Preeclampsia and Maternal Type-1 Diabetes: New Insights into Maternal and Fetal Pathophysiology

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PREECLAMPSIA AND MATERNAL TYPE-I DIABETES: NEW INSIGHTS INTO MATERNAL AND FETAL PATHOPHYSIOLOGY

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Abstract
Abnormal placentation is associated with preeclampsia and placental insufficiency, both of which increase the risk for fetal growth restriction. So far the early recognition of the risk population for preeclampsia has been problematic. The first hypothesis of this study was that in preeclampsia, the maternal serum proteomic profile is different from that in uncomplicated pregnancies, and this difference is detectable already in early pregnancy. The findings of this study demonstrate that in clinical preeclampsia the maternal serum proteomic profile is different from that in uncomplicated pregnancies with increased levels of placental proteins and antiangiogenic factors in pregnancies with clinical preeclampsia. Furthermore, the early pregnancy maternal serum proteomic profile in women who later develop preeclampsia revealed a distinct and different pattern compared with the profile in clinical preeclampsia. In early pregnancy, the differentially expressed proteins belong to placental proteins, vascular and/or transport proteins and matrix and/or acute phase proteins, while angiogenic and antiangiogenic proteins were not significantly expressed in early pregnancy.

Preeclampsia, placental insufficiency, fetal growth restriction and type-1 diabetes may have an impact on fetal cardiovascular hemodynamics. The second hypothesis in this thesis was that in placental insufficiency, abnormalities in fetal cardiovascular status correlate with biochemical markers of cardiac dysfunction and chronic hypoxia. In placental insufficiency, increases in fetal N-terminal pro-atrial (NT-proANP) and pro-B-type natriuretic peptide (NT-proBNP) and in fetal erythropoietin concentrations were related to increased pulsatility in the fetal umbilical artery and descending aorta. In addition, these fetuses demonstrated increased pulsatility in their systemic venous blood velocity waveforms. Thus, in placental insufficiency, biochemical markers of cardiac dysfunction and chronic hypoxia are associated with signs of increased fetal cardiac afterload and systemic venous pressure. Increased NT-proANP and NT-proBNP levels were also detected in fetuses of type-1 diabetic mothers with normal umbilical artery velocimetry. In these pregnancies, NT-proANP and NT-proBNP levels were related to poor maternal glycemic control during early pregnancy.

Keywords: cardiac function, Doppler, erythropoietin, fetal growth restriction, heart, hemodynamics, natriuretic peptides, physiology, placental insufficiency, preeclampsia, proteomics
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AEDV</td>
<td>Absent end diastolic velocity</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>AoI</td>
<td>Aortic isthmus</td>
</tr>
<tr>
<td>AoV</td>
<td>Aortic valve</td>
</tr>
<tr>
<td>A-wave</td>
<td>Atrial contraction wave</td>
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<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>CC</td>
<td>Cardiac circumference</td>
</tr>
<tr>
<td>CCO</td>
<td>Combined cardiac output</td>
</tr>
<tr>
<td>CNP</td>
<td>C-type natriuretic peptide</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross-sectional area</td>
</tr>
<tr>
<td>CSH1</td>
<td>Chorionic somatomammotropin hormone</td>
</tr>
<tr>
<td>DA</td>
<td>Ductus arteriosus</td>
</tr>
<tr>
<td>DAO</td>
<td>Descending aorta</td>
</tr>
<tr>
<td>DV</td>
<td>Ductus venosus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<tr>
<td>E-wave</td>
<td>Early ventricular filling wave</td>
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<tr>
<td>FHR</td>
<td>Fetal heart rate</td>
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<tr>
<td>FO</td>
<td>Foramen ovale</td>
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<tr>
<td>GA</td>
<td>Gestational age</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycosylated hemoglobin A1</td>
</tr>
<tr>
<td>IMP</td>
<td>Index of myocardial performance</td>
</tr>
<tr>
<td>IRT</td>
<td>Isovolumetric relaxation time</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior vena cava</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid-chromatography</td>
</tr>
<tr>
<td>LHV</td>
<td>Left hepatic vein</td>
</tr>
<tr>
<td>LVCO</td>
<td>Left ventricular cardiac output</td>
</tr>
<tr>
<td>LVeFo</td>
<td>Left ventricular ejection force</td>
</tr>
<tr>
<td>LVFS%</td>
<td>Left ventricular fractional shortening</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
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</table>
MV Mitral valve
mRNA Messenger ribonucleid acid
NT-proANP N-terminal peptide of pro-A-type natriuretic peptide
NT-proBNP N-terminal peptide of pro-B-type natriuretic peptide
PAPP-A1 Pappalysin-1
PAPP-A2 Pappalysin-2
PI Pulsatility index
PIV Pulsatility index for veins
PIGF Placental growth factor
PV Pulmonary valve
Q Volume blood flow (ml/min)
QDA Ductus arteriosus volume blood flow
QFO Foramen ovale volume blood flow
REDV Retrograde end diastolic velocity
RNA Ribonucleid acid
RVCO Right ventricular cardiac output
RVeFo Right ventricular ejection force
RVFS% Right ventricular fractional shortening
SD Standard deviation
SELDI Surface-enhanced laser desorption/ionization
sFlt-1 soluble Fms-like tyrosine kinase 1, soluble Vascular endothelial growth factor receptor 1
TC Thoracic circumference
TOF Time-of-flight
TR Tricuspid regurgitation
TV Tricuspid valve
TVI Time-velocity-integral
UA Umbilical artery
UtA Uterine artery
VEGF Vascular endothelial growth factor
List of original articles

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1 Introduction

Preeclampsia is a pregnancy-specific disorder, which has been known since the time of ancient Egypt and Greece (Purkerson et al. 1999). In the 21st century, preeclampsia is still a common obstetrical problem affecting approximately 5% of Finnish pregnancies (Koponen 2004). In developed countries, hypertensive disorders of pregnancy are the leading cause of maternal mortality with approximately 15 to 20% of maternal deaths worldwide (Sibai et al. 2005; Khan et al. 2006). In preeclampsia, the perinatal mortality is between 1 to 3% (Sibai et al. 2005; Xiong et al. 2007), the increment being even three-fold compared to uncomplicated pregnancies (Xiong et al. 2007). In fetal growth restriction, maternal vascular disease in association with preeclampsia is present in 30 to 40% of cases (Maulik 2006). Incidence of fetal growth restriction (fetal growth less than 10th percentile growth curve) is between 4 to 7% of all pregnancies (Gardosi et al. 1992). Intrauterine malnutrition can have long-term consequences with increased risk for diabetes, stroke, chronic hypertension, and death from coronary artery disease later in life (Barker 2006).

According to the present knowledge, preeclampsia is associated with abnormal implantation and development of placenta (Roberts et al. 2002; Jauniaux et al. 2006; Mohaupt 2007). Although new biochemical markers have been recruited to predict preeclampsia (Maynard et al. 2003; Levine et al. 2004), the early diagnosis of preeclampsia has been uncertain and the clinical care has focused on maternal symptoms. The lack of accurate knowledge of its aetiology and pathogenesis has made the prediction of preeclampsia and the development of new treatments for preeclampsia problematic.

Noninvasive Doppler ultrasonography has shown to be a valid method to examine placental and fetal hemodynamics (Rizzo et al. 1995a; Rasanen et al. 1997b; Rasanen et al. 1998; Makikallio et al. 2002b). Abnormal umbilical artery (UA) blood flow velocimetry is associated with a reduced number of arterioles in the placental tertiary villi (Giles et al. 1985). In placental insufficiency, redistribution of cardiac output, myocardial hypertrophy, altered fetal systolic and diastolic function, tricuspid regurgitation, cardiomegaly and abnormal systemic venous blood velocity waveforms can be found on fetal echocardiographic examination (Rasanen et al. 1997a). In addition, an increase in placental vascular resistance can change the fetal aortic isthmus blood flow profile (Bonnin et al. 1993; Fouron et al. 1999a). Retrograde aortic isthmus net blood flow is suggested to indicate decreased oxygen content of the blood entering the fetal brain (Fouron
et al. 1999a) and has been suggested to correlate with neurodevelopmental outcome (Fouron et al. 2005). Longitudinal Doppler studies on growth restricted fetuses have shown that abnormal ductus venosus (DV) velocimetry is one of the late ultrasonographic changes in severe placental insufficiency and related to adverse perinatal outcome (Hecher et al. 2001; Ferrazzi et al. 2002).

In type-1 diabetic pregnancies, poor maternal glycemic control in early pregnancy is associated with increased risk for fetal malformations and spontaneous abortions (American Diabetes Association 2003). Despite the improved care of type-1 diabetic pregnancies, these fetuses remain at increased risk for congenital heart defects and unexplained intrauterine death (Suhonen et al. 2000; Jensen et al. 2004). Newborn cardiac hypertrophy occurs in approximately 40% of type-1 diabetic pregnancies (Abu-Sulaiman et al. 2004). Factors leading to fetal cardiovascular changes in diabetic pregnancies are suggested to be fetal hyperglycemia, hyperinsulinemia and chronic hypoxia (Pedersen 1954; Susa et al. 1979; Widness et al. 1981; Salvesen et al. 1993; Teramo et al. 2004b).

The analysis of the protein component of biological material, the proteome, has recently proven to be a predictive tool for various cancers (Paweletz et al. 2001; Rai et al. 2002; Lehrer et al. 2003), rheumatoid arthritis (Doherty et al. 1998), Alzheimer’s disease (Choi et al. 2002) and intra-amniotic infection (Gravett et al. 2007; Pereira et al. 2007). Atrial (ANP) and B-type (BNP) natriuretic peptides are cardiac peptide hormones, released into the circulation mainly in response to increased cardiac pressure or volume load (Levin et al. 1998; Vuolteenaho et al. 2005). Previously, increased fetal cardiac ANP secretion has been detected in pregnancies with maternal hypertensive disorders (Makikallio et al. 2001) as well as in placental insufficiency with signs of elevated fetal systemic venous pressure (Makikallio et al. 2002b). Erythropoietin (EPO) is a hematopoietic and tissue protective glycoprotein hormone, released into adult and fetal circulation in response to hypoxia (Jelkmann 1992; Eckardt et al. 2005). Increased fetal cord plasma and amniotic fluid EPO levels are documented in type-1 diabetic pregnancies and in pregnancies with maternal hypertensive disorders (Teramo et al. 1987; Teramo et al. 2004b).

In this study, proteomic analysis was used to investigate changes in maternal serum proteome in preeclampsia. In addition, fetal cardiovascular responses to preeclampsia, placental insufficiency and type-1 diabetes were investigated with Doppler ultrasonography and with biochemical markers of fetal cardiac dysfunction and chronic hypoxia.
2 Review of the literature

2.1 Preeclampsia and placental insufficiency

Worldwide, preeclampsia is estimated to occur in about 5–8% of all pregnancies (Walker 2000; 2002) with similar prevalence numbers documented in Finnish pregnancies (Koponen 2004). Preeclampsia is an almost unique disorder to the human species with similar symptoms described only in ‘patas’ monkeys (Palmer et al. 1979) and guinea pigs (Seidl et al. 1979). Preeclampsia and associate diseases are not clinically present before the second trimester of pregnancy, but seem to originate already in early pregnancy. Clinical features of preeclampsia include maternal hypertension, proteinuria, uricemia and in severe cases, seizures (eclampsia) (Gynecologists 2002). Preeclampsia is primarily a disorder of first pregnancies. Other risk factors include diabetes mellitus, chronic hypertension, and preeclampsia in a previous pregnancy, antiphospholipid antibody syndrome, nephropathy, multifetal gestation, obesity and age of 40 years or older (Duckitt et al. 2005; Temple et al. 2006). Preeclampsia is associated with substantial maternal and neonatal morbidity and mortality. Women who develop preeclampsia are at an increased risk of placental abruption, acute renal failure, and more often have cerebrovascular and cardiovascular complications such as postnatal convulsions or pulmonary edema (Walker 2000). In addition, epidemiological data indicate that preeclamptic women are more likely to develop cardiovascular disease later in life (Bellamy et al. 2007). Worldwide, 10–15% of maternal deaths occurring annually are related to hypertensive disorders of pregnancy, mainly preeclampsia and eclampsia (Sibai et al. 2005; Khan et al. 2006). Placental insufficiency leading to fetal growth restriction is associated with preeclampsia in approximately 30% of cases (Walker 2000).

2.1.1 Pathophysiology of preeclampsia

Preeclampsia is associated with abnormal implantation and development of placenta. Observations in several countries indicate that genetic factors may play a role in the occurrence of preeclampsia (Cincotta et al. 1998; Arngrimsson et al. 1999). Rather than a single preeclampsia gene, preeclampsia is associated with several modifier genes along with environmental factors (Broughton Pipkin 1999; Roberts et al. 2002). The primary pathological process in preeclampsia is
suggested to be an excessive or atypical maternal immune response, which impairs the placentation process (Roberts et al. 1999; Matthiesen et al. 2005). Increased oxygen stress in the placenta of women with preeclampsia is well documented (Uotila et al. 1993; Morris et al. 1998; Walsh et al. 2000). It is thought that increased formation of free oxygen radicals leads to abnormal placentation (Jauniaux et al. 2006). Histological examination of the placental bed of preeclamptic women has demonstrated impaired invasion of cytotrophoblasts (Khong et al. 1986). In addition, insufficient remodeling of spiral arteries and trophoblast migration to line maternal spiral arteries has been found (Lim et al. 1997).

The current concept is that maternal endothelial dysfunction is caused by abnormal placentation (Fisher 2004; Kopcow et al. 2007). In preeclamptic women, blood flow is reduced to virtually all organs. The major pathological changes occur in the brain, liver, heart and kidney and in the decidual vessels. The low perfusion occurs secondary to intense vasospasm due to an increased sensitivity of vessels to pressor agents (Roberts et al. 2001). Tissue perfusion is further compromised by activation of coagulation cascade, especially platelets. Additionally, plasma volume in preeclampsia is decreased due to the vasoconstriction and an endothelial leak (Campbell et al. 1983). The morphological alterations in glomerular capillary endothelium suggest that vascular endothelial dysfunction is responsible for many of the changes in the preeclampsia (Roberts 1998). Similar changes, however, have also been seen in renal biopsies of healthy pregnant women (Wide-Swensson et al. 2007). Increased circulating concentrations of markers of endothelial activation and the alterations of endothelial function in vessels of women with preeclampsia have been documented (Poston 2006). Enhanced free radical formation secondary to tissue hypoxia also occurs in preeclampsia and may further augment endothelial damage (Walsh et al. 2000).

2.1.2 Prediction of preeclampsia and placental insufficiency

Abnormal uterine (UtA) and umbilical artery (UA) Doppler findings in early pregnancy are associated with an increased risk for preeclampsia or intrauterine fetal growth restriction (Harrington et al. 1997). In a recent study in low risk pregnancies at 11 to 13 gestational weeks, UtA PI values could predict preeclampsia in 41.1% of cases with 10% false-positive rate (Plasencia et al. 2007). In a similar study, the 95th percentile of mean UtA PI value at early
pregnancy predicted preeclampsia with the sensitivity of 23.1% (Pilalis et al. 2007). In both studies, the combination of maternal variables, such as history of previous preeclampsia, with the Doppler finding improved the prediction of preeclampsia (Pilalis et al. 2007; Plasencia et al. 2007). In unselected nulliparous women at 22–24 weeks, the sensitivity of abnormal UtA Doppler finding for prediction of preeclampsia varied between 41% to 50% (Papageorghiou et al. 2001; Subtil et al. 2003). The corresponding sensitivity of UtA pulsatility index (PI) value was 16% for fetal growth restriction, irrespective of preeclampsia (Papageorghiou et al. 2001). UtA Doppler assessment reached a better prognostic power for the prediction of preeclampsia in the second trimester among high risk patients (i.e. a history of preeclampsia) (Zimmermann et al. 1997). In high-risk pregnancies at 22 to 24 weeks of gestation, the bilateral notching of uterine arteries had a sensitivity of 62% and specificity of 89% for developing preeclampsia (Coleman et al. 2000).

A failure of normal trophoblast invasion in preeclampsia has been suggested to cause abnormal cytokine secretion (Poston 2006). The elevated plasma levels of interleukin-6 in preeclamptic patients have been documented (Vince et al. 1995). The levels of tumor necrosis factor-α are also increased in plasma, amniotic fluid and placenta of patients with symptomatic preeclampsia (Kupferminc et al. 1994; Wang et al. 1996). Elevated second trimester amniotic fluid levels of interleukin-6 and interleukin-8 in women with later developed preeclampsia have been documented (Nakabayashi et al. 1998). In other studies, however, amniotic fluid cytokine levels between 14 and 16 weeks of gestation (Heikkinen et al. 2001) and plasma tumor necrosis factor-α in the first trimester (Serin et al. 2002) have failed to predict preeclampsia.

Vascular endothelial growth factor (VEGF) and its placental counterpart, placental growth factor (PIGF), which promote angiogenesis via VEGF receptor (Fms-like tyrosine kinase 1 (Flt-1)), have been studied in the prediction of preeclampsia (Poston 2006; Mohaupt 2007). During normal pregnancy VEGF is essential to the proliferation of trophoblasts (Ferrara et al. 1997). Decreased free VEGF and PIGF concentrations have been documented in patients with symptomatic preeclampsia compared to normotensive pregnancies (Maynard et al. 2003; Levine et al. 2004). Significantly lower PIGF levels were found in women who later developed preeclampsia than in the controls at 13 to 16 weeks of gestation (Levine et al. 2004). Circulating levels of the soluble receptor of VEGF and PIGF (sFlt-1), on the other hand, are shown to be markedly increased already in the first trimester serum of women with late-onset preeclampsia.
(Baumann et al. 2008) as well as approximately five weeks before the onset of preeclampsia (Levine et al. 2004). In addition, soluble endoglin, another angiogenic protein, is markedly increased in the first trimester serum of women with late-onset preeclampsia (Baumann et al. 2008). Whereas another study has demonstrated increased soluble endoglin levels from 2 to 3 months before the clinical symptoms of preeclampsia (Levine et al. 2006). Alterations in the sFlt-1 and PIGF levels are found to be the highest in women with an early onset preeclampsia and in preeclampsia associated with fetal growth restriction (Levine et al. 2004).

In unselected pregnancies between 22 to 26 weeks of gestation, a decreased maternal plasma concentration of PIGF is an independent explanatory variable for the occurrence of preeclampsia with a sensitivity of 69.1% and specificity 51.4% (Espinoza et al. 2007). In addition, the decreased PIGF concentration contributed significantly to the identification of patients with early onset and severe preeclampsia when combined with abnormal UtA blood velocity waveform at 22 to 26 weeks of pregnancy. The combination of abnormal UtA blood velocity waveform finding and decreased PIGF concentration identified the patients at risk for early onset and severe preeclampsia with a sensitivity of 64% and specificity of 96% (Espinoza et al. 2007). However, in a multivariate logistic regression analysis, the increased UtA PI value at 23 weeks was the best predictor for preeclampsia compared to maternal plasma levels biomarkers including sFlt-1 (Parra et al. 2005).

2.1.3 Effects of preeclampsia and placental insufficiency on the fetus

In preeclampsia, the impaired uteroplacental hemodynamics after the abnormal placentation results in insufficient fetal nutrient availability and growth restriction. This, however, can remain subclinical until the third trimester if the fetal adaptation is successful (Baschat 2004a). Placental insufficiency leads to several metabolic disturbances such as fetal hypoglycemia, hypoxemia and hypoaminoacidemia, which have been documented through cordocentesis in human fetuses (Economides et al. 1991; Baschat 2004a). As a result of hypoxemia, increased human fetal EPO release has been found in pregnancies with hypertensive disorders (Teramo et al. 2004b).

In placental insufficiency, decreased oxygen content of the umbilical venous return leads to redistribution of blood flow in favor of the most important organs, the brain and the heart. Ultrasonographic studies have demonstrated increased
shunting through the DV and foramen ovale (FO) to the left ventricle and thereafter to the coronary circulation and brain (Kiserud et al. 2000b). In addition to increased placental vascular resistance, redistribution is seen as peripheral arterial vasoconstriction in the fetal trunk (Groenenberg et al. 1989; Hecher et al. 1995b; Baschat 2004a) and in the peripheral pulmonary arteries (Rizzo et al. 1991a). When the increased metabolic demands of cardiac work cannot be met, the cardiac function begins to decline in placental insufficiency. Increased pulsatility in fetal systemic venous blood velocity waveforms has been suggested as a marker of fetal congestive heart failure (Hecher et al. 1995a). Longitudinal ultrasonographic studies on growth restricted human fetuses have demonstrated that the amniotic fluid index and UA PI values are the first variables to become abnormal followed by the abnormal middle cerebral artery (MCA) and aortic blood flow velocity waveforms (Hecher et al. 2001; Ferrazzi et al. 2002). Thereafter short-term variation in fetal heart rate has been documented to diminish (Hecher et al. 2001). Late changes of fetal compromise in placental insufficiency include increased pulsatility in the DV and inferior vena cava (IVC) (Hecher et al. 2001) and abnormal pulmonary artery peak velocity (Ferrazzi et al. 2002). In longitudinal studies, the fetuses with the poorest outcome demonstrated abnormal DV blood velocity waveform pattern (Hecher et al. 2001; Ferrazzi et al. 2002).

A fetus with growth restriction is at increased risk for both intrauterine and early perinatal death (Kramer et al. 1990; Froen et al. 2004). After the birth, the adaptive complications such as perinatal acidosis, hypoglycemia, hypothermia, coagulation abnormalities and immunological deficiencies are more common in growth restricted fetuses (Pallotto et al. 2006). The preterm delivery is frequently associated with fetal growth restriction. It also adds to the morbidity of these fetuses and increases the risk of poor neurodevelopmental outcome later in life (Bardin et al. 2004; Kaukola et al. 2005). According to umbilical cord samples taken at birth, the infants of preeclamptic mothers have increased levels of biochemical risk factor markers of cardiovascular disease such as low-density lipoprotein, homocysteine and fibrinogen, compared with fetuses of normotensive pregnancies (Ophir et al. 2006). In addition, maternal hypertensive disorders are associated with increased fetal ANP secretion. The fetuses with placental insufficiency and elevated systemic venous pressure also have biochemical signs of myocardial cell damage (Makikallio et al. 2001; Makikallio et al. 2002b). These results are in line with the findings by Barker and colleagues, suggesting that low birth weight in relation to the length of gestation is associated with
increased rates of coronary heart disease, stroke, hypertension and type-2 diabetes later in life (Barker 2006).

2.2 Type-1 diabetic pregnancy

2.2.1 Characteristics of type-1 diabetic pregnancy

Type-1 diabetes is a pre-existing medical disorder in approximately 0.2–0.4% of all pregnancies (Engelgau et al. 1995; Suhonen et al. 2000; Temple et al. 2006). The pregestational diabetes was classified already in 1949 according to Priscilla White. White’s classification, in which the onset of diabetes, its duration, and the degree of vasculopathy are taken account, is still generally accepted (White 1949; White 1978). The White classes with the most severe degree of vasculopathy are shown to relate to adverse fetal outcome (Vaaramaki et al. 2000).

In type-1 diabetic pregnancies, the incidences of fetal congenital malformations (Suhonen et al. 2000; Jensen et al. 2004), preeclampsia (Hiilesmaa et al. 2000), premature delivery (Mimouni et al. 1988), perinatal mortality (Jensen et al. 2004) and fetal macrosomia (Casson et al. 1997) are increased compared to uncomplicated pregnancies. The current evidence indicates that the adverse pregnancy outcome in type-1 diabetic pregnancies is related to hyperglycemia already in early pregnancy (Fuhrmann et al. 1983; Suhonen et al. 2000; Temple et al. 2006). Accordingly, it has been shown that the pre-pregnancy care of type-1 diabetes will improve glycemic control in early pregnancy and reduce significantly adverse perinatal outcomes (Temple et al. 2006).

The metabolic disturbances of type-1 diabetic pregnant patients include increased concentrations of circulating metabolic fuels i.e. carbohydrates, proteins and fat. In addition to self-monitoring of blood glucose, retrospective glycemic status (4 to 8 weeks) of a diabetic woman is assessed by glycosylated hemoglobin A1c (HbA1c). HbA1c is a postsynthetic transformation of the native hemoglobin A0 with additional sugar moiety attached to it. HbA1c is expressed as a percentage of the total hemoglobin (O'Shaughnessy 1981). The goal in the treatment of pregnant type-1 diabetic mother with daily insulin-injections is to reach euglycemia without a significant risk of hypoglycemic episodes (Gabbe 1985). However, excessive transplacental maternal metabolic fuel passage contributes to the development of fetal macrosomia and related problems (Hagay 1999).
2.2.2 Fetus and type-1 diabetic pregnancy

Cardiac anomalies and myocardial hypertrophy occur about three times more often in the offspring of women with type-1 diabetes than in the normal pregnancies (Suhonen et al. 2000). The actual pathophysiologic pathways leading to fetal cardiac and circulatory changes are still unclear. The cardiac abnormalities have shown to occur even when the maternal glycemic control is optimal (Weber et al. 1991; Rizzo et al. 1991b; Jaeggi et al. 2001). However, it is generally accepted that fetal myocardial hypertrophy, macrosomia, and neonatal hypoglycemia are related to maternal diabetic control and associated with fetal hyperglycemia and hyperinsulemia (Pedersen 1954; Susa et al. 1979; Salvesen et al. 1993). Fetal chronic hypoxia, demonstrated by increased fetal plasma and amniotic fluid EPO levels (Widness et al. 1981; Teramo et al. 2004a) as well as neonatal polycytemia (Mimouni et al. 1986), may be involved in perinatal complications.

Fetal echocardiographic studies during the second and third trimesters have documented progressive fetal myocardial growth with increased thickness of interventricular septum (Rizzo et al. 1991b) and ventricular free walls (Weber et al. 1991; Veille et al. 1993). In type-1 diabetic pregnancies, altered fetal cardiac diastolic function with significantly lower E-wave and significantly higher A-wave velocity in ventricular inflow parameters have been documented (Rizzo et al. 1991b; Tsyvian et al. 1998). In addition, decreased right and left ventricular contractility and decreased left ventricular output in relation to fetal size in diabetic pregnancies have been shown (Rasanen et al. 1987). Longitudinal examination of fetuses of type-1 diabetic mothers has demonstrated that the weight-corrected total cardiac output in these pregnancies is higher than in the normal pregnancies (Lisowski et al. 2003). In addition, weight-indexed volume blood flow in the fetal aorta and the umbilical vein is higher in the early third trimester of diabetic pregnancies than in the normal pregnancies (Olofsson et al. 1987). Fetal myocardial hypertrophy which has also been demonstrated in an experimental model of gestational diabetes (Menezes et al. 2001) is found to resolve postnatally and is therefore considered relatively benign (Deorari et al. 1989). However, the role of cardiovascular abnormalities and the altered fetal autonomic function (Tincello et al. 2001) in high incidence of late stillbirths in type-1 diabetic pregnancies remain to be determined.
2.3 Cardiac atrial (ANP) and B-type (BNP) natriuretic peptides

2.3.1 Synthesis and release of ANP and BNP

In 1981, de Bold and associates demonstrated the endocrine function of heart by showing that extracts from atrial myocytes induced natriuresis and diuresis in rats (de Bold et al. 1981). Two years later the active factor, later called atrial natriuretic peptide (ANP), was purified and sequenced (Flynn et al. 1983). Soon after that an ANP-like peptide, named afterwards B-type natriuretic peptide (BNP), was found in porcine brain (Sudoh et al. 1988). Subsequent experiments revealed that the main source of BNP was cardiac myocytes and it shared peripheral receptors with ANP (Suga et al. 1992a). Also a third natriuretic peptide, C-type natriuretic peptide (CNP), is produced in the brain (Sudoh et al. 1990) and endothelium (Suga et al. 1992b), but apparently not in cardiac myocytes.

Cardiac natriuretic peptides, ANP and BNP, share a homologous structure, forming a ring with a disulfide bridge. The gene of both ANP and BNP is located in chromosome 1 in humans. Induction of ANP gene expression \textit{in vivo} is seen within one day after the initiation of increased cardiac overload (Ruskoaho 1992). However, because of the immediate release of ANP from atrial storage granules, the ANP levels in the circulation increase rapidly after the stimulus (de Bold \textit{et al.} 1996). Increased secretion of BNP is preceded by an increase in mRNA production. Thus, a wall stretch induces rapid activation of BNP gene expression within one hour in the atria (Mantymaa \textit{et al.} 1993) and the left ventricle (Magga \textit{et al.} 1994).
Fig. 1. Synthesis and secretion of ANP (left) and BNP (right) and their propeptides. Modified from (Ruskoaho 2003).

The transcription of the ANP gene forms a messenger mRNA species that encodes a 151-amino acid pre-proANP precursor containing a 25-amino acid signal sequence. This signal peptide is important for the translocation of pre-proANP from the ribosome into the sarcoplasmic reticulum. Pre-proANP is converted after cleavage of the signal peptide to a 126-amino acid proatrial natriuretic peptide, proANP₁₋₁₂₆, which is the principal storage form of ANP. The ProANP₁₋₁₂₆ is transported through the Golgi complex to secretory granules of atrial cardiocytes, and released by exocytosis to the extracellular space (Ruskoaho 1992). ProANP₁₋₁₂₆ is cleaved into the N-terminal fragment (proANP₁₋₉₈ = NT-proANP) and to the major biologically active C-terminal peptide ANP₉₉₋₁₂₆, more commonly known as hormone ANP₁₋₂₈ or ANP (Michener et al. 1986; Sundsfjord et al. 1988; Thibault et al. 1988). Cleavage of ProANP₁₋₁₂₆ into ANP and NT-proANP occurs during the exocytosis, presumably by the membrane-bound endonuclease Corin (Yan et al. 2000), producing equimolar amounts of these peptides. The circulating 28-amino acid ANP is the biologically active form in humans (Misono et al. 1984).

The human BNP gene consists of 3 exons and 2 introns. The mRNA is transcribed into a 108-amino acid prohormone proBNP₁₋₁₀₈ which coexists with ANP in some of the secretory granules of atrial and ventricular myocytes (Nakamura et al. 1991). The ProBNP₁₋₁₀₈ is cleaved into biologically active 32 amino acid hormone BNP and N-terminal fragment of proBNP (ProBNP₁₋₁₃ =
NT-proBNP) in equimolar amounts (Hunt et al. 1995). The exact mechanism of prohormone cleavage is unclear since small amounts of intact proBNP may be found in the circulation and split prohormone can be found in cellular extracts. In vitro experiments indicate that the proteolytic enzyme Furin is responsible for proBNP cleavage (Sawada et al. 1997). (Fig. 1)

The predominant site for ANP synthesis in adults is the atrium. BNP is synthesized, on the other hand, both within atrial and ventricular tissue (Yasue et al. 1994). During cardiac maturation, the ANP gene is actively expressed in fetal ventricle producing high levels of ANP mRNA from 17 to 19 weeks of gestation (Takahashi et al. 1992). Several studies have shown that ANP is also synthesized and secreted from the ventricles in adult patients with congestive heart failure (Saito et al. 1989; Yasue et al. 1989). Animal studies have demonstrated that cardiac fibroblasts also produce ANP and BNP (Cameron et al. 2000; Tsuruda et al. 2002). Although only the heart has been shown to secrete cardiac natriuretic peptides, gene expression and thus detectable levels of ANP and BNP mRNA have been found in various other human tissues such as the central nervous system, lung, adrenal gland, kidney and vascular tissue (Rosenzweig et al. 1991; Gerbes et al. 1994). Based on the information obtained at human autopsy, extracardiac levels of ANP and BNP transcripts are one or two orders of magnitude lower than in cardiac ventricular tissues in general (Gerbes et al. 1994). Although the murine placenta contains BNP mRNA (Cameron et al. 1996), a recent study found no evidence of BNP mRNA in human placentas from uncomplicated pregnancies (Halse et al. 2005).

**2.3.2 Regulation of release and elimination of ANP and BNP**

Cardiac pressure and volume overload which cause atrial and ventricular wall stress or an increase in wall dimension are physiologic stimuli for ANP and BNP release (Tokola et al. 2001). ANP is controlled by immediate release from atrial storage granules primarily in response to stretching of the atrium (de Bold et al. 1996). Hypoxia is also a potent stimulus for ANP release in adults (Lew et al. 1989), which can be mediated by atrial stretch, increased heart rate, sympathetic stimulus or metabolic factors (Ruskoaho 1992). Increased ANP release resulting from hyperosmolality with volume expansion has also been demonstrated (Arjamaa et al. 1985). A variety of humoral factors can increase the secretion of cardiac ANP. These include endothelin-1, a potent vasoconstrictor of vascular smooth muscle, which has been shown to induce ANP secretion directly from the
heart (Mantymaa et al. 1990). Moreover, endothelin-1 may also mediate atrial stretch-induced ANP release and effects of pressor hormones on the stress-activated release of ANP (Ruskoaho 1992). Endothelium- or endocardium-derived nitric-oxid may inhibit ANP secretion (Leskinen et al. 1995). In addition, catecholamines (Ruskoaho 1992), acetylcholine (Ruskoaho et al. 1985), angiotensin, arginine vasopressin, prostaglandins (Ruskoaho 1992) and both glucocorticoids and thyroid hormones increase circulating ANP levels (Rosenzweig et al. 1991).

BNP secretion is mainly controlled at the transcriptional level with increased secretion preceded by an increase in mRNA production (Magga et al. 1997). Integrins, proteins that mediate the cell adhesion, have been suggested to act as sensors of mechanical forces triggering the intracellular signaling that leads to elevated BNP mRNA levels (Liang et al. 2000). Local paracrine and autocrine factors may also be involved in stretch-induced BNP gene expression. In cultured myocytes, BNP production has been shown to be suppressed by endothelin-1 and angiotensin II receptor antagonists suggesting that these endocrine factors are involved in BNP gene expression (Harada et al. 1998).

Two pathways have been described for the clearance of circulating natriuretic peptides: 1) binding and internalization via the natriuretic peptide receptor C (NPRC) and 2) enzymatic degradation by neutral endopeptidase 24.11. The liver, lungs and especially the kidneys are major sites of extraction of cardiac natriuretic peptides (Cowie et al. 2002). Pharmacokinetic studies done on catheterized healthy human subjects have shown that BNP is cleared from the circulation more slowly than ANP, possibly attributed to lower binding affinity of BNP to clearance receptors and particularly to the neutral endopeptidase (Smith et al. 2000). In catheterized healthy subjects, the plasma half-time of infused BNP was 3.9 ± 0.23 minutes and the half-time of plasma ANP 1.7 ± 0.07 minutes, respectively (Mukoyama et al. 1991). Similar results have been demonstrated in other studies as well (McGregor et al. 1990; Ruskoaho 1992).

2.3.3 Physiologic effects of cardiac natriuretic peptides

ANP and BNP exert their physiological actions by binding to natriuretic peptide receptor A (NPRA). Altogether, three natriuretic peptide receptors have been identified (NPRA, NPRB, NPRC). Receptors A and B are receptor guanylate cyclases that synthesize cGMP in response to ligands, whereas the C-type receptor mainly acts as a clearance receptor. All natriuretic peptides are bound by
the NPRC receptor. ANP and BNP act through the NPRA and CNP through NPRB (Levin et al. 1998).

Cardiac natriuretic peptides counteract the renin-angiotensin-aldosterone system to control the extracellular fluid volume under normal and pathophysiological conditions. The biological effects of ANP and BNP are mainly exerted to vascular smooth muscle and kidneys. Both ANP and BNP reduce peripheral vascular resistance and lower blood pressure by relaxation of vascular smooth muscle (Levin et al. 1998). They act on the glomerulus and inner medullary collecting tubes to increase salt and water excretion, enhance capillary permeability and inhibit renin and aldosterone secretion. In addition, ANP can inhibit release or action of hormones like angiotensin II, endothelin and vasopressin (Ruskoaho 1992). Animal experiments have suggested that ANP and BNP also have protective autocrine effects in the heart such as inhibiting fibrosis and hypertrophy (Woods 2004). A rise in human adult ANP and BNP levels is detected in adult congestive heart failure, chronic renal failure, myocardial infarction, hypertrophic and dilated cardiomyopathy, tachycardias and severe essential hypertension (Sagnella 1998).

All three natriuretic peptides, ANP, BNP and CNP, are present in the human fetal circulation. Fetal ANP and BNP are found to respond to changes in fetal cardiac filling pressures (Stepan et al. 2000; Walther et al. 2001), with increased levels found in fetal distress of various reasons (Kingdom et al. 1992; Itoh et al. 1993; Ville et al. 1994). Increased plasma levels of ANP and BNP are found in children with clinical signs of heart failure due to congenital heart defects or cardiomyopathy (Westerlind et al. 2004).

2.3.4 N-terminal peptide of proANP and proBNP (NT-proANP and NT-proBNP)

N-terminal peptides of proANP and proBNP are secreted in equimolar amounts with ANP and BNP (Itoh et al. 1988; Rosenzweig et al. 1991; Gerbes et al. 1994; Hunt et al. 1995). NT-proANP and NT-proBNP have higher plasma concentration compared to ANP or BNP, and they are thus easier to measure to characterize the endogenous ANP and BNP secretion. The higher circulating concentrations are probably due to longer half-life (Thibault et al. 1988; Pemberton et al. 2000). Renal excretion is currently regarded as the main clearance mechanism for NT-proANP and NT-proBNP (Ruskoaho 1992; Goetze et al. 2006). Umbilical cord NT-proANP and NT-proBNP measurements have been used to assess the
peptide levels in human fetuses (Walther et al. 2001; Walther et al. 2004; Halse et al. 2005). Markedly increased NT-proANP and NT-proBNP levels are seen in healthy neonates during the first days of age, possibly due to increased ventricular pressure load resulting from neonatal lung expansion and elevated systemic vascular resistance (Mir et al. 2003). In adults, high circulating concentrations of N-terminal fragments of ANP and BNP are used to identify subjects with impaired cardiac function. They have been shown to be useful diagnostic and prognostic tools in heart failure and myocardial infarction (Sagnella 1998; de Lemos et al. 2001). Whether NT-proANP or NT-proBNP has biological effects of their own is currently unknown. It is likewise unknown whether there is a specific receptor for the N-terminal fragments (Ruskoaho 1992; Hall 2004).

2.3.5 Cardiac natriuretic peptides and pregnancy

No evidence of either ANP mRNA or BNP mRNA has been found in term human placentas of normal pregnancies (Inglis et al. 1993; Halse et al. 2005). However, conflicting data concerning the synthesis and storage of ANP and BNP in the placenta have been published (Inglis et al. 1993; Cameron et al. 1996). The lack of correlation between cord blood and maternal ANP or BNP, as well as their N-terminal fragment concentrations suggest that there is no placental exchange of these peptides (Walther et al. 2001; Hammerer-Lercher et al. 2005). Thus, the fetus has its own ANP and BNP production during development. Detectable human fetal ANP and BNP levels are documented from 16 gestational weeks onward (Ville et al. 1994; Walther et al. 2001). In mouse embryo, cardiac ANP and BNP mRNAs are present already at 8 to 9 days of gestation (Cameron et al. 2003).

Animal studies have suggested a possible role of ANP and BNP in the regulation of organogenesis of the heart and the cardiovascular system during fetal life (Cameron et al. 2003). In human fetuses, Walther et al. found elevated fetal ANP levels in cases with Rhesus isoimmunisation and documented a rapid increase in fetal BNP levels in these fetuses during intravascular blood transfusion (Walther et al. 2001). Furthermore, increased umbilical vein ANP and NT-proANP levels have been found in human pregnancies complicated by fetal growth restriction and maternal preeclampsia (Matzen et al. 1991; Furuhashi et al. 1994; Makikallio et al. 2001). Fetal plasma BNP concentrations are documented to be increased in pregnancies with maternal preeclampsia (Furuhashi et al. 1994) and in severe fetal distress (Itoh et al. 1993; Fleming et al. 2005).
2001). Increased neonatal plasma proBNP levels have also been found in type-1 diabetic pregnancies with suboptimal glycemic control before delivery (Halse et al. 2005). In addition, studies on rats have shown increased fetal ANP and BNP levels (Mulay et al. 1995) and increased neonatal ANP mRNA levels (Gopinath et al. 2004) in the hearts of neonates from diabetic dams. No significant effect of gestational age or mode delivery on cord blood ANP, BNP and N-terminal propeptide levels has been found (Ville et al. 1994; Bar-Oz et al. 2005; Halse et al. 2005).

2.4 Erythropoietin

2.4.1 Synthesis and release of erythropoietin

The control of erythropoiesis by a humoral factor (“hemopoietine”) was first suggested by Carnot and Deflandre in 1906 (Carnot 1906). It took nearly half a century, however, before the existence of this factor, erythropoietin, was proven conclusively (Erslev 1953; Fisher et al. 1961). The human EPO gene is located on chromosome 7 (Law et al. 1986). It exists as a single copy and is composed of five exons and four introns. The EPO gene encodes a single polypeptide chain consisting of 193 amino acids (Lin et al. 1985). A 27-amino-acid leader sequence at the N-terminal part and a carboxy-terminal arginine molecule are cleaved off during secretion, so that the mature protein contains 165 amino acids (Recny et al. 1987). Based on studies in fetal sheep, both the fetal liver and kidney contribute to the circulating plasma EPO levels until mid-gestation (60–80 days/term 140 days) (Moritz et al. 1997). EPO mRNA expression in the kidney of intact fetal sheep has been found already in early gestation (41 days/term 140 days) (Wintour et al. 1996). In addition, the yolk sac may contribute to EPO synthesis as seen in mouse embryos at 9 to 11 days of gestation (Yasuda et al. 2002). During maturation, the fetal EPO synthesis is gradually transitioned to the kidneys which produce most of the EPO in adults (Eckardt et al. 2005). Based on in situ hybridization studies, the EPO producing cells in the kidney are interstitial cells of the proximal tubules both in human fœtuses (Liapis et al. 1995) and adults (Eckardt et al. 1989b). In the liver, however, the majority of the cells producing EPO are hepatocytes (Koury et al. 1991). In addition, there is evidence that small amounts of EPO and its receptors are expressed in embryonic and adult
nonerythroid tissues, including brain, kidney, gut, muscle, uterus, pancreas, gonads and lung (Juul et al. 1998; Eckardt et al. 2005).

2.4.2 Regulation of release and elimination of erythropoietin

Tissue hypoxia is the major stimulus for EPO secretion, and thus the EPO concentrations largely reflect alterations of oxygen delivery to tissues (Jelkmann 1992; Eckardt et al. 2005). A measurable increase in plasma EPO has been documented already 1–2 hours after the initiation of anemia or hypobaric hypoxia in humans (Eckardt et al. 1989a). In fetal sheep, a significant and progressive increase in mean plasma EPO level has been observed during the 4th hour of acute hypoxemia (Widness et al. 1986). The human body contains no significant stores of EPO. Any change in the serum EPO concentration, therefore, reflects a change in the rate of production (Moritz et al. 1997). The production rate of EPO, in turn, is mainly determined by the amount of EPO mRNA in EPO-producing cells (Goldberg et al. 1991).

After a hypoxic stimulus, the regulation of EPO gene expression occurs mainly at the transcriptional level (Eckardt et al. 2005). A region in the 3´flanking region of the EPO gene has been found to function as a hypoxia-inducible enhancer. Transcription factors called “hypoxia-inducible factors” (HIFs) have been shown to bind to this region and to control the transcriptional activity of the EPO gene (Eckard 2005). Especially the HIF-2α –isoform induces transcription of the EPO gene in vivo (Warnecke et al. 2004). There is also evidence that the EPO gene can be downregulated by specific GATA transcription factors (Imagawa et al. 1996).

It has been shown that after a hypoxic stimulation, the EPO production continues for up to 2 hours (Cahan et al. 1990). However, the sites at which EPO are metabolized and cleared are not known either in the adult or in the fetus (Moritz et al. 1997). In human adults who were exposed to hypobaric hypoxia, plasma EPO was calculated to decline with an average half-life of 5.2 hours (Eckardt et al. 1989a). The mean serum half-life of recombinant human EPO in human adults is between 5 to 9 hours (Eckardt et al. 2005). The half-life of human fetal EPO is not known. When the endogenous human fetal plasma EPO was incubated in vitro at 37°C, a total of 69% of the initial concentration remained stable at 21 days (Schmidt et al. 2004). In infants born to mothers with preeclampsia, the mean half-life of EPO has shown to be 3.7 (± 0.9) hours (Ruth et al. 1990).
2.4.3 Physiologic effects of erythropoietin

Erythropoietin exerts its actions via the EPO receptor, a member of the cytokine receptor superfamily. The EPO receptor consists of a single membrane-spanning domain, an extra-cytoplasmic N-terminal part containing the EPO binding site, and a C-terminal cytoplasmic domain associated with signal transduction (Yoshimura et al. 1998). EPO acts primarily on colony-forming unit erythroid cells and induces these to proliferate and mature through the normoblast into reticulocytes and mature erythrocytes (Gregory et al. 1974). EPO proliferates the progenitor cells by inhibiting apoptosis and thus by decreasing the rate of cell deaths (Koury et al. 1990). In erythroid precursor cells, EPO increases both RNA and DNA synthesis, glucose uptake, globin gene expression, transferrin receptor expression and hemoglobin synthesis (Eckardt et al. 2005). In addition, EPO may also mitigate ischaemic and hypoxic damage under hypoxic conditions. Animal experiments have demonstrated that pharmacological doses of EPO protect tissue after certain ischemic injury in the nervous system, in the kidney and in the heart (Sakanaka et al. 1998; Yang et al. 2003; Lipsic et al. 2006).

2.4.4 Erythropoietin in pregnancy

During pregnancy, maternal plasma EPO levels increase gradually until term and decrease after the delivery (Milman et al. 1997). In the fetus, the liver and the kidney are the main production sites of EPO, but the EPO mRNA and EPO receptor have been found in all organs of human embryo during the first two trimesters (Juul et al. 1998). The studies on several animal species have demonstrated that during pregnancy, EPO does not cross the placenta in either direction, and thus fetal plasma EPO concentrations are thought to reflect the rate of synthesis and degradation of EPO in the fetus (Widness et al. 1995). Elevated fetal plasma EPO levels correlate inversely with UA pH and base excess in pregnancies complicated by fetal growth restriction, preeclampsia and maternal diabetes (Teramo et al. 1987; Jazayeri et al. 1999; Teramo et al. 2004b). Based on these observations, it has been suggested that fetal hypoxemia is the main stimulus of EPO synthesis. Because amniotic fluid EPO levels correlate directly with fetal plasma EPO levels before the onset of labor (Teramo et al. 1987), the amniotic fluid EPO measurements have even been proposed for clinical use to identify fetuses at risk for intrauterine chronic hypoxia (Teramo et al. 2004b).
Fetal sheep studies have shown that the placenta does not contribute to the circulating EPO levels under normoxemic conditions (Davis et al. 2003). However, in fetal chronic hypoxia, the placenta and the kidneys secrete large amounts of EPO into the fetal circulation with the placental EPO secretion exceeding that of the kidneys (Davis et al. 2003). Although the trophoblast cells of human placenta express EPO, the placental contribution to EPO secretion during hypoxemic challenge in human pregnancy is not known (Conrad et al. 1996).

It has been shown that fetal EPO secretion does not increase in the late second trimester or in the third trimester until the fetus becomes hypoxic (Voutilainen et al. 1989; Teramo et al. 2004b). Furthermore, a rise in EPO concentrations has been detected in uncomplicated singleton pregnancies reaching 41 weeks and beyond (Jazayeri et al. 1998; Manchanda et al. 1999). During vaginal delivery the uterine contractions may increase fetal EPO levels (Widness et al. 1984).

2.5 Proteomic analysis in perinatal medicine

Proteins regulate cellular actions and the alterations in their expression play a key role of understanding any biological process. Advances in proteomic technology have made it possible to analyze the protein component of biological material (the proteome). The primary aim in clinical proteomics is the identification of novel biomarkers for diagnostic, therapeutic and preventive purposes. So far, potential biomarkers for various cancers (Paweletz et al. 2001; Rai et al. 2002; Lehrer et al. 2003), rheumatoid arthritis (Doherty et al. 1998), Alzheimer’s disease (Choi et al. 2002) and intra-amniotic infection have been identified (Gravett et al. 2007; Pereira et al. 2007).

2.5.1 Methodology of proteomic analysis

Most of the analysis of complex protein samples is performed using mass spectrometry (MS). MS-based proteomic studies became possible after the availability of gene and genome sequence databases and development of protein ionization methods. The mass spectrometric measurements are performed in the gas phase on ionized analytes. A spectrometer consists of 1) an ion source which volatizes and ionizes the proteins or peptides, 2) a mass analyzer that measures
the mass-to-charge ratio of the ionized analytes, and 3) a detector that registers the number of ions at each mass-to-charge ratio value. (Aebersold et al. 2003)

The most commonly used ion sources in MS are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) techniques. Because the ESI ionizes the analytes out of a solution, it is normally coupled to liquid-chromatography (LC, liquid-chromatography ESI-MS or LC-MS) (Fenn et al. 1989; Aebersold et al. 2003). MALDI and its variant, surface-enhanced laser desorption/ionization (SELDI) MS, on the other hand, sublimate and ionize the samples out of a dry, crystalline matrix via laser pulses (Karas et al. 1988; Tanaka 1988; Hortin 2006). The mass analyzer generates information rich mass spectra from peptide fragments (tandem mass spectra or MS/MS). The four basic types of mass analyzers are ion trap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron (FT-MS) analyzers. These analyzers can be used alone or put together in tandem for more advantage. MALDI-ion source is usually coupled to TOF analyzers, whereas ESI has mostly been coupled to ion traps and triple quadrupole instruments (Aebersold et al. 2001).

The proteomic analysis requires different technical components for separating, identifying and quantifying the polypeptides of a complex protein sample, and tools for integrating and analyzing the data. MALDI-TOF is used to identify proteins by peptide mapping, also referred as peptide fingerprinting, in which proteins are identified by matching a list of experimental peptide masses with the calculated list of all peptide masses of a comprehensive protein database. This requires an essentially purified target protein and the technique is therefore commonly used in conjunction with prior protein fractionating using one- or two-dimensional gel electrophoresis (1D- or 2D-DIGE) and liquid chromatography (LC-MS) (Aebersold et al. 2003). The application of fractionation techniques before MALDI-TOF MS is necessary to also detect low molar abundance fragments, because the MALDI-TOF MS alone only detects small proteins and peptides with high molar abundance (Hortin 2006). The ESI-MS/MS instruments are based on collision-induced spectra of selected precursor ions. The peak pattern in the collision-induced spectrum provides information about the peptide sequence, although it is not readily convertible into a full, unambiguous peptide sequence. However, the collision-induced spectrum is a peptide sequence tag, which can be scanned against comprehensive protein sequence databases. When combined with the mass information, it is a specific probe to determine the origin of the peptide (Mann et al. 1994). The most commonly used protein sequence databases for searching tandem mass spectra include: Entrez Protein database.
from the US National Center for Biotechnology Information (NCBI), Reference Sequence database from NCBI; UniProt (consisting of UniProt and its supplement TrEMBL) and International Protein Index database by the European Bioinformatics Institute (Nesvizhskii et al. 2007).

Distinguishing the correct peptide assignments from false identifications among database search results can be achieved by manual verification of the peptide assignments by researchers with expertise in spectral interpretation. However, this is not feasible in the case of any larger data sets. Alternatively, researchers can apply filtering criteria based upon database search scores and other available data (Nesvizhskii et al. 2007). The computer programs utilized will estimate accurate probabilities indicating the likelihood of the presence of a peptide or a protein in the sample (Keller et al. 2002; Peng et al. 2003; Nesvizhskii et al. 2007).

2.5.2 Proteomic analysis in pregnancy complications

Proteomic analysis is expected to give new insights into the functional biochemistry of normal gestational tissues as well as to give information on the causation and molecular pathways of pregnancy complications. The association between novel protein biomarkers in maternal serum and fetal Down syndrome (Nagalla et al. 2007) suggest that proteomics may complement genomics for screening and prenatal diagnosis in the future.

The proteome of amniotic fluid and cervico vaginal fluid has been studied to find possible biomarkers of preterm birth. Gravett et al first demonstrated differential amniotic fluid proteomic profile in rhesus monkeys with intra-amniotic infection. This profile also detected subclinical intra-amniotic infection in a human cohort (Gravett et al. 2004). At the same time, differential expression of amniotic fluid proteins in a rabbit model with intra-amniotic infection was demonstrated (Klein et al. 2005). Recently, comprehensive proteomic analyses of both human amniotic fluid proteome (Michaels et al. 2007) and human cervico vaginal fluid proteome (Dasari et al. 2007) have been completed. In addition, the specific proteomic profile associated with intra-amniotic infection in humans has been demonstrated (Buhimschi et al. 2005). The findings of a significantly different proteomic profile in the cervico vaginal fluid in a primate intra-amniotic infection model and the differential expression of 17 proteins in women with preterm labor or spontaneous preterm birth suggest that these novel biomarkers
could be utilized in the noninvasive prediction of intra-amniotic infection or preterm delivery in the future (Gravett et al. 2007; Pereira et al. 2007).

The proteomic analysis of placental trophoblasts from preeclamptic women by MALDI-TOF-MS identified several proteins with significantly altered expression. This study found four proteins, protein disulfide isomerase precursor, endoplasmic reticulum resident protein, dihydrolipoyl dehydrogenase and TIM21-like protein, to be significantly up-regulated in preeclampsia (Sun et al. 2007). A pilot study of Myers et al demonstrated an up-regulation of five undefined proteins in the serum of women (n = 18) at 26 weeks’ gestation who subsequently developed preeclampsia (Myers et al. 2004). Similarly, consistent difference was found in the protein profiles (MALDI-MS analysis) of women with (n = 5) and without (n = 5) pregnancy induced hypertension in the third trimester (Bahtiyar 2003).

2.6 Fetal cardiovascular physiology

Some of the anatomic differences between the fetal and adult circulations were already described by Harvey in 1628 (Harvey 1628). The understanding of human fetal cardiovascular physiology has improved through physiologic studies on fetal sheep (Hirvonen et al. 1962; Peltonen et al. 1964; Rudolph et al. 1967). The difference between the fetal and newborn cardiovascular physiology lies in the unique characteristics of fetal myocardium and in the distribution of fetal circulation through various shunts. Ductus venosus, FO and ductus arteriosus (DA) are the three fetal anatomic shunts that permit the blood to bypass the liver and lungs during fetal life. In addition, DV and FO are important in fetal cardiovascular physiology by shunting the most oxygenated blood to the left ventricle to supply the fetal myocardium and the brain. From a physiologic point of view, the aortic isthmus (AoI) functions as an arterial shunt in the fetus, connecting the two parallel functioning cardiac ventricles. (Fig. 2)
Fig. 2. Central pathways of fetal circulation. Adopted by permission from Kiserud T. (Kiserud et al. 2000b). AO = aorta, CCA = common cerebral artery, LA = left atrium, LV = left ventricle, LP = left portal vein, MHV = middle hepatic vein, MP = main portal stem, PA = pulmonary artery, PV = pulmonary vein, RA = right atrium, RHV = right hepatic vein, RV = right ventricle, RP = right portal vein, SVC = superior vena cava.

2.6.1 Placental circulation

The placental circulation can be divided into uteroplacental circulation from the maternal side and umbilicoplacental circulation from the fetal side. Uteroplacental perfusion originates from the maternal uterine arteries which branch into the arcuate and radial arteries, thereafter reaching the placenta by the spiral arteries. Uterine volume blood flow has no autoregulatory reserve, but it seems to be directly proportional to perfusion pressure and inversely related to uterine vascular resistance (Greiss 1966; Berman et al. 1976).
Maternal nutrient delivery to the fetus depends on placental mass, villous surface, and fetoplacental mass. A total of 55% of fetuses with growth restriction were documented to have at least one type of uteroplacental lesions including placental infarction, chronic villitis, hemorrhagic endovasculitis, and placental vascular thromboses (Salafia et al. 1992). However, fetal growth restriction is thought to result from overall underperfusion of the placenta, rather than of any particular placental lesion (Salafia et al. 1995; Baschat 2004a). Abnormal UA blood flow velocity waveform findings correlate with a decreased number of small muscular arteries in placental tertiary stem villi (Giles et al. 1985).

2.6.2 Cardiac function

Contractility, the intrinsic ability of the myocyte to generate force and to shorten, is affected by the contractile state and by the preload and afterload of the heart. The contractile state of the myocyte depends on the number of cross-bridges formed between actin and myosin, which in turn is associated with the changing concentrations of calcium ions in the myocardial cytosol (Morad et al. 1980). Catecholamines, epinephrine and norepinephrine increase the availability of calcium at contractile sites and enhance the contractility of the heart. On the other hand, acetylcholine reduces the heart rate and lengthens the conduction time by increasing the potassium and chloride permeability (Pocock 1999).

Preload and afterload

Preload and afterload regulate the cardiac performance. Preload is defined as the stress imposed on the ventricular wall at the end of diastole. The wall stress is quantified in the law of Laplace: Wall stress = PR/2h, where P is pressure, R is the radius of the curvature, and h is the thickness of the ventricular wall. The cardiac muscle responds to the stretching of its fibers (preload) by increasing the strength of the subsequent contraction according to the Frank-Starling law. The Frank-Starling mechanism is known to also operate in the fetal heart (Faber 1968; Kirkpatrick et al. 1976; Lingman et al. 1984). However, the increases in mean atrial pressure above 5-7 mmHg result in only slight increases both in the fetal right (Thornburg et al. 1983) and left ventricular cardiac outputs (Thornburg et al. 1986). Under physiologic conditions, the fetal heart is therefore thought to operate near the plateau of its Frank-Starling curve. The ventricular stroke volume augmentation is explained by low compliance of the pericardium, lungs and chest
wall (Grant et al. 2001). However, if the mean arterial pressure is held constant, the fetal left ventricular stroke volume will continue to rise above mean atrial pressure of 10 mmHg (Hawkins et al. 1989). In addition, the right ventricular function is improved after 10 days of mild pressure loading in near-term fetal sheep (Pinson et al. 1991).

Afterload is defined as the ventricular wall stress opposing the ejection. The ventricles must eject blood against their own inertia and that of blood, the impedance of central blood vessels, and the resistance of peripheral vessels. When the afterload is increased, the shortening ability of the myocardium is decreased (Friedman 1972). Compared with the adult myocardium, the fetal myocardium is shown to shorten more slowly against the same relative load (Reller et al. 1987; Fouron 1999b). Approximately 65% of the blood ejected by the left ventricle perfuses the upper fetal body (Rudolph 1985). Therefore, the changes in the pressure or resistance in this area of the vasculature influences the afterload of the left ventricle. The afterload of the right ventricle is established by the descending aorta (DAo) with its various vascular beds, especially the placenta, which plays a key role as regards to right ventricular afterload. The similar fractional shortenings of right and left ventricle in normal human fetuses indicate that the ventricular afterloads are nearly the same (Sutton et al. 1991b).

Cardiac output, heart rate and blood pressure

The cardiac output (stroke volume x fetal heart rate (FHR)) is affected by contractility, peripheral resistance, blood volume and viscosity of the blood. Filling of the heart (preload) controls the cardiac output and the response of the heart to preload depends on the sympathetic stimulation and contractile state of the myocardium (Pocock 1999). The dynamics of blood flow in cardiac ventricles also have an effect on cardiac output. With increased intraventricular volume, a greater tension must be developed in the myocardium to produce higher pressure (Romero et al. 1972).

Fetal ventricular stroke volume, cardiac output and weight-indexed systemic vascular resistance increase with advancing gestation (Rasanen et al. 1996). The weight indexed combined cardiac output (CCO) of a human fetus is significantly higher than that of the human adult (Severi et al. 2000), remaining stable during the second half of pregnancy (400 ml/min/kg) (Kiserud et al. 2006a). There is no significant difference between the fetal left and right ventricular pressures because the ventricles pump in parallel to the fetal systemic circulation (Johnson
et al. 2000). Similarly, the pressure difference between the fetal atria is minimal (Thornburg et al. 1986; Reller et al. 1987). In human fetuses, the left and right ventricular systolic and end diastolic pressures increase linearly between 14 and 28 gestational weeks. The systolic pressure is documented to increase from 13 mmHg to 37 mmHg and end diastolic pressure from 3 mmHg to 10 mmHg (Johnson et al. 2000). Studies on fetal lambs have shown that the right ventricle ejects a larger stroke volume than the left ventricle, thus, representing a greater portion of the CCO (Rudolph 1985). In the case of diminished oxygenation in fetal lamb, the weight-indexed right ventricular cardiac output (RVCO) and CCO further increase (Makikallio et al. 2006).

In fetal lamb, the fetal heart rate has a direct influence on the left and right ventricular stroke volumes (Anderson et al. 1986; Anderson et al. 1987). Rapid pacing of the fetal heart will decrease the stroke volume as the filling time shortens (Anderson et al. 1986; Anderson et al. 1987). In addition, fetal heart rate is influenced by the balance between sympathetic and parasympathetic impulses (Walker et al. 1978). Throughout the gestation fetal heart rate remains relatively high compared with the adult heart rate, but it decreases towards term. The variability and the accelerations of fetal heart rate are the highest at the end of the third trimester (Park et al. 2001).

**Myocardial blood flow**

The fetal coronary arteries arise from the sinus in the base of the aorta. Coronary blood flow is known to be maximal in diastole (Ofili et al. 1993). Myocardial oxygenation and metabolism are critically dependent on coronary blood flow. It has been demonstrated that the fetus compensates for its relatively hypoxemic environment by a greater resting myocardial volume blood flow compared to the adult (Fisher et al. 1980; Thornburg et al. 1999). Resting myocardial blood flow is estimated to be 2–4% of fetal CCO (Rudolph 1985; Itskovitz et al. 1987; Jensen et al. 1991). The myocardial blood flow is primarily determined by the pressure difference between the ascending aorta and the right atrium. The myocardial perfusion is further affected by vascular tone, extravascular resistance (Baschat et al. 2002) and autoregulation which increases the myocardial blood flow by modulating the precapillary sphincter tone at areas of greatest oxygen demand (Reller et al. 1995; Fleming et al. 1996; Thornburg et al. 1999). Severe right ventricular pressure load in fetal sheep doubled the right and left ventricular myocardial blood flows in all regions of fetal heart (Reller et al. 1992b). Even
greater increases in myocardial blood flow may be triggered by fetal hypoxemia (Reller et al. 1992a; Reller et al. 1995) and adenosine infusion (Reller et al. 1992b).

**Diastolic function**

Diastolic performance, which reflects the ability of the heart to fill, is affected by cardiac loading conditions and ventricular compliance. Ventricular relaxation occurs in four phases: 1) isovolumetric relaxation, 2) early rapid filling, 3) late slow filling and 4) filling during atrial contraction. Echocardiographic studies suggest that atrial contraction is more important for ventricular filling in the fetus than in the adult (Tulzer et al. 1994; Veille et al. 1999; Makikallio et al. 2005). The ventricular inflow waveforms are documented to be monophasic before 9 weeks of gestation demonstrating only an A-wave (Makikallio et al. 2005). In addition, the biphasic inflow waveforms obtained across the atroventricular valves later in the pregnancy are characterized by a higher peak A wave than E-wave (Reed et al. 1986; Makikallio et al. 2005). Stiffness and impaired relaxation of the fetal myocardium are understood to alter the diastolic filling patterns of fetal heart. The ventricular stiffness, however, progressively decreases with advancing gestation (Veille et al. 1999).

**2.6.3 Fetal arterial and venous circulation**

Poiseuille’s law quantitatively relates the flow of a liquid through a rigid tube. The equation describing the relationship is: $Q = (P_0 - P_1) \times \left(\frac{\pi \times r^4}{8 \times \eta \times l}\right)$, where $Q$ is the flow rate, $P_0$ and $P_1$ are the pressure at the beginning and end of the tube, $r$ is the radius, $l$ is the tube length, and $\eta$ is the viscosity of the liquid. According to Poiseuille’s law, the flow of blood is proportional to the fourth power of the radius of the vessel and is inversely proportional to the viscosity. The viscosity of the blood depends on the number of circulating blood cells and thus varies with the hematocrit (Pocock 1999).

The blood flow through any part of the circulation is driven by the perfusion pressure. The resistance offered by the blood vessels to the blood flow is known as vascular resistance. Mathematically the relationship between blood flow ($Q$), perfusion pressure ($\Delta P$) and vascular resistance ($R$) is $Q = \Delta P / R$. The arterioles have the ability to regulate the resistance of systemic circulation. Sympathetic stimulus causes a tonic constriction in the majority of arterioles. In addition, the
internal pressure and the distensibility of a vessel affect the vessel diameter. The degree of distensibility varies with the thickness and composition of the vessel wall, and the degree of filling. The veins are thought to act as a reservoir for blood, or capacitance vessels, because of their distensibility. The arteries are referred as the pressure storers due to their ability to expand and recoil. (Pocock 1999)

**Ductus venosus and foramen ovale**

The ductus venosus allows the umbilical venous blood with the highest oxygen content to bypass the hepatic circulation and directly enter the IVC at its inlet into the heart. The “preferential streaming” of blood flow with the highest oxygenation and the highest kinetic energy, first described in animal studies (Edelstone *et al.* 1979), has been documented also in human fetuses (Kiserud *et al.* 1992). In the human fetus, the DV is a narrow trumpet-like structure with a width of only 2 mm at term (Kiserud *et al.* 1991). In fetal sheep, about 55% of umbilical venous blood passes through the DV while 45% is distributed to the hepatic circulation (Rudolph 1985). Fetal sheep studies have also shown that the fetus is able to redistribute umbilical venous return during hypovolemia and hypoxemia (Reuss *et al.* 1980; Itskovitz *et al.* 1982). In fetal sheep during hypoxemia, the proportion of umbilical venous blood that passes through the DV increased from 55% to about 65% whereas the percentage to the liver fell to 35% (Reuss *et al.* 1980). In growth restricted fetuses, DV shunting is reported to increase in proportion to placental insufficiency, the average DV shunting being 39% compared to 25% in the control fetuses (Kiserud *et al.* 2006b). In addition, the experiments performed on fetal sheep in vivo have shown that the tonically constricted DV inlet distended under the influence of nitroxide. The most extensive dilatation, however, was found in response to hypoxemia with a increase of 60% in the total diameter of the DV (Kiserud *et al.* 2000a).

The active regulation of umbilical blood flow to the DV has been suggested to be under neural (Pearson *et al.* 1969) and humoral influences such as adrenergic agents and prostaglandins (Coceani *et al.* 1988; Paulick *et al.* 1990). The ductus venosus constricts via α-adrenoreceptors and relaxes via β-receptors (Coceani *et al.* 1988). However, the in vitro experiments on fetal sheep suggest that vascular resistance and blood viscosity, flow, and pressure are major determinants of the blood distribution between the liver and the DV (Kiserud *et al.* 1997). Higher hematocrit was related to increased resistance for blood flow in
the DV. In addition, at very low umbilical pressures, the umbilical flow was exclusively directed through the DV (Kiserud et al. 1997).

Foramen ovale corresponds to an opening in the posterior lower part of the atrial septum of the fetal heart. In normal situations, this valve-like structure allows blood to pass only from the right to the left atrium. However, in the case of increased pressure on the left side, the enhanced diversion of blood is thought to be shifted into the right atrium (Kiserud 2005). The volume blood flow through the FO is difficult to assess because of the multiphasic blood velocity waveform and inaccurate calculations of the cross-sectional area of the FO (Phillipos et al. 1994; Rasanen et al. 1996). The volume blood flow through the FO is thus calculated by subtracting the volume blood flow through pulmonary arteries from the left ventricular cardiac output (LVCO) (Rasanen et al. 1996). Doppler investigations in human fetuses have estimated that the blood flow through the FO decrease from 34% to 18% of the CCO during the second half of pregnancy (Rasanen et al. 1996).

**Aortic isthmus and pulmonary circulation**

The aortic isthmus is a vascular segment between the left subclavian artery and the DA. In a unique way, the AoI establishes a link between the right and the left ventricles, which perfuse the upper and lower body in parallel. In systole, the direction of blood flow in the AoI is mainly dependent on the contributions of the right and left ventricular stroke volumes. In diastole, the direction of AoI blood flow reflects solely the downstream impedances of the right and left ventricles, indicating mainly placental and cerebral vascular impedances (Fouron 2003). Normally, the net blood flow in the AoI is antegrade and directed towards DAo. However, a short retrograde blood flow component in the AoI may be seen from 35 gestational weeks onward (Fouron et al. 1994). An increased AoI retrograde blood flow component is documented in conditions with decreased fetal upper body (cerebral arteriovenous fistulae) or increased lower body vascular resistance (placental insufficiency) (Patton et al. 1995; Sonesson et al. 1997). Fetal lamb studies have suggested that the oxygen content of the blood entering the brain is preserved as long as the net blood flow in the fetal aortic isthmus is antegrade (Fouron et al. 1999a).

The ductus arteriosus is a segment of the pulmonary arch, which participates in the combined output by channeling the right ventricular output toward the systemic circulation (Fouron 1999b). In human fetuses, 78% of RVCO and 46%
of the CCO is directed through the DA (Mielke et al. 2001), with no significant change during the second half of the gestation (Rasanen et al. 1996). The distribution of RVCO is heavily dependent on the impedance of the pulmonary vasculature. In human fetuses, pulmonary vascular resistance is shown to decrease almost 1.5-fold between 20 and 30 weeks of gestation and thereafter to increase significantly (Rasanen et al. 1996). This suggests that fetal pulmonary circulation is under acquired vasoconstriction after 30 weeks of gestation. Hypoxemia causes vasoconstriction in the fetal lamb pulmonary circulation (Lewis et al. 1976), whereas the effect of hyperoxia is vasodilatation also seen in human fetuses (Rasanen et al. 1998).

2.6.4 Chronic fetal hypoxemia

Chronic fetal hypoxemia has been studied in sheep models by decreasing the oxygen concentration of the air inspired by the ewe (Cohn et al. 1974), by restricting either uterine (Bocking et al. 1988; Jensen et al. 1991; Reid et al. 1991; Bocking et al. 1992) or umbilical blood flow (Itskovitz et al. 1987), and by disturbing the placental circulation by embolizations of the vasculature (Trudinger et al. 1987; Erkinaro et al. 2006). In fetal hypoxemia with preserved placental gas exchange, the fetal blood gases are dependent on maternal blood gas status. The fetal oxygen tension (pO2) of the umbilical venous return is lowered, whereas the fetal carbon dioxide tension (pCO2) is dependent on the maternal pCO2 and can be thus maintained normal (Cohn et al. 1974; Fouron 1999b). On the other hand, if the placental exchange is damaged like in placental insufficiency, fetal hypoxemia develops with increasing fetal pCO2 levels (Fouron 1999b; Baschat 2004a). Progressive hypoxemia and hypercapnia lead to accumulation of fetal acid metabolites and cause fetal acidemia (Low 1997).

Fetal hypoxemia causes bradycardia and an increase in arterial blood pressure (Cohn et al. 1974; Itskovitz et al. 1987; Jensen et al. 1991; Reid et al. 1991). In the hypoxic fetus, the redistribution of cardiac output in favor of the brain, heart and the adrenals has been demonstrated in several studies (Cohn et al. 1974; Itskovitz et al. 1987; Bocking et al. 1988; Block et al. 1990; Jensen et al. 1991; Reid et al. 1991). In addition, decreased blood flow to the fetal pulmonary, renal, splenic and gut tissues has been shown (Cohn et al. 1974; Rizzo et al. 1991a; Makikallio et al. 2006). The proportion of umbilical venous blood passing through the DV in hypoxic fetuses increases with preferential streaming of the DV blood flow through the FO (Edelstone et al. 1980; Itskovitz et al. 1987;
Jensen et al. 1991). According to most of the studies, CCO is maintained in hypoxemic fetuses (Cohn et al. 1974; Jensen et al. 1991; Reid et al. 1991), as long as the fetus does not become acidemic (Cohn et al. 1974). If the fetal hypoxemia is prolonged, the elevated umbilical blood flow is reduced back to the level of uncomplicated pregnancies (Bocking et al. 1992). The fetus is, however, able to maintain its oxidative metabolism for up to 24 hours when oxygen delivery is reduced by 40% and there is no progressive acidemia (Bocking et al. 1992). When the fetus becomes acidemic, the CCO decreases, while the preferential perfusion of the brain, heart, adrenal gland, and placenta may still be preserved (Block et al. 1990).

2.7 Ultrasonographic assessment of fetal and placental hemodynamics

2.7.1 Methodology of Doppler ultrasonography

The Doppler phenomenon was first described by Christian Doppler in the 19th century. According to the Doppler phenomenon, the pitch of sound waves of a moving object is changed when the distance between the observer and the source of sound changes. This change caused by a relative motion between the observer and the object is known as the Doppler shift. In ultrasonography, the Doppler shift is electronically calculated and converted into a graphic presentation called the blood flow velocity waveform, which is shown as a function of time. Doppler shift is dependent on the speed and direction of blood flow, the frequency of sound emitted from the Doppler transducer and the insonation angle between the transducer and the blood vessel. (Brody et al. 1974; Angelsen 1980; Gill 1985)

Continuous wave Doppler ultrasonography

In a continuous wave Doppler system, the ultrasound waves are continuously transmitted and received from two separate transducers, which are arranged in such a manner that their insonation axes intersect at a certain range. These transducers are not limited by the depth of the location or by the maximum detectable velocity range. However, the visualization of all Doppler signals from a certain range is impossible due to superimposed Doppler signals. (Gill 1985)
**Pulsed wave Doppler ultrasonography**

In pulsed wave Doppler ultrasonography, there is a single transducer, which transmits and receives the ultrasound waves in pulses. Because the velocity of sound waves is known, it is possible to analyze the back-scattered echo alone from a particular range allowing the blood flow analysis within a single vessel (Gill 1987). The axial length of a sample is determined by the time period the gate is open. However, the fact that a new pulse cannot be emitted before the last echo of the preceding pulse has arrived imposes a limit on the detectable maximum velocity of blood flow and on the depth of the object.

**Color Doppler ultrasonography**

Additional information is gained from the color-coded pulsed Doppler imaging where the flow toward the Doppler transducer is displayed in red and flow away from it is shown in blue. In the color Doppler image, the non-moving targets are presented in gray-scale. The saturation of the color is related to the velocity of the flow (Burns 1993). Limitations of color Doppler ultrasonography are the same as with pulsed Doppler.

**Analysis of Doppler spectra**

The physical, anatomical and morphological factors of blood flow velocity waveform differ from one vessel to another and can be analyzed qualitatively. Forward flow during diastole is seen in arteries supplying low-resistance vascular beds. As the peripheral impedance increases, the diastolic component disappears.

Quantitative measurements such as the velocity and acceleration can be assessed from the blood flow profiles. By semiquantitative assessment of blood flow profiles, several components can be evaluated at the same time, and the angle between the ultrasound beam and the longitudinal axis of the vessel becomes less important. One of the most common semiquantitative assessments in obstetric applications is the pulsatility index (PI = (peak systolic velocity − end diastolic velocity) / time-averaged maximum velocity over the cardiac cycle), which can be derived from the relationship between systolic and diastolic blood flow components. No correction for heart rate, if it is within normal physiologic range is required (Kofinas et al. 1989). PI is thought to reflect the downstream
flow impedance, which is resistance to pulsatile flow. This is, however, only an indirect estimation of the actual blood flow (Dickey 1997).

The volume flow \( Q \) (ml/min) through a fetal vessel can be calculated if the mean velocity \( V_{\text{mean}} = TVI \times FHR \) and the cross-sectional area (CSA) of the vessel are known according to formula: \( Q = TVI \times FHR \times CSA \). In this formula, the CSA is derived from the diameter \( d \) of the vessel using the formula \( CSA = \pi(d/2)^2 \). The time-velocity-integral (TVI) is calculated by planimetering the area underneath the Doppler spectrum. In the fetal heart, LVCO equals the volume blood flow through aortic valve \( (Q_{AoV}) \), RVCO equals the blood flow through pulmonary valve \( (Q_{PV}) \), and their sum is CCO.

The error in vessel diameter measurements becomes less as the vessel diameter increases. Therefore, volume blood flow analysis is recommended only for large vessels (Dickey 1997). However, the diameter assessment of small vessels is documented to be of high reproducibility when using a high-frequency ultrasound (Kiserud et al. 1999). At least three separate measurements of the vessel diameter from sequential cardiac cycles are recommended (Dickey 1997).

When measuring absolute velocity and volume, the angle of insonation is of great concern and should be kept at less than 30 degrees (Tessler et al. 1990). The PI calculations are considered to be reliable as long as the angle of insonation is less than 60 degrees (Gudmundsson et al. 1990).

### 2.7.2 M-mode echocardiography

M-mode imaging shows the position of one interface with respect to other interfaces along the selected transmission line. Monitored interfaces will change each time the selected transmission penetrates through the area. Each transmission collects and displays its interface data with its own time reference. Variations in the position of received echoes are thus recognized as motion displacements. M-mode measurements are possible without any physical changes of the transducer when the transducer is set to collect data from one direction and from only one line of transmission. M-mode is used in fetal cardiology to estimate the diameters of ventricular cavities and walls and the fractional shortenings of the ventricles. In addition, it is used in the diagnosis of fetal arrhythmias (DeVore et al. 1984; Reed et al. 1986).
2.7.3 Umbilicoplacental hemodynamics

Placental circulation is assessed by obtaining the UA blood velocity waveforms. The UA mean blood flow velocity increases significantly between 7 and 10 weeks of gestation, while UA PI values are documented to remain unchanged during that time (Makikallio et al. 2004). The UA diastolic blood flow component becomes present after 12 gestational weeks and should always be visible after 16 weeks in normal pregnancies (Wladimiroff et al. 1991). Umbilicoplacental volume blood flow in human fetuses consists of approximately 30% of CCO in midgestation (Sutton et al. 1991a; Kiserud et al. 2006a) and approximately 20% of CCO after 32 weeks of gestation (Kiserud et al. 2006a). It was previously believed that in normal human pregnancies, the weight-indexed umbilical venous volume blood flow remains constant at 110–120 ml/min/kg for most of the pregnancy (Sutton et al. 1990; Sutton et al. 1991a). However, the recent longitudinal study in low-risk pregnancies found that the umbilical volume blood flow increases to its maximum at the end of the second trimester and thereafter decreases throughout the third trimester to approximately 66 ml/min/kg at 40 weeks of gestation (Acharya et al. 2005a). In addition, the UA PI values decrease continuously during the second half of pregnancy, because the UA peak systolic velocity, the end diastolic velocity and the time-averaged maximum velocity progressively increase with gestational age (Acharya et al. 2005b).

A decrease in the number of small muscular arteries in the placental tertiary villi arterioles in humans is associated with abnormal UA blood velocity waveform pattern (Giles et al. 1985). Morphologic changes in placental vasculature affect placental oxygen and other nutritional transport and may lead to fetal growth restriction. Over 60% of the intraplacental fetal vasculature must be occluded before any significant change in UA blood velocity waveform pattern occurs (Thompson et al. 1990). According to experimental studies on fetal sheep, the UA PI value does not reveal changes in vascular resistance mediated by umbilical arterial vasoconstriction with angiotensin II, but reflects an increase in the resistance of cotyledons caused by placental embolizations (Adamson 1990). The UA PI value seems to reflect the hemodynamic and morphological events at the level of placental villi (Giles et al. 1985; Morrow et al. 1989), rather than indicate specific changes in the umbilical blood flow or resistance (Adamson 1990).

In the late second and third trimesters, the vascular impedance is considered increased if the systolic to diastolic velocity ratio is ≥ 3.5 (Forouzan et al. 1991).
Absent (AEDV) or retrograde (REDV, Fig. 3) diastolic velocity waveform in the UA indicates severely increased placental vascular impedance or change in the fetal arterial blood pressure (Faber et al. 2006). In monitoring low risk pregnancies, abnormal UA Doppler velocimetry is shown to correlate better with fetal acidosis than does a non-stress test (Arduini et al. 1991). However, some 50% of fetuses with AEDV in the UA can be non-acidemic (Nicolaides et al. 1988). The time interval between the first appearance of AEDV and abnormal fetal heart rate tracings is documented to range from 1 to 26 days (Arduini et al. 1993). In fetal growth restriction, an abnormal UA Doppler finding is one of the early changes preceding adverse perinatal outcome (Hecher et al. 2001; Ferrazzi et al. 2002). 50% of growth restricted fetuses demonstrated a UA AEDV finding at 15 to 16 days prior to delivery, indicated by non-reactive FHR tracing (Ferrazzi et al. 2002). In structurally normal human fetuses with an UA AEDV or REDV finding, the perinatal mortality rate was 19% before 30 weeks of gestation. A significantly higher mortality rate was found in fetuses with REDV (35.7%) than with AEDV (8.9%) (Kurkinen-Raty et al. 1997). In sheep fetuses, UA REDV was immediately followed by fetal death (Morrow et al. 1989).

![Fig. 3. Normal (left panel) and abnormal (right panel, retrograde end-diastolic velocity waveform, REDV) blood velocity waveforms in the umbilical artery during the third trimester.](image)

### 2.7.4 Fetal heart

**Cardiac output and its distribution**

In uncomplicated pregnancies, human fetal cardiac stroke volumes increase exponentially from 0.7 ml at 20 weeks of gestation to 7.6 ml at 40 weeks for the right ventricle and from 0.7 ml at 20 weeks to 5.2 ml at 40 weeks for the left ventricle (Kenny et al. 1986). Similarly, there is a 10-fold increase in right (RVCO), left (LVCO) and combined (CCO) ventricular outputs (Kenny et al. 1986; Rasanen et al. 1996). During the second half of pregnancy, the human fetal
CCO is approximately 400 ml/kg/min (Mielke et al. 2001; Kiserud et al. 2006a). From 13 to 41 weeks of gestation, the median proportions of RVCO and LVCO were 59% and 41% of the CCO (Mielke et al. 2001). According to another study in human fetuses, the proportion of RVCO of the CCO increased significantly from 20 to 38 weeks of gestation to 60% at term, while the proportion of LVCO of the CCO decreased to 40% at term (Rasanen et al. 1996). In growth restricted fetuses, the proportion of RVCO of the CCO has been demonstrated to be significantly lower than in normal fetuses (al-Ghazali et al. 1989). Furthermore, these fetuses demonstrated a relative increase in LVCO (al-Ghazali et al. 1989; Rizzo et al. 1991a) and a significant decrease in the CCO with advancing gestation (Rizzo et al. 1991a). In a longitudinal investigation of fetuses of type-1 diabetic pregnancies, a higher weight-indexed total cardiac output was documented (Lisowski et al. 2003). In addition, the fetuses of diabetic mothers did not show a normal increase in the RVCO/LVCO ratio between 15 and 40 weeks of gestation (Lisowski et al. 2003).

The distribution of human fetal CCO varies with advancing gestation. In human fetuses, the proportion of the CCO directed to the fetal pulmonary circulation increased from 13% to 25% between 20 to 30 weeks of gestation, decreasing thereafter to approximately 21% at term (Rasanen et al. 1996). Another study on human fetuses estimated that the pulmonary volume blood flow is 11% of the CCO irrespective of gestational age (Mielke et al. 2001). In human fetuses, the proportion of DA blood flow of the CCO varies between 32% and 40% during 20 to 38 weeks of gestation (Rasanen et al. 1996). Doppler investigations have estimated that the blood flow through human fetal FO decreases from 31–34% to 17–18% of the CCO between midpregnancy and 38 weeks of gestation (Rasanen et al. 1996).

**Systolic function**

Fetal ventricular systolic function can be assessed by calculating the right (RVeFo) and the left (LVeFo) ventricular ejection forces with the formula: $(1.055 \times \text{CSA} \times \text{TVI_{ac}}) \times (\text{PSV/\text{TTP}})$, in which TVI_{ac} is the time-velocity integral during the acceleration period of systole, PSV is the peak systolic velocity, and TTP is the time to peak velocity interval (Noble 1968; Sutton et al. 1991b; Rizzo et al. 1995b). Ventricular ejection force estimates the energy transferred from ventricular myocardial shortening to work done by accelerating the blood into the circulation (Sutton et al. 1991b). The ejection force calculation does not require...
estimation of ventricular volumes and is independent of ventricular configuration. Animal studies have suggested that the force exerted by the heart in early systole is proportional to the acceleration of blood. In addition, the early systolic flow is suggested to be less affected by changes in afterload and preload than the late systolic flow (Noble 1966; Noble 1968). In the human fetus, both left and right ventricular ejection forces increase more than 10-fold during second half of pregnancy and the ejection forces of both ventricles are equal (Sutton et al. 1991b; Rizzo et al. 1995b; Rasanen et al. 1997b). The human fetal RVeFo is shown to increase during chronic volume overload, but decrease significantly in the case of severely increased afterload such as occlusion of DA (Rasanen et al. 1997b).

The combined systolic and diastolic myocardial performance is described with an index of myocardial performance (IMP = (ICT + IRT)/ET, in which IRT is isovolumetric relaxation time, ICT is isovolumetric contraction time and ET is ejection time) (Tei et al. 1995; Tsutsumi et al. 1999). The IMP of the fetal left and right ventricles normally decreases during second half of the gestation suggesting improved myocardial performance. In pregnancies complicated by fetal growth restriction or maternal diabetes, the IMP is significantly greater during the late gestation than in the controls, suggesting abnormal global cardiac function (Tsutsumi et al. 1999). In fetal sheep with increased placental vascular resistance and metabolic acidosis, the proportion of ICT of the cardiac cycle increased but the IRT and ET proportions and the tissue Doppler Tei index did not change (Acharya et al. 2008).

**Diastolic function**

Isovolumetric relaxation time (IRT) is the time interval between the closure of the semilunar valve and the opening of the atrioventricular valve. During this time period the ventricles actively decrease the pressure from the systole to the atrial level, and the IRT is thus used to describe the early diastolic function of the heart. In uncomplicated pregnancies from 14 weeks of gestation onward, the IRT is shown to remain constant and therefore the changes in IRT have been suggested to reflect abnormal diastolic cardiac function (Tulzer et al. 1994; Tsyvian et al. 1998). In fetuses with placental insufficiency, the proportion of IRT from the total cardiac cycle (IRT%) is reported to be greater than in normal pregnancies (Makikallio et al. 2003).
Diastolic function of the fetal heart can be studied from the inflow blood velocity waveforms at the level of atrioventricular valves. Early ventricular filling is characterized by E-wave; filling during atrial contraction is characterized by A-wave. The E-/A-wave velocity ratio is similar between both atrioventricular valves, but both the E- and A-wave peak velocities are higher at the tricuspid valve than at the mitral valve. With advancing gestation the E/A ratio, the peak E- and A-wave velocities, and the A wave TVI/total TVI ratio increase significantly (Tulzer et al. 1994; Veille et al. 1999). The A wave TVI/total TVI ratio reflects the atrial contribution to ventricular filling and is greater at the tricuspid valve than at the mitral valve. These ratios do not normally change with advancing gestation (Tulzer et al. 1994). In a sheep model with increased placental vascular resistance, fetal metabolic acidosis was related to a reduction in tissue Doppler E-wave velocity, but, no significant change in the A-wave velocity was observed (Acharya et al. 2008).

**Afterload**

The ratio between cardiac circumference to thoracic circumference (CC/TC) and the ratio between cardiac and thoracic areas are used to evaluate the heart size. The fetal CC/TC -ratio is found to be normal when less than 50% (Huhta 2005). The mean ratio between cardiac and thoracic areas remains at 0.30 during the first half of normal pregnancy (Respondek et al. 1992). In pregnancy complications such as placental insufficiency and maternal diabetes, these ratios are demonstrated to be significantly greater (Respondek et al. 1992).

The ventricular diameters obtained by M-mode echocardiography are used in the calculation of left (LVFS) and right (RVFS) ventricular fractional shortenings by the formula: ventricular fractional shortening (%) = [(inner diastolic diameter \- inner systolic diameter)/inner diastolic diameter] \times 100 (DeVore et al. 1984). In normal human fetuses, the right/left ratio of the ventricular diameters remains constant throughout gestation (DeVore et al. 1984). In a fetal lamb, severely increased afterload due to ductal occlusion resulted in increased right ventricular systolic dimension and decreased RVFS (Tulzer et al. 1991). In addition, in human fetuses with intrauterine compromise, RVFS was found to be decreased, while the ratio of the right/left ventricular end-diastolic diameters was increased (Rasanen et al. 1989).

The prevalence of tricuspid regurgitation (TR) in fetuses with normal cardiac anatomy is 6.8% (Respondek et al. 1994). Tricuspid regurgitation is classified to be trivial (non-holosystolic, maximum velocity < 2m/s, duration > 72 ms) or
significant (holosystolic, maximum velocity > 2m/s) (Respondek et al. 1994). Fetal TR may indicate increased fetal right ventricular pressure due to elevated afterload like in cases with ductal constriction and severe placental insufficiency. In addition, TR is related to conditions with increased preload such as in increased volume overload (Respondek et al. 1994).

2.7.5 Fetal arterial hemodynamics

During normal pregnancy, there is a continuous forward flow in all cerebral arteries throughout the cardiac cycle (Kirkinen et al. 1987; van den Wijngaard et al. 1989). Normally the MCA PI slightly increases until 30–32 weeks of gestation (Konje et al. 2005) and then gradually decreases as a sign of decreased impedance of fetal cerebral circulation in late gestation (van den Wijngaard et al. 1989; Konje et al. 2005). Brain-sparing effect, the redistribution of fetal cardiac output in favor of cerebral circulation, may be seen in growth restricted and hypoxemic human fetuses as decreased impedance and increased blood velocities both in internal carotid and middle cerebral arteries (Kirkinen et al. 1983; Wladimiroff et al. 1987; van den Wijngaard et al. 1989; Hecher et al. 2001; Ferrazzi et al. 2002; Makikallio et al. 2002a). The opposite is documented to occur in the DAo (Rasanen et al. 1988; Hecher et al. 2001; Makikallio et al. 2002a). In growth restricted fetuses, increased MCA peak systolic velocity combined with reversed DV blood velocity waveform is reported to predict increased perinatal mortality (Mari et al. 2007) and the UA/MCA PI and DAo/MCA PI ratios to relate to adverse perinatal outcome (Wladimiroff et al. 1987; Harrington et al. 1999).

During the second half of pregnancy the blood flow in DAo is normally antegrade throughout the cardiac cycle because of the low vascular resistance in the placenta. The weight-indexed blood flow in DAo remains stable until 37 weeks of pregnancy, after which it slightly decreases (Marsal et al. 1987). In normal pregnancies, the total blood flow in DAo correlates well with cardiac size and left ventricular cardiac output (Rasanen et al. 1988). However, in pregnancies complicated by hypertensive disorders and fetal growth restriction, the aortic velocities are lower and the waveform indices higher than in normal pregnancies (Rasanen et al. 1988; Jouppila et al. 1989). In diabetic pregnancies, the weight indexed blood flow in the fetal aorta during the late third trimester has been found to be significantly decreased compared to normal pregnancies (Rasanen et al. 1988). No differences are found in the waveform indices of DAo (Olofsson et al. 2000).
1987; Rasanen et al. 1988), although one study reported a lack of normal decline in PI value of DAO in diabetic pregnancies (Grunewald et al. 1996).

The AoI waveform can be obtained either from a sagittal view of the aortic arch (Fig. 4) or from the three vessels and trachea view (Del Rio et al. 2005). Quantitative (velocities), semiquantitative (PI, isthmic flow index, antegrade TVI to retrograde TVI -ratio) and qualitative (presence of retrograde flow during diastole) measures have been used in the assessment of fetal AoI blood flow (Fouron et al. 1999a; Makikallio et al. 2002a; Fouron 2003; Fouron et al. 2005; Del Rio et al. 2008). In placental insufficiency, the abnormalities in Doppler waveform of AoI have been documented to occur earlier, at a less severe stage than the changes in the UA Doppler waveforms (Bonnin et al. 1993; Sonesson et al. 1997). In growth restricted human fetuses the absolute velocities in AoI are decreased (Del Rio et al. 2008). In addition, the growth restricted fetuses with antegrade net blood flow in AoI demonstrate a successful redistribution of RVCO from the pulmonary to the systemic circulation. However, the fetuses with retrograde AoI net blood flow (Fig. 4) fail to demonstrate these changes (Makikallio et al. 2003). Retrograde AoI net blood flow is related to a non-optimal human neurodevelopmental outcome at the age of 2–4 years (Fouron et al. 2005). In addition, fetal retrograde AoI net blood flow has been suggested to increase the risk of adverse perinatal outcome (Del Rio et al. 2008). The findings are in accordance with animal experiments showing that oxygen content of blood entering the cerebral circulation is diminished in the presence of retrograde AoI net blood flow (Fouron et al. 1999a).

In 10% of normal pregnancies, the fetal coronary circulation is visualized at 31 weeks of gestation. From 37 weeks onwards the fetal coronary circulation is visualized in 50% of normal pregnancies (Baschat et al. 1997). However, in severe fetal growth restriction, the coronary blood flow has been identified in 20% of cases at as early as 27 weeks of gestation (heart-sparing effect) (Baschat 1997). Increased coronary blood flow suggests autoregulation-mediated vasodilatation in response to hypoxemia, which is supported by poor perinatal outcome and increased mortality rate in these fetuses (Baschat et al. 1997; Baschat et al. 2000).
2.7.6 Fetal venous hemodynamics

In normal human fetuses between 20 and 38 weeks of gestation, the amount of umbilical volume blood flow shunted through the DV is reported to decrease significantly from 30–40% to 15–20%, while consequently the percentage of liver blood flow increases (Bellotti et al. 2000; Kiserud et al. 2000b). The right hepatic vein and IVC deliver the lowest oxygen saturated blood from the fetal lower body to the right atrium and right ventricle (Rudolph 1985). Altered distribution of fetal venous return is observed in stress conditions. In fetal sheep, the volume blood flow through the DV is shown to increase as much as 70% during fetal hypoxia or during reduced umbilical volume blood flow (Behrman et al. 1970; Edelstone et al. 1980). In human fetuses with growth restriction, an active dilatation and increased shunting through the DV with concomitant reduction in the umbilical blood supply to the fetal liver has been observed (Bellotti et al. 1998; Bellotti et al. 2004; Kiserud et al. 2006b).

Umbilical venous blood flow has pulsations up to 13 weeks of gestation after which pulsations gradually disappear (Rizzo et al. 1992). In cases of elevated end-diastolic right ventricular pressure, increased flow reversal during atrial systole has been reported in the IVC, hepatic veins and DV. In severe cases,
A-wave flow reversal can be identified in the umbilical vein (Fig. 5) (Huhta 1997). Umbilical venous pulsations, which decrease the umbilical venous velocity during atrial contraction, were first seen in fetal sheep experiments (Reuss et al. 1983) and later described in human fetuses with severe growth restriction and fetal hydrops (Indik et al. 1991; Reed et al. 1996). Abnormal end-diastolic umbilical venous pulsations have been reported in severely growth restricted fetuses with absent diastolic velocity in the umbilical arteries (Gudmundsson et al. 1996). It has been speculated that in placental insufficiency, umbilical venous pulsations are not transmitted retrograde from the right atrium but through the placenta (Gudmundsson et al. 1996; Huhta 1997). Umbilical venous pulsations, which occur locally and during ventricular systole, are documented during umbilical cord compression (Nakai et al. 1997).

In the DV, IVC and hepatic veins, the blood velocity waveforms are normally triphasic, reflecting ventricular systole (S-wave), early diastole (D-wave) and atrial contraction (A-wave). Normally, there is always forward flow throughout the cardiac cycle in the DV (Fig. 5). In the IVC and hepatic veins the flow can be towards the heart, zero or away from the heart during atrial contraction (Hecher et al. 1994). From the venous blood flow velocity waveforms, the pulsatility index values for veins (PIV = (PSV − velocity during atrial contraction)/time-averaged maximum velocity over the cardiac cycle) can be calculated (Hecher et al. 1994). The PIV values of the DV, IVC and right hepatic vein have been shown to decrease with advancing gestation (Hecher et al. 1994; Axt-Fliedner et al. 2004; Kessler et al. 2006). In fetal growth restriction, A-wave flow reversal in the DV and/or an increased DV PIV value are related to increased perinatal mortality (Hecher et al. 2001; Ferrazzi et al. 2002; Baschat et al. 2007). At 2 to 7 days before delivery, DV PIV value ≥ 2 SD occurred in 79% of growth restricted fetuses with adverse perinatal outcome whereas the corresponding value was 38% in growth restricted fetuses with normal outcome (Bilardo et al. 2004).

In growth restricted fetuses, abnormal UA ANP, NT-proANP and cardiac Troponin-T serum levels are related to significantly increased DV, left hepatic vein (LHV) and IVC PIV values (Capponi et al. 1997; Makikallio et al. 2002b). In addition, it has been demonstrated that growth restricted fetuses with abnormal umbilical venous Doppler waveforms have significantly lower pH and pO2 values and higher pCO2 values at cordocentesis than those with normal UV Doppler waveforms (Rizzo et al. 1995c). In another study, the best predictive tool for neonatal metabolic compromise (pH < 7.20) was the combination of abnormal IVC, DV and UV blood velocity waveforms with the sensitivity of 89%,
specificity of 48%, and negative predictive value of 92% (Baschat et al. 2004b). In addition, DV blood velocity waveform was reported to be the only statistically significant predictor of intact survival in neonates with birth weight greater than 600 grams and delivery beyond 29 weeks of gestation (Baschat et al. 2007).

Fig. 5. Normal blood velocity waveforms of ductus venosus and umbilical vein (non-pulsatile) (left panel). Abnormal flow reversal during atrial contraction in ductus venosus and end-diastolic umbilical venous pulsations in a fetus with placental insufficiency (right panel).
3 Hypothesis

Abnormal placentation is associated with preeclampsia and placental insufficiency, both of which increase the risk for fetal growth restriction. Early recognition of the risk population for preeclampsia would help the development of new treatments and improve both maternal and fetal outcome in preeclampsia. In this thesis, the first hypothesis was that in preeclampsia, the maternal serum proteomic profile is different from that in uncomplicated pregnancy and this difference is already detectable in early pregnancy. Specific aims were:

1. To determine whether the maternal serum proteomic profile in clinical preeclampsia is different from that in uncomplicated pregnancy (I).
2. To investigate whether the early pregnancy serum proteomic profile of women with later developing preeclampsia differs from the proteomic profile detected in women with uncomplicated pregnancies (I).

Preeclampsia, placental insufficiency, fetal growth restriction and maternal type-1 diabetes can significantly affect fetal cardiovascular hemodynamics. Therefore, the second hypothesis was that in placental insufficiency with or without preeclampsia, fetal hemodynamic abnormalities correlate with biochemical markers of cardiac dysfunction and chronic hypoxia. In addition, in maternal type-1 diabetes, the biochemical markers of fetal cardiac dysfunction are increased and a rise in these biochemical markers involves different mechanistic pathway compared with fetuses who suffered from placental insufficiency. More specific aims were (II–IV):

1. To investigate fetal concentrations of biochemical markers of cardiac dysfunction and chronic hypoxia in pregnancies complicated by placental insufficiency and type-1 diabetes (II–IV).
2. To determine Doppler ultrasonographic parameters related to biochemical evidence of cardiac dysfunction and chronic hypoxia in growth restricted fetuses (II–III).
4 Material and methods

4.1 Study populations

For the nested case-control study I, the subjects with clinical preeclampsia were recruited in the Department of Obstetrics and Gynecology, Oulu University Hospital, Oulu, Finland between years 2004 and 2006. The early pregnancy maternal serum samples for study I were collected from the Finnish Maternity Cohort serum bank at the National Public Health Institute (Table 1). The bank contains serum samples from over 98% of pregnant women in Finland since 1983. These samples are routinely drawn at the Finnish outpatient maternity clinics in the first trimester to screen congenital infections. After the screening, the remaining serum samples are stored at $-25 \, ^\circ C$. The Finnish Maternity Cohort samples used in study I were drawn at the maternity clinics in the area of Oulu University Hospital between years 2004 and 2006. Diagnostic criteria of maternal hypertensive disorder followed the American College of Obstetricians and Gynecologists guidelines (Gynecologists 2002).

The subjects for studies II–IV were recruited during the years 1998–2006 from the Department of Obstetrics and Gynecology, Oulu University Hospital, Oulu, Finland. In study II, the control subjects were recruited from the Department of Obstetrics and Gynecology, University Central Hospital in Helsinki, Finland during the years 1996–1997. The study designs (II–IV) were prospective and cross-sectional. The gestational age was confirmed by ultrasonography prior to 20 weeks of gestation. Cases with abnormal fetal karyotype and major structural anomalies were excluded.

Fetuses with birth weight below the 10th percentile growth curve of a Finnish standard population (Pihkala et al. 1989) and with normal UA blood velocity waveform pattern for gestational age (Acharya et al. 2005c) were considered small for gestational age (II–III). Fetal growth restriction was defined as birth weight below the 10th percentile growth curve (or below the -2SD of the mean) of a Finnish standard population and an abnormal UA blood velocity waveform pattern for gestational age (Acharya et al. 2005c) (II–III). Doppler ultrasonographic evaluation of fetal cardiovascular hemodynamics was performed within 7 days prior to delivery (median 4 (II) and 3 (III) days). The ultrasonographic data in studies II and III was obtained and analyzed by a single investigator blinded to biochemical data. The clinicians involved in the
management of the pregnancies were blinded to the results of the ultrasonographic examinations.

In study IV, women with pregestational type-1 diabetes were included in the study. Glycemic control of type-1 diabetic pregnancy was evaluated by maternal HbA1c (%) values in each trimester. A first trimester HbA1c value of 7.5% was used as a cut-off value when dividing the type-1 diabetic pregnancies into good (HbA1c < 7.5%) and poor glycemic control (HbA1c ≥ 7.5%) categories.

The local ethics committees approved all the study protocols (I–IV) and the subjects gave written informed consents before entering the study protocols (I–IV).
Table 1. Clinical characteristics of the groups in study.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Early pregnancy</th>
<th></th>
<th>Clinical preeclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control n = 96</td>
<td>Mild preeclampsia n = 33</td>
<td>Severe preeclampsia n = 45</td>
</tr>
<tr>
<td>Maternal age (year)</td>
<td>29.4 (18.4–42.3)</td>
<td>27.0 (19.2–38.2)*</td>
<td>30.1 (18.8–42.4)**</td>
</tr>
<tr>
<td>GA at enrollment (week)</td>
<td>12.4 (8.4–23.3)</td>
<td>11.4 (8.1–14.7)*</td>
<td>11.6 (8.3–17.9)*</td>
</tr>
<tr>
<td>GA at delivery (week)</td>
<td>N/A</td>
<td>39.0 (33.0–40.0)</td>
<td>33.0 (25.0–36.0)**</td>
</tr>
<tr>
<td>GA at the onset of preeclampsia (week)</td>
<td>N/A</td>
<td>39.0 (33.0–40.0)</td>
<td>33.0 (25.0–36.0)**</td>
</tr>
<tr>
<td>Birth Weight (g)</td>
<td>3532 (1884–4560)</td>
<td>3307 (990–4345)*</td>
<td>1940 (555–3835)†</td>
</tr>
<tr>
<td>Nulliparity (%)</td>
<td>64.6 (62/96)</td>
<td>69.7 (23/33)</td>
<td>64.4 (29/45)</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>8.3 (8/96)</td>
<td>9.1 (3/33)</td>
<td>0 (0/45)</td>
</tr>
<tr>
<td>Delivery &lt; 37 weeks (%)</td>
<td>2.1 (2/96)</td>
<td>6.1 (2/33)</td>
<td>75.6 (34/45)†</td>
</tr>
</tbody>
</table>

Data expressed as median (range) or percentage (proportion).

* p < 0.05 vs. Control
** p < 0.05 vs. Mild preeclampsia
† p < 0.001 vs. Control and mild preeclampsia
<table>
<thead>
<tr>
<th>Variable</th>
<th>Study II</th>
<th>Study III</th>
<th>Study IV</th>
</tr>
</thead>
</table>
| Included subjects | 1) Controls, n = 49  
2) Small for gestational age fetuses with normal UA PI and DV PIV, n = 13  
3) Growth restricted fetuses with abnormal UA PI and normal DV PIV, n = 15  
4) Growth restricted fetuses with abnormal UA PI and abnormal DV PIV, n = 14 | 1) Controls, n = 19  
2) Small for gestational age fetuses with normal UA PI and DV PIV, n = 9  
3) Growth restricted fetuses with abnormal UA PI and normal DV PIV, n = 18  
4) Growth restricted fetuses with abnormal UA PI and abnormal DV PIV, n = 11 | 1) Controls, n = 60  
2) Type-1 diabetic mothers with good glycemic control in the first trimester (HbA1c < 7.5%), n = 22  
3) Type-1 diabetic mothers with poor glycemic control in the first trimester (HbA1c ≥ 7.5%), n = 10 |
| Biochemical markers | UA NT-proANP  
UA NT-proBNP | UA EPO  
UA NT-proBNP | UA NT-proANP  
UA NT-proBNP |
| Ultrasonographic parameters | UA PI, DAO PI, MCA PI  
Retrograde Aol (%)  
CCO, LVCO, RVCO  
RVeFo, LVeFo  
IRT, IMP  
TV and MV TVI  
TR, RVFS, LVFS  
CC/TC -ratio  
IVC, LHV and DV PIV  
Pulsations in IA UV | UA PI, DAO PI, MCA PI  
Retrograde Aol(%)  
CCO, LVCO, RVCO  
RVeFo, LVeFo  
IRT, IMP  
TV and MV TVI  
TR, RVFS, LVFS  
CC/TC -ratio  
IVC, LHV and DV PIV  
Pulsations in IA UV | UA PI  
Retrograde Aol(%)  
CCO, LVCO, RVCO  
RVeFo, LVeFo  
IRT, IMP  
TV and MV TVI  
TR, RVFS, LVFS  
CC/TC -ratio  
IVC, LHV and DV PIV  
Pulsations in IA UV |
4.2 Analysis of biochemical markers

4.2.1 Maternal serum samples (I)

For a maternal serum sample at least 5ml of blood was drawn from the maternal cubital vein. The samples were centrifuged and the sera were stored at −80 °C until analyzed.

4.2.2 Fetal serum samples (II–IV)

The fetal biochemical markers were analyzed from UA blood samples (at least 1ml) collected immediately after delivery in serum tubes and centrifuged. Serum samples were stored at −80 °C until analyzed.

4.2.3 Proteomic analysis (I)

For proteomic approaches, 6 to 8 samples representing each group were pooled to perform in-depth protein identification and quantification using MS. A total of six sample groups were prepared for proteomic analysis. Three of the sample groups were in the clinical preeclampsia population (control, mild preeclampsia and severe preeclampsia) and, similarly, three sample groups were prepared for early pregnancy study group (control, mild and severe preeclampsia). Immunoassay validation of specific biomarkers was conducted on all samples individually to assess their performance.

Multidimensional Liquid Chromatography Tandem Mass Spectrometry (2D-LC-MS/MS)

Samples representing each group were subjected to 2D-LC-MS/MS and label-free quantification to identify differentially expressed proteins among the groups. A total of 1.0 mg protein from each sample was digested using trypsin. The resulting peptides were separated using strong cation exchange column into 32 fractions. These fractions were analyzed using an Agilent 1100 liquid chromatographer connected to a LTQ mass spectrometer (Thermo Finnegan, San Jose, CA). Peptides present in each sample were identified by searching corresponding mass spectra against a protein database containing forward and reverse entries of the Swiss-Prot human database (version 46.6), using two
independent search engines: TurboSequest (Thermo Finnegan, San Jose, CA) and X! Tandem. Peptide identifications from a sample were assembled into protein identifications using Scaffold software (v1.3.2, Proteome Software, Portland, OR). Protein identifications that had at least two independent peptide identifications were considered to be present in the sample. The total number of mass spectra matched to a particular protein was used to assess the relative abundance of a protein in a sample using a label-free quantification method.

**Enzyme-Linked Immunosorbent Assay**

Concentrations of 31 potential biomarkers in maternal serum were analyzed by enzyme-linked immunosorbent assay (ELISA). Antibodies and pure antigens from multiple vendors were used to develop specific sandwich immunoassays. Standard curves were developed using known quantities of recombinant proteins or standards provided by the manufacturer to reference sample concentrations. All assays were performed in triplicate. The inter-assay and intra-assay coefficient of variations ranged from 3–7%.

**4.2.4 N-terminal peptides of proANP and proBNP**

NT-proANP and NT-proBNP were determined in duplicates by specific radioimmunoassay from 50 and 25 µl unextracted serum, respectively, and reassayed if necessary, after appropriate dilution with the assay buffer as described in detail previously (Ala-Kopsala et al. 2004). Due to the development of the radioimmunoassay, the antisera of the assays utilized in studies II (year 2001) and IV (year 2003) were different. Because of the difference in the method, the absolute concentrations of the assay in study IV are approximately 30–40% lower than in study II (Ala-Kopsala et al. 2004).

In study II, the NT-proANP antiserum was specific to human NT-proANP79-98, and the NT-proBNP antiserum to human NT-proBNP324. The sensitivities of the NT-proANP and NT-proBNP assays were 30 and 63 pmol/L serum, respectively. In study IV, the NT-proANP and NT-proBNP antiseras employed in the assay recognized the sequences proANP46-79 and proBNP10-29, respectively. The detection limit for NT-proBNP was 40 pmol/L and for NT-proANP 60pmol/L (Ala-Kopsala et al. 2004; Ala-Kopsala et al. 2005). The 95th percentile values of UA NT-proBNP (Study II: 518pmol/L, Study IV: 370pmol/L) and NT-proANP (Study II: 1572pmol/L Study IV: 2048pmol/L) in the control groups of the studies
were used as a cut-off level for abnormal and normal categories. The within- and between-assay coefficients of variations were < 15% and 20%, respectively (Ala-Kopsala et al. 2004).

4.2.5 Erythropoietin

The EPO concentrations were analyzed from the UA serum samples in duplicates by a chemiluminescent immunological method (Immulite, Diagnostic Products, Los Angeles, CA). The intra- and inter-assay coefficients of variation were 6.4–9.9% and 8.8–13.2%, respectively. The 90th percentile value of UA EPO in the control group (29 mU/mL) was used to classify the studied fetuses into normal and abnormal categories.

4.3 Ultrasonographic examination (II–IV)

Ultrasonographic examinations were performed by image-directed color and pulsed Doppler equipment (Sequoia 512; Acuson, Mountain View, CA) with a 4- to 8-MHz convex or a 5-MHz sector probe. The high pass filter was set at minimum. The acoustic output of the system was controlled according to the ECMUS recommendations (ECMUS 1999; ECMUS 1999). An angle of less than 15 degrees between the vessel and the Doppler beam was accepted for analysis. All the ultrasonographic data were videotaped and analyzed afterwards with the ultrasound equipment’s own cardiac measurement package. Three consecutive cardiac cycles were analyzed and their mean values were used for further analysis.

4.3.1 Placental hemodynamics

Placental circulation was assessed by determining the UA PI values in a free loop of the umbilical cord.
4.3.2 Assessment of fetal cardiac function

**Cardiac size**

The cardiac (CC) and thoracic (TC) circumferences were measured at the level of a four-chamber view and the CC/TC ratio was calculated (Respondek et al. 1992).

**Volume blood flows**

For the volume blood flow calculations, the diameters of pulmonary (PV) and aortic (AoV) valves were measured in frozen real-time images during systole using the leading edge-to-leading edge method. The mean value of three separate valve diameter measurements was used for calculation of the cross-sectional area (CSA) of the valve. The calculations were based on the assumption that the annuli were circular. The PV and AoV blood velocity waveforms were obtained and their time-velocity integrals (TVI) were calculated by planimetricing the area underneath the Doppler spectrum. Volume blood flows (Q) across PV and AoV were calculated \( Q = CSA \times TVI \times FHR \). The LVCO equals \( Q_{AoV} \), the RVCO equals \( Q_{PV} \), and their sum is the CCO. The weight indexed CCO, RVCO and LVCO as well as the proportions of ventricular cardiac outputs (RVCO% and LVCO%) of CCO, were calculated. The actual birth weight was used for indexing purposes because the time interval between the ultrasonographic examination and delivery was 7 days or less.

**Time-intervals**

The left ventricular time intervals were assessed by obtaining simultaneously mitral valve (MV) and AoV blood velocity waveforms. The IRT was measured as the time period between the end of ejection and the onset of filling (Tulzer et al. 1994). The corresponding proportion of IRT (IRT%) of the total cardiac cycle was calculated. Global cardiac performance was evaluated by calculating the IMP (Tei et al. 1995; Tsutsumi et al. 1999).

**Inflow waveforms**

Inflow blood velocity waveforms were recorded at the level of the tricuspid (TV) and mitral (MV) valves. The TVI-ratio between early ventricular filling (E-wave)
and filling during atrial contraction (A-wave), and A-wave TVI to total TVI-ratio were calculated from both ventricles (Tulzer et al. 1994).

**Ventricular ejection forces**

The right (RVeFo) and left (LVeFo) ventricular ejection forces were calculated by the formula: \( (1.055 \times \text{CSA} \times \text{TVI}_{\text{ac}}) \times (\text{PSV}/\text{TTP}) \), in which TVI_{ac} is the time-velocity integral during the acceleration period of systole, PSV is the peak systolic velocity, and TTP is the time to peak velocity interval (Sutton et al. 1991b; Rizzo et al. 1995b). Ventricular ejection forces were weight indexed.

**Tricuspid regurgitation**

The presence of tricuspid regurgitation (TR) was noted and classified as trivial (non-holosystolic, ≥ 72 ms) or holosystolic (Respondek et al. 1994).

**M-mode recordings**

M-mode recordings were performed by placing the M-mode cursor perpendicularly toward the interventricular septum at the level of atrioventricular valves in a four-chamber view of the heart. The right (RVFS%) and left (LVFS%) ventricular fractional shortenings were calculated from M-mode recordings \([\text{ventricular fractional shortening } \% = (\text{inner diastolic diameter} - \text{inner systolic diameter})/\text{inner diastolic diameter} \times 100]\) (DeVore et al. 1984).

**4.3.3 Fetal arterial circulation**

Blood velocity waveforms of fetal MCA were obtained by placing the Doppler gate along the course of MCA. Blood flow in the DAo was assessed at the level of the diaphragm. Distribution of fetal arterial circulation was assessed by calculating the DAo/MCA and UA/MCA PI ratios. AoI blood velocity waveforms were recorded from a sagittal view, the TVIs of antegrade and retrograde AoI blood flow components were measured, and the ratio between the TVI components was calculated. Net blood flow in AoI was considered to be antegrade if the ratio was > 1 and retrograde if the ratio was < 1 (Fouron et al. 1999a). In addition, the visualization of coronary arteries demonstrating the heart sparing effect was noted (Baschat et al. 1997).
4.3.4 Fetal venous circulation

Pulsatility indices for veins were calculated from DV, IVC and LHV blood velocity waveforms. Pulsations occurring during atrial contraction in intra-abdominal umbilical vein and in free loops of umbilical vein were noted.

4.4 Statistical analysis

In study I, the statistical analysis of the data was performed using a SAS program (version 9.1). In the analysis of 2D-LC-MS/MS data, pair-wise comparisons were performed using either a 2 × 2 Chi-square test or Fisher exact test. Normalization of spectral counts to account for experimental variability was built into the pair-wise comparisons. Level of significance was set at 0.05. The fold expression change of differentially expressed proteins was quantified using the previously published equation (Old et al. 2005). Candidate protein biomarker concentrations (ng/ml) measured by ELISA experiments in control, mild preeclampsia, and severe preeclampsia samples were log transformed before statistical analysis. Independent pair-wise comparisons of between control and preeclampsia groups were performed using ANOVA test. The average value on the log-scale values was transformed back to original units (harmonic mean) for presentation. The Bonferroni correction was applied to adjust for multiple comparisons.

Simple logistic regression models with a binary dependent variable designating preeclampsia status (1 = preeclampsia n = 60, 0 = Control n = 58) were fit for each biomarker individually. The predicted values from these models were used to create Receiver Operating Characteristic (ROC) curves. ROC curves are plots of the true positive fraction of a test (sensitivity) versus the false positive fraction (1-specificity) across the entire continuum of observed values. The area under the curve (AUROC) should be between 0.5 (poor discriminant) to 1.0 (perfect discriminant), and can be expressed probabilistically as the probability that a randomly selected pair of preeclampsia and control subjects is correctly classified. Standard errors for the AUROCs were conducted based on percentiles of bootstrapped distributions.

To explore the possibility that two or more markers might be combined to improve classification accuracy, the multi-variable logistic regression models were fit to develop risk scores. Based on results from single proteins, the classification performance of several different combinations of 2, 3 or 4 proteins
was evaluated. ROC curves and other corresponding measures were computed based on each of the multi-protein models.

For studies II–IV the statistical analyses were made using the StatView for Macintosh (version 5.0; SAS Institute Inc., Chicago, IL, USA) and the SPSS for Windows (versions 13.0–16.0; SPSS Inc., Chicago, IL, USA) software packages. Comparisons between two groups were performed using Student’s t-test if the data were normally distributed or, otherwise, the Mann-Whitney U-test was chosen (IV). The analysis of variance (ANOVA) was used when the comparisons were made between more than two groups (II–III) and the data were normally distributed, otherwise, the nonparametric Kruskal-Wallis test was chosen. If statistical significance in ANOVA was shown, the Scheffe F-test was used for further analysis. Categorical data were compared using the chi-square test (II–IV). Linear regression analysis was used to show the relationship between the biochemical markers and ultrasonographic parameters (II–III). The non-parametric Spearman’s rho test was used to investigate the relationship between UA NT-proANP and NT-proBNP levels and maternal glycemic control in study IV. A p-value of 0.05 or less was considered statistically significant.
5 Results

5.1 Proteomic analysis in preeclampsia

5.1.1 Maternal serum proteomic profile in clinical preeclampsia

A total of 457 unique proteins were identified in the 2D-LC-MS/MS analysis. Label-free quantification and independent pair-wise comparisons for relative abundance of each protein between women with and without preeclampsia identified 38 maternal serum proteins to be differentially expressed. The potential biomarkers included pregnancy proteins such as choriogonadotropin and pappalysin-2 (PAPP-A2), and extracellular matrix signaling factors such as fibronectin. Sixteen of these potential biomarkers were selected for further validation by immunoassay, based on statistical significance, potential clinical relevance and/or availability of sensitive and specific immunoassays. As summarized in Table 3, several biomarkers showed statistically significant differences in concentrations among women with and without preeclampsia. Fibronectin, PAPP-A2, choriogonadotropin-ß, apolipoprotein C-III, cystatin-C and endoglin, had significantly higher concentrations in maternal serum from women with clinical preeclampsia. Matrix metalloproteinase-9 (MMP-9) was significantly lower in clinical preeclampsia. Fibronectin, PAPP-A2, endoglin, cystatin-C and apolipoprotein C-III had the best classification performance with AUROCs of 0.91, 0.89, 0.86, 0.77 and 0.76, respectively. A three-analyte model including fibronectin, PAPP-A2 and MMP-9 had an improved AUROC of 0.944 (95% CI 0.90–0.98). (Table 3)
Table 3. Clinical preeclampsia: Differences in 16 candidate protein biomarkers in serum samples from women with preeclampsia and from women with uncomplicated pregnancy. Early pregnancy serum proteomic profile in women who developed preeclampsia.

<table>
<thead>
<tr>
<th>Protein (ng/ml)</th>
<th>Geometric Mean Value for Each Group</th>
<th>Area Under ROC Curve (AUROC)</th>
<th>95% CI for AUROC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preeclampsia n = 60</td>
<td>Control n = 58</td>
<td>Preeclampsia vs. Control p-value</td>
</tr>
<tr>
<td>Apolipoprotein B-100</td>
<td>18237918</td>
<td>24945198</td>
<td>0.0028*</td>
</tr>
<tr>
<td>Cystatin-C</td>
<td>2116</td>
<td>1651</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>Endoglin</td>
<td>97</td>
<td>55</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1020441</td>
<td>228002</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>Plasma retinol-binding protein</td>
<td>20540</td>
<td>17573</td>
<td>0.0214</td>
</tr>
<tr>
<td>Apolipoprotein C-III</td>
<td>136861</td>
<td>92501</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>Chorionic somatomammotropin hormone (CSH1)</td>
<td>1803</td>
<td>996</td>
<td>0.0020*</td>
</tr>
<tr>
<td>Choriogonadotropin-ß</td>
<td>734</td>
<td>99</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>VEGF receptor 3</td>
<td>51</td>
<td>60</td>
<td>0.2594</td>
</tr>
<tr>
<td>Histidine-rich glycoprotein</td>
<td>221238</td>
<td>161983</td>
<td>0.0169</td>
</tr>
<tr>
<td>Insulin-like growth factor-binding protein 2</td>
<td>110</td>
<td>91</td>
<td>0.0088</td>
</tr>
<tr>
<td>MMP9</td>
<td>228</td>
<td>447</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>Pregnancy-specific beta-1-glycoprotein 1</td>
<td>35904</td>
<td>32534</td>
<td>0.2968</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>1196</td>
<td>1203</td>
<td>0.9748</td>
</tr>
<tr>
<td>Vascular endothelial growth factor receptor 1 (sFlt-1)</td>
<td>19</td>
<td>5</td>
<td>&lt; 0.0001*</td>
</tr>
<tr>
<td>Fibronectin + PAPPA2</td>
<td>0.927</td>
<td></td>
<td>(0.88–0.97)</td>
</tr>
<tr>
<td>Fibronectin + PAPPA2 + MMP9</td>
<td>0.944</td>
<td></td>
<td>(0.90–0.98)</td>
</tr>
<tr>
<td>Fibronectin+PAPPA2+MMP9</td>
<td>0.956</td>
<td></td>
<td>(0.92–0.99)</td>
</tr>
</tbody>
</table>

* Statistically significant difference between the groups after Bonferroni adjustment for multiple comparisons

In 2D-LC-MS/MS analysis, a total of 416 proteins in the maternal serum were identified for label-free quantification. 32 serum proteins showed significant differences between controls and women who developed preeclampsia.
subsequently. The potential biomarkers included placental proteins, choriogonadotropin-ß, chorionic somatomammotropin hormone (CSH1) and PAPP-A2. Other candidates include vascular or transport proteins, matrix and/or acute phase proteins such as tubulin beta-1 chain plasma protease C1 inhibitor, plasmin-2 and ADAMTS-13.

Immunoassays were performed for 15 available targets on all 178 subjects in this group. As shown in Table 4, complement factor D, vascular cell adhesion protein 1, PAPP-A1, cystatin-C, vasorin and ß-2-microglobulin differed significantly between the control and preeclamptic pregnancies. On the other hand, the early pregnancy maternal serum endoglin, VEGF, fibronectin and sFlt-1, which all are markers for active preeclampsia, did not differ between uncomplicated pregnancies and pregnancies that developed preeclampsia.

<table>
<thead>
<tr>
<th>Protein (ng/ml)</th>
<th>Geometric Mean Value for Each Group</th>
<th>Area Under ROC Curve (AUROC)</th>
<th>95% CI for AUROC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preeclampsia n = 78</td>
<td>Control n = 96</td>
<td>Preeclampsia vs. Control p-value</td>
</tr>
<tr>
<td>Apolipoprotein A-II</td>
<td>424120</td>
<td>436348</td>
<td>0.4836</td>
</tr>
<tr>
<td>Beta-2-microglobulin</td>
<td>1203</td>
<td>1103</td>
<td>0.0158</td>
</tr>
<tr>
<td>Complement factor D</td>
<td>2201</td>
<td>1914</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Varioxin</td>
<td>7759</td>
<td>7062</td>
<td>0.0207</td>
</tr>
<tr>
<td>Alpha-2-antiplasmin</td>
<td>1021</td>
<td>831</td>
<td>0.3814</td>
</tr>
<tr>
<td>Apolipoprotein C-III</td>
<td>52995</td>
<td>52687</td>
<td>0.9151</td>
</tr>
<tr>
<td>Vascular cell adhesion protein 1</td>
<td>12416</td>
<td>10794</td>
<td>0.0010*</td>
</tr>
<tr>
<td>Alpha-2-macroglobulin</td>
<td>1733583</td>
<td>1864547</td>
<td>0.2330</td>
</tr>
<tr>
<td>PAPP-A1</td>
<td>401</td>
<td>1935</td>
<td>0.0091</td>
</tr>
<tr>
<td>Apolipoprotein B-100</td>
<td>20721329</td>
<td>20942859</td>
<td>0.8119</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>703149</td>
<td>685658</td>
<td>0.8633</td>
</tr>
<tr>
<td>Plasma retinol-binding protein</td>
<td>21801</td>
<td>21942</td>
<td>0.8071</td>
</tr>
<tr>
<td>Lipopolysaccharide-binding protein</td>
<td>44266</td>
<td>40593</td>
<td>0.3203</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>3372</td>
<td>3019</td>
<td>0.4423</td>
</tr>
</tbody>
</table>

sFlt-1                           | 3                                   | 3                           | 0.4799          | 0.55 (0.47–0.68) |

* statistically significant difference between the groups after Bonferroni adjustment for multiple comparisons
5.2 Fetal cardiovascular responses to placental insufficiency

5.2.1 Clinical outcome

Perinatal data of studies II–IV is shown in Table 5. The growth restricted fetuses with either normal or abnormal DV PI values demonstrated significantly lower pO2 values compared to the control fetuses (II) and the small for gestational age fetuses (III).

Table 5. a) Clinical characteristics of the groups in studies II–IV. In studies II and III, group 1 represents small for gestational age fetuses, group 2 growth restricted fetuses with abnormal UA and normal DV velocimetries and group 3 fetuses had both abnormal UA and DV velocimetries. Values are given as mean (SD), median (range) or % (n).

<table>
<thead>
<tr>
<th>Study</th>
<th>GA at delivery (weeks)</th>
<th>Birth weight (grams)</th>
<th>UA pH</th>
<th>5 min Apgar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 49)</td>
<td>40.9 (1.0)</td>
<td>3711 (431)</td>
<td>7.24 (0.07)</td>
<td>9 (9–10)</td>
</tr>
<tr>
<td>Group 1 (n = 13)</td>
<td>37.9 (1.7)*</td>
<td>2525 (274)*</td>
<td>7.26 (0.05)</td>
<td>9 (7–10)</td>
</tr>
<tr>
<td>Group 2 (n = 15)</td>
<td>34.6 (3.6)**†</td>
<td>1778 (632)**†</td>
<td>7.28 (0.05)</td>
<td>8 (5–10)*</td>
</tr>
<tr>
<td>Group 3 (n = 14)</td>
<td>32.8 (4.2)**†</td>
<td>1432 (661)**†</td>
<td>7.24 (0.04)</td>
<td>8 (1–9)**†</td>
</tr>
<tr>
<td>Study III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (n = 9)</td>
<td>37.9 (1.1)</td>
<td>2367 (379)</td>
<td>7.25 (0.05)</td>
<td>9 (7–10)</td>
</tr>
<tr>
<td>Group 2 (n = 16)</td>
<td>33.8 (3.9)**</td>
<td>1735 (773)**</td>
<td>7.27 (0.05)</td>
<td>8 (3–10)</td>
</tr>
<tr>
<td>Group 3 (n = 11)</td>
<td>34.5 (3.2)**</td>
<td>1643 (497)**</td>
<td>7.22 (0.05)</td>
<td>9 (6–9)</td>
</tr>
<tr>
<td>Study IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 60)</td>
<td>40.4 (1.2)</td>
<td>3548 (418)</td>
<td>7.23 (0.07)</td>
<td>9 (7–10)</td>
</tr>
<tr>
<td>Good early pregnancy glycemic control (n = 22)</td>
<td>37.3 (2.1)**†</td>
<td>3682 (555)</td>
<td>7.22 (0.09)</td>
<td>9 (8–10)</td>
</tr>
<tr>
<td>Poor early pregnancy glycemic control (n = 10)</td>
<td>36.8 (1.7)**</td>
<td>4032 (559)*</td>
<td>7.22 (0.06)</td>
<td>9 (7–10)</td>
</tr>
<tr>
<td>Study</td>
<td>Cesarean section % (n)</td>
<td>NT-proANP (pmol/l)</td>
<td>NT-proBNP (pmol/l)</td>
<td>EPO (pU/l)</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------</td>
<td>--------------------</td>
<td>-------------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Study II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 49)</td>
<td>6.1 (3/49)</td>
<td>705 (258–1615)</td>
<td>311 (156–1870)</td>
<td></td>
</tr>
<tr>
<td>Group 1 (n = 13)</td>
<td>46.2 (6/13)*</td>
<td>1320 (732–2975)*</td>
<td>334 (156–2251)</td>
<td></td>
</tr>
<tr>
<td>Group 2 (n = 15)</td>
<td>73.3 (11/15)*</td>
<td>1660 (699–7500)*</td>
<td>453 (263–2498)</td>
<td></td>
</tr>
<tr>
<td>Group 3 (n = 14)</td>
<td>85.7 (12/14)**‡</td>
<td>2159 (1101–9574)** †</td>
<td>1263 (380–21000)<strong>,</strong>*</td>
<td></td>
</tr>
<tr>
<td><strong>Study III</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (n = 9)</td>
<td>33.3 (3/9)</td>
<td></td>
<td></td>
<td>83 (10–331)</td>
</tr>
<tr>
<td>Group 2 (n = 16)</td>
<td>66.7 (12/18)</td>
<td></td>
<td></td>
<td>476 (12–7470)</td>
</tr>
<tr>
<td>Group 3 (n = 11)</td>
<td>91.0 (10/11)</td>
<td></td>
<td></td>
<td>2975 (37–17100)<strong>,</strong>*</td>
</tr>
<tr>
<td><strong>Study IV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 60)</td>
<td>8.3 (5/60)</td>
<td>1100 (114–2357)</td>
<td>195 (49–414)</td>
<td></td>
</tr>
<tr>
<td>Good early pregnancy glycemic control (n = 22)</td>
<td>72.7 (16/22)*</td>
<td>2439 (259–8995)*,**</td>
<td>385 (166–2335)†</td>
<td></td>
</tr>
<tr>
<td>Poor early pregnancy glycemic control (n = 10)</td>
<td>80.0 (8/10)**</td>
<td>4613 (1980–11070)**</td>
<td>768 (145–1375)**</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05 vs. Controls, **p < 0.05 vs. Group 1 (Good early pregnancy glycemic control in study IV), ***p < 0.05 vs. Group 2, †p < 0.001 vs. Controls
5.2.2 Biochemical markers of fetal cardiac dysfunction and chronic hypoxia

Small for gestational age fetuses demonstrated increased UA NT-proANP levels compared to the control fetuses \((p < 0.001)\), with no significant difference in UA NT-proBNP and UA EPO concentrations (II). However, a total of 4 out of 9 (44%) small for gestational age fetuses demonstrated EPO concentrations exceeding the 90\(^{th}\) percentile value of the control group (29 mU/ml) (III), and a total of 4 out of 13 (31%) had NT-proANP levels higher than the 95\(^{th}\) percentile value of the control group (1572 pmol/l) (II). Growth restricted fetuses with normal DV PIV values demonstrated significantly increased UA NT-proANP and UA EPO concentrations compared to the control fetuses. In fetuses with abnormal UA and DV velocimetry, UA NT-proBNP levels were significantly higher than in control group and in groups 1 and 2 in fetuses with abnormal UA and DV velocimetry even exceeded these concentrations (II)(Fig. 6). In addition, these fetuses demonstrated higher UA EPO concentrations \((p < 0.05)\) than the fetuses with normal DV velocimetry (III). No difference was found in UA NT-proANP or UA EPO values between small for gestational age fetuses and the growth restricted fetuses with normal DV PI values (II, III).
5.2.3 Ultrasonographic parameters related to increased levels of biochemical markers

Fetal heart

UA NT-proBNP levels correlated positively with fetal weight-indexed LVCO \( (r = 0.33, \ p = 0.03) \) and the proportion of LVCO of the total CCO (LVCO%) \( (r = 0.39, \ p = 0.01) \), whereas, a negative correlation was found between UA NT-proBNP concentrations and the proportion of RVCO of the total CCO.
(RVCO%) (0.39, p = 0.01) (II). No significant difference was found in cardiac volumetric blood flows between the fetuses with either increased or normal UA EPO concentrations (III). Weight-indexed RVeFo and LVeFo were not associated with UA NT-proANP, NT-proBNP and EPO concentrations. The TV and MV total TVIs and their ratios as well as IRT% and IMP did not correlate with biochemical markers (II, III).

Significant negative correlation was found between UA NT-proBNP levels and LVFS% (r = 0.41, p = 0.02). However, no significant differences were found in the CC/TC ratio, RVFS% or LVFS% between the study groups. The incidence of TR was similar between fetuses with either normal or elevated UA NT-proBNP and UA EPO concentrations.

**Fetal arterial circulation**

Significant positive correlation was found between UA PI values and both UA NT-proBNP and UA LnEPO concentrations (II, III). The fetuses with the highest UA NT-proBNP and UA EPO levels had significantly increased DAo PI values and significantly greater UA/MCA PI and DAo/MCA PI ratios than other growth restricted fetuses (II, III). Growth restricted fetuses with abnormal DV velocimetry and increased UA NT-proBNP levels also had a higher visualization rate of coronary arteries (p < 0.05) compared to the growth restricted fetuses with normal DV velocimetry (II). The retrograde AoI net blood flow was present more frequently in growth restricted fetuses with elevated UA EPO levels (III). MCA PIs did not correlate with fetal UA EPO concentration. In addition, no differences were found in MCA PI values or in the incidence of retrograde AoI net blood flow as regards to UA NT-proBNP or UA NT-proANP levels (II). (Tables 6 and 7)

**Fetal venous circulation**

The fetuses with abnormal DV blood velocity waveform pattern demonstrated the higher UA NT-proANP, NT-proBNP and UA EPO concentrations compared to the fetuses with normal DV PIV values (II, III). DV PIV and LHV PIV values correlated positively with UA LnNT-proBNP (r = 0.70, p = 0.0001 and r = 0.63, p = 0.0001) (II) and UA LnEPO (r = 0.60, p < 0.0001 and r = 0.44, p = 0.006) (II) concentrations. The likelihood of having abnormal UA NT-proBNP value (> 95th percentile) in the presence of UV pulsations was 90%. The corresponding likelihood for abnormal DV PIV was 93% (II). (Tables 6 and 7)
Table 6. Ultrasonographic parameters of fetal hemodynamics and umbilical artery (UA) NT-proBNP concentrations in study II. Values given are mean (SD), median (range) or %.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Small for gestational age fetuses</th>
<th>Growth restricted fetuses with normal DV PIV</th>
<th>Growth restricted fetuses with abnormal DV PIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA NT-proBNP</td>
<td>334 (156–2251)</td>
<td>453 (263–2498)</td>
<td>1263 (380–21000)†</td>
</tr>
<tr>
<td>UA PI</td>
<td>0.94 (0.12)</td>
<td>1.61 (0.42)*</td>
<td>3.17 (0.78)**†</td>
</tr>
<tr>
<td>DAO PI</td>
<td>2.27 (0.34)</td>
<td>2.63 (0.64)</td>
<td>1.17 (0.78)*†</td>
</tr>
<tr>
<td>Retrograde AoI (%)</td>
<td>7.7</td>
<td>26.7</td>
<td>42.9</td>
</tr>
<tr>
<td>Visualization rate of coronary arteries (%)</td>
<td>7.7</td>
<td>13.3</td>
<td>50.0 †</td>
</tr>
<tr>
<td>IVC PIV</td>
<td>2.18 (0.17–2.99)</td>
<td>2.32 (1.03–3.35)</td>
<td>3.28 (2.22–144.51)**†</td>
</tr>
<tr>
<td>LHV PIV</td>
<td>2.52 (0.55)</td>
<td>4.05 (1.94)</td>
<td>5.91 (2.16)**†</td>
</tr>
<tr>
<td>DV PIV</td>
<td>0.43 (0.13)</td>
<td>0.54 (0.14)</td>
<td>1.10 (0.38)**†</td>
</tr>
<tr>
<td>UV pulsations (%)</td>
<td>0</td>
<td>6.7</td>
<td>64.3 **†</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. small for gestational age fetuses, **p < 0.01 vs. small for gestational age fetuses, †p < 0.05 vs. growth restricted fetuses with normal DV PIV

Table 7. Ultrasonographic parameters of fetal hemodynamics and UA EPO concentrations in study III. Values given are mean (SD), median (range) or %.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Small for gestational age fetuses</th>
<th>Growth restricted fetuses with normal DV PIV</th>
<th>Growth restricted fetuses with abnormal DV PIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA EPO</td>
<td>83 (10–331)</td>
<td>476 (12–7470)</td>
<td>2975 (37–17100)†</td>
</tr>
<tr>
<td>UA PI</td>
<td>0.95 (0.11)</td>
<td>1.77 (0.69)*</td>
<td>2.27 (1.22)*</td>
</tr>
<tr>
<td>DAO PI</td>
<td>2.39 (0.44)</td>
<td>2.67 (0.57)</td>
<td>3.37 (1.13)*†</td>
</tr>
<tr>
<td>Retrograde AoI (%)</td>
<td>33.3</td>
<td>29.4</td>
<td>72.7 **†</td>
</tr>
<tr>
<td>Visualization rate of coronary arteries (%)</td>
<td>11.1</td>
<td>11.1</td>
<td>36.4</td>
</tr>
<tr>
<td>IVC PIV</td>
<td>2.20 (1.60–3.30)</td>
<td>2.48 (1.03–3.49)</td>
<td>2.74 (1.93–144.51)</td>
</tr>
<tr>
<td>LHV PIV</td>
<td>2.30 (1.91–3.17)</td>
<td>3.36 (1.86–9.37)</td>
<td>4.02 (1.19–30.93)**†</td>
</tr>
<tr>
<td>DV PIV</td>
<td>0.45 (0.13)</td>
<td>0.53 (0.16)</td>
<td>1.10 (0.38)**†</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. small for gestational age fetuses, **p < 0.01 vs. small for gestational age fetuses, †p < 0.05 vs. growth restricted fetuses with normal DV PIV
5.3  Fetal cardiovascular responses in type-1 diabetic pregnancy (IV)

5.3.1 Clinical outcome

The neonates of type-1 diabetic mothers were delivered significantly earlier and more often by cesarean section (p < 0.001) than the neonates of the control group. In addition, the neonates of mothers with poor glycemic control in early pregnancy had significantly greater birth weight than the control neonates. No significant differences were found in UA pH, pO2, pCO2 and BE values or in the Apgar scores at 5 min between type-1 diabetic pregnancies and the control group. (Table 5)

5.3.2 Biochemical markers of fetal cardiac dysfunction

Newborn UA NT-proANP and NT-proBNP concentrations were significantly higher in type-1 diabetic pregnancies than in the control pregnancies (Table 5). In pregnancies with poor glycemic control during the first trimester UA NT-proANP levels were even higher (p < 0.05) than in the group with good glycemic control. Nine out of 10 (90%) newborns of mothers with poor glycemic control had UA NT-proANP and NT-proBNP concentrations exceeding the 95th percentile value in the control group, while the corresponding incidence was 12 out of 22 (55%) in the group with good glycemic control. UA NT-proANP levels correlated with maternal HbA1c values in the first (r = 0.421, p = 0.016), in the second (r = 0.414, p = 0.021) and in the third trimesters (r = 0.391, p = 0.029). No significant correlation was found between UA NT-proBNP levels and maternal HbA1c values. In all type-1 diabetic pregnancies, a normal UA velocimetry for gestational age was demonstrated.
6 Discussion

Based on meta-analysis of various studies, antiplatelet agents, mainly low-dose aspirin, moderately reduces the risk of preeclampsia and its consequences (Duley et al. 2004; Askie et al. 2007). In most of the studies, the antiplatelet treatment is started in the second trimester or even later (Duley et al. 2004; Askie et al. 2007). However, the present understanding is that the pathophysiologic insults leading to clinical preeclampsia may develop already during early pregnancy (Roberts et al. 1999; Matthiesen et al. 2005; Jauniaux et al. 2006), suggesting that early pregnancy would be the most beneficial time for antiplatelet or other preventive medication. Early recognition of the patients at increased risk for preeclampsia would be crucial for new pharmacological interventions and preventive measures in order to improve the outcome of these pregnancies.

Doppler ultrasonography allows the investigation of fetal cardiovascular hemodynamics and plays an important role in the evaluation of fetal wellbeing. In placental insufficiency, fetal protective mechanisms include i.e. redistribution of fetal cardiac output (brain-sparing and heart-sparing effects), increased oxygen extraction from erythrocytes and reduction in the fetal oxygen consumption (Fouron 1999b). Previous studies have demonstrated the importance of AoI Doppler in the evaluation of fetal wellbeing during chronic hypoxia (Fouron et al. 1999a; Makikallio et al. 2002a; Makikallio et al. 2003; Fouron et al. 2005; Del Rio et al. 2008). Increased systemic venous pressure is previously suggested to be a sign of inadequate fetal tissue perfusion (Hecher et al. 1995a). Longitudinal follow-up studies of growth restricted fetuses have demonstrated that abnormal DV velocimetry is a late sign of fetal distress (Hecher et al. 2001; Ferrazzi et al. 2002). Abnormal fetal cardiovascular hemodynamics have also been reported in type-1 diabetic pregnancies (Rasanen et al. 1987; Rizzo et al. 1991b; Tsyvian et al. 1998; Lisowski et al. 2003), suggesting altered fetal cardiac function in these pregnancies. The clinical significance of these cardiovascular changes has been unclear. Despite improved care and knowledge, the fetuses of diabetic mothers remain at increased risk for congenital heart defects and unexplained intrauterine death (Suhonen et al. 2000; Jensen et al. 2004).
6.1 Validation of maternal proteomic analysis

In this study, the previously described proteomic analysis methods were used (Gravett et al. 2004; Gravett et al. 2007; Nagalla et al. 2007; Pereira et al. 2007). For protein separation, 2D-LC-MS/MS method was used to make the protein identification more specific (Nesvizhskii et al. 2007). Statistical power of protein or peptide identification procedures are influenced by factors like the discriminative ability of the database search score, the quality of the spectra and the size of the database (Nesvizhskii et al. 2007). In this study, two independent search engines were used to confirm more specific protein discrimination. All ELISA assays were performed in triplicate, and interassay and intra-assay coefficient of variations were between 3 to 7%. The reproducibility of SELDI-TOF-MS proteomic analysis in the detection of prostate cancer was evaluated recently in a multi-institutional study and the “between laboratory” reproducibility was similar to “within-laboratory” reproducibility (Semmes et al. 2005).

6.2 Validation of fetal NT-proANP, NT-proBNP and EPO analyses

Previously described and well-characterized NT-proANP and NT-proBNP radioimmunoassays were used in the present study (Vuolteenaho et al. 1992; Ala-Kopsala et al. 2004; Ala-Kopsala et al. 2005). The detection limits of the NT-proANP assays were 30 (II) and 60 pmol/L (IV), and the corresponding limits for the NT-proBNP assays were 63 (II) and 40pmol/L (IV) (Ala-Kopsala et al. 2004). The within- and between-assay coefficients of variations were < 15% and 20%, respectively (Ala-Kopsala et al. 2004). The various assays correlate reasonably well with each other (Ala-Kopsala 2004). Based on findings in umbilical cord and maternal NT-proANP (Makikallio et al. 2001; Walther et al. 2001) and NT-proBNP (Bakker et al. 2004; Hammerer-Lercher et al. 2005) concentrations, it has been postulated that there is no significant transplacental exchange of these peptides and the fetus has its own cardiac natriuretic peptide production. In addition, the large molecular size of NT-proANP and NT-proBNP (98 and 76 amino acids) makes it very likely that they do not cross the placenta. As documented previously, the mode of delivery has no significant influence on umbilical cord NT-proBNP (Bakker et al. 2004; Bar-Oz et al. 2005) or umbilical artery or vein plasma ANP concentrations either (Kingdom et al. 1992).
The EPO immunometric assay of Diagnostic Products Corporation (Immulite, Diagnostic Products Corporation, Los Angeles, CA) is an automated immunoassay with chemiluminescent detection. The assay has been shown to be sensitive with ability to recognize absolute amount of EPO equivalent of 0.2 U/l (Owen et al. 2004). According to the previous studies, the Immulite EPO assay is reproducible and accurate (Benson et al. 2000; Owen et al. 2004; Mossuz et al. 2005). No significant interference has been noted with hemoglobin, bilirubin, or triglyceride (Benson et al. 2000). The intra- and interassay coefficients of variation in this assay were 6.4–9.9% and 8.8–13.2% (Teramo et al. 1987; Teramo et al. 2002; Teramo et al. 2004b), respectively, with similar results reported in other studies as well (Owen et al. 2004; Mossuz et al. 2005). According to studies on ovine fetuses, EPO does not cross the placenta and fetal plasma EPO concentrations reflect fetal EPO production and clearance (Widness et al. 1995). The half-life of human fetal EPO is not known. In newborns of mothers with preeclampsia, the mean half-life of EPO has been shown to be 3.7 (± 0.9) hours (Ruth et al. 1990). Previous studies have shown that fetal EPO secretion does not increase in the late 2nd trimester and in the 3rd trimester until the fetus becomes hypoxic (Voutilainen et al. 1989; Teramo et al. 2004b). In uncomplicated and singleton pregnancies, increased umbilical cord blood EPO levels have been reported in pregnancies beyond 41 gestational weeks (Jazayeri et al. 1998; Manchanda et al. 1999). The uterine contractions during vaginal delivery may increase fetal EPO levels (Widness et al. 1984). In the present study, however, 91% of the fetuses with elevated UA EPO concentrations were delivered by cesarean section (III). A subgroup analysis did not show any significant difference in EPO levels in relation to the mode of delivery either.

6.3 Validation of ultrasonographic measurements

In this study, the methodological errors of Doppler ultrasonographic measurements were minimized by keeping the angle of insonation at less than 15 degrees (Tessler et al. 1990) as well as by taking the mean value of three separate valve diameter measurements into CSA calculations (Beeby et al. 1991). In fetal echocardiographic measurements, interobserver errors have been shown to be consistently higher compared to the intraobserver errors (Simpson et al. 2002). In the present study, all the ultrasonographic measurements excluding the routine UA blood velocity waveform registrations in study IV, were performed and analyzed by a single observer. In a randomized blinded study on human fetuses,
the intraobserver variability of RVCO and LVCO measurements has been less than 9% (Rasanen et al. 1998) and in MV and TV total TVI calculations less than 6% (Makikallio et al. 2002b). In human fetuses, the intraobserver variability in RVeFo and LVeFo calculations during the second half of pregnancy has been reported to be less than 10% (Rizzo et al. 1995b; Rasanen et al. 1997b; Makikallio et al. 2002b). The interobserver variability in these measurements less than 13% (Rizzo et al. 1995b). In human fetal arterial PI calculations, the intraobserver variability has been shown to be less than 4% (Rasanen et al. 1998) and correspondingly in venous PIV calculations less than 6% (Makikallio et al. 2002b).

6.4 Evaluation of maternal serum proteomic profiles in preeclampsia (I)

In clinical preeclampsia, an antiangiogenic protein endoglin was overexpressed in maternal serum compared with gestational age-matched uncomplicated control pregnancies. This is in agreement with previously published studies, which have demonstrated increased soluble endoglin concentrations in serum in women with clinical preeclampsia (Levine et al. 2006; Romero et al. 2008). In fact, it appears that maternal serum antiangiogenic protein concentrations start to increase prior to clinical symptoms. It has been shown that maternal serum soluble endoglin levels are significantly higher at 17–20 gestational weeks in women who later develop clinical preterm preeclampsia than in the controls, and significantly increased levels at 25–28 gestational weeks were reported in women who develop term preeclampsia compared to the controls (Levine et al. 2006). In addition, increased sFlt-1 levels have been detected approximately 5 weeks before the onset of preeclampsia (Levine et al. 2004). In the present study, cystatin-C, fibronectin, plasma retinol-binding protein, apolipoprotein C-III, choriogonadotropin-ß, PAPP-A2, histidine-rich glycoprotein and insulin-like growth factor-binding protein-2 were overexpressed, and MMP-9 underexpressed in women with clinical preeclampsia compared with uncomplicated pregnancies. These proteins represent markers for renal dysfunction, endothelial dysfunction or are of placental origin, and all of these organs are affected in clinical preeclampsia (Roberts 1998; Poston 2006). In summary, in clinical preeclampsia, the maternal serum proteomic profile is different from that in uncomplicated pregnancies with overexpression of placental proteins and antiangiogenic factors.
The early pregnancy maternal serum proteomic profile demonstrated that placental proteins, vascular and/or transport proteins and matrix and/or acute phase proteins are differently expressed in women who develop preeclampsia later in pregnancy than in women with uncomplicated pregnancies. Angiogenic and antiangiogenic factors, which have been used to predict the development of preeclampsia in the second trimester of pregnancy, were not different from control pregnancies. Matrix and structural proteins seem to play more important role in the early placentation process than angiogenic proteins.

In the present study, early pregnancy maternal serum vascular cell adhesion protein 1 expression was increased in women who later developed preeclampsia compared to the controls. Intraplacenatal pO2 increases towards the end of the first trimester and it is accompanied by almost a parallel increases in mRNA concentrations and the activity of the major antioxidant enzymes in the villous tissue (Jauniaux et al. 2000). It is well known that oxidative stress in the placenta of women with preeclampsia is increased (Jauniaux et al. 2006). The disturbance in the oxidant-antioxidant balance renders the tissue more vulnerable to oxygen free radical injury. Lipid peroxides increase production of thromboxane A2 and the expression of cell adhesion molecules in the uteroplacental vasculature. This further leads to endothelial dysfunction.

Increased early pregnancy serum complement factor D was seen in women who later developed preeclampsia. An imbalance in the maternal immune response to the placenta has a significant role in the pathophysiology of preeclampsia. Extravillous trophoblast cells express major histocompatibility complex molecules on their cell surface, and malfunction in major histocompatibility complex molecules can lead to an increased cytolytic activation of decidual and blood leukocytes (Shorter et al. 1993). This could further contribute to the low trophoblast invasion and vascular abnormalities detected in preeclamptic placentas. In addition, the placental bed of women with preeclampsia is often infiltrated with activated macrophages, which can release molecules capable of reducing trophoblastic invasiveness and initiating apoptosis (Trundley et al. 2004).

In this study, PAPP-A1 expression was lower in women who later developed preeclampsia. This is in accordance with previously published studies demonstrating an increased risk for preeclampsia in patients with low first trimester PAPP-A1 levels (Spencer et al. 2008). PAPP-A is a syncytiotrophoblast-derived protease for insulin-like growth factor binding protein (Peterson et al. 2008). Its protease activity cleaves complexed growth factor, releasing it to
instigate mitogenic signaling pathways. In other words, low PAPP-A concentrations would leave the growth factors in the bound state, which would lead to decreased growth.

Vasorin is predominantly expressed in vascular smooth muscle cells and modulates the vascular response to injury by attenuating transforming growth factor-beta signaling (Ikeda et al. 2004). Moderate vasorin expression has been detected in the placenta. It is well established that transforming growth factor-beta contributes to neointimal formation by promoting fibrosis, and down-regulation of vasorin expression contributes to the fibroproliferative response to vascular injury. In the present study, early pregnancy maternal serum vasorin levels were increased in women who later developed preeclampsia. This could be a secondary response to inhibit fibrosis and fibroproliferative response to vascular injury.

Cystatin-C and β-2-microglobulin were elevated in early pregnancy maternal serum samples in women who developed preeclampsia later in pregnancy. Both proteins are sensitive markers of glomerular filtration and their plasma levels are increased in clinical preeclampsia (Kristensen et al. 2007). The results demonstrate that their concentrations are significantly higher already in early pregnancy.

In the present study, the early pregnancy maternal serum endoglin and sFlt-1 concentrations were not significantly different in women who later developed preeclampsia compared with uncomplicated pregnancies. The finding suggests that antiangiogenic proteins are not significantly involved in the primary pathophysiologic events leading to clinical preeclampsia. The results are in agreement with previous studies, which have shown that the first trimester maternal serum sFlt-1 concentrations in women who later develop preeclampsia are not markedly different from normal control patients (Thadhani et al. 2004; Powers et al. 2005). In fact, in women who develop preterm preeclampsia, the first trimester sFlt-1 concentrations are lower than in control subjects (Vatten et al. 2007). Also the serum soluble endoglin levels have been reported to be similar before 16 weeks of gestation between the women with later developed preeclampsia and uncomplicated pregnancies (Levine et al. 2006). On the other hand, Baumann et al recently reported increased serum soluble endoglin and sFlt1 levels in the first trimester in women with late onset preeclampsia (≥34 weeks) (Baumann et al. 2008). In the studies by Levine et al, the secretion patterns of antiangiogenic factors were different in groups of early onset (<37 weeks) and late onset (≥37 weeks) preeclampsia (Levine et al. 2004; Levine et al. 2006). Also in the present study, a difference in the proteomic profile was found between
mild and severe preeclampsia groups, already in the early pregnancy in women who later developed preeclampsia. Altogether, the findings suggest that there may be different disease entities in preeclampsia with distinctive secretion patterns of biochemical markers. Larger prospective studies will be required to investigate the antiangiogenic factors in different types of preeclampsia.

6.5 Fetal cardiovascular responses to placental insufficiency (II–III)

In this study, small for gestational age fetuses demonstrated increased NT-proANP levels compared to the control fetuses. The findings indicate that also the small for gestational age fetuses with normal placental and fetal hemodynamics in Doppler ultrasonography have increased cardiac ANP secretion. Previously, it has been estimated that over 60% of the intraplacental fetal vasculature must be occluded before any significant change in UA blood velocity waveforms occurs (Thompson et al. 1990). With successful placental and/or fetal adaptation to placental insufficiency, inadequate fetal nutrient availability may only be seen through its restrictive effect on exponential fetal growth in third trimester (Baschat 2004a). We suggest that in this study, at least some of the small for gestational age fetuses suffered from placental insufficiency, which we were unable to detect by Doppler ultrasonography. The finding that almost half of the small for gestational age fetuses had abnormal EPO concentrations supports this.

It can be suggested that a rise in placental vascular resistance increased cardiac wall stretch in these fetuses, which is the main stimulus for ANP and NT-proANP secretion from their storage granules (de Bold et al. 1996). Increased UA EPO concentrations and further elevated NT-proANP concentrations were found in fetuses with abnormal UA velocimetry. The increment in EPO levels in fetuses with abnormal UA velocimetry indicates that the placental villous damage caused fetal hypoxemia, the trigger of EPO secretion (Eckardt et al. 2005). In addition, placental insufficiency with increased placental vascular impedance leads to a rise in fetal cardiac afterload, especially in the right ventricle. Significant correlation between NT-proANP levels and UA PI values suggest that ANP secretion was increased due to rise in cardiac afterload and thus, pressure load. During cardiac maturation, ANP gene is actively expressed also in the fetal ventricles (Takahashi et al. 1992) and, thus, this increase in the NT-proANP levels was most likely due to ventricular secretion. Experimental studies on fetal sheep have shown that placental insufficiency caused by acute placental restriction or umbilicoplacental embolization also result changes in
cardiac myocytes (Louey et al. 2007; Morrison et al. 2007). In the case of acute placental restriction, the fetuses develop larger cardiomyocytes relative to heart weight (Morrison et al. 2007) and after 20 days of embolizations, the fetuses with placental insufficiency also had decreased myocardial cell-cycle activity (Louey et al. 2007). The biochemical evidence of fetal cardiac dysfunction in placental insufficiency found in the present study supports the previous results.

A rise in UA NT-proBNP levels was only seen in growth restricted fetuses with abnormal DV blood velocity waveform patterns. Cardiac pressure and volume overload causing ventricular wall stretch are the physiologic stimuli for BNP and NT-proBNP release (Tokola et al. 2001). In placental insufficiency, the increased cardiac afterload is suggested to result in elevated right ventricular end-diastolic pressure, which leads to increased venous Doppler indices as an evidence of elevated systemic venous pressure (Hecher et al. 1995a). The present finding of increased NT-proBNP and EPO in growth restricted fetuses with abnormal venous blood velocity waveforms supports the view. On the other hand, growth restricted fetuses with severe placental compromise are shown to have a reduced FO diameter compared to the normal fetuses (Kiserud et al. 2004). Previously, growth restricted fetuses with antegrade AoI net blood flow were shown to restrict their pulmonary volume blood flow and increase volume blood flow through FO, thus, distributing the RVCO towards systemic circulation (Makikallio et al. 2003). However, the fetuses with retrograde AoI net blood flow failed to demonstrate the redistribution (Makikallio et al. 2003). The redistribution of cardiac output from the right to the left side of the heart is seen also in asymmetrically growth restricted fetuses (al-Ghazali et al. 1989). In the present study, the NT-proBNP levels were found to correlate positively to fetal LVCO%, and on the other hand, negatively to fetal RVCO%, suggesting a reorganization of cardiac outputs in fetuses with severe placental insufficiency. In the case of impaired reorganization of cardiac outputs and with retrograde AoI net blood flow, the fetus supplies the cerebral circulation with additional blood from the right ventricle and DAo, which have lower oxygen content than the blood in the left ventricle. An experimental study in sheep fetuses has demonstrated that a gradual increase in placental vascular resistance leads to fetal retrograde AoI net blood flow and diminished oxygen content of the blood entering the cerebral circulation (Fouron et al. 1999a). In the present study, fetuses with elevated EPO concentrations had significantly higher incidence of retrograde AoI net blood flow, thus, supporting this concept. In addition, the growth restricted fetuses with increased NT-proBNP levels as well as the fetuses with increased EPO levels and
retrograde AoI net blood flow had increased Doppler venous indices. The results suggest that in placental insufficiency, the biochemical markers of cardiac dysfunction and chronic hypoxia are related to elevated ventricular end-diastolic pressure, possibly more in the right ventricle, due to rise in cardiac afterload.

The fetuses with abnormal DV velocimetry and a rise in NT-proBNP levels demonstrated the highest coronary artery visualization rate. The finding suggests a greater myocardial oxygen demand due to a rise in cardiac afterload and ventricular wall stress in these fetuses. In adults, increased NT-proBNP levels have a strong correlation to left ventricular dysfunction, which is mainly due to ischemic heart disease and inadequate coronary artery blood supply (Groenning et al. 2002; Corteville et al. 2007). However, in fetus, the coronary artery dilatation is a compensatory mechanism, which recruits all the available coronary blood flow reserves (Baschat et al. 1997). On the basis of unchanged fetal cardiac outputs and cardiac functional parameters in relation to increased NT-proBNP and EPO levels in this study, it seems that these fetuses were able to maintain adequate cardiac function. Previously, the fetal cardiac outputs and cardiac functional parameters were found unaffected although the fetuses had biochemical evidence of myocardial cell damage (Makikallio et al. 2002b), thus supporting the present findings. The fetal heart is known to have a remarkable ability to withstand hypoxia (Mott 1961). Part of the functional capacity of fetal heart has been explained by the ability of the fetal myocardium to extract oxygen maximally from the coronary blood flow. However, in sheep fetuses with increased placental vascular resistance and acute metabolic acidosis, the right and left ventricular cardiac outputs remained unchanged while decreased myocardial velocities during contractility and relaxation were noted (Acharya et al. 2008). Thus, fetal cardiac output is merely an end product of the ventricular function. In the present study, no significant difference was found in the echocardiographic parameters of fetal cardiac systolic and diastolic function even in the presence of increased UA NT-proBNP and EPO concentrations, demonstrating the functional capacity of the fetal heart.

6.6 Fetal cardiac responses to maternal type-1 diabetes (IV)

Increased UA NT-proANP and NT-proBNP levels were found in newborns of type-1 diabetic mothers with normal UA velocimetry. The finding is in line with the previous study on rats which documented increased ANP and BNP levels in the fetal hearts of diabetic dams (Mulay et al. 1995). Fetuses of diabetic mothers
are at risk for excessive growth, which can be explained by maternal hyperglycemia and increased amino acid transfer through placenta, which induce the fetal hyperinsulinemia and tissue growth (Persson et al. 1986; Eidelman et al. 2002). In addition, fetal hyperinsulinemia is suggested to lead to increased glycogen stores in fetal cardiac muscle resulting cardiac muscle hypertrophy (Veille et al. 1992). Hyperinsulinemia may also directly induce cardiac hypertrophy by binding to insulin-like growth factor -receptors (Eidelman et al. 2002). Increased cardiac wall stretch is the primary acute stimulus for release of ANP and BNP and their pro-peptides (Tokola et al. 2001). However, despite the different analysis method, which produces 30–40% lower concentrations of NT-proANP and NT-proBNP, the fetuses of type-1 diabetic pregnancies with normal placental hemodynamics demonstrated higher levels of these peptides than the growth restricted fetuses with increased cardiac afterload due to placental insufficiency. In adult type-1 diabetics, acute hyperglycemia increases plasma ANP levels (McKenna et al. 2000), which supports our finding of the correlation between UA NT-proANP levels and maternal HbA1c values in each trimester. A recent experimental study found that myocardial cell hypertrophy induced by insulin-like growth factor-2 -receptor signaling pathway also increased the expression of ANP and BNP genes (Chu et al. 2008), suggesting that the hyperinsulinemia alone could induce the ANP and BNP expression. In another experimental model, the neonates of diabetic dams had about 4-fold higher levels of ANP mRNA in their hypertrophied left ventricle than control neonates at birth (Gopinath et al. 2004). Altogether, the previous results support the present finding that increased ANP and BNP secretion in type-1 pregnancies is related to metabolic disturbances affecting the fetal cardiac cells rather than due to increased cardiac wall stretch like in pregnancies complicated by placental insufficiency.

A rise in newborn NT-proANP levels in type-1 diabetic pregnancies was related to poor maternal glycemic control in the first trimester of pregnancy, suggesting that the maternal metabolic state already in early pregnancy is associated with increased fetal cardiac natriuretic peptide secretion. The knockout mouse embryos that develop cardiac hypertrophy demonstrated increased atrial natriuretic factor gene expression from 14.5/21 days gestation onward (Thackaberry et al. 2003). In mouse embryo, cardiac ANP and BNP mRNA are present already at 8–9/21 days of gestation (Cameron et al. 1996). Echocardiographic studies have shown that fetal cardiac functional abnormalities in diabetic pregnancies may exist already at 12 weeks of gestation and can relate
to poor maternal glycemic control (Rizzo et al. 1995a). In addition, maternal hyperglycemia in early gestation is associated with adverse perinatal outcome later in type-1 diabetic pregnancies (Fuhrmann et al. 1983; Suhonen et al. 2000; Temple et al. 2006). The present finding of the highest newborn NT-proANP and NT-proBNP levels in pregnancies with poor glycemic control in the first trimester supports the previous results and suggests that the changes in fetal cardiac gene activation may take place early and can persist throughout the diabetic pregnancy.

Cardiac anomalies and myocardial hypertrophy are about three times more common among the neonates of type-1 diabetic mothers than in uncomplicated pregnancies (Suhonen et al. 2000). In the present study, type-1 diabetic pregnancies with fetal cardiac structural abnormalities and abnormal UA velocimetry were excluded before entering the study. Previous studies have reported decreased early ventricular filling/atrial contraction filling ratios in fetuses of type-1 diabetic pregnancies suggesting altered fetal cardiac diastolic function in these pregnancies (Rizzo et al. 1991b; Tsyvian et al. 1998). However, the indices of fetal cardiac diastolic and systolic function have also remained comparable between well-controlled type-1 diabetic pregnancies and the controls (Jaeggi et al. 2001). Another study in diabetic pregnancies reported a tendency to a smaller fetal right ventricular fractional shortening when the pulsatility in fetal DAo increased, suggesting a limited fetal cardiac functional capacity in these pregnancies (Rasanen et al. 1988). Currently, there are no studies that had reported about fetal ANP and BNP secretions in type-1 diabetic pregnancies complicated by fetal congenital heart defects. In addition, the exact etiology of echocardiographic alterations is unclear, although the fetal cardiac hypertrophy and metabolic disturbances have been suggested (Rizzo et al. 1991b; Veille et al. 1993). The correlation between NT-proANP levels and maternal glycemic control found in this study supports the view. Although, the fetal cardiac hypertrophy in diabetic pregnancies is known to resolve after birth (Deorari et al. 1989), the significance of cardiac dysfunction during maturation for the later life of these individuals is unclear. Because the cardiac natriuretic peptides are shown to possess antihypertrophic abilities (Woods 2004), the secretion of these peptides may acts as a compensatory mechanism against myocardial hypertrophy in fetuses of diabetic mothers. Altogether, the present findings suggest that in type-1 diabetic pregnancies, fetal heart is more sensitive to alterations in its loading conditions and fetal cardiac functional capacity can be limited.
6.7 Clinical implications

According to the results of this thesis, the serum proteomic profile in early pregnancy in women who develop preeclampsia is different from clinical preeclampsia. The angiogenic and antiangiogenic proteins, which are currently documented to increase before the clinical symptoms of preeclampsia, are not found in the early pregnancy serum of women with later developing preeclampsia. Further investigations are needed to demonstrate the predictive capabilities of early pregnancy maternal serum markers for preeclampsia.

A significant proportion of the small for gestational age fetuses had biochemical signs of cardiac dysfunction and chronic hypoxia. Although these fetuses are thought to be constitutionally small, the present finding suggests that at least some of the fetuses may have underlying placental pathology. The more specific recognition of these fetuses, such as using placental volume blood flow measurements, must be addressed in future studies.

In the presence of abnormal systemic venous blood velocity waveform patterns, the fetus has biochemical evidence of cardiac dysfunction and chronic hypoxia. In addition, the fetuses with abnormal DV blood velocity waveforms had more often retrograde net blood flow in AoI. In the presence of placental insufficiency and/or fetal growth restriction, it appears that NT-proANP secretion is activated first, and NT-proBNP secretion is increased when fetal cardiac afterload is severely increased and there are signs of elevated ventricular end-diastolic pressure.

Increased newborn NT-proANP and NT-proBNP levels in type-1 pregnancies with normal placental hemodynamics suggest that the secretion of fetal cardiac natriuretic peptides in diabetic pregnancies is related to metabolic disturbances affecting the fetal heart. These increased levels also suggest that fetuses of diabetic mothers may be at increased risk for cardiac dysfunction. Because the maternal glycemic control in the first trimester of pregnancy was related to increased NT-proANP and NT-proBNP levels, the changes in fetal cardiac gene activation may take already place in early pregnancy. Thus, euglycemia especially in pre- and early pregnancy would be important in avoiding adverse fetal cardiac outcome.
7 Conclusions

1. Early pregnancy maternal serum proteomic profile in women who later develop preeclampsia and in clinical preeclampsia are different from proteomic profiles in gestational age matched uncomplicated pregnancies. In addition, the serum proteomic profile in early pregnancy in women with later developing preeclampsia is a distinct and different from serum proteomic profile in clinical preeclampsia (I).

2. In placental insufficiency, elevated umbilical artery NT-proANP concentrations are related to increased fetal cardiac afterload (II). An increase in umbilical artery NT-proBNP and EPO levels is seen only in fetuses with more severe placental insufficiency and signs of elevated ventricular end-diastolic pressure (II–III). In addition, increased umbilical artery EPO levels are related to retrograde aortic isthmus net blood flow (III).

3. In type-1 diabetic pregnancies, fetal cardiac natriuretic peptide secretion is increased even in the presence of normal placental hemodynamics and NT-proANP secretion correlates with poor maternal glycemic control in early pregnancy (IV).
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