Acute atherosis and oxidative stress
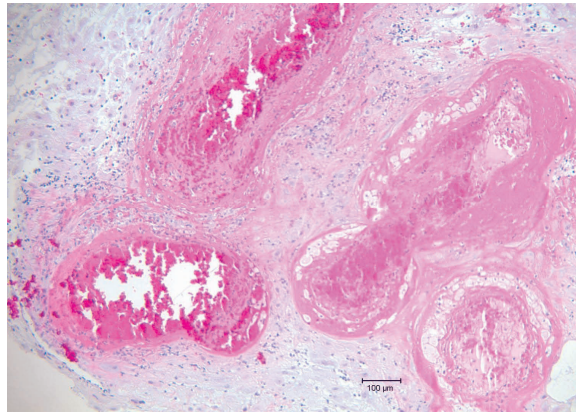
in preeclampsia

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Contents

1. Acknowledgements

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2. Publications included in the thesis

Paper I:

Harsem NK, Staff AC, He L, Roald B. *The decidual suction method: a new way of collecting decidual tissue for functional and morphological studies*. Acta Obstet Gynecol Scand 2004;83:724-30.

Paper II:

Harsem NK, Braekke K, Staff AC. *Augmented oxidative stress as well as antioxidant capacity in maternal circulation in preeclampsia*. Eur J Obstet Gynecol Reprod Biol 2006;128:209-15.

Paper III:

Braekke K, Harsem NK, Staff AC. *Oxidative stress and antioxidant status in fetal circulation in preeclampsia.* Pediatric Research 2006;60:560-64.

Paper IV:

Harsem NK, Roald B, Braekke K, Staff AC. *Acute atherosis in decidual tissue: not associated with systemic oxidative stress in preeclampsia*. Placenta 2007;28:958-64.

3. Abbreviations

AC	antioxidant capacity
ADRP	adipose differentiation related protein
AGE	advanced glycation end products
AT1-AA	angiotensin 1 autoantibody
8-isoprostane	8-isoprostaglandin $F_{2\alpha}$
BHT	butylated hydroxytoluene
BMI	body mass index
BPS	basal plate section
Carr U	Carratelli Units
CRP	C-reactive protein
CVD	cardiovascular disease
DIC	disseminated intravascular coagulopathy
DNA	deoxyribonucleic acid
d-ROM	diacron reactive oxygen metabolites
ELISA	enzyme- linked immunosorbent assay
FGR	fetal growth restriction
FRAP	ferric reducing ability of plasma
GC-MS	gas chromatography-mass spectrometry
GDM	gestational diabetes mellitus
HDL	high density lipoproteins
HELLP	hemolysis, elevated liver enzymes and low platelet count
HPLC	high performance liquid chromatography
IL-6	interleukin 6
IUGR	intrauterine growth restriction
MBRN	Medical Birth Registry of Norway
NO	nitric oxide
ORAC	oxygen radical absorbance capacity
OxLDL	oxidized low density lipoproteins
PAI-1	plasminogen-activator inhibitor-1
PBB	placenta bed biopsy
PlGF	placental growth factor

TBARS	tiobarbituric acid reactive substances
TEAC	trolox-equivalent antioxidant capacity
RAS	renin angiotensin system
ROM	reactive oxygen metabolites
ROS	reactive oxygen species
sFlt1	soluble fms-like tyrosine kinase 1 (also named sVEGFR-1)
TNF-alpha	tumor necrosis factor alpha
TPTZ	2,4,6-tripyridyl-s-triazine
VEGF	vascular endothelial growth factor

4. Introduction

4.1 Preeclampsia

Preeclampsia is a pregnancy-specific disorder defined clinically as de novo hypertension and proteinuria occurring after 20 weeks' gestation. Preeclampsia is a common complication in the second half of pregnancy and is potentially dangerous to both mother and fetus. It is the most important cause of maternal death in Scandinavia, Iceland, Finland, United Kingdom and the USA (1-3), and the incidence of preeclampsia has remained stable for the last fifty years. Preeclampsia occurs in 3-10% of pregnancies, and approximately 50 000 women worldwide die from this disease every year (4). In addition, the mortality of the offspring of a preeclamptic mother is also increased (5). In Norway, the most recent survey (2004) from the Medical Birth Registry of Norway (MBRN) reported an incidence of 3.7% (6).

The pathogenesis of preeclampsia is not fully understood. Considerable research over more than five decades has led to the notion of preeclampsia as a multifactorial syndrome with maternal and fetal interaction, with the participation of two genetically different individuals (7).

4.1.1 Definitions

There is no global agreement regarding the definition of preeclampsia or other hypertensive disorders in pregnancy. The clinical criteria of de novo hypertension and proteinuria are still the most generally accepted. According to the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (8), the following classifications are recommended and have been used in this PhD thesis:

Preeclampsia: A syndrome defined by hypertension and proteinuria. Hypertension is defined as a systolic blood pressure level of 140 mm Hg or higher or a diastolic blood pressure level of 90 mm Hg or higher occurring after 20 weeks' gestation in a woman with previously normal blood pressure. Proteinuria is defined as urinary excretion of 0.3 g protein or higher in a 24-hour urine specimen (or protein dipstick reading equal to or higher than 1+ on more than one midstream urine sample six hours apart).

Eclampsia: The occurrence of seizures in a preeclamptic woman, where the seizures cannot be attributed to other causes.

Gestational hypertension: Blood pressure elevation without proteinuria developing in a woman after 20 weeks' gestation, with blood pressure levels returning to normal postpartum.

Chronic hypertension: Hypertension that is observable before pregnancy or before 20 weeks' gestation. Hypertension that is primarily diagnosed during pregnancy and which persists beyond the 42nd day postpartum is also classified as chronic hypertension.

Superimposed preeclampsia: Preeclampsia developing either *on* chronic hypertension or *on* diabetes mellitus.

4.1.2 Clinical picture of preeclampsia

Preeclampsia is a maternal syndrome, unpredictable both in its onset, progression and severity. The clinical picture ranges from mild, late onset preeclampsia (with no clinical signs of the disease other than elevated blood pressure and proteinuria) to severe, early-onset preeclampsia, with maternal multiorgan failure, including disseminated intravascular coagulopathy (DIC), renal failure, cerebral hemorrhage and hepatic failure.

The HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome is discussed as a variant of preeclampsia (9). Eclampsia, a life threatening, although rare complication of preeclampsia, includes convulsions. Additionally, there is an increased risk of abruption of the placenta, a life-threatening situation for both mother and fetus (10). Other effects on the fetus include health consequences resulting from iatrogen premature delivery, such as neonatal death, persistent bronchopulmonal dysplasia, as well as placental insufficiency with consequent fetal growth restriction (11;12).

4.1.3 Risk factors for preeclampsia

Numerous maternal factors can predispose women to the syndrome, including genetic (13), behavioral or environmental factors. Some of the predisposing factors include diabetes mellitus (14), increased insulin resistance (14), hypertension (8), black race (15), and high body mass index (BMI) (15). Pregnancies with large placentas (as in twin pregnancies, diabetic pregnancies and hydatidiform moles), primigravidas, and a previous pregnancy with preeclampsia (16), increase the risk of developing preeclampsia. A new sexual partner and the use of barrier contraception are both associated with an increased risk of developing preeclampsia, whereas previous antigen exposure is protective (17). There are also reports of family clustering of preeclampsia (18;19), although no general, world wide genetic explanation has been confirmed. Skjærven et al (20), analyzing data from the

MBRN, found that the protective effect of a previous pregnancy against preeclampsia was associated with the time between the pregnancies, and probably not the paternal antigen exposure itself.

4.1.4 Preeclampsia and implications for health later in life

It has been proposed that pregnancy could be viewed as "a medical stress-test for life" (21). According to this concept, the increased physiological demands on the pregnant women in some cases are not met, resulting in gestational syndromes such as gestational hypertension and preeclampsia. The immediate remission is delivery of the baby, but in the long-term the problems reappear in the mother, especially with advancing age, as the reserves of the already "primed" organs diminish. If a pregnant woman does not develop gestational syndromes during her pregnancy, she is in a privileged position with a reduced risk of developing several diseases later in life as compared to a pregnant woman with a gestational syndrome (21).

There are several studies reporting increased maternal risk of cardiovascular disease later in life after a pregnancy with hypertensive disorders, including preeclampsia (5;22-25). In a long-term follow-up study by Irgens et al (5), including data from the MBRN, an eightfold elevated risk of dying from cardiovascular disease and a fivefold elevated risk of dying from stroke was found in women with preterm delivery and preeclampsia. A study by Smith et al (26) of 130 000 Scottish women who had developed cardiovascular disease (CVD), demonstrated a twofold risk of developing CVD later in life following a history of preeclampsia, as well as a sevenfold risk of CVD following preeclampsia and delivery of a baby below 2.5 kilos.

In addition, long-term effects for individuals exposed to an intrauterine preeclamptic environment are being increasingly studied. It seems that the offspring of preeclamptic mothers have an increased risk of adolescent hypertension (27;28), as well as a decreased risk of breast cancer (29), but the mechanisms are still unknown (30).

4.1.5 Treatment and prevention of preeclampsia

Preeclampsia is curable only by termination of the pregnancy and removal of the placental tissue. If parts of the placenta are retained after delivery of the baby, preeclampsia can persist (31). Symptomatic treatment with antihypertensive medication may prolong the pregnancy and reduce cerebral hemorrhagic episodes, but no other positive effects on maternal and fetal outcome have been documented (32). Glucocorticoids are administered to

women with threatening premature delivery prior to 34 weeks' gestation, including preeclamptic women. Glucocorticoids enhance fetal lung maturation and thereby reduce fetal mortality and morbidity. However, this treatment has no documented positive effect on the pregnant women (33).

As the etiology of preeclampsia is still unknown, and probably also multi-factorial, prevention of the disease is not yet within reach. Various approaches to preventing preeclampsia have been tried. Prophylactic treatment with aspirin has been shown to reduce the risk of preeclampsia by 19%, but there is still uncertainty as to which groups of pregnant women would benefit from such preventional therapy (34). Thus, there is still considerable controversy about the use of aspirin for prevention of preeclampsia (35;36). The Magpie Trial (37) reported that treatment with Magnesium sulphate in women with preeclampsia reduces the risk of eclampsia by 50% and probably also reduces the risk of maternal death.

There are a number of treatment and prevention- studies where results are less clear. Antioxidant supplementation, using vitamins E and C, has been shown to reduce the frequency of preeclampsia in a high-risk population (38;39). However, this effect was not verified in a randomized, multi-center trial recently published by Poston et al (40). On the contrary, the Poston study reported that the vitamin-treated group had more low birth neonates (<2500 grammes) than the non-vitamin treated group (28% versus 24% respectively), an increased need for neonatal intensive care and a higher incidence of stillbirth (1% versus 0.5%). In the Poston-study, the vitamin-treated group also had an increased risk of gestational hypertension and an increased need for MgSo4 and antihypertension treatment. The objection has been raised that the randomization of the patients in the study was too late in pregnancy and that the early damage caused by oxidative stress may be an early and irreversible event of pregnancy. It has therefore been hypothesized that earlier antioxidant intervention might have a positive effect (40). In light of the disappointing results to date of using antioxidants to prevent preeclampsia, this underlines the importance of evaluating both maternal and fetal outcome in intervention studies as well as markers of oxidative stress and antioxidants. It also highlights the importance of treating maternal complications and fetal outcome, rather than simply diagnosing preeclampsia (41).

Studies on calcium supplementation as a mean of preventing preeclampsia have shown this to be generally ineffective. It might possibly be effective in developing countries, however, where nutrition for pregnant women may be inadequate (42).

Finally, earlier observational studies have suggested a potential role for omega-3-fatty acids in decreasing the incidence of preeclampsia. This has not been confirmed in randomized, prospective trials, however (43).

4.1.6 Preeclampsia and defect placentation

A successful implantation is the end result of complex molecular interactions between the hormonally primed uterine endometrium and the mature blastocyst (44). The function of the uteroplacental unit is to secure an adequate exchange of nutrients, gases and metabolic end products. These functions are mediated by increased blood flow through the uterus. During the early phase of placental development, extravillous cytotrophoblasts (stemming from the outer cell-layer of the blastocyst) stream out of the anchoring villi, penetrating the syncytiotrophoblast layer, entering the decidua and invading and transforming the maternal spiral arteries. The major structural alterations occurring in these spiral arteries in early pregnancy (so-called "physiological changes") normally transform the arteries to vascular channels outside maternal vascular control, allowing an increased maternal blood supply to the placenta (45) in the last part of the pregnancy (figure I).

Trophoblast invasion of the decidua and maternal myometrium is thought to be a key event in early placentation (46). Reduced trophoblast invasion may represent one of the basic defects leading to preeclampsia (47). This reduced invasion of extracellular trophoblast is followed by a shallow transformation of the spiral arteries (46), leaving them narrow, tortuous and thick-walled. Besides remaining non-transformed, the spiral arteries in preeclampsia can have areas of lipid deposition in the vessel wall, a phenomenon known as acute atherosis. The name was given to the changes, first observed in 1945, as a result of the morphological resemblance of early phases of atherosclerotic lesions in vessels (48).

Figure I: Schematic drawing of a term placental structure. The arrows represent blood flow from the decidual arteries into the intervillous spaces and back into the venous blood (modified after Junqueira LC et al. Basic Histology. Appleton and Lange 1989: 452-458).
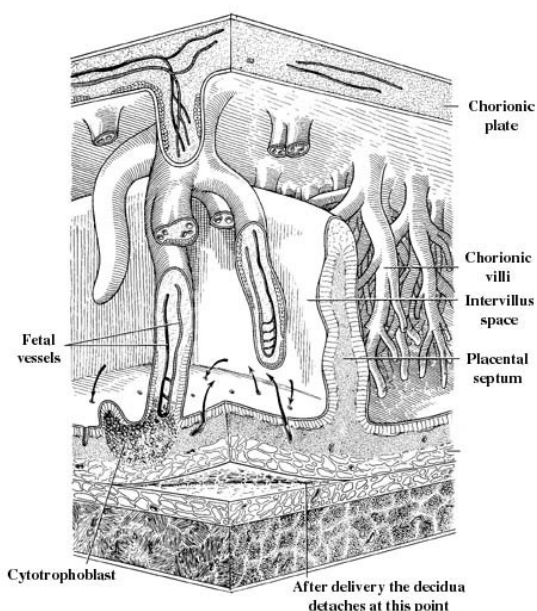
There is still some controversy and insufficient knowledge regarding the etiology and molecular mechanisms behind the formation of acute atherosis. This is partly due to the difficulty in obtaining sufficient and adequate tissue from the placenta bed, as well as a lack of relevant animal models.

Normal placental function relies on adequate maternal blood supply through the wholly or partially transformed spiral arteries in the placental bed (figure II). Any process that results in mural or occluding thrombi in these arteries reduces the flow and can lead to hypoxia and ischemia as well as infarcts in the placental tissue, i.e. reduced placental function, which is more common in preeclampsia than in uncomplicated pregnancies (49). A hypoxic placenta is believed to release toxic compounds to the maternal circulation, resulting in maternal endothelial dysfunction and oxidative stress (50;51). Acute atherosis in spiral arteries is associated with an increased risk of thrombosis and thereby placental ischemia and infarctions (49).

Bearing in mind the multifactorial aspects of preeclampsia, defect placentation additionally seems to be related to immunological processes such as decreased trophoblast expression of human leucocyte antigen-G (HLA-G) (52), as well as interaction with maternal lymphocytes such as uterine natural killer (NK) cells (53).

4.1.7 Inflammation and endothelial dysfunction

Endothelial cells represent a semipermeable barrier between the vessel wall and the blood flow in the vascular system (54). The endothelial lining plays a central role in the regulation of hemostasis via a series of receptors for proteins, hormones, lipid transports as well as through cell-cell interactions (55). "Endothelial dysfunction" has not been precisely defined, but the term is used to indicate change in endothelial properties with activation and abnormal function (56). Evidence suggests that endothelial activation and low grade maternal inflammation is present in all pregnancies, there being merely a different gradient in preeclampsia, with a stronger inflammatory response (57). The central pathological features of the maternal syndrome may thus include the presence of an excessive maternal inflammatory response, resulting in dysfunction, activation and peripheral vasoconstriction with reduced circulating volume, which in turn leads to oxidative stress (57).

4.1.8 Preeclampsia and the two-stage-model

The present concept of the pathogenesis of preeclampsia involves a two-stage model (figure II). Stage 1 refers to a defect and reduced trophoblast invasion into the maternal spiral

arteries (with reduced physiological arterial changes or remodeling) leading to reduced placental perfusion. Stage 2 refers to a generalized dysfunction and activation of the endothelium, and, finally, development of the maternal syndrome (58;59). Several factors have been proposed as *the* link between the two stages of preeclampsia. Among the substances suggested as being freed from the placenta to the maternal circulation are syncytiotrophoblast debris (60), oxidized lipids (61) and angiogenic factors (62-64). Recently, angiogenic associated factors, such as placental sFlt1 (soluble fms-like tyrosine kinase 1) (65) and soluble endoglin (66), have been proposed as possible circulating endothelial damaging factors originating in a hypoxic placenta in preeclampsia.
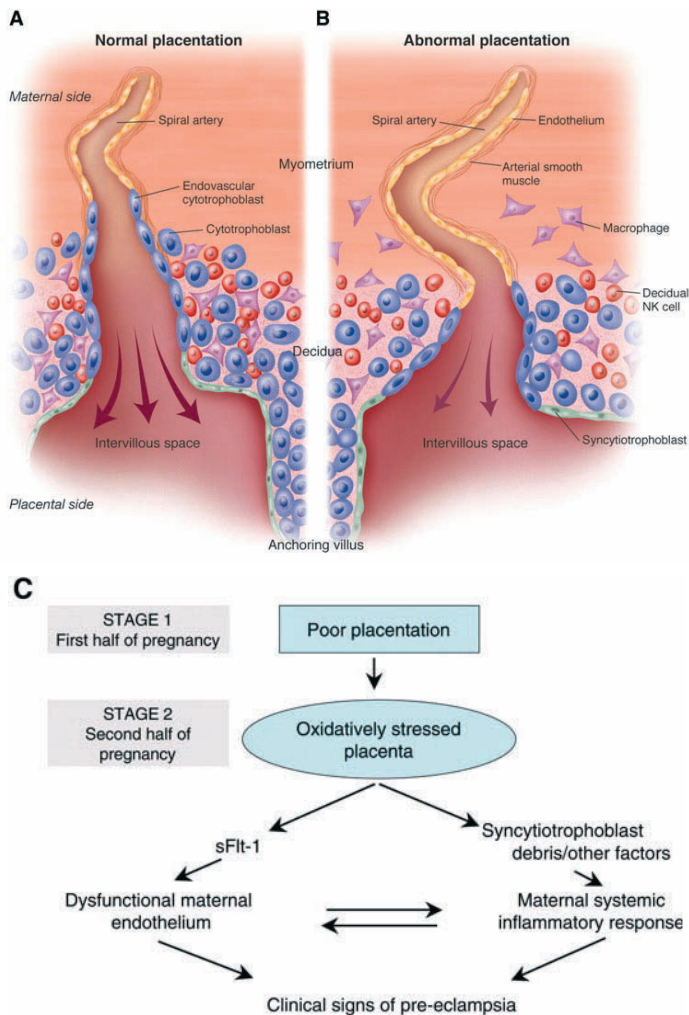

Figure II. Poor placentation and preeclampsia (Redman C & Sargent I. Science 2005;308:1592-4).

4.1.9 Oxidative stress in preeclampsia

Oxidative stress is defined as a biochemical imbalance between free radical damage and antioxidant protection. It arises from excessive generation of free radicals and/or inadequate endogenous antioxidant capacity (67;68). Reactive oxygen species (ROS) are constantly produced as by-products of normal oxidative metabolism in mitochondria and other cellular reactions (69;70). All organisms possess a range of enzymatic and non-enzymatic antioxidant systems, which serve to protect against the harmful oxidative reactions that occur as a consequence of this endogenous ROS production. Examples of antioxidant enzymes are superoxide dismutase, gluthatione reductase and catalase; examples of non-enzymatic antioxidants are vitamin C, vitamin E, vitamin A, glutathione and flavenoids (71). Under certain conditions, an increase in oxidants and a decrease in antioxidants cannot be prevented, and the oxidant/ antioxidant balance shifts towards the oxidative state. This may result in oxidative stress, which is implicated as a factor contributing to the endothelial dysfunction and pathological changes in preeclampsia (72-74).

Pregnancy itself is a condition of increased oxidative stress (75), and may contribute to endothelial dysfunction and the clinical development of preeclampsia (76;77). There is considerable evidence supporting an increase in products of oxidative stress in women with preeclampsia, demonstrated both in the placenta (61;78) and in maternal peripheral blood cells (73), as well as in the maternal circulation (79). There is still some controversy with respect to the results for latter compartment (80;81).

A number of circulating compounds may under certain circumstances enhance oxidative stress. They include lipoproteins, lipids, proteins, glucose and advanced glycation end products (AGEs).

An established method for measuring the end products of increased oxidative stress is to measure the degree of lipid peroxidation. Lipid peroxidation occurs when ROS interact with polyunsaturated fatty acids in membranes or lipoproteins (82). In preeclampsia, there is development of excessive maternal hyperlipidemia, and especially hypertriglyceridemia, as compared to normal pregnancy (83;84) and these changes are present long before the onset of preeclampsia (85). Oxidation of low density lipoproteins, which are prominent in preeclampsia, is one example of the measurable consequences of increased oxidative stress (86). There are several methods of examining lipid peroxidation products in biological samples, but most of them have both low specificity and low sensitivity in terms of measuring changes in free radical status. Among such lipid peroxidation products are the

tiobarbituric acid reactive substances (TBARS), lipid hydroperoxides and the isoprostanes (87-89).

There are also end products of oxidative stress, other than lipid peroxidation products, that can be measured as indices of oxidative stress. Direct damage to proteins by peroxidation of the amino acids can also give rise to protein carbonyls, which may serve as more general biomarkers of oxidative stress (90). Plasma protein carbonyls have been demonstrated to be elevated in preeclampsia as compared to controls (91).

Peroxinitrate, produced by the vasorelaxant nitric oxide (NO) reacting with superoxide anions (produced under conditions of oxidative stress) is also a potential marker of oxidative stress. Peroxinitrite is regarded as a marker of "nitrative stress" which, subsequent to oxidative stress, is seen in the placenta in preeclampsia and diabetes in association with altered placental function (92). NO is produced by endothelial cells and is also known to react with superoxide anions (produced under conditions of oxidative stress), yielding peroxynitrite that may impair vascular function (93).

4.1.10 Angiogenic factors in preeclampsia

Considerable attention has recently been focused on angiogenesis-related factors in the etiology of preeclampsia. It has been postulated that preeclampsia might be a syndrome of angiogenic disorders and that the angiogenic factor soluble fms-like tyrosine kinase 1 (sFlt1) plays a causative role in the development of preeclampsia (65;94). PlGF (placental growth factor) and VEGF (vascular endothelial growth factor) are growth factors necessary for normal endothelial function, and also important factors in the vasculogenesis and angiogenesis of early placentation (95). Recently the anti-angiogenic factor sFlt1, which binds soluble VEGF and PlGF and inhibits their effect on the vascular endothelium, was found to be up-regulated in preeclamptic placentas (65). High serum levels of sFlt have been found in preeclampsia (64;65), and elevated serum concentrations of sFlt1in the second trimester may predict onset of preeclampsia (63). A recent Norwegian study also showed that a low rise of maternal PlGF and a high rise of sFlt1 between the first and second trimester are strong predictors of early onset preeclampsia (96).

Recently, soluble endoglin (sEng) has been found in elevated concentrations in preeclamptic maternal serum (66). sEng cooperates with sFlt1 in inducing endothelial dysfunction in vitro, and severe preeclampsia-like illness can be induced in pregnant rats (65). sEng and sFlt1 block pro-angiogenic effects of TGF-β and VEGF in vitro, and show

additive effects, indicating that these soluble receptors may act in concert to cause endothelial dysfunction (66).

4.2 Preeclampsia and fetal aspects

The fetal well-being and development *in utero* during a pregnancy is dependent on both the fetal genes and the intrauterine environment. The in utero environment is mainly mediated through the placenta, which serves all the needs of the infant, including nutritional and oxygen supply, disposing of waste products as well as serving hormonal support for fetal growth and development.

In pregnancies complicated with preeclampsia, the consequences for the fetus may be both those according to prematurity, like lack of lung maturation and growth restriction, as well as the long term consequences for the infant, like increased risk of developing cardiovascular disease, dying from stroke and developing hypertension in adulthood, as described in chapter 4.1.4.

Maternal endothelial dysfunction is believed to be the common final pathway leading to the syndrome of preeclampsia (50;51). As described above, a dominating hypothesis is that agents produced by the relatively hypoxic placenta are transferred to the maternal circulation, causing endothelial dysfunction. To what extent such placental agents could affect the fetus, or are present in the fetal circulation at increased concentrations in preeclampsia, has not been fully explored. Only a few studies have addressed this issue, with conflicting results (97-100).

Prematurity has been associated with increased oxidative stress (101-103), and oxidative stress has also been implicated in several of the diseases of prematurity such as bronchopulmonary dysplasia, intraventricular hemorrhage and necrotizing enterocolitis (103-106). 8-isoprostane, an acknowledged marker of oxidative stress, has been demonstrated elevated in plasma of preterm infants compared to term infants in only one study (107), and also low concentrations of antioxidants in the fetal circulation have been demonstrated, possibly representing infants more vulnerable to oxidative injury (108-110). Since preeclampsia is associated with increased oxidative stress both in the maternal circulation and in the placenta, it is of interest to explore whether this imbalance between pro-and antioxidants also is present in the circulation of an infant born to a preeclamptic mother.

4.3 Diabetes mellitus and superimposed preeclampsia

Diabetes mellitus and preeclampsia share many pathophysiological features, including endothelial dysfunction, insulin resistance, oxidative stress, and inflammation (14;111;112). Both pre-gestational diabetes and gestational diabetes mellitus (GDM) are associated with a two to fourfold increased risk of developing preeclampsia in pregnancy (113-115), named superimposed preeclampsia. In this context, this includes women with diabetes mellitus (pre-existing or gestationally induced) with development of preeclampsia in the present pregnancy. The diagnosis of "superimposed preeclampsia" is also commonly used for women with pre-existing hypertension with a development of preeclampsia (development of proteinuria without hypertension induced nephropathy) after week 20 of pregnancy.

5. Aims of Study

The main aim of the present thesis was to investigate the relationship between placental acute atherosis and maternal circulating oxidative stress. Both features are assumed to be essential in preeclamptic pregnancies, but their relationship has not been extensively studied. The presence of circulating oxidative stress in maternal preeclampsia has also been debated, and the fetal situation is mainly unexplored.

Specifically, the aims were to:

1. evaluate histologically a new vacuum suction method designed to obtain decidual tissue from the placental bed, focusing on the yield of transformed decidual spiral arteries. We wanted to compare the tissue obtained by this method with two other well-established methods to obtain placental bed tissue, i.e. the placental bed biopsy method and the placental basal plate section method (Paper I)

2. study the phenomenon of acute atherosis in preeclampsia and controls in the decidual tissue from the vacuum suction method (Paper IV)

3. assess the maternal and fetal circulating levels of oxidative stress as well as antioxidant capacity in preeclamptic pregnancies, compared to uncomplicated pregnancies, using 8-isoprostane, a well-known marker of oxidative stress, and antioxidant indices (Paper II and III)

4. explore a potential correlation between maternal oxidative stress (measured as 8-isoprostane) and the maternal serum concentration of the placenta-produced angiogenic factor sFlt1. 8-isoprostane and sFlt1 are excessively produced by preeclamptic placentas as compared to placentas from uncomplicated pregnancies (65), and an association between these factors in the maternal circulation has not been explored previously (Paper II)

5. compare the presence of acute atherosis in decidual tissue spiral arteries, using our evaluated vacuum suction method, with the possible presence of hyperlipidemia and oxidative stress in the maternal circulation, both in uncomplicated and preeclamptic pregnancies (Paper IV).

6. Summary of Papers

Paper I: "Evaluation of the decidual suction method"

In this methodology paper, we wanted to evaluate and compare tissue from the placental bed obtained by three different methods: decidual tissue from our cesarean section biobank study, sampled using our own vacuum suction method (n=51), compared with archive material from placental bed biopsies (PBB, n=33) and placental basal plate sections (BPS, n=33). The success rate was defined as the proportion of cases in which one random tissue section contained at least one transformed decidual spiral artery with interstitial trophoblasts. The success rate of the decidual suction method (86%) was superior to the two other methods, the PBB method (61%) and the BPS method (48%). We also documented that the vacuum suction method was safe for the patient, both in the short-term and long-term.

Paper II: "Maternal oxidative stress in preeclampsia"

Our cesarean section biobank at Ullevål University Hospital was used for this study, including 21 women with preeclampsia and 38 women with uncomplicated pregnancies.

Evidence of increased oxidative stress, as assessed by a 62% elevated median 8-isoprostane concentration, was found in maternal circulation in the group of preeclamptic women compared to the group of women with uncomplicated pregnancies (median concentrations 354 pg/mL vs 218 pg/mL, P=0.02). For the total antioxidant capacity, measured as FRAP (ferric reducing ability of plasma), median concentration was 26% higher in the preeclampsia group as compared to the uncomplicated pregnancy group, demonstrating a relatively greater increase in oxidative stress (measured as 8-isoprostane) than in antioxidant capacity (measured as FRAP) in the maternal circulation in preeclampsia. The d-ROM (diacron reactive oxygen metabolites) test, another index of oxidative stress, did not show statistical significant differences between the control group and the preeclampsia group (457 Carr U vs 550 Carr U, P=0.06), and no difference in vitamin E levels was demonstrated between the control group and the preeclampsia group (35.8 µmol/L vs 35.8 µmol/L, P=0.7). We were also able to demonstrate a positive correlation between maternal angiogenic factor sFlt1 and maternal 8-isoprostane concentrations in the preeclampsia group (Spearman correlation 0.5, P=0.03).

Paper III: "Oxidative stress and antioxidant status in fetal circulation in preeclampsia"

Our cesarean section biobank at Ullevål University Hospital was also used for this study, including 19 women with preeclampsia (the same patient population used in Paper II, except for two extra patients included in Paper II) and 33 controls (the same patient population used in Paper II, except for five extra patients included in Paper II).

We demonstrated no statistical significant difference in median concentration of 8-isoprostane between the preeclampsia group and control group either in the umbilical vein (955 pg/mL vs 780 pg/mL, P=0.41) or in the umbilical artery (233 pg/mL vs 276 pg/mL, P=0.65), indicating no evidence of increased oxidative stress in fetal circulation in preeclampsia, as evaluated by 8-isoprostane concentrations. Concentration of 8-isoprostane was significantly higher in plasma from the umbilical vein than artery, suggesting the placenta as the source of fetal circulating 8-isoprostane. Fetal and maternal concentrations of 8-isoprostane (from Paper II) did not correlate, neither for the preeclampsia nor for the control group.

For the total antioxidant capacity, measured as FRAP, median concentration was higher in preeclampsia than in controls, both in the umbilical vein and artery. Also, there was a statistical significant positive correlation between fetal and maternal FRAP values.
For the preeclampsia group we demonstrated higher median vitamin E concentration in the umbilical vein compared to controls (5.7 µmol/L vs 3.6 µmol/L, P=0.001), but no difference between preeclampsia and controls was found in the umbilical artery (4.7 µmol/L vs 3.7 µmol/L, P=0.19). In the umbilical artery, median vitamin E concentration was approximately 10% of maternal value. Fetal and maternal concentrations did not correlate (for either umbilical artery or vein), neither in preeclampsia nor controls.

Paper IV: "Association between maternal oxidative stress and decidual acute atherosis"

Our cesarean section biobank at Ullevål University Hospital was again used for this study, including 49 women with preeclampsia (21 also used in Paper II and 19 used in Paper III), 46 controls (38 also used in Paper II and 33 used in Paper III) and 7 women with superimposed preeclampsia.

We demonstrated acute atherosis (defined as presence of CD68 positive foam cells and fibrinoid necrosis in the spiral artery wall) in 42% of the tissue from the preeclamptic

patients with identified spiral arteries. In addition, we demonstrated CD68 positive foam cells with fibrinoid replacement in 14% of the uncomplicated pregnancies.

Our study did not show any association between the presence of acute atherosis in decidual spiral arteries and elevated oxidative stress (assessed by an elevated 8-isoprostane concentration) or dyslipidemia (assessed by triglyceride, HDL-Cholesterol and total cholesterol) in the maternal circulation of the preeclampsia group.

We demonstrated elevated concentrations of 8-isoprostane in the maternal circulation of the diabetes group with superimposed preeclampsia compared to both the control group (691 pg/mL vs 218 pg/mL, P=0.03) and the preeclampsia group (691pg/mL vs 354 pg/mL, P=0.14). The group of patients with diabetes mellitus and superimposed preeclampsia is small (n=7) and the results thus need to be interpreted with caution. The study needs to be repeated with a larger patient cohort.

7. General Discussion

7.1 Subjects included

The patients included in the four papers of this PhD thesis were recruited at the Department of Obstetrics, Ullevål University Hospital, Norway. As well as being the largest general and referral hospital in Norway, it also has the largest delivery unit, with approximately 6500 deliveries per year. This represents 12% of all deliveries in Norway. The incidence of preeclampsia in the Ullevål population is 6.9% as opposed to 3.7% in the Norwegian population as a whole (6). Twenty-two percent of the preeclampsia patients are delivered by the cesarean route at our Department.

The recruitment of patients for our biobank study started in March 2001. All the patients in Papers I-IV are part of this ongoing biobank study, apart from the 33 subjects (Paper I) for whom placental bed biopsies and placental basal plate sections were taken from the archive routine diagnostic material from the Department of Pathology, Ullevål University Hospital. The biobank study includes women with uncomplicated as well as complicated pregnancies, the latter group comprising preeclamptic pregnancies and pregnancies complicated with diabetes mellitus.

The preeclampsia patients included were previously healthy women who were delivered by cesarean section because vaginal delivery was not considered appropriate due to disease progression and/or unfavorable cervical ripening. They were all normotensive prior to pregnancy. Controls were healthy, normotensive women undergoing cesarean section due to breech presentation or for psychosocial reasons. In Paper IV we included a small group of diabetes mellitus patients with superimposed preeclampsia (n=7), in addition to the preeclampsia group and the control group. This group of patients with superimposed preeclampsia was delivered by the cesarean route with the same clinical indications as the remaining preeclampsia group.

The time-consuming procedure of recruitment, collection and preparation of the biological material was principally performed by the two PhD students involved in the biobank study (Braekke and Harsem), both senior clinicians in their fields (pediatrics and gynecology/obstetrics, respectively). The patients were recruited mostly during the daytime, rarely on weekends and not during the summer or other holidays. It would have been preferable to include **all** patients delivered by cesarean section with a clinically verified diagnosis of preeclampsia during this study period. However, we do not believe the time-

point random selection of pregnancies included would have significantly biased patient selection.

Due to the composition of patient population at Ullevål University Hospital, the biobank material consists of a subgroup of more severe preeclamptic patients than normally distributed in the Norwegian population. As the clinically less severe preeclampsia patients had vaginal deliveries, our group of preeclamptic patients has more severe preeclampsia (and deliveries at a lower gestational age) than the total preeclampsia group at the hospital. Our preeclampsia group is therefore not representative of all women with preeclampsia, but is possibly the biologically most interesting preeclampsia group to study, as early and severe preeclampsia have most severe consequences for the infant.

All patients included had a clinical indication for cesarean section. Of the eligible women asked to participate in the biobank study, only three refused; one in the preeclampsia group and two in the control group. The majority of the pregnant women included in our studies are Caucasian Norwegian (90%), the remainder are non-Western immigrants from Pakistan, Somalia and India.

Since the biobank collection is ongoing, the numbers of patients included in the different studies (Papers I-IV) varies, depending on the number of patient samples available at the start of the various studies

In Paper I, we compared the spiral artery findings in the decidual tissue (n=51) from our biobank study with archive paraffin-embedded tissue material from the basal plate (n=33), as well as from the placental bed (n=33), retrieved from the files in the Department of Pathology. The sections were evaluated morphologically and immunohistochemically by two observers, Harsem and Roald, the latter a senior pathologist. Only anecdotal clinical information was available, but this is of no significance in our methodological study.

In Paper II we included 21 patients with preeclampsia and 38 controls. These patients are also part of the patient populations in Paper III and IV. The number of patients is higher in the latter paper because of additional patients being recruited at the start of these studies. The number of patients in Paper III is somewhat smaller because of technically difficulties in collecting samples from all compartments from the same pregnancy, especially lack of fetal samples. These technical difficulties include situations were the operating doctor forgot to clamp the umbilical cord according to the protocol as well as limited umbilical cord blood volume in the premature pregnancies.

Variations in oxidative stress during labor in vaginal deliveries could have affected the results in Papers II-IV. We thus believe cesarean section was a preferable delivery mode for answering the main question of the studies: What is the relationship between placental acute atherosis and maternal circulating oxidative stress? We consider our decision to include only patients undergoing cesarean section as a strength of our study, since vaginal delivery is associated with oxidant stress (75). Increased oxidative stress has also been demonstrated in the fetal circulation after vaginal delivery, both measured as augmented lipid peroxidation products (116) and gluthatione (117). Additionally, as shown in Paper I, it would not have been possible to collect adequate decidual tissue from the retroplacental area after a vaginal delivery. A blind curettage procedure following vaginal delivery was considered unethical, as it is a clinically unnecessary procedure with a risk of uterine perforation. In Paper I we examined the risk of complications after the vacuum suction procedure from the placental bed during cesarean section; the method was proven safe both in a short-term and long-term perspective.

A major advantage of our biobank selection is the quality of tissue material. This has been verified as non-degraded by mRNA studies and verified as site-relevant for the placental bed by histology (118). The bank also includes blood samples from the mother and fetus (umbilical cord) and extensive clinical information. All clinical information was personally and comprehensively collected from each patient by Harsem and Braekke, as well as from the medical chart and a national compulsory individual pregnancy chart following each pregnant woman in Norway. We therefore feel confident that the clinical information about the patients included is correct, and that the quality of the decidual tissue is not hampered due to time delay and poor tissue conservation.

Another advantage of our biobank study concept is the possibility of doing long-term follow-up studies related to maternal and fetal morbidity, as patients have consented to being contacted with regard to participation in future studies. The biobank material is also valuable for further studies relating to the etiology and pathophysiology of preeclampsia. As there is still uncertainty as to which organ or tissues play the primary pathogenetic role in preeclampsia, the sampling from different tissues and compartments could be beneficial for further research. In our comprehensive biobank we have also included a skeletal muscle and fat tissue from the mother, as well as amniotic fluid samples and fetal cord blood sampled separately from umbilical vein and umbilical artery blood, allowing fetal-maternal comparisons (64;99;118). The possibility of feto-maternal comparison was one of the

primary ideas of the biobank, and other feto-maternal results have also been published from our group (64;99;118;119).

Gestational length

One of the disadvantages of our study design is the lack of matching for gestational age. Since most of our preeclamptic patients have a severe disease, they are delivered early in gestation, as a result of disease progression. Our control group, however, consists of pregnant women delivered by cesarean section near term due to breech presentation or repeated cesarean section. It is clinically impossible to match the preterm preeclamptic cesarean delivered group with a prematurely delivered "control" group, since there would be extremely few clinical indications for a preterm cesarean delivery in uncomplicated pregnancies. Also, collection of umbilical cord blood with cordocentesis, in gestationally matched "controls", would not be ethically acceptable for research purposes only.

The gestational length and implications for interpretation of the results from the various analyses in this thesis are discussed in the method section. The gestational length of the study groups was not considered in Paper I, since this information and the diagnoses of the patients included were not known for the placental bed biopsies and placental basal plate sections, and the primary goal of the study was to compare the quality of the collected material, not considering the findings in accordance with clinical data.

We found only one study demonstrating an inverse correlation between gestational length and concentrations of umbilical vein plasma free-F2-isoprostanes, with higher levels in the premature deliveries (120). According to this publication, we could therefore have expected higher levels of total 8-isoprostane in the infants from preeclamptic pregnancies as compared to controls, due to their lower gestational length, but no such correlation was found, when analyzing the whole study group together or the preeclampsia group alone, neither in the umbilical vein nor in the artery.

Severity of disease

The most common ways of defining preeclampsia severity are either according to gestational length at delivery (severe preeclampsia: delivery prior to gestational week 34) or according to blood pressure level (systolic blood pressure level above 160 mm Hg as well as degree of proteinuria) (8).

In our biobank studies and in this thesis, the majority of patients included have severe preeclampsia, defined both according to gestational length and according to elevated blood pressure.

The severity of disease, as assessed according to the different analyses in the studies, is discussed further in the method section below. The differentiation of patient groups according to severity of disease was considered in Paper II, III and IV. In Paper I we included no information about gestational length and diagnoses.

7.2 Decidual tissue sampling and evaluation of acute atherosis

7.2.1 Tissue sampling

The collection of the decidual material was performed using the method originally devised by principal supervisor Staff and her collaborators (121). In this biobank study, the vacuum suction procedure was performed by a limited number of operating gynecologists. The localization of the placenta was known preoperatively from ultrasound findings and manual palpation, and a careful vacuum suction was performed on the entire uterine wall area underlying the removed placenta. We therefore believe that the decidual material harvested contains mainly spiral arteries delivering blood to the placenta, and not the non-transformed maternal arterioles from the membranous decidua. The method issue of harvesting is of some significance, since demonstration of spiral artery acute atherosis is one of the main issues in Paper IV.

It has previously been demonstrated that removing decidual tissue by post partum curettage results in a more rapid normalization of the blood pressure and clinical condition of the preeclamptic patient (122). Staff et al have previously demonstrated that the vacuum suctioned decidual material obtained by this method yielded larger amounts of tissue than the traditional placenta bed biopsies (121). Our aim in Paper I was therefore to evaluate decidual tissue, collected using the vacuum suction method, for use in studies of decidual pathomorphology in preeclampsia and diabetes mellitus. For comparison, the same quality assessment was undertaken on archive material from placental bed biopsies (PBB) and placental basal plate sections (BPS).

One major advantage of the decidual suction method, compared to the placenta bed biopsy method, is the tissue yield (121), which makes it possible to undertake both morphological as well as molecular studies on the material collected. The higher amounts of tissue collected, as compared to placental bed biopsies, make this technique valuable for studying pathological conditions in the decidua, such as acute atherosis. Another advantage is that decidual tissue is collected from the *whole* uterine wall underlying the placenta, not only from small areas of the placenta bed. A further advantage of the vacuum suction

method is that it is easy and rapid to perform, and does not lead to short-term or long-term complications (Paper I) when performed by experienced members of staff.

One of the disadvantages of the decidua suction method is the lack of topographical tissue orientation, in contrast to the placenta bed biopsy method in which the tissue orientation is well conserved. Another disadvantage is the relative lack of tissue from the myometrium (Paper I). Finally, the entire spiral artery is not presented in its whole length in the decidua suction method, which limits the interpretation of what is happening along its full length.

In terms of studying acute atherosis of the spiral arteries, a phenomenon most often seen in the decidual spiral arteries, and seldom in the myometrial part (123), we believe the decidua suction method is superior, or at least complementary to the placenta bed biopsy method. The latter has significant limitations compared to the vacuum suction methods with respect to tissue yield and focal representation, as well as potential clinical complications such as uterine wall perforation and bleeding, necessitating uterine wall suturing.

7.2.2   Acute atherosis

In spiral arteries of the maternal decidua in the placental bed a set of related pathological changes are described as decidual vasculopathy. They include defect remodeling (physiological change), acute atherosis and mural or occlusive thrombosis. Acute atherosis is defined as accumulation of foamy macrophages in spiral arteries with areas of necrosis. Acute atherosis has been regarded a phenomenon of preeclampsia, although is not specific to it (124;125). The etiology and biology of acute atherosis is not well understood. One reason for this is the difficulty of finding spiral arteries in the decidua attached to the membranes or placental bed even under normal conditions. There are about 100-150 spiral arteries in the placenta bed (70), and one is only able to harvest a very small proportion of them using a biopsy technique. Since the lesions in acute atherosis are focal (126), the phenomenon is technically difficult to study adequately. Much of the information on spiral artery changes comes from unique studies on intact hysterectomy specimens from early pregnancy (127). It has been argued that the hyperlipidemic profile in the maternal circulation in preeclampsia (84) may contribute to spiral artery lipid changes in the poorly transformed spiral arteries (128), resulting in a local endothelial dysfunction, which in turn contributes to the systemic disease. We found no evidence to support the validity of this theory for the entire group of preeclamptic women, since there was not significant

association between the local spiral artery acute atherosis and maternal lipid changes, or evidence of oxidative stress in the maternal circulation (Paper IV).

Additionally, the severity of preeclamptic disease, as measured by premature delivery or a more elevated blood pressure, did not differ between the preeclampsia groups with or without acute atherosis (Paper IV). As acute atherosis is focal and does not occur in all spiral arteries (124) there might be a sampling bias. In the absence of thrombosis they may not have a major impact on the total uteroplacental circulation and fetal growth.

In our decidual placental bed material we also lack the spiral arteries from the myometrium. This might be another sampling bias, as some authors believe that focal decidual changes have no major effect on the maternal circulation (124;129).

As we measured the presence of acute atherosis, we also made a quantification of the percentage of the transversal section of the vessel wall with CD68 positive foamy macrophages present. This was to investigate if there could be an association between a high level of CD68 positive foam cells in the spiral artery wall and the severity of the preeclamptic disease. We found no such association, however (Paper IV).

An interesting additional finding was the observation of CD68 positive foam cells and fibrinoid replacement in the subendothelial compartment of decidual arteries in 14% of non-preeclamptic patients in whom arteries could be identified (Paper IV). There are very few data on the exact nature of *fibrinoid* within the spiral artery wall, and further work on spiral artery *fibrinoid replacement* and *fibrinoid necrosis* is clearly necessary. PAS staining is a marker for matrix glycoproteins and "fibrinoid replacement", and these areas surrounding the lipid-laden foam cells may possibly represent secretion products from the trophoblast cells replacing the muscular wall, and do not equal "fibrinoid necrosis" morphologically. The term fibrinoid necrosis is believed to be an expression of cell death in the spiral artery wall and is usually demonstrated in arteries exposed to hypertension. The phenomenon of acute atherosis is defined as presence of CD68 positive foam cells *plus* the presence of fibrinoid necrosis (130;131). Fibrinoid necrosis was not demonstrated in the uneventful pregnant women with presence of CD68 positive foam cells, and could therefore not be defined as true acute atherosis. For all the four women, in whom the CD68 positive foam cells were present in the spiral artery walls, there were no clinical or biochemical pre-pregnant or intra-pregnant manifestations of disease and also no indication of restricted fetal growth in these pregnancies. It is possible to speculate that this group of uncomplicated pregnant women could have an unrevealed risk of cardiovascular disease later in life, and our biobank concept will allow us, or other associates, to study this on a long-term basis.

7.3 Methods for analyzing oxidative stress and antioxidant indices

7.3.1 Isoprostanes

As presented briefly in the introduction, there are several variables indirectly quantifying oxidative stress. As a measure of oxidative stress, we chose to measure the lipid peroxidation product 8-isoprostane, which is viewed as the "golden standard" for measuring oxidative stress (132), being more stable in terms of not undergoing further oxidative processes ex vivo, as long as the blood samples are treated adequately (adding indometacin to avoid platelet excretion of 8-isoprostane as well as butylated hydroxytoluene (BHT), a free radical scavenger, to avoid ex vivo formation of 8-isoprostane) (87). Gas chromatography mass spectrometry (GC-MS) is regarded as the superior method of analyzing 8-isoprostane, as it is highly specific and sensitive, though a complex and expensive method (132;133). It is also possible to measure the 8-isoprostane concentration with commercially available enzyme-linked immunosorbent assay (ELISA) kits, which are both inexpensive and measure 8-iso-prostaglandin relatively quickly, but this method cannot be regarded as a valid substitute for the more precise GS-MS assay (134).

The isoprostanes are stable products of free radical-catalyzed peroxidation of arachidonic acid in cell membrane phospholipids (135). 8-iso-prostaglandinF$_{2\alpha}$ (8-isoprostane) is one of the isoprostanes most often used for analysis; it is excreted in urine and its half-life is 20 minutes. The ratio of free (not cell-bound to phospholipids) to total 8-isoprostane (free and cell-bound 8-isoprostane) is 1:4 (132). The reason why we measured total, and not free, plasma 8-isoprostane in our studies (Papers II, III and IV), is because the concentration of total 8-isoprostane is higher than the free form and thereby easier to detect. This was therefore recommended by our collaborating laboratory in the UK (132).

8-isoprostane can be detected in biological samples such as plasma, tissues and urine (136), and increased concentrations have been found in conditions associated with oxidative stress (136). Increased levels of circulating oxidative stress have been demonstrated in cigarette smokers, but intervention with vitamin E has not shown any positive effect in reducing excretion of urine 8-isoprostane (137;138). In our study (Paper II), we could have measured urine 8-isoprostane instead of plasma 8-isoprostane, but as preeclampsia may possibly affect renal function, and thereby urine excretion of isoprostane, we considered plasma samples to be preferable.

In Papers II, III and IV, the blood samples from the patients were all collected and stored equally, the assays were performed equally and in a blinded manner, i.e. the

diagnoses were unknown to the technicians performing the assays. As blood samples were handled strictly according to the procedures, we believe that the difference in circulating 8-isoprostane levels found between the preeclampsia group and the control group in Paper II, and also the lack of difference in the fetal circulation between the preeclampsia group and the control pregnancies in Paper III, reflects the existence of maternal augmented oxidative stress present in the circulation in preeclampsia, as well as no true difference in the fetal circulation, as compared to uncomplicated pregnancies. However, there are reports indicating that there is not always a good correlation between various methods measuring oxidative stress in the circulation, evaluated as lipid peroxidation products (139), therefore other methods might conclude differently. Also, patient selection might impact on the results, as shown by a negative finding of oxidative stress by Barden et al in the maternal circulation (79), using the same 8-isoprostane methodology as in our papers.

The interpretation of the 8-isoprostane results is open to challenge, since the groups are not matched for gestational age. However, it has previously been demonstrated that the plasma 8-isoprostane concentration in uneventful pregnancies remains unaltered throughout pregnancy (140), which could indicate that the elevated 8-isoprostane concentration in the preeclampsia group (compared to the uneventful pregnancy group, Paper II) represents a true elevation for the preeclampsia group, and is not merely a result of a lower gestational length. One might, in fact, speculate that there could have been an even more pronounced difference in 8-isoprostane concentration if the preeclampsia group had been compared with a group of gestationally matched uneventful pregnancies. Severity of preeclamptic disease, as indicated by premature delivery prior to gestational week 34 (n=13), was associated with elevated median maternal 8-isoprostane concentrations compared to the preeclampsia group (n=8) delivered after 34 weeks gestation (465 pg/mL and 236 pg/mL, respectively, P=0.01). The apparent "gestational"-decline in 8-isoprostane concentrations in preeclampsia in our study (Paper II) is most likely to be confounded by the severity of disease. It is the latter which is the indication for cesarean section (and thereby, the blood sampling time), whereas late delivered (and late onset) preeclamptics are not delivered prematurely since the disease is not severe.

Few papers have previously been published regarding fetal isoprostane levels, and since different methods (such as ELISA or GC/MS) have been used, the results are difficult to compare. Also, the publications do not always indicate whether free or total 8-isoprostane concentrations are measured (141-144). A recently published study by Weinberger et al (145) report comparable results as we do in Paper III regarding plasma umbilical artery

concentrations of total 8-isoprostane in control infants (204 pg/ml measured with ELISA vs our result of 276 pg/ml measured with GC/MS, Paper III). The demonstration of a much higher concentration of 8-isoprostane in umbilical vein plasma compared to umbilical artery plasma in our study (Paper III), suggests that the placenta, and not the fetus, is the main source of fetal circulating 8-isoprostane, but such a difference between umbilical artery and vein 8-isoprostane was not found by Weinberger et al (145).

Exploring both the umbilical artery and the umbilical vein separately is beneficial in evaluating placental and fetal contribution to cord blood substances. Since some studies do not define whether mixed cord blood or separated cord blood is investigated, the results of the different publications are difficult to compare. However, the difference in concentrations of a substance between vein and artery cord blood gives an indication of whether there is a net uptake or production in the fetus. The venous umbilical cord blood mainly represents placenta derived blood to the fetus, whereas the arterial umbilical blood concentrations possibly better reflects the fetal situation, as the blood is circulated from the fetus towards the placenta. There are, though, technical challenges in collecting separated umbilical and vein cord blood, since especially the premature umbilical arteries are often fragile and constricted and difficult to handle as well as containing restricted volumes of arterial blood. The restricted volume is the reason for not having performed d-ROM analyses in the fetal circulation (Paper III), only in maternal circulation (Paper II). In addition, less fetal than maternal samples were available, therefore fewer patients were included in Paper III (requiring fetal samples) as compared to Paper II.

7.3.2 Antioxidant indices

Antioxidant capacity (AC) is defined as the measure of the moles of a given free radical scavenged by a test solution, independently of the antioxidant activity of any one antioxidant present in a given mixture (146). It is also regarded as a representative measure of the true balance between reactive oxygen species and antioxidant defense (146). Various methods have been developed for measuring total antioxidant status, but there is no accepted general reference method, nor one specifically for analyses in pregnancy. Widely used methods for measuring reactive oxygen species include colorimetric, fluorescence, chemiluminescence and electron spin resonance spectroscopy, but most methods are time-consuming, expensive, need sophisticated techniques and are not available in a routine laboratory (147). The total radical-trapping antioxidant parameter (148) is regarded as reliable, but is time consuming and has to be analyzed immediately with fresh plasma (149).

Oxygen radical absorbance capacity (ORAC) and Randox Trolox-equivalent antioxidant capacity (Randox-TEAC) assays are also commonly used measures of AC in the circulation (150). Unfortunately, in general the assays do not correlate with each other. Several of the assays have been applied to preeclampsia, with results varying from no change (97) to increases (151) and decreases (152) in plasma/serum antioxidant capacity compared to normal pregnancy. This is partly because the antioxidant capacity result depends upon which free radicals or oxidants are used in the measurements (153).

The method used in Paper II and III for measuring AC is the controversial ferric reducing ability of plasma (FRAP). The FRAP assay is one of several methods developed to assess the "total antioxidant capacity" of serum or plasma. It is a more recent method and it has (to our knowledge) not been used as a measure of AC in pregnancy before. The FRAP assay is regarded as highly reliable; it requires low blood volume, is not time-consuming and it is cheap (154).

The major objection against FRAP measurement in preeclampsia is the influence on the assay of uric acid (a potent antioxidant). This aspect is thoroughly discussed in Paper II and III, as well as in a discussion following the publication of Paper III (155). The role of uric acid in preeclampsia remains to be established: whether it actually exerts a net antioxidant effect, whether it has a role in the development of the syndrome or merely represents a marker of disease severity (156).

The d-ROM (reactive oxygen metabolites) test offers a complementary approach to the measurement of the total antioxidant capacity to evaluate the redox status, measuring ROM in serum. The test is regarded as both stable and precise, as well as being a reproducible method for the quantitative evaluation of oxidative stress (157). The method is discussed in Paper II, but was not used as a method in analyzing the fetal blood samples in Paper III due to limited blood volume. We have no reason to believe that the d-ROM test or the FRAP assay are superior markers, compared to other methods of analyzing AC, but they represent an additional approach to this difficult field of analysis.

The impact of the severity of preeclamptic disease, as indicated by premature delivery prior to gestational week 34, or defined according to the ACOG criteria, was investigated for FRAP or d-ROM in Paper II, demonstrating no statistical significant difference in FRAP- or d-ROM levels. We demonstrated unaltered vitamin E concentrations in maternal circulation in preeclampsia (Paper II), in agreement with some reports (158), but in contrast to others (38;159), as discussed in Paper II. For the fetal circulation, we believe that umbilical artery samples better reflect the fetal situation than the umbilical vein

samples. Therefore, we interpret our findings in Paper III of elevated concentration of vitamin E in umbilical vein and unaltered in umbilical artery for the preeclampsia group (as compared to the control group), as evidence for unaltered vitamin E concentration in the fetal circulation in preeclampsia. As discussed in Paper III, we only found one previous publication regarding vitamin E level in infants in preeclampsia, and the results were in accordance with our own (97).

7.4 Statistics

As not all the values in Papers II-IV were normally distributed, median and 95% confidence intervals of the median are reported. The Mann-Whitney U-test was used for testing differences between groups. Pearson's or Spearman's correlation was used to calculate correlation coefficients. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 10 (Paper I) and version 11 (Papers II-IV).

We did not perform detailed sample size analyses prior to the start of the sub-studies, as not all study variables had been analyzed previously, and we were therefore not aware of standard deviations. Our studies could therefore be considered as pilot studies. We were interested in finding at least one standardized difference between the groups, considered clinically relevant to detect. According to Altman (160), we would need at least 30 women (15 pregnant women with preeclampsia and 15 controls) in order to detect such a difference with a significance level of 0.05 and a power of 80%. In future studies, our results could be used for calculations of sample size in order to ensure sufficient power to detect differences between study groups.

Finally, multiple statistical testing is performed in Papers II-IV. The recruited pregnant women in Papers I-IV come from the same biobank study (apart from some tissue specimens in Paper I), hence methods such as Bonferroni's could have been used to avoid a type 1 error (incorrectly declaring a difference). We did not undertake a Bonferroni correction of the p-value level since we explored distinct hypotheses in each paper, each with different tests based on biologically plausible hypotheses. A Bonferroni correction would entail a loss of power and we might thus incorrectly not have detected a difference (type 2 error). Since there are correlations between the outcome variables, a Bonferroni's correction is considered too conservative in such as situation (161).

8. Conclusions

1. The placental bed vacuum suction method represents a significant improvement in obtaining relevant and sufficient decidual tissue for morphological, functional and molecular studies of extravillous trophoblasts, spiral artery morphology and other components of decidual tissue from the placental bed (Paper I). The method is superior to placental bed knife biopsies and tissue sections from placental basal plate.

2. The placental bed vacuum suction method can be used for studies of acute atherosis in spiral arteries in the decidual tissue in preeclamptic, diabetic and uncomplicated pregnancies (Paper I and IV).

3. Increased oxidative stress, measured as 8-isoprostane (Paper II and III) was found in the maternal circulation in preeclampsia, but not in the fetal circulation, compared to uncomplicated pregnancies. The concentrations of 8-isoprostane were higher in the umbilical vein than in the umbilical artery, indicating placental, rather than fetal, origin of this substance. The antioxidant capacity was elevated in the preeclampsia group in both the maternal and fetal circulation (measured as elevated FRAP, Paper II and III). On the other hand, another antioxidant index, d-ROM, was unaltered in maternal circulation (Paper II) and vitamin E was unaltered both in maternal (Paper II) and fetal circulation (Paper III).

4. The elevated maternal concentration of the angiogenic factor sFlt1 in preeclampsia was associated with augmented oxidative stress, measured as 8-isoprostane concentrations (Paper II).

5. Presence of acute atherosis in decidual spiral arteries was not necessarily paralleled by maternal augmented oxidative stress or hyperlipidemia, neither in preeclamptic nor in uneventful pregnancies (Paper IV).

In conclusion, pregnancies complicated by preeclampsia demonstrate large heterogeneity, both with regard to presence of augmented oxidative stress and placental pathology, such as acute atherosis. Even if both phenomena occur more frequently in preeclampsia than in uncomplicated pregnancies, they are not necessarily present simultaneously or in similar magnitude. Preeclampsia is a potentially life-threatening disease for mother and child, and does not seem to have one simple underlying cause, valid for all women developing the syndrome. Further research and understanding of the pathophysiology are required before there can be successful trials on intervention and prevention of the syndrome.

9. Future Projects

Our ongoing collection of biobank-samples from complicated and uncomplicated pregnancies at Ullevål University Hospital will enable us to continue our research on maternal-placental and fetal interactions and the pathophysiology of preeclamptic and diabetic pregnancies. Our sampling methods allow us to investigate various pathophysiological aspects concomitantly, both in diabetes and preeclampsia research projects.

We have several potentially interesting collaboration projects for future work, where inclusion of clinically well-defined patient populations is essential, as well as a thorough biobank sampling of extensive biological material from each participating subject. The biobank studies led by principal supervisor, Associate Professor Annetine Staff, will continue after the termination of my PhD period, and I intend to contribute to future studies, combining clinical and basic research methods.

1. Renin Angiotensin System (RAS)

In a collaboration with a research group in Berlin, led by researcher Ralf Dechend, we have recently been able to demonstrate novel aspects of the RAS system being linked to preeclampsia, including presence of the circulating agonist AT1 receptor autoantibodies (AT1-AA) in the fetus of preeclamptic pregnancies (118). In the future, we wish to explore further the function of RAS in preeclampsia, and also possible short-term and long-term consequences for fetal and maternal health.

2. The DIP study (decidual immunological disturbance)

Normal cellular interaction in pregnancy and knowledge of changes in the placental/decidual cellular interactions in women with preeclampsia are of pivotal importance in our understanding of pregnancy-related diseases. In this DIP study, a collaboration with the principal researcher Mette Holthe, we will compare distribution of local immune cells in decidual/placental tissue in preeclampsia and pregnancies using flow cytometry and vacuum suction collected decidual tissue. We will also compare circulating immune cells with decidual tissue immune cells and immune responses in preeclampsia and uncomplicated pregnancies.

3. Functional studies of placenta lipid transport proteins

In collaboration with researchers Kari Anne Risan Tobin, Guro M. Johnsen and Asim Dutta-Roy at the Department of Nutrition and Institute of Basic Medical Sciences, University of Oslo, we are currently exploring the roles of lipid droplet associated proteins, including ADRP (Adipose Differentiation Related Protein), the overall goal being to describe their role in the placenta. These studies will include isolation of human trophoblast cells, molecular studies and immunohistochemistry of decidual tissue sections from our biobank.

4. Diabetic pregnancies and decidual pathology

In collaboration with Professor Borghild Roald at the Department of Pathology at Ullevål University Hospital, we will compare placental and decidual findings to circulating markers of disease (such as angiogenic factors) in diabetic pregnancies as compared to uncomplicated pregnancies.

10. Publications during the PhD period, not included in the thesis

1. Ranheim T, Haugen F, Staff AC, Braekke K, Harsem NK, Drevon CA. Adiponectin is reduced in gestational diabetes mellitus in normal weight women. Acta Obstet Gynecol Scand 2004; 83:341-7.

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