Cardiovascular biomarkers in pregnancies complicated by preeclampsia or diabetes mellitus

PhD thesis
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Meryam Sugulle
LIST OF PAPERS


ABBREVIATIONS

AUC: Area Under Curve
BMI: Body Mass Index (kg/m²)
CRP: C-Reactive Protein
CT-proAVP: C-Terminal pro-Arginine Vasopressin
CVD: Cardiovascular Disease
ED: Endothelial Dysfunction
EDTA: Ethylenediaminetetraacetic Acid
GDF-15: Growth-Differentiation Factor-15
GDM: Gestational Diabetes Mellitus
HbA1C: Glycosylated Hemoglobin A1C
HDL: High Density Lipoprotein
LDL: Low Densitity Lipoprotein
LVH: Left Ventricular Hypertrophy
mRNA: messenger Ribonucleotide Acid
MR-proANP: Midregional pro-Atrial Natriuretic Peptide
MR-proADM: Midregional pro-Adrenomedullin
NT-proBNP: N-terminal pro-Brain Natriuretic Peptide
OGTT: Oral Glucose Tolerance Test
PCR: Polymerase Chain Reaction
PE: Preeclampsia
PIGF: Placental Growth Factor
ROC: Receiver Operating Characteristic
ROS: Reactive Oxygen Species
sEng: Soluble Endoglin
sFlt-1: Soluble fms-like tyrosine kinase-1
SGA: Small for Gestational Age
SHBG: Sex Hormone Binding Globulin
T1D: Type 1 Diabetes mellitus
T2D: Type 2 Diabetes mellitus
VEGF: Vascular Endothelial Growth Factor
SUMMARY
An increased risk for later cardiovascular disease has been observed in both women suffering from preeclampsia (PE) or diabetes mellitus (preexisting or gestational) and their in utero exposed offspring. The search for biomarkers predicting PE and gestational diabetes mellitus (GDM) has been intensified in obstetrical research for the last decade, but lesser focus has been directed towards biomarkers indicating an increased risk for development of future cardiovascular disease in women after pregnancies complicated by PE or diabetes mellitus.

The main aim of this PhD thesis was to explore and quantify biomarkers associated with cardiovascular disease outside pregnancy, namely growth-differentiation factor-15 (GDF-15), midregional pro-atrial natriuretic peptide (MR-proANP), neopterin, midregional pro-adrenomedullin (MR-proADM) and C-terminal pro-arginine vasopressin (CT-proAVP), in pregnant women with PE or any type of diabetes mellitus or both, as well as in the postpartum state.

The biological material (maternal and fetal blood, amniotic fluid and placental, decidual and fat tissues) used in Papers I-III originated from women in gestational week 24-42 recruited to a pregnancy biobank at the Department of Obstetrics at Oslo University Hospital, location Ullevål, by Professor Annetine Staff’s research group members since 2001, including the PhD candidate from 2007 onwards. Additionally, blood samples from a subgroup of these women recruited to a clinical follow-up study 5-8 years after their index delivery (as part of another PhD project in our research group) was investigated in Paper II. The placental villous explants were a contribution from collaborator Professor K. Bridget Brosnihan in USA (Paper II). The rat model for PE has been developed by co-supervisor Privat-Dozent Dr. Ralf Dechend’s group in Berlin, independently of this thesis, and all animal experiments were carried out by his researchers (Paper II). The remaining
laboratory work was carried out by our collaborators in Germany at the laboratory of Privat-Dozent Dr. Ralf Dechend, Berlin (Papers I and II), at the laboratory of Professor Kai C. Wollert, Hannover (Paper I) and at laboratory of the company B.R.A.H.M.S. Biomarkers, Hennigsdorf (Papers II and III) and by our research group in Oslo (Paper I).

The novel findings of this thesis are in summary:

- **GDF-15** is dysregulated in pregnancies complicated by PE and/or diabetes mellitus. The elevated GDF-15 protein concentrations in PE in both maternal and fetal circulation, as well as in amniotic fluid compared to healthy control pregnancies, may be related to an excessive placental production of GDF-15 in PE, and GDF-15 could represent a marker for placental oxidative stress. Possibly, extra-placental sources (i.e. the fat tissue) contribute to the increased circulating maternal levels of GDF-15 in diabetic pregnancies (Paper I).

- **MR-proANP** is significantly elevated in maternal circulation in PE compared to normotensive pregnancies, probably reflecting cardiovascular hemodynamic stress. This finding was confirmed in preeclamptic female rats (Paper II).

- MR-proANP shows a high discrimination between preeclamptic and normotensive control pregnancies which is only marginally inferior to the “preeclampsia biomarker” soluble fms-like tyrosine kinase-1 (sFlt-1) (Paper II).

- MR-pro ANP, MR-proADM and CT-proAVP differ in maternal circulation between healthy, pregnant controls and non-pregnant women. Altered hemodynamics and/or increased inflammation in normal pregnancy may influence the circulating levels of these markers (Papers II and III).

- **GDM** and type 2 diabetes mellitus show a similar cardiovascular biomarker profile for the biomarkers explored in this thesis (Paper III),
possibly attributable to common pathophysiological pathways in these two diseases, as well as shared risk factors for future cardiovascular disease.

- The increased MR-proANP concentrations observed in our study group of diabetic pregnancies further complicated by PE compared to the concentrations in the respective diabetes groups without PE seem to be related to PE and the associated cardiovascular hemodynamic stress, not to the underlying diabetes mellitus (Paper III).

- Despite the fact that women after pregnancies complicated by diabetes mellitus and/or PE have an increased risk of future cardiovascular disease in common, they differ during pregnancy in circulating biomarkers associated with cardiovascular disease (Papers I, II and III).

In a pathophysiological perspective, the cardiovascular biomarker distribution found in pregnancies complicated by PE or diabetes mellitus may not only be a consequence of such complicated pregnancy, but may also represent a contributing factor to the development of vascular-related disease, both in pregnancy and later in life. Whether the cardiovascular biomarkers explored in this thesis are potentially useful for the prediction and monitoring of women and possibly their offspring at increased risk for cardiovascular disease later in life, has to be studied further. Taking into account that some of the risk factors for preeclampsia, gestational diabetes mellitus, type 2 diabetes mellitus and cardiovascular disease are modifiable and that the population at risk consists of relatively young women, with potential influence on the lifestyle of their offspring, obstetrical research in the field of cardiovascular risk markers should be continued.
1. INTRODUCTION

1.1 Preeclampsia

1.1.1 Definitions, epidemiology and risk factors

Preeclampsia (PE) is defined as the development of de novo hypertension (blood pressure ≥140/≥90 mmHg) and proteinuria (≥0.3g protein/24 hours or dipstick ≥+1) after 20 weeks of gestation (1;2). Both “severe”, “non-severe”, “early-onset” and “late-onset” PE are terms regularly encountered in clinical practice and in the literature. Severe PE is characterized by blood pressure ≥160/≥110 mmHg and/or proteinuria ≥5 g/24 hours (dipstick ≥3+) on two separate occasions and/or the presence of signs of multiorgan affection in the mother (2).

The term “early-onset” PE refers to the time point in gestation when the disease manifests itself and the cut point is most commonly set as ≤34 weeks of gestation (3). In certain study contexts in the literature, delivery before or equal to 34 weeks gestation in a preeclamptic pregnancy is also referred to as “early-onset”, since the time of delivery may be easier to assess than the time of onset.

PE occurs in approximately 5% of healthy nulliparous women (4), and was in a large cohort study associated with delivery before 34 weeks’ gestation in 0.4% of primiparous women (5). In Norway, the prevalence of PE, which has been stable over the last decades, is 3% (6;7) among all births and 4% in first pregnancies (8).

Among the risk factors for PE development the following are of special relevance for this thesis: nulliparity, previous PE, advanced maternal age, high pre-pregnancy body mass index and pre-existing or gestational diabetes mellitus (6;9-11).
1.1.2 Pathophysiology

The heterogeneity of clinical PE has generated the concept of a placental type of PE and a maternal type of PE (12). The maternal type has been suggested to originate from preexisting disorders in the mother, such as diabetes, inflammatory disease and other risk factors for cardiovascular disease, rather than from impaired placentation, as in the placental type of PE (12). Although the detailed pathophysiology of PE is still unknown, for the placental type of PE, a 3 stage model has been suggested (see figure to the right, redrawn from (13)). In stage 1, dysregulated immunological factors are proposed to underlie an impaired trophoblast invasion resulting in a defective placentation and incomplete remodeling of maternal uteroplacental spiral arteries. Stage 2 comprises the ensuing altered placental circulation and subsequent oxidative and endoplasmatic reticulum stress, leading to the release of circulating factors into the maternal system. The maternal response to these factors is an excessive systemic inflammatory response and a generalized maternal endothelial dysfunction (ED) leading to the third stage, namely clinically overt PE (13).

Other factors and mechanisms suggested to contribute to ED in PE are insulin resistance (14-16), dyslipidimia (17;18), an imbalance between pro-
angiogenic and anti-angiogenic factors (19;20) and abnormal hemodynamics (21;22), all of which will be addressed more extensively in section 1.3.2.

1.2 Diabetes mellitus in pregnancy

1.2.1 Definitions, epidemiology and risk factors

The term “diabetes in pregnancy” as used in this thesis and in Papers I and III comprises both preexisting diabetes mellitus, i.e. type 1 and type 2 diabetes mellitus (T1D and T2D, respectively) and gestational diabetes mellitus (GDM). Preexisting diabetes mellitus is defined according to the current World Health Organization (WHO) recommendations (23). The probably first written report of a case of gestational diabetes was by H.G. Bennewitz, Berlin, in 1824 (24). Interestingly, nearly 200 years later, there is no globally valid consensus on the diagnostic criteria for GDM. Originally, GDM was defined as glucose intolerance first detected during pregnancy, independently of severity (25;26). This meant that previously undiagnosed, but in fact preexisting diabetes mellitus or cases in which the first occurrence of diabetes coincided with pregnancy, were included in the concept of GDM. Whether diabetes mellitus diagnosed in pregnancy resolves after delivery can first be stated several weeks postpartum. A certain proportion of women with a diagnosis of GDM will retrospectively prove to have had unrecognized T2D or T1D in pregnancy (27).

Current clinical practice aims at distinguishing between overt diabetes mellitus in pregnancy and GDM, reserving the term GDM for impaired fasting glucose and impaired glucose tolerance (26;28). In Norway, the national guidelines for the diagnosis of GDM follow the current WHO criteria for diabetes mellitus (23;29). The national guidelines for pregnancy controls do currently not include routine oral glucose tolerance test (OGTT) or fasting
plasma glucose screening for all pregnant women, but only for defined risk groups (29).

The risk criteria are the following (29):

- glucosuria in current pregnancy
- maternal age above 35 years
- adiposity/obesity (body mass index > 27 kg/m²)
- hereditary disposition
- ethnicity (women from South-East Asian and African countries)
- previous GDM
- previous macrosomic infant
- history of obstetric complications

The following table displays the criteria for the diagnosis of diabetes mellitus in pregnancy as used in this PhD thesis.

Criteria for the diagnosis of diabetes in pregnancy (29)

<table>
<thead>
<tr>
<th></th>
<th>Manifest diabetes</th>
<th>Gestational diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting plasma glucose</strong></td>
<td>( \geq 7.0 \text{ mmol/L} )</td>
<td>&lt; 7.0 mmol/L</td>
</tr>
<tr>
<td></td>
<td>or</td>
<td>and</td>
</tr>
<tr>
<td><strong>2-hour plasma glucose (OGTT 75 g)</strong></td>
<td>( \geq 11.1 \text{ mmol/L} )</td>
<td>( \geq 7.8 ) but &lt;11.1 mmol/L</td>
</tr>
</tbody>
</table>
The prevalence of GDM ranges from below 2% up to more than 13%, the wide range caused by differences in population characteristics and diagnosis criteria (30). In 2010, GDM occurred in 1.7%, T1D in 0.5% and T2D in 0.3% of all births in Norway (7). The prevalence of both pregestational and gestational diabetes in the pregnant population has worldwide been increasing over the last decades (30-32). Also in Norway the proportion of pregnant women with GDM has more than doubled during the last 15 years, from 0.7% of all births in 1995 to 1.7% in 2010 (7). The increase in T2D and GDM is parallel to that of increasing obesity among women in their fertile ages, especially in the youngest age groups (33). Apart from obesity, other risk factors for GDM include advanced maternal age, a family history of diabetes mellitus and non-white ethnicity (34;35).

1.2.2 Pathophysiology of gestational and preexisting diabetes mellitus

Normal pregnancy is an insulin resistant state, progressing throughout pregnancy and at levels resembling the insulin resistance of T2D (36;37). A normally functioning organism compensates for this insulin resistance by increasing insulin secretion by pancreatic islet β-cells (36).

In susceptible women, pregnancy unmasks a chronic pancreatic islet β-cell dysfunction in the presence of insulin resistance, with the inability to secrete sufficient amounts of insulin as required and hyperglycemia as the result (37). These women suffer from GDM.

In T2D, the islet β-cells secrete only insufficient amounts of insulin in response to varying degrees of overweight or obesity, and insulin resistance (38).

The shared pathophysiological feature of GDM and T2D is a relative insulin deficiency, in contrast to T1D, which is characterized by an absolute insulin deficiency of endogenous insulin due to pancreatic islet β-cell destruction (39).
1.2.3 Preeclampsia in diabetic pregnancies
An increased risk for PE was found in women with GDM compared to normoglycemic women, independently of body mass index (BMI) (40;41). For nulliparous women, a 3-fold increased risk for PE development in GDM has been reported (40). Two randomized trials have shown that treatment of GDM with either diet or insulin reduced the risk of PE development in these women (42;43).

A 5.5-fold increased risk for PE development in T1D pregnancies and a 2-fold increased risk in T2D pregnancies compared to healthy pregnancy has been reported (40;44).

1.3 Cardiovascular disease after pregnancies complicated by preeclampsia or diabetes mellitus

1.3.1 Cardiovascular disease: Definition, epidemiology and risk factors
The term cardiovascular disease (CVD) commonly comprises hypertension and ischemic heart disease. In Norway, as in other industrialized countries, CVD is the leading cause of death, accounting for about 33% of all deaths among women in 2010, thereof 11% ischaemic heart disease (45).

Some of the traditional risk factors for CVD are dyslipidemia, obesity, hypertension, diabetes mellitus and the metabolic syndrome (46;47). As will be addressed in section 1.3.4, an obstetric history of one or more prior pregnancies complicated by PE and/or GDM implies an increased risk for later CVD for the affected woman and should therefore be part of a complete CVD risk factor assessment in women. However, there still seems to be relatively little knowledge about the association between complicated pregnancies and subsequent risk for CVD among internists and obstetricians (48). In the following sections, the occurrence of two important risk factors for CVD after
pregnancies complicated by PE or GDM, namely T2D and the metabolic syndrome, will be described in more detail.

1.3.2 Type 2 diabetes after preeclampsia or gestational diabetes

T2D is an established independent risk factor for CVD (49;50). In women with prior PE, 4 times higher occurrence of development of T2D compared to women after normotensive pregnancies has been reported (51). However, an association with BMI seems to be influential, since another study reported an attenuation of the risk for development of diabetes after PE from an approximately 4-fold increased risk to a 3-fold increased risk after adjustment for current BMI (52). Preterm delivery before 37 weeks gestation in women with PE was associated with a further increased risk for subsequent development of T2D (51).

A recent systematic review and meta-analysis showed that women with prior GDM had an approximately 7.5 times increased risk of developing T2D, compared with those who had been normoglycemic during pregnancy (range of follow-up time in the included studies: 6 weeks -28 years) (53).

Another study described that 34% of women with prior diet-treated GDM had developed T2D 10 years after their index pregnancy (54). T2D occurred approximately 10 times more often in women after diet-treated GDM compared to the female background population in the corresponding age group (54). The same study showed that the risk of developing T2D after GDM had more than doubled during the course of a decade (ending in 2002) (54) parallel to an increase in BMI both prior to and following pregnancy in the study population, with an approximately 3-fold increased proportion of obese women (BMI≥30 kg/m²) (54).
1.3.3 Metabolic syndrome after preeclampsia or gestational diabetes

The metabolic syndrome, a risk factor for CVD (47), is defined as the concomitant presence of central obesity, dysglycemia, hypertension, hypertriglyceridemia, and low high-density lipoprotein (HDL) cholesterol (55). Already approximately 8 years after a pregnancy complicated by PE, a higher proportion of women displayed the metabolic syndrome compared to women after a normotensive index pregnancy (56).

Compared with women without prior GDM, a 2-fold increase in the prevalence of the metabolic syndrome in women with prior GDM at 3 months postpartum has been reported (57). At 10 years postpartum, the prevalence of the metabolic syndrome was 3 times higher in women with a history of diet-treated GDM compared to the general population (58).

1.3.4 Cardiovascular disease after preeclampsia

Epidemiological studies

The association between hypertensive pregnancy disorders and later CVD has been subject of systematic research during the last 80 years. Among the latest reports is a systematic review and meta-analysis of nearly 200 000 PE cases (59). The study showed that women with prior PE had an increased risk for hypertension, ischaemic heart disease and cerebrovascular stroke at 10 years or more postpartum, as well as for venous thromboembolism at 5 years postpartum, compared to women after normotensive pregnancies (59).

A large register-based study with a median follow-up time of about 13 years found that women with a history of PE had an approximately 3.5 to 6-fold increased risk for the development of hypertension, dependent on the severity of PE (51). The risk was independent of the development of T2D and increased further with recurrent PE (51). An association with increased risk for congestive heart failure, thromboembolism and stroke was also seen (51).
Another study described an association between a history of PE and the presence of risk factors for CVD 10 years after the affected pregnancy, including high BMI, high blood pressure, dyslipidemia and diabetes mellitus (52). The same group concluded in another study that prepregnancy risk factors for CVD are probably more important determinants of subsequent CVD risk factors than PE per se (60).

Another large epidemiological study showed an increased risk of death from cardiovascular causes among women after PE compared to women with a history of normotensive, term pregnancies (61). This risk was even more pronounced in women after a preeclamptic pregnancy resulting in a preterm delivery <37 weeks gestation (61).

Several studies have shown that the combination of PE and a preterm delivery (<37 weeks gestation), as well as severe PE and recurrent PE, further increases the risk of subsequent hypertension or ischemic heart disease compared to preeclamptic women delivering at term (51;62;63). These epidemiologic findings of further increased CVD risk in women with PE resulting in a preterm delivery indicate that early-onset disease implies a greater risk for subsequent CVD than late-onset PE.

Functional studies
Several functional studies have shown that signs of impaired cardiovascular function are present in asymptomatic, clinically healthy young women during and after pregnancies complicated by PE. Global diastolic dysfunction and an adaptive left-ventricular remodeling have been observed in women with PE (64). A recent prospective case-control study of women with prior PE showed that at 1 year postpartum, echocardiographically proven moderate to severe left-ventricular functional or geometric abnormality persisted in over 50% of women after preterm PE, compared to 14% after PE and 8% of normotensive controls (65). In previously preeclamptic women, reduced stress-induced
forearm blood flow as a sign of impaired endothelial function compared to normotensive women at 10-16 months postpartum has been described (66;67).

1.3.5 Cardiovascular disease after gestational diabetes

Epidemiological studies
As described in section 1.3.2, the progression rate from GDM to T2D is high and T2D is an important risk factor for the development of CVD (49;50).

A large cohort study of young women with GDM and a median follow-up period of 11.5 years found a 1.7-fold increased risk of CVD development compared to normoglycemic, matched controls (68). Adjustment for development of T2D attenuated the CVD risk in these women, indicating that the increased CVD risk was mostly attributable to concomitant T2D (68). In contrast to this report, another study showed an increased risk for development of hypertension in women with prior GDM, independently of pregnancy hypertension or subsequent T2D (69).

It has long been known that patients with either T1D or T2D diabetes are at increased risk for later CVD. The Framingham study showed an 8-fold increased risk of chronic heart failure in women with diabetes compared to women without diabetes (49). Even in relatively young women (<40 years of age) with T1D, mortality risk from ischaemic heart disease is high (70).

Functional studies
Similarly to the situation in PE, there is evidence of impaired left ventricular function in women with GDM compared to healthy pregnant women (71). In pregnant women with either GDM or T2D, increased systemic arterial stiffness was detected by applanation tonometry (72). Another study showed impaired endothelial function evaluated by brachial artery flow-mediated dilatation in
women with GDM in third trimester of pregnancy compared to healthy controls (73).

1.3.6 Cardiovascular disease in offspring
An increased prevalence of risk factors for CVD has been found in offspring exposed to PE or diabetes mellitus in utero. Since offspring cardiovascular health after complicated pregnancies has not been the focus of this thesis, only a brief overview of this topic will be given in the following paragraphs.

The “fetal origin hypothesis” suggests that fetal adaptive responses to an adverse intrauterine environment, as a result of maternal malnutrition or placental insufficiency, may have long term effects on health later in life (74). Observational studies on which this concept is based on revealed an increased risk for CVD and T2D in adults who had been growth restricted in utero (75;76).

Since there at present does not exist any causal medical therapy for PE, the only measure in case of deterioration of the mother’s and/or the fetus’ state is to deliver the fetus in order to prevent PE-related maternal and/or neonatal morbidity and mortality. Especially in early-onset PE, the result is often a preterm infant with low birth-weight, often not only a result of low gestational age, but also due to intrauterine growth restriction (77). Low birth weight is associated with increased risk of coronary disease, hypertension and T2D in adulthood (75;76). Children born after preeclamptic pregnancies tend to have higher blood pressure in early adolescence than offspring of normotensive pregnancies (78;79). A study by our own group showed a significantly reduced endothelial function in children aged 5-8 years born small for gestational age after preeclamptic pregnancies compared to children with a birth weight appropriate for gestational age (80).

In general, the fetoplacental impairment caused by GDM and preexisting diabetes mellitus, and thereby also the effects on intrauterine programming of
diseases in the offspring, seem to be similar (81). Studies indicate an increased risk for overweight in offspring of pregnancies complicated by preexisting diabetes mellitus or GDM (82-85). Large for gestational age offspring (birth weight >90th percentile for gestational age) born of mothers with GDM had a high risk of developing insulin resistance or the metabolic syndrome during childhood and adolescence (86;87). Higher systolic and diastolic blood pressure in adolescent offspring of GDM mothers has been observed (84). Moreover, several studies have reported a higher incidence of T2D in children born of mothers with either T2D or T1D (88).

In summary, there is evidence of accumulation of CVD risk factors in children born of mothers with PE or with any type of diabetes during pregnancy.

1.3.7 Common pathophysiological mechanisms

The exact pathophysiological mechanisms linking pregnancies complicated by PE and/or diabetes mellitus with future cardiovascular health are presently unknown.

Normal pregnancy is characterized by increased inflammatory activity (89), insulin resistance (90;91), hyperlipidemia (92;93) and oxidative stress (94) compared to the non-pregnant state. These alterations are thought to be of importance for the adaptation of the maternal organism to pregnancy, for the maintenance of pregnancy and for fetal growth (93).

Metabolic and vascular alterations that are present in normal pregnancies are found to be enhanced in complicated pregnancies, as will be described in detail in the following paragraphs. Persistence of these alterations may contribute to the increased risk for later CVD (95).

In CVD, inflammatory processes and oxidative stress as well as metabolic alterations such as insulin resistance and dyslipidemia, contribute to the development of endothelial dysfunction (ED) and atherosclerosis (96-100).
Thus, there is a substantial overlap in the pathophysiological mechanisms present in pregnancies complicated by PE or diabetes and CVD. The common resultant of these pathologies is endothelial dysfunction, which is of importance in all three conditions.

The overlap in risk factors and pathophysiological mechanisms in CVD, PE and diabetes mellitus is illustrated in the following figure.
Endothelial dysfunction

Endothelial dysfunction (ED) is the earliest stage of alteration in endothelial function and structure caused by reduced bioavailability of nitric oxide (101). ED is an early marker for atherosclerosis (102) and an independent predictor of CVD in the general population (103).

There is evidence from studies of circulating markers, as well as from histological and functional studies that ED is present in women both preceding the onset of PE, during PE and up to several years after PE (66;67;104-107).

Also in women with GDM there is functional and biochemical evidence of abnormal vascular endothelial function during pregnancy (108) and up to several years postpartum (109-111). Impairment of vascular responsiveness in pregnant women with T1D as compared to nondiabetic pregnant women has been described (112). Current publications on endothelial health in pregnant women with T2D could not be identified. However, in non-pregnant patients with either T2D or T1D, endothelial dysfunction is a well-described pathophysiological feature (113;114).

Factors involved in the development of ED are inflammation, insulin resistance, dyslipidemia and oxidative stress, and their presence in pregnancies complicated by PE or diabetes mellitus will be described in the following paragraphs.

Inflammation

Inflammatory processes are of major importance for the development of atherosclerosis (97). In PE there is evidence of exaggerated maternal systemic inflammatory response (115) as well as histological proof of uteroplacental atherosis, similar to the early stages of atherosclerosis (116-118).

Markers of inflammatory activity such as leucocyte count and C-reactive protein (CRP) were found increased in first trimester in women who later on developed GDM (119). In women with established GDM in late second/early
third trimester, obesity was identified as a major confounding factor for increased CRP levels (120). Persistent increased inflammatory activity 4 years postpartum was found in nondiabetic women with a GDM in their index pregnancy compared to women without a history of GDM (110).

A study of pregnant women with T1D did not show any differences in markers of inflammation throughout pregnancy and postpartum compared to pregnant, non-diabetic women (121). Publications on inflammatory activation in pregnant women with T2D could presently not be identified. However, in non-pregnant patients, T2D and T1D have been associated with increased inflammatory activity (122-124).

**Insulin resistance**
Insulin resistance impairs endothelial function (114), and might also directly contribute to hypertension by increased renal sodium reabsorption and stimulation of the sympathetic nervous system (125). In PE increased insulin resistance resulting in pronounced hyperinsulinemia compared to normal pregnancy has been described (15;126). At 3 months postpartum, insulin sensitivity had improved in formerly preeclamptic women compared to their situation in pregnancy, but they were still more insulin resistant than women who had been normotensive in their pregnancy (126). A study of formerly preeclamptic women 17 years postpartum detected mild hyperinsulinemia compared to women after normotensive pregnancies (127).

As described in section 1.2.2, insulin resistance and resultant hyperinsulinemia are main characteristics of GDM and T2D (37;38). Persistent insulin resistance was found at average 2 years postpartum in normoglycemic women with prior GDM (128). Furthermore, obesity, a common risk factor for both PE, GDM and T2D, is per se associated with insulin resistance (129).
Dyslipidemia

Dyslipidemia, which is defined as an abnormal serum lipid profile and may include high total cholesterol, high triglycerides, low levels of high density lipoprotein (HDL) cholesterol, and elevated (small) low density lipoprotein (LDL) cholesterol, is associated with ED and atherosclerosis (96). Direct and indirect effects of oxidized lipids are thought to impair endothelial function (130).

In PE, increased circulating levels of increased triglycerides and free fatty acids (18;126), lower levels of HDL (131;132), as well as a shift towards small, dense LDL-particles have been found (133). Small, dense LDL-particles are known to be more susceptible to peroxidation and have been associated with increased risk for CVD in non-pregnant individuals (133).

A shift towards small dense LDL has also been observed in any type of diabetes in pregnancy, however most pronounced in T2D in pregnancy (134;135). In GDM and pregnant women with T2D, both higher (136-138) and unchanged triglyceride concentrations (134) compared to the physiologically increasing triglyceride concentrations throughout pregnancy, have been reported. Triglyceride levels in T1D pregnant women did not differ substantially from healthy, pregnant women (134;136).

Oxidative stress

Oxidative stress is defined as a disturbance in the pro-oxidant/anti-oxidant balance in favor of the former, i.e. an accumulation of reactive oxygen species (ROS) with potentially damaging properties, able to cause cell injury or cell death (139). Oxidative stress has been associated with endothelial dysfunction, atherosclerosis and CVD (99;100).

In PE oxidative stress is assumed to be of vascular origin (reviewed in (140). Incomplete remodeling of the spiral arteries is proposed to result in intermittent placental perfusion and thereby in an ischemia-reperfusion type
injury, leading to increased production of ROS (140). However, there is some controversy related to the concept of oxidative stress in PE. Several studies, also by our group, have shown increased circulating maternal as well as placental concentrations of markers indicative of oxidative stress in PE, such as 8-isoprostane and advanced glycation end products (141-143), whereas others have not (144). A hyperglycemic environment is in general associated with oxidative stress (145). In women with GDM, a variety of markers indicative of increased oxidative stress have been detected in maternal circulation as well as in placental tissues (146;147). Lower total antioxidant capacity measured in maternal circulation has been reported for any type of diabetes in pregnancy, but most markedly for T2D (134).

Interestingly, large randomized clinical trials of vitamin C and E supplementation initiated in late first/early second trimester in women at either low or high risk (including women with diabetes) for PE development did not show any beneficial effect with regard to prevention of PE or adverse perinatal outcome (148;149). Similarly, a trial of vitamin C and E supplementation in pregnant women with T1D did not prevent PE (150).

1.4 Biomarkers

1.4.1 Definition

A biomarker is defined as a characteristic that can be objectively measured and evaluated as an indicator of a normal biological or pathogenic process or a pharmacological response to a therapeutic intervention (151). Biomarkers measured in body fluids such as serum or urine are commonly referred to as soluble biomarkers. Biomarkers are potentially useful for screening, early identification, diagnosis, monitoring of the response to therapy, staging or prognosis of a disease (151).
1.4.2 Biomarkers in preeclampsia and diabetes mellitus

The search for biomarkers predicting PE and GDM has been a focus in obstetrical research for the last decade. The aim is to be able to identify women at risk of developing PE or GDM in order to tailor antenatal care and therapy and thereby to improve maternal and neonatal outcome. Lesser focus has been directed towards the identification of biomarkers indicating an increased risk for development of future cardiovascular disease in women after pregnancies complicated by PE or diabetes mellitus. Taking into account that some of the risk factors for PE, T2D and CVD are modifiable, that the population at risk consists of relatively young women, and on the backdrop of the global obesity and diabetes mellitus epidemics, such research should not be neglected. Furthermore, there are possible repercussions for the next generation, since women have an important impact on the life-style (including diet and physical activity) of their children.

**Biomarkers during and after pregnancy complicated by PE**

Angiogenesis is the formation of new vessels from preexisting vessels and endothelium, and plays an important role in placentation (152). The current 3-stage model of PE, depicted in section 1.1.2, includes the release of circulating factors by the placenta in response to oxidative and endoplasmatic reticulum stress (13). Angiogenic factors are proteins involved in the process of angiogenesis, and have been recognized as biomarkers for the prediction of PE (153). The antiangiogenic peptide soluble fms-like tyrosine kinase-1 (sFlt1) (154) binds the pro-angiogenic molecules vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) and thereby antagonizes vasorelaxation and contributes to the development of hypertension (154). Another anti-angiogenic peptide, soluble endoglin (sEng), is expressed in large quantities by the placenta in PE. sEng inhibits endothelial capillary tube formation and promotes vascular permeability regulating vascular tone (155).
Both placental sFlt-1 and sEng mRNA expression is upregulated in PE (154;155).

Both sFlt-1 and sEng concentrations have been found elevated in maternal circulation in PE and concentrations correlated with disease severity (154-158). There are reports of reduced free VEGF in maternal circulation in PE (19;154;159), whereas other studies, including one by our own group, conclude that the very low free VEGF levels in pregnancy are not detectable (158;160). A study by our group and collaborators showed that a change in the sFlt-1/PIGF ratio between first and second trimester was a strong predictor of early-onset PE (161). Increased sEng as well as an increased sFlt-1/PIGF ratio in second trimester were found to predict the later onset of the disease in healthy nulliparous women at risk for developing PE (20). However, a recent systematic review and meta-analysis concluded that maternal circulating angiogenic factors were not suitable as sole predictors for PE in clinical practice due to low test accuracies together with modest (although significant) differences in sFlt-1, PIGF and sEng concentrations before 30 weeks gestation in PE (162).

Postpartum studies, including a study by our own group, have reported increased maternal levels of sFlt-1 in women with a history of PE compared with those without PE at average 1.5 years (163) and 6.7 years after index pregnancy (80).

Interestingly, circulating concentrations of sFlt-1 have been found increased in patients with acute myocardial infarction (164). Moreover, the sFlt-1 level on admission was associated with the development of severe acute heart failure after myocardial infarction (164). Also, sFlt-1 was described as an early indicator of endothelial hypoxia in acute coronary occlusion (165). Studies on PIGF in relation to CVD have led to the suggestion that PIGF may prove useful as a marker of plaque instability and myocardial infarction as well as a prognostic marker in acute coronary syndrome (166).
Biomarkers during and after pregnancies complicated by diabetes mellitus

Early screening with an OGTT at 16 weeks gestation had a good negative but not a good positive predictive value (167) with regard to later GDM development. A strong association between high fasting glucose levels (which still were within a non-diabetic range) and later GDM development was found in women tested at average 9.5 weeks gestation (168). In maternal circulation of normoglycemic women in first trimester, lower plasma adiponectin (169;170), lower serum sex hormone-binding globulin (SHBG) concentrations (171) and increased plasma insulin concentrations (170) were associated with later GDM development. A recent study identified apolipoprotein CIII, which is important for lipid metabolism, as a potential predictive biomarker for GDM, with increased levels of this lipoprotein at 16 to 20 weeks gestation in women who developed GDM (172). Recently a first trimester screening model for GDM, combining maternal risk factors with the measurement of maternal serum adiponectin and SHBG (finding decreased levels), has been suggested, with a reported detection rate of 65% for nulliparous women and parous women without prior GDM (173).

1.4.3 Biomarkers in cardiovascular disease

Creatine kinase MB fraction, C-reactive protein (CRP), N-terminal pro–brain natriuretic peptide (NT-pro-BNP) and troponin T are well-known biomarkers for acute cardiac events (reviewed in (174;175). During the recent years, several new biomarkers for CVD have been identified. Recent practice guidelines emphasize prevention of CVD and thus the current focus in CVD biomarker research focus concentrates on biomarkers which can predict CVD in persons free from disease (176).

Growth-differentiation factor-15 (GDF-15), midregional pro-atrial natriuretic peptide (MR-proANP), neopterin midregional pro-adrenomedullin
(MRproADM) and the C-terminal part of the arginine vasopressin prohormone (CT-proAVP) are novel markers associated with cardiovascular disease (177-182) and will be described in the following paragraphs.

**Growth-differentiation factor-15 (GDF-15)**

GDF-15, in early studies referred to as macrophage-inhibitory cytokine-1, is a stress-responsive transforming growth factor-β-related cytokine (183). Expression of GDF-15 mRNA is upregulated in cardiomyocytes by ischemia/reperfusion injury and nitrosative stress (184). GDF-15 has been ascribed a cardioprotective role in vivo in the adult heart (185). GDF-15 is a prognostic biomarker in patients with acute coronary syndrome (181), myocardial infarction (179), and chronic heart failure (180). An association between increased circulating GDF-15 concentrations and an augmented risk of future CVD has been observed in healthy elderly women (186). In a population-based study of elderly persons, GDF-15 has been proposed to have a substantial predictive value with respect to clinical manifestations of CVD, superior to the predictive value of traditional CVD risk factor assessment (187).

**Midregional pro-atrial natriuretic peptide (MR-proANP)**

MR-proANP is the midregional part of the pro-atrial natriuretic peptide (proANP), which in turn, is the precursor of atrial natriuretic peptide (ANP) (188;189). ANP is a diuretic, natriuretic, and vasodilatory cardiac hormone which is released into the circulation after atrial distension (190-192). Circulating MR-proANP is considered a diagnostic marker for acute heart failure and atrial fibrillation independently of conventional risk factors (182;193). An association between plasma MR-proANP levels and arterial stiffness as well as severity of hypertension has been found (194). A recent study showed higher MR-proANP concentration in patients with hypertension
and left ventricular hypertrophy (LVH), an indicator for subsequent CVD, than in hypertensive patients without additional LVH (195). A recent study found an increased risk of T2D development in patients with a low baseline MR-proANP during a mean follow-up time of 16 years and suggested a primary role of low (MR-pro)ANP in the development of T2D (196).

**Neopterin**

Neopterin, a marker of cell-mediated immunity, is produced by activated monocytes/macrophages following pro-inflammatory stimuli (197). Neopterin has been ascribed pro-oxidant functions and an involvement in the promotion of cell death (198). Plasma neopterin has been shown to increase with atherosclerotic plaque formation (198). Circulating neopterin concentrations were not only elevated in patients with angina pectoris, acute myocardial infarction and coronary artery disease (199-201), but have also proven to be of prognostic value in CVD with regard to future adverse outcome such as recurrent cardiac events and death (178;202-205).

**Midregional pro adrenomedullin (MR-proADM)**

MR-proADM is the midregional fragment of the precursor peptide pre-pro-adrenomedullin and is synthesized together with mature adrenomedullin (ADM) (206). ADM, a potent vasodilator, is present in a variety of organs such as the adrenal medulla, myocardium, lungs, and kidneys (207). Vascular endothelial cells and vascular smooth muscle cells are considered the main source of circulating ADM (208;209). ADM expression is upregulated by inflammatory and mechanical stimuli as well as in response to oxidative stress (210). Animal studies indicate that ADM may be involved in the regulation of ventricular remodeling (211) and it has been suggested that ADM has a cardioprotective role in the development of, or in response to, CVD (209;210). Circulating MR-proADM concentrations in adults with hypertension were
associated with pulse-pressure, left ventricular mass index, and albuminuria (212). Based upon these findings, MR-proADM has been suggested as a biomarker for target organ damage in hypertension (212). In a study of MR-proADM and NT-proBNP in patients after acute myocardial infarction, both biomarkers were equally strong predictors for cardiovascular death or heart failure (213). Furthermore, MR-proADM is considered a predictive marker for the survival of heart failure patients (182).

**C-terminal pro-arginine vasopressin (CT-proAVP)**

The C-terminal portion of provasopressin (CT-proAVP), also named copeptin, is derived together with arginine vasopressin (AVP) from a larger precursor peptide (pre-provasopressin) (214). AVP, which is identical with the antidiuretic hormone, is produced in the hypothalamus and released from the neurohypophysis into the circulation in order to regulate blood volume and cardiovascular homeostasis (215;216). AVP exerts vasoconstrictive effects on smooth muscle cells (215). In patients with acute myocardial infarction, the combination of CT-proAVP and troponin T measurements resulted in a high diagnostic accuracy shortly after first admission to the emergency department (217). Increased circulating CT-proAVP is considered a prognostic marker of death or heart failure in patients with acute myocardial infarction, independently of established conventional risk factors (218). Furthermore, CT-proAVP was found to be a predictor of adverse outcome in advanced heart failure patients (177;219).

**1.4.4 Cardiovascular biomarkers in preeclampsia or diabetes in pregnancy**

It is of great interest to study biomarkers associated with CVD in pregnancies complicated by PE or diabetes mellitus. Firstly, information on the presence and concentration of such markers could potentially increase insight into the
pathophysiology of PE and diabetes in pregnancy. Secondly, the relation of PE and diabetes in pregnancy and increased future CVD risk could be elucidated and thereby future directions for research and intervention strategies could be pointed out. The novel cardiovascular biomarkers introduced in section 1.4.3 have at present only been subject to limited research in obstetrics. Although the mature peptides ANP, ADM and AVP have been studied in healthy and complicated pregnancies for several decades, studies on the precursor fragments MR-proANP, MR-proADM are lacking for pregnancies complicated by PE or diabetes. CT-proAVP has recently been investigated in pregnancy, showing increased concentrations in maternal circulation in PE compared to normotensive pregnancies (220).

Previous studies of ANP in maternal circulation in pregnancy have provided contradictory results, with reports of increased (221-224), as well as similar (225-227) circulating ANP concentrations in PE compared to normotensive pregnancies. Similarly, studies on circulating concentrations of ADM (228-230) showed both higher, lower and unchanged concentrations in maternal circulation compared to normotensive pregnancies. Circulating AVP in PE has been found unchanged compared to normotensive pregnancies (231-233).

In PE, neopterin has previously been reported as either increased (234) or unchanged (235) in maternal circulation compared to normotensive pregnancy.

For pregnancies complicated by GDM or T2D, studies of mature ADM are limited (236), and lacking for ANP, neopterin and AVP.

An earlier study of GDF-15 in maternal circulation reported similar concentrations in normotensive and preeclamptic pregnancies (237). For pregnancies complicated by any type of diabetes, studies on GDF-15 are lacking.
In summary, data on novel cardiovascular biomarkers in pregnancies complicated by PE, preexisting diabetes or GDM are either scarce or lacking.
2. AIMS OF THE THESIS

An increased risk for later cardiovascular disease (CVD) has been observed in women suffering from preeclampsia (PE) or diabetes mellitus (preexisting or gestational) and their in utero exposed offspring.

The main aim of this project was to explore and quantify biomarkers associated with CVD outside pregnancy in these women, both during pregnancy and postpartum. Dysregulation of cardiovascular biomarkers may not only be an expression or a consequence of a complicated pregnancy, but possibly also participate in its pathophysiology. Characterization of cardiovascular biomarker profiles during pregnancy may increase our understanding of the pathophysiology for CVD following pregnancy complicated by PE or diabetes mellitus. The long-term goal of cardiovascular biomarker research in obstetrics is the early identification of women (and their offspring), who are at increased risk for future CVD following a pregnancy. This would enable targeted prevention, monitoring and intervention prior to the clinical manifestation of CVD or in early disease stages and possibly improve clinical outcome.

Specifically, we explored whether:

- Growth-differentiation factor-15 (GDF-15) concentration in maternal and fetal circulation or amniotic fluid and GDF-15 expression in placental tissue or maternal fat tissue is altered in pregnancies complicated by PE and/or diabetes mellitus, compared to normotensive pregnancies.

- Midregional pro-atrial natriuretic peptide (MR-proANP) concentration in maternal circulation and placental tissue expression during and after pregnancies complicated by PE is altered compared to normotensive pregnancies and whether our hypothesis of a cardiac source of
circulating MR-proANP in human pregnancy could be confirmed in a transgenic rat model for PE.

- MR-proANP, neopterin, midregional pro-adrenomedullin (MR-proADM) and C-terminal pro-arginine vasopressin (CT-proAVP) concentrations are altered in pregnant women with gestational diabetes or type 2 diabetes mellitus with or without PE as compared to non-diabetic, normotensive or preeclamptic women.
3. MATERIAL AND METHODS

3.1 Patient selection and biological samples

3.1.1 Pregnancy biobanks

Most samples investigated in this thesis were collected at the Department of Obstetrics at Oslo University Hospital, location Ullevål, which is the largest in Norway with approximately 7000 deliveries annually. The Department functions both as a primary delivery unit for pregnant women, as well as a tertiary referral center for rural obstetric departments in the county. The “Oslo pregnancy biobank” comprises biological samples (maternal and fetal blood, amniotic fluid, placental and decidual tissue, fat and muscle biopsies) and extensive clinical maternal and neonatal information. Patient inclusion to the biobank started in 2001 and is ongoing. Patients defined as eligible for the studies presented in this thesis met the following inclusion criteria:

- Singleton pregnancy (from 24+0 weeks of gestation onwards)
- Diagnosis of either:
  - preeclampsia
  - pregestational or gestational diabetes mellitus with or without preeclampsia
  - normotensive, non-diabetic pregnancy

Exclusion criteria were:

- preexisting hypertension
- heart or kidney disease
- rupture of membranes
- clinical signs of infection
- regular uterine contractions as sign of commencing labor
- impossibility to give informed written consent
Women scheduled for cesarean section, admitted to the high risk maternity unit or attending the outpatient clinic were recruited by the PhD candidate(s) or the biobank technician of Professor Annetine Staff’s research group. From the women who were included during pregnancy and who later on delivered vaginally, only maternal blood samples were collected. In case of cesarean section, umbilical cord blood, amniotic fluid and samples from maternal subcutaneous fat, maternal pyramidal muscle, placental and decidual tissue were collected in addition to maternal blood. Collection and storage of the samples is described in more detail in section 3.2.

**Placental villous explants**

The placental villous explants were a contribution from Professor K. Bridget Brosnihan, Hypertension and Vascular Disease Center, Wake Forest University School of Medicine, Winston-Salem, USA. The method for the isolation of placental villous explants has been developed earlier and independently from this PhD-project and is described in detail elsewhere (238;239). Briefly, placental tissue sections obtained from normotensive and preeclamptic pregnancies within 15 minutes after cesarean delivery were further dissected in order to isolate the chorionic villi. The chorionic villi were incubated for 0, 2, 4, or 16 hours in serum-free media plus cell lysis buffer. The conditioned media were collected at the different time points and frozen at -80°C for further analysis (238;239).

**3.1.2 Postpartum follow-up**

As part of another PhD-project in our research group, women recruited to the “Oslo pregnancy biobank” with their index pregnancy during the years 2001-2004 were invited to a clinical follow-up study in 2008-2009 (240). For Paper II, postpartum blood samples of women with prior normotensive (n=11) and
preeclamptic (n=14) pregnancies were available for biomarker analyses and comparison to biomarker concentrations in their respective index pregnancy.

3.2 Collection and storage of biological samples

Maternal venous blood was obtained from an antecubital vein, and prior to start of intravenous infusion in case of cesarean section. A subcutaneous fat tissue biopsy from the maternal abdominal wall was taken after incision of the skin. Amniotic fluid was either aspirated with a syringe after hysterotomy or directly collected into vials in case of accidental rupture of membranes during hysterotomy. Directly after the safe delivery of the baby, the umbilical cord was double clamped and blood from the umbilical arteries and the vein was drawn separately and immediately after the delivery of the placenta. Decidual tissue from the placental bed was collected using a vacuum suction method previously developed and evaluated by our group (241;242). Placental biopsies were taken from a macroscopically normal looking, centrally located cotyledon, omitting the decidual layer. The vial containing amniotic fluid as well as the maternal and fetal blood samples collected into EDTA- and citrate vials were immediately kept on ice before centrifugation at 4°C and storage at -80°C until assay. All tissue samples were collected in plastic freezer vials, snap frozen in liquid nitrogen directly after collection and stored at -80°C until further processing.

3.3 Laboratory analyses and animal studies

Details on the laboratory assays and references are given in Papers I-III. A brief summary of the analyses carried out in relation to this thesis is provided in the following section for each paper separately.


**Paper I**

**Immunooassay for growth-differentiation factor-15 (GDF-15)**  
GDF-15 concentration in maternal and fetal plasma, amniotic fluid and conditioned media of placental villous explants was measured by an immunoradiometric sandwich assay at the laboratory of professor Kai C. Wollert, Department of Cardiology and Angiology, Hannover Medical School, Hannover, Germany, where the assay was developed (243). The assay specifics are given in the table at the end of section 3.3.

**GDF-15 mRNA expression studies**  
We previously identified in an Affymetrix screening study (performed by coauthor F.H., Berlin), GDF-15 as potentially upregulated in placental tissue of preeclamptic pregnancies (244). As detailed in Paper I, we therefore confirmed GDF-15 mRNA expression with a TaqMan procedure in placental, decidual and fat tissue (performed by coauthors M.S.W.F and G.M.J, Oslo).

**Immunoblotting**  
Quantification of GDF-15 protein levels in homogenized placenta tissue by immunoblotting was carried out at the laboratory of coauthor professor Kai C. Wollert, Hannover (184;243). The affinity-purified polyclonal goat anti-human GDF-15 IgG antibody from R&D Systems (Catalog No. AF957) was used to determine GDF-15 expression levels in human placenta. An antibody against β-actin was obtained from Cell Signaling Technology (Danvers, MA).
Paper II

**Immunoassay for midregional pro-atrial natriuretic peptide (MR-proANP)**

The analyses of the (MR-proANP) was carried out at the laboratories of B.R.A.H.M.S Biomarkers, Clinical Diagnostics Division, Thermo Fisher Scientific, Hennigsdorf, Germany, where the method has been developed (188). The assay specifics are given in the table at the end of section 3.3.

**Rat model of preeclampsia**

The rat model of preeclampsia has been developed by our collaborating research group of Privat-Dozent Dr. Ralf Dechend at the Experimental and Clinical Research Center, Berlin, Germany, independently of and years prior to the present PhD project (245-247) and all animal experiments were carried out at Privat-Dozent Dr. Ralf Dechend’s laboratory. Briefly, female Sprague-Dawley rats, transgenic for the human angiotensinogen gene, were crossed with male rats transgenic for the human renin gene. During pregnancy, this model shows preeclamptic phenotype, consisting of hypertension, proteinuria and intrauterine growth restriction in the pregnant dams.

**Atrial natriuretic peptide mRNA expression studies**

This work was carried out at the laboratory of Privat-Dozent Dr. Dechend at the Experimental and Clinical Research Center, Berlin, Germany. Total mRNA was isolated from human decidua and placenta samples collected in the “Oslo pregnancy biobank” from a subgroup of patients. Human RNA from heart and kidney was obtained from Human Total RNA Master Panel (BD Bioscience). Total RNA was isolated from kidney, heart, placenta and mesometrial triangle of the transgenic PE rat. Analyses were performed by realtime quantitative PCR.
**Paper III**

The analyses of the midregional pro-atrial natriuretic peptide (MR-proANP), the midregional pro-adrenomedullin (MRproADM), the C-terminal part of the arginine vasopressin prohormone (CT-proAVP) and neopterin assays were carried out at the laboratories of B.R.A.H.M.S Biomarkers, Clinical Diagnostics Division, Thermo Fisher Scientific, Hennigsdorf, Germany, where the assays have been developed (248;249). The assay specifics are given together with those of the cardiovascular biomarkers investigated in Papers I and II in the following table.
Cardiovascular biomarker assay characteristics \(^a,b\)

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Type of assay</th>
<th>Intra-assay coefficients of variation</th>
<th>Inter-assay coefficients of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDF-15</td>
<td>Immuno-radiometric sandwich assay</td>
<td>(\leq 10.6%)</td>
<td>(\leq 12.2%)</td>
</tr>
<tr>
<td>MR-proANP</td>
<td>Immuno-fluorescent assay</td>
<td>(&lt;2.5%)</td>
<td>(&lt;6.5%)</td>
</tr>
<tr>
<td>Neopterin</td>
<td>Radio-immuno-assay</td>
<td>(2.9%)</td>
<td>(8.5%)</td>
</tr>
<tr>
<td>MR-proADM</td>
<td>Immuno-fluorescent sandwich assay</td>
<td>(\leq 10% \text{ (0.2-0.5 nmol/L)})</td>
<td>(\leq 20% \text{ (0.2-0.5 nmol/L)})</td>
</tr>
<tr>
<td>CT-proAVP</td>
<td>Immuno-luminometric sandwich assay</td>
<td>(&lt;5%)</td>
<td>(&lt;10%)</td>
</tr>
</tbody>
</table>

\(^a\) For the GDF-15-assay: according to co-author professor K.C. Wollert’s laboratory information

\(^b\) For the MR-proANP-, neopterin-, MR-proADM- and CT-proAVP-assays: according to manufacturer’s information
3.4 Statistical analyses

In this thesis, biomarker concentrations were reported as median values and 95% confidence intervals of the median and differences between groups were tested by non-parametric Mann-Whitney tests. Demographic and pregnancy data as well as tissue gene expression in humans and rats (Papers I-III) and immunoblot calculations (Paper I) were presented as mean and standard deviation of the mean (±SD), as these variables were normally distributed. Differences between groups were tested by Student’s t-test. For statistical analyses, the Statistical Package for the Social Sciences (version 15.0, SPSS Inc., Chicago, IL, USA) was used in Papers I and II and Predictive Analytics Soft Ware (version 18.0.1, SPSS Inc., Chicago, IL, USA) in Paper III. Spearman’s correlation was used to calculate correlation coefficients. Receiver operating characteristic (ROC) curve was constructed for maternal circulating MR-proANP and sFlt-1 as discriminator between PE and normotensive pregnancy (Paper II). MR-proANP concentrations during and after pregnancy were compared by Wilcoxon signed ranks test (Paper II). Linear regression analyses were conducted to adjust for potential confounders. A probability level of <0.05 was considered statistically significant.

3.5 Legal and ethical aspects

The Declaration of Helsinki (250) was respected in all aspects of research conducted in relation to this thesis. The “Oslo pregnancy biobank” has all required formal external (by the Regional Committee of Medical and Health Research Ethics in South-Eastern Norway) and internal (Oslo University Hospital boards) approvals. Informed written consent was obtained from each woman at study inclusion. The women agreed to review of their own as well as their offspring’s medical journal by the researchers and to the storing of study information in a de-identified manner. Furthermore, they consented to blood and tissue sampling and storage for research purposes in the field of PE,
diabetes in pregnancy and related pregnancy complications, and were aware of the fact that not all analyses to be performed were pre-defined at inclusion. Additionally, the women were asked to state whether or not they agreed to be potentially contacted for inclusion to new research projects during a time span up to 25 years, still being able to decline participation in such a case. All women who agreed to inclusion in the pregnancy biobank have the possibility to withdraw consent at any time without reprisal, including destruction of remaining stored biological samples and deletion of non-published study information related to their samples.

The animal study protocol complied with criteria outlined by the American Physiological Society (251) and was approved of by local authorities in Berlin, Germany (permission number 0268/06) (Paper II).
4. SUMMARY OF RESULTS

4.1 Paper I
Circulating and placental growth-differentiation factor-15 in preeclampsia and in pregnancy complicated by diabetes mellitus

In this study we investigated whether growth-differentiation factor-15 (GDF-15) was dysregulated in pregnancies complicated by preeclampsia (PE) and/or diabetes mellitus compared to healthy pregnancies.

Circulating GDF-15 concentrations in maternal plasma from normotensive, non-diabetic control pregnancies (n=59), pregnancies complicated by preeclampsia (PE, n=85) or diabetes mellitus [n=112; gestational diabetes mellitus (GDM) n=55, type 1 diabetes (T1D) n=46 or type 2 diabetes (T2D) n= 11] as well as diabetic pregnancies further complicated by PE (n=11) were investigated and yielded the following results:

- In PE, median GDF-15 concentration in maternal plasma was higher than in controls (99 124 vs 79 875 ng/L), although the difference was not statistically significant (P=0.1). Maternal GDF-15 concentration correlated however positively with gestational age at sampling time in PE (Spearman’s correlation 0.5, P<0.001). Median maternal GDF-15 concentration was higher in PE ≥37 weeks gestation compared to control pregnancies ≥37 weeks gestation (127 061 vs 80 319 ng/L; P<0.001).

- In diabetes mellitus in pregnancy, median maternal GDF-15 concentration was significantly elevated compared to the control group (91 549 vs 79 875 ng/L, P=0.02). When analysed separately, the group of pregnant women with T1D had significantly elevated median maternal GDF-15 compared to the control group (93 129 vs 79 875 ng/L, P=0.03), whereas the other diabetes groups, although with markedly elevated median maternal GDF-15 concentration, did not differ statistically significantly from controls (GDM: 89 728 ng/L, P=...
0.08; T2D: 98 923 ng/L, \(P= 0.2\)). There were no significant differences in median maternal GDF-15 concentrations between the diabetes groups (\(P=0.8\)).

- In general, fetal plasma GDF-15 concentrations were much lower than maternal concentrations, whereas GDF-15 concentrations in amniotic fluid were intermediate between maternal and fetal concentrations. Still, fetal GDF-15 concentrations were almost 10-fold higher than circulating GDF-15 reported for elderly persons or non-pregnant healthy, fertile women.

In fetal circulation (total n=72) and amniotic fluid (total n=99) we found the following:

- Elevated GDF-15 in PE and in diabetic pregnancies complicated by PE compared to controls (for fetal plasma: 5978 and 6002 vs 3710 ng/L, \(P<0.001\) and \(P=0.001\), respectively; for amniotic fluid: 52 775 and 59 595 vs 29 565 ng/L, \(P<0.001\) and \(P=0.001\), respectively).

In maternal tissue samples we observed:

- Placental tissue: elevated mean GDF-15 mRNA expression in PE (n=29) compared to controls (n=33, \(P=0.002\)), but not in pregnancies complicated by diabetes mellitus with PE (n=6) or without additional PE (n=10).

- Fat tissue: elevated GDF-15 expression in the diabetes mellitus group (n=10) as compared to controls, although not statistically significant (\(P=0.2\)), and unaltered expression in PE.

- Placental immunoblots of control (n=8) and PE (n=10) pregnancies confirmed a single GDF-15 protein band, however, differences between the PE and the control group were not statistically significant (\(P>0.1\)).

- Conditioned media from the placental villous explants showed a time-dependent increase in GDF-15 protein production, however, differences
between the PE (n=6) and the control group (n=6) were not statistically significant ($P>0.1$).

4.2 Paper II

**Cardiovascular biomarker midregional pro-atrial natriuretic peptide during and after preeclamptic pregnancies**

In this study we explored the cardiovascular biomarker midregional pro-atrial natriuretic peptide (MR-proANP) in maternal circulation and pregnancy tissues as well as in a transgenic rat model for PE. EDTA-plasma from normotensive, non-pregnant women (n=49), normotensive, pregnant women (n=77) and preeclamptic women (n=107) was analysed by immunoassay for MR-proANP. Of these women, 25 were included to a postpartum follow-up at 5 to 8 years after index pregnancy (normotensive pregnancies n=11, PE n=14). Placental and decidual atrial natriuretic peptide mRNA expression levels were analysed by quantitative real-time PCR in 21 normotensive and 23 preeclamptic pregnancies, as well as in human heart and kidney samples. Circulating MR-proANP and expression studies of MR-proANP were carried out in a transgenic rat model for PE.

We found during pregnancy:

- Elevated median MR-proANP in maternal plasma in PE compared to normotensive pregnancies (135 vs 56 pmol/L, $P<0.001$). In the PE group, MR-proANP concentration correlated inversely with gestational age at sampling time (Spearman's rho -.3, $P=0.001$), however, after correcting for gestational age, the difference in median MR-proANP concentration between preeclamptic and normotensive pregnancies remained significant ($P<0.001$).

- No difference in median MR-proANP between severe (n=54) and non-severe (n=53) PE cases (149 vs 116 pmol/L, $P=0.09$).
Higher median plasma MR-proANP in the preeclamptic women delivering small for gestational age (SGA, <10% birth weight percentile) babies (n=51) as compared to the preeclamptic women delivering non-SGA infants (n=56; 177 vs 114 pmol/L, \( P<0.001 \)).

The sensitivity and specificity of plasma MR-proANP in discriminating between preeclamptic and normotensive pregnant controls close to delivery was high, with an area under the curve (AUC) of 0.85 (95% CI: 0.79- 0.90, \( P<0.0001 \)). Maternal sFlt-1 performed only slightly better than MR-proANP as discriminator between PE and normotensive pregnancy, with an AUC of 0.94 (95% CI: 0.91- 0.98, \( P<0.0001 \)).

We found at postpartum follow-up 5-8 years after the index pregnancy:

- No residual difference in circulating median maternal MR-proANP between the formerly preeclamptic group and the formerly normotensive group (53 vs 49 pmol/L, \( P=0.5 \)).
- Formerly preeclamptic women had significantly lower median plasma MR-proANP concentrations compared to concentrations during their pregnancy (53 vs 135 pmol/L, \( P=0.001 \)), whereas the median value for the formerly normotensive women was unaltered (49 vs 56 pmol/L \( P=0.2 \)).
- Median MR-proANP concentrations in formerly preeclamptic and formerly normotensive women did not differ from median MR-proANP concentrations in non-pregnant women (53 and 49 vs 46 pmol/L, \( P=0.7 \) and \( P=0.2 \)).

In the preeclamptic rat model and human tissues:

- Circulating MR-proANP protein differences between preeclamptic and normotensive pregnancy (10.9 vs 4.3 pmol/l, \( P=0.05 \)).
- Atrial natriuretic peptide (ANP) expression was high in the heart, but negligible in the uteroplacental unit in both normotensive humans and rats (both \( P<0.0001 \) vs all other tissues), whereas ANP expression in
maternal and fetal hearts in the preeclamptic rats was significantly increased, compared to controls (both $P<0.001$).

4.3 Paper III
Cardiovascular risk markers in pregnancies complicated by diabetes mellitus or preeclampsia

In this study we explored circulating concentrations of midregional pro-adrenomedullin (MR-proADM), C-terminal pro-arginine vasopressin (CT-proAVP) and neopterin in EDTA-plasma from 262 women in gestational week 24-42 (healthy pregnancies n=71, preeclampsia n=105, type 2 diabetes n=17, gestational diabetes n=61, diabetes with preeclampsia n=8). The diabetes groups were also analysed for midregional pro-atrial natriuretic peptide (MR-proANP), and compared to the MR-proANP concentrations reported in Paper II for healthy, pregnant controls (n=77), preeclamptic women (n=107) and non-pregnant, premenopausal women (n=49).

Our findings were as follows:

- Median circulating MR-proADM and CT-proAVP were lower in the non-pregnant group compared to all pregnancy groups (all $P<0.001$). In both healthy and preeclamptic pregnancies, median circulating neopterin concentration was in the upper reference level of healthy non-pregnant controls reported in the literature (252).

- In the GDM group, median plasma MR-proANP was significantly lower compared to healthy, pregnant controls ($P=0.002$). Median neopterin was higher compared to the healthy pregnancy group and to the total PE group (both $P<0.001$), whereas the latter two groups did not differ from each other ($P=0.4$). Median MR-proADM and CT-proAVP were elevated in both GDM and in PE, compared to healthy, pregnant controls (GDM: $P=0.007$ and $P=0.02$; PE: $P=0.007$ and $P<0.001$).
- In GDM, adjustment for either prepregnancy or gestational body mass index (BMI) resulted in a maintained significant lower median MR-proANP concentration in GDM (both \(P=0.02\) vs healthy pregnancies, both \(P<0.001\) vs PE). Also, the results for neopterin and MR-proADM remained unaltered (neopterin: \(P=0.004\) and 0.002; MR-proADM: \(P=0.002\) and 0.001), whereas the difference in CT-proAVP between GDM and healthy, pregnant controls was no longer significant (\(P=0.2\) for both).
- In GDM, adjustment for gestational age resulted for CT-proAVP a loss of significance for the difference between GDM and healthy, pregnant controls (\(P=0.2\)).
- In pregnant women with T2D, median neopterin was significantly elevated compared to healthy, pregnant controls (\(P=0.01\)). The other biomarkers did not differ significantly between pregnant women with T2D and healthy, pregnant controls. None of the biomarkers differed significantly between the T2D and the GDM group.
- In PE, neopterin correlated positively with gestational age at blood sampling (Spearman’s correlation 0.4, \(P<0.001\)), and we found a significantly higher median neopterin concentration in PE included near term (≥ gestational week 37) compared to healthy, pregnant controls in the women (all included near term) (n=31 and n=62, 10.2 nmol/L vs 9.2 nmol/L, \(P<0.001\)).
- Additional PE development in a GDM or type 2 diabetic pregnancy resulted in increased median concentrations of MR-proANP compared to GDM (\(P=0.01\)) or T2D mellitus (\(P=0.03\)) without PE, but not compared to only PE.
The following table summarizes the findings of all CVD biomarkers in maternal circulation in pregnancies complicated by PE and/or diabetes mellitus investigated in Papers I-III of this thesis.
Synopsis of the alteration of cardiovascular biomarkers in maternal circulation investigated in the present thesis\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>PE</th>
<th>GDM</th>
<th>T2D in GDM or T2D</th>
<th>T1D</th>
<th>DM (total group)</th>
<th>PE in DM (total group)</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDF-15</td>
<td>↑</td>
<td>(†)</td>
<td>n.a.</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>I</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>MR-proANP</td>
<td>↑</td>
<td>↓</td>
<td>(↓)</td>
<td>(†)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>II and III</td>
</tr>
<tr>
<td>Neopterin</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR-proADM</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
<td>↔</td>
<td>n.a.</td>
<td>n.a.</td>
<td>III</td>
</tr>
<tr>
<td>CT-proAVP</td>
<td>↑</td>
<td>↑</td>
<td>(†)</td>
<td>(†)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>III</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Arrows indicate alteration of median biomarker concentration as compared to healthy, pregnant controls as follows:↑: higher (statistically significant), (↑): higher, but not statistically significant, ↓: lower, (↓): lower, but not statistically significant, ↔: unaltered

\textsuperscript{b}Abbreviations: n.a.: not analyzed; GA: Gestational Age; C: normotensive, non-diabetic pregnant women, named Controls; PE: Preeclampsia; GDM: Gestational Diabetes Mellitus; T2D: Type 2 Diabetes mellitus; T1D: Type 2 Diabetes mellitus; DM: total group of pregnant women with any type of diabetes mellitus
5. DISCUSSION

5.1 Methodological considerations

5.1.1 Study design and study populations

Patient selection and inclusion
All studies in this thesis have a cross-sectional design. For practical reasons (capacity problems due to conflicting clinical duties), not all eligible patients were asked to participate in the study. Very few patients declined to take part in the studies (only 3 patients from 2001 until 2007 (253)). Due to ethical concerns, the Regional Medical Research Ethics Committee did not allow us to keep research record on clinical data of patients who declined to participate.

We are of the opinion that our inclusion method does not imply a general patient selection bias between our study population and other women with healthy, preeclamptic or diabetic pregnancies who were admitted to and/or delivered by cesarean section at our hospital. For all patients included in our studies, cesarean section was either medically indicated or, in case of 46% (Paper I) and 53% (Papers II and III) of the healthy, normotensive controls, granted upon “maternal request” (including nulliparous women with breech presentation who chose not to deliver vaginally). In either case, the decision for cesarean section was made independently of the research projects of this thesis, (i.e. by another physician than the PhD candidate or the main supervisor), and prior to patient inclusion to the study. Also, admittance of patients to the high-risk maternity unit and scheduled appointments at the outpatient clinic occurred independently of the research projects and were medically indicated.
Number of included patients and available biological samples

As described in section 3.1, inclusion to our pregnancy biobank has been ongoing since 2001. Therefore, the number of patients included in the three studies of this thesis varies, and is the result of which type of biological sample (i.e. maternal and fetal blood, amniotic fluid, placental, decidual and fat tissue) was needed in order to answer the respective study questions, as well as of the sample availability, since not all patients had complete sample sets or sufficient amounts of blood samples available for all performed analyses. The reasons for incomplete samples sets were either medical (e.g. oligohydramnios, consideration of to the newborn’s need of own umbilical cord blood in case of prematurity, the surgeon’s duty to secure maternal and fetal well-being in case of unexpected course of operation, making the task of providing biological samples for research projects unimportant) or technical (e.g. hemolysis, limited amount of umbilical cord blood or failed maternal venous punctions, or simply “used up samples” for previous research project analyses).

Mode of delivery

All women included in the studies were recruited prior to labor onset, i.e. none had regular uterine contractions prior to or at inclusion to the studies. This is in our view crucial, since labor contractions and variation in labor duration may cause variations in placental oxidative stress, with effects on tissue expression (254) and possibly on circulating protein concentrations.

The overall cesarean section rate at Ullevål in 2010 was 20.3% as opposed to the nationwide mean of 16.8% (7). The aim for our pregnancy biobank was to recruit women scheduled to deliver by elective cesarean section in order to obtain all types of biological samples from the same pregnancy, giving us the possibility to analyse our proteins of interest in several compartments (Papers I and II). This inclusion policy led to a selection bias towards women delivered by cesarean section in the healthy control
(Paper I: 95% and Papers II and III: 90%) and in the preeclampsia (PE) group (Paper I: 71% and Papers II and III: 58%), compared to the situation in the group of diabetic pregnancies without further complications, in which elective cesarean section was less frequent (Paper I: 12.5% and Paper III: 20%). For the PE group, this selection bias resulted in more severe, early-onset PE cases in our study population than in the general population, as discussed in more detail in the following paragraph. For the healthy controls and the diabetes groups, our study conclusions (i.e. differences in biomarker concentrations between the study groups) are unlikely to be influenced by variations in mode of delivery.

**Preeclampsia group**

As mentioned in section 3.1, our obstetric department is a tertiary referral center with approximately 7000 deliveries annually. Qualified pediatric service in a neonatal intensive care unit (NICU) enables us to deliver fetuses from 24+0 weeks gestation if inevitable. More rural hospitals in our region have a lower gestational admittance limit of 26 or 28 weeks.

Independently of diagnosis, the proportion of preterm delivered babies <37 weeks gestation is 7.4% of all births at Ullevål in 2010 as opposed to the nationwide mean of 6.5% of all births (7). Early-onset PE more often results in a cesarean delivery compared to late-onset PE, where vaginal delivery is attempted by induction of labor.

Collectively, these circumstances led to a selection bias towards more preterm and severe PE cases in our study population compared to the general distribution of PE in healthy nulliparous women with late-onset disease in more than 75% of cases (6). In the present studies, 75% (Paper I) and 71% (Papers II and III) of the PE cases had a gestational age below 37 weeks. A gestational age below 34 weeks at inclusion to the studies was noted in 52% (Paper I) and 43% (Papers II and III) of PE cases. However, severe PE is more likely to be associated with later cardiovascular disease (CVD) or risk factors
for CVD in both the mother and offspring (51;61;62;75;76), and represents therefore the group of greatest research interest.

**Diabetes mellitus groups**

In Paper I, we merged all forms of diabetes mellitus in pregnancy into one “diabetes mellitus in pregnancy” group and all diabetic pregnancies further complicated by PE into one “diabetic PE” group. We are aware of the fact that type 1 diabetes mellitus (T1D), type 2 diabetes mellitus (T2D) and gestational diabetes mellitus (GDM) differ with respect to etiology and pathophysiology. However, abnormal glucose homeostasis is the crucial common pathophysiologic momentum in all types of diabetes in pregnancy and contributes to the similarly increased risk for PE and later CVD in the affected women and their offspring. As the main aim of Paper I was to characterize the assumed dysregulation of GDF-15 during pregnancy in women and their fetuses with increased risk for later CVD due to PE and/or diabetes mellitus, we allowed us to comprise the different types of diabetes into one group, despite different etiology.

In Paper III, we chose not to include patients with T1D because our main focus was to compare the cardiovascular risk marker profile of conditions limited to the duration of pregnancy and their possible implications for later CVD. We included, however, the group of pregnant women with T2D since GDM and T2D are closely interrelated with regard to risk factors, the common pathophysiological feature of relative insulin deficiency and the high proportion of women with GDM progressing to T2D during the postpartum years. With background in these similarities, in addition to a similar increased risk for PE in GDM and T2D in pregnancy, we comprised the group of women with GDM and PE and the group of women with T2D and PE into one group in Paper III, resulting in higher case numbers.
Pregnant control group
Normotensive, pregnant women without glucosuria, chronic disease, medication on a regular basis or intercurrent disease at time of inclusion were in our study defined as healthy, pregnant controls.

As described in detail in section 1.2.1, the national guidelines for routine health care during pregnancy do not include a general screening for diabetes mellitus for all pregnant women in Norway, but is limited to those women who display certain risk factors (29). It is therefore theoretically possible that women with undiagnosed GDM were included in our healthy pregnant control group. However, Norway has free-of-charge pregnancy follow-up with regulated visit intervals, including urine dipstick check, and the national guidelines for (repeated) testing of glucose homeostasis are relatively broad, as described in section 1.2.1. Therefore, most GDM cases should be detected. Furthermore, the mean gestational age for healthy controls at study inclusion was 38.0 weeks (Paper I) and 37.5 weeks (Papers II and III) and it is therefore unlikely that women with impaired glucose tolerance would not have been identified either by positive urine dipstick or other clinical findings (maternal or fetal symptoms) by this time in pregnancy. Most importantly, the medical journal of all included women was reviewed by the PhD student at least once after delivery. A diagnosis of diabetes mellitus after blood sampling would have been noted and consequently led to the exclusion of the patient as a control from the current studies. However, such exclusion did not occur during the study period, supporting that none of our control patients had undetected GDM.

Postpartum follow-up
In Paper II, we were able to analyse a small group of women with prior normotensive or preeclamptic pregnancy for MR-proANP 5 to 8 years postpartum. As previously published by our group (240), there was a selection
bias towards preterm delivered PE cases (<34 week of gestation) compared to the general distribution of PE in healthy nulliparous women. This reflects the same selection bias as described for the PE group in the pregnancy biobank, as discussed further above. An additional attendance bias cannot be ruled out, i.e. that the women with the most severe, preterm PE during index pregnancy are more likely to participate in a follow-up study compared to women with less severe disease, in order to contribute to research in the field and/or to receive an additional health check at their former obstetrical department.

By the time of the study conducted in relation to Paper I, patient inclusion to the postpartum follow-up study had not yet started. For Paper III, postpartum blood samples of 10 women with GDM in their index pregnancy would have been available for analyses. However, the follow-up cohort did not contain any women with a prior pregnancy complicated by T2D, which made an analysis of postpartum samples for Paper III not expedient.

**Confounders**

**Parity**

In this thesis, both nulliparous and multiparous women were included to the studies. PE is considered “a disease of first pregnancies”, which is why one might argue that PE research should concentrate on nulliparous women. Thorough assessment of parity is of major importance with regard to correct interpretation of the obtained results (255). In our studies, parity was assessed for every woman. Most of the women with PE were nulliparous (Paper I: 65.8%; Papers II and III: 64.5%), whereas for women with diabetes and PE, the proportion of nulliparity was varying (Paper I: 72.7%; Paper III 56.3%).

In Paper I, the finding of elevated GDF-15 in maternal plasma observed for the PE group (>37 weeks gestation) compared to healthy, pregnant controls remained significant, when only data of nulliparous women were analysed (P=0.004). Also for fetal plasma and amniotic fluid, the increase in GDF-15 in
nulliparous PE (n=14), and nulliparous diabetic pregnancies with PE (n=6) compared to nulliparous healthy pregnancies (n=18) remained significant. We believe that parity is not a major confounder for the conclusions of this thesis. In addition, as preeclampsia does not only occur in primiparous women, nor only in parous women with previous PE, we believe that including parous women to this biomarker study is better than excluding them.

**Gestational age**

As described in the paragraph “Preeclampsia group” in this section, there was a selection bias towards preterm PE in our study population with a gestational age below 37 weeks in 75% (Paper I) and 71% (Papers II and III) of the PE cases. Admission to hospital and/or preterm delivery does not occur in women with healthy pregnancies except in case of pathology, as for example threatening preterm labor, preterm rupture of membranes or chorioamnionitis. These cases did not fulfill the inclusion criteria to our studies. Cesarean section was a prerequisite for our pregnancy tissue based studies (Papers I and II), in order to obtain concomitant blood and tissue samples. Obviously, cesarean section in preterm pregnancies, either healthy or diabetic, does not occur without an (additional) pregnancy pathology making such an intervention necessary. Therefore, matching for gestational age with regard to the study aim of comparing biomarker expression in pregnancy tissues (Papers I and II) was not feasible.

Maternal blood samples from otherwise healthy women with a singleton pregnancy and isolated cervical insufficiency, i.e. without contractions or signs of infection were found suitable for study inclusion, and were included as preterm, healthy controls. However, these cases were scarce (Paper I: 3/59 <34 weeks; 4/59 <37 weeks; Papers II and III: 7 <34 weeks; Papers II and III: 9 <37 weeks).
In order to account for a potential influence of gestational age on significant differences in cardiovascular biomarker concentrations between study groups, linear regression analyses was carried out and accounted for in Papers II and III since in these studies, mainly circulating biomarkers were investigated. For the studies in which only circulating biomarkers were explored, a longitudinal blood sampling over all gestational ages for the study groups would have been preferable, but was not possible in our clinical research biobank setting.

**Body mass index (BMI)**

In adults, normal weight is defined as BMI (weight/height in meters$^2$) from 19 to 25 kg/m$^2$. Adiposity is defined as BMI from 25 to 30 kg/m$^2$, whereas obesity is defined as BMI from 30 kg/m$^2$ (256). In our pregnancy biobank population, both prepregnancy BMI and current gestational BMI (i.e. at blood sampling) are recorded. Increased BMI is associated both with increased risk for PE and GDM and T2D (9;34;35). In our study populations, women in the PE, GDM and T2D groups had significantly higher prepregnancy and current gestational BMI compared to healthy controls (Papers I-III). In Papers II and III correlations between the investigated biomarkers and BMI were tested and adjusted for when indicated. Such adjustment for BMI did not alter the conclusions of study group differences for 4 of the 5 investigated biomarkers (Paper III: CT-proAVP in the GDM group being the exception), which is why we do not consider BMI as a major confounder in our studies.

*Circadian variation*

Possible circadian variations in circulating biomarker concentrations are unlikely to affect our analyses, since all samples were collected at daytime, with the majority during routine morning laboratory rounds. For MR-proADM and CT-proAVP, concentrations in plasma have been found unaffected by
timing of sampling during daytime (206;249). We did not find any reports on circadian variation for the other biomarkers explored in this thesis.

**Fasting state**

The majority of our patients in the healthy, pregnant control group, in the PE group and in the diabetic PE group were fasting for at least 6 hours at blood and tissue sampling (Papers I-III: ≥85% for all three groups). In the diabetes groups, the proportion of fasting women at blood sampling was low, explained by the preponderance of outpatients in these study groups (Papers I and III: ≤20%). Except for MR-proADM in the PE group, where we found a lower concentration for the non-fasting group, there were no significant differences in maternal cardiovascular biomarkers concentrations between fasting and non-fasting women in any of the diagnosis groups (data not shown). Interestingly, MR-proADM has previously been reported not to be influenced by fasting state in non-pregnant subjects (206). We therefore interpret our finding as a selection bias towards less severe PE in the non-fasting patient group, as compared to the fasting PE patients, who were recruited prior to a cesarean delivery. We did not find any reports on effects of fasting for the other biomarkers explored in this thesis.

**Renal function**

Increased circulating biomarker concentrations could be caused by impairment of renal function, as previously shown for neopterin (257) and CT-proAVP (258). However, as described in section 3.1.1, a diagnosis of kidney disease was an exclusion criterion in our studies, i.e. none of the women had a diagnosis of nefropathia. Furthermore, none of the PE patients in Paper II and only 4 of the PE patients in Paper I had a serum creatinine above normal range (50-90 μmol/L) and median serum creatinine in women with PE was similar in all three studies (Paper I: 62 μmol/L, Paper II and III: 61 μmol/L). Although
data on microalbuminuria were missing for the GDM and T2D groups in Paper III, only four patients had proteinuria as evaluated semi-quantitatively by urine dipstick. A major impact of evident differences in renal function between our study groups on study group differences in biomarker concentrations seems unlikely.

**Placenta weight**

As the placenta might be the source of the circulating investigated biomarkers, it is relevant to investigate correlations between placenta weight and circulating biomarker concentrations. Even though placenta weight is to be recorded in all deliveries at our delivery unit, the recordings for the patients included in our biobank studies are partly lacking up to the year 2005. Medical records were reviewed by the PhD student in 2011, but not all placenta weight data could be retrieved. Patient inclusion to the studies (and thereby blood sampling) could occur several weeks prior to delivery. Not all of these women underwent cesarean section, but delivered vaginally at a later date. Since time of blood sampling and delivery not necessarily were identical, correlation of circulating biomarker concentration with placenta weight seemed not expedient in these cases.

We cannot, however, conclude from any positive correlation findings per se that placenta is the source for these maternally circulating biomarkers in maternal circulation. As samples from cardiovascular organs for obvious ethical reasons cannot be obtained from healthy, young women during pregnancy, animal models such as the preeclamptic rat model may be used in order to investigate tissue sources of circulating maternal biomarkers in pregnancy (Paper II). Also comparison of pregnancy and immediate postpartum samples could indicate whether the placenta is a likely origin for circulating biomarkers in pregnancy. Postpartum blood sampling was however not feasible in our clinical research biobank setting.
**Ethnicity**

Due to ethical considerations, women eligible for participation in our studies had to be able to communicate in Norwegian or English, since other languages common among immigrants delivering at our Department were not spoken by the patient recruiters. Translations of informed written consent to several languages could have been provided. However, our experience from previous study collaboration at our Department was that translation of written study information had no stimulating effect on recruitment of patients without knowledge of Norwegian or English. As a consequence, the ethnical distribution in our study groups may not actually mirror the ethnical distribution of these patient groups in general at our Department. In the studies presented in this thesis, the majority of women in the healthy, pregnant control, PE and T1D groups were of Caucasian origin (≥80% for all these groups in Papers I-III), whereas the proportion of Caucasians was lower in the group of women with GDM or T2D in pregnancy (Paper I: GDM: 58%, T2D: 36%; Paper III: GDM: 57%, T2D: 35%). The remainder of the women in these groups originated from South Asia or Africa. We cannot rule out that ethnical group differences through genetic or environmental variations influence circulating biomarker concentrations. For the GDM and T2D groups, adjustment for ethnicity may also remove the cause of group differences per se, namely the state of diabetes, therefore such adjustment was not performed.

**5.1.2 Collection of clinical data**

A strength of the studies included in this thesis is the thorough assessment of the clinical data which was achieved by means of individual patient interview by the PhD student or research biobank technician directly at inclusion time, supported by a standardized study questionnaire. Additionally, each patient’s official pregnancy chart (“helsekort for gravide”) and hospital medical files were thoroughly reviewed by the PhD student. Additionally, the newborns’
hospital files were consulted in order to confirm parameters such as gestational age at delivery and neonatal weight.

5.1.3 Assessment of soluble biomarkers

Collection and storage of biological samples

A strength of our pregnancy biobank is the precise and constant method of biological sample collection, further sample processing and storage according to a standardized laboratory protocol valid since the foundation of the “Oslo pregnancy biobank” in 2001, as described in section 3.1. Precise and rapid tissue collection and cord blood sampling after placental delivery is of major importance in order to reduce possible post partum ex vivo effects to a minimum. All surgeons involved in biological sample collection during cesarean section were instructed and supervised by the PhD student. We are of the opinion that this strategy minimized the risk of incorrect sample collection and accidental contamination or destruction of the samples.

Maximum storage time was 7 years for biological samples at -80°C analysed in Paper I and 9 years for samples analysed in Papers II and III. There is no published documentation available regarding the stability of the investigated cardiovascular biomarkers during long-term storage. For MR-proADM stability in plasma at 20°C for at least 12 months has been reported (206). In all three studies, the proportion of samples stored for more than 5 years was evenly distributed between the diagnosis groups. Possible changes in absolute concentrations due to storage time should have affected all study groups equally. Therefore, we think it is unlikely that storage time is a crucial confounder for the conclusions drawn in this thesis.
Laboratory analyses

GDF-15 concentration has been shown to be independent of the added anticoagulant matrix (243), which is why maternal citrate plasma and fetal EDTA-plasma concentrations could be compared to each other in Paper I.

As described in Papers II and III, the analytical methods used for the analyses of the cardiovascular biomarkers MR-proANP, MR-proADM and CT-proAVP used in this thesis are thought to better reflect the circulating concentrations of the respective mature hormones ANP, ADM and AVP than assays used in previous studies. The theoretical background for this is described in the following paragraphs.

ANP is stored in the cardiac atrial granules as proANP, which is upon secretion split into a biologically inactive N-terminal fragment (NT-proANP) and mature ANP in equimolar amounts (259). NT-proANP is more stable in human plasma as compared to mature ANP, which is why the former has been used as an analyte in ANP assays (189;260). However, based on reports of early degradation of crucial epitopes at the extreme ends of proANP (189), it has been remarked that NT-proANP assays used in previous studies may have underestimated the quantity of the circulating mature ANP (188;189). The midregion of proANP (MR-proANP) is more stable in the circulation than the N-terminal part of proANP (189). Analysis of circulating MR-proANP has been carried out in several other studies in the field of CVD in the recent years (182;193;194;261).

Midregional pro-ADM (MR-proADM) is synthesized together with the mature adrenomedullin (ADM) from the precursor peptide preproADM (206). Mature ADM has a very short half-life in the circulation (262) and binding to other proteins makes a reliable measurement difficult (206;263). As a consequence, previous studies analysing ADM may have underestimated the quantity of mature ADM the circulation. In contrast to mature ADM, MR-proADM is stable in the circulation (206) and is used as a surrogate for plasma
levels of ADM (264). Analysis of circulating MR-proADM has previously been carried out in several other studies in the field of CVD in the recent years (182;212;219;265;266).

The C-terminal portion of provasopressin (CT-proAVP) is derived together with arginine vasopressin (AVP) from a larger precursor peptide (pre-provasopressin) (214). AVP has a short half-life in the circulation (267), which is why the reliability of previous assays analysing mature AVP has been questioned. In contrast, CT-proAVP is stable in plasma. Due to its stochiometric generation, it is an appropriate surrogate marker for mature AVP (249). Similarly to the assays for MR-proANP and MR-proADM, the CT-proAVP assay used in Paper III has been applied in several CVD studies over the recent years (177;193;217;268).

5.1.4 Statistical analyses

Sample size
The number of included patients in several of our study groups is very small, including the groups of pregnant women with T2D or with diabetes mellitus further complicated by PE, as well as the postpartum groups. This results in low statistical power with subsequent risk of type II errors, i.e. not rejecting the null hypothesis when, in fact, it should be rejected (false negative results). As accounted for in section 1.4.4, previous reports on the cardiovascular biomarkers in pregnancies complicated by PE and/or diabetes mellitus investigated in this thesis are either limited (GDF-15, CT-proAVP, neopterin) or lacking (MR-proANP and MR-proADM). Therefore, pregnancy concentrations of these biomarkers and related standard deviations were not available for power calculations for our studies.

In conclusion, although the number of patients included in Papers I-III surpassed most of the comparable publications, the studies presented in this
thesis should be regarded as pilot studies and could be used for future power calculations when analysing these biomarkers in pregnancy.

**Multiple testing**

The Bonferroni method for adjustment for multiple comparisons in order to avoid a type I error (falsely rejecting the null hypothesis based on the finding of a difference in biomarker concentrations between study groups which just occurred by chance), was not carried out in any of the studies in this thesis. The hypotheses of all the studies were based on biologically plausible considerations. Furthermore, the tested variables were relevant to the research questions and not randomly chosen.

**Linear regression analyses**

As described in the above sections, linear regression analyses were applied when appropriate in order to adjust for potential confounders. However, with regard to adjustment for gestational age, it has to be remarked that it may not necessarily be biologically correct to do so, since women with preterm, early-onset PE have a more severe disease than women with late-onset PE close to term. As a consequence, pregnancies complicated by early-onset PE have a shorter duration than pregnancies complicated by late-onset PE due to the need of obstetric intervention. Therefore, a comparison of biomarker concentrations in late phases of severe PE to healthy controls cannot be made, since the former group is not existent in countries with sufficient health care.
5.2 Discussion of results

Paper I: Circulating and placental growth-differentiation factor-15 in preeclampsia and in pregnancy complicated by diabetes mellitus

Dysregulation of GDF-15 in PE: Marker of placental stress or mediator of disease?

Placental expression of GDF-15 is high and strongly induced by oxidative and nitrosative stress (269). In cultured cardiomyocytes GDF-15 expression is induced by simulated ischemia/reperfusion injury (184;270). Increased placental oxidative and nitrosative stress as well as ischemia/reperfusion are also characteristics of preeclampsia (PE) (94;140;141;271;272). In monocytoid cells expression of GDF-15 is upregulated by interleukin-1β and tumor necrosis factor-α (183), both circulating cytokines that are elevated in PE (273). Based on these considerations, our finding of elevated GDF-15 concentrations in PE in maternal and fetal circulation, as well as in amniotic fluid, may be caused by excessive placental production of GDF-15 in PE. We found higher median maternal plasma GDF-15 concentration in PE compared to normotensive, pregnant controls, irrespective of gestational age, however, the difference was only statistically significant for late-onset PE close to term (≥gestational week 37). We found a positive correlation between maternal GDF-15 concentration and gestational age in PE. In our study, pregnancies complicated with PE had a significantly shorter median pregnancy duration than normotensive pregnant controls. Therefore, differences in gestational age between these two study groups could explain why the difference in median maternal GDF-15 in the total PE group as compared to the control group was not statistically significant. Circulating GDF-15 concentrations have previously been shown to increase with gestational age in normal pregnancy (274). Therefore, we would have expected an even larger difference between the PE and control groups if they had been matched for gestational age. In Paper I, linear regression analysis
in order to correct for gestational age as a possible confounder was not carried out. We argued that adjustment for gestational age would not take into account the biological situation, i.e. that preterm, early-onset PE is a more severe disease than PE close to term. Longitudinal blood sampling in the control group would have been preferable to adjustment for gestational in order to confirm this hypothesis. However, our clinical pregnancy biobank research studies are not designed for such longitudinal blood sampling.

There was a large heterogeneity within our recruited PE group regarding GDF-15 concentrations, which may also be the reason why a previous, smaller study could not demonstrate any significant difference of maternal GDF-15 in PE compared to healthy, normotensive pregnancy (237).

The function of GDF-15 in pregnancy, irrespective of PE development, is presently unknown. In our study, there was no evidence of more severe PE (as defined by ACOG criteria (2)) being associated with more elevated maternal GDF-15 concentrations.

Even if circulating GDF-15 only represents a marker for placental stress in PE, reflecting part of the underlying pathophysiology, and not necessarily serves as a mediator of disease, increased GDF-15 in mother and fetus during pregnancy could possibly assist in identifying individuals at increased risk of CVD later in life. Longitudinal studies are needed in order to explore this hypothesis.

*Dysregulation of GDF-15 in diabetes in pregnancy: Extraplacental origin?*

We are not aware of studies of circulating GDF-15 or GDF-15 tissue expression in pregnancies complicated by diabetes mellitus prior to our study. We found elevated circulating GDF-15 in pregnancies complicated by diabetes mellitus compared to healthy, pregnant controls. In contrast to the findings for our PE group, we did not find increased GDF-15 expression levels in the placental tissues from the diabetic group as compared to controls. We
observed, however, increased GDF-15 expression levels in fat tissue in diabetic pregnancies, as opposed to the situation in PE, although absolute GDF-15 expression levels were higher in placental tissue compared to fat and decidual tissues. A study of non-pregnant, obese women with T2D, published after our Paper I, showed increased GDF-15 concentrations compared to non-diabetic, obese women and healthy non-obese controls (275). An endocrine function of adipose tissue has been demonstrated, and the secretion of several metabolically active proteins such as leptin, resistin, and adiponectin, termed adipokines, has been described (276). An earlier study showed GDF-15 expression and release from adipocytes, as well as its contribution to increasing adiponectin production (277). The increased GDF-15 mRNA expression in fat tissue in pregnancies complicated by diabetes found in our study, rather than in placental tissue, may contribute to the increased circulating maternal concentrations of GDF-15 in diabetic pregnancies. This could also explain why we did not find elevated GDF-15 concentrations in amniotic fluid and fetal blood from diabetic pregnancies, as opposed to the situation in PE. However, the net contribution of fat tissue to the elevated circulating maternal GDF-15 levels in diabetic pregnancies remains unknown.

**GDF-15: Associated with increased risk for CVD after PE and diabetic pregnancies?**

Evidence of acute atherosis of the uteroplacental maternal spiral arteries in PE, resembling early stages of atherosclerotic lesions, has previously been reported, also in our pregnancy biobank cohort (116-118). Endothelial dysfunction is perceived as an early marker for atherosclerosis (102) and is characteristic of pregnancies complicated by PE (66;104-107), GDM (108-110) and preexisting diabetes mellitus (105;113;114). GDF-15 is expressed in macrophages in the human atherosclerotic plaque (278), and its secretion has been associated with increased inflammatory response in atherosclerotic vessel
walls (186). Higher baseline GDF-15 concentrations were found in healthy, elderly women who subsequently suffered from cardiovascular events (186).

Whether the elevated maternal and fetal GDF-15 in pregnancies complicated by PE or diabetes mellitus shown in our study remain elevated after pregnancy and birth, and whether increased circulating GDF-15 may play a role in the increased risk for CVD following these conditions, should be investigated in longitudinal studies including assessment of cardiovascular function.

**Paper II: Cardiovascular biomarker midregional pro-atrial natriuretic peptide during and after preeclamptic pregnancies**

*Dysregulation of MR-proANP in PE: Cause or consequence?*

We found that preeclamptic women have significantly elevated circulating MR-proANP, a marker of heart failure, most likely reflecting substantial cardiovascular hemodynamic stress present in PE (22;64). The MR-proANP values we observed in young women with PE were similar to those reported in elderly patients with acute ischemic stroke (279), but lower than in patients with acute unstable heart failure (265).

Our study was not designed as a predictive PE study with longitudinal pregnancy samples. A previous study found higher ANP concentrations in second trimester in women who later developed PE compared to normotensive women (280). The MR-proANP Receiver Operating Characteristic (ROC) curve showed a high discrimination between preeclamptic and normotensive pregnancies and was only marginally inferior to that of soluble fms-like tyrosine kinase-1 (sFlt-1) (19), which was very high in our cohort. The combination of a MR-proANP and sFlt-1 ROC curve resulted in a small, but not significant improved area under curve (AUC).
Whether MR-proANP could represent a predictive biomarker for PE and whether the combination of sFlt-1 and MR-proANP would increase the possibility to predict PE could be clarified by large longitudinal studies.

**MR-proANP: Indicative of increased risk for CVD after PE pregnancies?**

We found that in women 5-8 years after their preeclamptic index pregnancy, circulating MR-proANP was no longer elevated, compared to women after a normotensive index pregnancy. A previous longitudinal study of circulating postpartum ANP concentrations had a follow-up period limited to a few weeks (223). In this study, a significant decrease of ANP concentrations postpartum was found both in women with prior PE and women with a prior normotensive pregnancy and there were no residual differences between the groups (223).

Longitudinal studies of MR-proANP with long-time follow-up and objective assessment of cardiovascular function and risk factors are needed in order to explore whether the women with the highest circulating MR-proANP concentrations in pregnancy also are in the highest risk group of developing cardiovascular disease later in life.

**Paper III: Cardiovascular risk markers in pregnancies complicated by diabetes mellitus or preeclampsia**

**Dysregulation of cardiovascular markers in pregnancies complicated by diabetes: Cause or consequence?**

The notion that pregnancy per se is a state of increased inflammation (89) and hemodynamic alterations (21;281) is underscored by our finding of elevated circulating maternal concentrations of MR-proANP, MR-proADM and CT-proAVP, as well as of median neopterin concentration in the upper reference level (252) in healthy pregnancies compared to the non-pregnant state.
In our study, median plasma MR-proANP was significantly lower in GDM, compared to healthy, pregnant controls, contrasting the finding of elevated median MRproANP in PE in Paper II. Low levels of MR-proANP in non-pregnant elderly women and men have previously been shown to predict insulin resistance and T2D development (196).

A protective effect of ANP against oxidant-induced injury in cardiomyocytes has been proposed (282). Recently, a study on the interaction between adipose tissue and cardiomyocytes in an animal model showed the inhibition of ANP mRNA expression in cardiomyocytes by adipose tissue (283). The authors suggested that adipose tissue prohibits the functional differentiation of cardiomyocytes (283). In our study, women with GDM were more obese than healthy, pregnant controls, but not more obese than women with PE. We suggest that our observations of lower circulating MR-proANP in GDM (which was sustained when adjusted for body mass index (BMI)) compared to normotensive pregnancy may be related to obesity, as opposed to the situation in PE, where increased circulating MR-proANP mainly seems reflect increased hemodynamic stress (Paper II).

Interestingly, we found relatively high neopterin concentrations in women with GDM, T2D or PE near term, similar to concentrations found in patients with acute coronary syndrome, who subsequently died or had a recurrent cardiac event (178).

We found that neopterin was associated with gestational age in PE. Adjustment for gestational age resulted in significantly elevated median neopterin concentration also in PE, compared to healthy, pregnant controls. Our finding of increased neopterin in PE at term as compared to normotensive control pregnancies is in accordance with a previous study (234).

Neopterin as a marker of cell-mediated immunity might reflect the subclinical low-grade inflammation present in GDM and T2D mellitus (284;285), as well as the pronounced inflammatory response seen in PE (115). The
inflammatory response in GDM has been closely linked to obesity (285). However, the previously reported association between neopterin and BMI outside pregnancy (286) could not be confirmed in our study of pregnant women with PE, GDM or T2D.

In pregnant women with T2D, we saw a similar trend in biomarker distribution as in GDM, although only neopterin was significantly elevated as compared to non-diabetic controls. We did not find any differences in biomarker concentrations between the GDM and the T2D group and we propose that this finding at least partly may be attributable to the similar inflammatory response, degree of endothelial dysfunction, and/or alterations in hemodynamics during pregnancy in these two patient groups (108;284;285).

In the patients with either GDM or T2D and additional PE development in our study, the cardiovascular biomarker profile was altered compared to pregnancy with diabetes alone, but not compared to only preeclampsia. We therefore conclude that in patients with diabetes mellitus and superimposed PE, PE may be the driving pathophysiological event for the altered circulating biomarkers, rather than the underlying diabetes mellitus.

In pregnancies complicated by diabetes, we did not find any associations of CVD related markers and treatment modality (insulin- and diet- or metformin-treated GDM), glycemic control (serum glycosylated hemoglobin A1c below or above 6% in any trimester) and duration of T1D or T2D (data not shown). Surprisingly, in our group of women with T1D only 13% had an HbA1c below 6% in first trimester, indicating that periconceptional blood sugar regulation was not good in our group of pregnant women with T1D. Also in other populations, such dissatisfying tendency towards suboptimal glycemic control in first trimester has been reported (287). We cannot rule out that populations with a general better glycemic control in first trimester, may have a different cardiovascular biomarker profile during pregnancy than shown for our cohort.
Cardiovascular risk markers during pregnancy: Associated with increased risk for CVD after PE and diabetic pregnancies?

Despite a similar increased risk for cardiovascular disease later in life (50;59;68;70), the cardiovascular biomarker profile as found in our study in pregnancies complicated by diabetes differed substantially from the one observed in PE (Papers II and III). This difference was somewhat attenuated when diabetic pregnancies were further complicated by PE.

The progression rate of GDM to T2D is high (49;50), and T2D is an independent risk factor for CVD (49). Interestingly, we found that the cardiovascular biomarker profile was similar in pregnancies complicated by GDM or T2D mellitus.

The increased risk for CVD in women after pregnancies complicated by PE or GDM may be mediated through enhanced chronic inflammation. Related to the findings in our study, neopterin, which was elevated in diabetic pregnancies, could represent a candidate biomarker for longitudinal studies assessing cardiovascular risk for women with a pregnancy affected by GDM or a preexisting T2D mellitus. In contrast, neopterin does not seem to represent a useful CVD risk marker in preeclamptic women, as the median value for early onset PE (representing the women with the highest cardiovascular risk epidemiologically) did not differ from the normotensive pregnancies.

For both pregnancy complications, also MR-proADM should be further investigated as a potential marker for increased CVD risk, whereas CT-proAVP only seemed of value in PE, since in GDM it appeared to be a function of obesity, rather than of GDM itself.

Whether the cardiovascular biomarkers analysed in this thesis are useful for the identification of women at increased risk for subsequent CVD after pregnancy complications such as PE and or GDM, remains to be investigated in longitudinal follow-up studies including assessment of cardiovascular function.
6. CONCLUSIONS

The maternal concentrations of cardiovascular biomarkers growth-differentiation factor-15 (GDF-15), midregional pro-atrial natriuretic peptide (MR-proANP), neopterin, midregional pro-ADM (MRproADM) and C-terminal pro-arginine vasopressin (CT-proAVP) investigated in this thesis were altered in pregnancies complicated by preeclampsia (PE) or diabetes mellitus with or without PE development compared to normotensive pregnancies. Postpartum analyses of circulating maternal concentrations of MR-proANP (Paper II) did not show a persisting elevation after pregnancies complicated PE.

In order to achieve the long-term goal of identification of those women with complicated pregnancies who are at increased risk for later cardiovascular disease (CVD), future suitably powered longitudinal studies of cardiovascular biomarker concentrations together with assessment of surrogate markers for impaired cardiovascular function (such as evaluation of endothelial function, e.g. arterial stiffness by non-invasive measurements (80)) and clinical endpoints (such as diagnosis of hypertension, coronary artery disease, heart failure or eventually CVD-related death) should be conducted.

The conclusions of this thesis are the following:

- Growth-differentiation factor-15 (GDF-15) is dysregulated in pregnancies complicated by PE and diabetes mellitus (Paper I).
- The finding of intermediate GDF-15 concentrations in amniotic fluid compared to the relatively high maternal plasma concentrations and the relatively low fetal plasma concentrations support the notion that the placenta is the primary source of GDF-15 in pregnancy (Paper I).
- It has previously been shown that GDF-15 is strongly induced by oxidative stress and nitrosative stress, and increased placental oxidative
and nitrosative stress is a feature of PE. Therefore, the observed elevated GDF-15 concentrations in PE in both maternal and fetal circulation, as well as in amniotic fluid, may be related to an excessive placental production of GDF-15 in PE, and GDF-15 could represent a marker for placental stress (Paper I).

- GDF-15 placental mRNA expression was not upregulated in the diabetes mellitus group, whereas GDF-15 mRNA expression levels in the fat tissue were increased, although not statistically significant. Possibly, maternal fat tissue contributes to the increased circulating maternal levels of GDF-15 in diabetic pregnancies (Paper I).
- GDF-15 stemming from fat tissue, rather than from the placenta itself, could also explain why GDF-15 in amniotic fluid and fetal blood from diabetic pregnancies are not increased. This hypothesis is also underscored by the fact that GDF-15 concentrations in the fetal circulation and amniotic fluid are increased in the cases of PE in diabetes mellitus, i.e. in the situation of increased oxidative stress (Paper I).
- Midregional pro-atrial natriuretic peptide (MR-proANP) is altered in maternal circulation in PE with significantly elevated median plasma concentrations compared to normotensive pregnancies. This finding was confirmed in preeclamptic female rats (Paper II).
- The MR-proANP ROC shows a high discrimination between preeclamptic and normotensive pregnancies and is only marginally inferior to soluble fms-like tyrosine kinase-1 (sFlt-1) (Paper II).
- The finding of high atrial natriuretic peptide (ANP) expression in the heart of both normotensive humans and rats but negligible expression in the uteroplacental unit, and the observation of significantly increased ANP expression in maternal and fetal hearts in preeclamptic rats compared to normotensive controls rats leads us to conclude that the
heart is the main source of circulating MR-proANP in pregnancy (Paper II).

- Our findings in human and rat pregnancy suggest that circulating MR-proANP, a prognostic marker in heart failure, is a serviceable biomarker in PE probably reflecting cardiovascular hemodynamic stress (Paper II).

- Postpartum, 5-8 years after index pregnancy, decreased circulating MR-proANP concentrations in formerly preeclamptic women compared to pregnancy levels, indicate that there is no maintained hemodynamic stress. Furthermore, there were no residual differences in MR-proANP concentrations between former preeclamptic, formerly normotensive and non-pregnant women (Paper II).

- Whether the women with the highest circulating MR-proANP concentrations in pregnancy also are in the highest risk group of developing CVD later in life remains to be explored.

- The cardiovascular biomarkers MR-proANP, MR-proADM and CT-proAVP differ in maternal circulation between healthy, pregnant controls and non-pregnant women, suggesting that altered hemodynamics and/or increased inflammation in normal pregnancy influence the circulating levels of these markers (Papers II and III).

- Pregnant women with either gestational diabetes mellitus (GDM) or type 2 diabetes mellitus (T2D) show a similar cardiovascular biomarker profile for the biomarkers explored in Paper III. We suggest that a similar inflammatory response, endothelial dysfunction, and/or alterations in hemodynamics during pregnancy for these two patient groups contribute to their similar cardiovascular biomarker profile (Paper III).

- The finding of lower circulating MR-proANP in GDM compared to normotensive pregnancy may be related to obesity (Paper III).
- The increased MR-proANP concentrations observed in our study group of diabetic pregnancies further complicated by PE compared to the concentrations in the respective diabetes groups without PE seem to be related to PE and the associated cardiovascular hemodynamic stress, not to the underlying diabetes mellitus (Paper III).

- Neopterin does not seem to represent a useful risk marker in preeclamptic women for future cardiovascular disease, since the median value for early onset PE (representing the women with the highest cardiovascular risk epidemiologically) did not differ from the normotensive pregnancies (Paper III).

- Collectively, we conclude that although women after pregnancies complicated by PE/ and or diabetes mellitus have an increased risk for future CVD in common, they differ during pregnancy in circulating biomarkers associated with CVD (Papers I, II and III).
7. FUTURE WORK AND CLINICAL PERSPECTIVES

- **MR-proANP**: The next step in this project is to investigate MR-proANP concentrations in fetal circulation and amniotic fluid from the same preeclamptic and normotensive pregnancies included in Paper II.

- **Continued recruitment to the pregnancy biobank and extended cardiological assessment**: Inclusion of pregnant women to our pregnancy biobank is ongoing and will continue also after the present PhD project. A future goal is extended cardiological assessment (e.g. echocardiogram, echocardigraphy, additional blood sample analyses) of the included women. Our aim is also to include patients with preexisting chronic or pregnancy induced hypertension, in order to possibly clarify effects on measured parameters caused by hypertension in pregnancy versus effects of PE as a syndrome.

- **Continuation of postpartum follow-up** of both women and offspring after pregnancies complicated by PE and diabetes mellitus, such as in our “CHASE” (Cardiovascular health in mother and offspring after pregnancy complications) study (240).
8. ERRATA

- Paper I: Table 1: ranges are given instead of confidence interval for both the clinical data and GDF-15 plasma concentrations.

- Paper I: Methods section on placental villous explants: “The explants were prepared as described previously,(238) and the conditioned media were collected at different time points (0, 2, 4, and 8 hours).” should read: “The explants were prepared as described previously,(238) and the conditioned media were collected at different time points (0, 2, 4, and 16 hours).”

- Paper II: Figure legends of figure 3B and 3C have been switched and should read as follows:
  Figure 3B. Circulating MR-proANP levels in preeclamptic rats (PE) and normotensive control rats (SD).
  Figure 3C. ANP expression in rat tissue of heart, kidney, placenta and mesometrial triangle is shown (*P<0.0001 vs. all other tissues).

- Paper III: The minus in front of -0.4 is incorrect and the sentence that reads “In PE, both MR-proADM and neopterin correlated positively with gestational age at blood sampling (Spearman’s correlation for both -0.4, P<0.001), …” should read “In PE, both MR-proADM and neopterin correlated positively with gestational age at blood sampling (Spearman’s correlation for both 0.4, P<0.001), …”

Further corrections in thesis in bold:

- Page 6: “…at the Department of Gynaecology and Obstetrics, Lillehammer for giving me a solid education in obstetrics and gynaecology,…”
• Page 12: “MR-proANP shows a high discrimination between preeclamptic and normotensive control pregnancies which is only marginally inferior…”

• Page 16: “…such as diabetes, inflammatory disease and other risk factors for cardiovascular disease, rather than…”

• Page 20: “As will be addressed in section 1.3.4,…”

• Page 23: “…women after PE compared to women with a history of normotensive, term pregnancies (61).”

• Page 55: “…was analysed by immunoassay for MR-proANP.”

• Page 60: Deleted “+” from column heading “PE in GDM or T2D”

• Page 87: “…leads us to conclude that the heart is the main source of circulating MR-proANP in pregnancy (Paper II).”

• Page 87: “Postpartum, 5-8 years after index pregnancy, decreased circulating MR-proANP concentrations in formerly preeclamptic women compared to pregnancy levels, indicate that there is no maintained hemodynamic stress.”
9. OTHER PUBLICATIONS DURING THE PhD PERIOD

1. Weedon-Fekjaer MS, Johnsen GM, Anthonisen EH, Sugulle M, Nebb HI, Duttaroy AK, Staff AC.

2. Sugulle M, Kvehaugen AS, Brække K, Harsem NK, Staff AC.

   Intrauterine CYP2J2 expression and circulating epoxyeicosatrienoic acid levels in preeclampsia. *In revision April 2012.*
10. REFERENCE LIST


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Braekke K. Preeclampsia - biomarkers in fetal circulation. Oxidative stress, inflammation, homocysteine and angiogenic factors. Faculty of Medicine, University of Oslo; 2007.


11. PAPERS I-III