertensive women. The result confirms the hypothesis that pregnancy-induced hypertension and preeclampsia represent, at least to some degree an inflammatory response to the pregnancy(Can, et al. 2011).

However, the markers of inflammation were measured from systemic blood samples and thus the results do not necessarily represent the actual concentrations within various tissues as the cytokines are often released locally.

This study is the first of its kind to show that in adequately managed gestational diabetes, endothelial function remains good despite the metabolic disorder. In the years 2003-2008 when the patients were recruited from Kuopio University Hospital the OGTT was considered abnormal when 0-1-2 hour values were ≥ 4.8-11.2-9.9 mmol/l measured from venous plasma. The fasting glucose value was pathological in 91.7 % of GDM patients in the present study. The low cut-off value for fasting glucose may explain partly the result: the endothelial function was not attenuated because part of the GDM patients would be considered normal according the diagnostic criteria for GDM today (glucose ≥ 5.3 − 10.0 − 8.6 mmol/l from capillary plasma). Somewhat contradictory findings have also been published; FMD% was attenuated in ten women with GDM and also in 13 women with reduced insulin sensitivity when compared to normal pregnant women(4.1 ± 0.9 vs. 7.6 ± 1.1 v.s. 10.9 ± 1.1) (Paradisi, et al. 2002). In that study OGTT and assessment of FMD% were completed simultaneously, so the effect of treatment might explain the different results. In addition to its actions in glucose and lipid metabolism insulin is involved in regulation of NO production in endothelial cells and when insulin is administered intravenously it acts as weak vasodilator(Yki-Järvinen, Utriainen. 1998) Resistance to insulin action associates with endothelial dysfunction(Yki-Järvinen. 2003). According to our results it seems that in GDM, if the newly appearing insulin resistance is well controlled by diet or insulin therapy, then the mild hyperglycemia does not cause endothelial dysfunction in the conduit arteries during pregnancy. While this can be viewed as good news for the mother, even mild hyperglycaemia can cause adverse consequences to the foetus, such as foetal demise and macrosomia and complications at birth or during the neonatal period(Reece. 2010).

One very interesting finding was that the good metabolic control during pregnancy in GDM women was lost already three months after delivery. This was reflected by the diminished weight loss, elevated blood pressure and worsened lipid profile and elevated fasting blood glucose level, increasing the future risks for type 2 diabetes and cardiovascular disease. Insulin therapy was terminated when the baby was born. It is evident that after parturition, the women were no longer adhering to a stringent diet anymore.

The levels of proinflammatory cytokines were higher during pregnancy than postpartum especially in normal but also in GDM women. Although GDM women had greater BMI values, they had significantly lower Hs-CRP levels than normal pregnant women. After pregnancy, the differences disappeared and the circulating levels of the proinflammatory cytokines declined. Both normal pregnant women and women with metabolic disorder displayed an inflammatory response in pregnancy, but this did not seem to correlate with the endothelial function.

It seems that endothelial function is attenuated after severe preeclampsia and IUGR but not after mild to moderate preeclampsia and hypertensive or GDM pregnancy. The association between CVD risk factors and endothelial dysfunction cannot be expressed in simple ways, because there are still unknown vasculoprotective or genetic factors that can alter the results. These variables may be related to pregnancy and they seem to protect the endothelium even in complicated pregnancy.

7 *Summary and future research needs*

This study confirms that in normal pregnancy, endothelial function is enhanced, despite the many associated risk factors that in nonpregnant state would lead to endothelial dysfunction and signs of preclinical arteriosclerosis. Asymmetric dimethylarginine (ADMA) concentrations were significantly lower in pregnant women but this did not explain the improved FMD in the correlation analysis. Endothelial function in normal pregnancy was not attenuated despite the significant increase in hsCRP, and pregnancy related changes in the concentrations of TNF-α and IL-6 were non-significant.

Women with third trimester hypertensive pregnancies and mild-to-moderate preeclampsia and also GDM women exhibited similar flow mediated vasodilation during and three months after pregnancy when compared to normal pregnant women. GDM patients displayed metabolic disturbances and increased BMI compared to controls after pregnancy.

The precise mechanisms behind the enhanced endothelial function in pregnancy are still unclear, though a considerable amount of studies have been published in the field of normal and complicated pregnancy and endothelial function These studies have included in vitro work, where the endothelial cells from the vessels of pregnant or preeclamptic women have been examined and the eNOS and and NO measured, or Ca^{++}-concentrations in the cell or gap junctions investigated. Clinical studies with pregnant women are conducted mostly in very small study populations because of ethical and practical difficulties and thus the outcomes can be confusing since selection and publication biases probably affect the results. Experimental animals do not experience preeclampsia, probably due to negative selection, i.e. the animal models cannot be extrapolated to preeclamptic women; though similar NO mediated vasodilation in pregnancy can be detected in animals. A genetic predisposition to preeclampsia is evident and studies in different populations have identified polymorphisms in PE candidate genes in women with pre-eclampsia(Valenzuela, et al. 2012). Identifying how the endothelial cells in normal pregnancy are reprogrammed would help to reveal the pathophysiology in preeclamptic pregnancies and perhaps improve the treatment of this disease. In general, the enhancement of endothelial function encountered during pregnancy could also be applied to other common forms of hypertension.

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Heli Saarelainen

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Attenuated endothelial function is associated with cardiovascular risk factors and is considered as preclinical sign of cardiovascular disease. Pregnancy related complications such as hypertensive disorder in pregnancy, preeclampsia and gestational diabetes and cardiovascular disease share many risk factors. This study was undertaken to explore endothelial function in normal pregnancy as well as in pregnancy related complications. Endothelial function was assessed with brachial artery flow mediated dilation as a part of the on-going population based Cardiovascular Risk Factors in Young Finns Study and in the Complicated Pregnancy Study in Kuopio University Hospital conducted in 2003-2008.

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