FIRST TRIMESTER SCREENING FOR MATERNAL AND FETAL DISEASE

A search for novel biomarkers

Sylwia Kuć

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A search for novel biomarkers

EERSTE TRIMESTER SCREENING
OP MATERNALE EN FOETALE ZIEKTE
Een zoektocht naar nieuwe biomarkers (met een samenvatting in het Nederlands)

BADANIA PRZESIEWOWE CHORÓB MATKI I PŁODU
W PIERWSZYM TRYMESTRZE CIĄŻY
Poszukiwanie nowych biomarkerów (ze streszczeniem po polsku)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
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door

Sylwia Kuć
geboren op 10 november 1980 te Warschau, Polen

Promotoren:	Prof. dr. G.H.A. Visser
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Co-promotoren:	Dr. M.P.H. Koster
	Dr. P.C.J.I. Schielen

Learn the past, research the presence and predict the future.

(Hippocrates c. 460 BC – c. 370 BC)

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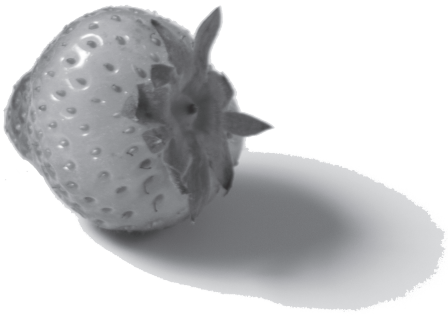
GENERAL INTRODUCTION



BIOMARKERS IN DISEASE MANAGEMENT

Over the past decades, substantial investment has been made in the early detection of different disorders such as cancer or cardiovascular diseases.[1, 2] The quest for early disease detection has been the driving force for the search for novel biomarkers. An ideal disease biomarker is a substance or a characteristic that can be objectively measured and evaluated in a patient as an indicator of pathological processes, but is undetectable or detectable at significantly different levels in a healthy person.[3] Biomarkers may be found in any biological fluid such as serum, plasma, urine and cells. They can be biological products such as metabolites, genetic markers or personal characteristics. Biomarkers provide a powerful and dynamic approach in understanding the underlying pathogenesis of the disease. Biomarkers can be broadly applied to improve health management through many different facets such as:

- early identification of the "high risk" individuals
- early diagnosis of the disease
- stratification of patients depending on disease severity
- prognosis of the disease
- identification of "high risk" patients for long term complications after the manifestation of a particular disease.[4]

To be clinically applicable a diagnostic biomarker should ideally have a sensitivity and specificity close to 100% and be measured within a non-invasive (urine/saliva) or minimal-invasive (venapuncture) collected specimen.[5] Moreover, a diagnostic test measuring the biomarker(s) should be accurate, low-cost, easy to perform and highly reproducible. An example of an ideal biomarker is the measurement of human Chorionic Gonadotropin (hCG) in the urine as an early indicator of pregnancy. This is a simple, highly accurate and inexpensive test and therefore appropriate for broad usage.

BIOMARKERS IN PRENATAL SCREENING

Biomarkers have become very valuable in prenatal screening and diagnosis. Alpha-fetoprotein (AFP), reported in 1972, was the first recognised biomarker in prenatal diagnosis.[6] Measured in amniotic fluid, AFP appeared to be significantly higher in women carrying a child with neural tube defect (NTD). Two years later the association between high AFP levels and NTD was also found in second trimester maternal serum.[7, 8] This discovery led to the development of a first non-invasive screening method in pregnancy.[9] Later on measurements of AFP were extended to the Down Syndrome (DS) screening as it was found that low levels of AFP were associated to this chromosomal disorder.[10, 11] Since then, prenatal screening has become standard practice in many countries. It also underwent an evolution in relation to the application of novel markers, the timing of the screening as well the purpose of the screening. Over a time span of more than forty years numerous biomarkers have been described and subsequently applied, well-known examples being human Chorionic Gonadotropin (hCG) [increased in DS], unconjugated

estriol (uE_3) [decreased in DS] and inhibin-A [increased in DS].[12, 13] DS screening meanwhile made a shift from the second to the first trimester of the pregnancy whereas NTD screening remained in the second trimester and is at present solely based on advanced ultrasound techniques. Current first trimester DS screening is based on the assessment of a fetal sonographic marker (nuchal translucency; NT) measurement [enlarged in DS] and evaluation of two biochemical markers: free β-subunit of human Chorionic Gonadotropin (fβ-hCG) [increased in DS] and Pregnancy Associated Plasma Protein-A (PAPP-A) [decreased in DS] in maternal serum.[14] Recently, DS screening was extended with two other aneuploidies: trisomy 13 (Patau syndrome) and 18 (Edwards syndrome).

INNOVATIONS IN CURRENT PRENATAL SCREENING: PREECLAMPSIA AND GROWTH PATHOLOGIES

Already for more than a decade there are strong indications that the biomarkers currently used in first trimester prenatal screening as well a number of novel biomarkers are associated to other pregnancy complications than aneuploidies, namely preeclampsia, intrauterine growth restriction (IUGR) or preterm birth.[15-17] Consequently, there is a growing scientific and social need, relevance and urgency to extend current first trimester screening for pregnancy diseases. Such a screening may offer the possibility of early selection of high risk pregnancies and facilitate preventive or therapeutic interventions to improve fetal and maternal health in a wider perspective.

One of the priorities in prenatal care is preeclampsia (PE). PE is a serious disorder that occurs only during pregnancy and in the post partum period. PE affects approximately 2% of pregnant women and is a leading cause of maternal and perinatal morbidity and mortality worldwide, particularly when it occurs before 34 weeks of gestation.[18-20] PE is associated with substantial fetal risks due to frequently accompanying intrauterine growth restriction (IUGR), iatrogenic prematurity, placental abruption and stillbirth.[19, 21] It is also associated with cardiovascular disease of both mother and child later in life.[22] PE is a rapidly progressing condition characterized by high blood pressure and the presence of protein in the urine. These parameters however are only the terminal features of the cascade of events initiated already early in pregnancy. Therefore, early recognition of patients at high risk and timely intervention ahead of the clinical onset of the disease might enable suitable pregnancy care and better pregnancy outcomes for both mother and child.[23, 24]

Currently, only pre-existing risk factors such as a previous pregnancy with PE or chronic hypertension serve as markers to predict PE.[25] Although the prevalence of PE among the women with a priori risk is higher, prior risk by itself is not accurate enough to predict PE, also since most women who will develop PE will not fall in this risk category. Measurements of a variety of biomarkers strongly associated with the underlying pathophysiology of PE in early pregnancy, may lead to the development of a highly sensitive and specific screening tool. Although the precise aetiology of PE still needs to be completely elucidated, the last two decades of research brought considerable knowledge about pathophysiology of this disease. Interactions between placental factors,

maternal constitution, and unfavourable adaptive changes to pregnancy most likely lead to the clinical syndrome of PE (Figure1).[26-29] These interactions predominantly involve the cardiovascular and inflammatory systems resulting in diffuse maternal endothelial dysfunction and organ damage due to vascular compromise.[26-29] Generally two types of PE are recognized, placental and maternal. Placental PE is considered to arise from poor placentation. It develops mostly early in pregnancy and is a result of impaired trophoblast invasion into the spiral arteries.[27, 30] In consequence, spiral arteries do not convert into low-resistance structures, leading to substantially reduced placental blood supply, hypo-perfusion and oxidative stress.[31] In maternal PE, it is likely that the contribution of reduced placental perfusion and maternal constitutional factors are balanced differently. Placental formation is expected to be normal while maternal constitution plays a crucial role in the development of the latter disease. Obesity, microvascular disease, chronic hypertension and diabetes belong to the specific maternal constitution and strongly predispose to PE.[31] It is widely known that women who develop PE already early in life share the microvascular dysfunction phenotype with adults suffering from atherosclerosis. These women appear to be at increased risk of cardiovascular disease later in their lives and pregnancy apparently evokes in them diffuse vascular stress. Their at risk constitution finally leads to a maternal syndrome of PE. The maternal variant develops mostly in the course of the third trimester of pregnancy. Maternal PE is considered more as an adverse systemic response of the mother to the pregnancy than otherwise. From the screening point of view however, most PE cases are a combination of both forms and result from the misbalance of the three already mentioned components. The search for potential PE biomarkers, nevertheless, cannot be based on anything else than the current knowledge of the pathophysiology of PE. Due to the heterogeneous character of PE it is likely that the final prediction model will be based on a combination of several biomarkers rather than on a single biomarker only. The three components of the pathophysiology of PE should inspire to find a suitable predictive combination of biomarkers:

- signs of the poor placentation and placenta dysfunction. Here we should look for biomarkers derived from the placenta, which may inform us about the condition of this organ. Placental proteins, hormones or metabolites should be prioritised,
- maternal characteristics and constitution. These can already be assessed during the first prenatal visit: BMI (body mass index), blood pressure, age, smoking status, family history of hypertensive pregnancy disorders, and
- unfavourable adaptive changes to pregnancy involving the cardiovascular and inflammatory systems. Doppler measurements of maternal vessels like uterine artery or the concentration of angiogenic factors in maternal blood should be considered.

Additionally, early screening for PE through the monitoring of placental development and functionality may facilitate the identification of other pregnancy complications. The placenta is an organ mainly responsible to provide nutrients for the developing fetus. It provides the fetus with a wide range of endocrine signals, cytokines and growth factors thereby regulating its intrauterine development and growth.[32] There is accumulating evidence that growth anomalies such as IUGR (isolated or in combination with PE) and macrosomia may

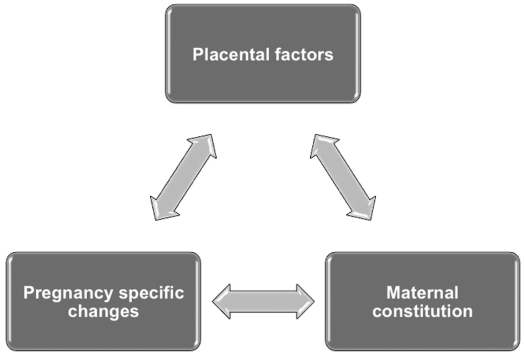


Figure 1. Proposed model for the pathogenesis of preeclampsia.

be related to placental (dis)function already early in pregnancy.[17, 32, 33] Accordingly, this knowledge offers the possibility, perhaps with additional specific biomarkers, to organise prenatal screening in such a way that screening for PE could be combined with other pathologies related to placental dysfunction.

PHASES OF BIOMARKER DEVELOPMENT FOR EARLY DETECTION OF DISEASE

To be able to extend the current prenatal screening to other diseases and in particular to PE, sets of (novel) biomarkers are needed that are capable to detect the presence of pathologic conditions already early in the pregnancy. The development of new techniques such as gene expression arrays, immunohistochemistry-, and high throughput techniques (proteomics or metabolomics) offer outstanding approaches for the identification of potential screening biomarkers. Nevertheless the entire process from the discovery phase to the final application of the screening test may be long and time consuming. Discovery of potential biomarkers is just the first step in the entire process. It is followed by validation of the biomarker and finishes with the evaluation of the impact of the biomarker on clinical outcome.[34] Multiple studies are usually involved in each stage. Sullivan Pepe et al., proposed five phases in the biomarker development for early detection of cancer and recently this scheme was adapted for other diseases (Table 1).[35] The phases of research are generally ordered according to the strength of evidence that each provides in favour of the biomarker, from weakest to strongest. The results of earlier phases are generally necessary to design later phases.[35]

Phase 1 is an exploratory phase, which helps to identify the unique signature of the disease. This phase focuses mostly on differences between the individuals prone to develop the disease and the ones who remain healthy. The search may be based on the histological studies, literature studies or high-throughput techniques. The objective of this phase is to generate a putative list of potentially overexpressed or underexpressed biomarkers present in the subjects destined to develop the disease, and give the directions for further research.

Phase 2 focuses on the development and evaluation of an assay, which is able to non-invasively measure the promising biomarkers generated in the phase 1. The major aim of this phase is to examine the assay performance regarding the distinction between at risk subjects and the healthy controls. The primary outcome measures applied are the detection rate (DR), false positive rate (FPR) and receiver operating characteristics (ROC) curve for this particular assay.

Phase 3 involves large validation studies in retrospective cohorts. The cohorts include well-documented specimens (serum, urine or sputum) of patients who subsequently developed disease and of healthy controls. Screening tests must be capable to identify the cases ahead of the clinical onset of the disease with high sensitivity and specificity. As in Phase 2 the screening performance of the test is presented in DR, FPR and ROC's. The DR is generally established at a certain percentage of FPR. This particular percentage of FPR becomes therefore a threshold for the test and will be applied to define screen positivity in following phase.

Phase 4 applies the assay in a prospective cohort of living patients to monitor the predictive value of the test when compared to the final clinical diagnosis for each subject. The major aims of this phase are:

- evaluation of the performance of the test in classification of patients in high and low risk groups
- assessment of practical feasibility of the screening program in the current clinical setting
- evaluation of present diagnostics after the patient has been screened as high risk for this specific condition and
- evaluation of possible treatment / preventive treatment

The 5th phase evaluates the performance of the test in the general population in terms of the reduction of the disease and health benefits. It provides the cost-effectiveness analysis of the screening and treatment following it. This phase provides the insight and conclusive evidence on the actual impact of this screening.[35]

Table 1. Phases of biomarker development.[35]

Preclinical exploratory	Phase 1	Promising directions identified
Assay validation	Phase 2	Assay detects disease
Retrospective screening	Phase 3	Biomarkers detect disease before it becomes clinical
Prospective screening	Phase 4	Disease detected by the test and the false referral rate is identified
Control	Phase 5	Impact of screening on reducing the burden of disease on the population is quantified

AIMS AND OUTLINE OF THIS THESIS

Aims of this thesis

(i) A search for (novel) potential first trimester biomarkers in the detection of preeclampsia and growth pathologies = phase 1 and 2.
(ii) Assessment of the screening capacity of first trimester biomarkers in the detection of preeclampsia and other diseases before the clinical diagnosis = phase 3.
(iii) To evaluate prospectively the potential biomarkers in relevant populations = phase 4.

Part 1

In Part 1 of this thesis we tried to capture the two first phases of the biomarker development model: exploratory and assay validation phases for first trimester preeclampsia screening (**Chapters 2-4**). At first we captured and recapitulated the already existing knowledge on first trimester biomarkers. Secondly, we explored a new approach to identify biomarkers unique to PE that may lead to ideas for a clinical screening test. Finally we appraised the detection value of the possible biomarkers.

Chapter 2 is a systematic review of the recent literature on the early pathophysiology of PE and on the evaluation of the predictive value and use of already described first trimester biomarkers in the assessment of PE risk. In this review we evaluated the detection rate of seven serum biomarkers in combination with uterine artery Doppler measurements and maternal characteristics for the first trimester prediction of PE.

In **Chapter 3** a bioinformatics approach was applied to create a set of candidate first trimester PE biomarkers. The search strategy was based on the pathophysiology of PE. We assessed the large gene and protein database systems for tissue specific products in maternal blood that are highly associated with the PE aetiological mechanism.

In **Chapter 4** a novel high-throughput technique – metabolomics – was applied for the first trimester detection of novel metabolic PE biomarkers. With this approach we tried to create a specific metabolite profiling of women who subsequently develop PE and to evaluate the detection rate of the selected metabolites.

Part 2

In Part 2 of this thesis the predictive value of known first trimester biomarkers was evaluated in a retrospective manner (**Chapters 5-6**). For this purpose we used the specimens (first trimester serum and medical records) collected from the subjects before the clinical diagnosis was made and compared them with those from control subjects. In this manner we provided the evidence regarding the capacity of the biomarker to detect preclinical disease. This part of the thesis was not limited to the prediction of PE; we also studied the predictive value of the biomarkers for IUGR and macrosomia at birth.

In **Chapter 5** we investigated the predictive value of the first trimester placental biomarkers in combination with first trimester mean arterial pressure and maternal characteristics for the identification of early and late onset PE. Furthermore we assessed the placental function in PE pregnancies with regard to birthweight (centiles) of the fetuses, highlighting IUGR.

In **Chapter 6** we assessed the early development of the placenta and studied the value of first trimester placental biomarkers in the prediction of macrosomia at birth in women with pregestational diabetes mellitus (PGDM).

Part 3

Part 3 of this thesis describes the physiology of the biomarkers measured longitudinally (**Chapters 7-10**). In this part we aimed to capture the fourth prospective phase of the biomarkers development and to provide the baseline of biomarker values in time. The measurements were made four times in early pregnancy at the gestational weeks 6-7, 8-9, 10-11 and 12-13 in four different populations of pregnant women.

In **Chapter 7** the changes of biomarkers are shown in physiological pregnancies and in **Chapters 8-10** in three groups of a priori high risk pregnancies: pregnancies of women who had an earlier pregnancy complicated by PE (**Chapter 8**); pregnancies of women suffering from PGDM (**Chapter 9**); and pregnancies after assisted reproductive technologies (**Chapter 10**; IVF- in vitro fertilisation and ICSI – intra cytoplasmatic semen injection).

This thesis concludes with a general discussion. The discussion embraces the 5th phase of biomarker development model. We recapitulate the implications of the presented studies and discuss suggestions for future research, implementation of new first trimester prenatal screening and the consequences of this particular screening for the broader population.

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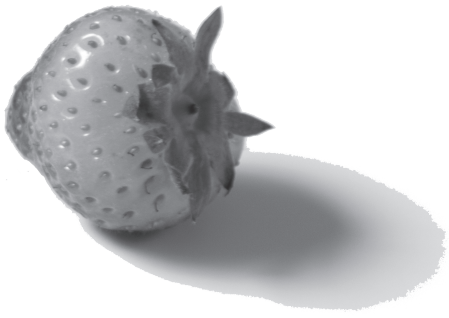
PART 1
PRECLINICAL EXPLORATORY AND ASSAY VALIDATION – PHASE 1 AND 2

II

EVALUATION OF SEVEN SERUM BIOMARKERS AND UTERINE ARTERY DOPPLER ULTRASOUND FOR FIRST TRIMESTER PREDICTION OF PREECLAMPSIA

A systematic review



ABSTRACT

Preeclampsia (PE) affects 1% to 2% of pregnant women and is a leading cause of maternal and perinatal morbidity and mortality worldwide. The clinical syndrome of PE arises in the second half of pregnancy. However, many underlying factors including defective placentation may already be apparent in the first and early second trimester in many patients. In clinical practice, there is currently no reliable screening method in the first trimester of pregnancy with sufficient accuracy to identify women at high risk to develop PE. Early identification of high risk pregnancy may facilitate the development of new strategies for antenatal surveillance or prevention and thus improve maternal and perinatal outcome. The aim of this systematic review was to study the literature on the predictive potential of first trimester serum markers and of uterine artery Doppler velocity waveform assessment (Ut-A Doppler). Literature on the 7 most studied serum markers (ADAM12, fβ-hCG, Inhibin A, Activin A, PP13, PlGF, and PAPP-A) and Ut-A Doppler was primarily selected. In the selected literature, a combination of these markers was analyzed, and where relevant, the value of maternal characteristics was added. Measurements of serum markers and Ut-A Doppler were performed between week 8 + 0 and 14 + 0 gestational age (GA). Low levels of PP13, PlGF, and PAPP-A and elevated level of Inhibin A have been found to be significantly associated with the development of PE later in pregnancy. The detection rates of single markers, fixed at 10% false positive rate, in the prediction of early onset PE were relatively low, and ranged from 22% to 83%. Detection rates for combinations of multiple markers varied between 38% and 100%. Therefore, a combination of multiple markers yields high detection rates and is promising to identify patients at high risk of developing PE. However, large-scale prospective studies are required to evaluate the power of this integrated approach in clinical practice.

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INTRODUCTION

Preeclampsia (PE) is a serious complication of pregnancy that affects approximately 1% to 2% of pregnant women worldwide.[1,2] It is a leading cause of maternal and perinatal morbidity and mortality, particularly when it occurs at a gestational age (GA) of less than 34 weeks.[3,4] Although its presentation is predominantly late term with a mild clinical course, severe maternal complications of PE do occur and include: renal failure, hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome, liver hemorrhage and rupture, eclampsia, cerebral hemorrhage, and maternal death. In addition, PE is associated with substantial risk of perinatal morbidity and mortality due to concomitant intrauterine growth restriction, iatrogenic prematurity, placental abruption, and stillbirth.

PE is considered to result from a complex interaction between placental factors, maternal constitutional factors, and pregnancy-specific vascular and immunologic adaptation.[5-8] These interactions predominantly involve the cardiovascular and inflammatory systems, resulting in marked maternal endothelial dysfunction and organ damage due to vascular compromise.[5,6,8,9] PE is a heterogeneous syndrome that likely does not always develop by the same pathophysiologic pathway, and the causative complex of interacting factors may differ from patient to patient.[5,6,8,9] Some authors distinguish between the two types of PE, maternal and placental PE. Placental PE is considered to result from impaired trophoblast invasion into the spiral arteries and their failure to remodel.[6,10] Narrow spiral arteries lead to placental ischemia and generate oxidative stress conditions.[11] Early onset PE (EO-PE), developing relatively early in pregnancy and necessitating delivery before 34 weeks' gestation, is more frequently associated with this defective placentation than late onset (LO-PE) disease. Conversely, maternal PE, i.e., resulting predominantly from maternal constitutional factors such as high blood pressure, obesity, impaired glucose tolerance, and dyslipidemia, has been suggested to be the predominant type in late pregnancy.[11] However, genuine placental and maternal PE, although attractive from a conceptual point of view, may be rare and most cases of PE are likely to be of mixed etiology, i.e., resulting from interplay between factors of more than one of the three earlier mentioned categories.

Despite a current lack of effective preventive strategies, risk assessment for PE early in pregnancy may be of benefit for both pregnancy outcome and optimization of resource utilization in antenatal care.[12] Stratification of women by risk category, as early as the first trimester of pregnancy, could enable intensified antenatal surveillance, timely intervention and better outcomes in those who are at high risk, and less intensified antenatal care and additional testing in those at low risk. However, so far we have no reliable single screening test to identify women who are at high risk before the clinical manifestation of PE.[12,13]

Placental factors that have been studied for their predictive potential for PE include A Disintegrin And Metalloproteinase 12 (ADAM12), free β subunit of human Chorionic Gonadotropin (fβ-hCG), Inhibin A, Activin A, Placental Protein 13 (PP13), Placental Growth Factor (PlGF), and Pregnancy Associated Plasma Protein-A (PAPP-A).[14-18] Second, maternal (constitutional) factors such as parity, age, body mass index, blood pressure, and medical history are also thought to be useful in identifying high risk women. Additionally, abnormal vascular adaptation to pregnancy, such as unfavorable adaptive changes of the

uterine artery (Ut-A), may contribute to the pathogenesis of PE.[19] Based on the current knowledge of its pathogenesis, it may be expected that optimal strategies for stratification of overall PE risk will include factors from all three categories.

The aim of this systematic review was to appraise the recent literature on the development of PE in the first trimester; evaluate the predictive value of the most studied single and combined first trimester placental serum markers, Ut-A Doppler measurements, and maternal characteristics; and finally rank the use of these first trimester markers in PE risk assessment.

METHODS

Definitions of PE

For PE, we included only studies that used the definition according to the International Society for the Study of Hypertension in Pregnancy: gestational hypertension beyond 20 weeks GA in previously normotensive women with a systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on at least 2 occasions 4 hours apart with the presence of proteinuria of ≥ 300 mg in 24-hour collection or ≥ 2+ by dipstick on a spot urinalysis with or without generalized edema.[20]

EO-PE was defined as requiring delivery before 34 weeks of gestation, LO-PE requiring delivery at or after 34 weeks of gestation.[21]

Literature Search

A systematic literature search of PubMED, EMBASE, CINAHL, and Cochrane was performed on October 21, 2010. In a single comprehensive search, we aimed to find all primary studies reporting on the accuracy of screening tests in the first trimester of pregnancy using serum markers in maternal blood and Ut-A Doppler. To find relevant articles, various terms within the determinant (e.g., first trimester and serum markers or first trimester uterine artery Doppler) and outcome (e.g., PE) were combined. The domain was not included in the search filter to prevent retrieval and reporting bias. The syntax is listed in Table 1. Both determinant and outcome were searched in title and abstract in all search engines. After filtering doubles, 2 authors (S.K., E.J.W.) separately screened titles and abstracts of all selected studies to manually identify the articles investigating the value of first trimester serum markers and Ut-A Doppler in the prediction of PE. Authors used inclusion and exclusion criteria shown in Figure 1. The full text of the remaining articles was retrieved. Subsequently, more extensive inclusion and exclusion criteria were applied.

Quality Assessment

The methodological quality of the finally selected studies (n = 35) was appraised independently by 2 reviewers (S.K. and E.J.W.) using the validated Quality Assessment of Diagnostic Accuracy Studies tool (Fig. 2).[22]

Data Analysis

Pregnancies complicated by PE were divided into 3 groups: (I) EO-PE, (II) LO-PE, and (III) PE. PE was used for those studies in which the PE subgroups definition was different from the one applied in this review or the studies in which the PE subgroups were not specified.

Table 1. Search syntax.

Search syntax 21.10.2010	
Search	Synonyms
#1	(11+0 AND 13+6) OR (11+0 AND 14+0) OR (11-13) OR (11-14) OR ("First trimester" OR "first-trimester" OR ((first OR early OR initial OR inaugural OR premier OR primal OR primary OR prime) AND ((trimester OR trimesters OR quarter OR quarters) OR (pregnancy OR conception OR conceptions OR gestation OR gestations OR gestosis OR gravidity OR gravidities))) OR (placental AND (phase OR phases OR stage OR state OR states))) AND (serum OR blood OR marker OR markers OR factor OR factors OR screening OR (Doppler OR ultrasonography OR ultrasonographies OR sonography OR sonographies OR ultrasound OR ultrasounds OR (blood AND (flow OR flows OR flowing OR flood OR inflow OR inflows OR move OR moves OR pass OR passes OR run OR runs OR stream OR streams) AND (velocity OR rapidity OR rate OR swiftness OR tempo)) AND ((artery OR arteries OR arterias OR arterial) AND (uterine OR uterus OR uteri OR womb))))
#2	(PE OR Preeclampsia OR "pre-eclampsia" OR (pre AND eclampsia) OR eclampsia) OR (((pregnancy OR conception OR conceptions OR gestation OR gestations OR gestosis OR gravidity OR gravidities) OR complex OR (syndrome OR syndromes)) AND (toxaemia OR toxemia OR toxicosis) OR ((edema OR oedema OR edemas OR oedemas OR dropsy) AND (proteinuria OR proteinurias OR albuminuria OR albuminurias) AND (hypertension OR hypertensions OR (high AND blood AND pressure))))
#3	#1 AND #2

In this review, we were interested in the detection rate (DR) of the tests at a fixed 10% false positive rate (FPR). Where the appropriate data could be derived from the reports, it was incorporated into this review. Otherwise, we personally approached the authors of the studies for additional information. When, even after various attempts we were not able to retrieve the appropriate data, we derived DR from the receiver operating characteristics (ROC) shown in the articles, where applicable. Otherwise the studies were discarded. If the 95% confidence interval (95% CI) of the DR was not cited, we calculated it with a web calculator (available at: http://www. causascientia.org/math_stat/ProportionCI.html).

Initially, we wanted to analyze the screening performance of first trimester serum markers and Ut-A Doppler measurements only. In a number of selected studies, however, one or more maternal characteristics (MC) were enclosed as a separate variable. MC in all studies contained combination of characteristics such as maternal age, maternal weight, race, parity, cigarette smoking, family history of PE, method of conception, medical history, and medication during pregnancy. Separate description and analysis of individual risk factors reaches beyond the scope of this review. However, when MC were included in selected studies, they were regarded as a separate variable in the current review.

Upon final selection of the studies, authors considered the performance of:

- Serum markers either individually or combined
- Ut-A Doppler measurements
- Ut-A Doppler measurements and serum markers combined
- Maternal characteristics
- Maternal characteristics combined with serum markers or with Ut-A Doppler

Results are presented as forest plots of DR at 10% FPR with 95% CIs for EO-PE, LO-PE, and PE, respectively.

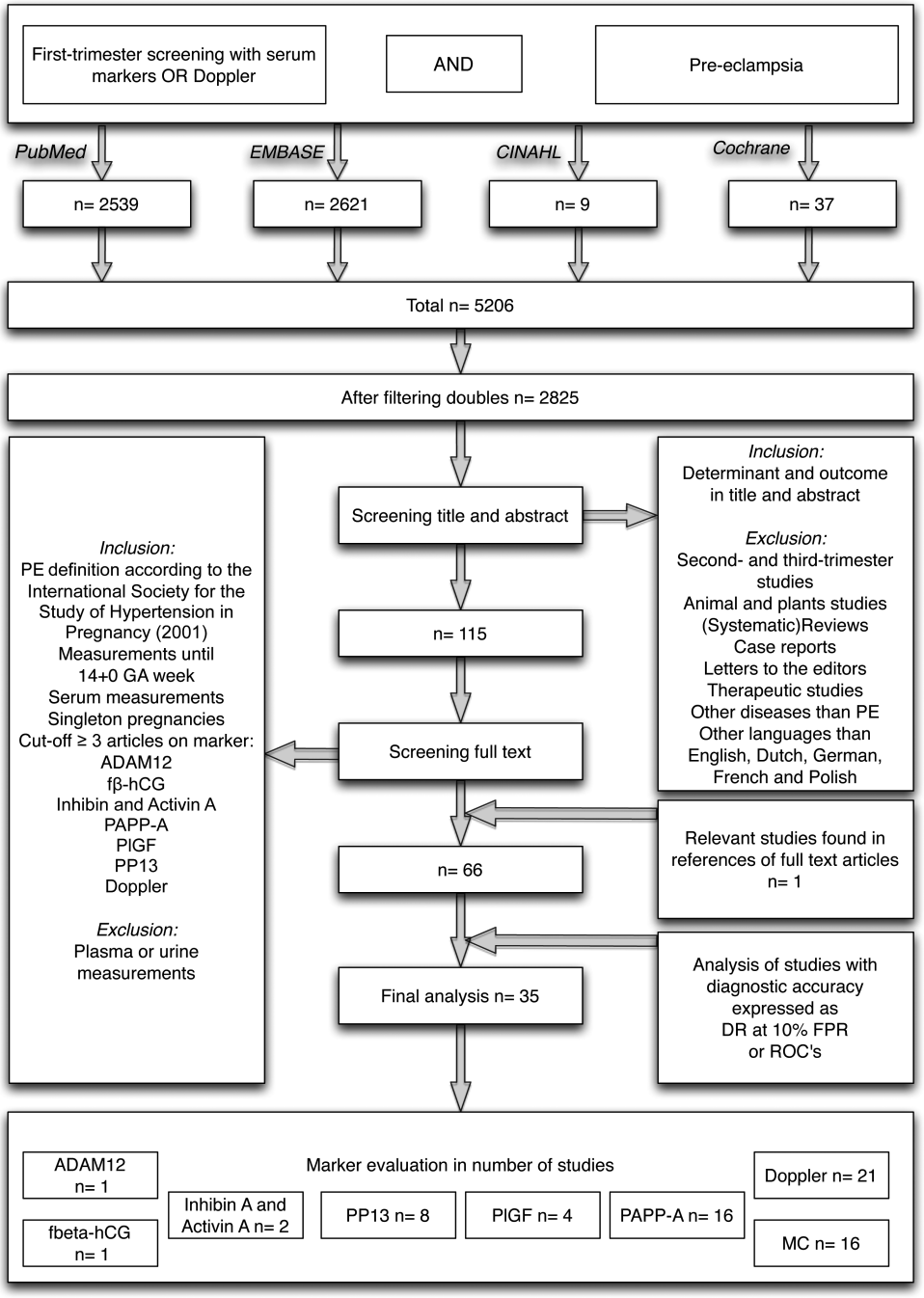

Figure 1. Flow chart of included studies on first trimester markers used to predict PE. GA indicates gestational age; ROC, receiver operating characteristic.

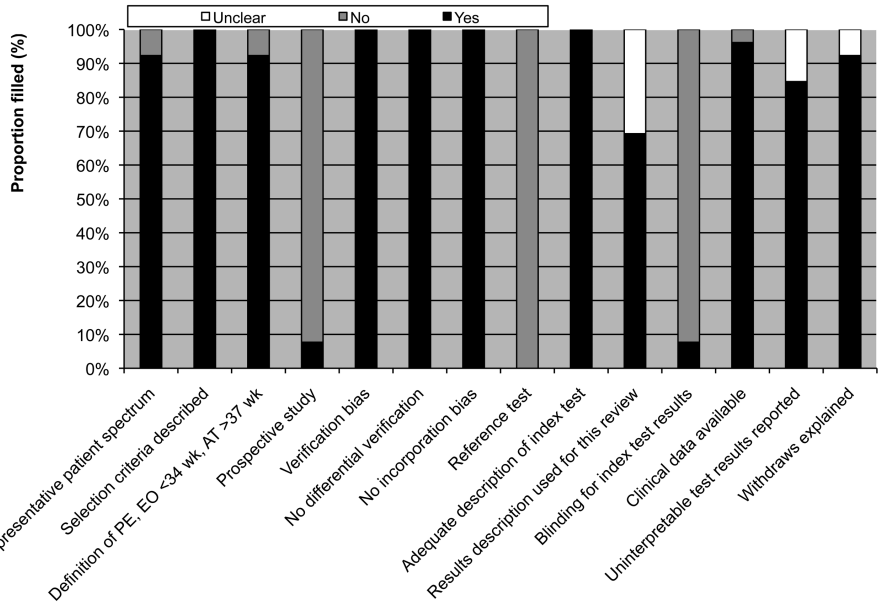

Figure 2. Summary of quality assessment (QUADAS).

RESULTS

Figure 1 gives an overview of the selection process for relevant literature. The literature search yielded 5.206 articles, after filtering doubles 2.825 were left for screening of titles and abstracts. After screening for title and abstract using specific inclusion and exclusion criteria, 115 articles were retrieved for full text reading. Given the large number of the proposed serum markers tested for their potential role in screening for PE, an additional exclusion criterion comprising a cut off value of ≥ 3 articles describing a specific serum marker in the first trimester of pregnancy was added. As a consequence, seven maternal serum biomarkers were selected: ADAM12, fβ-hCG, Inhibin A, Activin A, PP13, PlGF, and PAPP-A together with Ut-A Doppler measurements. This step resulted in 66 studies. One additional study meeting all selection criteria was found after reading the references of selected full-text articles, and was also included. This study was not captured by the primary search because of lack of PE or other synonyms of the disease in title and abstract.

To be able to compare the performance of the selected markers, only studies containing the diagnostic accuracy of the markers presented as DR at fixed 10% FPR or as ROCs reached the final analysis (n = 35; Fig. 1). Therefore, although markers such as ADAM12 were described more than 3 times in the literature, only a single study on this particular marker met the diagnostic accuracy criterion.[17] All 35 studies were case control or cohort studies in which the cases were included after PE was clinically diagnosed. Among the selected studies, 8 studies evaluated PP13 separately or in combination with

other markers[15,23-29], 4 evaluated PlGF[14,15,30,31], 16 considered PAPP-A[14-17,23,29-39], and 21 Ut-A Doppler.[14,23,26,27,30,31,36,37,40–52] Inhibin A was evaluated in 2 studies and ADAM12, fβ-hCG and Activin A (in combination with Inhibin A) were evaluated in a single study each.[17,18,35,42] In 16 studies, MC were added to the analysis of the markers.[14,16,23,28,30,31,33,36,37,40,46–48,50,51,53] Table 2 lists the finally selected studies, test characteristics, and study population.

In total, we included 138,571 women of whom 3654 developed PE (2.6%). Measurements of both serum markers and Ut-A Doppler were performed between week 8 + 0 and 14 + 0 GA in all studies. The study population comprised women with low prior risk, except for 3 studies.[26,40,43]

ADAM 12

ADAM12 is a placenta-derived member of the ADAM protein family.[54] It is present in the syncytiotrophoblast and is thought to be involved in placental growth and development. Gack et al., demonstrated that ADAM12 was the most upregulated transcript in placental tissues of women with PE.[55] This finding led to speculations as to whether ADAM12 could serve as an early biomarker for hypertensive disorders of pregnancy.[55] There are 5 published studies that have measured serum levels of ADAM12 between 8 and 14 weeks of gestation.[15,17,31,56,57] In these studies, the percentage of PE cases was 15.5% (n PE = 88), 1.5% (n PE = 64), 4.5% (n PE = 40), 33% (n PE = 160), and 11.2% (n PE = 128), respectively. Laigaard et al., and Spencer et al., have shown reduced ADAM12 levels in pregnancies complicated by PE, and in both studies ADAM12 was suggested to be a potential PE marker (median ADAM12 MoMs were 0.86, P = 0.008 and 0.49 P = 0.0001, respectively).[17,56] In contrast, Poon et al., Audibert et al., and Wortelboer et al., reported no alteration of first trimester ADAM12 levels in women developing PE.[15,31,57] The median MoMs in these studies were not significantly different from controls (0.95, 1.04, and 1.02, respectively). In only one study could a DR of 37% for unspecified PE be derived from the ROC curve (Fig. 5).[17]

Free Beta Subunit of hCG

The fβ-hCG is secreted by the syncytiotrophoblast cells. Its primary function is to maintain the decidual spiral arteries and the vascular supply of the placenta during pregnancy.[58] In normal pregnancies, the concentration of fβ-hCG increases exponentially until 8 to 10 weeks, decreasing afterwards. In the second trimester, fβ-hCG has been reported to be elevated in women who later develop PE.[59-61] Studies that have retrospectively evaluated fβ-hCG have shown no evidence for a predictive value of this marker for hypertensive disorders of pregnancy.[16,31,33,35,38,39,62-65] Only in one study, a DR of 22% for unspecified PE was mentioned (Fig. 5).[35]

Inhibin A and Activin A

The fetoplacental unit is an important source of Inhibin A and Activin A, and it has been suggested that both are involved in a feedback loop regulating hCG levels during pregnancy.[66,67] Muttukrishna et al., have shown that the third trimester maternal serum concentrations of both markers were about 10-fold higher in women with severe PE as compared to controls.[68] Several studies have shown that Inhibin A and Activin A were

significantly elevated in the first trimester in women with PE.[18,31,65,69,70] In these studies, the incidence of PE was 33% (n PE = 52), 4.5% (n PE = 40), 33%(n PE = 30), 1.2% (n PE = 9),and 21% (n PE = 64), respectively. In only the study by Spencer et al., was the DR for Inhibin A and Activin A for unspecified PE stated; 35% and 20%, respectively (Fig. 5).[18] Further separate analysis of Inhibin A was made by Audibert et al.,; however in that study the DRs were presented only in combination with that of other markers.[31]

Placental Protein 13

PP13 is produced predominantly by the syncytiotrophoblast and is thought to play a major role in the implementation of the blastocyst.[71] PP13 has also been suggested to be involved in the remodeling of the common fetomaternal blood-spaces through binding to proteins between the placenta and endometrium.[72-74] From the first trimester onwards, levels of PP13 slowly increase in healthy pregnancies. First trimester concentrations of PP13 have been shown to be significantly lower in the first trimester, but higher in the second and third trimesters in association with PE.[72] The reasons for this are currently not known. More specifically, 5 studies reported significant differences between the median PP13 MoM of EO-PE and control pregnancies in the first trimester (Table 3).[15,23,26,27,75] In these studies, the DR of PP13 as a single marker was 36% to 80% (Fig. 3). In PE pregnancies, DRs and MoMs were comparable to those of EO-PE (Fig. 5).[24-26,28,29]

PlGF and other Angiogenic Factors

An imbalance between pro- and antiangiogenic factors before and after the onset of PE is suggested to play a crucial role in its pathogenesis.[7] It is thought that the poorly implanted placenta becomes ischemic and subsequently secretes antiangiogenic factors such as sFlt-1 (soluble Fms-like Tyrosine Kinase-1) also known as soluble vascular endothelial growth factor receptor-1 and sEng (soluble endoglin) into the maternal circulation, which later antagonize a number of proangiogenic factors, such as PlGF and vascular endothelial growth factor.[76] It is hypothesized that as a consequence, the concentration of important angiogenic and PlGFs in the maternal circulation is reduced, leading to impaired endothelial function and subsequently EO-PE.

These studies concentrated mostly on the second half of the pregnancy in which a clear difference in the concentrations of both antiangiogenic and proangiogenic factors in PE pregnancies was shown when compared with controls (antiangiogenic factors elevated[77,78], proangiogenic factors decreased.[78-80] Major studies in the first trimester, however, have shown that plasma sFlt-1 and sEng were constant throughout the first trimester and that their concentrations in normal pregnancy might be equal to or possibly lower than those in pregnancies destined to be complicated by PE.[30,75,78,81-84] From the perspective of first trimester screening for PE, studies on antiangiogenic factors have been indeterminate and so far do not support inclusion of these factors as new markers for risk assessment for PE. Moreover, there were not enough studies on these markers tested in the first trimester serum; therefore, neither sFlt-1 nor sEng was included in this review.

Proangiogenic factors, particularly PlGF, can be measured as early as 9 weeks of gestation, whereas vascular endothelial growth factor concentrations are too low to be

detected in the first trimester.[78,85] Consequently, the majority of reports considering the proangiogenic factors in the first trimester concentrate on PlGF. PlGF concentration are found to increase throughout normal pregnancy beginning in the first trimester. In contrast, in pregnancies destined to develop PE, PlGF increases less or remains low throughout pregnancy.[14,30,31,75,78,81-83,86] The DR of PlGF alone in first trimester measurements for predicting EO-PE was 41% to 59%, and for LO-PE 33% (Fig. 3).[14,15,30] The median MoMs for PlGF were lower for both EO-PE and LO-PE (Table 3 and Fig. 4).[14,15,30,31]

PAPP-A

PAPP-A is produced by the developing syncytiotrophoblast.[87,88] It regulates the bioavailability of free IGF at the placental-decidual interface during human implantation.[89,90] It is thought to play a major role in the autocrine and paracrine regulation of trophoblast invasion into the deciduas.[91] Low concentrations of PAPP-A in the first trimester of pregnancy are highly associated with chromosomal aneuploidies. In pregnancies with a normal karyotype, low PAPP-A has been shown to be an indicator of increased risk for various pregnancy complications.[38] The majority of the published studies have shown that low concentrations of PAPP-A are significantly associated with EO-PE[14,15,23,29,30,35,37,39] (Table 3), with DR ranges from 22% to 43%[14,15,23,30] (Fig. 3). The median MoMs for PAPP-A for EO-PE, LO-PE, and PE were lower than in controls[14,23,29–31,35,37,39] (Table 3).

Uterine Artery Doppler Velocity Waveform Patterns

Ut-A Doppler has become a valuable tool for the study of uteroplacental circulation.[92] The development of trophoblast invasion can be followed by measurement of impedance to flow in the uterine arteries. In normal pregnancies, impedance to flow progressively decreases between 6 and 24 weeks' gestation and remains constant thereafter.[93,94] Hence, abnormal development of the placental vasculature related to PE can be detected by sustained impedance to flow in the maternal uterine vessels.[92,95,96]

This observation has led to the idea of using Ut-A Doppler as a screening tool in predicting adverse pregnancy outcomes. Abnormal uterine artery waveforms seem to be a good predictor of PE. The DR for EO-PE has varied from 29% to 83% in published studies (Fig. 3).[14,23,27,30,37,40,41,46,48,50–52] For LO-PE and PE, reported DR have ranged between 5% and 62% (Figs. 4, 5).[14,36,37,40-49,52] Median MoMs of Ut-A Doppler have been shown to be markedly higher in all variants of PE as compared with healthy pregnancies (Table 3).[14,23,29–31,35,37,39,40,48,50,51]

Maternal Characteristics

PE is considered to be a multifactorial disease with various maternal constitutional factors contributing to its pathogenesis. Risk factors include nulliparity, maternal age ≥ 40, body mass index > 29, previous PE, family history of PE, chronic hypertension, and race.[97-99] Separate description and analysis of these risk factors are beyond the scope of this review. However, several of the analyzed articles used one or more risk factors of MC to improve first trimester screening.[14,16,23,28,30,31,36–38,40,46–48,50,51] In this review, all maternal risk factors were analyzed together under the section called maternal characteristics. DR of MC alone ranged from 23% to 56% in EO-PE, LO-PE, and PE (Figs.3-5).[14,16,23,28,30,31,36–38,40,46–48,50,51]

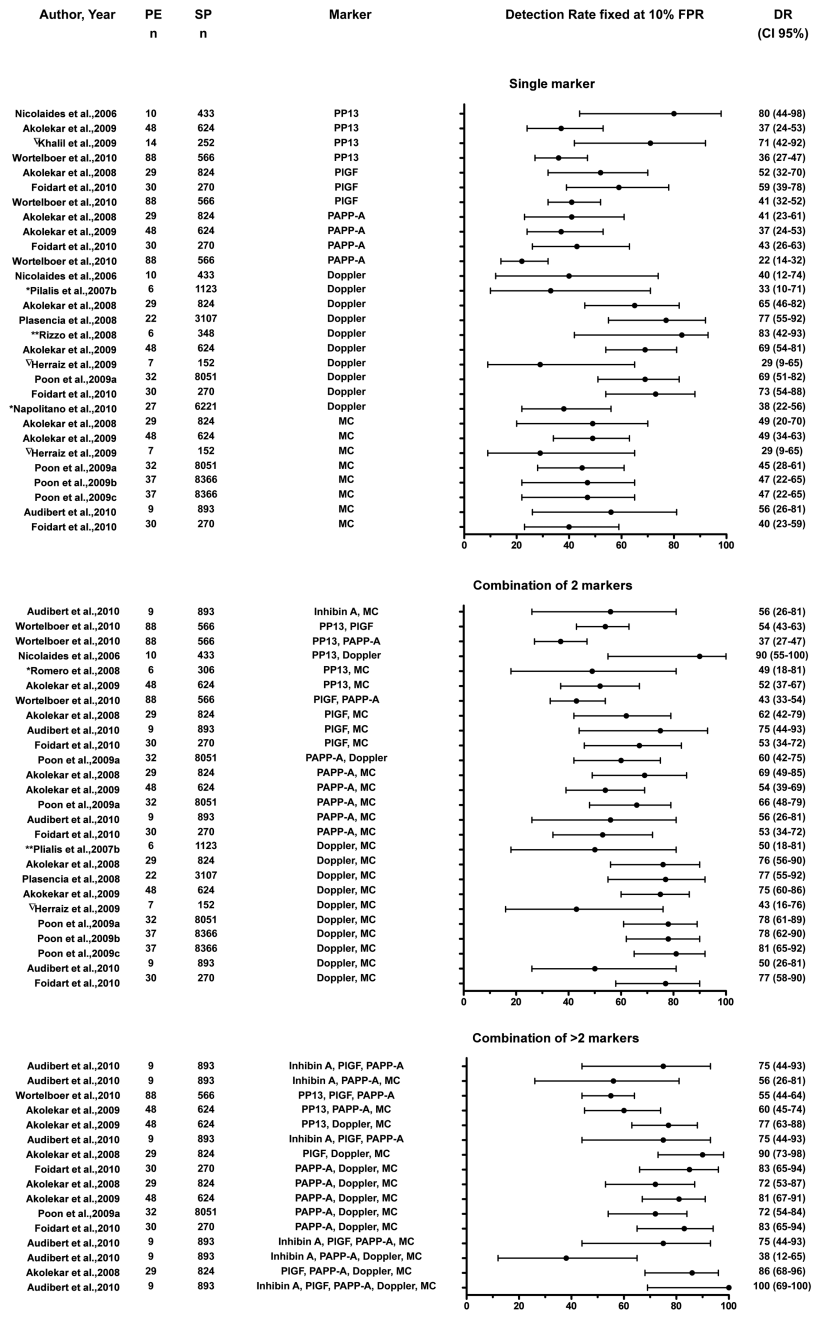

Figure 3. Forest plot with the detection rates (DR) of the screening tests fixed at 10% false positive rates (FPR) in prediction of early onset PE (EO-PE) in the first trimester, with 95% confidence intervals (CI). PE indicates number of preeclampsia cases; SP, study population; MC, maternal characteristics. High risk population; * Adapted from ROC curve; ** contact with the author of the study.

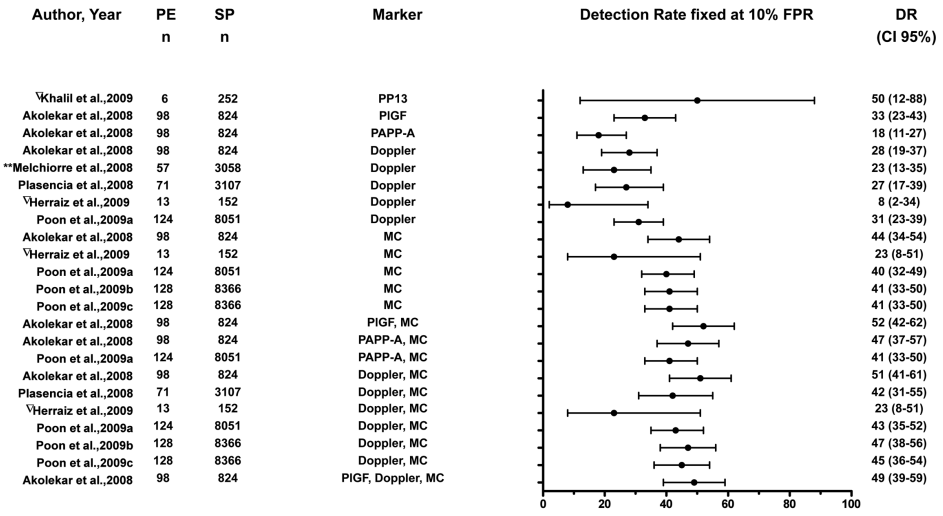

Figure 4. Forest plot with the detection rates (DR) of the screening tests fixed at 10% false positive rates (FPR) in prediction of late onset preeclampsia (LO-PE) in the first trimester, with 95% confidence intervals (CI). PE indicates number of preeclampsia cases; SP, study population; MC, maternal characteristics. High risk population; ** contact with the author of the study.

Combined Screening

In all reviewed studies, markers were combined in different ways, including a combination of serum markers only, of serum markers in combination with Ut-A Doppler or/and with MC. In this review, we separately analyzed the combination of 2 markers and the combination of more than 2 markers. Among the studies, which combined 2 markers, the best DR was reached for the combination of PP13 and Ut-A Doppler (90% and EO-PE n = 10).[27] Furthermore, combination of Ut-A Doppler with MC appears promising, ranging between 51% and 81%.[14,23,30,31,37,40,46,48,50,51] DR for the combination of more than 2 markers varied between 38% and 100% with the best results seen with the combination of 5 markers (Inhibin A, PlGF, PAPP-A, Ut-A Doppler, and MC).[14,15,23,30,31,37]

DISCUSSION

Early screening for risk of PE would be of benefit in identifying patients at high risk for maternal and perinatal complications later in the pregnancy. In this systematic review, we have found that fβ-hCG is not suitable for the prediction of PE. In all 10 studies concerning this particular marker, no difference in the levels of fβ-hCG between PE cases and controls was found. The data regarding ADAM12 or Activin A are too contradictory (ADAM12) or limited (Activin A) to determine their predictive value. More studies on these 2 markers have to be conducted to evaluate their possible role in the PE screening. In contrast, low first trimester levels of PP13, PlGF, and PAPP-A and increased levels of

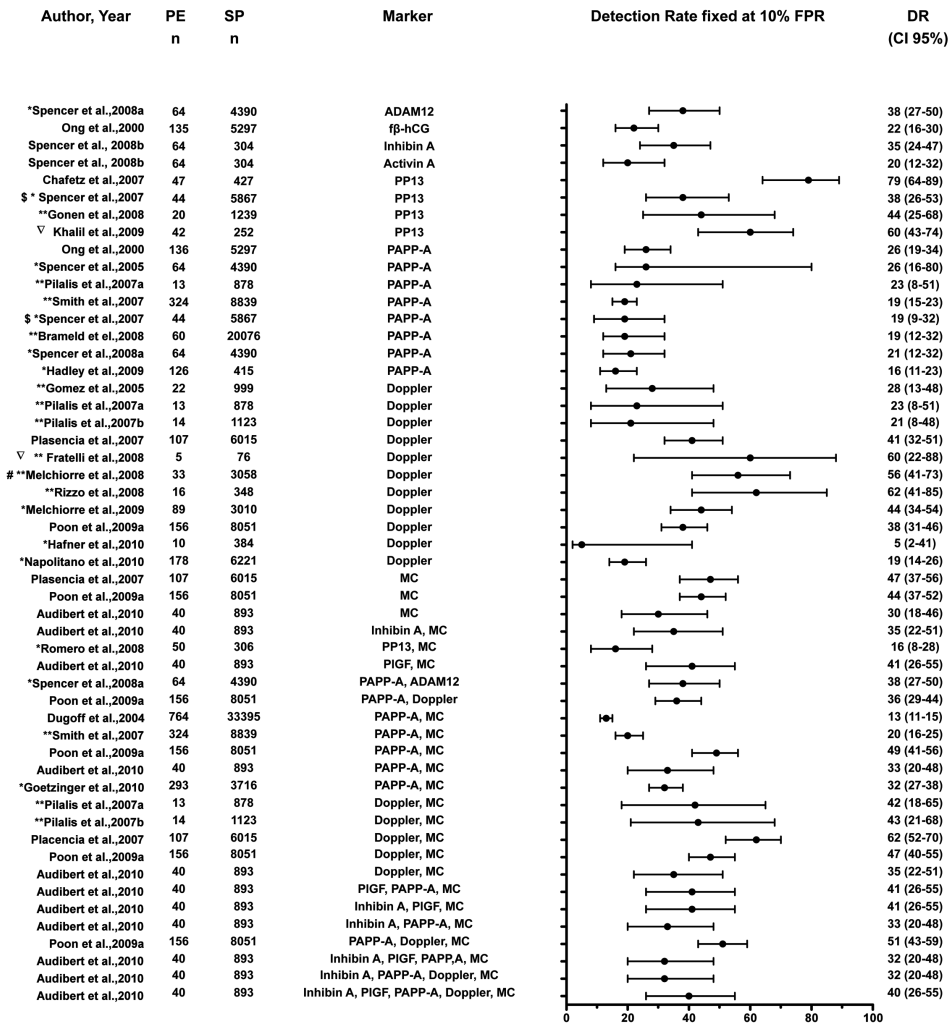

Figure 5. Forest plot with the detection rates (DR) of the screening tests fixed at 10% false positive rates (FPR) in prediction of PE (PE cases/not specified in the studies) in the first trimester, with 95% confidence intervals (CI). PE indicates number of preeclampsia cases; SP, study population; MC, maternal characteristics. $, delivery < 35 weeks; ∇, high-risk population; #, delivery < 37 week; * Adapted from ROC curve; ** contact with the author of the study.

Inhibin A were significantly associated with the development of PE later in pregnancy. However, none of these 4 serum markers seems to be promising on its own. The screening potential of each single serum marker was limited by only modest DRs at a FPR of 10%. For screening of unselected populations and implementation in the clinic, much higher DRs are needed so that a substantial number of high risk cases are not missed. Therefore, single marker screening is unsuitable for clinical practice. Combinations of best performing

serum markers with maternal constitutional characteristics and/or Ut-A Doppler yield higher DRs and are, therefore, more promising. The DRs appeared to be lower for LO-PE as compared to EO-PE, which seems logical given the mostly normal placentation in the former.[14,23,37,40,46,48,50,51] These findings are clinically relevant, especially because EO-PE is associated with multiple maternal and fetal complications.

Regarding the prediction of EO-PE, data are promising with DRs ranging between 55% and 100%. So far the highest DR (100%) is reached through the combination of 5 markers (Inhibin A, PlGF, PAPP-A, Ut-A Doppler, and MC). However, it should not be forgotten that this particular combination of markers was studied so far only once and in a study with a very low number of PE cases. Nevertheless, the combination of Ut-A Doppler, MC, and multiple serum markers appears promising because the DRs in all studies reached 70% to 90%. However, all current studies have been underpowered, with very low numbers of PE cases (highest number: 88 cases); moreover, the great majority of studies was retrospective. Large prospective studies are needed to confirm these reported associations and to validate the potential utility of combinations of markers in different populations/cohorts.

Other markers, such as nucleic acids, proteins, peptides, or cellular metabolites, are also the subject of current investigation. Recent data report that a combination of 14 markers quantified with the metabolomics technique resulted in a predictive model with an area under the curve of > 0.9 and a DR of 73% to 77% at 14 to 16 weeks of gestation in the original study and in a replica study.[100] It could be of great potential utility if this strategy were found to be effective at an earlier gestational age. Using proteomics, Rasanen et al., were able to accurately discriminate between patients who developed PE and controls using a combination of 16 proteins (DR 88% derived from area under the curve).[101] However, when serum was taken between 8 and 14 weeks of gestation, the DR of PE was considerably lower (exact DR was not given). [101] The number of patients who developed PE in this study was relatively small and a validation of the use of proteomics in a larger population is, therefore, still needed.

A best potential for a first trimester screening test appears to involve a multiple marker blood test combined with measurements of uterine artery Doppler, since the latter is still the most promising screening tool. Nevertheless, measurements of uterine artery Doppler are difficult and need to be standardized before being broadly implemented. Large prospective studies using standardized measurement methods are needed to evaluate the potential combination strategies.

Implications

A clinically relevant first trimester screening test for PE remains a major challenge. Such a test could potentially push current antenatal care into a new era. It would help early identification of women who are at high risk of PE, who consequently shift from routine visits to a more individualized approach. Close maternal and fetal surveillance, starting from the first weeks of pregnancy, may greatly improve their pregnancy outcome.

Unfortunately, there are, as yet, no effective preventive treatment strategies, and low-dose aspirin only reduces the risk of early onset PE by about 10% in high risk

women.[102] However, there is evidence that the prophylactic use of aspirin starting before 16 weeks of gestation may result in a 50% reduction of the incidence of PE (RR: 0.48; 95 CI: 0.33–0.68), although data are rather limited thus far.[103] If this is confirmed in the future trials, early identification of patients at risk for PE would be of even greater benefit.

An effective screening may therefore lead to a better utilization of resources in antenatal care. It will help in the selection of suitable patients for future randomized controlled trials and with investigations into potential preventive measures, such as low-dose aspirin, to be started early in the pregnancy.[102,103] This could lead to the prevention and/or earlier diagnosis of the clinical signs of the disease and avoid serious complications.

Limitations

This systematic review has several limitations. First, the number of affected women in the studies included was relatively small; clearly large-scale studies are needed. The majority of the studies in our review did not specify the timing of onset of PE. Only 15 studies with a relatively small number of patients (max n = 88) evaluated the risk for early onset disease. Second, although the quality of included studies was generally sufficient, they had several shortcomings. In particular, only 2 studies were prospective. The great majority of studies were performed after PE had been diagnosed clinically. Moreover, the results varied considerably between the studies, with large differences in reported MoM values of the markers. The latter may be explained by the use of different immunoassay kits with different test characteristics with respect to variability and validity. In addition, discordant results may have been caused by differences in cutoff points for abnormality of the biomarker assays, differences in reporting of uterine artery Doppler measurements (pulsatility or resistance index, PI and RI, respectively), and differences in statistical models used for multiple prediction. Because of this variability, a formal meta-analysis with estimated overall relative risks was not feasible. We dealt with this by presenting the DR at a fixed 10% FPR, which allows for recognition of a trend in presented results.

Conclusion

After completion of this review, physicians should be better able to appraise the recent literature on the development of PE in the first trimester, evaluate the predictive value of first trimester markers and their use in assessment of PE risk.

Although this review showed that a combination of serum makers, uterine artery Doppler measurements, and maternal characteristics may help to identify high risk patients, currently there is no validated screening tests that may accurately predict PE early in the pregnancy. New techniques will hopefully provide sets of multiple markers, which will lead to a screening program with clinically relevant performance. Early detection of women at high risk may enable trials of interventions that will lead to prevention or treatment of PE with a better pregnancy outcome. A major continuing search for new potential screening makers and therapeutic opportunities is needed.

Table 2. Study characteristics of included studies for prediction of PE.

Early Onset PE						
Author, year	Study design	Population	Measurements GA weeks	EO-PE n (%)	Controls n	Study population
Nicolaides 2006(29)	1	LR	11 – 14	10 (2.3)	423	433
Pilalis 2007b(48)	1	LR	11 – 14	6 (0.5)	n.a.	1123
Akolekar 2008(17)	1	LR	11 – 14	29 (3.5)	609	824
Plasencia 2008(50)	1	LR	11 – 14	22 (0.7)	2595	3107
Rizzo 2008(54)	1	LR	11– 14	6 (1.7)	n.a.	348
Romero 2008(30)	1	LR	8 – 14	6 (2.0)	250	306
Akolekar 2009(25)	1	LR	11 – 14	48 (7.7)	416	624
Herraiz 2009(43)	1	HR	11 – 14	7 (4.6)	132	152
Khalil 2009(28)	1	HR	11 – 14	14 (5.5)	210	252
Poon 2009a(39)	1	LR	11 – 14	32 (0.4)	7895	8051
Poon 2009b(52)	1	LR	11 – 14	37 (0.4)	8061	8366
Poon 2009c(53)	1	LR	11 – 14	37 (0.4)	8061	8366
Audibert 2010(33)	1	LR	11 – 13	9 (1.0)	833	893
Foidart 2010(32)	1	LR	11 – 14	30 (11.1)	180	270
Napolitano 2010(42)	1	LR	11 – 14	27 (0.4)	6043	6221
Wortelboer 2010(18)	1	LR	8 – 14	88 (15.5)	478	566
Late Onset PE						
Author, year	Study design	Population	Measurements GA weeks	LO-PE n (%)	Controls n	Study population
Akolekar 2008(17)	1	LR	11 – 14	98 (11.9)	609	824
Melchiorre 2008(47)	1	LR	11 – 14	57 (1.9)	2968	3058
Plasencia 2008(50)	1	LR	11 – 14	71 (2.3)	2595	3107
Herraiz 2009(43)	1	HR	11 – 14	13 (8.6)	132	152
Khalil 2009(28)	1	HR	11 – 14	6(2.4)	210	252
Poon 2009a(39)	1	LR	11 – 14	124 (1.5)	7895	8051
Poon 2009b(52)	1	LR	11 – 14	128 (1.5)	8061	8366
Poon 2009c(53)	1	LR	11 – 14	128 (1.5)	8061	8366
Foidart 2010(32)	1	LR	11 – 14	60 (22.2)	180	270
PE not specified						
Author, year	Study design	Population	Measurements GA weeks	PE n (%)	Controls n	Study population
Ong 2000(37)	1	LR	11 – 14	135 (2.5)	4297	5297
Smith 2002(55)	1	LR	8 – 14	324 (3.7)	n.a.	8839
Dugoff 2004(19)	1	LR	10+3 – 14	764 (2.3)	n.a.	33395
Gomez 2005 (46)	1	LR	11 – 14	22 (2.2)	932	999
Spencer 2005(41)	1	LR	11 – 14	64 (1.5)	3999	4390

Table 2. *Continued*

PE not specified						
Author, year	Study design	Population	Measurements GA weeks	PE n (%)	Controls n	Study population
Chafetz 2007(26)	1	LR	9 – 12	47 (11.0)	289	427
Pilalis 2007a(38)	1	LR	11 – 14	13 (1.5)	715	878
Pilalis 2007b(48)	1	LR	11 – 14	14 (1.2)	924	1123
Plasencia 2007(49)	1	LR	11 – 14	107 (1.8)	5041	6015
Spencer 2007(31)	1	LR	11 – 14	44 (0.7)	446	5867
Brameld 2008(36)	1	LR	11 – 14	60 (0.03)	n.a.	20076
Fratelli 2008(45)	1	HR	11 – 14	5 (6.5)	n.a.	76
Gonen 2008(27)	1	LR	6 – 10	20 (1.6)	1178	1239
Melchiorre 2008(47)	1	LR	11 – 14	33 (1.1)	2533	3058
Rizzo 2008(54)	1	LR	11 – 14	16 (4.5)	n.a.	348
Romero 2008(30)	1	LR	8 – 13	50 (16.3)	250	306
Spencer 2008a(20)	1	LR	11 – 14	64 (1.5)	3999	4390
Spencer 2008b(21)	1	LR	11 – 14	64 (21.0)	240	304
Khalil 2009(28)	1	HR	11 – 14	42 (16.6)	210	252
Melchiorre 2009(51)	1	LR	11 – 14	89 (3.0)	2445	3010
Poon 2009a(39)	1	LR	11 – 14	156 (1.9)	7895	8051
Audibert 2010(33)	1	LR	11 – 13	40 (4.5)	833	893
Goetzinger 2010(35)	1	LR	11 – 14	293 (7.9)	3423	3716
Hafner 2010(44)	1	LR	11 – 14	10 (2.6)	374	384
Hedley 2010(34)	1	LR	10 – 14	126 (30.4)	289	415
Napolitano 2010(42)	1	LR	11 – 14	178 (2.9)	6043	6221

Abbreviations: LR low-risk population, HR high-risk population, n.a. not available.

Table 3. Median MoMs of first trimester markers.

Early Onset PE							
			Preeclampsia		Controls		
Author, year	Marker	GA	n	MoM Median	n	MoM Median	*p* value
	ADAM12						
Audibert 2010(33)		11 – 13	9	1.04	833	0.95	n. sign.
	fb-hCG						
Audibert 2010(33)		11 – 13	9	0.83	833	1.06	n. sign.
	Inhibin-A						
Audibert 2010(33)		11 – 13	9	1.67	833	1.03	<0.05
	PP13						
Nicolaides 2006(29)		11 – 14	10	0.07	423	1.00	<0.001
Romero 2008(30)		8 – 13	6	0.26	250	1.00	0.002
Akolekar 2009(25)		11 – 14	48	0.83	416	1.02	<0.0167
Audibert 2010(33)		11 – 13	9	0.99	833	1.02	n. sign.
Wortelboer 2010(18)		8 – 14	88	0.68	478	0.99	<0.0001
	PlGF						
Akolekar 2008(17)		11 – 14	29	0.61	609	0.99	<0.0001
Audibert 2010(33)		11 – 13	9	0.68	833	0.94	n. sign.
Foidart 2010(32)		11 – 14	30	0.61	180	1.01	<0.0167
Wortelboer 2010(18)		8 – 14	88	0.73	478	1.00	<0.0001
	PAPP-A						
Akolekar 2008(17)		11 – 14	29	0.53	609	1.07	<0.0001
Akolekar 2009(25)		11 – 14	48	0.55	416	1.08	<0.0167
Poon 2009a(39)		11 – 14	32	0.55	7895	1.00	<0.001
Audibert 2010(33)		11 – 13	9	0.79	833	1.04	n. sign.
Foidart 2010(32)		11 – 14	30	0.56	180	1.01	<0.0167
Wortelboer 2010(18)		8 – 14	88	0.82	478	0.99	<0.02
	Doppler						
Nicolaides 2006(29)		11 – 14	10	1.43	423	1.00	<0.001
Akolekar 2008(17)		11 – 14	29	1.52	609	1.03	<0.001
Plasencia 2008(50)		11 – 14	22	1.49*	2595	0.99*	<0.001
Akolekar 2009(25)		11 – 14	48	1.61	416	0.97	<0.0167
Herraiz 2009(43)		11 – 14	7	1.13	132	0.91	n. sign.
Poon 2009a(39)		11 – 14	32	1.49	7895	1.01	<0.001
Poon 2009b(52,53)		11 – 14	37	1.51	8061	1.01	<0.0001
Poon 2009c(52,53)		11 – 14	37	1.51	8061	1.01	<0.0001
Audibert 2010(33)		11 – 13	9	1.28	833	1.10	n. sign.
Foidart 2010(32)		11 – 14	30	1.65	180	1.00	<0.0167

Table 3. *Continued*

Late Onset PE							
			Preeclampsia		Controls		
Author, year	Marker	GA	n	MoM Median	n	MoM Median	*p* value
	PP13						
Akolekar 2009(25)		11 – 14	160	0.96	416	1.02	n. sign.
	PlGF						
Akolekar 2008(17)		11 – 14	98	0.82	609	0.99	<0.0001
Foidart 2010(32)		11 – 14	60	0.82	180	1.01	<0.0167
	PAPP-A						
Akolekar 2008(17)		11 – 14	98	0.93	609	1.07	<0.05
Akolekar 2009(25)		11 – 14	124	0.84	416	1.08	<0.0167
Poon 2009a(39)		11 – 14	160	0.91	7895	1.00	0.03
Foidart 2010(32)		11 – 14	60	0.93	180	1.01	n. sign.
	Doppler						
Akolekar 2008(17)		11 – 14	98	1.22	609	1.03	<0.0001
Plasencia 2008(50)		11 – 14	71	1.03*	2595	0.99*	0.04
Akolekar 2009(25)		11 – 14	160	1.25	416	0.97	<0.0167
Herraiz 2009(43)		11 – 14	13	0.84	132	0.91	n. sign.
Poon 2009a(39)		11 – 14	124	1.19	7895	1.01	<0.001
Poon 2009b(52,53)		11 – 14	128	1.19	8061	1.01	<0.0001
Poon2009c(52,53)		11 – 14	128	1.19	8061	1.01	<0.0001
Foidart 2010(32)		11 – 14	60	1.31	180	1.01	<0.0167

PE not specified							
			Preeclampsia		Controls		
Author, year	Marker	GA	n	MoM Median	n	MoM Median	*p* value
	ADAM12						
Spencer 2008a(20)		11 – 14	64	0.71	4390	1.0	<0.0001
Audibert 2010(33)		11 – 13	40	1.02	833	0.95	n. sign.
	fb-hCG						
Ong 2000(37)		11 – 14	135	0.88	4297	1.05	0.012
Audibert 2010(33)		11 – 13	40	0.85	833	1.06	n. sign.
	Inhibin A						
Spencer 2008b(21)		11 – 14	64	1.24	240	1.0	0.0006
Audibert 2010(33)		11 – 13	40	1.11	833	1.03	n. sign.
	Activin A						
Spencer 2008b(21)		11 – 14	64	1.17	240	1.0	0.0276

Table 3. *Continued*

PE not specified							
			Preeclampsia		Controls		
Author, year	Marker	GA	n	MoM Median	n	MoM Median	*p* value
	PP13						
Chafetz 2007(26)		9 – 12	47	0.20	289	1.00	<0.01
Gonen 2008(27)		6 – 10	20	0.3	1178	1.01	<0.001
Romero 2008(30)		8 – 13	50	0.59	250	1.00	<0.001
Khalil 2009(28)Ñ		11 – 14	42	0.4	210	1.00	<0.001
Audibert 2010(33)		11 – 13	40	1.00	833	1.02	n. sign.
	PlGF						
Audibert 2010(33)		11 – 13	40	0.74	833	0.94	<0.05
	PAPP-A						
Ong 2000(37)		11 – 14	135	0.90	4297	1.05	<0.001
Spencer 2005(41)		11 – 14	64	n.a.	3999	1.00	0.039
Spencer 2007(31)$		11 – 14	44	0.89	446	1.00	0.042
Audibert 2010(33)		11 – 13	40	0.89	833	1.04	n. sign.
Hedley 2010(34)		10 – 14	126	0.97*	289	1.12*	n. sign.
	Doppler						
Plasencia2007(49)		11 – 14	107	1.19*	5041	1.00	<0.001
Audibert 2010(33)		11 – 13	40	1.24	833	1.10	n. sign.

* Calculated from $\log_{10}$ MoM to MoM

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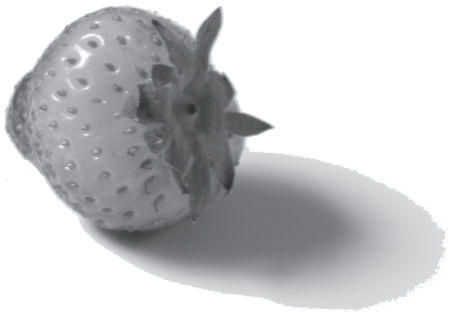
III

INTEGRATIVE DATA MINING TO IDENTIFY NOVEL CANDIDATE SERUM BIOMARKERS FOR PREECLAMPSIA SCREENING



ABSTRACT

Objective

Preeclampsia (PE) is a serious complication that affects approximately 2% of pregnant women worldwide. At present, there is no sufficiently reliable test for early detection of PE in a screening setting that would allow timely intervention. To help future experimental identification of serum biomarkers for early onset PE (EO-PE), we applied a data mining approach to create a set of candidate biomarkers.

Methods

We started from the disease etiology, which involves impaired trophoblast invasion into the spiral arteries. Based on this, we used a three-stage filtering strategy consisting of selection of tissue-specific genes, textmining for further gene prioritization, and identifying blood-detectable markers.

Results

This approach resulted in 38 candidate biomarkers. These include the best three first trimester serum biomarkers for PE found to date LGALS13 (PP13), PAPPA (PAPP-A), and PGF (PlGF), as well as five proteins previously identified as biomarker beyond the first trimester or disease onset. This substantiates the effectiveness of our approach and provides an important indication that the list will contain several new biomarkers for PE.

Conclusions

We anticipate this list can serve in prioritization of future experimental studies on serum biomarkers for EO-PE.

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INTRODUCTION

Preeclampsia (PE) is a severe disorder that occurs only during pregnancy and the postpartum period. It affects approximately 2% of pregnant women worldwide and is the leading cause of maternal and perinatal morbidity and mortality, particularly when it occurs before the 34th week of gestation (early onset PE; EO-PE).[1] Renal failure, HELLP syndrome (hemolysis, elevated liver enzymes, and thrombocytopenia), cerebral or liver hemorrhage, eclampsia, and maternal death are the most common complications of PE. Furthermore, PE is strongly associated with intrauterine growth restriction, iatrogenic prematurity, placental abruption, and stillbirth of the child.

Despite great efforts, the exact pathophysiology of PE remains unraveled. Several different hypotheses have came into light and numerous theories have been postulated.[2,3,4] As yet, PE is considered to be a result of an interplay between placental factors, maternal constitution and inadequate adaptive changes to pregnancy predominantly involving the cardiovascular and inflammatory systems.[2,5,6] Likewise, the clinical syndrome of PE does not develop along the same pathophysiological pathway and the causative complex of interacting factors may differ from patient to patient.[3,7] A number of specialists in the field distinguish between two types of PE, placental and maternal. The first type is considered to be a result of impaired trophoblast invasion into spiral arteries and their failure to remodel.[2] Narrow spiral arteries lead to placental ischemia and generate oxidative stress conditions.[8,9] EO-PE, developing early in the pregnancy and necessitating delivery prior to 34 weeks' gestation, is more frequently associated with this defective placentation than late onset disease. On the other hand, the late onset PE is considered as "maternal", where an abnormal maternal response rather than an abnormal pregnancy occurs.[2,10]

Because of serious health consequences of PE, risk assessment and identification of women at risk early in the pregnancy remain a major challenge in prenatal care.[11] Because widespread serum alterations are expected to forego the clinical onset of PE, there is great interest in the identification of early predictive biomarkers. A number of candidate biomarkers have been proposed for prediction of disease, including placental hormones and angiogenic factors.[12-14] Additionally, maternal characteristics combined with the measurements of uterine artery Doppler appear useful in PE risk stratification.[15-18] However, to date, no serum or maternal marker (either single or in combination) has emerged with the necessary specificity and sensitivity to be of clinical use. In consequence, clinicians are so far unable to offer targeted intensified antenatal surveillance and potential preventive therapies to women at high risk. Therefore, development of a clinically relevant prenatal screening test, preferably as early as the first trimester of pregnancy, is of a great importance to enable timely intervention where needed, thereby improving outcome for mother and child as well as general antenatal care efficiency.

In a previous study from our laboratory, we used a bioinformatics approach to identify novel screening markers for Down Syndrome (DS) screening by a three-step strategy, namely: http://www.simplyrecipes.com/recipes/eggs_nested_in_sauteed_chard_and_mushrooms/ (1) selection of genes highly expressed in relevant tissues, (2) limiting this set to genes more specifically associated with the disorder, and (3) identifying blood-detectable markers.[19] In

this study, we will apply a similarly structured strategy for identification of potential serum biomarkers for predominantly EO-PE, taking the etiological mechanism as a starting point.

METHODS

Analysis of tissue-specific gene expression data

To compare gene expression data across various human tissues we used data from the Human U133A/GNF1H dataset as available on the BioGPS website (http://biogps.gnf.org, formerly known as SymAtlas).[20,21] This dataset includes gcRMA normalized microarray gene expression data from 84 human tissues. For our analysis, we excluded data from cancer (cell lines) as well as testis and prostate tissues, as these were considered less relevant for screening in pregnant women. Gene expression data for the remaining 72 tissues were imported in R and used to compare the expression in placenta or endothelial cells to the expression in all other tissues. Here we used essentially the same approach as described before.[19] For each gene, the expression in placenta or endothelial cells was compared to the median expression among all other tissues, thereby providing a ratio for each gene. Next, we determined for different ratio thresholds how many genes had a ratio at least as large as that threshold. The resulting data distribution was assessed to determine the nonspecific underlying trend over lower ratio stringency levels. As we described before, this trend could be approximated with a power law distribution, where a two-fold increase in the threshold led to a four-fold decrease in the number of genes expressed above that threshold.[19] We refer to this trend as the nonspecific underlying trend. For higher stringencies, the number of genes began to decrease at a slower rate, indicating enrichment for tissue-specific genes over the nonspecific trend. Based on this finding, a threshold was determined that yielded approximately 10 times more tissue-specific genes than could be estimated based on the nonspecific underlying trend. This threshold was determined at 30 times the median gene expression level. Genes exceeding this threshold were used in subsequent analysis steps.

Textmining

To determine textmining associations between genes and pathogenesis-associated search terms we used two different textmining tools, which are complementary in nature, to restrict the chance of false negatives. The first of these tools is Anni 2.1 (http://www.biosemantics.org/anni/), which provides an ontology- and thesaurus-based interface to Medline and retrieves associations for several classes of biomedical concepts (*e.g.* genes, drugs, and diseases).[22] These concepts are given a concept weight, which indicates their relevance to the applied search term. The second application is Polysearch (http://wishart.biology.ualberta.ca/polysearch), which supports information retrieval queries against several different types of text, scientific abstract or bioinformatic databases such as PubMed, OMIM, DrugBank, SwissProt, the Human Metabolome Database (HMDB), the Human Protein Reference Database (HPRD), and the Genetic Association Database (GAD).[23] The relevancy scores of the obtained genes or proteins are expressed as Z scores, *i.e.* as standard deviations above the mean.

In our study on Down Syndrome biomarkers we found that the combined use of Anni and PolySearch offers a better search performance, as these tools use different approaches to search partially different databases.[19]

The two textmining applications were searched for genes associated with the terms "placentation", "placental villus", "chorionic villus", "uterine artery", "spiral artery", "Doppler ultrasound/ultrasonography", "pulsatility index", and "blood flow velocity". Significance criteria were based on a minimally tenfold enrichment over the statistically determined distribution of the concept weight (Anni) or a software documentation suggested Gaussian distribution (PolySearch). Gene lists obtained for the search terms were combined and subsequently manually curated to resolve ambiguous or redundant gene symbols.

Assessing applicability for blood-based detection

To determine if putative biomarkers identified by gene expression and text mining analysis are potentially blood-detectable, they were cross-checked against two different data resources. Proteins were considered blood-detectable if they had at least one of the Gene Ontology (GO) annotation terms "extracellular region", "extracellular region part", or "extracellular space"; or if they were included in the Human Plasma Proteome (HPP) list. GO (http://www.geneontology.org) annotations are partially based on computational predictions whereas the HPP list is based on a combination of experimental methodologies.[24,25] As it has been found that these resources are complementary, their results were combined.[19,24]

RESULTS

Identification of tissue-specific candidate genes

Current insights into EO-PE indicate that its pathogenesis primarily occurs in the transitional area between the placenta and the endometrial spiral arteries.[7,9] Therefore, as a first step in our data mining approach, we identified genes with expression specific to either of these tissues. Using the BioGPS gene expression data set for 72 human tissues, we compared placenta and endothelial cells (the latter as a substitute for spiral artery tissue which was not included as a tissue in the data set) to other tissues in the dataset. For each gene, the ratio was calculated between the expression in either of these tissues to the median expression in all other tissues. By using various stringencies, we first determined the number of false positive genes exceeding the ratio at several lower stringency levels. Next, we extrapolated the trend in these values to higher stringency levels. At a threshold of 30 times the median tissue expression, we found the number of positive genes being ten times higher than the number of expected false positives, and therefore 90% of the positive genes for either placenta or endothelial cells can be considered to be specifically derived from that tissue and not be a statistical artifact. Using these criteria, we found 268 genes specifically expressed in placenta and 170 in endothelial cells, which when combined add up to 433 non-redundant genes (Figure 1).

Applying additional relevance criteria

In the next step, we applied textmining to determine which genes are functionally associated with the mechanism of PE pathogenesis. This was done using two different textmining

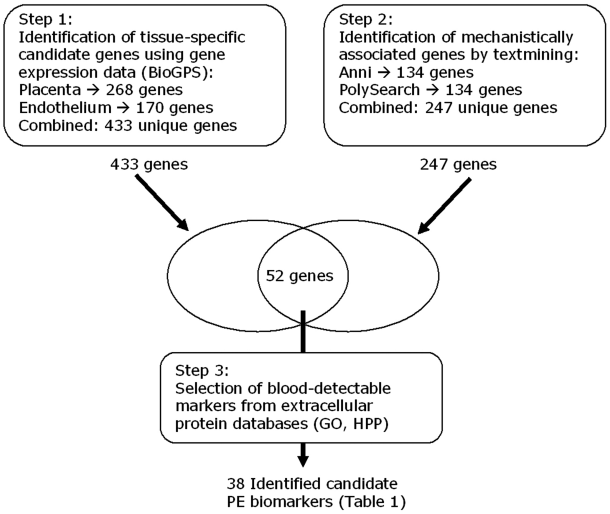

Figure 1. Graphic overview of our data mining approach and the number of markers identified per step.

tools (Anni and Polysearch) to select genes associated with several PE pathogenesis terms (placentation, villi, uterine spiral arteries) or diagnostic characteristics (Doppler, pulsatility index, blood flow). This resulted in a total number of 247 non-redundant genes (134 using Anni, also 134 using PolySearch). As can be expected for a textmining search, genes found using both methods are mostly those that are frequently mentioned in the literature on pregnancy or placental development. Some of such genes are also found in step 1 (*e.g.* PAPPA, LGLAS13, PGF, INHBA) although some are not found in step 1 as sufficiently tissue specific (*e.g.* AFP, ERVWE1, FLT1, PPARG).

We determined the overlap between the 433 tissue-specific genes identified in step 1 and the 247 functionally PE-associated genes in step 2, which resulted in a list of 52 unique genes that met both of our relevance criteria (Figure 1).

Selection of blood-detectable markers

For the 52 genes selected based on the previous steps, we identified the ones potentially detectable as proteins in plasma or serum. Such detectability is a necessary prerequisite for biomarkers to be measured by immunoassays that are currently used for routine screening programs. We checked which of the 52 genes had either a GO annotation as being extracellular or were part of the experimentally derived Human Plasma Proteome list. This led to a final list of 38 potential biomarkers detectable in human plasma or serum, and as such relevant for biomarker analysis follow-up studies (Figure 1, Table 1).

Functional analysis

To evaluate functionality of the list, we compared our list with PE markers assessed by others (Table 1). We found that 21 of the final 38 proteins were tested by others as

potential biomarker for PE of which 8 were specifically tested as first trimester marker for PE (Table 1). Over-representation analysis of biological processes within the list of 38 proteins showed that the most notable GO terms were those related to hormone activity (ADM, CGA, CGB5, CSH1, CSH2, INHA, INHBA, INSL4, IGF2, PRL) and other forms of growth regulation (DLK1, DKK1, GPC3, HMOX1, HTRA1, IGFBP1, IGFBP3, PAPPA, PAPPA2, PGF, PLA2G2A, PLAU, SPP1). The enrichment is mainly found among markers for which evidence as a potential biomarker has been found. Other represented functions known to be involved at this pregnancy stage are cell adhesion molecules (COL15A1, FN1, PECAM1, VCAM1), proteases (HTRA1, PAPPA, PAPPA2, PLAU, PRSS8) and their inhibitors (GPC3, SPINT1, TFPI2, TIMP1), and the IGF pathway (IGF2, IGFBP1, IGFBP3, PAPPA, PAPPA2). Five of the seven genes not annotated in one of the above categories, (ABP1, ALPP, LGALS13, PLAC1, PSG5) are associated with the placenta and its early development, although their functions cannot be assigned to a common denominator. The remaining two proteins (CD55, HBB) are primarily known as blood associated proteins.

DISCUSSION

PE is a serious disease burden to pregnant women, and therefore there is much need for a reliable early screening program that can be offered on a routine basis. Although serum biomarkers for PE have been identified, their overall prediction accuracy is not yet sufficient for implementation in a screening setting.[12,14,18] By identifying novel PE serum biomarkers and combining these – with current serum markers, uterine artery Doppler measurements, and additional maternal history – into a single prediction method, it may be possible to create a screening program with clinically relevant performance. This would allow for improved surveillance of women with high risk pregnancies and preventive measures such as antihypertensive medication or induced delivery before clinical signs of serious complications occur.

This study was set up to identify novel potential PE markers, to subsequently be tested in biomarker discovery approaches. By combining data from different publicly available data resources into a three-step approach we identified 38 potential PE serum biomarkers (Figure 1, Table 1).

Among the markers given in Table 1 are PAPPA (PAPP-A), LGALS3 (PP13) and PGF (PLGF), which to date represent the most promising biomarkers for PE.[14] The finding that these markers are also identified by our approach demonstrates that our approach has the potential to identify other promising biomarkers as well. For another 18 markers we found literature studies that examine their potential as a PE biomarker. For 5 of these (FN1, HMOX1, PAPPA2, PRL, VCAM1) there is moderate evidence for their use as a PE biomarker.[26-35] These markers were informative beyond the first trimester of pregnancy or already after the clinical onset of the disease. For the other 13, the evidence was either lacking, inconsistent, or contradictory between studies.

In the context of first trimester screening, it is important whether the selected markers are able to distinguish between women at risk for developing PE and those with healthy pregnancies already so early in the pregnancy. So far, most of the markers are shown to be distinctive in the third trimester after the onset of the disease, and as such provide little

Table 1. Identified candidate biomarkers for EO-PE.

Marker	Description	Overall potential [a]	First trimester potential [b]	Source tissue
ABP1	amiloride binding protein 1 (amine oxidase, copper-containing)			Placenta
ADM	adrenomedullin	Tested [c]	Tested	Placenta
ALPP	alkaline phosphatase, placental (Regan isozyme)	Tested		Placenta
CD55	CD55 molecule, decay accelerating factor for complement			Placenta
CGA	glycoprotein hormones, alpha polypeptide			Placenta
CGB5	chorionic gonadotropin, beta polypeptide 5	Tested		Placenta
COL15A1	collagen, type XV, alpha 1			Placenta
CSH1	chorionic somatomammotropin hormone 1 (placental lactogen)	Tested		Placenta
CSH2	chorionic somatomammotropin hormone 2 (placental lactogen)	Tested		Placenta
DKK1	dickkopf homolog 1 (Xenopus laevis)			Placenta
DLK1	delta-like 1 homolog (Drosophila)			Placenta
FN1	fibronectin 1	Evidence		Placenta
GPC3	glypican 3			Placenta
HBB	hemoglobin, beta			Endothelial
HMOX1	heme oxygenase 1	Evidence		Endothelial
HTRA1	HtrA serine peptidase 1			Placenta
IGF2	insulin-like growth factor 2 (somatomedin A)	Tested		Placenta
IGFBP1	insulin-like growth factor binding protein 1	Tested	Tested	Placenta
IGFBP3	insulin-like growth factor binding protein 3	Tested		Placenta
INHA	inhibin, alpha	Tested	Tested	Placenta
INHBA	inhibin, beta A	Tested	Tested	Placenta
INSL4	insulin-like 4 (placenta)	Tested	Tested	Placenta
LGALS13	lectin, galactoside-binding, soluble, 13 (PP13)	Strong evidence	Strong evidence	Placenta
PAPPA	pregnancy-associated plasma protein A, pappalysin 1	Strong evidence	Strong evidence	Placenta
PAPPA2	pappalysin 2	Evidence		Placenta
PECAM1	platelet/endothelial cell adhesion molecule	Tested		Placenta
PGF	placental growth factor (PlGF)	Strong evidence	Strong evidence	Placenta
PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)			Placenta
PLAC1	placenta-specific 1			Placenta
PLAU	plasminogen activator, urokinase			Placenta
PRL	prolactin	Evidence		Placenta
PRSS8	protease, serine, 8			Placenta
PSG5	pregnancy specific beta-1-glycoprotein 5			Placenta
SPINT1	serine peptidase inhibitor, Kunitz type 1			Placenta
SPP1	secreted phosphoprotein 1 (osteopontin)	Tested		Placenta
TFPI2	tissue factor pathway inhibitor 2			Placenta
TIMP2	TIMP metallopeptidase inhibitor 2			Placenta
VCAM1	vascular cell adhesion molecule 1	Evidence		Endothelial

[a] Potential PE markers described as examined in the literature, with indicated their level of evidence.
[b] Potential PE markers also examined in the first trimester, with indicated their level of evidence.
[c] The word "Tested" is used here to denote proteins which have been tested as PE biomarker but for which (to date) insufficient evidence has been found.

use in preventing PE development by intervention strategies. Only eight of the markers described in the literature were tested in the first trimester (Table 1). It may be assumed that the marker profiles of women with clinically confirmed PE are different from the profiles of healthy women. Therefore it is of major importance that the selected markers also have the potential of differentiating between healthy individuals and women at risk even before the onset of the disease. Larger prospective or case-control studies will be needed to provide an answer to this matter.

Among the 38 proteins in Table 1, several over-representations of biological processes can be observed. The most notable of these are GO terms related to hormone activity and other forms of growth regulation. The enrichment for these functional terms can be attributed to early development of the placenta, which involves the production of growth factors, hormones, and metalloproteases.[36] The enrichment is mainly found among markers for which evidence for usage as a potential biomarker already exists. This suggests that markers, which are not tested yet and have a similar function, might deserve priority in the following experimental stages of biomarker identification. More specifically, this would lead to prioritization of HTRA1 and DKK1. Indeed, it has recently been found that down-regulation of total HTRA1 can be correlated to placental (*i.e.* early onset) as opposed to maternal (*i.e.* late onset) PE, making it a very interesting candidate marker. Also, aberrant expression of DKK1 has been associated with impaired embryonic attachment and implantation.[37,38]

Among the 17 genes that have not yet been tested as candidate biomarkers for PE, functional enrichment was strongest for proteases (HTRA1, PLAU, PRSS8) or protease inhibitors (GPC3, SPINT1, TFPI2, TIMP2). Such proteins are involved in early placental development through their function in either tissue remodeling, but also by regulating concentrations of IGF binding proteins and thereby IGF levels.[36] This latter aspects might especially be relevant for prioritizing these markers, as PAPPA is a well known example of a protease acting upon IGFBP4.[39] Given this, the further interest in the IGF pathway among previously tested markers, and the knowledge that HTRA1 also acts upon IGF binding proteins, HTRA1 also might deserve future priority from this functional point of view (Table 1).[40]

The importance of the placenta in the etiology of PE is further underlined by the finding that 35 out of the 38 markers in Table 1 are of placental origin, with only HMOX1, VCAM1 and HBB being of endothelial origin. The predominance of placental genes among the identified markers suggests that this tissue might outperform endothelial cells as a relevant source for novel PE biomarkers. To some extent, this can be explained by the fact that when compared to placenta, endothelial tissue has less specific (literature) association with pregnancy. This is reflected in the finding that the textmining step keeps more placental genes (47 out of 268) than endothelial (5 out of 170) genes. However, both HMOX1 and VCAM1 have been described as markers for PE, which suggests that genes of endothelial origin are nevertheless interesting enough to warrant further study.

One marker with endothelial origin, hemoglobin beta (HBB), meets the criteria used in our approach. Interestingly, fetal hemoglobin (containing alpha and gamma chains) has recently been described as a promising PE biomarker.[41,42] However, this has not been described for the (maternal) HBB chain. Additionally, it can be assumed that HBB concentrations in plasma or serum will be affected by variations in erythrocyte lysis during

sample handling. Therefore, we expect that this particular marker might not be very valuable in clinical practice. This might serve as an illustration of how data mining can identify potential markers, yet additional clinical background experience can help in giving lower (HBB) or higher (HTRA1) priority for further clinical testing.

To summarize, we have used integrative data mining to identify a set of 38 candidate early PE screening biomarkers. Among the list are three markers, which have been shown to be validated first trimester clinical PE biomarkers (strong evidence). Five markers have literature evidence, however are not yet tested in the first trimester. Thirteen other markers have been examined in other studies, from which 5 in the first trimester, leaving approximately half of the markers in Table 1 still interesting and open for further examination as potential biomarkers. Given the number of confirmed biomarkers among those that have been examined, and taking into account that the first two gene selection steps in our approach are both based on a minimally ten-fold enrichment over the background, we expect there will be several novel, useful PE biomarkers among those that have not yet been examined. Additional case-control serum analysis experiments will be necessary and initiated by us, to determine which of these candidate biomarkers have differential serum levels in PE versus normal pregnancies as early as the first trimester. Moreover, before a set of serum biomarkers can be combined with parameters such as maternal history or uterine Doppler measurements in a risk stratification algorithm, larger cohort studies need to be performed. These are necessary to further determine how these markers interrelate and whether sufficiently reliable prediction accuracy can be obtained before a large-scale PE screening program can be introduced. Such validation experiments with the PE screening biomarkers reported in this manuscript will be the subject of forthcoming research.

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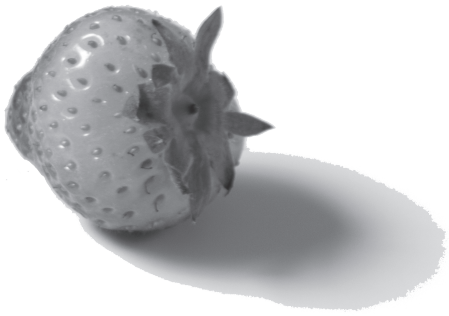
IV

METABOLOMICS PROFILING FOR IDENTIFICATION OF NOVEL POTENTIAL MARKERS IN EARLY PREDICTION OF PREECLAMPSIA



ABSTRACT

Objective

To investigate if specific signatures of metabolic biomarkers significantly differ in the first trimester serum of women who subsequently developed preeclampsia (PE) compared to that of healthy controls. The performance of selected metabolites was evaluated for use during the first trimester screening of PE.

Methods

This was a case-control study of maternal serum samples collected between 8+0 and 13+6 weeks of gestation from 167 women who subsequently developed PE (early onset PE [EO-PE] n = 68; late onset PE [LO-PE] n = 99) and 500 controls with uncomplicated pregnancies. Metabolomics profiling analysis was performed using two methods. One has been optimized to target eicosanoids/oxylipins, which are known inflammation markers and the other targets compounds containing a primary or secondary biogenic amine group. Logistic regression analyses were performed to predict the development of PE using metabolites alone and in combination with first trimester mean arterial pressure (MAP) measurements.

Results

Two metabolites were significantly different between EO-PE and controls (taurine and asparagine) and one in case of LO-PE (glycylglycine). Taurine appeared the most discriminative biomarker and in combination with MAP predicted EO-PE with a detection rate (DR) of 55%, at a false-positive rate (FPR) of 10%.

Conclusion

Our findings suggest a potential role of taurine in both PE pathophysiology and first trimester screening for EO-PE.

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Submitted

INTRODUCTION

Preeclampsia (PE) remains a leading complication of pregnancy and affects approximately 2% of women worldwide.[1,2] Early onset PE (EO-PE), requiring delivery before 34 weeks of gestation (GA), is considered the most severe form of the disease in contrast to late onset PE (LO-PE; delivery ≥ 34 weeks GA).[3,4] Apart from perinatal problems, PE is also associated with substantial health problems later in life. Both women who suffered from PE and their children have a substantially elevated risk of chronic hypertension, cardiovascular disease and diabetes mellitus type-2.[5] The diagnosis of PE is based on clinical symptoms, such as proteinuria and *de novo* hypertension. These symptoms however are only the terminal features of a cascade of events initiated during the first trimester of pregnancy.[6,7] Therefore early recognition of patients at high risk and timely intervention ahead of the clinical onset of the disease would enable suitable pregnancy care and hopefully better pregnancy outcomes for both mother and child.

It becomes more and more clear that PE is caused by interactions between complex pathophysiological mechanisms, individual genes and environmental factors. Some features of the pathophysiology of PE have been elucidated already. Underlying mechanisms comprise impaired early placentation and trophoblast invasion in the spiral arteries, placental hypoxia and endothelial dysfunction.[8-11] However, there is currently no complete view on the pathophysiology of PE. Classical approaches to discover novel PE biomarkers are hypothesis-driven and concentrate mostly on the early placental development and maternal adaptation to pregnancy. Other approaches which include novel classes of methods, such as "omics technologies" are "integrated system-based" methods which study general chemical processes in the whole organism to subsequently focus on differences in concentrations of individual molecules, their interactions and role in the pathogenesis.

One of these "omics" technologies is metabolomics. Metabolomics has been successfully applied as biomarker discovery tool for the early detection of diseases such as cancer and cardiovascular disease.[12]

Early prediction of PE by using metabolomics analyses on samples derived from maternal blood has been investigated by other groups (Table 1).[13-16] The markers identified differ between studies as do the detection rates, and ranged from 50% in a heterogeneous PE group to 82.6% in an EO-PE group when metabolites are combined with classical PE screening markers such as maternal characteristics, uterine artery Doppler velocity and fetal crown-rump-length (CRL) measurements.[13-16]

In our large nested case-control study we have divided the PE group in two subgroups (EO-PE and LO-PE), as it is known that PE is a heterogeneous syndrome and the pathophysiology of both entities may differ.[9,17,18] In order to cover the biologically relevant pathways, we selected two metabolomics profiling methods. One has been optimized to target eicosanoids/oxylipins, which are known inflammation markers and the method targets a wide variety of biogenic amines, including the amino acids.

These groups of metabolites are associated with PE as well other pregnancy complications such as placental abruption, IUGR and preterm birth.[19-23] Furthermore both groups are also strongly associated with different inflammatory processes in the cell and endothelial

Table 1. Studies that assessed preeclampsia screening using metabolomics techniques.

Study	Sample taken (weeks)	Body fluid	Numbers		Gestational age at delivery		Number of metabolites	DR (at FPR)
			Controls	PE	Controls	PE		
Kenny et al[13]								
Discovery study	15 ± 1	plasma	60	60	40.1 (1.1)	37.5 (2.8)	14	77% (10%)
Validation study	15 ± 1	plasma	40	39	38.1 (2.3)	40.0 (1.3)	14	73% (10%)
Odibo et al[14]	12.1 (0.6)	serum	41	41	39.0 (2.8)	35.3 (4.1)	4	50% (10%)
Bahado-Singh et al[15]	11+0 – 13+6	plasma	60	30	"unaffected"	< 34 weeks	4 + MC 3 + MC + UtA + CRL	75.9% (4.9%) 82.6% (1.6%)
Bahado-Singh et al[16]	11+0 – 13+6	serum	119	30	"unaffected"	≥ 37 weeks	2	56.7% (5%)

PE: preeclampsia; DR: detection rate; FPR: false positive rate; MC: maternal characteristics; UtA: Uterine artery Doppler measurement; CRL: crown-rump-length.

dysfunctions, which are common for PE and cardiovascular disease later in life.[24-26] The above associations make them potentially suitable candidate markers for prediction of PE.

The study has two aims: the first aim is to investigate specific signature patterns of metabolites significantly altered in PE. The second aim of this study is to examine the predictive performance of selected metabolites in first trimester screening of both EO-PE and LO-PE.

MATERIAL AND METHODS

Study population

This was a nested case-control study derived from a large cohort of women participating in the routine Dutch first trimester Down syndrome screening between 2007 and 2009. In this context maternal age, sample date, gestational age (GA) at sampling, maternal weight, method of conception, history of diabetes, and smoking status were recorded by a midwife or gynaecologist. As part of the screening, maternal serum concentrations of two standard placental markers (pregnancy-associated plasma protein-A [PAPP-A] and free β-subunit of human Chorionic Gonadotropin [fβ-hCG]) were measured in serum of blood sampled at 8^{+0} - 13^{+6} weeks GA. Samples were subsequently aliquoted and stored at -80ºC until metabolomics analysis. Where applicable, GA was calculated based on first trimester CRL measurement at ultrasound examination using the formula of Robinson and Fleming (1975)[27]; otherwise the first day of the last menstrual period was used. Pregnancy outcomes, including chromosomal disorders, date of birth, birthweight and hypertensive pregnancy complications (PE, HELLP syndrome or pregnancy induced hypertension), were collected through self-reporting by the participating women. Six months after the estimated delivery date, a reminder letter was sent to these women to collect missing data. This way, 75% of all pregnancy outcomes could be recorded. For the current study women with a multiple pregnancy, women who delivered before 24 weeks and women who gave birth to a child with a chromosomal abnormality were excluded.

By follow up of self-reported cases of EO-PE we confirmed the diagnosis of EO-PE in 68 pregnancies at the participating hospitals. Moreover, from a larger cohort of women who developed LO-PE we randomly selected 99 cases. From all EO-PE and LO-PE cases we collected missing data on maternal characteristics i.e. medical history, parity, weight, height, first trimester mean arterial pressure (MAP) and pregnancy outcome i.e. GA at delivery, birthweight and fetal sex. The control group, consisting of 500 women having delivered phenotypically and chromosomally normal neonates at term (37^{+0} – 42^{+0} weeks) and not having developed any pregnancy complication, was randomly selected from the two largest ultrasound centres participating in the routine Dutch first trimester Down syndrome screening program (Universitair Verloskundig Centrum Utrecht and De Poort Leiden). The outcomes of these pregnancies were confirmed in the midwifery practices and missing maternal characteristics and first trimester MAP were collected.

Outcome measures

PE was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy as: gestational hypertension beyond 20 weeks GA in previously

normotensive women with a systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on at least two occasions four hours apart, with the presence of proteinuria of ≥ 300 mg in 24-hour collection or at least 2+ by dipstick on a spot urinalysis.[6] Early onset PE (EO-PE) was defined as PE in pregnancies delivering <34 weeks GA, and late onset PE (LO-PE) as PE in pregnancies delivering ≥ 34 weeks. Pregnancy at term was defined as delivery ≥ 37 weeks of GA.

MAP was calculated from the formula *DP + 1/3 (SP – DP)*, where DP represents diastolic blood pressure and SP -systolic blood pressure.

Sample analysis

Amine measurements were performed using the method based described previously by Noga et al., 2012.[28] The amine platform covers amino acids and biogenic amines employing an Accq-tag derivatization strategy adapted from the protocol supplied by Waters (Etten-Leur, The Netherlands). 5 µL of each plasma sample was spiked with an internal standard solution, followed by deproteination by addition of MeOH. The supernatant was transferred to a deactivated autosampler vial (Waters) and dried under N_2. The residue was reconstituted in borate buffer (pH 8.5) with 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) reagent (Waters). After reaction, the vials were transferred to an autosampler tray and cooled to 10°C until the injection (1.0 µL) of the reaction mixture into the UPLC-MS/MS system.

An ACQUITY UPLC system with autosampler (Waters) was coupled online with a Xevo Tandem Quadrupole mass spectrometer (Waters) operated using Masslynx data acquisition software (version 4.1; Waters). The samples were analyzed by UPLC-MS/MS using an Accq-Tag Ultra column (Waters).

The Xevo TQ was used in the positive-ion electrospray mode and all metabolites were monitored in Selective Reaction Monitoring (SRM) using nominal mass resolution.

Acquired data were evaluated using Quanlynx software (Waters), by integration of assigned SRM peaks and normalization using proper internal standards. For analysis of amino acids their $^{13}C^{15}N$-labeled analogs were used. For other amines, the closest-eluting internal standard was employed. Blank samples were used to correct for background, and in-house developed algorithms were applied using the pooled QC samples to compensate for drift in the sensitivity of the mass spectrometer with and over different batches.[29]

Measurements of eicosanoids/oxylipins was performed as described earlier by Strassburg et al.[30] After thawing the 250 µL serum aliquots on ice, the samples were treated immediately with antioxidants (0.2 mg 3,5-di-t-butyl-4-hydroxytoluene [BHT]/ Ethylenediaminetetraacetic acid [EDTA]) and spiked with a set of 34 isotopically labeled internal standards (ISTDs). Compound extraction was performed with solid phase extraction using Oasis Hydrophilic-lipophilic-balanced reversed-phase sorbent for acids [HLB] (60 mg/30 µm). Oxylipins were eluted with 2 mL ethyl acetate after wetting the cartridge with 0.5 mL methanol. The eluent was reduced under nitrogen. The dried extract was subsequently reconstituted in 50 µL solution of methanol and acetonitrile (1:1) containing 100 nM 1-cyclohexyluriedo-3-dodecanoic acid (CUDA) as a quality marker for the analysis. Afterwards, the extract was filtered by centrifugation using Amicon Ultrafree-MC Durapore PVDF filter (pore-size 0.1 µM; Millipore, Bedford, MA).

Samples were analyzed by liquid chromatography (LC-MS; Agilent 1260, San Jose, CA, USA) coupled to electrospray ionization on a triple quadrupole mass spectrometer (Agilent 6460, San Jose, CA, USA). For analysis 5µL of the extract was injected. The auto sampler was cooled at 10°C. Chromatographic separation was achieved on an Ascentis Express (2.1×150 mm, 2.7 µm particles; Sigma-Aldrich Supelco) column using the solvents A, 0.1 % acetic acid, and B, 90:10 *v/v* acetonitril/isopropanol. Electrospray ionization was performed in the negative ion mode.

To detect the individual oxylipins, MRM in negative ion mode was performed with individually optimized fragmentor voltage and collision energies (Optimizer application, MassHunter, Agilent). Optimal MRM settings were obtained from flow injection analysis of pure standards using the optimizer application and compared to literature when available. Dynamic MRM was used, assuring optimal dwell time and sufficient data points per peak.

Peak determination and peak area integration was performed automatically with MassHunter Quant (Agilent, Version B.04.00) while auto-integration was manually inspected and corrected if necessary. The obtained peak areas of targets were corrected by appropriate ISTD and calculated response ratios were used throughout the analysis. In-house developed algorithms were applied using the pooled QC samples to compensate for shifts in the sensitivity of the mass spectrometer over different batches.[29]

Statistical analysis

Only metabolites detected in more than 80% of the samples were included in statistical analysis. For other analytes, data below the detection limit, where the LC-MS software provided a missing value, were imputed as half the lowest detectable value for that individual analyte. This replacement was made to get a more realistic value in further calculations and was a compromise between substituting a zero value, which would be an underestimation, and truncating these values at the lowest measurable level, which would be an overestimation of their actual concentrations.

In accordance with the statistical approach described below, the data set was divided into sets for training, testing (evaluation) and validation, respectively. For each group (controls, EO-PE, LO-PE), samples were randomly assigned to the training (40%), test (30%) or validation set (30%). Overall samples assignment was as follows: training set 200 controls, 27 EO-PE, 40 LO-PE; test set 150 controls, 20 EO-PE, 30 LO-PE; validation set 150 controls, 21 EO-PE, 29 LO-PE.

After this random assignment we confirmed that there were no significant differences in maternal characteristics between the three sets (results not shown). The concentration of the (remaining) metabolites and MAP of the training set were expressed as multiples of the gestation-specific normal medians (MoMs). Normal medians were obtained by regression analysis of the median concentration for each completed gestational week in the controls of the training set, weighted for the number of women tested. Values of MoM were adjusted for variables such as gestation, weight, smoking and ethnicity where MoM values differed significantly between groups (within controls), following the standard methods described by Cuckle and Wald, with curve fitting based on the training set.[31]

Maternal characteristics (i.e. medical records, parity, weight and length) were used to calculate prior risks for PE in multiple logistic regression models. The steps undertaken to develop the models for prior risk for EO-PE and LO-PE were described earlier in detail in our recent article.[32]

In the next step, metabolites MoM data were compared between controls and either EO-PE or LO-PE, using a Student's t-test. Values were corrected for multiple testing by calculating the False Discovery Rate (FDR).

The potential of MAP and metabolites with FDR < 15% as part of a PE prediction model was further tested using logistic regression in R statistical software. Training models were based on training set data (controls and either EO-PE or LO-PE cases) for prior risks and log-MoM data for each significant metabolite as well as all possible metabolites combinations. Models were then tested on test set data for the corresponding metabolites. Models were evaluated based on their predicted Detection Rate [DR] (sensitivity) in the test set for a fixed 10% False Positive Rate [FPR] (1-specificity).

Finally, the model with the best performance was validated on the validation set.

Statistical analyses were performed using SPSS (release 20.0; Chicago, IL), SAS software package (release 9.2; SAS Institute, Cary, NC, USA) and R programming language version 2.15 (http://www.r-project.org).

Ethics Statement

This study approach was approved by the Scientific Ethical Committee of the University Medical Centre of Utrecht (METC Utrecht), the Netherlands (protocol number: 11-002). All participating women had given written informed consent at enrolment in the first trimester Down syndrome screening.

RESULTS

Baseline characteristics of the study population are shown in Table 2. Women who developed PE had higher BMI (EO-PE 24.7, $p < 0.0001$; LO-PE 23.7, $p = 0.005$), were more often smokers (EO-PE 11.8% vs 4.2%, $p = 0.008$), and more often had a history of hypertensive pregnancy disorders compared to controls (EO-PE 5.9%, $p = 0.009$; LO-PE 10.1%, $p < 0.0001$). Furthermore, there were more nulliparous women among the cases (both EO-PE 80.9% and LO-PE 72.7%, $p < 0.0001$).

Metabolite pre-selection

Using the data in the training set, we analyzed a total number of 105 potential variables (58 amines, 46 oxylipins and MAP) for statistically significantly different levels between controls and cases of both EO-PE and LO-PE. MAP proved significant for both of these comparisons, with a 10% (MoM ratio case and controls [MoMR] = 1.10) and 7% (MoMR 1.07) increase in EO- and LO-PE, respectively, and both p-values less than 10^{-4} (Table 3). For the amines, taurine and asparagine were significantly different (FDR < 15%) between EO-PE and controls, with approximately 20% reduced levels (MoMR 0.79 and 0.84, respectively). Glycylglycine showed a significant reduction in LO-PE of 28%,

Table 2. adapted from Kuc et al., 2013 – Study population baseline characteristics in control and PE pregnancies. Values are presented as median (IQR) or number (%).[32]

Characteristics	Controls n = 500	EO-PE n = 68	LO-PE n = 99
Maternal age (y)	33 (30-35)	34 (30-37)	33 (30-36)
Maternal weight (kg)	65.5 (60.0-73.0)	70.0 (62.0-81.5)*	67.5 (62.0-75.0)
Maternal BMI (kg/m2)	22.8 (20.7-24.8)	24.7 (21.9-29.3)*	23.7 (21.3-26.5)*
Nulliparity	233 (46.6)	55 (80.9)*	72 (72.7)*
Smoking	21 (4.2)	8 (11.8)*	6 (6.1)
Assisted reproduction	0 (0)	3 (4.4)	8 (8.1)
Gestation at sampling (days)	88 (84-91)	85 (76-89)*	85 (79-89)*
History of hypertensive pregnancy disorders	4 (0.8)	4 (5.9)*	10 (10.1)*
Gestation at birth (wk)	40 (39-41)	31 (30-32)*	37 (36-39)*
Birthweight (gr)	3544 (3243-3800)	1300 (1045-1609)*	2650 (2130-3110)*
Birthweight centile	57.0 (33.1-78.4)	25.0 (13.4-50.4)*	13.8 (3.8-46.0)*
Sex, n male (%)	244 (48.8)	34 (49.7)	53 (53.5)

A Pearson's chi square test and Mann-Whitney U test, both with *post hoc* Bonferroni correction were used for statistical analysis. Adjusted significance value p <0.016 (*). EO-PE: early-onset preeclampsia; LO-PE: late-onset preeclampsia; IQR: interquartile range; BMI: body mass index.

Table 3. Selection of the markers significantly different between controls and cases (EO-PE or LO-PE) based on training set.

Type marker	Variable	p-value	FDR	MoM ratio case/control
EO-PE				
Blood pressure	MAP	<0.0001	<0.0001	1.10
Amine	Taurine	0.0015	0.07	0.79
Amine	Asparagine	0.0043	0.10	0.84
LO-PE				
Blood pressure	MAP	<0.0001	<0.0001	1.07
Amine	Glycylglycine	0.0002	0.01	0.72

Student's t-test was used for statistical analysis. Significance value FDR < 15%. EO-PE: early-onset preeclampsia; LO-PE: late-onset preeclampsia; MoM: multiple of the median; FDR: false discovery rate; MAP: mean arterial pressure.

MoMR 0.72 (Table 3). None of the oxylipins showed a significant (FDR 15%) difference for any group.

Model selection

Prediction models were fitted based on the training set, using the prior risk and one or more of the significant markers.

Comparison of the performance of these models on the test set indicated that for EO-PE the highest DR was obtained for the model with prior risk, MAP and taurine. This model gave a DR of 88% at a FPR of 10%, which is a 19% gain on the DR obtained using only the prior risk. We selected this model for further validation. Another model using prior risk, MAP, taurine and asparagine also gave a DR of 88% (Table 4). However, this latter model was not selected, as adding asparagine to the model did not result in further improvement of the DR as compared to the former model.

For LO-PE, the best predicting model included prior risk and MAP. This model did not include any metabolites (Table 4).

Validation

Applying the selected models to independent data validated the EO-PE model. This model gave a DR of 55%, which was a 25% improvement to the DR obtained using only the prior risk (Table 4). This gain of 25% was also obtained for the training set.

The LO-PE model resulted in a DR of 17% (Table 4). Neither metabolic marker nor MAP improved this model. Final models for both EO-PE and LO-PE are shown in Figure 1.

Table 4. Model predicted early preeclampsia detection rate (95% CI) for FPR of 10% with prior risk, MAP, taurine, asparagine and glycylglycine in control and preeclampsia groups.

	Training set		Test set		Validation set	
	DR at 10% FPR (95% CI)	AUC	DR at 10% FPR (95% CI)	AUC	DR at 10% FPR (95% CI)	AUC
EO-PE						
Prior risk	30 (16-49)	0.74	69 (48-85)	0.92	30 (14-50)	0.73
Prior risk + MAP	55 (37-72)	0.88	81 (58-92)	0.91		
Prior risk + taurine	48 (31-66)	0.80	65 (43-82)	0.90		
Prior risk + asparagine	36 (22-56)	0.77	70 (48-85)	0.91		
Prior risk + MAP + taurine	55 (37-72)	0.88	88 (70-97)*	0.93	55 (36-76)	0.78
Prior risk + MAP + asparagine	55(37-72)	0.87	75 (53-89)	0.91		
Prior risk+ MAP + taurine + asparagine	55 (37-72)	0.87	88 (70-97)	0.93		
LO-PE						
Prior risk	37 (24-53)	0.75	38 (22-55)	0.70	17 (8-35)	0.55
Prior risk + MAP	43 (28-58)	0.81	46 (30-64)*	0.79	17 (8-35)	0.65
Prior risk + glycylglycine	46 (31-60)	0.79	38 (21-59)	0.72		
Prior risk + MAP + glycylglycine	53 (37-67)	0.83	42 (27-61)	0.78		

DR: detection rate; FPR: false positive rate; MAP: Mean Arterial Pressure; CI: confidence interval; AUC: area under curve, EO-PE: early-onset preeclampsia; LO-PE: late-onset preeclampsia. *The best model selected for further validation.

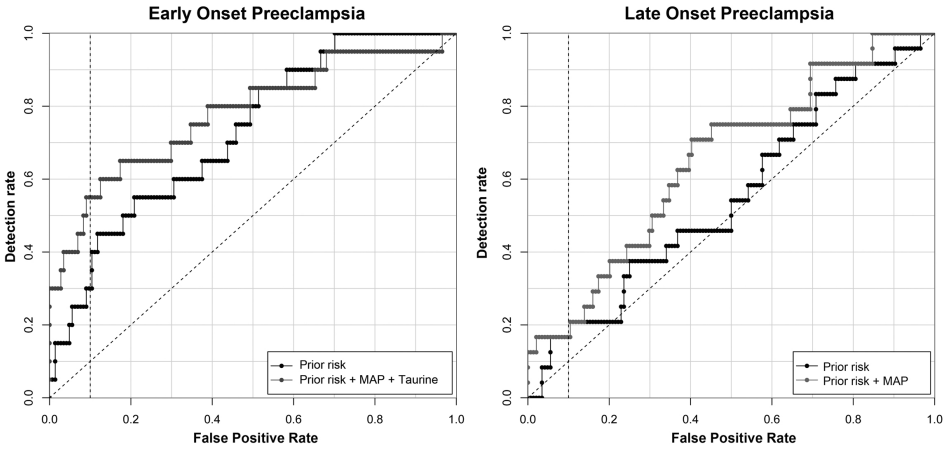

Figure 1. Receiver operating characteristic curves (ROCs) with prediction models for early onset preeclampsia and late onset preeclampsia used on the validation set. Prior risk of preeclampsia containing maternal characteristics (black line) and prior risk for preeclampsia combined with different markers (grey line). MAP: mean arterial pressure.

DISCUSSION

Early detection of PE would allow the development and application of targeted intervention. Detection, however, has been restricted to clinical parameters such as high blood pressure and presence of protein in the urine until recently. When these parameters are present, the condition has already progressed. An assay-detecting women at risk to develop PE at an early stage would therefore be extremely helpful in preventing its severe symptoms at the end of the pregnancy. To be able to develop such an effective first trimester prenatal screening test, sets of biomarkers are needed that are capable of detecting the presence of pathologic conditions in maternal serum with high sensitivity and specificity.

In this study we applied LC-MS based metabolomics to assess the diagnostic potency of metabolites such as amines and oxylipins to predict EO-PE en LO-PE from first trimester maternal serum. Using standardized metabolomics analytical techniques and an established statistical approach we identified two molecules of which the serum concentrations were significantly decreased in the first trimester maternal serum of women who subsequently develop EO-PE, and one metabolite with decreased levels in LO-PE compared to controls. All three compounds (taurine, asparagine and glycylglycine) are amino acids or amino acid derivates. Altered levels of amino acids in maternal fluids have earlier been observed in preeclampsia and fetal growth restriction.[33-37] Interestingly, they reported a clear association of the properties of taurine and glycylglycine and the possible pathogenesis of PE.

Taurine is a sulfur-containing amino acid-like endogenous compound found in substantial amounts in mammalian tissues. Among its many functions, taurine is an important regulator of antioxidation and membrane stabilization.[38] It is the most abundant free amino acid – derivative in human placenta.[39,40] Through its cytoprotective role and as a regulator of cell

volume, taurine is thought to be involved in placental trophoblast development, during the remodeling of the spiral arteries early in the pregnancy.[40] Reduced activity of taurine transporters in the placental trophoblast with shortage of taurine in placental tissue is strongly associated with impaired trophoblast invasion into the spiral arteries.[40] Impaired placental trophoblast invasion is one of the most important components of PE pathology.[9,17] Furthermore, taurine has also been associated with cardio- and vasoprotective effects through the influence of renin-angiotensin-aldosterone system.[38] From different animal model studies taurine appears to have antihypertensive effects and to reduce total peripheral vessel resistance. Spontaneously hypertensive rats supplemented with taurine show dosage dependant blood pressure reduction.[41] Taurine supplementation during pregnancy in rats leads to reduction of hypertension in their offspring as well.[41-43]

Glycylglycine is a dipeptide of glycine. The latter one appears to be an essential amino acid in pregnancy and fetal cardiovascular development since its supplementation prevents elevation of blood pressure in rats.[44] Furthermore, glycine is an important component of the S-amino acid metabolic pathway as it antagonises homocysteine levels.[45,46] Through the biological feedback loop, lower levels of glycine lead to higher levels of homocysteine. Hyperhomocysteinemia is a known risk factor for endothelial dysfunction, PE and, also cardiovascular disease.[47-50]

So far, this is the first study associating lower levels of asparagine to PE or any other aspect of cardiovascular disease. Although the role of asparagine in PE development is not yet known, our results may suggest that the shortage of asparagine may be a risk factor for development of EO-PE. Further research is needed to evaluate this particular finding.

None of the significant metabolites that we found have been brought to light by previous metabolomics studies.[13-16] From all 105 metabolites we tested, five were reported to be important in previous studies (methylhistidine, alanine, phenylalanine, methionine and valine). However, in our study these five did not differ between PE and control groups. There are hardly matches in significant metabolites selected by different groups.[13-16] This strengthens the assumption of the major complexity of the syndrome and the origins of its pathology. The causative complex of interacting mechanistically blameworthy factors as well as the likely presence of confounding factors in a system that inherently changes over time, may therefore lead to differences not only between two variants of the disease (EO-PE vs LO-PE), but also to differences between subsets of patient and control cohorts and even from one study to the next.

The prediction model using a combination of prior risk with MAP and taurine provided a DR of 55% at a fixed 10% FPR in the case of EO-PE, which is comparable to the results obtained in training. Addition of asparagine did not further improve the results (Table 4). For LO-PE a model using prior risk performed best and it gave a DR of 15% on the validation set. Addition of MAP did not improve the DR, however the AUC increased. Glycylglycine, although significantly decreased in LO-PE versus controls (Table 3), did not turn out to be sufficiently additionally informative to become included in the final prediction model. Therefore, taurine remains the single predictive metabolite marker of this study. Because of its narrow affiliation with placentation, hypertension and cardiovascular disease, a potential screening role of taurine is conceivable.

As a next step, it would be desirable to combine taurine serum levels with first trimester classical markers: pregnancy-associated plasma protein A (PAPP-A), A Disintegrin And Metalloprotease 12 (ADAM12), Placental Protein 13 (PP13), Placental Growth Factor (PlGF) and with artery uterine Doppler measurement in a large-scale study. Prediction results of those markers are very promising, particularly in case of EO-PE and even more in EO-PE complicated by the growth restriction.[32] Given our results, it is plausible that the DR of PE would increase if taurine would be added, although the added value in such a large-scale screening setting, also in relation to increased costs and logistics, would need to be established before further recommendations can be made.

In conclusion, three markers out of 105 were significantly different between women who developed PE and healthy individuals, but after adequate statistical analysis only taurine remained as a predictive marker in the screening model. However, given the possible role of taurine in hypertension treatment, its role as a possible screening marker or maybe even as a diet supplement early in pregnancy remains very interesting.

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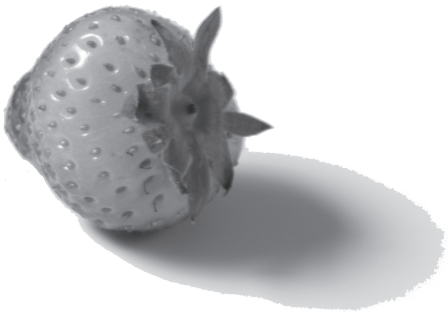
PART 2
RETROSPECTIVE SCREENING – PHASE 3

V

MATERNAL CHARACTERISTICS, MEAN ARTERIAL PRESSURE AND SERUM MARKERS IN EARLY PREDICTION OF PREECLAMPSIA



ABSTRACT

Objectives

In a previous study, we have described the predictive value of first trimester pregnancy-associated plasma protein-A (PAPP-A), free β-subunit of human chorionic gonadotropin (fβ-hCG), Placental Growth Factor (PlGF) and A Disintegrin And Metalloprotease 12 (ADAM12) for early onset preeclampsia (EO-PE; delivery < 34 weeks). The objective of the current study was to obtain the predictive value of these serum makers combined with maternal characteristics and first trimester maternal mean arterial blood pressure (MAP) in a large series of patients, for both EO-PE and late onset PE (LO-PE; delivery ≥ 34 weeks).

Methods

This was a nested case-control study, using stored first trimester maternal serum from women who developed EO-PE (n=68) or LO-PE (n=99), and 500 uncomplicated singleton pregnancies. Maternal characteristics, MAP and pregnancy outcome were collected for each individual woman and used to calculate prior risks for PE in a multiple logistic regression model. Models containing prior PE risks, serum markers and MAP were developed for the prediction of EO-PE and LO-PE. The model-predicted detection rates (DR) for fixed 10% false positive rates were calculated for EO-PE and LO-PE with or without the presence of a small-for-gestational age infant (SGA, birth weight <10th centile).

Results

The best prediction model included maternal characteristics, MAP, PAPP-A, ADAM12 and PlGF, with DR of 72% for EO-PE and 49% for LO-PE. Prediction for PE with concomitant SGA was better than for PE alone (92% for EO-PE and 57% for LO-PE).

Conclusion

First trimester MAP, PAPP-A, ADAM12 and PlGF combined with maternal characteristics and MAP are promising markers in the risk assessment of PE, especially for EO-PE complicated by SGA.

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INTRODUCTION

Preeclampsia (PE) affects approximately 2% of pregnant women worldwide and is a leading cause of maternal and perinatal morbidity and mortality, in particular when resulting in a delivery before 34 weeks of gestation.[1-3] The latter is called early onset PE (EO-PE). It is associated with insufficient placentation and consequently often with severe perinatal morbidity and mortality due to concomitant fetal growth restriction, iatrogenic preterm birth, placental abruption and stillbirth.[4,5] The exact pathophysiology of PE remains to be elucidated. Nevertheless it is widely acknowledged that PE is a syndrome that may arise by different pathophysiological pathways in which impaired placentation, maternal constitution and abnormal circulatory and immunological adaptation to pregnancy may play a role. Depending on these pathways PE may occur early in pregnancy with impaired placentation and with fetal growth restriction ('placental-PE'), or late in pregnancy without fetal growth restriction ('maternal-PE').[6-8]

First trimester screening for PE may prove useful to identify high risk patients for subsequent increased surveillance and/or early preventive treatment with low dose aspirin.[9,10] Screening with markers of placentation is likely to identify early placental PE, whereas maternal characteristics may be related to both EO- and LO-PE.

In 2010, we performed a nested case-control study in 568 singleton pregnancies at 8^{+0} – 13^{+6} weeks of gestation, including 88 women who developed EO-PE.[11] In that study we investigated the maternal serum concentrations of serum markers: pregnancy-associated plasma protein-A (PAPP-A), free β-subunit of human Chorionic Gonadotropin (fβ-hCG), Placental Growth Factor (PlGF), Placental Protein 13 (PP13) and A Disintegrin And Metalloprotease 12 (ADAM12). For a fixed 10% false positive rate we were able to identify 54% of EO-PE pregnancies. Furthermore, in pregnancies complicated by PE and with an infant small-for-gestational age (SGA, birth weight <10^{th} centile) all serum marker levels were significantly lower as compared to those of pregnancies complicated by PE alone.

Several maternal characteristics are also known to be related to the risk of developing PE. Such factors are nulliparity, higher maternal age, high body mass index (BMI), PE in previous pregnancy, family history of hypertensive pregnancy disorders and chronic hypertension. Also, the mean arterial pressure (MAP) in the first trimester may provide valuable information.[1,12,13]

It was the aim of this study to examine the performance of first trimester screening for both EO-PE and LO-PE based on maternal characteristics, first trimester MAP, and placenta derived serum makers in a new patient population. Prospective studies on markers for PE require large data sets given the low 1-2% incidence of PE. We therefore conducted a nested case-control study using stored first trimester serum. This way we were able to study 68 EO-PE, 99 LO-PE and 500 controls.

MATERIAL AND METHODS

Ethics Statement

This study approach was approved by the Scientific Ethical Committee of the University Medical Center of Utrecht (METC Utrecht), the Netherlands (protocol number: 11-002).

All participating women in this manuscript have given written informed consent during the first trimester Down syndrome screening.

Study population

This was a nested case-control study derived from a large cohort of women participating in the routine Dutch first trimester Down syndrome screening between 2007 and 2009. In this context maternal age, sample date, gestational age (GA) at sampling, maternal weight, method of conception, history of diabetes, and smoking status were recorded by a midwife or gynaecologist. As part of the screening, maternal serum concentrations of PAPP-A and fβ–hCG were measured in serum of blood sampled at 9^{+0} - 13^{+6} weeks GA. Samples were subsequently stored at -80ºC. An ultrasound measurement of fetal crown-rump length (CRL) and nuchal translucency (NT) thickness were performed between 11^{+4} and 13^{+6} GA. All ultrasound examinations were performed by certified sonographers using standardized techniques. Where applicable, GA was calculated based on first trimester CRL measurement at ultrasound examination using the formula of Robinson and Fleming (1975)[14]; otherwise the first day of the last menstrual period was used. Pregnancy outcomes, including chromosomal disorders, date of birth, birthweight and hypertensive pregnancy complications (PE, HELLP syndrome or pregnancy induced hypertension), were collected through self-reporting by the participating women. Six months after the estimated delivery date, a reminder letter was sent to these women to collect missing data. This way, 75% of all pregnancy outcomes could be recorded. For the current study women with a multiple pregnancy, women who delivered before 24 weeks and women who gave birth to a child with a chromosomal abnormality were excluded.

By follow up of self-reported cases of EO-PE we confirmed the diagnosis of EO-PE in 68 pregnancies at the participating hospitals. Moreover, from a larger cohort of women who developed LO-PE we randomly selected 99 cases; 50 cases with an infant appropriate for gestational age at birth and 49 cases with a SGA infant. From all these cases we collected missing data on maternal characteristics i.e. medical history, parity, weight, height, first trimester mean arterial pressure (MAP) and pregnancy outcome i.e. GA at delivery, birthweight and fetal sex. For the control group 500 women having delivered phenotypically and chromosomally normal neonates at term (37^{+0} – 42^{+0} weeks) and not having developed any pregnancy complication, were randomly selected from the two biggest ultrasound centres participating in the routine Dutch first trimester Down syndrome screening program (Universitair Verloskundig Centrum Utrecht and De Poort Leiden). The outcomes of these pregnancies were confirmed in the midwifery practices and missing maternal characteristics and first trimester MAP were collected.

This was the first time this large cohort of women participating in the routine Dutch first trimester Down syndrome screening (2007-2009) was used for any research purpose. The data set presented in this study has never been used before in any of our previous studies.

Outcome measures

PE was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy as: gestational hypertension beyond 20 weeks GA in previously normotensive

women with a systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on at least two occasions four hours apart, with the presence of proteinuria of ≥ 300 mg in 24-hour collection or at least 2+ by dipstick on a spot urinalysis.[2] EO-PE was defined as PE in pregnancies delivering < 34 weeks GA, and LO-PE as PE in pregnancies delivering ≥ 34 weeks. Pregnancy at term was defined as delivery ≥ 37 weeks of GA.

First trimester blood pressure was collected from the medical records at the hospitals and midwifery practices. It were measured following the standard method of measuring blood pressure in the pregnancy described in the guidelines for gynaecologists and midwives (NVOG: http://www.nvog-documenten.nl and KNOV: http://www.knov.nl/docs). Blood pressure was measured with validated hand devices, calibrated yearly. The pregnant women were in the sitting position for at least 2-3 minutes and preferably their right arm was supported, with the upper arm at heart level. Either a normal or large adult cuff was used depending on the mid-arm circumference. Korotkov V was used to determine the diastolic pressure and the value was noted with a 2 mmHg accuracy. MAP was calculated from the formula *DP + 1/3 (SP – DP)*, where DP represents diastolic blood pressure and SP - systolic blood pressure.

Growth charts corrected for gestational age, sex and parity according to the Dutch Perinatal Registry were used to calculate the birthweight Z-scores (http://www.perinatreg.nl [15]). Weight for GA at the 50th centile was used as the mean of the population and the average standard deviation (SD) was calculated by the formula *(-1 SD + 1 SD)/2)*. Subsequently, the Z-score was converted into an exact centile for each studied infant. Small-for-gestational age (SGA) was defined as occurring when the birthweight was under the 10th centile.

Sample analysis

The concentrations of PlGF and ADAM12 were measured in serum samples thawed once. AutoDELFIA time resolved assays (Perkin Elmer, Turku, Finland) were used to measure all serum marker concentrations. The detection limit (DL) of PlGF was 5.9 pg/mL, all measurements under the DL were discarded (serum samples of 6 controls and 3 cases). For ADAM12 (DL: 6.0 ng/ml), PAPP-A (DL: 5.0 mU/L) and fβ–hCG (DL; 1.5 ng/ml; together with PAPP-A already measured as part of the routine first trimester Down syndrome screening), none of the measurements was lower than the DL. Before analysis extensive validation was performed for PlGF and ADAM12 assays. Mean intra-assay and inter-assay coefficients of validation for the assays were below 5% at all levels. Due to insufficient stored serum, ADAM12 measurements were not available for 9 controls and 9 cases and PlGF measurements for 12 controls and 14 cases.

Statistical analysis

The comparison between PE groups (EO-PE and LO-PE) and controls was made by chi-square test for categorical variables and Mann Whitney-U test for continuous variables, both with *post hoc* Bonferroni correction. The Bonferroni correction was applied to counteract the use of multiple comparisons. Therefore the critical statistical significance was set at $p < 0.0167$.

The following steps described previously by Akolekar *et al.* were undertaken to develop the models for prior risk for EO-PE and LO-PE based on maternal characteristics.[1] 1) The association of various variables (height [19 mean imputations], logarithmically

transformed weight [one mean imputation], nulliparity, age, and smoking status with EO-PE and LO-PE was evaluated to establish whether this was a linear or non-linear relationship. If necessary, variables were logarithmically transformed to provide normal distributions. 2) Univariate analysis was done to explore whether the individual maternal characteristics contributed significantly to EO-PE and LO-PE by assessing their odds ratios (ORs) and 95% confidence intervals (CI). 3) Logistic regression analysis with backward stepwise elimination of variables was used to develop the prior risk models for EO-PE and LO-PE. 4) Shrinkage factors (applied to all the parameters in the models to adjust for overfitting) were calculated using the equation $[x^2 - (\textit{degrees of freedom} - 1)]/x^2$ where x^2 is the model chi-square derived from the log-likelihood statistic. 5) The patient-specific risks for EO-PE and LO-PE were calculated using formula: odds/(1 + odds), where odds = exp(Y) and Y was derived from the logistic regression analysis.

Serum marker levels and MAP were expressed as multiples of the gestation-specific normal medians (MoMs). Normal medians were obtained by regression analysis of the median concentration for each completed gestational week in controls, weighted for the number of women tested. If MoMs were significantly correlated with maternal weight (PAPP-A, fβ–hCG, ADAM12 and MAP), the observed MoM value was divided by the expected value for the maternal weight based on regression analysis in the controls. When MoMs were significantly different between smoking and non-smoking women a correction factor for smoking was applied (PlGF). The distributions of serum marker MoMs and MAP MoMs were made Gaussian by logarithmical transformation ($\log_{10}$). Likelihood ratio's (LR) were calculated for all markers and subsequently multiplied with the prior risk for EO-PE and LO-PE to calculate posterior risks. Correlation coefficients for the markers were calculated using the $\log_{10}$ (all serum markers) and normal (MAP) concentrations/values and gestation, after excluding outliers exceeding 3 standard deviations from the median.

The posterior risks for EO-PE and LO-PE were calculated using different combinations of variables: (1) prior risks based on maternal characteristics, serum markers, and MAP separately, (2) prior risks combined with serum markers or MAP, and (3) prior risk combined with serum markers and MAP. The distributions of the prior and posterior risks were then used to calculate detection rates (DR) and false positive rates (FPRs) at different risk cut-offs by receiver operating characteristic (ROC) curves analysis. Moreover, the EO-PE and LO-PE models were used for prediction of PE cases complicated by SGA (EO-PE with SGA and LO-PE with SGA).

Statistical analyses were performed using SPSS (release 20.0; Chicago, IL) and SAS software package (release 9.2; SAS Institute, Cary, NC, USA).

RESULTS

Baseline characteristics of the study population are shown in *Table 1*. Women who developed PE had higher BMI (EO-PE $p < 0.0001$; LO-PE $p = 0.005$), were more often smokers (EO-PE $p = 0.008$), and more often had a history of hypertensive pregnancy disorders compared to controls (EO-PE $p = 0.009$; LO-PE $p < 0.0001$). Furthermore, there were more nulliparous women among the cases (both EO-PE and LO-PE $p < 0.0001$).

Table 1. Study population baseline characteristics in control and PE pregnancies. Values are presented as median (IQR) or number (%).

Characteristics	Controls n = 500	EO-PE n = 68	LO-PE n = 99
Maternal age (y)	33 (30-35)	34 (30-37)	33 (30-36)
Maternal weight (kg)	65.5 (60.0-73.0)	70.0 (62.0-81.5)*	67.5 (62.0-75.0)
Maternal BMI (kg/m2)	22.8 (20.7-24.8)	24.7 (21.9-29.3)*	23.7 (21.3-26.5)*
Nulliparity	233 (46.6)	55 (80.9)*	72 (72.7)*
Smoking	21 (4.2)	8 (11.8)*	6 (6.1)
Assisted reproduction	0 (0)	3 (4.4)	8 (8.1)
Gestation at sampling (days)	88 (84-91)	85 (76-89)*	85 (79-89)*
History of hypertensive pregnancy disorders	4 (0.8)	4 (5.9)*	10 (10.1)*
Gestation at birth (wk)	40 (39-41)	31 (30-32)*	37 (36-39)*
Birthweight (gr)	3544 (3243-3800)	1300 (1045-1609)*	2650 (2130-3110)*
Birthweight centile	57.0 (33.1-78.4)	25.0 (13.4-50.4)*	13.8 (3.8-46.0)*
Sex, n male (%)	244 (48.8)	34 (49.7)	53 (53.5)

A Pearson's chi square test and Mann-Whitney U test, both with *post hoc* Bonferroni correction were used for statistical analysis. Adjusted significance value p <0.016 (*). EO-PE: early onset preeclampsia; LO-PE: late-onset preeclampsia; IQR: interquartile range; BMI: body mass index.

Multiple regression analysis in the control group resulted in the following median equations for placental marker and MAP:

Log_{10} expected PAPP-A = (0.52405 + 0.03234 x GA at sampling in days) / (-0.19564 + 76.2423/ maternal weight in kg).

Log_{10} expected fβ-hCG = (2.64423 - 0.01236 x GA at sampling in days) / (0.2947 - 0.00429 x maternal weight in kg).

Log_{10} expected PlGF = (0.4999 + 0.0118 x GA at sampling in days) / 1.33 [if smoking]; or (0.4999 + 0.0118 x GA at sampling in days) / 0.991 [if no smoking]

Log_{10} expected ADAM12 = (-493.422 + 10.6898 x GA at sampling in days) / (0.29637 - 0.00442 x maternal weight in kg).

Expected MAP = 101.052 - 1321.65 / maternal weight in kg.

Table 2. shows the distribution of median MoM values of all markers in controls and PE groups (with and without SGA). In case of EO-PE, PlGF MoM was significantly lower (0.94 MoM, p=0.014) and MAP was significantly higher (1.04 MoM, p<0.0001) compared to controls. Lower PlGF MoM and higher MAP MoM were also found in LO-PE pregnancies, however the difference in PlGF MoM did not reach statistical significance (MAP – 1.05 MoM, p<0.0001; PlGF – 0.90, p=0.024). In EO-PE pregnancies complicated by SGA there was a clear tendency for all serum markers to be even more reduced. MAP was again statistically significantly increased in the EO-PE SGA group as compared to controls (1.04 MoM, p = 0.01).

Table 2. Median MoM (IQR) of marker concentrations in control and PE groups.

			EO-PE				LO-PE PE			
	Controls		All cases n=68		With SGA infant n=13		All cases n=99		With SGA infant n=49	
Variables	n	MoM	n	MoM	n	MoM	n	MoM	n	MoM
PAPP-A	500	1.01 (0.70-1.48)	68	0.89 (0.53-1.45)	13	0.56 (0.35-1.22)	99	1.04 (0.62-1.49)	49	0.95 (0.54-1.44)
Free β–hCG	500	0.99 (0.70-1.44)	68	0.92 (0.56-1.57)	13	0.88 (0.57-1.06)	99	1.04 (0.70-1.46)	49	1.04 (0.66-1.38)
ADAM12	491	1.00 (0.80-1.20)	63	0.93 (0.68-1.15)	12	0.68 (0.57-1.03)*	95	1.02 (0.85-1.28)	48	0.99 (0.81-1.19)
PlGF	482	1.00 (0.81-1.25)	59	0.94 (0.68-1.16)*	12	0.83 (0.42-1.15)	91	0.90 (0.65-1.22)	47	0.81 (0.56-1.15)
MAP	497	0.99 (0.93-1.05)	58	1.08 (1.02-1.17)*	13	1.04 (1.00-1.12)*	85	1.05 (1.00-1.14)*	37	1.01 (0.98-1.10)*

A Mann-Whitney U test, with *post hoc* Bonferroni correction were used for statistical analysis. Adjusted significance value p <0.016 (*).
MoM: multiple of the median; IQR: interquartile range; PAPP-A: Pregnancy-Associated Plasma Protein-A; fβ–hCG: free β–human Chorionic Gonadotropin; ADAM12: A Disintegrin And Metalloprotease 12; PlGF: Placental Growth Factor; MAP: Mean Arterial Pressure; EO-PE: early onset preeclampsia; LO-PE: late-onset preeclampsia.

We studied the distribution of all markers throughout the first trimester. The difference in median MoM PlGF between both PE groups and controls appeared to be more distinctive ≥ 11 weeks of gestation (*Figure 1*). Corresponding median MoM values of PlGF ≥ 11 weeks were substantially lower: 0.77 ($p<0.0001$) and 0.89 ($p=0.005$) for EO-PE and LO-PE, respectively. This trend was only found for PlGF, since there were no differences in the MoM distributions of the other markers (data not shown).

Patient specific prior risks for EO-PE and LO-PE (with and without SGA) were based on maternal age, weight, height, parity, and smoking status (*Table 3*). The shrinkage coefficients for EO-PE and LO-PE models were both 0.94. The analysis resulted in the following equations:

Prior risk EO-PE = -6.790-0.119 x maternal height (cm) + 4.8565 x Ln maternal weight + 1.845 x nulliparity [1] + 0.086 x maternal age (years) + 1.353 x smoking [1].

Prior risk LO-PE = -14.374 + 2.300 x Ln maternal weight + 1.303 x nulliparity [1] + 0.068 x maternal age (years).

Model-predicted detection rates for EO-PE and LO-PE (with or without SGA), for fixed false positive rates are shown in *Table 4*. The detection rates based on maternal characteristics only were 56% and 31% for EO-PE and LO-PE, respectively. The best predictive model contained the combination of markers: maternal characteristics, MAP, PAPP-A, ADAM12 and PlGF (EO-PE: DR=72% for 10% false positive rate, area under the curve [AUC] =0.88). The DRs for SGA groups were even more pronounced, particularly in case of EO-PE. The highest DR (92%) for EO-PE with SGA was obtained by the same combination of markers: maternal characteristics, MAP, PAPP-A, ADAM12 and PlGF, AUC=0.95 (*Figure 2*).

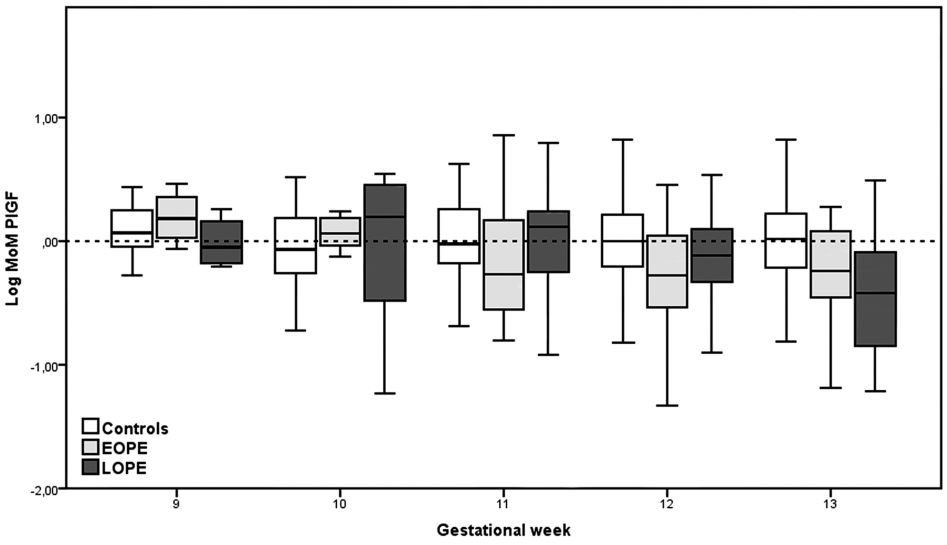

Figure 1. Distribution of logarithmically transformed MoM PlGF through gestation weeks 9^{+0}–13^{+6}. MoM: multiple of the median.

Table 3. Multivariate logistic regression analysis of the factors defining the prior risk for the prediction of EO-PE and LO-PE by maternal characteristics.

Independent variable	EO-PE			LO-PE		
	OR	95% CI	p	OR	95% CI	p
Age	1.095	1.006-1.193	0.036	1.075	1.008-1.147	0.026
Ln Weight	175.634	22.921-1345.844	<0.0001	11.437	2.619-49-949	<0.0001
Height (cm)	0.881	0.835-0.930	<0.0001	-	-	-
Nulliparity	7.1	3.106-16.229	<0.0001	3.981	2.31-6.858	<0.0001
Smoking	4.210	1.543-11.487	0.005	-	-	-

OR: odd ratios; CI: confidence interval; *p*: significance value; Ln: natural logarithm; EO-PE: early onset preeclampsia; LO-PE: late onset preeclampsia

DISCUSSION

This large retrospective study, showed that serum markers, combined with maternal characteristics such as age, weight, height, nulliparity, smoking status and first trimester MAP, are a powerful tool to predict PE in the first trimester. Prediction was better for EO-PE than for LO-PE and for PE complicated by SGA (both early and late).

In 2010 we reported on the results of a combination of three placenta derived serum markers (PAPP-A, PlGF and PP13) and calculated a detection rate for EO-PE of 55% at 10% FPR.[11] In the present study inclusion of maternal characteristics and first trimester MAP resulted in a higher detection rate (72%), which is in line with other publications.[3,16] This seems logical given the different pathways that may result in PE. Serum markers are related to placentation, MAP is related to the maternal vascular adaptation and maternal characteristics determine the susceptibility of the mother.[7,8,17,18]

In the current study the DR of the combined placental markers for EO-PE was lower than in our previous study (31% and 55% at 10% FPR, respectively). This might be due to differences in the laboratory assays (ADAM12 and PlGF) and to the absence of the marker PP13, which is commercially unavailable at this moment.

PlGF is a placenta derived pro-angiogenic factor and its concentrations increase throughout pregnancy.[19,20] In pregnancies destined to develop PE, concentrations of PlGF are significantly lower compared to healthy controls.[11,19,21] We found that < 11^{+0} weeks of GA MoM values of PE cases were comparable to MoM values of controls. Starting from 11^{+0} weeks onwards, MoM values of PlGF appeared to be significantly lower in PE cases, more dominantly in EO-PE than in LO-PE cases. A systematic literature search resulted in eight studies in which PlGF was measured in the first trimester of pregnancy.[1,11-13,21-24] Six out of the eight studies measured PlGF from 11^{+0} weeks onwards and median MoM values in EO-PE cases varied between 0.59 and 0.69.[1,12,13,21,22,24] In the other two studies measurements were carried out from 8^{+0} weeks onwards. The median MoM values in case of EO-PE in these two studies were 0.73 and 0.86, respectively.[11,23] Based on these publications and on our data it has to be concluded that signs of impaired placentation

Table 4. Model predicted early preeclampsia detection rate (95% CI) for FPR of 5 and 10% with PAPP-A, fβ–hCG, ADAM12, PlGF and MAP in control and preeclampsia groups.

	Detection rate (95% confidence interval) for fixed FPR							
	EO-PE				LO-PE			
	All n=68		with SGA infant n= 13		All n=99		with SGA infant n= 49	
Screening test	5%	10%	5%	10%	5%	10%	5%	10%
Maternal characteristics	40 (29-52)	56 (44-67)	39 (18-65)	69 (42-87)	22 (16-32)	31 (23-41)	27 (16-30)	41 (28-55)
PAPP-A	12 (6-22)	19 (12-31)	31 (13-58)	46 (23-71)	8 (4-15)	14 (9-22)	8 (3-19)	16 (9-29)
Free β–hCG	7 (3-16)	21 (13-32)	8 (2-34)	15 (4-43)	6 (3-13)	14 (9-22)	4 (1-14)	12 (6-24)
ADAM12	13 (7-23)	16 (9-27)	25 (9-54)	33 (14-61)	11 (6-18)	18 (12-27)	10 (5-22)	17 (9-30)
PlGF	17 (10-29)	25 (16-38)	25 (9-54)	33 (14-61)	15 (9-24)	25 (17-35)	23 (14-37)	36 (24-51)
MAP	31 (21-44)	40 (28-53)	15 (5-43)	31 (13-58)	27 (19-37)	31 (22-41)	19 (10-34)	19 (10-34)
PAPP-A and PlGF	17 (10-29)	32 (22-45)	33 (14-61)	42 (19-68)	18 (11-27)	24 (17-34)	28 (17-42)	36 (24-51)
PAPPA, ADAM12 and PlGF	20 (12-32)	31 (20-43)	42 (19-68)	42 (19-68)	19 (12-28)	30 (21-40)	28 (17-42)	40 (28-55)
Maternal characteristics plus								
PAPP-A	47 (36-59)	62 (50-72)	69 (42-87)	69 (42-87)	23 (16-32)	34 (26-44)	29 (18-42)	41 (28-55)
ADAM12	40 (29-52)	60 (48-71)	58 (32-81)	75 (46-91)	19 (12-28)	31 (22-40)	21 (12-34)	38 (25-52)
PlGF	51 (38-63)	58 (45-69)	67 (39-86)	75 (46-91)	22 (15-32)	35 (26-45)	34 (22-48)	53 (39-67)
MAP	50 (37-62)	64 (51-75)	39 (18-65)	62 (35-82)	27 (19-37)	45 (35-55)	24 (13-40)	41 (26-57)
Maternal characteristics plus combination of markers								
MAP and PAPP-A	53 (41-66)	71 (58-81)	69 (42-87)	69 (42-87)	32 (23-42)	46 (36-56)	32 (20-49)	41 (26-57)
MAP and PlGF	54 (40-67)	68 (54-79)	67 (39-86)	75 (46-91)	35 (25-46)	56 (45-66)	46 (30-62)	63 (46-77)
MAP, PAPP-A and PlGF	54 (40-67)	70 (57-81)	50 (25-75)	83 (55-95)	38 (28-49)	52 (41-63)	49 (33-65)	60 (43-74)
MAP, PAPPA, ADAM12 and PlGF	56 (42-69)	72 (59-83)	67 (39-86)	92 (64-98)	40 (30-51)	49 (38-60)	49 (33-65)	57 (41-72)

FPR: false positive rate; PAPP-A: Pregnancy-Associated Plasma Protein-A; fβ–hCG: free β–human Chorionic Gonadotrophin; ADAM12: A Disintegrin And Metalloprotease 12; PlGF: Placental Growth Factor; MAP: Mean Arterial Pressure; EO-PE: early onset preeclampsia; LO-PE: late-onset preeclampsia; SGA: small-for-gestational age.

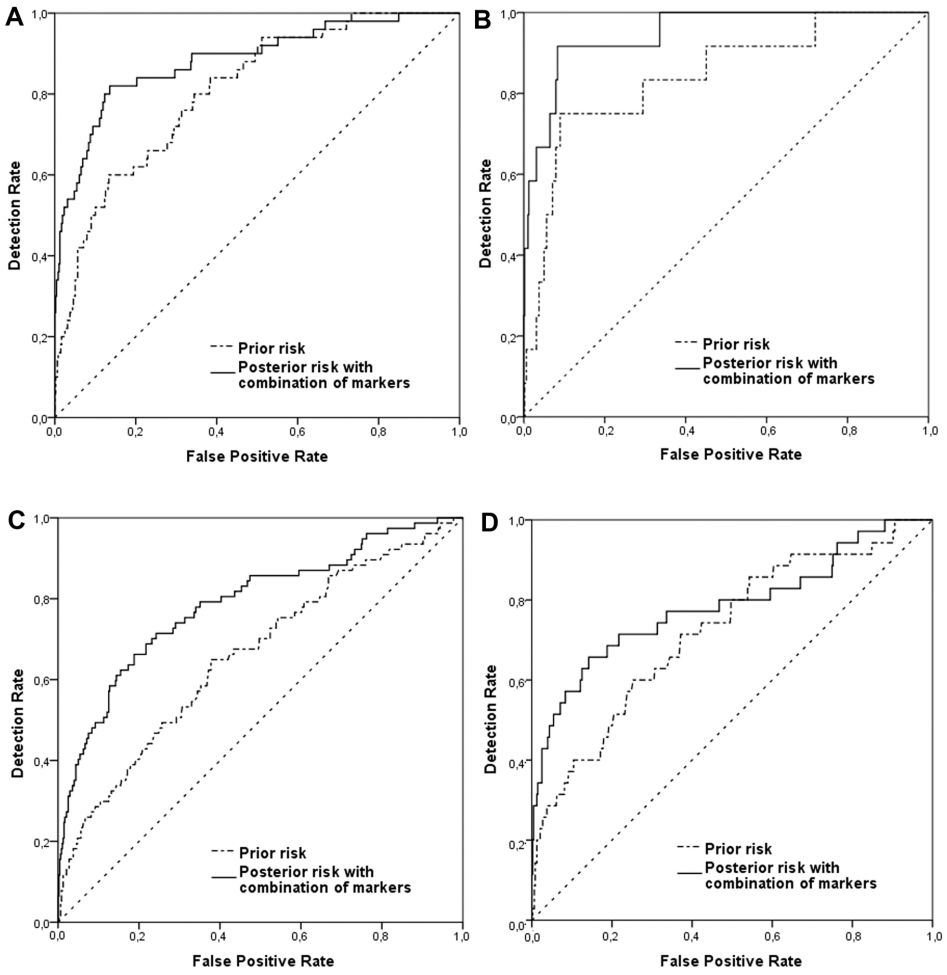

Figure 2. Receiver operating characteristic curves (ROCs) with prediction models for a) EO-PE, b) EO-PE with SGA, c) LO-PE, d) LO- PE with SGA. (- - - -) Prior risk of preeclampsia containing maternal characteristics, (_____) prior risk for preeclampsia combined with all markers: Pregnancy-Associated Plasma Protein-A (PAPP-A), A Disintegrin And Metalloproteinase 12 (ADAM12), Placental Growth Factor (PlGF) and Mean Arterial Pressure (MAP).

should be investigated after 11 weeks GA. We are currently conducting a study to investigate the longitudinal course of several placenta derived serum markers during the first trimester.[20] Results from this study might provide more insight in the predictive value of PlGF throughout the first trimester.

Maternal characteristics based on routine medical information were the most informative predictors for EO-PE. For LO-PE maternal characteristics, MAP and serum markers were of equal importance. These data are in agreement with those of Poon et al.[13] Others, however, have found that maternal characteristics were better in case of LO-PE.[25]

In our analysis we found that maternal smoking was independently associated with EO-PE. This was an unexpected and most likely a chance finding, given the literature on a possible preventive effect of smoking on PE.[26-28]

Identification of PE with SGA was better than for PE without SGA, both for EO-PE and LO-PE. This was due to a better predictive value of both maternal characteristics and serum makers, but not of MAP. The relationship of SGA with serum markers seems logical given the fact that these markers are related to early placentation. Also in our previous study in EO-PE we found lower placental markers in PE complicated by SGA.[11] Surprisingly, placental serum markers of the 10% of SGA infants in the control group were similar to those of appropriate-for-gestational age infants (data not shown). This implies that SGA with PE after 34 weeks constitutes a different entity, with signs of impaired early placentation, although not as evident as SGA with EO-PE. In this context it is important to note that late PE consisted of women delivered after 34 weeks and PE at 34-37 weeks is still related to SGA, although less than at earlier gestation.[29-31] SGA at term, in the absence of PE, is apparently not related to early impaired placental development.

First trimester MAP appears to be one of the most important predictors of PE. A considerable shortcoming of our study may be the fact that this parameter, as the rest of maternal characteristics, was derived from the medical records at the hospitals and midwifery practices in the retrospective manner. However, in the Netherlands blood pressure in pregnancy is measured following the standard method described in the guidelines for gynaecologists and midwives. Therefore we strongly believe that MAP provided here is enough accurate to be able to draw the conclusions from. Standard use of accurate automatic devices might increase its prognostic value in the future.

In this study we did not measure first trimester uterine artery Doppler. Uterine artery Doppler is known to be one of the best first trimester PE markers.[3] Addition of this marker to our prediction model may well increase the detection of PE even more. However, uterine artery Doppler measurements should preferably be measured between 11 and 13 weeks of GA and women in the Netherlands do not routinely receive an ultrasound examination at that time. However, we have shown that even without the measurement of uterine artery Doppler detection rates for PE are quite high. The addition of more placenta derived serum markers, such as PP13, may also increase detection rates.[3,20]

Currently there is emerging evidence that early risk assessment for PE could play an important role in the prevention of PE and subsequent adverse pregnancy outcome. Low-dose aspirin is thought to improve the placental vasculature and therefore reduce the risk for PE. A recent meta-analysis has shown that the prophylactic use of aspirin, started before 16 weeks of GA, may reduce the risk of EO-PE up to 50% and even 90% in case of severe PE, with a 55% reduction in early fetal growth restriction.[9,10,32] Limited data on LO-PE have not (yet) shown beneficial effects of aspirin, emphasizing differences in aetiology.[5,7,33] Early identification of PE may also prove useful in differentiation prenatal care, with increased surveillance in high risk women and less intensive surveillance in low-risk women

In conclusion, we developed a prediction model of PE using a combination of placenta derived markers PAPP-A, ADAM12 and PlGF and first trimester MAP and maternal characteristic. External validation of this model is needed to confirm that it is a powerful tool for the early

prediction of women at risk to develop PE, in particular to predict cases of EO-PE complicated by SGA. Addition of first trimester uterine Doppler is likely to further improve the model.

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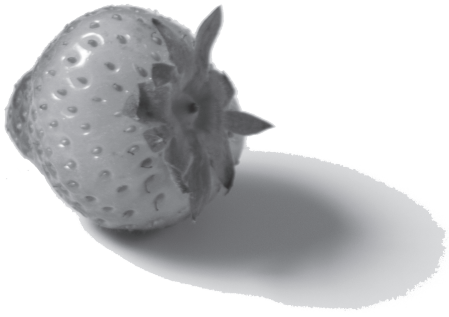
VI

PREDICTION OF MACROSOMIA AT BIRTH IN TYPE-1 AND 2 DIABETIC PREGNANCIES WITH BIOMARKERS OF EARLY PLACENTATION



ABSTRACT

Objective

To evaluate the value of first trimester placental biomarkers (fb-hCG, PAPP-A, ADAM12, PP13 and PlGF) and fetal nuchal translucency (NT) in the prediction of macrosomia at birth in pregestational type-1 and type-2 diabetes (PGDM).

Design: Nested case–control study.

Setting: Routine first trimester combined test.

Population: A total of 178 PGDM and 186 control pregnancies.

Methods

ADAM12, PP13 and PlGF concentrations were measured in stored first trimester serum, previously tested for fβ-hCG and PAPP-A. All concentrations were expressed as multiples of the median (MoM). Where applicable, the median MoMs of PGDM and control pregnancies were compared in relation to birthweight centiles (≤ 90th centile, non-macrosomic, versus >90th centile, macrosomic). Model-predicted detection rates for fixed false positive rates were obtained for statistically significant markers, separately and in combination.

Main outcome measures: Prediction of macrosomia in diabetic pregnancies.

Results

In the PGDM group, median ADAM12 MoM (0.88; P = 0.007) was lower than in the controls. Subgroup analyses showed that median MoMs of PAPP-A (0.65), ADAM12 (0.85), PP13 (0.81) and PlGF (0.91) were only reduced in the PGDM non-macrosomic birthweight subgroup (n = 93) compared with other weight subgroups. In the PGDM macrosomic birthweight subgroup (n = 69), MoMs of all markers were comparable with the control birthweight subgroups. The screening performance for macrosomia at birth in the PGDM group provided a detection rate of 30% for a 5% false positive rate (FPR) and 43% for a 10% FPR.

Conclusions

Macrosomia at birth in PGDM pregnancies may be predicted by normal levels of PAPP-A, ADAM12, PP13 and PlGF already in the first trimester of pregnancy. Fetal birthweight in PGDM offspring is partially determined by placental development during the first trimester of pregnancy. The present increase in fetal macrosomia may be related to better early glycemic control and placentation.

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INTRODUCTION

Pregestational type-1 and type-2 diabetes mellitus (PGDM) in pregnancy is strongly associated with increased fetal morbidity and mortality, compared with the general pregnant population.[1-3] Diabetes-induced metabolic disorders are thought to interfere with embryonic organogenesis and early placentation, and hyperglycaemia is thought to trigger excessive fetal growth.[4] The use of new glucose monitoring techniques, new insulins and new methods of insulin administration have enabled ever better levels of glycemic control to be achieved, and therefore have significantly improved the outcomes of PGDM pregnancies.[5] However, the incidence of macrosomia at birth (birthweight above the 90th centile) remains markedly high and – surprisingly – has even seemed to increase over time, with 38–56% of infants currently weighing more than the 90th centile, and up to 34% of infants being delivered with severe macrosomia of greater than the 97.7th centile.[1,2,6-10] Currently, macrosomia at birth is one of the most concerning complications of a PGDM pregnancy. During labour, macrosomic infants suffer higher rates of shoulder dystocia, brachial plexus injuries and intrapartum asphyxia compared with nonmacrosomic infants.[1-3] After delivery, these infants are at increased risk for hypoglycaemia, infant respiratory distress syndrome (IRDS), hyperbilirubinaemia, cardiomyopathy and hyperviscosity.[2] Macrosomia at birth predisposes babies to childhood obesity and to increased morbidity, including insulin resistance, hypertension and diabetes.[11]

Fetal growth is strongly linked to maternal factors and placental function.[12] Although the association between fetal macrosomia and PGDM in pregnancy is widely recognized, very little is known about the role of placental function in determining macrosomia. In particular, the contribution of early placentation is unknown.

In the Netherlands, the first trimester combined test [maternal serum concentrations of pregnancy-associated plasma protein A (PAPP-A) and the free β subunit of human chorion gonadotropin (fβ-hCG), measured between 8 and 14 weeks of gestation, first trimester ultrasound measurement of the fetal nuchal translucency (NT) and maternal age] is routinely used in screening for aneuploidy. However, other potential markers emerge. Serum levels of biomarkers such as A disintegrin and metalloproteinase 12 (ADAM12), placental protein 13 (PP13) and placental growth factor (PlGF) have been associated with placental growth and development.[13-15] Altered levels of these markers have been reported to be associated with fetal growth restriction, macrosomia at birth and maternal preeclampsia in nondiabetic pregnancies.[13-18] Therefore, concentrations of these markers measured in maternal blood at 8–14 weeks of gestation may help to predict macrosomia at birth, and provide important information about early placental function in PGDM pregnancies.

The aim of this study was to evaluate the value of five first trimester placental biomarkers (fβ-hCG, PAPP-A, ADAM12, PP13 and PlGF) and fetal NT for the prediction of macrosomia at birth in type-1 and type-2 diabetic pregnancies.

METHODS

Serum samples together with NT measurements were collected at the Dutch National Institute for Public Health and the Environment (RIVM) between 2005 and 2007, as part of

the national first trimester Trisomy 21 screening program. Serum samples were collected between 8 and 14 weeks of gestation, and analysis of serum concentrations fβ-hCG and PAPP-A was performed. Fetal NT and crown–rump length (CRL) were measured by accredited sonographers using standardized techniques. The duration of the pregnancies was determined according to either the last menstrual period (LMP) or ultrasound dating. For all women taking part in the screening, sample date, maternal age, maternal weight, smoking and medication use were recorded by the investigator. Pregnancy outcome, including pregnancy duration, date of birth, pregnancy complications, chromosomal disorders, gender and weight of the child were collected after delivery.

From this cohort, serum samples from women with PGDM were selected and retrieved from storage. Women were classified as having PGDM if they had been registered as using insulin in the first trimester of pregnancy. In current first trimester Trisomy 21 screening there is no distinction between pregestational type-1 or type-2 diabetes. One control serum from an uncomplicated singleton pregnancy was matched to each PGDM case, for the same day of gestational age at sampling (±1 week), maternal weight (±5 kg), maternal age (±1 year) and for sample date (±6 months).

Serum concentrations of PP13, PlGF and ADAM12 were measured using an automated time-resolved fluorescence assay (autoDELFIA or DELFIA Xpress; PerkinElmer, Turku, Finland).

Serum marker levels were expressed as multiples of the gestation-specific normal medians (MoMs). Normal medians were obtained by regression analysis of the median concentration for each completed gestational week in the controls, weighted for the number of women tested. NT was expressed as a multiple of the CRL-specific normal median (MoM) in the same way. When MoMs were significantly correlated with maternal weight (fβ-hCG, PAPP-A and ADAM12), the observed MoM value was divided by the expected value for the maternal weight based on regression analysis in the controls. When MoMs were significantly different between smoking and non-smoking women (only for PlGF), a correction factor for smoking was applied. Median MoMs and $\log_{10}$ standard deviations (SDs) were calculated in cases and controls and statistically compared using a Mann–Whitney U-test (two-tailed). Correlation coefficients for all markers were calculated using the $\log_{10}$ concentrations and gestation, after excluding outliers exceeding three standard deviations from the median.

The correlations between marker concentrations and birthweight centiles were assessed by Pearson correlation coefficients. Statistical analyses were performed using SPSS v17.0 (SPSS, Chicago, IL, USA). Differences with $P < 0.05$ were considered to be statistically significant.

Growth charts corrected for gestational age, sex and parity according to the Dutch Perinatal Registry were used to calculate the birthweight Z-scores (www.perinatreg.nl).[19] Weight for gestational age at the 50th centile was used as the mean of the population, and the average SD was calculated by the formula (1 SD + 1 SD)/2. Subsequently, the Z-score was converted into an exact centile for each studied subject with a Z-score to centile web calculator (www. measuringusability.com/pcalcz.php).

Macrosomia was defined as a birthweight above the 90th centile.[19] PGDM and control groups were divided in non-macrosomic (control non-macrosomic; PGDM non-macrosomic) and macrosomic (control macrosomic; PGDM macrosomic) subgroups. In the

next step, the entire PGDM and control group were both divided according to four centile groups: <10th; 10–49th; 50–90th; and >90th.

Model-predicted detection rates (DRs) for fixed false positive rates (FPRs) were obtained for each marker and different combinations of markers by numerical integration.[20] This assumes multivariate log Gaussian distributions to fit both macrosomic and non-macrosomic birthweights in PGDM pregnancies. The theoretical range of MoMs was then divided into a number of equal sections, thus forming a 'grid' in multi-dimensional space. The Gaussian distributions were used to calculate for each section (square for two markers, cube for three etc.) the proportion of macrosomic and non-macrosomic birthweights in PGDM pregnancies in the section and the likelihood ratio (LRs) between them. The appropriate centiles of LR (90th and 95th centiles, respectively) of the non-macrosomic distribution were determined, and the proportion of infants with a birthweight above these centiles was the predicted DR. Data were analysed using SAS (SAS Institute, Cary, NC, USA).

RESULTS

Serum from 186 PGDM and 186 control pregnancies was selected for analysis. Eight samples from the PGDM group were excluded because there was not enough serum for analysis.

The baseline characteristics of the study population are shown in Table 1. Postpartum information on birthweight was available in 100% (186/186) for the control group and in 91% (162/178) for the PGDM group, respectively. Infants from control and PGDM groups had median birthweights of 3600 and 3520 g, respectively. Median birthweight centiles were significantly higher in the PGDM group than in the control group (89th and 72nd centiles, respectively; $P < 0.0001$), and significantly more PGDM infants were macrosomic (42.6% and 18.3%, respectively; $P < 0.0001$), concomitant with a shorter GA at delivery (264 versus 280 days, PGDM and control group, respectively; $P < 0.0001$).

The median ADAM12 MoM was significantly lower (0.88; $P = 0.007$) in the PGDM group compared with the control group. The median PAPP-A MoM was also lower in the PGDM group; however, the difference did not reach statistical significance (0.93; $P = 0.056$; Table 2). Median MoMs of fβ-hCG, PP13, PlGF and NT did not differ between PGDM and control groups.

Table 3. shows the median MoMs for all markers according to macrosomia at birth in both control and PGDM groups. Median MoMs were on average lower in the PGDM non-macrosomic subgroup compared with all other subgroups, and the median MoMs of PAPP-A and ADAM12 in this subgroup were significantly lower as compared with the other subgroups (Figure 1). For PP13, the difference between the PGDM non-macrosomic and control non-macrosomic subgroups was not significant ($P = 0.082$). The median MoM of PlGF in the PGDM non-macrosomic subgroup was also lower compared with all other subgroups, but only reached statistical significance between both PGDM subgroups ($P = 0.005$). The median MoMs of fβ-hCG and NT were comparable between all birthweight subgroups.

When birthweight centiles were divided into four centile groups (<10th, 10–49th, 50–90th and >90th), marker values only appeared to be higher in macrosomic PGDM infants, with no trend in the non-macrosomic centile groups. To illustrate the phenomenon present for PAPP-A, ADAM12, PP13 and PlGF, as an example, the data for PAPP-A is shown in Figure 2.

Table 1. Study population baseline characteristics in control and PGDM pregnancies.

N†	Controls 186	PGDM 178	*P* value*
Maternal age at test (years)‡	34.6 (22-42)	34.5 (22-43)	0.790
Maternal weight (kg)‡	76.0 (49-135)	76.0 (46-144)	0.765
Smoking (%)	13 (7)	24 (13.4)	0.041*
GA at sampling (days)‡	82 (59-97)	82 (57-97)	0.906
Post-partum information available (%)	186 (100)	162 (91)	0.530
NT measurement available (%)§	120 (64.5)	119 (66.8)	0.831
Sex (%)			
male	93 (50)	84 (51.9)	0.690
female	93 (50)	78 (48.1)	0.690
GA at birth (days)‡	280 (235-294)	264 (199-290)	<0.0001*
Birthweight (g)‡	3600 (2140-5000)	3520 (755-5585)	0.03*
Birthweight centile ‡	72 (1-100)	89 (1-100)	<0.0001*
Non-macrosomic weight (%)	152 (81.7)	93 (57.4)	<0.0001*
Macrosomic weight (%)	34 (18.3)	69 (42.6)	<0.0001*

A Pearson's chi test and a Mann-Whitney *U* Test were used for statistical analysis.
* $P < 0.05$ compared to controls.
†Each case was separately matched to one control sample. Eight samples from the PGDM group were excluded since there was not enough serum for analysis.
‡Values are presented as median (range).
§Data on the NT measurement was not always available to our laboratory, because some applicants performed a combined risk calculation on-site.

The screening performance for macrosomia at birth in the PGDM group with four different serum markers, individually and in combination, is shown in Table 4. The combination of four markers together provided a DR for macrosomia in the PGDM group of 30% for a 5% FPR, and a DR of 43% for a 10% FPR.

DISCUSSION

This study showed that the first trimester placental marker ADAM12 was significantly reduced in the total PGDM population. However, birthweight subgroup analyses showed that levels of PAPP-A, ADAM12, PP13 and PlGF were on average lower only in the non-macrosomic PGDM subgroup. There was no difference in marker concentrations between the general population and the PGDM macrosomic subgroup.

It is clear that PAPP-A, ADAM12, PP13 and PlGF allow distinguishing between non-macrosomic and macrosomic subgroups in PGDM pregnancies. While looking at their concentrations in the PGDM group, there seems to be a significant clear cut-off increase above the 90th centile in the concentration of these markers in macrosomic infants (Figure 2). Therefore PAPP-A, ADAM12, PP13 and PlGF appear to be promising predictors of macrosomia

Table 2. Median MoMs and standard deviation (SD) of $\log_{10}$ MoMs of all first trimester markers in control and PGDM pregnancies.

	Median MoM		
	Controls (SD)	PGDM (SD)	
N	186	178	*P* value*
fβ-hCG	0.99 (0.24)	0.99 (0.29)	0.763
PAPP-A	0.98 (0.27)	0.93 (0.28)	0.056
ADAM12	1.00 (0.23)	0.88 (0.22)	0.007*
PP13	1.00 (0.21)	0.97 (0.22)	0.156
PlGF	1.00 (0.15)	0.95 (0.19)	0.785
N	120	119	
NT	1.00 (0.11)	0.99 (0.12)	0.970

* $P < 0.05$

Table 3. Median MoMs and standard deviation (SD) of $\log_{10}$ MoMs of all markers according to macrosomia at birth in control and PGDM pregnancies.

	Median MoM			
	Control non-macro (SD)	Control macro (SD)	PGDM non-macro (SD)	PGDM macro (SD)
N	152	34	93	69
fβ-hCG	0.98 (0.24)	1.03 (0.22)	0.96 (0.29)	1.06 (0.25)
PAPP-A	0.98 (0.27)	0.93 (0.24)	0.65 (0.27)	1.07 (0.24)
ADAM12	0.99 (0.23)	1.04 (0.25)	0.85 (0.21)	1.03 (0.24)
PP13	0.97 (0.22)	1.04 (0.18)	0.81 (0.23)	1.01 (0.24)
PlGF	1.04 (0.15)	1.00 (0.13)	0.91 (0.19)	1.03 (0.18)
N	100	20	67	52
NT	0.97 (0.12)	1.03 (0.08)	0.98 (0.13)	1.01 (0.11)

Control non-macrosomic birthweight (Control *non-macro*), Control macrosomic birthweight (Control *macro*), PGDM non-macrosomic birthweight (PGDM *non-macro*), PGDM macrosomic birthweight (PGDM *macro*).

at birth in this particular high risk population. The use of these four first trimester screening markers as predictors for macrosomia at birth in PGDM pregnancies provides a DR of 43% for a fixed 10% FPR. Two remaining markers, fβ-hCG and the fetal NT, appeared not to be related to the birthweight in any of the groups. This is in agreement with other studies, in which no association between NT or fβ-hCG and diabetes, or fetal growth has been found.[21] None of the maternal characteristics, such as weight or age, were correlated with macrosomia at birth in the PGDM group; therefore, they were not included in the prediction model.

The fact that marker levels tend to be lower in the non-macrosomic PGDM subgroup suggests an impaired placentation in this subgroup, as reduced levels of PAPP-A,

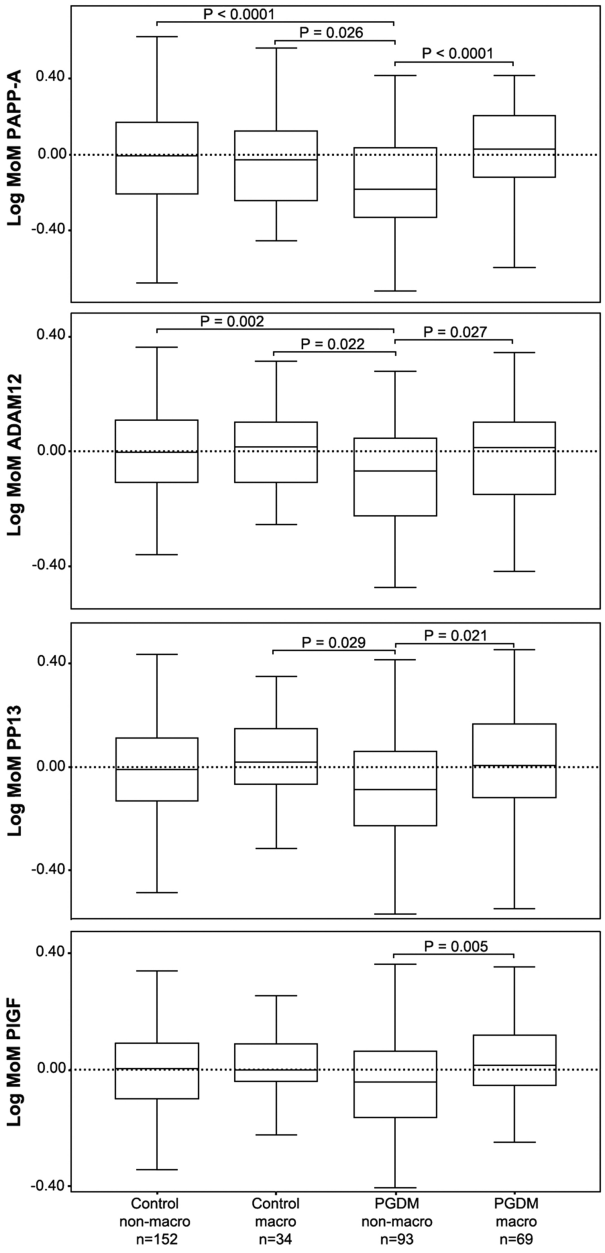

Figure 1. Boxplots showing the differences between non-macrosomic and macrosomic infants of control and PGDM pregnancies in the log MoMs of PAPP-A, ADAM12, PP13 and PlGF. Plotted are the median, quartiles and minimum/maximum values. Control non-macrosomic birthweight (Control non-macro), Control macrosomic birthweight (Control macro), PGDM non-macrosomic birthweight (PGDM non-macro), PGDM macrosomic birthweight (PGDM macro). Groups were compared with Mann-Whitney *U* tests. P values are indicated in the figures.

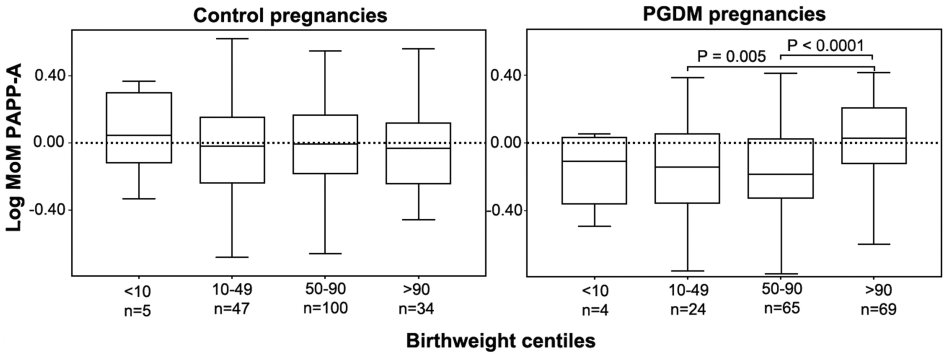

Figure 2. Boxplots showing the differences in the log MoM values of PAPP-A between four birthweight centile subgroups (<10th, 10-49th, 50-90th and > 90th centile) of control and PGDM pregnancies by plotting the median, quartiles and minimum/maximum values. Subgroups were compared with Mann-Whitney *U* tests. P values are indicated in the figure. * Post-partum information on birthweigt available: 100% (n = 186) in control group and 91% (n=162) in PGDM group.

Table 4. Detection rate for 5% and 10% falsepositive rate (FPR) for the prediction of macrosomic birthweight in PGDM pregnancies and with confidence intervals (CI 95%).

	Detection rate	
First trimester markers	5 % FPR (CI 95%)	10% FPR (CI 95%)
PAPP-A	18 (10-28)	30 (21-42)
ADAM12	15 (8-25)	23 (15-34)
PP13	9 (4-18)	17 (10-28)
PlGF	7 (2-14)	14 (8-25)
PAPP-A and ADAM12	22 (14-33)	34 (23-45)
PAPP-A and PP13	18 (10-28)	30 (21-42)
PAPP-A and PlGF	25 (16-36)	38 (27-49)
ADAM12 and PP13	24 (15-34)	34 (23-45)
ADAM12 and PlGF	15 (8-25)	24 (16-36)
PP13 and PlGF	13 (7-23)	23 (15-34)
PAPP-A, ADAM12 and PP13	25 (16-36)	37 (27-49)
PAPP-A, ADAM12 and PlGF	28 (18-39)	40 (30-52)
PAPP-A, PP13 and PlGF	26 (17-37)	38 (27-49)
ADAM12, PP13 and PlGF	19 (11-30)	29 (20-40)
PAPP-A, ADAM12, PP13 and PlGF	30 (21-42)	43 (32-55)

ADAM12, PP13 and PlGF are known to be implicated in the pathogenesis of impaired placentation.[13-16] In other words, this study indicates that normal birthweight in PGDM offspring is related to impaired early placentation, and that macrosomia at birth is related to normal placentation. In both instances fetal overgrowth occurs during pregnancy (poor placentation, normal birthweight; normal placentation, increased birthweight) only in the case of adequate placental development leading to macrosomia. This assumption may help to explain the weak association observed between indices of glycemic control during pregnancy (i.e. $HbA1_c$) and birthweight.[22] Apparently, events early in pregnancy contribute to the eventual birthweight, and maternal and fetal hyperglycemia contributes to fetal growth acceleration in all cases in the course of a PGDM pregnancy.

The present increase in macrosomia at birth in infants of PGDM women has been attributed to a decreased prevalence of microangiopathy, thus enabling a better placentation.[1] It may also be because of better glycemic control around the time of conception in early pregnancy. In this study the levels of placental markers in the macrosomic PGDM subgroup appeared comparable with the controls. PAPP-A and ADAM12 are components of the insulin growth factor (IGF) axis.[23-26] The IGF-axis is associated with a degree of glycemic control, and is negatively correlated with $HbA1_c$.[27] The IGF-axis components are known to be reduced in poorly controlled diabetic patients.[23,27-31] Optimal glycemic control is needed to support normal concentrations of IGF-axis components, presumably including both PAPP-A and ADAM12.[27,29,32] Therefore, one could speculate that PGDM mothers of macrosomic infants have a better metabolic control, reflected in concentrations of PAPP-A and ADAM12 that are similar to those of normal pregnancies. In consequence, we may expect better embryonic growth and development in this particular group, as normalized levels of IGF-axis components are crucial for these processes.[27,31,33]

A limitation of our study is the lack of distinction between type-1 and type-2 PGDM pregnancies. Coding only included the use of insulin during the first trimester of pregnancy; however, at present, fetal macrosomia occurs in a similarly high percentage in both groups.[1,3,5] Despite our effort to gain additional information at hospitals where first trimester screening participants were delivered, data on birthweight was not available in 9% of the PGDM cases. These missing data, although limited, may have skewed rates of macrosomia in this study. Because of the screening setting of our study, we did not have the information about glycemic control of PGDM patients. We also lacked data on placental size and histology at birth. Furthermore, in the PGDM group there were significantly more smoking pregnant women compared with the control group. It is broadly known, that smoking may influence the development and function of the placenta. Where relevant, we corrected the markers for smoking (PlGF). However, because of the relatively small number of smokers in both groups and the lack of placenta-specific information in current data, we were unable to further investigate this particular influence. Therefore, distinction between type-1 and type-2 diabetes, glycemic control, including $HbA1_c$ measurements, histological information on placental size and influence of smoking may be highly relevant material for future studies.

CONCLUSION

Macrosomia at birth in PGDM pregnancies may be predicted by normal levels of PAPP-A, ADAM12, PP13 and PlGF already in the first trimester of pregnancy. Fetal growth and birthweight in PGDM pregnancies are partly related to markers of early placentation. The present increase in fetal macrosomia might well be related to improved glycemic control around conception and during the first trimester of pregnancy, and to a better early placentation.

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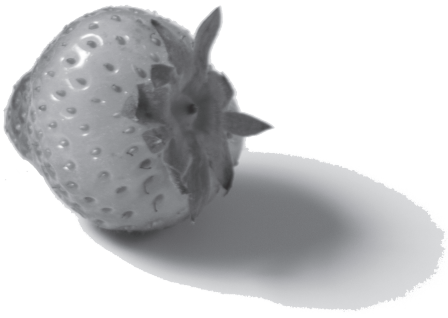
PART 3
PROSPECTIVE SCREENING – PHASE 4 – MARKERS OF (UN)COMPLICATED PREGNANCIES IN 1ST TRIMESTER STUDY (MUPPIT-STUDY)

VII

LONGITUDINAL TRENDS IN FETOPLACENTAL BIOCHEMICAL MARKERS, UTERINE ARTERY PULSATILITY INDEX AND MATERNAL BLOOD PRESSURE DURING THE FIRST TRIMESTER OF PREGNANCY



ABSTRACT

Objective

To assess trends in levels of biochemical markers, uterine artery (UtA) pulsatility index (PI) and maternal blood pressure changes over time and study their relationships in uncomplicated first trimester pregnancies.

Methods

The study population comprised 86 women with singleton pregnancies. In each woman, a blood sample was collected at 6–7, 8–9, 10–11 and 12–13 weeks' gestation. At the same visit blood pressure was measured and ultrasound examination was performed to measure the crown–rump length and Doppler flow velocity waveform patterns of both UtAs. Serum concentrations of Pregnancy-Associated Plasma Protein-A (PAPP-A), free β-human Chorionic Gonadotropin (fβ-hCG), A Disintegrin And Metalloprotease 12 (ADAM12), Placental Protein 13 (PP13) and Placental Growth Factor (PlGF) levels were measured in thawed specimens using an automated time-resolved fluorescence assay. Summary curves were created to describe normal ranges and trends over time. The data were analyzed with a linear mixed model with the log-transformed marker values as dependent variables. This allowed for flexible modeling of patterns over time.

Results

Sixty-eight pregnancies had an uneventful outcome, with the birth of an appropriate-for-gestational-age (AGA) infant. In these pregnancies serum PAPP-A, ADAM12, PP13 and PlGF levels increased with gestational age. The UtA-PI decreased and the mean arterial blood pressure remained constant. There were no significant correlations between maternal age, birthweight percentile, gender and blood pressure and any of the biochemical markers. The serum markers were highly correlated with each other except for free β-hCG. A negative correlation was found between most biomarkers and UtA-PI, especially from 10 weeks onwards. Serum concentrations of ADAM12 and PP13 were lower in a small-for-gestational age (SGA) subgroup born at term ($n = 6$), the former statistically significantly ($P = 0.031$), the latter non-significantly ($P = 0.054$), whereas UtA-PI was significantly higher ($P = 0.02$). Biomarker concentrations in 12 women delivering a large-for-gestational age infant did not differ from those delivering AGA neonates.

Conclusion

There is a relationship between biochemical markers of early placentation and downstream resistance to flow in the UtAs in low risk uncomplicated pregnancies, indicating differences in placentation. In a small series of SGA infants born at term we could demonstrate differences as compared to normal pregnancies, with potential value for screening.

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INTRODUCTION

At present in most countries in the developed world the combined test (maternal serum concentrations of Pregnancy-Associated Plasma Protein-A (PAPP-A) and free β-human Chorionic Gonadotropin (free β-hCG) measured at between 8 and 14 weeks' gestation and nuchal translucency measurement at 11 to 13 + 6 weeks) is the first trimester Down syndrome screening test of choice. Besides these markers, other potential serum markers are being identified and the scope of first trimester screening is widening to include identification of other fetal aneuploidies and pregnancies at risk for preeclampsia, fetal growth restriction and/or preterm delivery. PAPP-A and other serum biomarkers such as A Disintegrin And Metalloprotease 12 (ADAM12), Placental Protein 13 (PP13) and Placental Growth Factor (PlGF), all of which have been associated with placental growth and fetal development, have been suggested as indicators of increased risk for pregnancy complications.[1-5] Similarly, maternal cardiovascular factors and their adaptation in early pregnancy have been found to be related to early intrauterine growth restriction and preeclampsia. This holds for blood pressure and Doppler velocity waveform patterns of the uterine arteries (UtAs), both of which are indicative of downstream resistance.[6-8]

The relationships between markers in early pregnancy used for screening for fetal aneuploidies and adverse pregnancy outcome have been poorly studied, and normal ranges and trends throughout the first trimester are lacking for some of the most recent ones. It was the aim of this longitudinal study to assess the trends and to study the interrelationships of the maternal serum markers PAPP-A, free β-hCG, ADAM12, PP13, PlGF, maternal blood pressure and UtA pulsatility index (PI) in normally evolving first trimester pregnancies without risk factors and with a favorable outcome.

METHODS

Study design

The study population in this longitudinal observational study comprised 108 healthy low risk women with singleton pregnancies. Estimation of gestational age was based on the first day of the last menstrual period (LMP) or on ultrasound measurement of the crown–rump length (CRL) (in case of an irregular cycle, or when the difference between age determined by LMP and CRL was > 7 days).

Venous blood samples were collected at approximately 6–7, 8–9, 10–11 and 12–13 weeks' gestation. During the same visit an ultrasound examination was performed to measure CRL and Doppler flow velocity waveform patterns (i.e. pulsatility indices (PIs)) of both UtAs. The transducer was placed in the lower lateral quadrant of the abdomen. A sagittal section of the uterus was obtained and the cervical canal and cervical os identified. The transducer was angled to the side and color Doppler ultrasound was used to identify each artery along the side of the cervix and uterus (www.fetalmedicine.com). For this study the lowest PI measured was used since this appears to perform best as a screening tool for subsequent preeclampsia.[9] Blood pressure in both arms was measured according to the protocol of the British Hypertension Society with a Maxi Stabil (Welch Allyn, Benelux) aneroid device.[10] Mean arterial pressure (MAP)

was defined as DP + 1/3 (SP – DP), where DP = diastolic blood pressure and SP = systolic blood pressure. Maternal weight and height were measured and maternal history was documented. Data on pregnancy outcomes (chromosomal disorders, date of birth, birth weight, pregnancy complications and gender) were collected through self-reporting of the participating women using a questionnaire and verified through their medical records or by telephone call. All women gave their written informed consent and the project was approved by the Scientific Ethics Committee of the University Medical Centre, Utrecht, The Netherlands.

Reference values of biomarkers were based on pregnancies resulting in the birth of an appropriate-for-gestational age (AGA) infant. Data from large-for-gestational age (LGA; birth weight > 90th percentile) and small-for-gestational age (SGA; birth weight < 10th centile) subgroups were used to study possible differences from the 'normal' (AGA) controls.[11]

Sample collection and immunoassays

Blood samples were centrifuged and serum was aliquoted before storage at –80°C. Serum concentrations of PAPP-A, free β-hCG, ADAM12, PP13 and PlGF were measured in thawed specimens using a time-resolved fluorescence assay (autoDELFIA and DELFIA Xpress, PerkinElmer, Turku, Finland). Prior to analysis extensive validation was performed for all assays; mean intra- and interassay coefficients of variance for the assays were below 5% at all levels. The lowest standard concentrations of the assays were 9.4 mIU/L for PAPP-A, 2.1 ng/mL for free β-hCG, 6 ng/mL for ADAM12 (analytical sensitivity), 7 pg/mL for PlGF (limit of quantification) and the limit of detection for PP13 was 3.8 pg/mL.

Statistical analysis

Analyses were performed to assess the changes in serum concentrations of PAPP-A, free β-hCG, ADAM12, PP13 and PlGF over time. Summary curves were created to describe the longitudinal changes in serum concentrations, UtA-PI and maternal blood pressure. A linear mixed model was used for analysis with the log-transformed marker values as dependent variable. This is a common statistical method for analyzing repeated measurements on the same patient taking into account variation between and within patients. The average pattern of the log(marker) values as a function of gestational age (in days) was represented by a restricted cubic spline function with four knots (at the 5th, 35th, 65th and 95th percentile values of the marker). This data analysis allows a two-level structure with four repeated measures to depict the average marker pattern over time and the 95% limits of variation that exist between women. The between-patient variation around the average pattern was modeled through a random effect on the intercept.

Correlations were studied for maternal age, MAP, body mass index (BMI), UtA-PI and the biochemical markers for all sampling periods. Pearson correlation coefficients were calculated using the $\log_{10}$ concentrations. Statistically significant correlations were considered meaningful. Statistical analysis was performed using SPSS software (release 17.0; Chicago, IL, USA) and the R-program and $P < 0.05$ was considered statistically significant.

Growth charts corrected for gestational age, sex and parity according to the Dutch Perinatal Registry were used to calculate the birth weight *Z*-scores (http://www.perinatreg.nl). Weight for gestational age at the 50th percentile was used as the mean of the population

and the average SD calculated from the mean of the 16th (–1 SD) and the 84th (+1 SD) percentiles. Subsequently, the *Z*-score was converted into an exact percentile for each studied subject with a *Z*-score-to-percentile web calculator (www.measuringusability.com).

RESULTS

Eighty-six women had an uncomplicated pregnancy, delivering a healthy infant at > 37 weeks' gestation. Sixty-eight of these infants were AGA, 12 were LGA and six were SGA. The other 22 patients of the original 108 were excluded from this study because of missed miscarriage (n = 8), gestational diabetes (n = 7) or other complications such as preterm delivery or a genetic anomaly (n = 7). The demographic and clinical characteristics of the study population are shown in Table 1.

In the 68 women with a normal pregnancy and birth of an AGA infant serum PAPP-A, ADAM12, PP13 and PlGF increased between 6 and 13 weeks. Serum free β-hCG concentration followed a bell-shaped curve (Figure 1). The lowest UtA-PI decreased, whereas the MAP remained constant. In women with a higher BMI the concentrations of PAPP-A and ADAM12 were significantly lower (P = 0.022 and 0.036, respectively). Parous women had significantly higher PlGF concentrations (P < 0.0001). While the PAPP-A and free β-hCG assays are known to be sensitive and reliably measured as early as 6 weeks' gestation, ADAM12, PP13 and especially PlGF concentrations were generally lower than the lowest standard concentration before week 8 (ADAM12 and PP13) or week 10 (PlGF).

There were no significant correlations between maternal age, exact birth-weight percentile, gender and blood pressure and any of the biochemical markers. The Pearson correlation

Table 1. Demographic and clinical characteristics of the study groups.

Parameter	AGA group n = 68	LGA group n = 12	SGA group n = 6
Maternal age (years)	33.8 (19.8-42.7)	32.3 (28.1-37.0)	33.3 (22.4-36.1)
Gestational age at sample (days)	68 (40-100)	69 (42-93)	67 (43-88)
Maternal height (cm)	173 (155-184)	176 (162-183)	169 (165-187)
Maternal weight (kg)	66 (50-110)	72 (63-95)*	75 (57-120)
Body mass index (kg/m^2)	23 (19-35)	23 (21-31)	25 (20-43)
Birthweight (g)	3445 (2795-4250)	4290 (3890-4900)*	2943 (2740-3030)*
Birthweight centile	47 (11-90)	95 (91-100)*	8 (5-10)*
Gestational age at delivery (days)	280 (260-297)	281 (268-288)	278 (276-283)
Nulliparous	31 (45.6)	3 (25.0)	2 (33.3)
Smoker	1 (1.5)	0	2 (33.3)
Male : female ratio	40 : 28	6 : 6	3 : 3

Data are given as median (range), n (%) or n. *Statistically significantly different from normal pregnancies. AGA, appropriate-for-gestational age (birth weight between 10th and 90th percentiles); LGA, large-for-gestational age (birth weight > 90th percentile); SGA, small-for-gestational age (birth weight < 10th percentile).

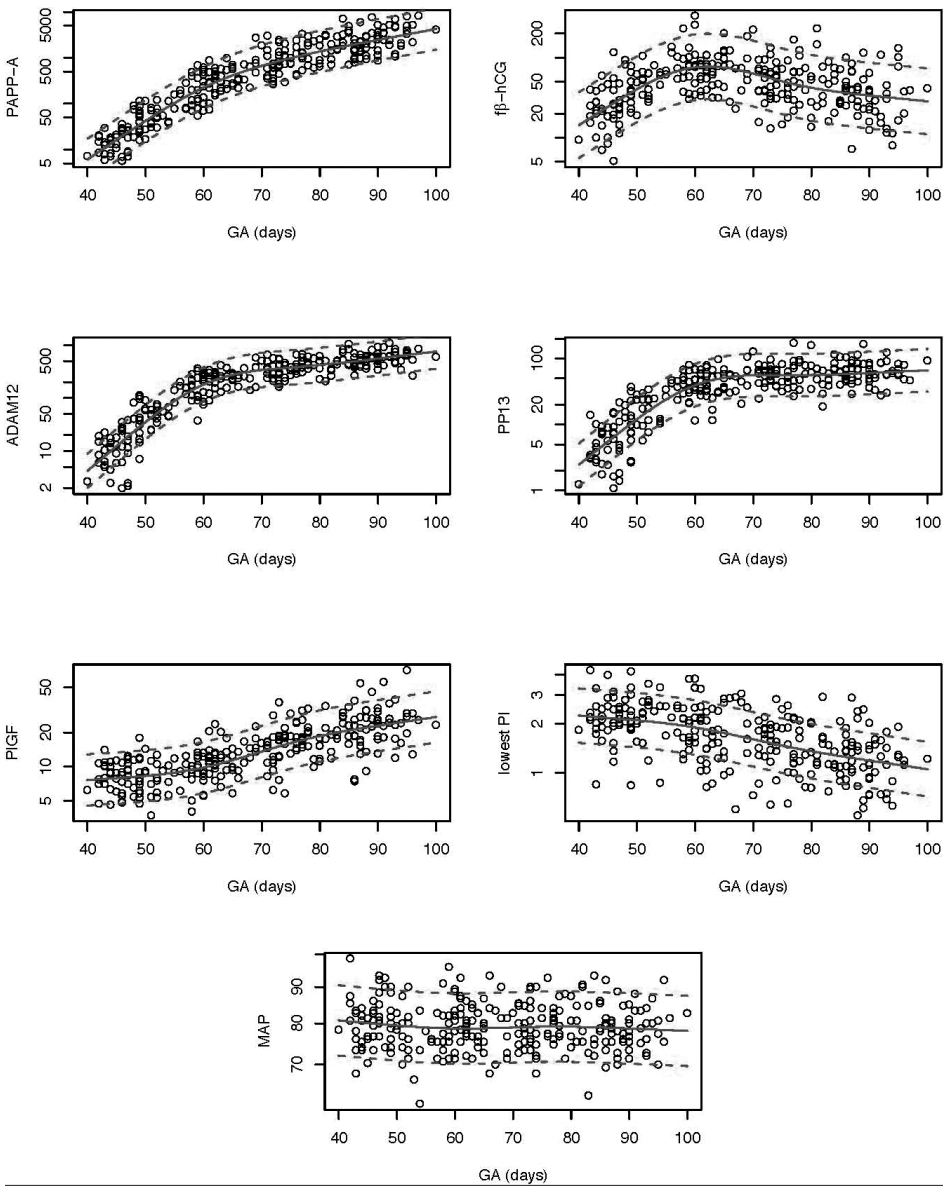

Figure 1. Maternal serum concentrations of: (a) Pregnancy-Associated Plasma Protein-A (PAPP-A), (b) free β-human Chorionic Gonadotropin (fβ-hCG), (c) A Disintegrin And Metalloprotease 12 (ADAM12), (d) Placental Protein 13 (PP13) and (e) Placental Growth Factor (PlGF); and (f) lowest uterine artery pulsatility index (UtA-PI) and (g) Mean Arterial Pressure (MAP) in 68 physiological (normal) pregnancies. Mean values over time (**----**) and 95% CIs of between-patient variation (- - -) are shown.

coefficients between the serum markers in each gestational age window (6 – 7, 8 – 9, 10 – 11 and 12 – 13 weeks) for all 68 in the AGA group are shown in Figure 2. The serum markers were highly correlated except for free β-hCG. For PlGF the correlations were very low before 10 weeks, in contrast to those of the other biomarkers. A negative correlation was found between most biomarkers and UtA-PI, especially from 10 weeks onward (Table 2).

Table 2. Correlations (Pearson's rho) between serum markers (log_{10} concentration) and uterine artery lowest pulsatility index in different gestational-age windows

Figure 3 shows preliminary data comparing patients who had an AGA infant with those having either an SGA or an LGA infant. Serum concentrations of ADAM12 and PP13 were reduced in the SGA group as compared to the AGA group (P = 0.031 and 0.054, respectively). The UtA-PI was significantly higher in the SGA group than in the AGA group (P = 0.02). Data from the LGA group did not differ from those in the AGA group.

DISCUSSION

This is the first study to investigate longitudinal changes in concentrations of five serum markers, UtA-PI and maternal blood pressure in normal physiological pregnancies during the first trimester. PAPP-A, ADAM12, PP13 and PlGF increased with gestational age and

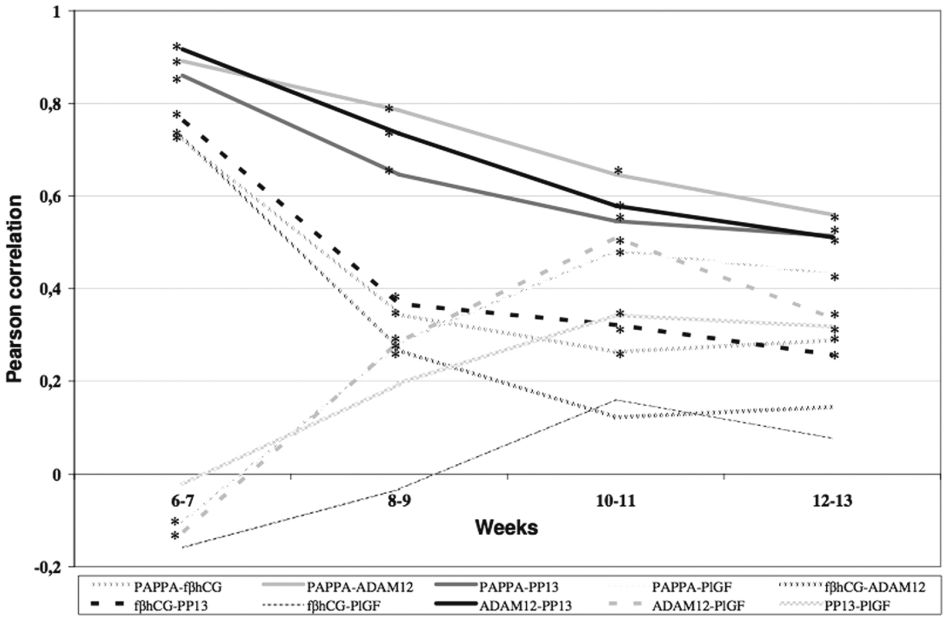

Figure 2. Pearson correlations (rho) between log^{10} concentration of the biochemical markers Pregnancy-Associated Plasma Protein-A (PAPP-A), free β-human Chorionic Gonadotropin (fβ-hCG), A Disintegrin And Metalloprotease 12 (ADAM12), Placental Protein 13 (PP13) and Placental Growth Factor (PlGF). *Significant correlations in gestational age windows 6–7, 8–9, 10–11 and 12–13 weeks.

Table 2. Correlations (Pearson's rho) between serum markers (log_{10} concentration) and uterine artery lowest pulsatility index in different gestational age windows.

Serum marker	Gestational age (weeks)			
	6-7	8-9	10-11	12-13
PAPP-A	0.053	-0.332*	-0.244*	-0.292*
fβ-hCG	-0.019	0.070	0.068	0.150
ADAM12	-0.034	-0.296*	-0.408*	-0.202
PP13	-0.039	-0.236	-0.366*	-0.340*
PlGF	0.013	-0.168	-0.252*	-0.263*

*Significant correlations. ADAM12, A Disintegrin and Metalloprotease protein-12; β-hCG, β-human Chorionic Gonadotropin; PAPP-A, Pregnancy-Associated Plasma Protein-A; PlGF, Placental Growth Factor; PP13, Placental Protein-13.

were negatively correlated with UtA-PI at one or more gestational ages studied, with a tendency for the extent of the correlation to increase with gestational age. The pairwise correlations between the serum markers changed with gestation.

Collecting serial samples of blood from the same woman starting very early in pregnancy is difficult, as the majority of women without known risk factors who become pregnant spontaneously do not book for testing very early, but we managed to achieve this for 108 low- risk pregnant women. Of these only 68 (63.0%) had a completely uneventful course and outcome of pregnancy.

The longitudinal changes in serum concentrations of PAPP-A, free β-hCG, ADAM12, PP13 and PlGF were described using a mixed model (Figure 1). This represents a new approach to looking at the relationship between gestational age and serum concentrations compared with the calculation of trends with gestation based on large numbers of cross-sectional and unrelated serum concentrations.

The spread in the range of distribution of the serum markers can be explained by many factors, one of which may be BMI. When BMI increased, the concentrations of PAPP-A and ADAM12 decreased significantly (P = 0.022 and 0.036, respectively). Furthermore, parity could be an explanatory factor for the range of distribution of PlGF, since in parous women a significantly higher PlGF concentration was found ($P < 0.0001$). Another factor may be the reliability of measurements, determined by the serum concentrations of biomarkers and the analytic capabilities of the assays. Performing serial measurements early in the first trimester thus provides an indication as to when a marker can be measured reliably. PAPP-A and free β-hCG assay kits are known to be very sensitive. In combination with the fact that free β-hCG and PAPP-A are present in the ng/mL range even early in the first trimester, they can be measured as early as 6–7 weeks' gestation. ADAM12 and PP13 are present in serum in the pg/mL range, but can be measured quite reliably early in the first trimester, as most concentrations are above the limit of detection or quantification. However, PlGF cannot be measured reliably until at least 10 weeks, which might explain its low correlations with the other biomarkers before 10 weeks.

We found that UtA-PI fell with gestation, which is in agreement with previous research.[12-14] Blood pressure is also known to decrease in the first half of gestation, but differences

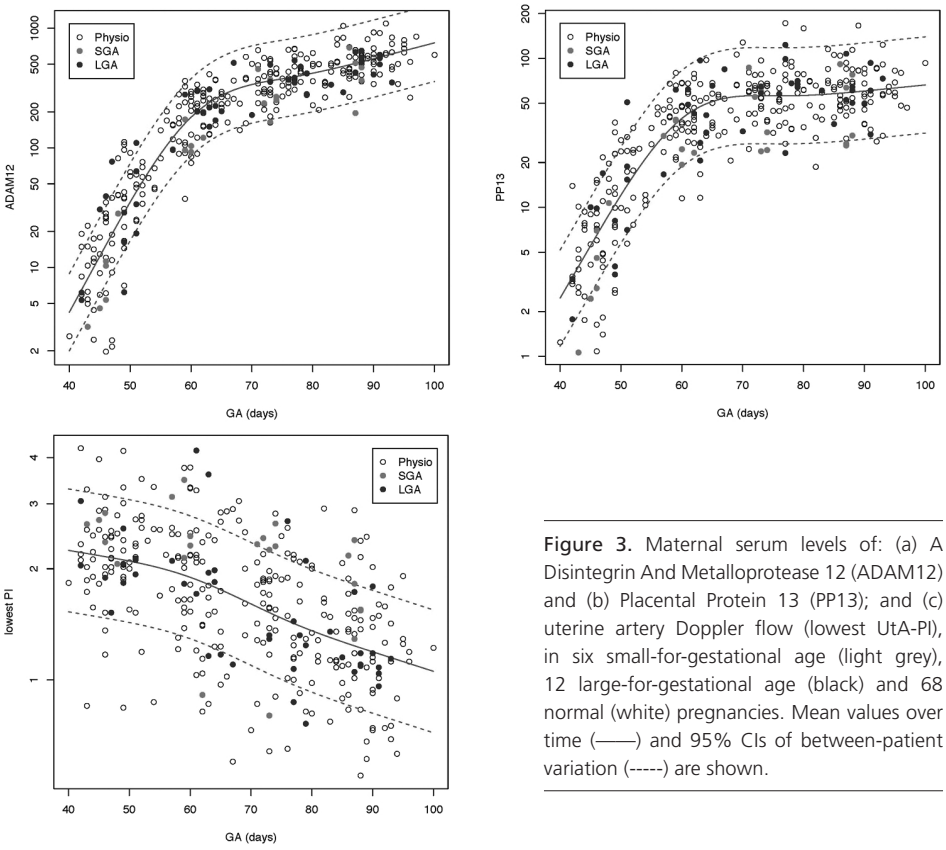

Figure 3. Maternal serum levels of: (a) A Disintegrin And Metalloprotease 12 (ADAM12) and (b) Placental Protein 13 (PP13); and (c) uterine artery Doppler flow (lowest UtA-PI), in six small-for-gestational age (light grey), 12 large-for-gestational age (black) and 68 normal (white) pregnancies. Mean values over time (——) and 95% CIs of between-patient variation (-----) are shown.

between 7 and 12 weeks were not significant is this population.[8,15,16] In our population there was a negative correlation between the biomarkers and the UtA-PI, especially after 10 weeks. PlGF is a member of the angiogenic vascular endothelial growth factor family, and produced predominantly by trophoblast cells it is able to cause endothelial cell proliferation, migration and activation.[17,18] Impaired trophoblast invasion leads to insufficient vascular remodeling of the spiral arteries, therefore a negative correlation between UtA-PI and this biomarker seems physiologically plausible. The biomarkers ADAM12, PP13 and PlGF are also known to be related to early placentation.[2-5] The fact that biochemical markers of early placentation and downstream resistance to flow in the UtAs are correlated in low risk uncomplicated pregnancies points towards differences in placentation, even within the 'physiological' range. Correlations between the biochemical markers have been described before, but not to this extent and not for separate gestational weeks.[5,19-21]

The serum markers we investigated are considered to be involved in early placentation, and reduced serum concentrations presumably reflect impaired placentation.[2-4,18,22,23] We found that ADAM12 was significantly reduced in the SGA group, with an almost significant reduction in PP13. ADAM12 is an insulin-like growth factor-binding protein

(IGFBP)-protease, with specificity for IGFBP-3 and -5.[23] It is thought to be of importance in fetal growth, just as is PAPP-A.[24] Low levels of these markers may explain placenta-related complications such as intrauterine growth restriction.[24,25] PP13 is predominantly produced by the syncytiotrophoblasts and is thought to play a major role in the implantation of the blastocyst. Moreover, it is possibly involved in remodeling of the common fetomaternal blood spaces through binding to proteins between placenta and endometrium.[26,27] In preeclampsia and pregnancies complicated by intrauterine growth restriction first trimester concentrations of PP13 have been found to be significantly reduced.[5,28]

The principal aim of this study was to provide a baseline of normal values of serum markers of adverse pregnancy outcome for future investigation and risk assessment. The search for new potential markers in maternal serum that are capable of detecting the presence of pathological conditions early in pregnancy with a high sensitivity and specificity is still ongoing. Early screening for fetal and maternal health would provide early reassurance and a low-frequency antenatal visit schedule for women at low risk, whereas for those at increased risk intensified antenatal surveillance is advisable. Even in the small series of pregnancies resulting in the birth of an SGA infant at term we could demonstrate differences from pregnancies with an AGA infant, which shows the potential value of these markers in screening for abnormal placentation. This finding is particularly interesting, since all six SGA infants in this cohort were born at term without serious perinatal complications. Currently, we are investigating a larger number of pathological pregnancies. It remains to be established if serial first trimester measurements constitute a better screening method than does a single measurement.

In conclusion, this study presents longitudinal data on biophysical and biochemical markers of pregnancy development in the first trimester, starting from as early as 4 weeks after implantation. These data provide unique information on the physiological changes associated with normal placentation.

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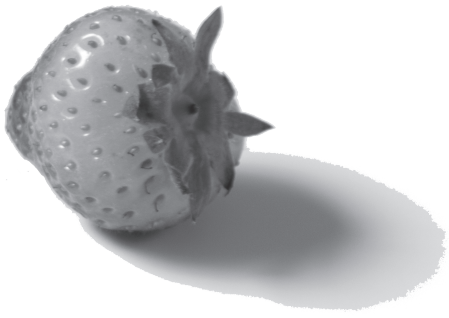
VIII

LONGITUDINAL FIRST TRIMESTER TRENDS IN FETOPLACENTAL BIOCHEMICAL MARKERS, UTERINE ARTERY PULSATILITY INDEX AND MATERNAL MEAN ARTERIAL BLOOD PRESSURE IN WOMEN WITH PREECLAMPSIA IN THEIR PREVIOUS PREGNANCY



ABSTRACT

Objective

To assess trends in levels of biochemical markers, uterine artery (UtA) pulsatility index (PI) and maternal mean arterial blood pressure (MAP) changes over time and study their relationships in the first trimester of a-priori high risk pregnancies of women who had a preeclampsia (PE) in their previous pregnancy.

Methods

The study population comprised of a low-risk control group (n= 85) and a high risk (n= 27) group. During four visits at 6 – 7, 8 – 9, 10 – 11 and 12 – 13 weeks of gestation maternal serum concentrations of Pregnancy-Associated Plasma Protein-A (PAPP-A), free β-human Chorionic Gonadotropin (fβ-hCG), Placental Growth Factor (PlGF) and A Disintegrin And Metalloprotease 12 (ADAM12), maternal mean arterial blood pressure (MAP) and Doppler flow velocity waveform patterns of both UtAs were measured. Summary curves were created to describe the course of the markers over time. The data were analysed with a linear mixed model with the log-transformed marker values as dependant variables. The courses of the makers were compared between the two groups.

Results

MAP was the only first trimester marker that was significantly higher in women with a PE in their previous pregnancy. MAP increased gradually in the high risk group depending on the severity of the outcomes in the current pregnancy - lowest in uncomplicated outcome, highest in early onset PE.

Conclusion

MAP measured longitudinally is constantly significantly higher in high risk pregnancies through the entire first trimester of pregnancy compared to control pregnancies.

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INTRODUCTION

While preeclampsia (PE) is generally thought to be a disease of the first pregnancy, women with PE in their history are known to be at higher risk to develop it again in a next pregnancy compared to women with an uncomplicated first pregnancy.[1-4] The estimated recurrence risk of PE differs considerably between studies, ranging from 5.0% up to 65.0% depending on the gestational age (GA) at the onset of the disease, the definition of PE and the study population.[3-8]

Although the course of re-occurring PE is milder and outcomes are generally much better, these pregnancies still ought to be considered as a-priori high risk.[4,7]

Currently, much effort is put into predicting PE in early pregnancy. The most promising approach is the expanded version of first trimester Down syndrome screening. In addition to the classical maternal serum markers free β-human Chorionic Gonadotropin (fβ-hCG) and Pregnancy-Associated Plasma Protein-A (PAPP-A), new markers are introduced. These are: serum Placental Growth Factor (PlGF) and A Disintegrin And Metalloprotease 12 (ADAM12), as well as the uterine artery (UtA) pulsatility index (PI) and maternal mean arterial pressure (MAP).[9-14] Serum markers tend to be significantly lower whereas UtA PI and MAP are significantly higher in women who subsequently develop PE. In combination, they are able to predict 50% to 90% of the PE cases in the first trimester of pregnancy.[9-14]

From Down syndrome screening studies it is known that there is a considerable correlation between serum markers in the index high risk pregnancy and following pregnancies.[15-18] This would suggest that maternal and genetic factors affect the level of these markers. The same may hold for women who experienced PE in the first pregnancy, whereby aberrations leading to the development of PE in the first pregnancy might also be present in subsequent pregnancies.

In a previous study we established first trimester longitudinal serum concentrations of potential markers for PE in low risk pregnancies.[19] It was the aim of this study to assess whether these markers differ in pregnant women who had a PE in their previous pregnancy.

METHODS

Study design

The study population in this longitudinal observational study comprised of two groups: 1) women with a-priori low risk single pregnancy with an uneventful course and delivery of a healthy infant at term (control group, n= 85) and 2) women with PE in their obstetric history (high risk pregnancies, n = 27). Data for this study was collected between 2009 and 2012 at the University Medical Centre in Utrecht, the Netherlands. Results of the study regarding low-risk women have been published before, albeit that the present data set was somewhat larger.[19]

Estimation of gestational age (GA) was based on the first day of the last menstrual period (LMP) or on ultrasound measurement of the crown-rump length (CRL) in case of an irregular cycle. During out-patient visits at 6 – 7, 8 – 9, 10 – 11 and 12 – 13 weeks of gestation we recorded maternal characteristics and measured blood pressure with a manual device (Maxi Stabil, Welch Allyn, Benelux). A blood sample was taken by venous puncture, an ultrasound scan was performed to measure the crown-rump length (CRL)

and Doppler flow velocity waveform patterns were obtained of both UtAs (i.e. pulsatility indices [PIs]). Data on pregnancy outcomes were collected through self-reporting by the participating women using a questionnaire and were verified though their medical records or by telephone call. Written informed consent was obtained from the women agreeing to participate in the study, which was approved by the Scientific Ethics Committee of the University Medical Center, Utrecht, the Netherlands (protocol nr 07-222).

Definitions

Mean arterial pressure was calculated from the formula *DP + 1/3 (SP – DP)*, where DP represents diastolic blood pressure and SP - systolic blood pressure.

Pregnancy induced hypertension (PIH) was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy as: gestational hypertension beyond 20 weeks GA in previously normotensive women with a systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on at least two occasions four hours apart.[20]

Preeclampsia (PE) was defined as PIH with the presence of proteinuria of ≥ 300 mg in 24-hour collection or ≥ 2+ by dipstick on a spot urinalysis with or without generalized oedema (REF). Early onset PE (EO-PE) was defined as PE requiring delivery < 34 weeks GA, late onset PE (LO-PE) as PE requiring delivery ≥ 34 weeks GA.

Growth charts corrected for gestational age, sex and parity according to the Dutch Perinatal Registry were used to calculate the birthweight Z-scores (http://www.perinatreg.nl;[21]). Weight for gestational age at the 50th centile was used as the mean of the population and the average standard deviation (SD) was calculated by the formula *(-1 SD + 1 SD)/2*. Subsequently, the Z-score was converted into an exact centile for each studied subject.

The reference values of the control group were based on 85 uncomplicated pregnancies after which a healthy infant without congenital malformations was born after 37 weeks of gestation. The course of these pregnancies had been uneventful, i.e. without occurrence of pregnancy induced hypertension, gestational diabetes etc.[19] For this study we included all birth weight centiles, i.e also pregnancies ending in the birth of a large- or small-for-gestational age infant.

Sample analysis

Blood samples were centrifuged and serum was aliquoted before storage at -80ºC. Serum concentrations of PAPP-A, fβ–hCG, ADAM12 and PlGF were measured in thawed specimens using a time-resolved fluorescence assay (autoDELFIA, PerkinElmer, Turku, Finland). Prior to analysis extensive validation was performed for all assays. The results of this validation have been published elsewhere.[19]

Statistical analysis

The single comparisons between control and high risk pregnancies groups were made by chi-square test for categorical variables and Mann Whitney-U test for continuous variables.

Analyses were performed to assess the changes of the first trimester markers over time and to further compare these between our two study groups. Summary curves were created to describe the longitudinal course of the serum markers as well the measurements of

UtA-PI and maternal mean arterial pressure. A linear mixed model was used for analysis with the log-transformed marker values as dependent variable. The average pattern of the log(marker) values as a function of gestational age in days was represented by a restricted cubic spline function with four knots (at the 5th, 35th, 65th and 95th percentile values of the marker). The between patient variation around the average pattern was modelled through a random effect on the intercept. The difference between the groups was estimated as a fixed effect. Since this difference is assessed on the log-scale, it was back transformed into a Fold Change (FC) reflecting the relative (ratio) difference between levels of the markers in control and high risk pregnancies groups. Where applicable, these analyses were corrected for maternal age, BMI, smoke status and parity as potential confounders. The critical statistical significance was set at p-value < 0.05. Bonferroni correction was applied, to counteract the problem of multiple comparisons on 7 different outcomes. Therefore the critical statistical significance in the case of multiple comparisons was set at $p < 0.007$. Statistical analyses were performed using SPSS (release 20.0; Chicago, IL) and R-program (www.r-project.org).

RESULTS

The control group consisted of 85 women and the high risk group of 27 women. The latter group consisted of 17 women who had experienced an EO-PE and 10 women who had experienced a LO-PE in their previous pregnancy. In the current index pregnancy 17 women had an uncomplicated outcome (high risk uncomplicated). Ten women developed a hypertensive complication of pregnancy (37%; EO-PE n=3, LO-PE n=3, PIH n=4; high risk complicated). In women with a previous EO-PE, 41% again developed a hypertensive complication; in women with a previous LO-PE this was 30% (Figure 1). All infants were healthy without congenital malformations and had an uneventful neonatal course.

We compared the characteristics of the high risk pregnancies to those of the control group. The characteristics of the study groups are shown in table 1a. Women in the high risk group delivered significantly earlier (275 days vs. 281 days; $p = 0.003$) and their neonates were on average 115 grams lighter; however, birthweight centiles were comparable. Maternal characteristics such as age, weight, BMI and smoking status did not differ between the groups, except for parity since women in the high risk group were never nulliparous. The marker levels of the control group were compared to those of the entire high risk group regardless the outcome of these pregnancies (Table 2). Only MAP was significantly higher in the high risk group (FC 1.10, $p < 0.0001$). The median MAP over the entire first trimester in the control group was 78.2 (IQR 74.7-82.3) vs. 85.3 (IQR 79.7-94.3) in the high risk group (Figure 2).

In a subgroup analysis we studied whether any differences in maternal characteristics and first trimester markers existed within the high risk group with regard to previous pregnancy outcomes (EO-PE vs. LO-PE in previous pregnancy). There were no statistically significant differences between the maternal and fetal characteristics of the two subgroups: maternal age ($p = 0.09$), weight ($p = 0.54$), BMI ($p = 0.84$), smoking ($p = 0.12$), gestational age at sampling ($p = 0.92$).

While MAP was already significantly increased in the entire high risk group, MAP was even higher in women with EO-PE in their previous pregnancy compared to those who

Table 1. Study population baseline characteristics. Values are presented as median (IQR) or number (%). a) women with an uncomplicated pregnancy (controls) versus women with a high risk pregnancy

Characteristics	Controls n = 85	High risk pregnancies n = 27	p-value
Maternal age (y)	33 (31-36)	34 (32-37)	0.174
Maternal weight (kg)	67.0 (63.0-75.0)	68.5 (61.5-76.0)	0.965
Maternal BMI (kg/m2)	23.0 (20.9-24.7)	24.0 (21.5-27.1)	0.272
Nulliparity	36 (42.3)	9 (0)	<0.0001*
Smoking	3 (3.5)	1 (3.7)	0.966
Gestation at sampling	68 (54-83)	70 (58-83)	0.239
Gestation at birth (days)	281 (276-285)	275 (264-282)	0.003*
Birthweight (gr)	3520 (3230-3890)	3405 (2850-3875)	0.124
Birthweight centile	61 (38-83)	60 (36-87)	0.672
Male : female ratio	48 : 37	12 : 15	0.934

b) women with an uncomplicated pregnancy in the high risk group (high risk uncomplicated) versus complicated high risk pregnancy (high risk complicated)

Characteristics	High risk uncomplicated n = 17	High risk complicated n = 10	p-value
Maternal age (y)	34 (32-38)	33 (31-37)	0.359
Maternal weight (kg)	65.0 (59.3-72.5)	76.5 (63.0-85.3)	0.059
Maternal BMI (kg/m2)	22.5 (20.9-24.9)	27.1 (23.0-28.8)	0.015*
Smoking	1 (5.9)	0 (0)	0.824
Gestation at sampling	70 (61-83)	69 (54-83)	0.755
Gestation at birth (days)	270 (275-284)	258 (228-269)	0.001*
Birthweight (gr)	3485 (3060-3995)	3010 (1949-3530)	0.04*
Birthweight centile	57.1 (31.3-88.8)	60.7 (50.3-83.6)	0.902
Male : female ratio	8 : 9	6 : 4	0.786

A Pearson's chi square test and Mann-Whitney U test, were used for statistical analysis. IQR: interquartile range; BMI: body mass index; (*): statistically significant at $p < 0.05$.

had LO-PE ($p<0.001$; FC 1.09). The median MAP over the entire first trimester in the EO-PE group was 91.8 (IQR 83.7-95.7) vs. 81.8 (IQR 77.8-85.6) in the LO-PE group.

Next, we compared high risk uncomplicated and high risk complicated subgroups (Table 1b; Figure 2). Women who again developed PE or PIH (high risk complicated) had a significantly higher BMI than their peers who did not develop any pregnancy complications in their current pregnancy (high risk uncomplicated; 27.1 kg/m^2 vs. 22.5 kg/m^2, respectively; $p = 0.015$). Also, they delivered about 12 days earlier and the birthweight of their children was lower (258 days vs. 270 days; $p = 0.001$; 3405 grams vs. 3520 grams, respectively; $p = 0.04$), but birthweight centiles were comparable. MAP was lowest in the controls and gradually increased, with a FC of 1.06 for uncomplicated high risk

Table 2. Linear mixed regression analysis comparing the control group (n = 85) with the entire high risk group (n = 27). Markers were adjusted for maternal age, BMI, smoking and parity.

	FC	p-value
PAPP-A	0.95	0.73
Free β-hCG	1.08	0.52
ADAM12	1.12	0.21
PlGF	1.00	0.99
CRL	0.96	0.19
Lowest Ut-A-PI	1.04	0.50
MAP	1.10	<0.001*

PAPP-A: Pregnancy-Associated Plasma Protein-A; Free β-hCG: free β-human Chorionic Gonadotropin; ADAM12: A Disintegrin And Metalloprotease 12; PlGF: Placental Growth Factor; CRL: crown-rump length; Lowest Ut-A-PI: lowest uterine artery pulsatility index; MAP: mean arterial pressure; FC: fold change. Significance value p < 0.007 (*) because of multiple testing.

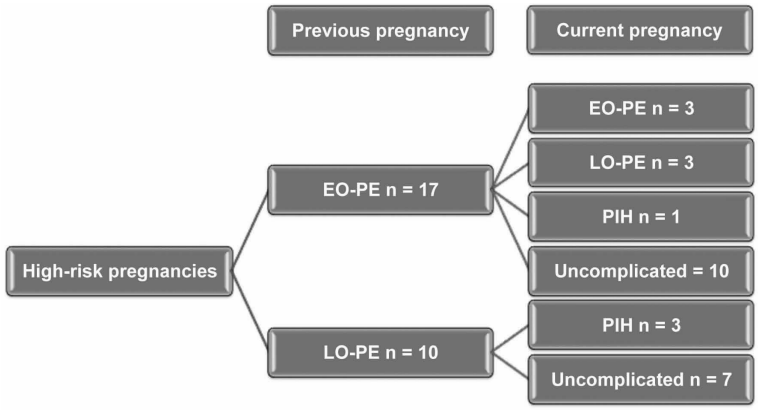

Figure 1. A-priori high risk group characteristics and distributions in the group depending on the variables: previous and current pregnancies outcomes. EO-PE: early onset preeclampsia; LO-PE: late onset preeclampsia; PIH: pregnancy induced hypertension.

pregnancies and a FC of 1.15 for complicated high risk pregnancies. The median MAP over the entire first trimester in controls was 78.2 (IQR 74.7-82.3), in high risk uncomplicated pregnancies 82.0 (IQR 78.0-89.3) and high risk complicated pregnancies 93.8 (86.5-96.3). The differences between all subgroups were statistically significant (Table 3).

Finally, despite numbers were low, we studied the trend of markers in the high risk group depending on the severity of the outcomes starting from uncomplicated outcome through PIH, LO-PE and EO-PE. Again MAP appeared to increase gradually (p < 0.001).

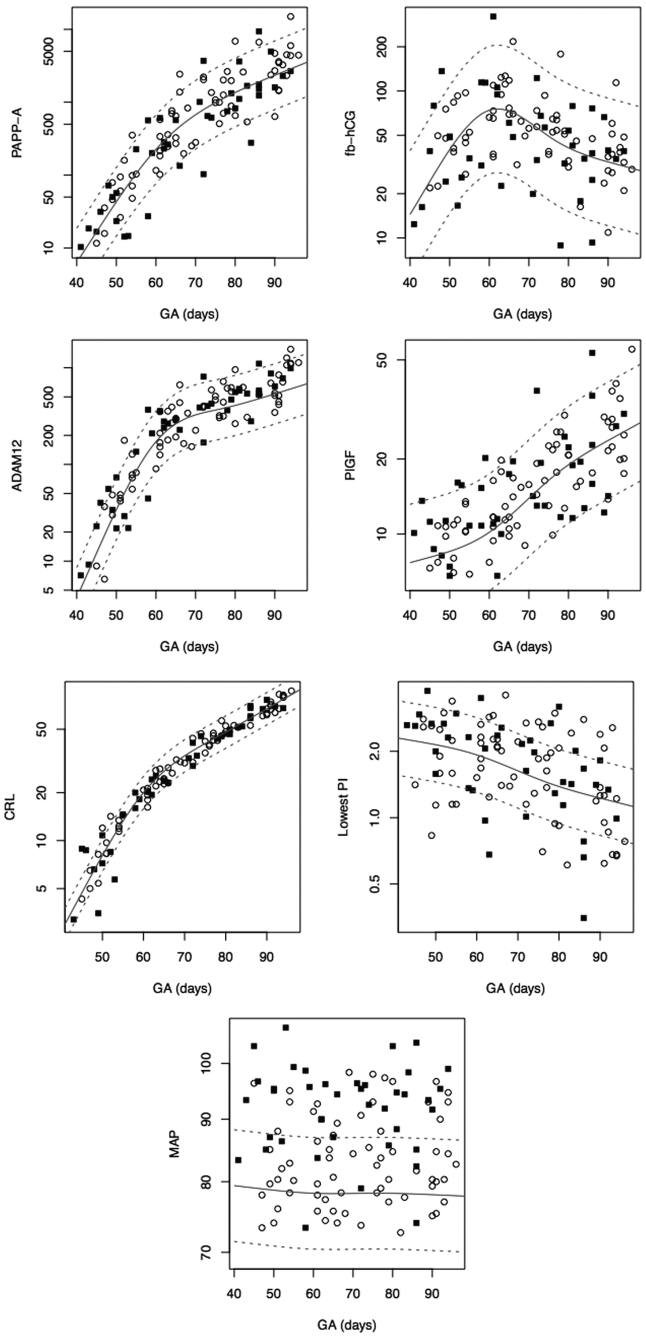

Figure 2. The distribution of the first trimester markers in the 27 high risk pregnancies. The reference lines are based on the values from the control group. High risk uncomplicated pregnancies are marked with white dots, high risk complicated cases are marked with black squares.

Table 3. Linear mixed regression analysis comparing the control group (n = 85) with the high risk uncomplicated and complicated subgroups (n = 20 and n = 10, respectively). MAP was adjusted for maternal age, BMI, smoking and parity.

	Controls vs high risk uncomplicated	Controls vs high risk complicated	High risk uncomplicated vs High risk complicated
MAP	FC = 1.06	FC = 1.15	FC = 1.08
p value	p < 0.001*	p < 0.001*	p = 0.014*

MAP: mean arterial pressure; FC: fold change. Significance value p < 0.0167 (*).

DISCUSSION

This is the first study, in which longitudinal changes of several first trimester markers were investigated in high risk pregnancies and compared to those of uncomplicated pregnancies. MAP of women with a PE in their previous pregnancy was significantly higher compared to controls starting from 6 weeks of gestation onwards. Other first trimester markers did not differ between groups.

A total of 37% of these women again developed a hypertensive complication (PIH or PE) in their current pregnancy, with the highest risk in women who previously experienced an EO-PE. The latter group also had the highest MAP and BMI in the first trimester of their current pregnancy. The subgroup of women who experienced EO-PE in a previous pregnancy appears to be at highest risk to develop PE or PIH again. From the beginning of pregnancy their blood pressure was higher compared to the other groups and in this EO-PE subgroup pregnancy complications occurred the most often. In the LO-PE group none of the women developed PE again and PIH was the only complication. Although we did not construct a prediction model in this study due to the relatively limited number of cases, first trimester MAP may inform us about the risk to develop recurrent PE or PIH fairly well. Maternal characteristics such as maternal weight and BMI may additionally help in distinguishing women at risk. These findings are in agreement with earlier studies where MAP combined with maternal characteristics appeared to effectively predict PE.[11-13]

In women who had PE in a previous pregnancy we clearly see that routine measurement of blood pressure combined with maternal characteristics during the first antenatal visit of the successive pregnancy have a potential to foresee the development of hypertensive pregnancy disorders several months later. Moreover, getting information on maternal characteristics and measuring MAP are cheap and generally accessible. At this point of pregnancy eventual ultrasound and/or maternal serum screening with emerging serum markers may be added.[10,12] However, according to our current study, markers such as PAPP-A, PlGF, ADAM12 and uterine artery pulsatility index did not appear to be associated with PE/PIH in a second pregnancy. Presumably, we did not find any alteration of these markers in our study population due to the limited number of recurrent EOPE cases. The other women developed LOPE or PIH and first trimester makers are known to be less suitable for the detection of these diseases. We may also speculate that the prognostic

value of these markers is lower in multiparous women. Studies on serum markers in multiparous women only have, to the best of our knowledge, not been performed before.

Since serum markers did not differ from controls it seems unlikely that these markers are correlated between first and subsequent pregnancies, which is in contrast to findings in pregnancies complicated by Down syndrome.[15-18] However, this should be confirmed in another study in which markers from separate pregnancies of the same women can be compared.

The strength of our study is its longitudinal approach. A longitudinal approach seems superior to the measurements based on large numbers of cross-sectional and unrelated measurements since repetitive measurements of the same study subjects provides greater accuracy. The benefit of a longitudinal study is the possibility to detect developments or changes in the measurements of the target population at both the group and the individual level. However, such studies are difficult to conduct and inclusion proved to be difficult, which is a limitation.

A perceptible step to a further level would be longer follow-up study of these women. This particular research could focus on the risk profiling of the women who recurrently develop PE or PIH. Hypertensive pregnancy disorders together with high maternal weight and BMI predispose these women to the development of chronic hypertension and cardiovascular diseases (CVD).[8,22-24] The magnitude of the risks occurs related to the severity of hypertensive pregnancy disorders and the time of onset of the disease.[25] Recurrent PE compared with PE in only the first pregnancy has been associated with a three and sevenfold increased risk of future hypertension.[22,26] The pathology of the events leading to future hypertension, and also CVD, are poorly understood; however, there are some indications that permanent damage of the endothelium, persistent high blood pressure, higher maternal weight and already existing genetical predisposition play a role.[8,22,27]

In conclusion, the longitudinal course of first trimester MAP is highly associated with recurrent preeclampsia in a successive pregnancy. The role of serum markers and UtA Doppler may well be limited.

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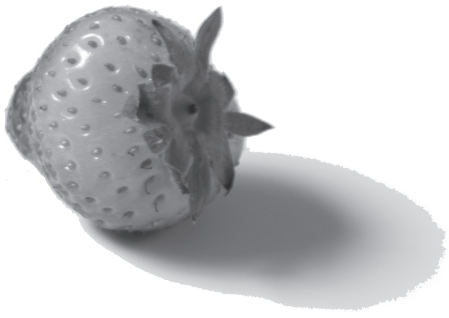
IX

LONGITUDINAL TRENDS IN FETOPLACENTAL BIOCHEMICAL MARKERS, UTERINE ARTERY PULSATILITY INDEX AND MATERNAL BLOOD PRESSURE DURING THE FIRST TRIMESTER IN PREGNANCIES WITH PREGESTATIONAL DIABETES MELLITUS INCLUDING ONE FETAL DEATH AT 34 WEEKS



ABSTRACT

Objective

To assess trends in levels of biochemical markers, uterine artery pulsatility index (UtA-PI) and maternal mean arterial pressure (MAP) in the first trimester of pregnancy in women with pregestational diabetes mellitus (PGDM).

Methods

The study population comprised of a low-risk control group (n = 85) and a PGDM group (n= 16). During four examinations at 6 – 7, 8 – 9, 10 – 11 and 12 – 13 weeks' gestation maternal serum concentrations of Pregnancy-Associated Plasma Protein-A (PAPP-A), free β-human Chorionic Gonadotropin (fβ-hCG), Placental Growth Factor (PlGF) and A Disintegrin And Metalloprotease 12 (ADAM12), maternal mean arterial pressure and Doppler flow velocity waveform patterns of both UtAs were measured. Summary curves were created to describe the course of the markers over time. The data were analysed with a linear mixed model with the log-transformed marker values as dependant variables. The courses of the makers were compared between the groups.

Results

Median birth weight in the PGDM group was the 92nd centile. After application of adjustments (maternal age, BMI, smoking and partity) and *post hoc* Bonferroni correction there were no differences in first trimester markers between control and PGDM group. In one case of intrauterine fetal death (IUFD, birth weight 58th centile) both PAPP-A and ADAM12 were very low combined with high $Hba1_c$.

Conclusion

In PGDM pregnancies resulting in the birth of infants > 50th centile, levels of the first trimester markers did not differ from those of priori low risk control pregnancies. Low values of PAPP-A and ADAM12 in a case of IUFD may be indicative of poor placentation in early pregnancy followed by fetal overgrowth later in gestation.

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Submitted

INTRODUCTION

Recently we have shown that in women with type-1/type-2 diabetes low concentrations of fetoplacental biomarkers in the first trimester of pregnancy are associated with normal birthweight at term, whereas normal concentrations are associated with fetal macrosomia.[1] Significantly lower first trimester values of Pregnancy-Associated Plasma Protein-A (PAPP-A) and A Disintegrin And Metalloprotease 12 (ADAM12) were found in women with diabetes who subsequently delivered an appropriate-for-gestational age (AGA) infant as compared to women in the control group who delivered an AGA infant. These data suggest that fetal birthweight in women with pregestational diabetes mellitus (PGDM) is partially determined by placental development during the first trimester of pregnancy. The presence of fetal overgrowth in these pregnancies may be explained by increased glucose levels later on in gestation in the majority of cases. Longitudinal ultrasound data on head-to-abdomen ratio have shown decreased ratios in both AGA and large-for-gestational age (LGA) fetuses of women with diabetes, indicating fetal overgrowth in both weight categories in the course of gestation.[2] Others have described an inverse association between first trimester PAPP-A and $HbA1_c$ in early pregnancy, indicating impaired placentation in case of poor glycemic control.[3] Likewise, from a large data set on women with type-1-diabetes from Northern England it was concluded that a high $HbA1_c$ in early pregnancy was associated with impaired fetal growth, whereas a high $HbA1_c$ in the third trimester was associated with fetal macrosomia.[4] However, explained variance of $HbA1_c$ was relatively low in both instances. Nevertheless all these data suggest that poor glycemic control in early pregnancy results one way or the other in relatively low values of first trimester biomarkers of placentation and impaired fetal growth at that time, whereas poor glucose control late in pregnancy results in fetal overgrowth.

Earlier we have published longitudinal first trimester data on the course of fetoplacental biomarkers in normal pregnancies.[5] In the present study we measured these biomarkers longitudinally in 16 women with (PGDM), to investigate their time course and intra-individual consistency. Moreover we included Doppler measurements of the uterine artery and maternal mean arterial blood pressure (MAP).

METHODS

Study design

The study population in this longitudinal observational study comprised two groups: 1) women with a priori low risk single pregnancy with an uneventful course and delivery of a healthy infant at term (control group, n= 85) and 2) pregnant women with PGDM (n = 16). Data on the control group, albeit in a slightly smaller population, have been published earlier.[5]

Estimation of gestational age (GA) was based on the first day of the last menstrual period (LMP) or on ultrasound measurement of the crown-rump length (CRL) in case of an irregular cycle. During out-patient visits at 6 – 7, 8 – 9, 10 – 11 and 12 – 13 weeks' gestation we recorded maternal characteristics and measured blood pressure with a manual device (Maxi Stabil, Welch Allyn, Benelux). Blood samples were obtained in all women by venapuncture and all women had an ultrasound scan to measure the crown-

rump length (CRL) and Doppler flow velocity waveform patterns (i.e. pulsatility indices [PIs]) of both UtAs. Data on pregnancy outcomes were collected through self-reporting of the participating women using a questionnaire and were verified through their medical records or by telephone call. Written informed consent was obtained from the women agreeing to participate in the study, which was approved by the Scientific Ethics Committee of the University Medical Center, Utrecht, the Netherlands (protocol nr 07-222).

Mean arterial pressure was calculated from the formula *DP + 1/3 (SP – DP)*, where DP represents diastolic blood pressure and SP - systolic blood pressure.

Growth charts corrected for gestational age, sex and parity according to the Dutch Perinatal Registry were used to calculate birthweight Z-scores (www.perinatreg.nl).[6] Weight for gestational age at the 50th centile was used as the mean of the population and the average standard deviation (SD) was calculated by the formula *(-1 SD + 1 SD)/2*. Subsequently, the Z-score was converted into an exact centile for each studied subject.

The intraclass correlation coefficient (ICC) is used to quantify the degree of similarity between two or more repeatedly measured values on continuous scale. It can be interpreted as follows: 0-0.2 indicates *poor* agreement: 0.3-0.4 indicates *fair* agreement; 0.5-0.6 indicates moderate agreement; 0.7-0.8 indicates *strong* agreement; and >0.8 indicates *very strong* agreement.

Sample analysis

Blood samples were centrifuged and serum was aliquoted before storage at -80ºC. Serum concentrations of PAPP-A, fβ–hCG, ADAM12 and PlGF were measured in thawed specimens using a time-resolved fluorescence assay (autoDELFIA, PerkinElmer, Turku, Finland). Prior to analysis extensive validation was performed for all assays. The results of this validation have been published elsewhere.[5]

Statistical analysis

The single comparisons between control and PGDM group was made by chi-square test for categorical variables and Mann Whitney-U test for continuous variables.

Analyses were performed to assess the changes of the first trimester markers over time and to further compare these between our two study groups. Summary curves were created to describe the longitudinal course of the serum markers as well the measurements of UtA-PI and maternal mean arterial pressure. A linear mixed model was used for analysis with the log-transformed marker values as dependent variable. The average pattern of the log(marker) values as a function of gestational age in days was represented by a restricted cubic spline function with four knots (at the 5th, 35th, 65th and 95th percentile values of the marker). The between patient variation around the average pattern was modelled through a random effect on the intercept. The difference between the groups was estimated as a fixed effect. Since this difference is assessed on the log-scale, it was back transformed into a Fold Change (FC) reflecting the relative (ratio) difference between levels of the markers in control and PGDM group. Where applicable, these analyses were corrected for maternal age, BMI, smoke status and parity as potential confounders. The critical statistical significance was set at p-value < 0.05. Bonferroni correction was applied, to counteract the problem of multiple

comparisons on 7 different outcomes. Therefore the critical statistical significance in the case of multiple comparisons was set at $p < 0.007$. Statistical analyses were performed using SPSS (release 20.0; Chicago, IL) and R-program (www.r-project.org).

RESULTS

The control group consisted of 85 women. The PGDM group consisted of 16 women. Fourteen PGDM women had an uncomplicated pregnancy; however six of them delivered between 34 and 36 weeks of gestation. Two pregnancies in this group were complicated: a) one woman developed pregnancy induced hypertension and delivered spontaneously at 34 weeks, b) one woman experienced an unexpected IUFD at 34 $^{4/7}$ weeks GA. She delivered a stillborn boy, weighing 2435 gr (58th centile). The placenta showed a delayed maturation and weighed 271 gr, which is far below the 10th centile. Her diabetes was poorly controlled in early pregnancy with a $HbA1_c$ of 9.8% (84 mmol/l) and was subsequently treated with continuous subcutaneous insulin infusion.

The characteristics of the two study groups are shown in Table 1. Detailed characteristics of the PGDM group are shown in Figure 1. This group, compared to the control group had a higher BMI (26.9 vs. 23.7; $p = 0.014$) contained more smokers (18.8% vs. 3.5%; $p = 0.019$), delivered significantly earlier (257 days vs. 280 days; $p < 0.0001$) and their neonates had higher birthweight centiles (91.7 vs. 58.0; $p = 0.001$). Macrosomia was significantly more frequent in the PGDM group (56.3% vs. 14.1%; $p < 0.0001$). The median $HbA1_c$ in PGDM group was 6.9% (5.6 – 7.2) / 51.5 mmol/l (37.8 – 54.6). All live born PGDM infants had an uneventful neonatal course.

Table 1. Study population baseline characteristics in uncomplicated pregnancies and PGDM pregnancies. Values are presented as median (IQR) or number (%).

Characteristics	Controls n = 85	Diabetes n = 16
Maternal age (y)	33 (20-43)	31 (23-40)
Maternal weight (kg)	69.6 (50.0-120.0)	74.3 (56.0-120)
Maternal BMI (kg/m2)	23.7 (18.7-43.0)	26.9 (21.0-45.7)*
Nulliparity	36 (42.3)	4 (25.0)
Smoking	3 (3.5)	3 (18.8)*
Gestation at sampling (days)	68 (40-100)	71 (42-99)
Gestation at birth (days)	280 (260-297)	257 (238-275)*
Birthweight (gr)	3588 (2740-4900)	3510 (2350-4125)
Birthweight centile	58 (7-100)	91.7 (57.9-99.7)*
Macrosomia at birth	12 (14.1)	9 (56.3)*
Male : female ratio	48 : 37	11 : 5

A Pearson's chi square test and Mann-Whitney U test, were used for statistical analysis. Significance value $p < 0.05$ (*).; IQR: interquartile range; BMI: body mass index.

Subsequently we compared the distribution of the first trimester markers (PAPP-A, free β-hCG, ADAM12, PlGF, CRL, Lowest UtA-PI, MAP) between the two groups. A linear mixed model analysis of six markers showed no statistically significantly difference in the marker levels between the two groups (Table 2). Distinction between macrosomic and non-macrosomic infants in control group and PGDM cases did not show differences in the first trimester markers between any of the outcome subgroups neither (data not shown).

We also did not find a correlation between first trimester $HbA1_c$ levels and any markers or birthweight centile except for MAP (Table 3).

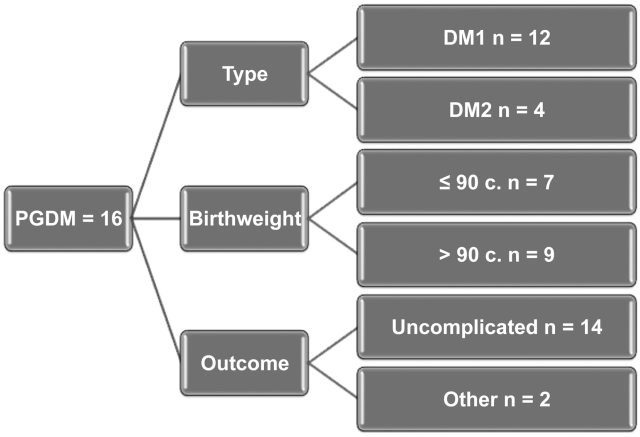

Figure 1. PGDM group characteristics and distributions in the group depending on the variables: type diabetes; birthweight; outcome of the pregnancies, respectively. Other outcomes: one woman developed PIH and there was one intrauterine fetal death.

Table 2. Linear mixed model comparing control group (n = 85) with PGDM group (n = 16). Markers are corrected for age, BMI, smoke and parity.

	FC	p-value
PAPP-A	0.88	0.44
Free β-hCG	0.96	0.81
ADAM12	0.88	0.23
PlGF	0.99	0.98
CRL	0.96	0.28
Lowest Ut-A-PI	0.86	0.03
MAP	1.07	0.0077

PAPP-A: Pregnancy-Associated Plasma Protein-A; Free β-hCG: free β-human Chorionic Gonadotropin; ADAM12: A Disintegrin And Metalloprotease 12; PlGF: Placental Growth factor; CRL: crown-rump length; Lowest Ut-A-PI: lowest uterine artery pulsatility index; MAP: mean arterial pressure; FC: fold change. Significance value $p < 0.007$ (*) because of multiple testing.

Both in de control group and in the PGDM group there was a moderate to very strong intraclass correlation coefficient (ICC) with time in PAPP-A, free β-hCG, ADAM12 and PlGF (Table 4; Figure 3 PAPP-A and ADAM12 in PGDM pregnancies, IUFD in black squares). This indicates that measurements within one study subject were strongly related to each other.

Data on the biochemical markers of the IUFD pregnancy are shown in Figure 2 (black squares). Levels of PAPP-A and ADAM 12 were very low compared to other group peers especially from 8-9 weeks onwards (Table 5). Individual longitudinal values of PAPP-A and ADAM12 are shown in Figure 3.

Table 3. Correlations between $HbA1_c$ and first trimester markers in PGDM group.

Marker	Correlation with $HbA1_c$ (rho)	p-value
PAPP-A	-0.165	0.193
Free β-hCG	-0.152	0.231
ADAM12	-0.235	0.062
PlGF	0.104	0.412
CRL	-0.067	0.605
Lowest Ut-A-PI	-0.067	0.607
MAP	-0.403	0.001*

PAPP-A: Pregnancy-Associated Plasma Protein-A; Free β-hCG: free β-human Chorionic Gonadotropin; ADAM12: A Disintegrin And Metalloprotease 12; PlGF: Placental Growth Factor; CRL: crown-rump length; Lowest Ut-A-PI: lowest uterine artery pulsatility index; MAP: mean arterial pressure; Significance value $p < 0.007$ (*) because of multiple testing.

Table 4. ICC for first trimester markers in control and PGDM group.

	Intraclass correlation coefficient (ICC)	
Marker	Controls n = 85	PGDM n = 16
PAPP-A	0.71	0.81
Free β-hCG	0.66	0.78
ADAM12	0.52	0.58
PlGF	0.58	0.73
CRL	0.30	0.13
Lowest Ut-A-PI	0.32	0.20
MAP	0.57	0.47

PAPP-A: Pregnancy-Associated Plasma Protein-A; Free β-hCG: free β-human Chorionic Gonadotropin; ADAM12: A Disintegrin And Metalloprotease 12; PlGF: Placental Growth Factor; CRL: crown-rump length; Lowest Ut-A-PI: lowest uterine artery pulsatility index; MAP: mean arterial pressure; ICC: 0-0.2 *poor* agreement: 0.3-0.4 *fair* agreement; 0.5-0.6 moderate agreement; 0.7-0.8 *strong* agreement; >0.8 *very strong* agreement.

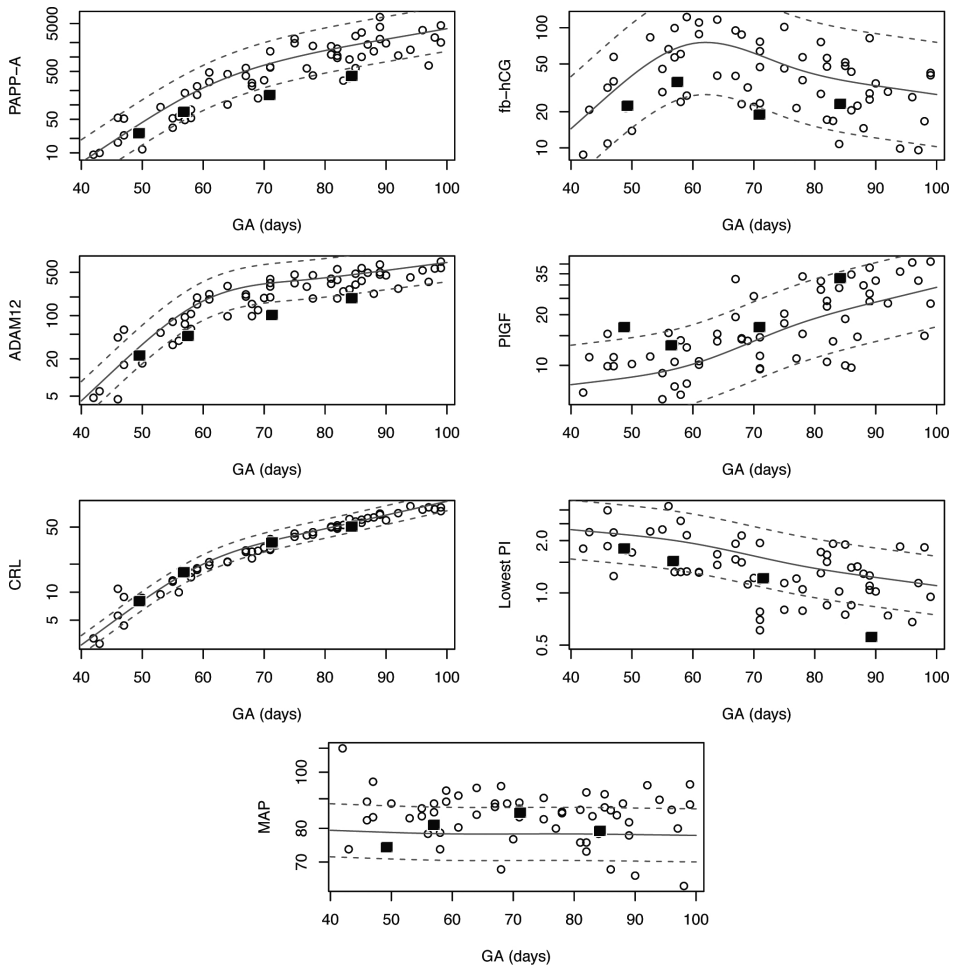

Figure 2. The distribution of the first trimester markers in the 16 PGDM pregnancies. The reference lines are based on the values from the control group. The IUFD case is indicated with black squares.

DISCUSSION

In this first longitudinal study on biomarkers in PGDM women in early pregnancy, we found no differences in PAPP-A and ADAM12 values as compared to controls. This contradicts earlier findings by us and others.[1,3] This may at least partly be explained by the small population studied and by the high average birth weight in this diabetes population (median centile 91.7, lowest centile 58 [IUFD]). In an earlier study we showed that first trimester biomarkers in PGDM mothers of LGA infants were within the normal range.[1] Women in our current study population were on average well controlled (median first

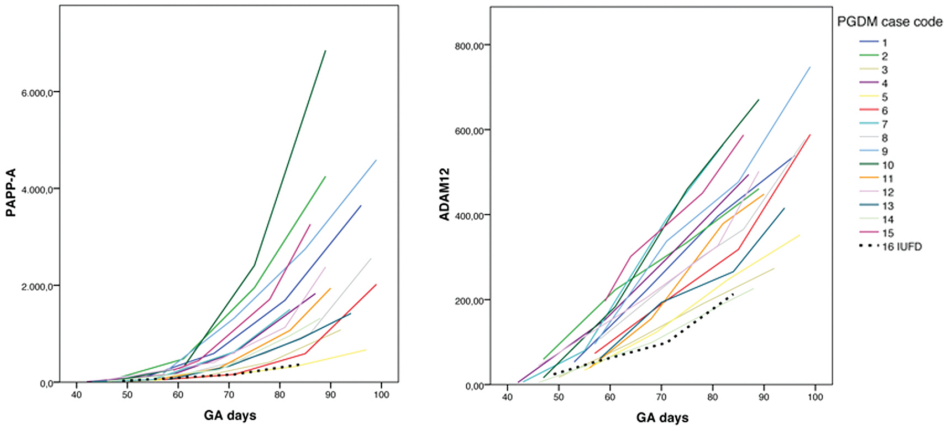

Figure 3. Longitudinal values of PAPP-A and ADAM12 in PGDM group. PAPP-A: Pregnancy-Associated Plasma Protein-A; ADAM12: A Disintegrin And Metalloprotease 12; IUFD: intrauterine fetal death.

Table 5. PAPP-A and ADAM 12 values in the case of IUFD as compared to controls and the other PGDM cases.

	Control median (IQR)	PGDM median (IQR)	IUFD
Visit 1			
PAPP-A	25.8 (13.3 - 41.2)	50.4 (18.2 – 74.4)	28.2
ADAM12	19.2 (9.0 – 40.6)	41.9 (16.3 – 70.3)	24.2
Visit 2			
PAPP-A	281.0 (154.5 – 491.5)	295.5 (143.5 – 469.8)	73.2
ADAM12	203.9 (128.8 – 269.8)	188.3 (104.6 – 222.5)	53.0
Visit 3			
PAPP-A	986.0 (615.0 – 1630.0)	985.5 (593.3 – 1705.0)	160.0
ADAM12	374.3 (286.1 – 470.0)	330.8 (251.9 – 394.9)	98.0
Visit 4			
PAPP-A	2270.0 (1310.0 – 3630.0)	1980.0 (1345.0 – 3552.5)	365.0
ADAM12	524.2 (424.7 – 649.7)	498.3 (368.0 – 585.0)	212.0

trimester $HbA1_c$ 6.9%) and therefore we may expect better placentation with similar levels of first trimester serum markers to those of controls and larger infants.

Interestingly the case of IUFD had a notable combination of very high $HbA1_c$ and very low PAPP-A and ADAM12 values in early pregnancy. This clearly suggests impaired placentation in association with poor glycemic control in early pregnancy. A birth weight at the 58th centile together with evidence of poor placentation in early pregnancy may well indicate fetal

overgrowth in the second half of gestation resulting in fetal demise. The fetal/placental weight ratio was 8.8, whereas this should have been around 1:5-6 at this GA (personal communication P.G.J. Nikkels, MD, PhD). Intrauterine death in pregnancies complicated by type1/type-2 diabetes still occurs in 1-3 % of cases and usually in between 34 and 39 weeks.[7-9] There is no evidence that such an IUFD predominantly occurs in macrosomic fetuses although data are limited. The early biomarkers in this case may in retrospect explain the pathophysiological origin of IUFD and may also explain similar cases in other studies. Measurement of PAPP-A and ADAM12 in the first trimester may help to identify cases at risk for later IUFD, although it has to be realized that our assumption is only based on one case. However, our earlier findings of a relationship between these biomarkers and birthweight (centile) do strengthen our assumption.

The discrepancy between PAPP-A and ADAM12 on the one hand and PlGF on the other hand is noteworthy, both in the total PGDM population as in the case of IUFD. Also earlier we found that only the former two were significantly lower in a PGDM population. Low PAPP-A levels in women with type-1 diabetes have also been found by others.[3,10,11] PAPP-A and ADAM12 are primarily synthesized by the placenta and are components of the insulin-like growth factor (IGF)–axis.[12-14]. The IGF-axis regulates cell proliferation and differentiation.[15] The bioavailability of IGF-axis is strongly related to a degree of glycemic control and is known to be reduced in diabetes.[16-19] PAPP-A and $HbA1_c$ have been found by others to be negatively related.[3] Therefore, lower values of these markers seem logical in case of non-optimally regulated PGDMs. In the present study we could not confirm this association, maybe related to the small study population and on average good and better glycemic control of the PGDM women than in the study by Matsen et al.[3]

PlGF is not part of the IGF-axis, but is also a determinant of placental growth and development. The current data suggest at least in PGDM a different impact of this marker on placental development, but the pathophysiology remains to be elucidated.[20]

Although not significantly different, MAP appeared relatively higher in PGDM pregnancies (FC 1.07; Figure and Table 2). The vascular changes in PGDM women may contribute to the explanation of this phenomenon. The correlation between $HbA1_c$ values and MAP and personal characteristics of this group (higher BMI and frequenter smoking status) may confirm this hypothesis.

The moderate to very strong ICC of the first trimester serum markers confirms the individual consistency of the marker levels throughout the first trimester. The measurements within one study subject appeared strongly related to each other. This may be helpful in case only one first trimester measurement is available.

The longitudinal approach, which we used here, seems superior to the measurements based on large numbers of cross-sectional and unrelated measurements since repetitive measurements of the same study subjects provides greater accuracy. The benefit of a longitudinal study is the possibility to detect developments or changes in the measurements of the target population at both the group and the individual level. However, such studies are difficult to conduct and inclusion and continued participation proved to be difficult.

Concluding, the course of fetoplacental biomarkers in PGDM pregnancies did not differ from that in a low risk control group. Notably, levels of PAPP-A and ADAM12 were extremely low in a case of IUFD.

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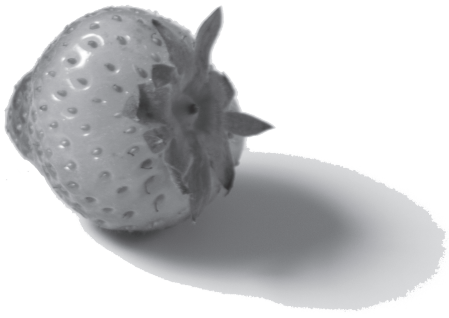
LONGITUDINAL TRENDS IN FETOPLACENTAL SERUM MARKERS, UTERINE ARTERY PULSATILITY INDEX AND MATERNAL BLOOD PRESSURE DURING THE FIRST TRIMESTER IN PREGNANCIES FOLLOWING IVF/ICSI



ABSTRACT

Objective

Earlier studies have shown altered first trimester placental markers for Down syndrome screening in pregnancies following in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). It was the aim of this study to investigate whether these markers and markers for risk assessment of early onset preeclampsia are different from controls in IVF/ICSI pregnancies conceived following mild ovarian stimulation.

Methods

The study population comprised of 85 women who had a naturally conceived uncomplicated singleton pregnancy and 42 women with pregnancies following IVF/ICSI. At 6 – 7, 8 – 9, 10 – 11 and 12 – 13 weeks of gestation, maternal serum concentrations of Pregnancy-Associated Plasma Protein-A (PAPP-A), free β-human Chorionic Gonadotropin (fβ-hCG), Placental Growth Factor (PlGF) and A Disintegrin And Metalloprotease 12 (ADAM12), maternal blood pressure and Doppler flow velocity waveform patterns of both uterine arteries (UtAs) were measured. Summary curves were created to describe the course of the markers over time. Data were analysed using a linear mixed model with the log-transformed marker values as dependent variables. The courses of the markers were compared between the groups.

Results

There were no differences in any of the first trimester marker levels between naturally conceived pregnancies and pregnancies following IVF or ICSI. GnRH downregulation method, fresh embyo transfer in a stimulated cycle versus cryo embryo transfer in a natural cycle did not influence the levels of these markers either.

Conclusion

In IVF/ICSI pregnancies conceived following mild ovarian stimulation levels of the first trimester markers for fetal chromosomal anomalies or for risk assessment for early onset preeclampsia or fetal growth restriction do not differ from controls.

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Submitted

INTRODUCTION

First trimester screening for fetal chromosomal abnormalities like Down syndrome, trisomy 13 and 18 is based on maternal serum levels of Pregnancy-Associated Plasma Protein-A (PAPP-A) and the free β-human Chorionic Gonadotropin (β-hCG) and on maternal age and fetal nuchal translucency thickness (NT). Recently, several studies have indicated that other first trimester serum markers such as Placental Growth Factor (PlGF) and A Disintegrin And Metalloprotease 12 (ADAM12), in combination with the uterine artery pulsatility index (UtA-PI) and first trimester maternal blood pressure, are potent predictors of (early onset) preeclampsia (PE) and fetal growth restriction.[1-3] Regarding Down syndrome screening several studies have reported that first trimester biomarkers are different in pregnancies following IVF/ICSI although data are conflicting.[4] Altered first trimester markers may indicate altered early placentation and embryonic development. The differences mainly concerned PAPP-A levels, which were either lower in both IVF/ICSI pregnancies, lower in ICSI pregnancies only or lower in fresh embryo transfer in a cycle accompanied by hormone treatment and not in a natural cycle.[4-9] fβ-hCG has been found to be either decreased or increased.[4,10-14] Specific group characteristics such as maternal age, cause of subfertility and different methods of hormonal ovarian stimulation have been suggested as factors that may affect the levels of screening markers in these pregnancies. Altered levels of the first trimester markers may lead to inaccurate estimation of the risks for chromosomal abnormalities and consequently to unnecessary invasive procedures such as amniocentesis. Accordingly, in several countries correcting factors for IVF/ICSI pregnancies have been introduced when assessing the risk for Down syndrome.[4] To the best of our knowledge, first trimester data on biomarkers predicting PE in women following IVF/ICSI are lacking.

It was therefore the purpose of this study to assess trends in levels of serum markers, uterine artery pulsatility index (UtA-PI) and maternal mean arterial pressure (MAP) over time in the first trimester of pregnancies following current mild ovarian stimulation techniques as compared to controls. Such data is important both for Down syndrome screening and for risk assessment of preeclampsia.

METHODS

Study design

The population in this longitudinal observational follow-up study comprised of two groups: 1) women with a naturally conceived low risk singleton pregnancy, and 2) subfertile women who became pregnant following IVF/ICSI, also only singleton pregnancy. Data for this study was collected between 2009 and 2012 at the University Medical Centre in Utrecht, the Netherlands. Results of the study regarding low risk women have been published before, albeit that the present data set was somewhat larger.[15]

Estimation of gestational age (GA) in the first group was based on the first day of the last menstrual period (LMP) or on ultrasound measurement of the crown-rump length (CRL) in case of an irregular cycle. In the IVF/ICSI group the day of follicle aspiration plus two weeks was used. Data were obtained at outpatients' visits at 6 – 7, 8 – 9, 10 – 11 and 12 – 13 weeks' gestation. The workup of the visits was described in detail in a previous publication on first

trimester biomarkers in low risk pregnancies.[15] Written informed consent was obtained from the women agreeing to participate in the study, which was approved by the Scientific Ethics Committee of the University Medical Centre, Utrecht, the Netherlands (protocol nr 07-222).

Mean arterial pressure was calculated from the formula *DP + 1/3 (SP – DP)*, where DP represents diastolic blood pressure and SP - systolic blood pressure.

Growth charts corrected for gestational age, sex and parity according to the Dutch Perinatal Registry were used to calculate the birthweight Z-scores (http://www.perinatreg.nl;[16]). Weight for gestational age at the 50th centile was used as the mean of the population and the average standard deviation (SD) was calculated by the formula *(-1 SD + 1 SD)/2*. Subsequently, the Z-score was converted into an exact centile for each studied subject.

The reference values of the control group were based on 85 uncomplicated pregnancies in which a healthy infant without congenital malformations was born after 37 weeks of gestation. The course of these pregnancies had been uneventful, i.e. without occurrence of pregnancy induced hypertension, gestational diabetes etc.[15] For this study we included all birthweight centiles, i.e also pregnancies ending in the birth of a large- or small-for-gestational age infant.

The pregnancies following IVF/ICSI all came from our department of Reproductive Medicine. Patients were asked to participate in this study when they attended the obstetrical outpatient clinic for the first time. In all cases the local fertility work-up protocol of the University Medical Centre Utrecht (UMCU) had been followed. Details of this population are given in Figure 1. Patients underwent IVF or ICSI depending on the cause of their subfertility, according to local standards. In both cases the ovarian stimulation protocol was the same: recombinant *follicle-stimulating hormone* (rec-FSH*) in dosage of 150 IU once a day (with a maximum of 300 IU once a day)* combined with a gonadotropin-releasing hormone (GnRH) agonist protocol (Leuprolide or Triptorelin 100 microgram) or a GnRH antagonist protocol (Ganirelix/Cetrorelix 0.25 mg/day) was used. Final oocyte maturation was achieved by the administration of 10,000 IU *human Chorionic Gonadotropin* (hCG) when 3 or more follicles of more than 16 mm were present. Oocyte retrieval was carried out 36 hours after hCG administration. In case of fresh embryo transfer luteal phase supplementation consisted of 600 mg natural micronised progesterone vaginally, in three separate dosages (Utrogestan®/ Progestan® 100 mg 3x2/day) starting in the evening after oocyte retrieval and continued until 18 days after ovum pick-up. In case of previous cryopreservation (cryo embryos), embryos were transferred in a natural cycle, which consisted of a 5000 IU hCG trigger when 1 follicle of ≥ 16 mm and an endometrial thickness ≥ 7 mm were present on ultrasound.

Primarily we studied the presence of possible differences within the IVF/ICSI group, since the laboratory procedure (IVF versus ICSI), method of downregulation (GnRH agonist versus GnRH antagonist) or embryo transfer (fresh versus cryo) may influence the levels of the first trimester markers.

Secondly we compared IVF/ICSI pregnancies with the control group to study whether there are any significant differences in the first trimester markers between these groups.

Sample analysis

Blood samples were centrifuged and serum was aliquoted before storage at -80°C. Serum concentrations of PAPP-A, fβ–hCG, ADAM12 and PlGF were measured in thawed

specimens using a time-resolved fluorescence assay (autoDELFIA, PerkinElmer, Turku, Finland). Prior to analysis extensive validation was performed for all assays. The results of this validation have been published elsewhere.[15]

Statistical analysis

The single comparisons between control and IVF/ICSI groups were made by chi-square test for categorical variables and Mann Whitney-U test for continuous variables.

Analyses were performed to assess the changes of the first trimester markers over time and to further compare these between our two study groups. Summary curves were created to describe the longitudinal course of the serum markers as well the measurements of UtA-PI and maternal mean arterial pressure. A linear mixed model was used for analysis with the log-transformed marker values as dependent variable. The average pattern of the log(marker) values as a function of gestational age in days was represented by a restricted cubic spline function with four knots (at the 5^{th}, 35^{th}, 65^{th} and 95^{th} percentile values of the marker). The between patient variation around the average pattern was modelled through a random effect on the intercept. The difference between the groups was estimated as a fixed effect. Since this difference is assessed on the log-scale, it was back transformed into a Fold Change (FC) reflecting the relative (ratio) difference between levels of the markers in control and IVF/ICSI groups. Where applicable, these analyses were corrected for maternal age, BMI, smoke status and parity as potential confounders. The critical statistical significance was set at p-value <0.05. Bonferroni correction was applied, to counteract the problem of multiple comparisons on 7 different outcomes. Therefore the critical statistical significance in the case of multiple comparisons was set at $p < 0.007$. Statistical analyses were performed using SPSS (release 20.0; Chicago, IL) and R-program (www.r-project.org).

RESULTS

The control group consisted of 85 women. The IVF/ICSI group consisted of 42 women of whom 36 had an uncomplicated pregnancy. Six pregnancies were complicated by: a spontaneous preterm birth (n=2; at $36^{2/7}$ and $36^{6/7}$ weeks, respectively); pregnancy-induced hypertension requiring labor induction at term (n=2; at $39^{3/7}$ weeks and $40^{1/7}$ weeks, respectively); or development of gestational diabetes (n=2; both women delivered at term).

All infants were healthy without congenital malformations and had an uneventful neonatal course. The characteristics of both study groups are shown in *Table 1*. The detailed characteristics of IVF/ICSI group are shown in Figure 1.

Population and outcome data of the IVF/ICSI group only differed from the control group on parity (nulliparous: 66.7% vs. 42.3%, respectively; p = 0.001) and GA at birth (276 days vs. 281 days, respectively; p = 0.017). Women pregnant after IVF/ICSI had their first blood samples taken at the same time as the control group; median GA 69 and 68 days, respectively.

In the first step of our analyses the marker levels of the IVF group were compared to those in the ICSI subgroup. Both the first visit (6 –7 week GA; *Table 2a*) as well as the whole study period (6 – 13 week GA; *Table 3a*) were analysed. After application of *post*

Table 1. Study population baseline characteristics in uncomplicated pregnancies and IVF/ICSI pregnancies. Values are presented as median (IQR) or number (%).

Characteristics	Controls n = 85	IVF/ICSI n = 42	p-value
Maternal age (y)	33 (31-36)	35 (31-38)	0.138
Maternal weight (kg)	67.0 (63.0-75.0)	69.0 (64.0-80.0)	0.158
Maternal BMI (kg/m2)	23.0 (20.9-24.7)	24.2 (22.2-26.4)	0.051
Nulliparity	36 (42.3)	28 (66.7)	0.001*
Smoking	3 (3.5)	2 (4.8)	0.738
Gestation at sampling (days)	68 (54-83)	69 (56-82)	0.395
Gestation at birth (days)	281 (276-285)	276 (272-281)	0.017*
Birthweight (gr)	3520 (3230-3890)	3478 (3100-3770)	0.428
Birthweight centile	61 (38-83)	60 (44-85)	0.374
Male : female ratio	48 : 37	21 : 21	0.493

A Pearson's chi square test and Mann-Whitney U test, were used for statistical analysis. Significance value $p < 0.05$. IVF/ICSI: in vitro fertilisation/intracytoplasmic sperm injection; IQR: interquartile range; BMI: body mass index; (*): statistically significant at $p < 0.05$.

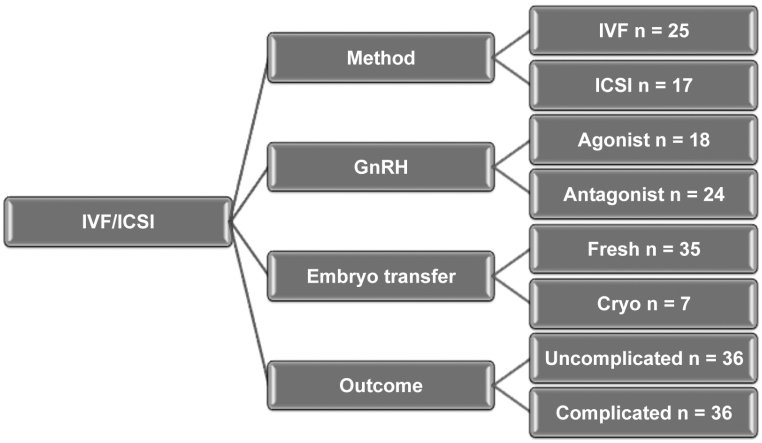

Figure 1. IVF/ICSI group characteristics and distributions in the group depending on the variables: method of fertilisation; method of GnRH downregulation; method of embryo transfer; outcome of the pregnancies respectively. Other outcomes: two preterm births, two cases with pregnancy-induced hypertension and two cases with gestational diabetes mellitus.

hoc Bonferroni correction for multiple comparisons there were no significant differences between the marker levels in the subgroups.

In the second step we compared the method of GnRH downregulation in IVF/ICSI group, since it has been found that LH and E2 concentrations in the early follicular phase

are higher in the antagonist regime as compared to the agonist regime.[17] We did not find any differences between these subgroups either, nor at 6-7 weeks (Table 2b), nor during the whole study period (Table 3b). The same held for a comparison between fresh and cryo embryo transfer (Table 2c and 3c).

Table 2. Concentration of the first trimester markers during the first visit (6 – 7 week GA) in the study population.
a) IVF and ICSI group

	Median (IQR) 1st visit		
	IVF n = 25	ICSI n = 17	p-values
PAPP-A	32.6 (17.1-61.3)	19.2 (11.7-44.1)	0.18
Free β-hCG	28.9 (20.8-55.3)	20.7 (14.1-47.1)	0.16
ADAM12	32.6 (16.5-55.2)	13.5 (8.2-39.9)	0.07
PlGF	9.4 (8.2-13.0)	9.4 (8.3-11.4)	0.79
CRL	9.6 (6.7-11.6)	6.7 (3.2-9.1)	0.02
Lowest Ut-A-PI	2.3 (1.7-2.9)	2.0 (1.7-2.2)	0.31
MAP	79.3 (76.2-82.2)	78.7 (74.5-81.7)	0.39

b) GnRH agonist and GnRH antagonist group

	Median (IQR) 1st visit		
	GnRH Agonist n = 18	GnRH Antagonist n = 24	p-values
PAPP-A	18.2 (11.2-34.4)	35.4 (22.3-69.4)	0.02
Free β-hCG	20.4 (13.8-45.6)	30.2 (21.1-56.6)	0.08
ADAM12	12.5 (7.3-23.6)	34.7 (24.0-70.1)	0.01
PlGF	8.8 (7.8-10.7)	8.6 (10.9-13.0)	0.10
CRL	6.2 (3.4-8.8)	9.6 (7.8-11.6)	0.02
Lowest Ut-A-PI	1.9 (1.6-2.1)	2.5 (1.8-3.0)	0.03
MAP	79.0 (75.8-85.3)	78.5 (74.4-81.8)	0.45

c) fresh and cryo embryos

	Median (IQR) 1st visit		
	Fresh n = 35	Cryo n = 7	p-values
PAPP-A	21.5 (13.8 – 45.0)	39.1 (33.4 – 73.5)	0.05
Free β-hCG	23.6 (18.0 – 52.4)	37.9 (27.6 – 52.8)	0.17
ADAM12	20.3 (9.0 – 12.0)	42.7 (26.6 – 73.6)	0.11
PlGF	9.3 (8.1 – 12.0)	9.8 (9.3 – 13.1)	0.45
CRL	8.4 (4.6 – 10.9)	8.7 (7.5 – 9.6)	0.82
Lowest Ut-A-PI	2.0 (1.7 – 2.6)	2.6 (1.8 – 2.9)	0.49
MAP	79.0 (75.7 – 82.3)	78.7 (74.0 – 82.0)	0.84

PAPP-A: Pregnancy-Associated Plasma Protein-A; Free β-hCG: free β-human Chorionic Gonadotropin; ADAM12: A Disintegrin And Metalloprotease 12; PlGF: Placental Growth Factor. IQR: interquartile range
Significance value $p < 0.007$ (*).

Table 3. Linear mixed regression analysis comparing the different study subgroups over the total study period from 6-13 weeks of gestation. Markers were adjusted for maternal age, BMI, smoking and parity.
a) IVF group (n = 25) vs. the ICSI group (n = 17)

	FC	p-value
PAPP-A	1.05	0.75
Free β-hCG	0.88	0.39
ADAM12	1.03	0.82
PlGF	0.94	0.45
CRL	0.96	0.12
Lowest Ut-A-PI	0.93	0.36
MAP	0.98	0.22

b) GnRH agonist group (n = 18) vs. GnRH antagonist group (n = 24)

	FC	p-value
PAPP-A	0.95	0.75
Free β-hCG	0.97	0.86
ADAM12	0.99	0.93
PlGF	0.93	0.37
CRL	0.97	0.27
Lowest Ut-A-PI	0.84	0.02
MAP	0.99	0.68

c) fresh embryo group (n = 35) vs. cryo embryo group (n = 7)

	FC	p-value
PAPP-A	1.03	0.90
Free β-hCG	1.15	0.45
ADAM12	0.97	0.81
PlGF	1.13	0.39
CRL	1.03	0.31
Lowest Ut-A-PI	0.83	0.16
MAP	0.99	0.64

PAPP-A: Pregnancy-Associated Plasma Protein-A; Free β-hCG: free β-human Chorionic Gonadotropin; ADAM12: A Disintegrin And Metalloprotease 12; PlGF: Placental Growth Factor; CRL: crown-rump length; Lowest Ut-A-PI: lowest uterine artery pulsatility index; MAP: mean arterial pressure; FC: fold change. Significance value $p < 0.007$ (*).

Since there were no differences between the IVF and ICSI group we compared the combined IVF/ICSI group to the controls (Figure 2, Table 4a and 4b). There were no significant differences between IVF/ICSI and control group (Table 4a). The 6 (mild) pregnancy complications did not influence the outcome either (Table 4b).

Table 4. Linear mixed regression analysis comparing the control group with the IVF/ICSI group (n = 42) over the total study period from 6-13 weeks of gestation. Markers were adjusted for maternal age, BMI, smoking and parity.
a) control group (n = 85) vs. IVF/ICSI group (n = 42)

	FC	p-value
PAPP-A	1.00	0.97
Free β-hCG	0.88	0.88
ADAM12	0.99	0.85
PlGF	1.11	0.05
CRL	1.11	0.62
Lowest Ut-A-PI	1.02	0.73
MAP	0.99	0.82

b) control group (n = 85) vs. IVF/ICSI group (uncomplicated outcomes only) (n = 36)

	FC	p-value
PAPP-A	0.97	0.82
Free β-hCG	0.99	0.94
ADAM12	0.95	0.52
PlGF	1.07	0.23
CRL	1.02	0.50
Lowest Ut-A-PI	1.01	0.90
MAP	0.99	0.57

PAPP-A: Pregnancy-Associated Plasma Protein-A; Free β-hCG: free β-human Chorionic Gonadotropin; ADAM12: A Disintegrin And Metalloprotease 12; PlGF: Placental Growth Factor; CRL: crown-rump length; Lowest Ut-A-PI: lowest uterine artery pulsatility index; MAP: mean arterial pressure; FC: fold change. Significance value $p < 0.007$ (*).

DISCUSSION

In this study we longitudinally explored trends of first trimester classical and new markers in pregnancies following IVF/ICSI, starting from 6 weeks of gestation onwards. To our knowledge this is a novelty since longitudinal measurements of classical or new markers in IVF/ICSI pregnancies are lacking. The longitudinal trends in serum markers, CRL, UtA-PI and MAP were described using a mixed model incorporating the relationships between repetitive measurements of the same study subject.

Before we compared the IVF/ICSI group as a homogenous group to the controls we undertook a number of detailed steps to study whether any differences in the first trimester markers within the IVF/ICSI group existed with respect to the laboratory procedure (IVF versus ICSI), method of downregulation (GnRH agonist versus GnRH antagonist) or embryo transfer (fresh versus cryo) used. Practically, only during the first visit (week 6 and 7 of GA) differences in some first trimester markers in different subgroups were present. However, after a post hoc Bonferroni correction for the multiple comparisons these differences

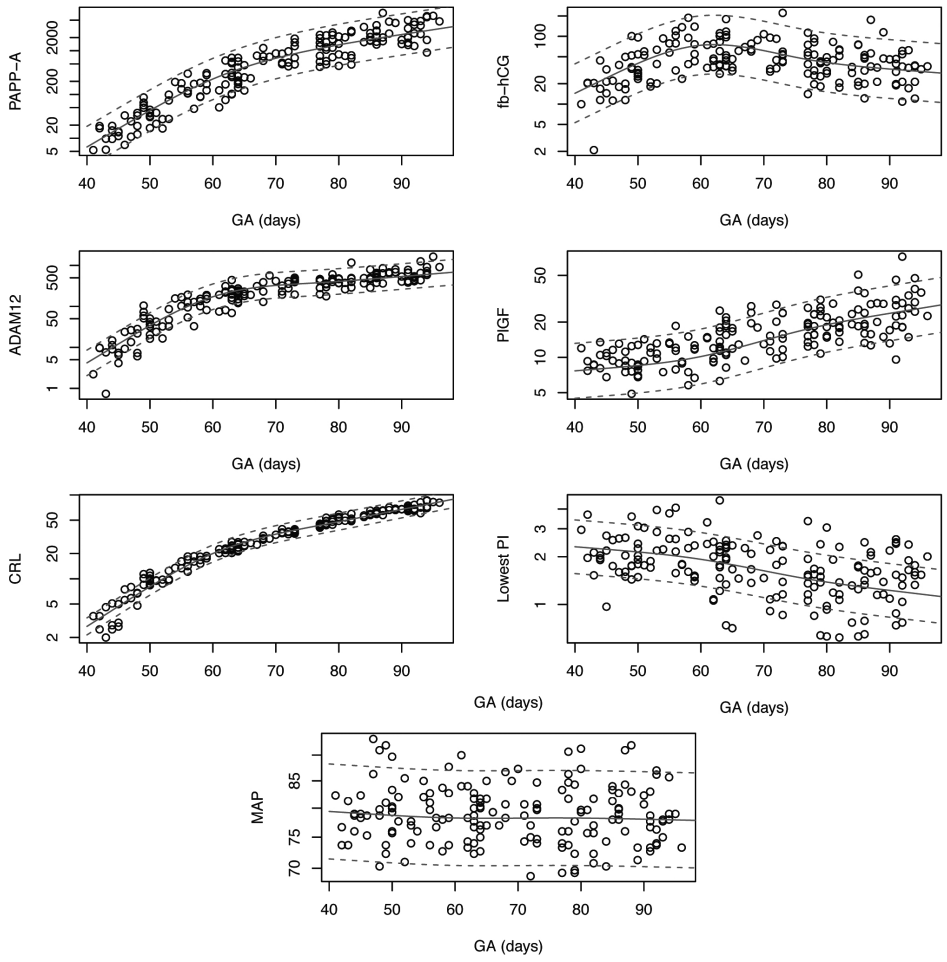

Figure 2. The distribution of the first trimester markers in the 42 IVF/ICSI pregnancies. The reference lines are based on the values from the control group (5th, 50th and 95th centile, respectively).

disappeared and may therefore be considered as non-existing. Therefore comparison between the complete IVF/ICSI and control groups seemed legitimate and trustworthy.

There were no differences in the markers of early fetal and placental development, nor of UtA-PI and MAP between IVF/ICSI and spontaneous pregnancies in the course of the study. These findings are contradictory to earlier studies, where overall first trimester biomarkers levels in IVF/ICSI pregnancies were significantly different compared to spontaneous pregnancies.[4] In some studies lower levels of first trimester markers were only found after fresh embryo transfer, in others only after ICSI- fresh embryo transfer but not following IVF.[4] Here we cannot confirm these findings.

Lower levels of PAPP-A and higher levels of β-hCG are related to higher risk for Down syndrome. The finding that IVF/ICSI may affect these levels has led to recommendations to use a correction factor in the pregnancies to avoid unnecessary concern and inappropriate invasive diagnostic tests like amniocentesis or chorion villus sampling. Our data indicate that in case of IVF/ICSI pregnancies corrections are unnecessary. Markers for early PE and fetal growth restriction, including Doppler waveform patterns of the uterine artery, did not differ either. So, also for such a screening no correction of values seems necessary.

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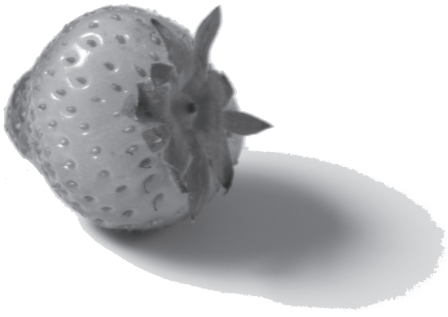
PART 4
FUTURE PROSPECTIVES OF CLINICAL VALIDATION – PHASE 5

XI

SUMMARY AND GENERAL DISCUSSION



Currently, in the Netherlands there is no routine screening program for preeclampsia syndrome (PE) or related pathologies. PE is a serious disorder that occurs only during pregnancy and the post partum period.[1] It is a rapidly progressive condition characterized by high blood pressure and the presence of protein in the urine. PE affects 1-3% of pregnant women and is a leading cause of maternal and perinatal morbidity and mortality worldwide, particularly when it occurs before 34 weeks of gestation (GA).[2-5] Most severe maternal complications include hemolysis, elevated liver enzymes and low platelets (HELLP) - syndrome, renal failure, liver hemorrhage and rupture, eclampsia, cerebral hemorrhage and maternal death. In addition, PE is associated with substantial risks of perinatal morbidity and mortality due to concomitant intrauterine growth restriction (IUGR), iatrogenic prematurity, placental abruption and stillbirth.[3,6] It is also associated with substantial health problems later in life. Both women who developed PE and their children have substantially elevated risks of chronic hypertension, cardiovascular disease and diabetes mellitus type 2.[7,8]

Worldwide PE contributes to about 50.000-76.000 maternal and 500.000 infant deaths per year and approximately $7 billion in total costs (Preeclampsia Foundation 2009). In the Netherlands, annually about 2.000 women develop PE and of the 20 maternal deaths per year PE is the cause in about half of them.[9,10]

In the Netherlands the incidence of maternal mortality due to PE is greater than in other West European countries.[10,11] Analysis of all PE-related maternal deaths reported to the Dutch maternal mortality Committee (MMC) between 2000 and 2004 revealed that in 96% (26/27) of the cases, delay of the diagnosis and substandard care were the major cause.[9] Although in this study the focus was put on the PE-related maternal deaths, the clinical practice strongly suggests that, in case of surviving patients (again, about 2.000 per year), the timing of recognition, referral to a specialized centre and start of treatment is correlated to the severity of the complications.

It is therefore of major importance, to identify patients at high risk already at the beginning of the pregnancy through an adequate screening test, thereby detecting the disease ahead of its clinical onset, enabling suitable pregnancy care, and ensuring better pregnancy outcomes for both mother and child. Such a screening test, incorporating maternal risk factors, performed at the first antenatal visit, would define a pregnancy-specific risk.

While close monitoring of high risk pregnancies itself may allow for substantial health gain in terms of prevented suffering and costs, additional therapeutic interventions may be available in the near future to add to this. There is strong evidence from recent meta-analysis studies that timely (i.e. early) prediction of PE and low-dose aspirin treatment may substantially reduce the incidence of PE and IUGR.[12,13] Low dose aspirin treatment started before the 16th week of gestation in a priori high risk women was associated with up to 50% reduction of PE.[13] In the case of severe early onset PE (EO-PE), the health benefit was even higher and reached up to 90%.[13] Aspirin prophylaxis started after 18-20 week, only reduced the risk of EO-PE with about 10%, which supports/underlines the need of early screening and risk assessment for PE.[13]

The necessity to develop PE screening biomarkers prompted this thesis. This thesis captures four out of five phases of biomarker development for early detection of PE and associated pathologies, as outlined in the introduction of this thesis (Figure 1). In this

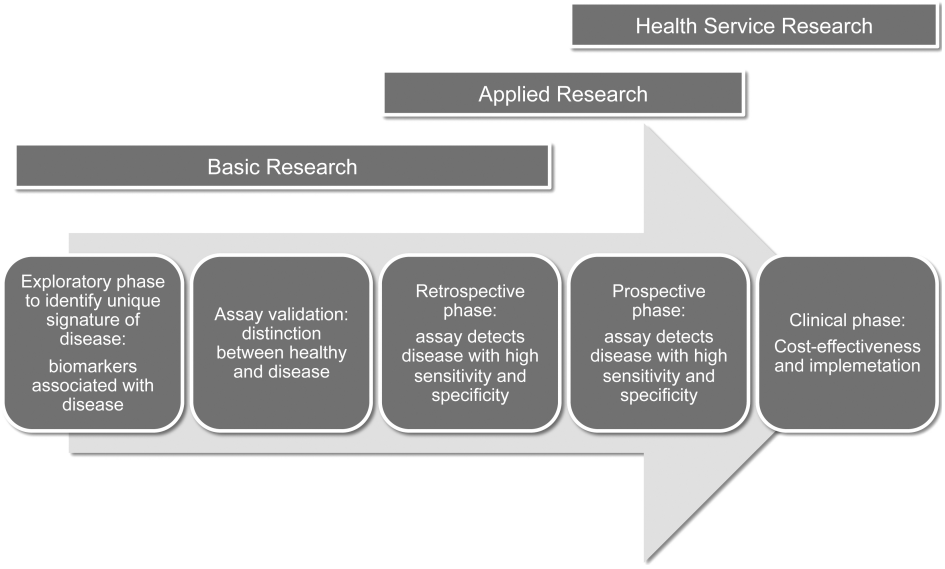


Figure 1. Five phases of biomarker development for early detection of PE.

general discussion section the key objectives of each phase and our studies performed to address these objectives will be recalled.

PROMISING DIRECTIONS IDENTIFIED

The first phase focuses on the search for biomarkers. It starts with the exploration of pathophysiology of the disease and seeks the characteristics unique to disease that may lead to ideas for clinical tests. Broad literature search and data-mining are widely used for this purpose. This approach is known as "knowledge based". Furthermore, new techniques such as metabolomics that yield information regarding expression of thousands of metabolites based on mass spectroscopy may inform the researcher about the pathology of the disease and distinguish the levels of metabolites between healthy and (potential) sick individuals. This approach is known as "disease based".

Searching the literature is a standard requirement of evidence-based medicine. A systematic literature search goes beyond the search for information and includes the identification and articulation of relationships between literature and our own field of research. In this thesis we assessed most of the currently studied first trimester screening biomarkers (**chapter 2**). After a broad search seven promising serum biomarkers were selected: A Disintegrin and Metalloproteinase 12 (ADAM12), free beta subunit of human Chorionic Gonadotropin (fβ-hCG), Inhibin A, Activin A, Placental Protein 13 (PP13), Placental Growth Factor (PlGF) and Pregnancy-Associated Plasma Protein-A (PAPP-A) together with uterine artery (Ut-A) Doppler and maternal characteristics (MC). The selection of serum markers, Ut-A Doppler

and MC appeared appropriate and in agreement with the pathophysiology of PE. After thorough analysis it appeared that low levels of PP13, PlGF and PAPP-A and elevated level of Inhibin A combined with high pulsatility index (PI)/resistance index (RI) of Ut-A Doppler and MC are significantly associated with the development of PE later in pregnancy.

Data-mining is an interdisciplinary field initiated for discovering patterns in large data sets of e.g. genes and proteins as well literature databases such as PubMed. The overall goal of data-mining is to find novel associations between function of genes/proteins and disease. Recent studies using data-mining for identification of blood based tissue-specific cancer biomarkers successfully have demonstrated the feasibility of this approach.[14,15] In this part of this thesis, we used data from the literature and publicly available databases to identify potential biomarkers for PE (**chapter 3**). Data-mining strategies were based on the pathology of PE, and on the origin of the biomarkers already described by others. This also included a prediction of the serum detectability of the markers identified by data-mining, as this is a necessary condition for implementation in a screening setting. Moreover, data mining approaches allowed us for a ranking in testing priority based on the strength of available evidence. This facilitates an efficient testing of available samples. The data-mining approach resulted in 38 blood-detectable candidate biomarkers. Between the markers with the strongest evidence featured the already known PP13, PlGF and PAPP-A. An additional five markers arose as putative screening markers: Fibronectin 1 (FN1), Heme Oxygenase 1 (HMOX1), Pregnancy-Associated Plasma Protein-A Lysin 2 (PAPP-A2), Prolactin (PRL) and Vascular Cell Adhesion Molecule 1 (VCAM1). The latter markers were so far found to be informative beyond the first trimester of pregnancy or after the clinical onset of the disease and are presumably less functional in first trimester screening. However, they are noteworthy for further research for putative PE screening markers.

Metabolomics is a new powerful approach to detect evidence of disease based on a panel of small molecules derived from the global analysis of metabolic profiles of samples such as serum. Metabolomics utilizes high-resolution analytical methods such as mass spectrometry (MS) for the quantitative analysis of hundreds of small molecules (less than ~ 1,000 Da) present in the samples. The high sensitivity of metabolite profiles can provide the means to detect the early onset of various biological perturbations in real time. In order to identify new potential biomarkers for the screening of PE we performed metabolomics analysis of first trimester serum of women who developed PE later in pregnancy and controls (**chapter 4**). After sampling variability and thorough statistical cross-validation three metabolites were significantly lower in PE cases compared to controls (taurine and asparagine for early onset PE; glycylglycine for late onset PE). Interestingly, for two markers (taurine and glycylglycine) there was a clear association between properties of the markers and the pathogenesis of PE making them putative makers for screening. Our study was, thus far, the first one associating low asparagine with PE.

ASSAY VALIDATION

The results of the first phase are frequently used in the second phase to validate promising biomarkers. The assay must be able to distinguish with high sensitivity and specificity subjects

with disease from those who remain healthy in order to be considered promising for screening. The primary results of the assay are presented as Detection Rates (DR), False Positive Rates (FPR) or Receiver Operating Characteristic (ROC) curves. DR (or sensitivity) measures the proportion of actual sick subjects, which are correctly identified as having chance for PE. FPR (or 1- specificity) gives a number or a percentage of healthy subjects incorrectly identified as being at risk for PE. ROC curves are graphical plots, which illustrate the performance of a screening test. They are created by plotting the fraction of DR versus the fraction of FPR at various threshold settings. ROC analysis provides a adequate tool to select optimal screening tests or models.

In the systematic review (**chapter 2**) the DR of single markers, fixed at 10% FPR were relatively low, and ranged from 22% to 83%. However, DR for combinations of multiple markers yielded better DR between 38% and 100% and therefore appeared attractive to carry on with the PE biomarker quest.

In **chapter 4** after a selection of three promising metabolites (taurine, asparagine and glycyglycine) we constructed prediction models to evaluate our findings. The best prediction model included only taurine in combination with prior maternal risk (maternal characteristics) and measurement of first trimester mean arterial pressure (MAP). The DR was 55% at a fixed 10% FPR. Therefore, from 105 potential biomarkers, taurine remained as the single putative metabolite predicting PE, which may be considered a disappointing result.

RETROSPECTIVE SCREENING

In the third phase, specimens are collected in retrospective manner from large serum banks. Based on the information about the clinical outcomes in large and related databases it is possible to make a distinction between, and compare the healthy cases to, those with a disease. When the levels of the biomarkers in the cases are noticeably different compared to controls the biomarker's potential is established. The major aim of this particular phase is to evaluate the predictive value of already known markers in the preparation for a prospective study.

In this thesis we were able to study first trimester serum from a large serum bank of women participating in the first trimester prenatal screening. Maternal characteristics and pregnancy outcomes were collected and we compared the levels of promising PE biomarkers in the first trimester serum of women who subsequently developed PE (with or without fetal growth restriction) to those from controls with uneventful pregnancies. First we tested markers known from previous phases/studies on the total PE cohort; subsequently we distinguished between women who developed PE with or without intra-uterine fetal growth restriction (**chapter 5**). Finally, we examined whether promising PE markers might be suitable to predict other pregnancy complications. For that reason we conducted a study on first trimester serum from women with pregestational diabetes mellitus (PGDM) in order to predict fetal macrosomia, i.e. overgrowth at birth (**chapter 6**).

In case of PE (**chapter 5**) levels of PlGF were significantly lower and those of MAP significantly higher than in controls. The differences were even more outspoken in case of women with PE who additionally delivered growth restricted infants. The best prediction model, using PAPP-A, ADAM12, PlGF, MAP and maternal characteristics was able to detect 72% of PE cases (EO-PE in particular). Prediction for PE with concomitant growth

restricted infants was better than for PE alone and reached 92%, which is encouraging for the next phase of biomarkers research.

In PGDM pregnancies (**chapter 6**) we found that first trimester serum markers such as PAPP-A, ADAM12, PP13 and PlGF are able predict macrosomia at birth. The DR reached 43% without inclusion of maternal characteristics or maternal mean arterial pressure. A very interesting discovery of this particular study was that, in case of macrosomic infants, the serum biomarkers levels were comparable to that of the controls whereas these levels were significantly lower in the PGDM non-macrosomic subgroup. This implies that fetal growth in PGDM pregnancies is partly determined by early placentation and indicates, therefore, a broader usability of the first trimester placental serum markers than only for the detection of PE.

PROSPECTIVE SCREENING

The retrospective phase of the marker quest helps to determine whether it is possible for the disease to be detected adequately and early enough, i.e. timely before the symptoms become clinically evident, to be able to therapeutically intervene. The shortcoming of this phase is the fact that outcome is already known, even before the study has been started. In a prospective screening study the method is applied to living individuals without this knowledge on outcome. Hereby the individuals at risk should be timely recognized and subsequently narrowly surveyed. A frequent shortcoming of this phase is the low number of cases due to a low prevalence of the disease in a prospective cohort. Therefore a large sample size is required for such studies.

In this thesis we also covered the fourth phase of prospective PE biomarkers evaluation. The main purpose of our study was to assess the first trimester concentration trends of already established PE biomarkers throughout a prospective and longitudinal follow-up of the study subjects. This was done to provide a baseline of normal marker values for future investigation and PE risk assessment and establish the most appropriate gestational age for measurement. Due to small number of PE cases we were not able to conduct any prediction model yet at this point of our research.

First of all we conducted a study on low risk pregnant women to establish the concentration charts of first trimester markers in uncomplicated pregnancies and explore their interrelationships (**chapter 7**). The following steps were to assess the marker distribution in different at-risk pregnancy groups and to compare them to those with low risk pregnancies.

The first high risk group consisted of women who had developed PE in previous pregnancy (**chapter 8**). Women who previously developed PE are at higher risk to develop PE again.[16-19] In this group of women MAP appeared to be significantly higher throughout the first trimester compared to women with uncomplicated pregnancies. Interestingly, MAP appeared to gradually augment depending on the severity of the pregnancy outcomes. It was lowest in women who did not develop any pregnancy complication in their current pregnancy and highest in women who developed the most severe complication, i.e. EO-PE. Unfortunately none of the other markers appeared different between control and this at-risk group. Presumably the group of severe outcomes was too small to establish any differences (n = 3).

The second high risk group consisted of women with pregestational diabetes mellitus (PGDM) (**chapter 9**). The main purpose of this study was to examine whether the first

trimester PE markers are influenced by the presence of PGDM. In this group there were no differences in first trimester markers between control and PGDM group. No correlations between the marker levels and birth weight of the newborns were found either. However, in this study there was one intra-uterine fetal death at 34 weeks of gestation (IUFD). Interestingly this particular case had the lowest PAPP-A and ADAM12 and the highest $HbA1_c$ values in early pregnancy. Together with the small placenta (< 10^{th} centile) and normal birth weight of the baby we suggest that there was a severely impaired placentation already early in pregnancy, with a relative fetal overgrowth in the course of gestation due to increased glucose levels. Therefore extremely low levels of PAPP-A and ADAM12 and high $HbA1_c$ throughout the first trimester may be alarming and compel the leading obstetrician to narrow surveillance of such a case.

The third high risk group consisted of women who conceived with the aid of IVF/ICSI methods (**chapter 10**). So far the literature has been inconclusive with regard to the levels of first trimester Down syndrome screening markers, whereby altered levels have resulted in correction factors in several countries. Data on early PE markers in pregnancies following ICSI/IVF are lacking. We studied all possible factors, which could be of influence on the first trimester markers: laboratory workup of the conception (IVF/ICSI), method of the GnRH downregulation and manner of embryo transfer. We did not find any differences in the levels of the markers between control and IVF/ICSI group. None of the treatment methods affected the levels of the first trimester markers, thus, PE risk calculation may be safely applied in these women in the future.

CLINICAL VALIDATION OF THE BIOMARKERS

The last phase of biomarker development evaluates the effectiveness and efficacy of the screening before it is implemented for broader group of patients. This phase examines whether the screening test is able to diminish the prevalence of disease in a selected patient population. The most appropriate study method to conduct this phase is a standard randomized controlled trial (RCT) with two groups: the first one undergoing the new screening procedure and the second treated in a classical manner. Together with the assumed benefit of the screening method other aspects of this particular method can be studied. For instance, effectiveness of the screening program, availability and effectiveness of the treatment and economic aspect of the screening such as the cost effectiveness.

This phase of the biomarker development is not covered by this thesis. A RCT with larger series of patients is currently being prepared in our department and belongs to the future prospectives of PE screening.

FUTURE PROSPECTIVES OF PE SCREENING

The data presented in this thesis covering first trimester PE screening is rather encouraging; however the quest for the best screening markers is not finished yet. Recent meta-analyses suggest that prevention of PE with low dose aspirin, if started before 16 weeks of gestation, is highly effective in a high risk group and reduces up to 50% of PE cases.[13] Nevertheless,

more research is needed before standard screening programs for PE with adequate risk assessment followed by drug intervention may be implemented in daily practice. A fully applicable prediction model combining maternal characteristics, serum biomarkers and measurement of uterine artery Doppler does not exist yet, however international literature is promising.[20] The different prediction models presented in this thesis still need to be evaluated prospectively. Even more ideally, such a prediction model should be evaluated and validated in a population other than the population in which the model has been developed. Thereby, for the most reliable results the study population of this particular project should be large since the prevalence of PE is relatively low. Statistically, in the ideal situation every variable taken in a prediction model requires about 10 cases of PE. Taking into account that the prevalence of PE is about 1-2%, in case of a prediction model containing for instance maternal characteristics, MAP, one or two serum markers and artery uterine Doppler, 2500 – 5000 pregnant women are needed in such a model evaluation.

At this point the University Medical Center, Utrecht, is conducting a study, which fulfills the needs of the last phase of the biomarker validation. The RESPECT study (Risk Selection for Pregnancy Complications to provide Tailored care) focuses on improving the current risk selection of pregnant women and validation of a prediction model for detection of PE. The goal of this study is to include 4000 women in the first trimester of pregnancy. The first phase of this study will validate a prediction model for PE through collection of maternal characteristics of pregnant women, medical records, blood pressure, serum (first trimester) and measurement of uterine artery Doppler (second trimester). In the second phase of the study, the prediction model will be implemented in the obstetric care system and the maternal and fetal outcomes will be evaluated. Through the application of a prediction model for every woman customized and personalized care will be possible: regular obstetric care for low risk women and high obstetric care surveillance for high risk women.

After a prediction model has been evaluated in a broader group of pregnant women a RCT on an even larger cohort is required. In this setting preventive treatment with low dose aspirin should be studied. A desirable detection rate (DR) should be about 90% fixed at a 10% false positive rate (FPR). Currently this rate is generally the most favorable in preeclampsia screening programs. This approach originates from prenatal Down syndrome screening studies, where most studies accept 5% FPR, i.e not so high as to induce anxiety in a large number of unaffected pregnant women but sufficiently large to identify a significant percentage of the target condition. In fact a false positive result in a Down syndrome screening program involves an invasive procedure with a risk of miscarriage. A higher false positive rate in PE screening is commonly accepted because it would only involve increased surveillance and prophylactic aspirin therapy. Suppose we need about 100 PE cases per RCT arm, than a population of at least 10000 (nulliparous) women per study arm would be needed. Nicolaides and colleagues from King's College, London, UK currently prepare such a RCT (www.fetalmedicine.com). The ASPRE (Aspirin for Evidence-Based Preeclampsia Prevention) study will be a multicentre RCT conducted through several countries in Europe (31,000 inclusions). Screening for PE at 11-13 weeks' gestation will include a combination of maternal medical history and characteristics, maternal serum PAPP-A, and PlGF, MAP and uterine artery PI. Women who are deemed to be at high risk

for PE (about 10% of the population) will be offered the opportunity to participate in the aspirin vs. placebo trial. Participants will be randomized to one tablet per night of either aspirin 150 mg or matching placebo tablet and treatment will continue until 36-37 weeks. The preventive effect of low dose aspirin started in the first trimester of pregnancy on the occurrence of PE will be evaluated. Such a study is an immense undertaking requiring not only outstanding logistics but also large investments.

GENERAL INTERPRETATION OF RESULTS

The knowledge of PE pathogenesis has led to the discovery of several first trimester biomarkers and the development of different mathematical models that combine these markers in order to predict PE.[20] The findings of this thesis as well as work of others confirm that the combination of measurements of uterine artery Doppler, maternal characteristics, MAP and multiple serum markers appears promising, especially regarding the prediction of EO-PE and concomitant intra-uterine growth restriction.[20-25]

First trimester measurements of uterine artery Doppler have so far the best potential. In several studies the DR of uterine artery Doppler for EO-PE reaches up to 83%.[26-36] This DR further improves after combination with other first trimester markers such as a.o. PAPP-A, PlGF, PP13 and ADAM12.[26-36] However, it is important to realise that measurements of uterine artery Doppler are only reliable if carried out correctly. This measurement is rather difficult and needs lots of effort and training to be accurately conducted.

PE is considered to be a multifactorial disease with various maternal constitutional factors contributing to its pathogenesis.[37-39] Many studies provide evidence that PE may be well predicted by algorithms combining maternal racial origin, BMI, parity and personal or family history of PE[24,26-29,32-35,40-47]. Solely maternal characteristics detect up to 56% of cases, the DR value improving with the combination of other markers.

First trimester MAP may inform us about the maternal vascular health status and its adaptation to pregnancy. It appears to be one of the most important predictors of PE.[23,24,26-28,39-41,48,49] Routine measurement of blood pressure during the first antenatal visit has the potential to foresee the development of PE several months later. Thereby, measurement of MAP is the cheap and generally accessible.

Several well-established serum biomarkers (e.g. PAPP-A, PP13, PlGF and ADAM12) have shown potential as predictors of PE. Individually or in combination, biomarkers are able to predict a high proportion of pregnancies destined to develop PE.[20,23,24,49] However, a high detection rate (up to 90%) and positive predictive value as mentioned in different studies cannot be achieved by a serum makers approach only. Serum markers need to be combined with earlier mentioned uterine artery Doppler measurements and maternal characteristics.[20,23,24,49]

Novel markers such as nucleic acids, proteins, peptides, or cellular metabolites, are also the subject of current investigation. Recent studies using metabolomics technique, including ours, report novel, potential markers for prediction of PE.[49-53] Studies present DRs of the assays reaching up to 83%.[49-53] It should be noted however that although different studies use similar metabolite platforms, each study presents a different set of markers. This shows the major complexity in the quest for novel PE markers. PE is very

complex and the causes of the pathology may differ not only between different variants of the disease (EO-PE / LO-PE / HELLP syndrome) but also between subsets of patient and control cohorts and even from one study to the next.

Last but not least, from this thesis and a large number of other studies, we know that measurements of uterine artery Doppler, maternal characteristics, MAP and serum biomarkers are of great importance in PE risk assessment. Results are promising, applicability however is still limited. Large prospective cohort studies and RCTs are needed to evaluate the already known biomarkers. Novel and even better biomarkers are searched for. Therefore, the great expedition for the best set of biomarkers to detect PE early in pregnancy is not yet finished.

GENERAL CONCLUSIONS

PE remains a major health problem and the possibilities of PE prediction are promising however still limited.

- There is not one ideal biomarker to predict PE; a combination of several biomarkers is needed.
- Ideal biomarkers for PE:
  - play a central role in the pathogenesis of the disease and are specific for the condition
  - are detectable long before the clinical manifestation of disease and are not detectable or detectable at different levels in normal pregnancies
  - should be easy and cheap to measure
  - show a high sensitivity and specificity
  - correlate with the severity of the condition.
- Maternal characteristics, first trimester serum markers, measurement of uterine artery Doppler and MAP may be of a great value to predict PE in the first trimester of pregnancy.
- Large prospective cohort studies and RCTs are needed to evaluate already existing prediction models and the prophylactic value of low dose aspirin treatment of women at risk to develop PE.
- Biomarkers assessed in early pregnancy may also be of great value to detect early onset fetal growth restriction. Regarding pre-existing diabetes, they are related to fetal growth and may be of value to identify cases a risk for fetal death.
- First trimester biomarkers in pregnancies following IVF/ICSI do not differ from controls and may, therefore, also in these pregnancies be used for early PE risk assessment.

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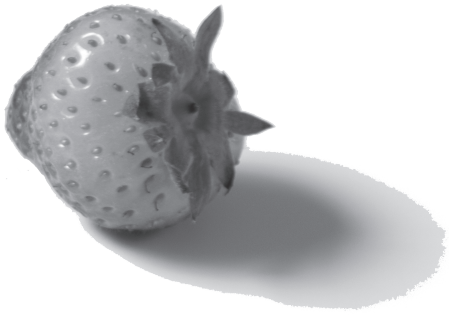
XII

NEDERLANDSE SAMENVATTING



INTRODUCTIE

Biomarkers spelen al een aantal decennia een belangrijke rol in de vroege opsporing van verschillende ziekten, zoals kanker en hart- en vaatziekten. Een ideale biomarker is een stof of een karakteristiek die objectief gemeten en geëvalueerd kan worden bij een patiënt maar bij een gezond persoon niet detecteerbaar is, of in andere hoeveelheden te vinden is. Biomarkers kunnen worden toegepast voor de vroege identificatie van hoog-risicopatiënten om tijdig een geschikte behandeling in te kunnen zetten. Daarnaast kunnen biomarkers, afhankelijk van de ernst van de ziekte, ons meer vertellen over de prognose van een individuele patiënt. Om biomarkers in de kliniek te kunnen toepassen, moeten ze een hoge sensitiviteit en specificiteit hebben, eenvoudig te bepalen zijn en daarbij niet te duur zijn.

Al vanaf 1972 worden biomarkers breed toegepast in de prenatale screening en diagnostiek. Een van de eerste, was alfafoetoproteïne een eiwit gemeten tijdens de zwangerschap, dat gebruikt werd voor het opsporen van foetussen met spina bifida (open ruggetje). Tegenwoordig worden andere biomarkers gebruikt voor de eerste trimester screening op downsyndroom waarbij er een risicoschatting wordt gemaakt op basis van moederlijke leeftijd, meting van de foetale nekplooi en bepaling van twee bloedserummarkers: de vrije β-humaan Chorion Gonadotropine (fβ-hCG) en het met zwangerschap geassocieerde plasma eiwit–A (PAPP-A). Het is al langer bekend dat deze biomarkers, samen met een aantal nieuwe biomarkers, ingezet kunnen worden bij de screening naar zwangerschapsaandoeningen zoals preeclampsie (zwangerschapsvergiftiging; PE), intra-uteriene groeirestrictie en vroeggeboorte. Dit vermogen is vanuit klinisch oogpunt interessant, omdat de eerste trimester screening daardoor ook de mogelijkheid biedt op een vroege selectie van zwangere vrouwen met een' hoog-risico zwangerschap. Aan deze vrouwen kan vervolgens preventie en therapie worden aangeboden met als einddoel verbetering van de gezondheid van moeder en kind tijdens zwangerschap en geboorte.

Preeclampsie is een ernstige aandoening die alleen tijdens de zwangerschap en vlak na de geboorte optreedt. PE treft ongeveer 2% van de zwangere vrouwen en is wereldwijd een van de belangrijkste oorzaken van maternale en perinatale morbiditeit en mortaliteit. PE veroorzaakt dus aanzienlijke risico's voor zowel kind als moeder. Hierbij moet worden gedacht aan foetale groeirestrictie, iatrogene vroeggeboorte of intra-uteriene vruchtdood, maar ook aan hart- en vaatziekten in het latere leven van moeder en kind. PE wordt gekenmerkt door een hoge bloeddruk en de aanwezigheid van eiwit in de urine. Deze parameters treden echter pas op als de ziekte volledig ontwikkeld is, terwijl de gebeurtenissen die tot deze verschijnselen leiden al vroeg in de zwangerschap aanwezig zijn. Vroegtijdige herkenning van hoog risicopatiënten voordat de ziekte optreedt, is daarom zeer belangrijk, net als het vervolgens tijdig toepassen van therapie om uiteindelijk betere zorg te kunnen bieden en betere zwangerschapsuitkomsten te bereiken.

De identificatie van verschillende biomarkers die geassocieerd zijn met de pathofysiologie van PE in de vroege zwangerschap, kunnen leiden tot de ontwikkeling van een gevoelige en specifieke screeningsmethode. Hoewel de exacte achtergrond van PE nog niet volledig bekend is, hebben de laatste twee decennia van onderzoek ons een schat aan kennis over deze ziekte gebracht. Wij weten inmiddels dat interacties tussen (1) placentaire factoren,

(2) moederlijke karakteristieken en (3) ongunstige adaptieve veranderingen tijdens de zwangerschap leiden tot de verschillende klinische symptomen van PE. De zoektocht naar potentiële biomarkers voor PE zal daarom gebaseerd moeten worden op de huidige kennis van de pathofysiologie van PE en de eerder genoemde drie facetten. Omdat PE een heterogene aandoening is, is het te verwachten dat een screeningstest gebaseerd zal zijn op een combinatie van meerdere biomarkers in plaats van één enkele.

Dit proefschrift beschrijft de zoektocht naar biomarkers voor PE. Deze zoektocht bevat vijf fasen die het gehele proces van de ontwikkeling van een klinisch toepasbare screeningsmethode omvatten. Dit proces begint bij het verzamelen van informatie over de pathofysiologie van PE (fase 1). Deze eerste fase geeft richting aan het onderzoek waarin de unieke handtekening van de ziekte te herkennen is en verschaft inzicht in de wijze waarop potentiële biomarkers geïdentificeerd kunnen worden. Fase 2 richt zich op de ontwikkeling van een test die potentiële markers bevat die naar voren gekomen zijn uit fase 1. Vervolgens wordt deze test op kleine schaal geëvalueerd. De resultaten worden beschreven in termen van sensitiviteit en specificiteit van de test. In fase 3 wordt de ontwikkelde test geëvalueerd in grote retrospectieve, goed gedocumenteerde groepen (cohorten) van zwangerschappen met een aandoening en ongecompliceerde zwangerschappen en wordt gekeken of een dergelijke test al vroegtijdig een adequaat onderscheid kan maken tussen gezonde en zieke mensen. In fase 4 wordt de test toegepast op een prospectief cohort van patiënten. De test wordt geëvalueerd zo gauw het bloedmonster beschikbaar is en er wordt gekeken naar de voorspellende waarde van de test in een niet-geselecteerde populatie. In de laatste fase (fase 5) worden de prestaties van de test bekeken in termen van vermindering van de ziekteprevalentie (na behandeling van de door de test geïdentificeerde hoog risicogroep) en algemene gezondheidsvoordelen. Deze fase kent ook een kosten-batenanalyse en biedt de beste aanwijzingen voor de werkelijke voordelen en impact van de ontwikkelde screening.

FASE 1

In fase 1 van dit proefschrift is een systematisch literatuuronderzoek verricht naar reeds bekende eerste trimester biomarkers voor PE (**hoofdstuk 2**). Na een uitgebreide zoektocht werden zeven veelbelovende serumbiomarkers (ADAM12, fβ-hCG, PP13, PlGF, Inhibine A, Activine A en PAPP-A) geïdentificeerd, samen met Doppler-onderzoek van de arteriae uterina (meet de bloeddoorstromingsweerstand in de twee slagaders naar de baarmoeder) en maternale karakteristieken. Na een grondige analyse bleek dat lage concentraties van PP13, PlGF en PAPP-A en verhoogde waarden van Inhibine A en pulsatility index van de Doppler samen met een aantal maternale karakteristieken al in het eerste trimester van de zwangerschap sterk geassocieerd te zijn met de ontwikkeling van PE later in de zwangerschap.

Voor een verdere zoektocht naar potentiële biomarkers werd er een 'dataminingmethode' toegepast (**hoofdstuk 3**). Deze methode houdt in dat, op basis van de pathofysiologie van PE meerdere (genetische) databases doorzocht worden naar associaties tussen de ziekte en over- of onderexpressie van genen of eiwitten. Deze aanpak resulteerde in 38 kandidaat biomarkers die in het bloed te detecteren zijn. Tot de meest veelbelovende markers

behoorden naast de al bekende PP13, PlGF en PAPP-A ook vijf potentiële nieuwe: FN1, HMOX1, PAPP-A2, PRL en VCAM1. Deze laatste biomarkers zijn tot op heden beschreven als potentieel onderscheidend in een later stadium in de zwangerschap, maar zijn vermoedelijk minder functioneel in de eerste trimesterscreening. Echter, verder onderzoek naar hun potentie in de eerste trimesterscreening zal nog verricht moeten worden.

In **hoofdstuk 3** werd op metabolomics-niveau gezocht naar potentiële biomarkers. Dat betekent dat er niet gekeken wordt naar verschillen in eiwit-niveaus, maar naar producten uit verschillende stofwisselingsprocessen tussen zwangeren met PE en gezonde zwangerschappen. Na het testen van twee grote panels met amines en oxylipines werden drie metabolieten geselecteerd: taurine, asparagine en glycylglycine. Uit de literatuur blijkt dat er een duidelijk verband is tussen de eigenschappen van deze biomarkers en de pathofysiologie van PE. Deze biomarkers lijken dan ook waardevol te zijn voor de toekomstige screening.

FASE 2

Markers die geselecteerd zijn bij het systematisch literatuuronderzoek (**hoofdstuk 2**) en de metabolomics aanpak (**hoofdstuk 4**) werden in deze fase geëvalueerd. Gekeken werd naar hun potentie als screeningmarkers in termen van sensitiviteit en specificiteit van de test. Uit het systematische review in **hoofdstuk 2** bleek dat de sensitiviteit van enkele markers varieerde van 22% tot 83% (dus tussen de 22 en 83% van alle gevallen werden correct voorspeld door de test). De sensitiviteit voor combinaties van markers bleek nog beter te zijn en varieerde tussen 38% en 100%. Daarbij bleek de sensitiviteit voor vroeg optredende PE (< 34^{e} week van de zwangerschap) beter te zijn dan die van de laat optredende variant. In **hoofdstuk 4** werd naar de drie eerder genoemde metabolieten (taurine, asparagine en glycyglycine) gekeken. Er werden verschillende voorspelmodellen gecreëerd om deze biomarkers te evalueren. Het beste voorspelmodel bevatte taurine in combinatie met moederlijke karakteristieken en de moederlijke bloeddruk in het eerste trimester van de zwangerschap. Voor vroege PE bleek de sensitiviteit van deze test 55% te zijn.

FASE 3

Tijdens deze fase is er onderzoek gedaan met een selectie van serummonsters uit duizenden ingevroren serummonsters (serumbank) die verzameld zijn in het kader van de eerste trimesterscreening op downsyndroom. De zwangerschappen behorend bij die serummonsters waren goed gedocumenteerd (databank). Deze databank bevat informatie over de lichamelijke karakteristieken van de deelnemende vrouwen en de uitkomsten van hun zwangerschappen. De analyses van de eerste trimester sera van vrouwen met ongecompliceerde zwangerschappen werden vergeleken met die van zwangerschappen waarbij uiteindelijk PE optrad (**hoofdstuk 5**). Daarbij werd in detail gekeken naar PE-zwangerschappen met intra-uteriene groeirestrictie als complicatie. De markers die in deze fase zijn getest waren PAPP-A, PlGF, ADAM12 en de moederlijke bloeddruk. PlGF bleek significant lager en de moederlijke bloeddruk significant hoger bij vrouwen die uiteindelijk PE hebben ontwikkeld. Nog meer uitgesproken waren de verschillen bij vrouwen met een PE met foetale groeirestrictie. Het beste voorspelmodel

bevatte PAPP-A, ADAM12, PlGF, moederlijke bloeddruk en moederlijke karakteristieken en de sensitiviteit van dit model was 72%. De voorspelling van PE met foetale groeirestrictie was zelfs beter dan voor PE alleen en bereikte een sensitiviteit van 92%. In **hoofdstuk 6** werd gekeken naar een andere zwangerschaps- en groeipathologie, namelijk foetale macrosomie (een te grote en te zware foetus) in zwangerschappen van vrouwen met insuline afhankelijke diabetes mellitus (DM-suikerziekte). In deze zwangerschappen bleken de eerste trimester serummarkers PAPP-A, ADAM12, PP13 en PlGF foetale macrosomie te kunnen voorspellen. De sensitiviteit van deze test was 43%. Deze waarde was berekend zonder de toevoeging van moederlijke kenmerken.

FASE 4

In deze fase werden de meest veelbelovende biomarkers in prospectieve cohorten op longitudinale wijze geëvalueerd door in het eerste trimester om de week bloedmonsters te nemen. Het belangrijkste doel van deze fase van het onderzoek was het bepalen van het eerste trimester concentratieverloop in bloed van deze biomarkers. De eerste stap hierin was de bepaling van trends en het concentratieverloop in ongecompliceerde zwangerschappen (**hoofdstuk 7**) om ze vervolgens te vergelijken met de concentraties in a-priori hoog risicozwangerschappen (**hoofdstukken 8-10**). Ongecompliceerde zwangerschappen werden vergeleken met die van vrouwen die in hun vorige zwangerschap PE hadden ontwikkeld (**hoofdstuk 8)**. Daaruit bleek dat de moederlijke bloeddruk in het eerste trimester al significant hoger is bij vrouwen die eerder een PE hebben doorgemaakt. Daarbij hebben deze vrouwen een aanzienlijk verhoogd risico om opnieuw PE te ontwikkelen. In **hoofdstuk 9** werden de ongecompliceerde zwangerschappen vergeleken met die van vrouwen met DM. Hierbij werd bepaald of de aanwezigheid van DM van invloed kan zijn op de concentraties van de eerste trimester biomarkers. In deze studie werden geen verschillen gevonden in biomarkers tussen de controlegroepen en de DM-groepen. Opvallend was echter een geval van een intra-uteriene sterfte in de DM-groep waarbij de concentraties van PAPP-A en ADAM12 aanzienlijk lager waren in vergelijking met alle andere zwangerschappen. De derde groep met een a-priori hoog risico bestond uit vrouwen die met behulp van IVF/ICSI zwanger waren geworden (**hoofdstuk 10**). Tot op heden is het onduidelijk of de concentraties van de tot dusver gebruikte eerste trimesterscreening biomarkers in IVF/ICSI zwangerschappen gelijk zijn aan die van spontaan tot stand gekomen zwangerschappen. In de studie beschreven in dit hoofdstuk werden geen verschillen gevonden in concentraties van de markers tussen de controlegroep en de IVF/ICSI-groep. Geen van de IVF/ICSI-(hormoon-)behandelmethoden leek van invloed te zijn op de concentraties van de biomarkers. Dat betekent dat de PE-risicobepaling ook in deze groep toegepast kan worden.

FASE 5

De vijfde en tevens laatste, fase van de zoektocht naar biomarkers bevat idealiter een standaard gerandomiseerd onderzoek (randomized controlled trial; RCT) met twee

groepen. De eerste groep ondergaat de nieuwe screeningsmethode inclusief bijbehorende zorgpaden afgestemd op de verschillende risicocategorieën en de tweede groep wordt zorg geboden op een klassieke manier. Samen met de voordelen van het screeningprogramma worden andere aspecten, zoals de kosten-batenanalyse van de nieuwe werkwijze geëvalueerd. Het onderzoek in dit proefschrift bevat deze fase van de zoektocht naar de biomarkers niet. Dergelijke grote RCT's met een groot aantal patiënten worden op dit moment wel voorbereid vanuit het UMC Utrecht (RESPECT studie) en in Europees verband (ASPRE project). Op basis van deze implementatiestudies zullen we meer te weten komen over het toekomstperspectief van de PE-screening.

TOEKOMSTPERSPECTIEF

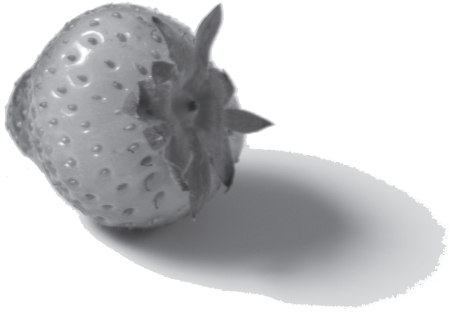
De resultaten betreffende de eerste trimesterscreening naar PE, zoals gepresenteerd in dit proefschrift, zijn bemoedigend. Hoewel de voorspellende waarde van enkelvoudige biomarkers bescheiden blijkt te zijn, biedt de combinatie van verschillende biomarkers samen met maternale karakteristieken de mogelijkheid tot accurate voorspelling van PE. Daarbij suggereren recente meta-analyses dat preventie van PE met lage doses aspirine, mits gestart bij een zwangerschapsduur van minder dan 16 weken, effectief is bij groepen met een hoog risico. Er moet echter nog duidelijker bewijs komen vanuit de hiervoor genoemde RCT's waarin eerste trimesterscreening naar PE met eerder genoemde biomarkers geïmplementeerd wordt en vervolgd wordt door een, al dan niet medicamenteuze, behandeling van vrouwen met een hoog risico. De voorspellende waarde van de modellen die biomarkers zoals PAPP-A, PP13, PlGF, ADAM12 bevatten samen met de Doppler in de arteriae uterina en maternale karakteristieken, moeten nog in grote ongeselecteerde populaties geëvalueerd worden. Tot de evaluatie van nieuwe predictiemodellen zal ook een kosten-batenanalyse dienen te behoren.

CONCLUSIE

- Preeclampsie is een belangrijk gezondheidsprobleem; er bestaat geen ideale biomarker die PE kan voorspellen, hiervoor is een combinatie van verschillende biomarkers nodig;
- Moederlijke karakteristieken, eerste trimester serum biomarkers, de moederlijke bloeddruk en een Doppler meting van de arteriae uterina zijn van grote waarde voor het voorspellen van PE in het eerste trimester van de zwangerschap;
- Grote gerandomiseerde onderzoeken zijn nodig om bestaande predictiemodellen en de waarde van profylactische (voorzorgs-) aspirinebehandeling bij hoog risico zwangerschappen te evalueren;
- Bekende biomarkers kunnen in de toekomst ook toegepast worden voor de voorspelling van groeipathologieën, zoals intra-uteriene groeirestrictie en macrosomie.

XIII

STRESZCZENIE PO POLSKU



WPROWADZENIE

Już od kilkudziesięciu lat biomarkery grają ważną rolę we wczesnym wykrywaniu różnych chorób, takich jak rak czy choroby serca. Idealnym biomarkerem jest zarówno substancja, którą można łatwo wykryć w płynach fizjologicznych takich jak krew czy mocz, jak i cecha danego człowieka taka jak wiek, waga czy wzrost. Sama obecność biomarkera w organizmie, albo też jego ilość (niższa lub wyższa niż norma) pozwala na wykrycie stanu chorobowego. Biomarkery mogą przyczynić się do poprawy stanu zdrowia pacjenta na wiele różnych sposobów: miedzy innymi poprzez wczesną identyfikację pacjentów wysokiego ryzyka. Ponadto, biomarkery mogą nam wiele powiedzieć na temat przebiegu choroby u danego pacjenta i jego rokowań. Aby móc zastosować biomarkery w klinice, muszą być one wykrywalne w prosty i tani sposób, do tego muszą mieć wysoką czułość i być specyficzne dla danej choroby. Przykładem idealnego biomarkera jest hCG stosowany we wczesnych testach ciążowych.

Biomarkery są szeroko stosowane w diagnostyce prenatalnej. Już w 1972, jednym z pierwszych biomarkerów była alfa-fetoproteina, której używano do wykrywania płodów z rozszczepem kręgosłupa. Obecnie głównym testem przesiewowym we wczesnej ciąży jest test na zespół Downa. Ocena ryzyka na posiadanie dziecka z zespołem Downa dokonywana jest na podstawie wytycznych: wieku matki, pomiarów przezierności karku płodu oraz pomiaru dwóch biomarkerów pobieranych z krwi matki - fβ-hCG i PAPP-A. Już od jakiegoś czasu wiadomo, że te biomarkery, wraz z kilkoma nowymi biomarkerami, mają zdolności do wykrycia również innych zaburzeń ciąży takich jak stan przedrzucawkowy (także nazywany preeklampsią), opóźnienie wzrostu wewnątrzmacicznego i porodu przedwczesnego. Możliwość wczesnego wykrycia tych zaburzeń jest bardzo interesująca z klinicznego punktu widzenia, gdyż selekcja ciężarnych wysokiego ryzyka już w tak wczesnej ciąży pozwala na adekwatną profilaktykę i terapię a co za tym idzie ogólne polepszenie stanu zdrowia matki i dziecka.

Stan przedrzucawkowy jest bardzo poważną chorobą, która występuje tylko w okresie ciąży i okresie poporodowym. Około 2% kobiet zapada na tę chorobę. Preeklampsia jest główną przyczyną śmiertelności i ciężkich powikłań u młodych matek i noworodków. Preeklampsia charakteryzuje się wysokim ciśnieniem krwi i obecnością białka w moczu. Parametry te objawiają się jednak dopiero w momencie, kiedy choroba jest już bardzo zaawansowana. Dlatego wczesna identyfikacja ciężarnych wysokiego ryzyka jest tak ważna. W ten sposób można zapobiec zaawansowanej chorobie.

Wykrycie i pomiar biomarkerów związanych z patofizjologią preeklampsji we wczesnej ciąży wydaje się być bardzo dobrym kandydatem do testów przesiewowych. Choć dokładne pochodzenie preeklampsji nie jest do końca poznane, w ciągu ostatnich dwudziestu lat wiele się już dowiedzieliśmy na jej temat. Wiemy już, że interakcje między (1) łożyskiem, (2) cechami charakterystycznymi matki (3) i patologiczną reakcją organizmu matki na stan ciąży w końcu prowadzą do preeklampsji. Dlatego też poszukiwanie nowych biomarkerów opiera się na tych trzech aspektach choroby. W wyniku skomplikowanej patofizjologii preeklampsji należy się spodziewać, że test przesiewowy na preeklampsię będzie się opierał raczej na pomiarze wielu różnych biomarkerów, a nie tylko jednego z nich.

Celem tej pracy doktoranckiej było stworzenie nowego testu przesiewowego wykrywającego preeklampsie już w pierwszym trymestrze ciąży. Praca ta opisuje poszukiwania

nowych biomarkerów i obejmuje 4 z 5 etapów, które składają się na całą kliniczną procedurę zastosowania metody przesiewowej. Proces ten zaczyna się od zbierania informacji na temat patofizjologii preeklampsji - faza 1. Ten pierwszy etap nadaje kierunek badaniom oraz informuje nas o podłożu choroby. Faza 2 koncentruje się na ustaleniu testu, który wykryje potencjalne biomarkery zidentyfikowane w fazie 1. Następnie, możliwości i czułość tego testu są analizowane w małej skali. Wyniki zostają opisane w odniesieniu do czułości i swoistości testu. W fazie 3 test jest analizowany w dużych bazach danych posiadających informację na temat ciężarnych, tych które w przeszłości przeszły preeklampsie i tych bez powikłań. W taki sposób możemy sprawdzić czy test jest w stanie odróżnić kobiety zdrowe od chorych. W fazie 4 test jest sprawdzany w klinicznych warunkach w czasie rzeczywistym, kiedy jeszcze nie wiemy jaki będzie finał ciąży. Patrzymy na wrażliwość testu w wykryciu kobiet wysokiego ryzyka. W końcowej fazie (faza 5) wyniki testu są analizowane pod względem profilaktyki tej choroby oraz ogólnych korzyści dla zdrowia. W fazie tej analizuje się również koszty i korzyści takiego testu na większą skalę.

FAZA 1

W tej fazie doktoratu został napisany artykuł przeglądowy na temat znanych już biomarkerów wykrywających preeklampsię w pierwszym trymestrze ciąży (rozdział 2). Po wielu poszukiwaniach w literaturze medycznej zidentyfikowane zostalo siedem obiecujących biomarkerów. Biomarkery te można wykryć w krwi matki już w pierwszym trymestrze ciąży - ADAM12, fβ-hCG, PP13, PlGF, Inhibina A, Aktywina A, PAPP-A wraz z pomiarem przepływu krwi w tętnicy macicznej (Doppler) oraz specyficznymi charakterystykami matek takimi jak wiek, waga czy wzrost. Po gruntownej analizie okazało się, że niskie stężenie PP13, PlGF i PAPP-A wraz z podwyższonym poziomem Inhibiny A i nasilonym efektem Dopplera, jest w stanie przewidzieć rozwój preeklampsji już w pierwszym trymestrze ciąży. W rozdziale 3, do poszukiwania nowych biomarkerów została zastosowana technika eksploracji danych. Metoda ta opiera się na patofizjologii preeklampsji i dzięki specyficznym programom komputerowym umożliwia przeszukiwanie wielkich baz danych zawierających informację na temat związków między chorobą i wytwarzaniem specyficznych genów/białek. Ta metodyka doprowadziła do odkrycia 38 potencjalnych biomarkerów. Pomiędzy nimi znalazły się już wcześniej znane PP13, PlGF i PAPP-A oraz pięć nowych, obiecujących: FN1, HMOX1, PAPP-A2 ,PRL i VCAM-1. W rozdziale 4 do poszukiwania nowych biomarkerów została użyta technika „metabolomics". Na tym etapie zostały przetestowane dwa panele metabolitów związanych z preeklampsią. Ostatecznie 3 biomarkery okazały się obiecujące: tauryna, asparaginę i glicyloglicyna.

FAZA 2

Biomarkery wyselekcjonowane w fazie 1 (rozdział 2 i 4) zostały poddane analizie pod względem ich czułości w wykryciu preeklampsji w pierwszym trymestrze ciąży. Biomarkery pochodzące z rozdziału 2 miały specyfikę sięgającą 22% - 100%. Wyniki wykazały, że im więcej biomarkerów jest ze sobą łączonych, tym lepsza jest czułość testu. Dodatkowo

zostało ustalone, że biomarkery z rozdziału 4 mają mniejszą potencję w wykrywaniu choroby – tylko informacja o taurynie w połączeniu z informacją o ciśnienieniu krwi matki i jej specyficznych cechach fizycznych pomogła w wykryciu preeklampsji. Czułość tego testu wynosiła 55% (test był w stanie przewidzieć preeklampsię u połowy kobiet, spośród tych, które ostatecznie zapadły na tę chorobę).

FAZA 3

W tej fazie badania zostały przeprowadzone na dużych i dobrze udokumentowanych bazach danych. W tej fazie dane kobiet oraz ich krew z pierwszego trymestru ciąży były dostępne wraz z informacją dotyczącą przebiegu ich ciąży. Od początku było wiadomo, które z tych kobiet w końcu zapadły na preeklampsie, a których ciąża przebiegła bezproblemowo (rozdział 5). Podczas tego badania wynikło, że u kobiet z preeklampsią już w pierwszym trymestrze niektóre z biomarkerów są obniżone: PAPP-A, PlGF, ADAM12, a ciśnienie krwi wyższe. Na tej podstawie skonstruowany został model matematyczny, który był w stanie wyselekcjonować grupę wysokiego ryzyka, w którego skład wchodziły wyżej wymienione markery. Dzięki temu modelowi wyselekcjonowano aż do 72% kobiet wysokiego ryzyka. Interesujące okazało się, że test był w stanie wyselekcjonować do 92% kobiet z preeklampsią, u których dzieci były opóźnione we wzroście. W tej fazie zostało sprawdzone również, czy test jest w stanie przewidzieć nie tylko dzieci z opóźnieniem we wzroście, ale również dzieci przerośnięte. Dzieci przerośnięte – makrosomy – stanowią niebezpieczeństwo u kobiet z cukrzycą (rozdział 6). Czułość testu używającego tych samych markerów wynosiła 43% w przypadku makrosomów.

FAZA 4

Podczas tej fazy, najbardziej obiecujące biomarkery zostały ocenione na grupach ludzi w czasie rzeczywistym. Kobiety te były czterokrotnie badane podczas ciąży – w tygodniach 6-7, 8-9, 10-11 i 12-13. Kobiety te zostały podzielone na 4 grupy:

- grupa bardzo niskiego ryzyka – w tej grupie przeanalizowano fizjologiczny profil markerów, i ich poziom w zdrowej ciąży.
- grupa, w której kobiety zapadły na preeklampsię w poprzedniej ciąży – tu okazało się, że kobiety te mają wielokrotnie wyższe ryzyko ponownego zapadnięcia na preeklampsię oraz, że ich ciśnienie krwi jest o wiele wyższe już na samym początku ciąży w porównaniu z kobietami niskiego ryzyka.
- grupa kobiet chorych na cukrzycę – tu markery nie różniły się od grupy niskiego ryzyka.
- grupa, w której kobiety zaszły w ciążę dzięki zapłodnieniu *in vitro*. W tej grupie również nie wykryto żadnych różnic pomiędzy kobietami niskiego ryzyka a tą grupą.

Z badań tych wynikło, że kobiety, które w poprzedniej ciąży zapadły na preeklampsię należą do wysokiego ryzyka, zaś w grupie kobiet z cukrzycą bądź *in vitro* – badania przesiewowe mogą zostać wykonane bez obaw o nieprawidłowe wyniki testu.

FAZA 5

Piąty i ostatni etap poszukiwania biomarkerów zawiera standardowe badanie kliniczne randomizacyjne: oznacza to, że badani ludzie poprzez losowanie są przydzielani do dwóch grup: grupę otrzymującą standardowe badanie i leczenie oraz grupę, która jest selekcjonowana poprzez nowy test i leczona w nowatorski sposób. W tej fazie, oprócz korzyści wynikających z programu badań przesiewowych, badane są inne aspekty nowej metody, takie jak analiza kosztów i ogólne korzyści. Doktorat ten nie obejmuje tej fazy badań, niemniej jednak departament w którym został ten doktorat napisany właśnie rozpoczął badania do fazy 5.

Reasumując: wyniki badań dotyczących biomarkerów pierwszego trymestru ciąży potrzebnych do wykrycia preeklampsji są bardzo obiecujące. Choć wartość prognozyjna pojedynczych biomarkerów wydaje się być niewielka, połączenie kilku biomarkerów daje możliwość wykrycia preeklampsji już we wczesnej ciąży. Ponadto, niedawne badania terapeutyczne sugerują, że można zapobiec 50% przypadków preeklampsji poprzez podawanie małych dawek aspiryny w grupie wysokiego ryzyka. Aspiryna działa jednak jedynie w momencie kiedy zostaje podana przed 16-ym tygodniem ciąży. Jeśli jest podana później, nie ma ona już żadnego wpływu na przebieg ciąży. Dlatego wczesne wykrycie kobiet wysokiego ryzyka jest tak ważne w tym przypadku.

XIV

LIST OF CO-AUTHORS
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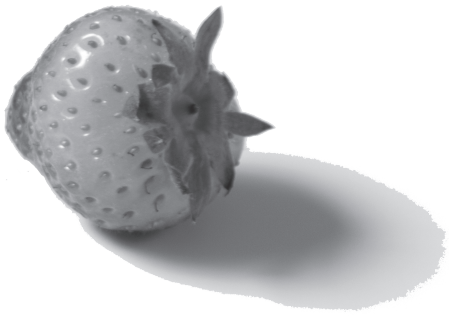
DANKWOORD/
PODZIĘKOWANIE



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