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KASPAR RATNIK

Development of predictive multimarker test for preeclampsia in early and late pregnancy

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Development of predictive multimarker test for preeclampsia in early and late pregnancy

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Human Genetics Research Group, Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

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2. Hanson, Ele; Rull, Kristiina; Ratnik, Kaspar; Vaas, Pille; Teesalu, Pille; Laan, Maris (2022). Value of soluble fms-like tyrosine kinase-1/placental growth factor test in third trimester of pregnancy for predicting preeclampsia in asymptomatic women. Journal of Perinatal Medicine, 1−8. DOI: 10.1515/jpm-2022-0127.
3. Ratnik, Kaspar; Rull; Kristiina; Aasmets, Oliver; Kikas; Triin; Hanson, Ele; Kisand; Kalle; Fischer, Krista (2022). Novel early pregnancy multimarker screening test for preeclampsia risk prediction. Frontiers in Cardiovascular Medicine, 9 (932480), 1−11. DOI: 10.3389/fcvm.2022.932480.
4. Kikas, Triin; Inno, Rain; Ratnik, Kaspar; Rull, Kristiina; Laan, Maris (2020). C-allele of rs4769613 near flt1 represents a high-confidence placental risk factor for preeclampsia. Hypertension, 76 (3), 884−891. DOI: 10.1161/HYPERTENSIONAHA.120.15346.

Contribution of the author to the preparation of the original publications:

Study 1. Participated in the overall design and led the experimental setup of the study. Performed all the experiments. Contributed critically to the development of the concept for the data analysis and modelling, analysed the data and had a key role in interpreting the outcome. Prepared the first draft of the manuscript and contributed significantly to its revision and finalization.

Study 2. Participated in the collection and processing of serum samples for the biomarker measurement. Contributed to the data interpretation and discussion, and critical reading of the manuscript.

Study 3. Participated in the overall design and led the experimental setup of the study. Performed all the experiments. Contributed critically to the development of the concept for the data analysis and modelling, analysed the data and had a key role in interpreting the outcome. Prepared the first draft of the manuscript and contributed significantly to its revision and finalization.

Study 4. Participated in the collection and processing of serum samples for biomarker measurement. Contributed to the data interpretation and discussion, and critical reading and improving of the manuscript.

ABBREVIATIONS

ACOG	The American College of Obstetricians and Gynecologists
ADAM12	disintegrin and metalloproteinase domain-containing protein 12
ALSPAC	Avon Longitudinal Study of Parents and Children
AUC	area under the curve
BMI	body mass index
CARET	R package: Classification And REgression Training
CBA	cytometric bead array
CLIA	Clinical Laboratory Improvement Amendments
DR	detection rate
ELISA	enzyme-linked immunosorbent assay
EOPE	early-onset preeclampsia
EVT	extravillous trophoblast
fc	fold change
FGR	fetal growth restriction
FIGO	International Federation of Gynecology and Obstetrics
FLT1	fms related receptor tyrosine kinase 1 gene
FMF	Fetal Medicine Foundation
FPR	false positive rate
g.day	gestational day
GAD	General Assay Diluent
GD	gestational diabetes
GH	gestational hypertension
GLM	generalized logistic regression models
GWAS	genome-wide association studies
HAPPY PREGNANCY	Development of novel non-invasive biomarkers for fertility and healthy pregnancy
HPV	Human Papillomavirus
HTN	hypertension
ISSHP	International Society for the Study of Hypertension in Pregnancy
IVD	“in vitro” diagnostics
LGA	large for gestational age
LoD	limit of detection
LOOCV	leave-one-out cross-validation
LOPE	late-onset preeclampsia
MAP	mean arterial pressure
MERS-CoV	Middle East Respiratory Syndrome coronavirus
MoM	multiple of median
NICE	National Institute for Health and Care Excellence
NIPT	non-invasive prenatal testing
NPE	non-preeclamptic
PAPP-A	pregnancy-associated plasma protein A

PE	preeclampsia
PlGF	placental growth factor
PTX3	pentraxin 3
REPROMETA	REPROgrammed fetal and/or maternal METAbolism
RIA	radioimmunoassay
ROC	receiver operating characteristic
sENG	soluble endoglin
sFlt-1	soluble fms-like tyrosine kinase-1
SGA	small for gestational age
SMCf	Serum Matrix Coefficient
SNP	single nucleotide polymorphisms
TRL	technology readiness level
TR-FRET	time-resolved fluorescence resonance energy transfer
uNK	uterine natural killer
UtA-PI	uterine-artery pulsatility index
VEGF	vascular endothelial growth factor

1. INTRODUCTION

Pregnancy is considered as a continuous period of intrauterine development in mammals that in its successful outcome ends with the delivery of a newborn. The average gestational time for humans is 40 weeks (280 days) counted from the date of last menstrual period.

Preeclampsia (PE) is a complex disorder affecting globally 3–5% of pregnancies and is responsible for >50,000 maternal deaths and >500,000 fetal and newborn deaths annually (GBD, 2015). PE is considered as the disease originating from placental malfunction leading to both fetal and maternal complications. Placenta is a unique and core organ responsible for the delivery of nutrients and oxygen to the fetus, and eliminating its generated waste. It also has a critical role in adjusting maternal physiology to the pregnancy and in contributing to the fetal developmental programming. Placental derived factors like placenta growth factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt-1) and maternal response mediators have been identified as circulatory factors relevant to pregnancy progression and outcome (Aplin et al. 2020).

PE prediction has a vital part in disease prevention and management. Traditional clinical risk factors such as parity, maternal age, previous pregnancy history, ethnic or racial background are straightforward to use but deliver moderate prediction rate with false risk estimations. The more complex modelling of PE risk in early pregnancy incorporates ultrasonography and laboratory analysis of maternal serum biomarkers PlGF or PAPPA and enables close to 90% correct prediction for early PE cases (at <34 weeks' gestation) but much less accurate prognosis for the late PE cases (Magee et al., 2022).

Immunoassays are widely used methods in basic life sciences and in clinical diagnostics. With developments from mid-20th century the protocols and chemistry have evolved generating more accurate, robust, scalable, etc., solutions. One of the used methods is microsphere based Luminex® xMAP protocol that with sandwich approach enables multiplexing and quantification of biomarkers (Graham et al., 2019).

The main aim of this thesis was to develop a novel Luminex® xMAP based multimarker 6PLEX immunoassay targeting several PE related biomarkers in a single test tube, and to generate alternative high-accuracy PE prediction models applicable either in early or late pregnancy settings.

2. LITERATURE REVIEW

2.1. Development of the placenta

Placenta is a mammalian-specific organ with a restricted average lifespan of 9 months at the start of the life of a new conceptus. It provides oxygen, nutrients to the growing fetus, and is a critical source for a range of signalling molecules, hormones and stem cells (e.g. hematopoietic) driving the fetal development and pregnancy maintenance. It also removes and transfers the fetal waste to maternal organism. Placental development is initiated as soon as implantation begins, about a week after conception with the differentiation of the first trophoblast lineages (Ruane, P. T. et al. 2017). Low oxygen environment *in utero* changes by the end of first trimester where maternal arterial circulation promotes manifold increase in oxygen concentration (Burton et al., 2010). At the beginning of the second trimester the placental maturation is not fully complete, but sufficient to support the progress of pregnancy until delivery, including fetal development, placental function, and maternal gestational physiology.

Maternal-fetal interface is a unique functional structure enabling active communication and material exchange between the mother and the fetus. Trophoblast is the outermost cell type of the placenta and is directly exposed to the maternal environment through the gestation period. These cells are further classified as cytotrophoblast or extravillous trophoblast (EVT) (Aplin et al., 2008). During the anchoring, a collums of EVT cells assuredly connects the placenta into the decidual placental bed (Baines et al., 2017). Within the endometrial invasion at ca 3rd week of gestation, the EVTs have escaped from the placenta and the penetration of maternal stroma has started. This invading step is an inflammatory process that is actively controlled by endometrium. After implementation, the anti-inflammatory phase sets off to prevent the rejection of the fetus (Mor et al., 2011). The remodelling results in a tight placental-endometrium structure containing maternal spiral arteries providing all the necessary nutrients and oxygen to intervillous space through secretion glands and waste products in the opposite direction (Aplin et al., 2008; Aplin & Ruane, 2017). Floating villus covered with protecting syncytiotrophoblasts (barrier to toxins, pathogens and most macromolecules) portals the fetal blood vessels (**Figure 1**).

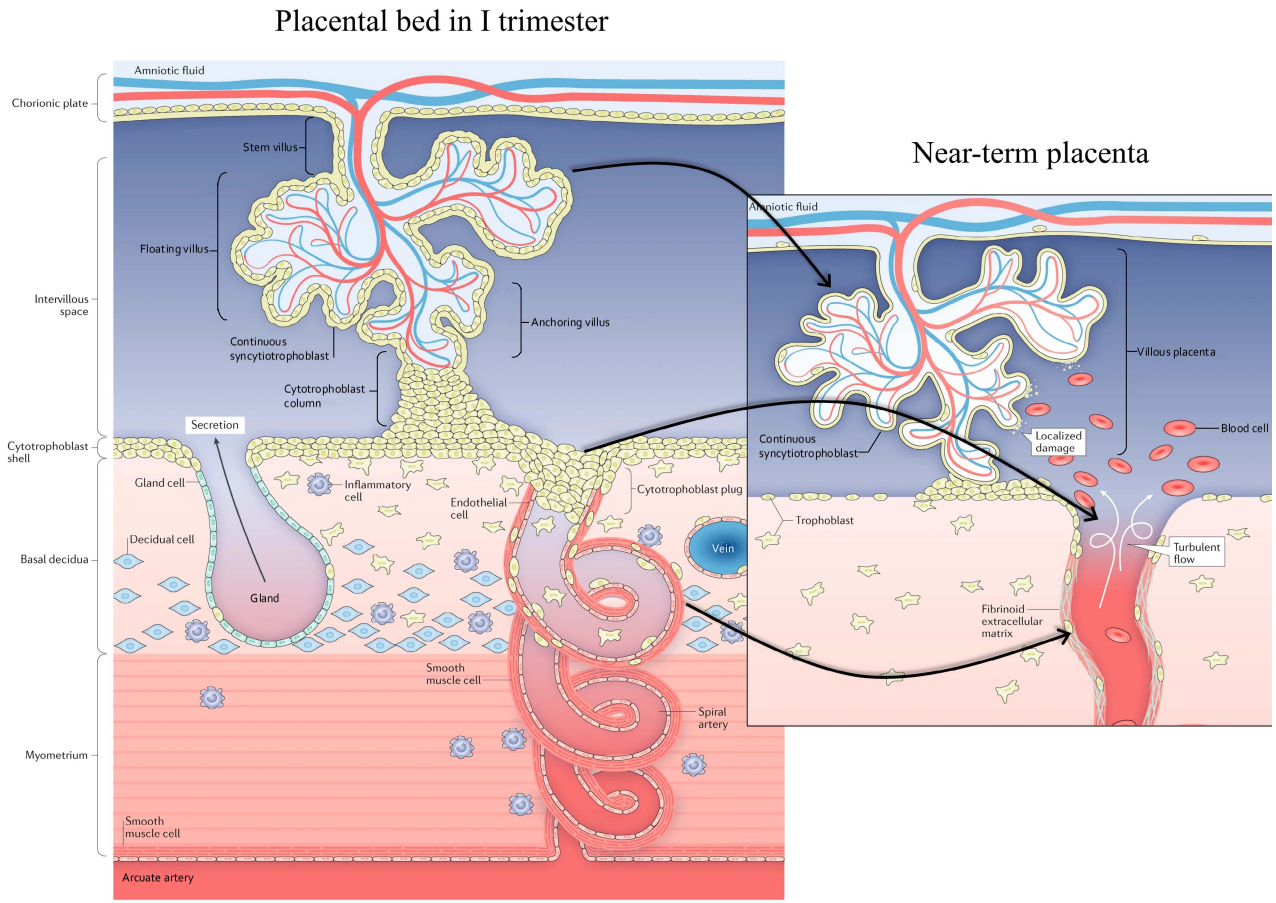

Figure 1. The structure of placental bed in the first trimester compared to near-term placenta in case of PE with turbulent blood flow into intervillous space. Adapted from Aplin et al., 2020.

In the first trimester of pregnancy the cytotrophoblast plug blocks the flow of blood cells and restricts the movement of the blood into the intervillous space (Weiss et al., 2016). From the 11th week of gestation onwards the initial remodelling of the decidual segments of spiral arteries is changing. The uterine natural killer (uNK) cells (members of innate lymphoid cells) initiate the damage of vascular extracellular matrix where the invasive trophoblasts further penetrate the basal decidua (Smith et al., 2016). Displacement of trophoblastic plugs results in maternal blood access to intervillous space and the placental interface becomes haemochorial (**Figure 1**). This remodelling continues in the second and third trimester and reaches as far as the innermost third of the myometrium. The blood vessel wall transformation is relevant in the context of potential pathological changes. If the final structure is not correct, it renders a threat to maternal hypertension (Alpin et al., 2020). The correct conversion and depth of spiral arteries allows blood to access the intervillous space at high volume but low velocity. The reduced trophoblast invasion into deeper arterial segments is widely reviewed in preeclampsia (PE) cases but not being the only cause of the syndrome (Pijnenborg et al., 2006).

2.2. Preeclampsia – the placental disease

2.2.1. Clinical presentation

Preeclampsia (PE) is considered as a disease of placental origin. It is defined as mother' s new hypertension (≥140 mmHg systolic blood pressure or ≥90 mmHg diastolic blood pressure arising after 20 weeks of gestation) with an additional feature of uteroplacental dysfunction and/or haematological (thrombocytopenia) or biochemical abnormalities (hepatic or renal function changes) indicative of widespread maternal endothelial dysfunction. The most observed associated features are fetal growth restriction (FGR) (up to 1/3 of cases) and proteinuria (75% of the cases) (ACOG, 2019; NICE, 2019). The only current known resolution to PE is childbirth.

PE affects in total close to 50,000 maternal deaths and 500,000 fetal and newborn deaths annually with overall 3–5% prevalence (GBD, 2015). The disease stress is more biased to women in low- to middle-income countries or otherwise disadvantaged (Burton et al., 2019). In Estonia with circa 13,000 live births in recent years, the PE prevalence has been decreasing from 2016 with 272 cases (1.9% from live births) to 171 cases (1.3%) in 2020 (Estonian Health Statistics and Health Research Database; www.statistika.tai.ee).

Two subtypes of PE are distinguished based on the of presentation of symptoms (Lisonkova & Joseph, 2013). Early-onset PE (EOPE) is defined with the onset between 20–34th gestational week whereas late-onset (LOPE) is considered from 34th gestational week onward (Tranquilli et al, 2013). LOPE cases form most of the patients, generally accounting for 75% of all cases. However, the remaining 25% of EOPE incidences are far more life-threatening or fatal and clearly linked with poor invasion of trophoblast resulting in insufficient conversion of spiral arteries in placental-endometrium structure (Magee et al., 2022) (**Figure 2**).

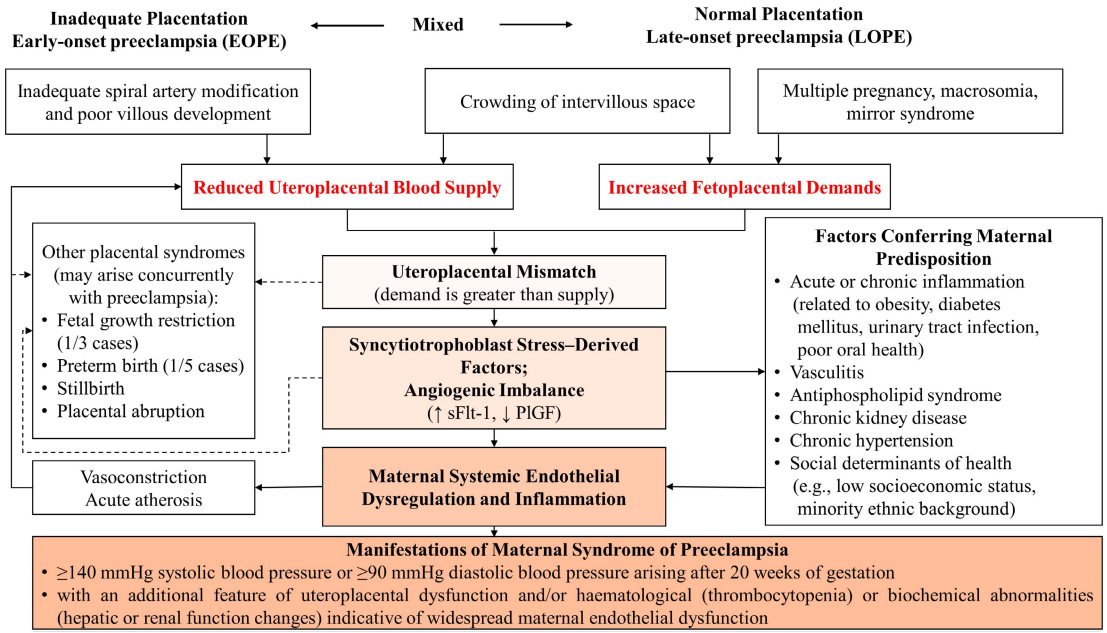

Figure 2. Pathogenesis of preeclampsia concluding the mechanism, feedbacks and additional factors boosting the syndrome symptoms. PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1
Adapted from Magee et al., 2022.

2.2.2. Pathophysiology

PE is characterized by the systemic activation of maternal vascular endothelium (linking to poor maternal immunological adaptation, conferring maternal predisposition factors, etc.) leading to inadequate spiral artery modification and poor villous development. Specifically, cytotrophoblast is secreting proangiogenic molecules such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) and their cell surface receptors (VEGFR and PlGFR accordingly) that are expressed by the villous trophoblast as well as by the endothelium in placental vessels (Zhou et al., 2002; Bushway et al., 2014). The VEGF is expressed in many isoforms, one of them being soluble fms-like tyrosine kinase-1 (sFlt-1) that also binds PlGF (Cebe-Suarez et al., 2006). Risen concentrations of sFlt-1 and extremely low levels of PlGF have been found in severe EOPE cases reflecting their crucial input to placental-maternal angiomodulatory communication. Contrary to EOPE where most of the pathology is explained by placental bed issues LOPE holds fairly normal placenta (Leavey et al., 2016).

2.3. Current approaches to predicting a risk of developing PE

Prognosis or predicting tests in medical diagnostics are clearly proven tools in most healthcare settings. An effective PE predictive test would facilitate early diagnosis, targeted surveillance, and timely delivery (MacDonald et al., 2022). Such tests could identify women who may benefit from increased clinical surveillance and carefully timed birth, both needed to reduce the PE outcome risks short and long term. Also, the test could confidently select the ones in low risk and thus safely reduce the number of antenatal visits. In addition, treatment or prophylaxis could be offered to those in the high-risk group.

Currently, there are two commonly used concepts to screening for women with increased susceptibility to PE:

a) Risk estimation based on maternal clinical and health history factors;
b) Risk estimation based on statistical modelling combining clinical, Doppler ultrasound, and maternal biomarker measurement data.

2.3.1. PE prognosis based on solely maternal clinical characteristics

Traditional screening markers for PE prediction have been used for years and integrated into most clinical practice guidelines (Scott et al., 2022). Usually, they include maternal ethnic or racial background, body mass index (BMI), blood pressure, parity, mother's age, and conception method (**Table 1**). The best PE prediction model by Poon et al. (Poon et al., 2010) reached to area under the curve (AUC) 0.79 (95% CI 0.72–0.87) for the EOPE and AUC 0.80 (95%

CI 0.76–0.83) for the LOPE just by assessing race, chronic hypertension (HTN), parity, and conception method. The same model was validated in 2013 by Park et al. (Park et al., 2013) with slightly poorer performance, AUC 0.76 (95% CI 0.74–0.77) and AUC 0.68 (95% CI 0.66–0.69) respectively. No exact sensitivity or specificity were detailed, but receiver operating characteristic (ROC) curves indicate a sensitivity of more than 70% to predict preeclampsia could be achieved at specificity of 70%.

Table 1. Maternal characteristics associated with the risk to preeclampsia.

Risk factor	Increase in the risk to develop PE	References
Parity	3-fold increased risk in nulliparous women	Duckitt et al., 2005; Hernandez-Diaz et al., 2009
Maternal age	3-fold increased risk in women aged >40 years	Duckitt et al., 2005; Khalil et al., 2013
PE in previous pregnancy	15 to 30-fold increased risk to EOPE in cases with the history of a previous PE pregnancy	Hernandez-Diaz et al., 2009
BMI	2 to 4-fold increased risk in women with pre-pregnancy BMI ≥30 kg/m^2	Poon et al., 2019
Ethnic or racial background	2 to 3-fold higher risk in women with the African, Caribbean and South Asian ethnic background	Knuist et al., 1998
Multiple pregnacy	2 to 3-fold higher risk in multiple compared to singleton pregnancy	Singh et al., 2014

2.3.2. PE prognosis using models combining clinical, ultrasound and biomarker data

As one of the known pathological errors of PE has been the disturbed transport of nutrients and oxygen flow by spiral arteries, then many prognosis models have incorporated ultrasonographic assessment of the uterine-artery pulsatility, issued as index (UtA-PI). UtA-PI or the same as transvaginal Doppler ultrasonography provides a measure of uteroplacental perfusion and high PI implies impaired placentation. The use of such method has increased the PE prediction performance in development studies to a range of AUC 0.91–0.95 (Al-Rubaie et al., 2018). The best validated study in early 2010s including predictors such as parity, history of preeclampsia, race, maternal age, family history of preeclampsia, BMI, mean arterial pressure (MAP), and UtA-PI achieved AUC 0.93 (95% CI 0.88–0.98), sensitivity 85% at fixed specificity of 90% in case of LOPE (Farina et al., 2011). A comprehensive multicentre study in 2017 with 8,775 singleton pregnancies determined the accuracy of proposed models (sampling at 11–13 gestational week) in prospective way with the best combination of MAP, UtA-PI, and PlGF achieved AUC 0.987 with detection rate (DR) of 94% (95% CI 71–100) at 5% false positive rate (FPR) for PE with delivery <32 weeks. The

same exact model reached AUC 0.792 with DR of just 32% (95% CI 25–39) at 5% FPR for PE with delivery ≥37 weeks (O'Gorman et al., 2017).

2.3.2.1. Maternal serum sFlt-1/PlGF ratio in predicting PE

PlGF and sFlt-1 have proven their use both in early and late pregnancy situation for PE prediction. PlGF, secreted by placental trophoblast cells, is detectable in lower concentrations in case of PE situation compared to non-preeclamptic (NPE) cases (Levine et al. 2004). This phenomenon is identifiable already in early pregnancy phase (during I trimester screening) and is associated with placental deficiency. The measurement done by sensitive immunoassay is expressed as multiple of median (MoM) and enables PE DR of 51% and 32% EOPE and LOPE respectively (both at a 10% false-positive rate) when assessed at gestational week 11–13 (Akolekar et al., 2008). Meta-analysis has revealed the usable PlGF cut-off 80–120 pg/mL to have PE prediction pooled sensitivity of 78% with specificity of 88% (Agrawal et al., 2019).

The sFlt-1 circulating levels in maternal blood correlate with PE symptoms intensity in late pregnancy and can be 8x higher compared to healthy cases (Levine et al. 2004). Thus, the sFlt-1/PlGF ratio has been taken into use as diagnostic tool in trimester III. First validated sFlt-1/PlGF ratio ≤33 cut-off ruled out EOPE at the time of the test with a sensitivity of 95.0% and specificity of 94% (Verlohren et al., 2014). At the same time, the ratio ≥85 predicted EOPE with a sensitivity of 88.0% and a specificity of 99.5%. In case of LOPE, the rule out cut-off (sFlt-1/PlGF ≤33) at the time of the test reached a sensitivity of 89.6% and a specificity of 73.1%. A higher sFlt-1/PlGF ratio ≥110 diagnosed LOPE with a sensitivity of 58.2% and a specificity of 95.5%. More recent PROGNOSIS study reported that applying a sFlt-1/PlGF ratio cut-off of ≤38 ruled out the onset of PE for up to 4 weeks with a high negative predictive value of 94.3%, thus listed as useful marker to isolate PE from another hypertension situation (Zeisler et al., 2019). The sFlt-1/PlGF ratio has also been used as a monitoring tool to plan time for delivery. Specifically, the sFlt/PlGF ratio ≥85 at admission to hospital predicts delivery in mean 6 days compared to ratio of <85 with respective mean of much longer 14 days (Baltajian et al., 2016). The daily increase of the sFlt/PlGF in case of PE is associated with the worsening of the pregnancy outcome, with absolute change per day for sFlt-1/PlGF being 15.1 versus 2.7 between adverse and non-adverse outcomes. In case of twin pregnancy, there is less proof to use the same sFlt-1/PlGF ratios for ruling out or in. Healthy twin pregnancies in trimester III have significantly higher levels of sFlt-1 and lower PlGF levels, thus higher sFlt-1/PlGF ratios compared to singletons. At the same time, the PE twin pregnancies had no difference in sFlt-1, but significantly higher PlGF levels compared to singletons, thus driving the sFlt-1/PlGF ratio to lower levels (49 vs. 158, $p = 0.002$) (Saleh et al., 2018).

2.3.2.2. Other maternal serum-based biomarkers of PE

Pregnancy-associated plasma protein A (PAPP-A) serum concentration is routinely used to evaluate Down syndrome in the first trimester screening, a decrease compared to healthy pregnancy is an indication of chromosomal anomalies and adverse pregnancy outcomes (Kalousová et al., 2014). By means of its proteolytic activity, PAPP-A functions as a regulatory protein in the insulin-like growth factor system, known to be important for placental formation and regulation of fetal growth (Kirkegaard et al., 2010). PAPP-A levels $<10^{th}$ percentile during trimester I screening estimate the relative risk of 3.27 to develop PE, with high-risk estimation of 9.26 to EOPE (Luewan et al., 2018). Combining the PAPP-A measurements from the first trimester with sFlt-1/PlGF ratio from mid-pregnancy period can enhance the PE DR to 87.5% at fixed FPR of 10% (Park et al., 2014). Pentraxin 3 (PTX3), mainly expressed by vascular endothelium and smooth muscle cells, regulates inflammation, activating and interacting with multiple components of the complement system (Haapsalo & Meri, 2019). PTX3 has been proposed as a novel biomarker predicting placental failure with higher levels compared to healthy pregnancy (Zhou et al., 2012, Garg et al., 2018). A disintegrin and metalloproteinase-12 (ADAM12) regulates trophoblast migration and invasion into the uterus during placental development and its reduced concentrations have been associated with pregnancies resulting in small for gestational age (SGA), fetal growth restriction (FGR) and PE (Andres et al., 2022). Soluble endoglin (sENG), a homodimeric transmembrane glycoprotein shedding from endothelial cell surface into maternal circulation, has antiangiogenic effects in preeclampsia (Luft et al., 2006). Higher levels of sENG have been reported to correlate with seriousness of PE and shorter time to delivery (Leaños-Miranda et al., 2019). Leptin has been associated with PE through insufficient energy homeostatic compensatory mechanisms. Highly linked with FGR, most studies have found upregulated leptin levels in mid to late pregnancy phase (de Knegt et al., 2021).

2.3.3. Guidelines for PE management and risk assessment

2.3.3.1. International guidelines and recommendations

There are several guidelines set for management of hypertensive disorders during pregnancy. Most recent and widely used are:

- Hypertension in pregnancy: diagnosis and management; National Institute for Health and Care Excellence (NICE, 2019);
- The hypertensive disorders of pregnancy: ISSHP classification, diagnosis & management recommendations for international practice; International Society for the Study of Hypertension in Pregnancy (Brown et al., 2018);
- Gestational Hypertension and Preeclampsia; The American College of Obstetricians and Gynecologists (ACOG, 2020).

In addition to covering the definitions and symptoms of PE, they all include paragraphs of assessment and management. It is common today to assess PE

based on Fetal Medicine Foundation (FMF) model suggesting screening at gestational weeks 11–13+6 and measuring preferably MAP, UtA-PI, PAPP-A and/or PlGF (O'Gorman et al., 2017). PAPP-A and/or PlGF concentration measurements have been agreed to be accepted from only three platforms: DELFIA Xpress system (PerkinElmer Life and Analytical Sciences), Cobas e411 system (Roche Diagnostics), B·R·A·H·M·S KRYPTOR compact PLUS (Thermo Fisher Scientific). If belonging to risk group, then aspirin usage (≥100 mg per day) is recommended on top of active lifestyle. Prophylaxis with aspirin is based on its activity to inhibit cyclooxygenase, the enzyme responsible for converting arachidonic acid into prostaglandin and thus promoting the vasodilation in placental-endometrium structure (Atallah et al., 2017). Starting uptake of low-dose aspirin between gestational weeks 12–16 reduced EOPE incidence 82%, preterm PE incidence 62%, but aspirin had no significant effect on term PE incidence (Rolnik et al., 2017). Daily calcium uptake (1–2 grams per day) also reduces the risk of PE by lowering the blood pressure (Hofmeyr et al., 2018). Simple method as exercising to maintain health and appropriate body weight are recommended (Brown et al., 2018).

2.3.3.2. PE risk assessment and management in Estonia

In2019 the adaptation of FMF model for PE prediction was initiated and in 2021 a new national guideline for assessment and management of gestational hypertensive disorders was released with aiming first trimester screening at gestational weeks 11–13+6 and assessing preferably MAP, UtA-PI, PAPP-A and/or PlGF (Rull et al., 2021).

2.4. Principles and methodological aspects of immunoassay development

Immunoassays (IA) are bioanalytical methods in which the detection of the analyte depends on the reaction of an antigen (analyte) and an antibody, essentially providing the quantitative, semiquantitative, or qualitative detection of analytes. The importance and widespreadness of IA methods in analysis are attributed to their essential specificity, high-throughput, and high sensitivity for the analysis of a wide range of analytes in biological samples (Darwish, 2006). Early methods already available from early 1960s and since the development of monoclonal antibodies by Georges Köhler and César Milstein, resulting in Nobel Prize in 1984, a new area began (Wu, 2006). Most of the IAs, developed several decades ago, were based on radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). The recent two decades have witnessed tremendous innovation in ELISA – automatisation and format change from 96-well to higher scale have significantly decreased the assay turn-around time and improved the cost-effectiveness. One of the latest developments has been the wash-free homogenous colorimetric immunoassay where gold nanoparticles

aggregation results in a red-to-blue (or -purple) color change of the bulk solutions (Liu et al., 2016). The other trend seen is multiplexing, having high potential in in vitro diagnostics (IVD) and bioanalytical sciences. Enabling multiple parameter detection from a single reaction compartment simultaneously enables both time and instrument resource savings, less sample volume needed, and added clinical value when assessing not only single analyte (e.g., characterisation of biomarker profiles to accommodate greater diagnostic resolution between closely related disease phenotypes) (Tighe et al., 2015).

The Clinical Laboratory Improvement Amendments (CLIA) and other regional regulatory bodies require validation of novel or modified multiplex assays prior to their enlistment in routine clinical diagnostics for the use of IVD landscape (Tighe et al., 2015). The whole scope of fundamental requirements is extensive and has been recently even more conditioned in the Europe Union IVD regulation (EU) 2017/746 (Cobbaert et al., 2021).

In short, the basic metrics needed to report are:

- inter- and intra-assay reproducibility;
- analytic sensitivity and specificity;
- addressing limit of detection (LoD);
- reportable reference ranges.

Inter- and intra-assay reproducibility are metrics showing coefficient of variation respectively. Analytic sensitivity and specificity characterize the accuracy of the assay protocol to measure the analyte of interest. LOD is the lowest analyte concentration likely to be reliably distinguished from the background signal and at which detection is feasible (Armbruster, 2008). Reference ranges indicate the quantitation range of analyte with the protocol in use (Cox et al., 2012). Challenges in IA are: 1) selection of antibodies and their stability, 2) immobilization of antibodies and securing this process standardization from batch to batch, 3) multiple steps of incubation and wash cycles are inevitably required in a typical immunoassay method, thus the used detergents and protocol must be optimal for the best outcome.

2.5. Use of Luminex® xMAP in diagnostic settings

The Luminex® xMAP system is a multiplexed microsphere-based suspension array platform enabling detection of up to 500 targets from single reaction. Using the patented xMAP beads the platform enables detection of nucleic acids, proteins, antibodies. The open architecture has enabled its implementation in a variety of applications such as pathogen detection, biomarker discovery and validation, personalized medicine, transplant medicine, drug discovery, vaccine development, neurodegeneration, and cancer research (Graham et al., 2019). In principle the system is based on xMAP beads and corresponding instrument enabling digital fluorescence signal detection. Covalently covering the beads with target complementary analyte (ssDNA, antibody or antigen) enables capturing

of the analyte of interest by incubation or hybridization following qualitative or quantitative detection via streptavidin-phycoerythrin conjugate (**Figure 3**).

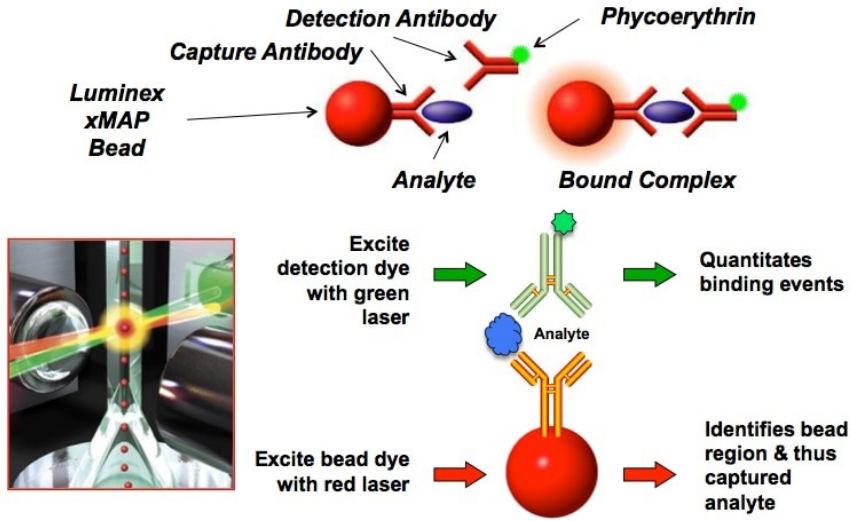

Figure 3. Luminex® xMAP principle in sandwich immunoassay.
Adapted from https://www.luminexcorp.com/.

For example, a six-plex respiratory virus panel was developed for sensitive detection of influenza virus type A and type B, para-influenza virus type 3, respiratory syncytial virus, human metapneumovirus and Middle East Respiratory Syndrome coronavirus (MERS-CoV) (Yan et al., 2017). A 27-plex Human Papillomavirus (HPV) DNA detection assay was used to assess the genotype prevalence in cervical cancer patients (García et al., 2011). Single nucleotide polymorphisms (SNPs) have been analyzed in pharmacogenetics using the Luminex® xMAP system (Spierings & Dunbar, 2013). A group from Italy used the platform for HLA-typing in donor blood testing (Guarene et al., 2018). Luminex based cytokine assay was compared to multiple other immunoassays (such as enzyme-linked immunosorbent assay (ELISA), time-resolved fluorescence resonance energy transfer (TR-FRET), cytometric bead array (CBA)), and concluded to have superior performance in the sensitivity and dynamic range (Platchek et al., 2020).

2.6. Rationale of the doctoral thesis project

Growing knowledge about the pathogenesis of PE and recent advances in biomarker detection have enabled the development and clinical implementation of various PE prediction models. These models are gradually getting more precise and specific, permiting early identification, prophylaxis, and management options. Still, a prediction relying on complex ultrasound methods cannot be made accessible everywhere and thus easy and cost-friendly solutions are welcome. Also, the high rate of false positive predictions especially in LOPE in the current early pregnancy PE screening approaches drives clinicians and scientists to look for novel methods to provide the most optimal PE screening tools. There is a need to raise the clinical decision confidence and also patients' satisfaction with judgment.

3. AIMS OF THE PRESENT STUDY

The overall aim of this thesis was to explore the dynamics and value of proposed serum biomarkers of PE in predicting the risk for the disease, and to combine informative biomarkers and clinical data to develop novel PE prediction models applicable either in early or late pregnancy.

The specific aims of this thesis were:

1. To develop a novel multiplex immunoassay protocol 6PLEX incorporating the measurements of multiple PE biomarkers in a single test tube.
2. To exploit 6PLEX assay measurements for the development of PE prediction model applicable during 2nd half of pregnancy and to compare its performance with the currently clinically used sFlt-1/PlGF ratio-based approach.
3. To combine 6PLEX immunoassay data with clinical and genetic risk factors to develop novel PE prediction models applicable during 10–14th gestational weeks.

4. MATERIAL AND METHODS

4.1. Ethics statement

The protocols for the HAPPY PREGNANCY (full name "Development of novel non-invasive biomarkers for fertility and healthy pregnancy") and REPROMETA (full name "REPROgrammed fetal and/or maternal METAbolism") studies were reviewed and accepted by the Ethics Review Committee of Human Research of the University of Tartu, Estonia (permissions 221/T-6, 17.12.2012; 286/M-18, 15.10.2018).

All subjects provided written informed consent. All procedures and methods have been carried out in compliance with the guidelines of the Declaration of Helsinki.

4.2. Study subjects

All study subjects analyzed in the framework of this doctoral project have been recruited at Women's Clinic, Tartu University Hospital. Overview of the pregnancy-related samples analyzed in this study is provided in **Table 2**.

Table 2. Sample sets used in the studies.

Sample set	Study 1. Ratnik et al., 2020	Study 2. Hanson et al., 2022	Study 3. Ratnik et al., 2022	Study 4. Kikas et al., 2020
		HAPPY PREGNANCY		
Maternal cases	22 PE/31 NPE	24 PE/12 GH / 142 NPE	22 PE/31 NPE	18 PE/135 NPE
Placental DNA	n.a.	n.a.	22 PE/29 NPE	44 PE/1724 NPE
		REPROMETA		
Placental DNA				52 PE/227 NPE

n.a., not applicable; GH, gestational hypertension; NPE, non-preeclampsia; PE, preeclampsia

Detailed data of HAPPY PREGNANCY samples used in Studies 1 and 3 is characterized in **Table 3**. Detailed facts for Study 1 (29 PE/32 NPE serum samples) can be seen in publication by Ratnik et al., 2020 and Study 3 (60 PE/ 72 NPE serum samples) by Ratnik et al., 2022 Table 1.

Table 3. Clinical characteristics and preeclampsia risk factors of analyzed pregnancies.

Parameter	Analyzed index gestations	
	Controls with no PE	PE pregnancies
Number of subjects	31	22
Number of serum samples	72	60
Maternal age (years)	28.5 ± 5.1 28 (18 – 39)	28.0 ± 5.2 26.5 (20 – 39)
Maternal BMI at conception (kg/m2)	25.7 ± 4.8 25.5 (16.1 – 38.9)	26.8 ± 6.2 26.3 (18.0 – 42.9)
Mean arterial blood pressure at first visit (mm Hg)	84.3 ± 7.7 85.0 (66.0 – 96.6)	86.9 ± 9.1 86.6 (73.3 – 103.3)
Gravidity (n)	1.9 ± 1.3 2 (1 – 6)	1.7 ± 1.2 1 (1 – 4)
Smoking during pregnancy	1	1
Maximum SBP (mm Hg)	128.9 ± 12.1 125 (100 – 50)	159.1 ± 11.6* 160 (135 – 177)
Maximum DBP (mm Hg)	80.2 ± 12.5 80 (60 – 100)	101.6 ± 8.9* 100 (80 – 122)
Nulliparity (n), %	16 (52%)	17(77%)
Obesity, BMI >30 kg/m2 (n)	5 (16%)	6 (27%)
Previous PE or hypertensive disorder/multiparous women (n)	4/12	3 /5
Pre-existing hypertension	2	0
Family history of gestational hypertension and PE	0	2
Pre-existing diabetes / gestational diabetes	0/3	1/0
Pregnancy after IVF	0	1
Hypertension or single hypertensive measurement during the pregnancy (n), %	15 (48%)	22 (100%)*
Proteinuria during the pregnancy (n), %	1 (3%)	22 (100%)*
Gestational age at birth (days)	279.6 ± 11.3 283 (236 – 292)	257.0 ± 20.8* 261.5 (199 – 284)
Preterm delivery <37 gest. weeks (n)	1 (3%)	7 (32%)*
Newborn weight (g)	3579 ± 581 3558 (1510 – 4442)	2643 ± 728* 2642 (814 – 4274)
SGA newborn (n)	3 (10%)	9 (41%)*
Newborn's sex (F/M)	16/15	10/12
Labor induction (n)	3 (10%)	11 (50%)*
Delivery mode (vaginal/C-section)	27/4	15/7*

Data are presented as mean ± standard deviation and median (min-max values), except where indicated differently.
*Statistically significant difference ($p<0.05$). Chi-squared test for categorical and Mann-Whitney U-test for non-categorical variables were applied.

4.2.1. Recruitment and serum sampling in the HAPPY PREGNANCY cohort study

Participants of HAPPY PREGNANCY study were recruited at the Women's Clinic of Tartu University Hospital, Estonia during 2013–2015 (Archimedes Foundation, grant no 3.2.0701.12-0047 to Maris Laan). In total 2,334 pregnant women were recruited prospectively during their first antenatal visit. The patients were asked to fill out three questionnaires throughout their pregnancy concerning epidemiological data, reproductive history, parental lifestyle, and additional pregnancy course and outcome data collected from the medical records. HAPPY PREGNANCY consisted of 61 PE pregnancies in total. Placental samples were available from 1,768 singleton deliveries (44 PE and 1724 NPE).

Blood samples from the HAPPY PREGNANCY study participants were collected into Becton Dickinson Vacutainer® SST™ Serum Separation Tubes containing spray-coated silica and a polymer gel (Becton Dickinson Company, Franklin Lake, NJ, USA) during routine blood tests throughout pregnancy. Serum was separated in the service laboratory (United Laboratories, Tartu University Hospital) using routine procedures according to manufacturer's instructions (centrifugation at 1,800 g for 10 min at room temperature, RT). Serum samples were kept at -80°C before further aliquoting and subsequent analysis.

4.2.2. Recruitment and characteristics of the REPROMETA study

Participants of REPROMETA (full name "REPROgrammed fetal and/or maternal METAbolism") study were recruited at the Women's Clinic of Tartu University Hospital, Estonia during 2006–2011 (grant #55005617 from Howard Hughes Medical Institute HHMI and ETF9030 from Estonian Science Foundation to M.L.). All 377 participants were of Caucasian ancestry and focussing on recruitment of extreme cases of selected PE (n=53), gestational diabetes (GD) (n=50), small for gestational age (SGA) (n=72) and large for gestational age (LGA) (n=97) and healthy (n=105). Pregnancy outcome data was acquired from the medical records. Placental samples were available for 366 cases.

Cases with known fetal anomalies, chromosomal abnormalities, inherited diseases, and pre-existing diabetes mellitus, chronic hypertension, or chronic renal disease were excluded from the REPROMETA study.

4.2.3. Definition of pregnancy-related phenotypes

PE cases were defined as in ISSHP (Brown et al., 2018). Gestational hypertension (GH) group was distinguished in study 2 (Hanson et al., 2022) as a new onset hypertension after 20 gestational week without proteinuria or another maternal organ dysfunction. In other sub-studies in this dissertation, GD patients were included in the healthy subgroup, defined as NPE pregnancy. Prenatal growth of newborns was evaluated in the context of the Estonian Medical Birth Registry growth standards (Sildver et al., 2015).

4.3. Luminex® xMAP based 6PLEX immunoassay

Development of the Luminex® xMAP based assay was targeted at previously established maternal serum biomarkers for PE prediction: PlGF, sENG, sFlt-1, leptin, ADAM12 and PTX3 (**Table 4**). Initial development and quality assessment of the Luminex® singleplex and multiplex immunoassays were performed using a commercially available standardized pool of human serum samples ('Serum Matrix'; S1-100ML, Human Serum; Merck KGaA, Darmstadt, Germany).

Table 4. List of antibodies and reference proteins utilized in the developed Luminex® 6PLEX immunoassay.

Biomarker	Uniprot #	Standard protein	Capture antibody	Detection antibody
ADAM12	O43184	Recombinant Human ADAM12, CF, #4416-AD-020 (R&D Systems)	Anti-ADAM Monoclonal Mouse IgG Clone #632525 (R&D Systems)	Biotinylated Anti-human ADAM12 Antibody Sheep IgG BAF4416 (R&D Systems)
Leptin	P41159	Recombinant Human Leptin, CF, #398-LP-01M (R&D Systems)	Anti-Leptin Monoclonal Mouse IgG Clone #44802 (R&D Systems)	Human Leptin Biotinylated Antibody Monoclonal Mouse IgG BAM398 (R&D Systems)
PTX3	P26022	Recombinant Human Pentraxin 3/TSG-14, CF, #1826-TS-025/CF (R&D Systems)	Anti-Pentraxin 3 Monoclonal Mouse IgG Clone #247911 (R&D Systems)	Human Pentraxin 3 Biotinylated Antibody Polyclonal Goat IgG BAF1826 (R&D Systems)
PlGF	P49763	Recombinant Human PlGF, CF, #264-PG-010/CF (R&D Systems)	Anti-PlGF Monoclonal Mouse IgG Clone #37203 (R&D Systems)	Human PlGF Biotinylated Antibody Polyclonal Goat IgG BAF264 (R&D Systems)
sENG	P17813	Recombinant Human Endoglin/CD105, CF, #1097-EN-025/CF (R&D Systems)	Anti-ENG Monoclonal Mouse IgG Clone #166713 (R&D Systems)	Human Endoglin Biotinylated Antibody Polyclonal Goat IgG BAF1097 (R&D Systems)
sFlt1	P17948	Recombinant Human VEGF R1/Flt-1 Fc Chimera, CF, #321-FL-050/CF (R&D Systems)	Anti-Flt1 Monoclonal Mouse IgG Clone #49560 (R&D Systems)	Antigen Affinity-purified Human Flt-1 Polyclonal Goat IgG Biotinylated BAF321 (R&D Systems)

PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; sENG, soluble endoglin; ADAM12, disintegrin and metalloproteinase domain-containing protein 12; PTX3, pentraxin-related protein 3, TNF-inducible gene 14 protein

The applied Luminex® sandwich immunoassay protocol followed the manufacturer's guidelines (The xMAP® Cookbook, https://www.luminexcorp.com/). Experiments and the estimation of analytical accuracy, Serum Matrix Coeffi-

cient (SMCf), LoD, intra- and inter-assay variability are detailed in the Results section and in **Study 1**. All measurements were carried out on Luminex® MAGPIX instrument.

4.4. Measurement of sFlt-1 and PlGF with the B·R·A·H·M·S Kryptor assay

The analyzed serum samples had been stored in -80° C with no thawing for maximum 1.5 years. Prior immunoassay applications, all serums were thawed on ice and aliquoted.

For study 1, 61 serum samples were shipped on dry ice to accredited medical laboratory service provider Synlab Germany (Leinfelden, Germany) offering the B·R·A·H·M·S sFlt-1 Kryptor (#845.075) / PlGF (#859.075) plus Kryptor PE ratio test (Thermo Fisher Scientific). Same aliquot was subjected to analysis with the Luminex® 6PLEX assay followed the developed protocol.

For study 2, aliquots of clinical serum samples were shipped on dry ice to SYNLAB Germany (Leinfelden, Germany) for sFlt-1 and PlGF measurements at the B·R·A·H·M·S platform (Thermo Fisher Scientific). Concentrations of sFlt-1 and PlGF were measured simultaneously for all 239 shipped samples. Study 1 analyzed 61 samples representing 180–275 g.days and Study 2 included 178 pregnant women sampled during 195–254 g. days. In both studies, the sFlt-1 and PlGF measurements were performed 'blindly', and the service provider had no access to the information to the preeclampsia/not preeclampsia status of the analyzed cases.

4.5. Placental collection for DNA research and genotyping

Collection of placental samples, extraction and genotyping of placental DNAs was carried out by other team members and is in detail described in Study 4. Briefly, collected placental tissues were washed with 1xPBS (pH~7.4) to remove maternal blood. A full-thickness block of 2 cm was taken from the middle region of each placenta and stored in dry tube at -80°C for DNA extraction. For genotyping of placental rs4769613 T/C premade Taqman Genotyping Assay was used according to manufacturer's protocol (Applied Biosystems, Foster City; Assay ID: C__32231378_10).

4.6. Statistical analysis and data modelling

Summary estimates of the data were calculated, and all statistical tests were implemented using the STATA software ver. 13.1 (StataCorp TX, USA) or the R 3.3.3 language and environment (Free Software Foundation, Boston, MA, USA, http://www.r-project.org). To compare groups, Mann-Whitney U-test was used

for continuous variables and Chi2 test for categorical variables. $P<0.05$ was considered statistically significant.

Generalized logistic regression models (GLM) were applied to investigate associations between biomarker measurements and clinical onset of PE during the index pregnancy. All biomarker values were centred and scaled before modelling for data normalization and standardization. For 3rd trimester study (Study 1), the models were developed using prospective measurements of 56 serum samples drawn after 179th g.day from cases who developed after 4–62 days PE (n=24) and from healthy pregnant women (n=32). Models were built using three settings: 1) only biomarker measurements; 2) biomarker data corrected for gestational age at the blood draw and leptin to maternal weight; 3) biomarker data, gestational age and maternal weight at sampling.

The predictive power of the best models was assessed using the AUC with the threshold AUC > 0.7. For every model, a corresponding formula was developed along with the calculated threshold value for PE prediction, and coefficients for the included biomarkers and clinical characteristics. The function 'predict' in the package 'stats' was used to obtain individual predictions from a fitted GLM model objects (for individual cases). Application of the formula generates the PE prediction value p(i) for the analyzed patient across a forthcoming period up to two months. The p(i) equal or superior to a threshold value indicates that the subject will develop PE or has PE, whereas the p(i) inferior to a threshold value rules out PE development.

For 1st and 2nd trimester study (Study 3), the first step was automated computational pre-filtration for identification of the optimal prediction model with stepAIC selection method (generalized linear model with stepwise feature selection) in package CARET (Classification And REgression Training). This machine learning strategy in combination of leave-one-out cross-validation (LOOCV) allows to select the statistically most significant prediction model and to pick the complexity parameters that are associated with the optimal resampling statistics. Pre-filtration was carried out by using following input variables: measured concentrations of six biomarkers (ADAM12, Leptin, Pentraxin3, sENG, sFlt-1, PlGF) and maternal characteristics of blood sampling time in g.days, maternal weight at blood draw and parity as binary variable (nulliparity defined as 0 and multiparity defined as 1). As an output of this procedure, all possible models generated from these input parameters were automatically ranked based on their AUC estimates. The best predicted model by the LOOCV + stepAIC approach was developed further by alternatively replacing and/or adding biomarker measurements to trial the model performance using GLM package in R. Additionally, statistical models were built combining parameter combinations with the placental genotypes of the SNP rs4769613 T/C (in additive manner, defined as variables 0, 1 or 2 according to genotypes TT, CT and CC respectively) either by replacing parity with the SNP data or considering them both.

The predictive power of the models was assessed using the ROC curve and AUC. For every fitted model, model-based individual predictions were obtained, as estimated probabilities of PE (during the index pregnancy until term),

denoted as p(i) with Epi::ROC(). The p(i) represents the probability thresholds at the maximum Youden's J index on the curve. The p(i) equal or superior to a fitted model optimal cut-off point value indicates that the subject will develop PE or has PE, whereas the p(i) inferior to a fitted model optimal cut-off point value predicts that PE will not develop.

The difference in median *FLT1* or sFlt-1 between PE and NPE individuals was assessed using the Student t-test with Bonferroni correction (Study 4).

5. RESULTS

5.1. Luminex® immunoassay based 6PLEX test for PE prediction (Study 1)

The first study (Study 1) developed and tested the analytical performance and properties of the new 6PLEX multiplex assay in predicting the risk of developing preeclampsia. This forms the core part of the doctoral thesis where comprehensive optimization of assay was conducted followed by applying the protocol to HAPPY PREGNANCY cohort.

5.1.1. Experimental development of a multiplex immunoassay

Development of the Luminex® xMAP based multiplexed assay was targeted to six previously established maternal serum predictive biomarkers for preeclampsia: ADAM12, PlGF, sENG, sFlt-1, PTX3 and Leptin (**Materials and Methods, Table 4**). All reagents were commercially purchased, and the assay optimization was carried out based on data available from scientific literature and according to CLSI Guidelines.

For all biomarkers, the optimization series tested 20 alternative combinations of 0.24–6 µg capture antibody (bound to 1.25x106 magnetic beads) and 1–8 µg/mL monoclonal or polyclonal detection antibody to capture the expected concentration range of the biomarker based on the literature reports. The best performing antibody combinations for each biomarker detection is shown in **Table 5**.

Table 5. Expected concentration ranges of biomarkers and the optimised concentration ranges of the used antibodies in the final assay protocol.

Biomarker	Expected concentration range in sera	Optimised conc of capture antibody bound to $1.25x10^6$ Luminex xMAP® magnetic microspheres	Optimised conc of detection antibody
sFlt-1	500–50,000 pg/ml	6 µg	8 µg/mL
PlGF	10–2,000 pg/ml	6 µg	2 µg/mL
ADAM12	30–1,500 ng/mL	6 µg	4 µg/mL
sENG	3–200 ng/ml	1.2 µg	1 µg/mL
leptin	10–40 ng/ml	6 µg	4 µg/mL
PTX3	0.4–40 ng/mL	6 µg	0.5 µg/mL

PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; sENG, soluble endoglin; ADAM12, disintegrin and metalloproteinase domain-containing protein 12; PTX3, pentraxin-related protein 3, TNF-inducible gene 14 protein

The optimal multiplex setup resulted in good analytical performance to comparable singleplex measurement with Spearman' s correlation of 0.83–1.00. Also, the sample' s serum matrix effect (represented as Serum Matric Coefficient factor (SMCf)) in multiplex format was estimated to be low, varying between 77–104% in 1:10 and 72–146% in 1:20 serum dilution (**Table 6**). LoD metrics assessed in 1:20 serum dilution was following: sFlt-1 164.20 pg/µL, PlGF 7.19 pg/µL, ADAM12 0.06 ng/µL, sENG 0.05 ng/µL, leptin 0.44 ng/µL and PTX3 1.30 ng/µL.

Table 6. SMCf in measuring biomarker concentrations in human serum in multiplex assay format. SMCf represents the ratio of the median fluorescence intensity measurements of the reference proteins spiked into the commercial Serum Matrix relative to the respective analytes diluted in General Assay Diluent (GAD).

Biomarker	Parameter	Concentration level of the spiked biomarker		
		High	Medium	Low
ADAM12	Spiked biomarker [C] (ng/mL)	200	20	2
	SMCf 1:10 dilution	91%	97%	91%
	SMCf 1:20 dilution	102%	98%	95%
Leptin	Spiked biomarker [C] (ng/mL)	10	1	0.1
	SMCf 1:10 dilution	78%	90%	88%
	SMCf 1:20 dilution	106%	128%	146%
PTX3	Spiked biomarker [C] (ng/mL)	10	1	0.1
	SMCf 1:10 dilution	77%	97%	104%
	SMCf 1:20 dilution	89%	72%	78%
PlGF	Spiked biomarker [C] (ng/mL)	500	50	5
	SMCf 1:10 dilution	85%	82%	81%
	SMCf 1:20 dilution	106%	97%	133%
sENG	Spiked biomarker [C] (ng/mL)	10	1	0.1
	SMCf 1:10 dilution	84%	84%	79%
	SMCf 1:20 dilution	99%	97%	97%
sFlt-1	Spiked biomarker [C] (ng/mL)	10,000	1,000	100
	SMCf 1:10 dilution	88%	85%	83%
	SMCf 1:20 dilution	100%	115%	115%

PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; sENG, soluble endoglin; ADAM12, disintegrin and metalloproteinase domain-containing protein 12; PTX3, pentraxin-related protein 3, TNF-inducible gene 14 protein; SMCf, Serum Matric Coefficient factor

Intra-assay reproducibility was estimated in 1:20 serum dilution with HAPPY PREGNANCY samples in duplicate with variation of ca 3–20%. Inter-assay metrics were obtained with spiking of reference protein on two parallel plates with variation of ca 2–8% (**Table 7**).

Table 7. Intra-assay and inter-assay coefficients of variation of the biomarkers measured using the developed Luminex® 6PLEX assay.

Biomarker	Intra-assay			Inter-assay	
	P[a]	CV%	mean [C]	CV%	mean [C]
ADAM12	P10	10.08%	578.73 ng/mL	5.29%	396.85 ng/mL
	P10-P90	9.99%	1,295.32 ng/mL		
	P90	3.23%	2,601.80 ng/mL		
Leptin	P10	7.08%	9.04 ng/mL	1.88%	35.21 ng/mL
	P10-P90	5.83%	39.59 ng/mL		
	P90	5.14%	102.54 ng/mL		
PTX3	P10	n.d.	n.d.	2.00%	8.37 ng/mL
	P10-P90	22.26%	0.86 ng/mL		
	P90	8.80%	4.68 ng/mL		
PlGF	P10	15.06%	305.12 pg/mL	7.84%	860.69 pg/mL
	P10-P90	11.11%	2,010.91 pg/mL		
	P90	3.21%	15,116.94 pg/mL		
sENG	P10	4.15%	2.39 ng/mL	5.60%	18.37 ng/mL
	P10-P90	5.39%	6.81 ng/mL		
	P90	6.46%	18.02 ng/mL		
sFlt-1	P10	2.74%	3,790.10 pg/mL	4.18%	2413.63 pg/mL
	P10-P90	5.28%	11,632.10 pg/mL		
	P90	5.78%	32,755.20 pg/mL		

ADAM12, disintegrin and metalloproteinase domain-containing protein 12; CV%, coefficient of variation%; n.d., not detectable; PlGF, placental growth factor; PTX3, pentraxin-related protein 3; sENG, soluble endoglin; sFlt-1, soluble fms-like tyrosine kinase-1

[a] P10, <10%; P10–90, within 10–90%; P90, >90%

5.1.2. Comparison of Luminex® 6PLEX and B·R·A·H·M·S Kryptor assays

Several commercial companies provide immunoassay-based tests for the measurement of maternal serum concentrations of two well-established PE biomarkers, sFlt-1 and PlGF (DELFIA Xpress system by PerkinElmer Life and Analytical Sciences), Cobas e411 system by Roche Diagnostics, B·R·A·H·M·S KRYPTOR compact PLUS by Thermo Fisher Scientific™). In this study, parallel serum samples of the same clinical cases were selected for 'blind' analysis of sFlt-1 and PlGF serum concentrations using outsourced B·R·A·H·M·S Kryptor immunoassay platform (Thermo Fisher Scientific™) to compare these data with the Luminex® 6PLEX measurements.

Parallel analysis revealed differences in detected concentration ranges – sFlt-1 on Luminex® 2,207–39,417 pg/mL vs. on B·R·A·H·M·S 817–11,010 pg/mL; and PlGF 7.19–28,031 pg/mL vs. 12–998 pg/mL, accordingly (**Figure 4**).

However, the sFlt-1/PlGF ratio estimates had high correlations between the two platforms (Spearman's r=0.93, $P<0.0001$). sFlt-1/PlGF based PE rule-in evaluation was successful with both assays among asymptomatic pregnant women within 27 days after serum sample collection, group III vs group I ($P<0.0005$; Mann-Whitney U-test). sFlt-1/PlGF ratios of both tests also discriminated equally well the patients with isolated clinical symptoms that progressed to PE onset within 4–43 days ($P<0.0005$; Mann-Whitney U-test).

Take home message: Study 1 described the novel 6PLEX assay basic characteristics being precise and having low intra- and interassay variations. Furthermore, comparison with commercially IVD validated B·R·A·H·M·S Kryptor platform expressed high correlation and similar discrimination power based only on sFlt-1/PlGF.

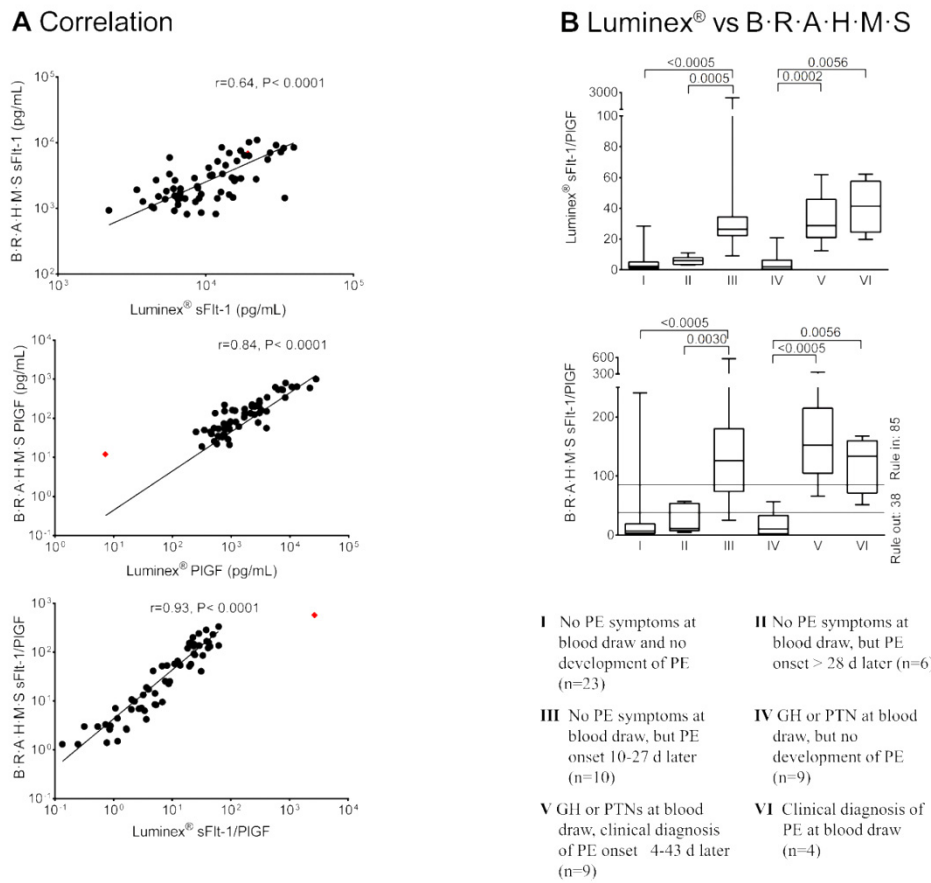

Figure 4. Luminex® based 6PLEX assays show good correlation in PlGF and sFlt-1 measurements from maternal serum. The discrimination power of sFlt-1/PlGF to different patient groups is equally good in case of Luminex® based 6PLEX and B·R·A·H·M·S Kryptor.

5.2. PE prediction using prospectively collected serum samples from late pregnancy

5.2.1. Limitation of the currently used sFlt-1/PlGF test (Study 2)

B·R·A·H·M·S Kryptor based sFlt-1/PlGF analysis for late pregnancy situation was assessed in order to gain a more detailed insight into its prediction power using a broader asymptomatic sample set. Serum sFlt-1 and PlGF were measured in serum samples collected at mean gestational age of 229 days (range 206–257) from 178 pregnant women participating in the HAPPY PREGNANCY study (Study 2, Table 1). The measurements were performed at the

commercial B·R·A·H·M·S Kryptor immunoassay platform (Thermo Fisher Scientific) from the same aliquote-batches as 6PLEX measurements.

All results were formalized as sFlt-1/PlGF ratio and divided into low-risk (sFlt-1/PlGF≤38) and-risk risk (sFlt-1/PlGF>38) cases, 149 and 29 respectively (**Table 8**). The estimated sFlt-1/PlGF ratio >38 was able to distinguish two critical pregnancy parameters. Median time from sampling to delivery was significantly shorter for cases with sFlt-1/PlGF ratio >38 compared to the low-risk group (29 vs 50 days; $P=2.0\times10^{-7}$). Also, the cases with measured sFlt-1/PlGF ratio >38 had earlier gestational age at delivery compared to pregnant women with sFlt-1/PlGF≤38 (median of 265 vs 282 g.day; $P=3.0\times10^{-6}$).

The detection rate (DR) of PE prediction development with sFlt-1/PlGF ratio cut-off value 38 was calculated to be 83.3% (with FPR of 3.0%) in case of limiting the PE prediction period to 30 days. The general DR including all samplings was moderate, 58.3% (with FPR 9.7%).

Table 8. Maternal characteristics and pregnancy outcome stratified by sFlt-1/PlGF.

Parameter	Ratio ≤ 38	Ratio > 38	*P* – value
Study subjects (n)	149	29	
Gestational age at sampling (days)	227 (206 – 251)	230 (210 – 257)	0.2
Time analysis until delivery (days)	50 (29 – 78)	29 (12–64)	2.0×10^{-7}
Gestational age at delivery (days)	282 (263-292)	265 (233 – 289)	3.0×10^{-6}

Modified from Hanson et al., 2022. Data are given as median (5th–95th percentiles).
GH, gestational diabetes; PE, preeclampsia; PlGF, placental growth factor; sFlt-1, soluble fmf-like tyrosine kinase-1

Take home message: Study 2 confirmed the limits of B·R·A·H·M·S sFlt-1/PlGF ratio-based approach performance for PE prediction in asymptomatic women. Satisfying performance is only reached within short timeframe assessment (PE diagnosis in 30 days) that is not acceptable for clinical management.

5.2.2. PE prediction models using the Luminex® 6PLEX assay based multimarker measurements (Study 1)

Multifactorial PE prediction models based on logistic regression were generated to understand the compound effect of each biomarker. As shown in Study 2, relying only on two biomarkers allows for limited prediction. Thus, the dataset acquired with 6PLEX assay was admitted to challenge the current sFlt-1/PlGF ratio-based approach.

Retrospective utilization of the 6PLEX measurements enabled together 30 alternative models with AUC values from 0.910–0.993. Notably, the best per-

formance (**Figure 5**) was achieved with inclusion of five parameters (sFlt1, PlGF, ADAM12, sENG, leptin) and adjustment for gestational age and maternal weight at the blood draw (Model 3A) resulting in AUC of 0.993 (95% CI 0.97–1.00), sensitivity 100% (95% CI 100–100%) and specificity 96.9% (95%CI 91–100%). 55 out of 57 cases (96.5%; 95% CI 87.9–99.6%) were correctly prognosed in regard to PE development in general. To note, prediction model 1C without cofactors and leptin yielded 87.7% accuracy (95%CI 76.6–93.9%) with a total of 7 false predictions (AUC 0.958, 95%CI 0.90–0.99). In case of B·R·A·H·M·S measurements, the PE predictions outcome was AUC 0.872 (95%CI 0.76–0.96%) or 0.867 (95%CI 0.76–0.95%) accordingly with and without cofactors.

6PLEX based PE prediction model 3A resulted in only two false-positive cases without any false-negatives. One developed gestational hypertension 49 days after blood draw, shortly followed by an elective C-section due to fetal breech position. The second had minimal elevation of 0.0004 points over model threshold value of 0.175. Model 1C resulted in one false-negative case (sample drawn 62 days before the onset of PE) and six false positive cases. The B·R·A·H·M·S sFlt-1/PlGF assay failed in two false positive, five false negative, and eight inconclusive test outcomes (sFlt-1/PlGF ratios between 40–65).

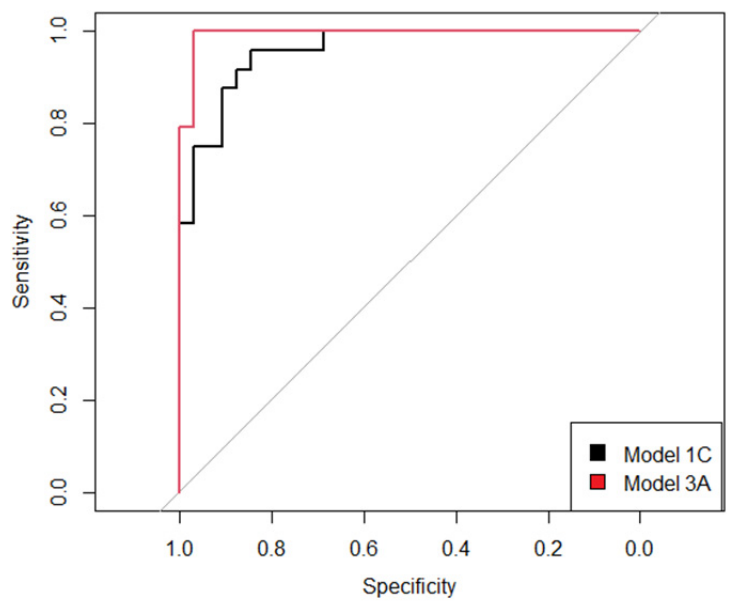

Figure 5. ROC analysis of two novel 6PLEX PE prediction models 1C with AUC= 0.958 (sFlt1, PlGF, ADAM12, sENG with adjustment for gestational age) and 3A with AUC=0.993 (adapted from 1C with leptin inclusion and adjustment for gestational age and maternal weight at the blood draw). Adapted from Ratnik et al., 2020.

Take home message: The novel 6PLEX assay offers value that, together with modelling to PE prediction in late pregnancy stages, facilitates good performance with a PE prediction accuracy of 96.5%. Compared to sFlt-1/PlGF ratio-based approach, the new alternative model is categorical without any borderline interpretation.

5.3. PE prediction models for early and mid-pregnancy

Final part of the study investigated the gestational dynamics of maternal serum biomarkers measured by the 6PLEX assay, and developed and explored PE prediction models for early and mid-gestation period.

5.3.1. Gestational dynamics of the investigated biomarkers (Study 3)

As covered in literature overview, the biomarkers have been used in early phase PE prediction due to some of them having important functional roles in controlling the prompt placental bed formation or other regulatory characteristics. The 6PLEX assay measuring PE-linked biomarkers ADAM12, PTX3, PlGF, sFlt-1, sENG, and leptin was used to analyze 132 serum samples covering a vast gestational period (drawn from 53 pregnant women between 70 and 275 g.days, **Table 2**). Conclusive analysis revealed (**Figure 6**) that both sFlt-1 and sENG have an increasing tendency specifically in late pregnancy duration for PE vs NPE cases with median fold change (fc) of 2.47 and 2.19 (P<0.0001). Inversely, PlGF had decreasing course (fc=3.81; P<0.0001). PTX3 serum levels preserve stable levels with decreasing movement in both groups, with the exception of 1.59-fold (P<0.05) increased levels in PE group in early gestational age. ADAM12 has overall increasing dynamics through all three trimesters with a modest but significant rise in PE group during early and mid-pregnancy (P<0.05).

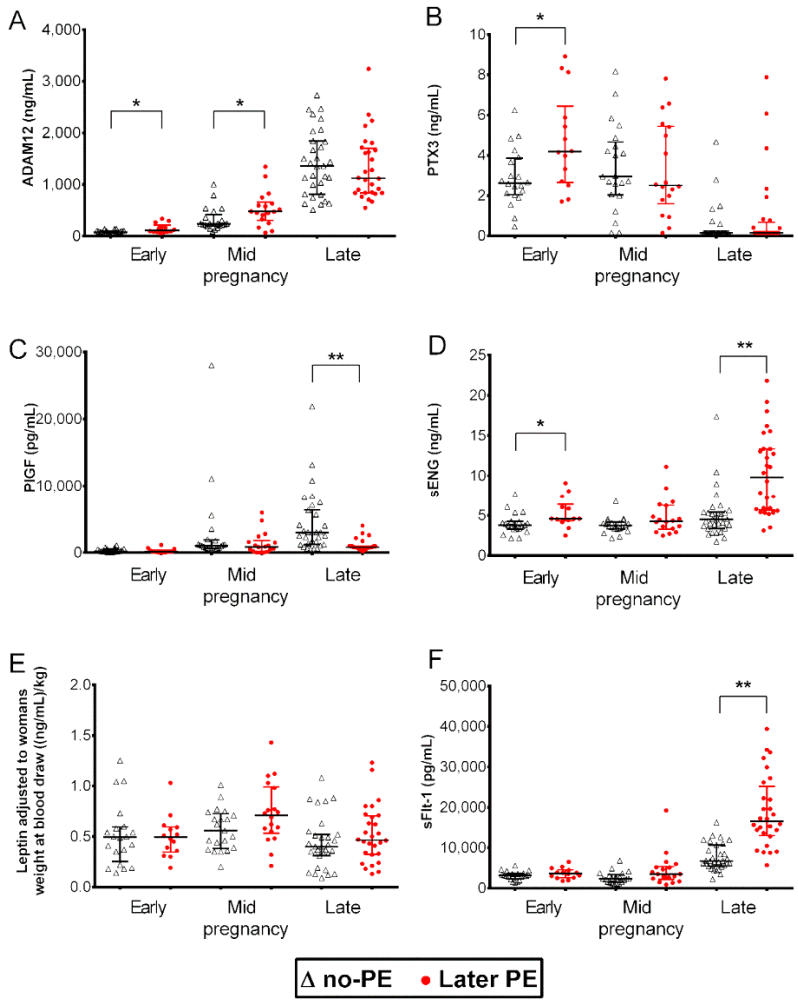

Figure 6. Dynamics of PE related biomarkers throughout the gestation in healthy and preeclamptic pregnancies. Luminex® 6PLEX assay measurement data of concentrations of ADAM12, PTX3, PlGF, sENG, sFlt-1 and leptin (adjusted to woman's weight) in 132 serum samples collected from pregnant women. Whiskers on the plot show median with interquartile range. Statistical difference in biomarker distributions between PE and control cases was compared using Mann-Whitney U-test, * representing $P<0.05$ and ** $P<0.0001$. ADAM12, ADAM Metallopeptidase Domain 12; PlGF, placental growth factor; PTX3, Pentraxin3; sENG, soluble endoglin; sFlt-1, soluble fms-like tyrosine kinase 1. Adapted from Ratnik et al., 2022.

5.3.2. PE prediction model using early pregnancy data

More sophisticated modelling was practiced compared to the first study, aiming at the 3rd trimester PE prediction. Narrow sampling window from 10–14 gestational weeks was selected from HAPPY PREGNANCY cohort to match tri-

mester I screening timing. 34 serum samples (14 PE/20 NPE samples) in total were incorporated into modelling (Study 3, Table1).

Leave-one-out cross-validation based approach together with stepwise selection pointed out the model 1A comprised of PTX3, sFlt-1, and ADAM12 measurements, the subject's parity, and gestational age at sampling (**Table 9**; AUC 0.936 (95%CI 0.843–0.993)). Between 70–98th g.days this approach enabled correctly "ruled in" or "ruled out" the onset of PE for 30 of 34 analyzed samples (accuracy 88.2% (95%CI 73.4–95.3)). With 100% sensitivity (95%CI 92.9–100.0) but 80% specificity (95%CI 65.0–100.0) four false-positive PE predictions were made.

Table 9. Performance characteristics of PE prediction models developed.

Model	Correct prognosis (Accuracy%[95%CI)]	AUC [95%CI]	Sensitivity % [95%CI]	Specificity % [95%CI]
Early pregnancy data modelling (70–98th g.days) [a]				
Models combining biomarkers, gestational age and parity				
1A[b]	**30/34 (88.2% [73.4–95.3])**	**0.936 [0.843–0.993]**	**100.0 [92.9 – 100.0]**	**80.0 [65.0–100]**
1B	29/34 (85.29% [69.9–93.6])	0.914 [0.804–0.989]	100.0 [78.6–100.0]	80.0 [60.0–100.0]
1C	30/34 (88.2% [73.4–95.2])	0.932 [0.839–0.993]	100.0 [92.9–100.0]	80.0 [65.0–100.0]
Models combining biomarkers, gestational age, parity and rs4769613 T/C placental genotype				
3A	29/31 (93.5% [79.3–98.2])	0.969 [0.882–1.000]	100.0 [83.3–100.0]	94.7 [89.5–100.0]
3B	29/31 (93.5% [79.3–98.2])	0.947 [0.851–1.000]	100.0 [83.3–100.0]	89.5 [84.2–100.0]
3C	29/31 (93.5% [79.3–98.2])	0.969 [0.895–1.000]	100.0 [100.0–100.0]	94.7 [89.5–100.0]
Mid-pregnancy data modelling (100–182th g.days)				
Model combining PlGF serum measurement, gestational age and parity				
4[b]	29/39 (74.4% [58.9–85.4])	0.784 [0.634–0.912]	73.7% [36.8–94.7]	76.2% [19.0–85.7]
Model combining PlGF data, gestational age, parity and rs4769613 T/C placental genotype				
5	23/32 (71.9% [54.6–84.4])	0.786 [0.613–0.932]	78.6% [28.6–92.9]	68.4% [36.8–100]

[a] Early pregnancy model A includes serum measurements of ADAM12, PTX3, sFlt1-1; model B measurements of ADAM12, PTX3, sENG and model C ADAM12, PTX3, sFlt1-1 and sENG.

[b] selected as statistically most significant model using the automatic computational prefiltration strategy

AUC, area under curve; g.days, gestational days

Modified from Ratnik et al., 2022.

5.3.3. Placental C-allele of the genetic variant rs4769613 near *FLT1* is significantly associated with the risk to late-onset PE (Study 4)

Large genome-wide association study (GWAS) targeting placental genotypes reported a genetic variant rs4769613 T/C near the *FLT1* gene as a risk factor for PE in populations of European descent (McGinnis et al., 2017). Meta-analysis across two Estonian pregnancy cohorts (REPROMETA and HAPPY PREGNANCY; 96 PE/2001 non-PE cases; see **Materials and Methods**) replicated robustly the genome-wide association study outcome (Bonferroni corrected $P=4\times10^{-3}$; odds ratio, 1.75 [95% CI, 1.23–2.49]) (Study 4, Table 3).

In the framework of the current thesis project, association between the placental *FLT1* variant rs4769613 and serum sFlt-1 levels was tested. Prospectively collected late pregnancy serums (198 – 258 g.days) were stratified by both, PE and non-PE status as well as the placental rs4769613 genotypes. Significantly higher sFlt-1 levels in PE compared to no-PE pregnancies were measured in the CC (fold change (fc)=3.1; P=0.01) and CT (fc=3.5; $P=4.4\times10^{-5}$), but not in the TT group (fc=2.1; P=0.08) (**Table 10**).

Table 10. Differentiation of sFlt-1 levels between PE and healthy cases in contrast to placental genotype.

Placental genotype	CC		CT		TT	
PE status	NPE	PE	NPE	PE	NPE	PE
No of samples	16	6	80	9	39	3
G.days median (range)	230 (205 - 250)	230 (211 - 256)	231 (200 - 258)	225 (213 - 240)	224 (198 - 252)	222 (206 - 249)
sFlt-1 in maternal serum log2(pg/mL)	10.6 (10.3 - 11.1)	12.1 (10.5 - 13.1)	10.3 (9.8 - 11.1)	12.1 (10.6 - 12.8)	10.2 (9.8 - 10.9)	11.2 (11.1 - 11.2)
Fold change (*P*-value)	3.1 (0.01)		3.5 (4.4×10^{-5})		2.1 (0.08)	

sFlt-1 concentration level expressed in median with interquartile range.
g.days, gestational days; PE, preeclampsia; NPE, no preeclampsia; Flt-1, soluble fms-like tyrosine kinase-1
Adapted from Kikas et al., 2020

5.3.4. Incorporating *FLT1* C/T variant rs4769613 genotype data to the PE prediction models (Study 3)

As the placental C-allele of the genetic variant *FLT1* rs4769613 was identified as a placental risk factor for PE, the early pregnancy PE prediction model was further improved by incorporation of this variant. An alternative PE prediction model 3C was generated combining PTX3, sFlt-1, sENG, and ADAM12 measurements with the parity, gestational age and placental FLT1 rs4769613 additive genotype data (AUC of 0.969 (95%CI 0.895–1.000)) (**Table 9**). The specificity of the previous 1A model improved from 80% to 94.7% (95%CI 89.5–100.0), only two false-positive PE predictions were made for pregnancies that remained normotensive.

Early prediction of PE using novel 6PLEX assay offers superb performance with 88.2% DR that in combination with the placental *FLT1* rs4769613 data achieves 93.5% correct prediction. No PE true positive cases were missed in either predictions.

5.3.5. PE prediction in mid-pregnancy

For mid-pregnancy the same LOOCV together with stepwise selection was applied to 39 samples (18 PE/21 NPE) (Study 3, Table 1). Analysis in this gestational age (100–182 g.days) detected moderate prediction models with machine-learning method. The only biomarker of age-adjusted PlGF with parity demonstrated any significant input to the prediction yielded to two alternative models 4 and 5 (including *FLT1* rs4769613 data) with both reserved performance with accuracies of 74.4% (95%CI 58.9–85.4) and 71.9% (95%CI 54.6–84.4) respectively.

Take home message: Early prediction of PE using novel 6PLEX assay offered superb performance with 88.2% DR that in combination with the placental *FLT1* rs4769613 data achieves 93.5% correct prediction. No PE true positive cases were missed in either predictions. For mid-pregnancy period the 6PLEX assay with used modelling approach was only able to deliver moderate PE prediction accuracy.

6. DISCUSSION

This thesis presents innovative experimental research aiming to develop and apply multiplex immunoassay measurement of PE related biomarkers in either early or the 2nd half of pregnancy. Development of such an assay was facilitated by careful methodological experimentation aiming to measure six biomarkers in a single test tube, and robust implementation of the Luminex® xMAP platform. Availability of the prospectively collected HAPPY PREGNANCY cohort serum samples allowed the monitoring of the biomarker levels in early, mid- and late gestation and sophisticated modelling of the PE prediction in early and late pregnancy. Innovatively, disease risk modelling considered simultaneously biomarker measurements, maternal clinical characteristics and placental genotype data of the PE-associated genetic variant.

6.1. Added value of measuring multiple PE-linked signature molecules in a single test-tube

The new 6PLEX assay is a novel tool to investigate confidently established PE related biomarkers with a cost-effective and time saving approach. As it is based on a sandwich immunoassay, it has great potential to be developed further on technology readiness level (TRL) scale to the degree required for diagnostically suitable solutions. Many of the metrics needed on CLSI and European Union IVD regulation (EU) 2017/746 have been described, but obviously a lot of work is ahead to reach the required level and robustness.

Understanding the pathology of a disease is the key for its surveillance and management. The collected evidence has shown that using just single PE associated biomarker as diagnostic parameter is not informative enough for disease prediction or confirmation (Magee et al., 2022). Pregnancy, together with placental development, is a complex ecosystem challenging the mother and the fetus to find a balanced course. Two-way signalling, a pregnancy specific secretome, is deriving both from the fetus and mother and their counteraction defines the gestational success. Angiomodulatory imbalance is clearly shown in case of PE, but also in FGR (Aplin et al., 2020). Still there is no consensus and understanding evidence if this is the cause of placental dysfunction or a resulting reflection. PlGF, predominantly expressed in cytotrophoblast cells of placenta, has vastly rising levels in the course of healthy pregnancy, and by binding to VEGF receptors (VEGFR), it promotes the angiogenesis needed for placental vascular system (Chau et al., 2017). sFlt-1 counteractive mechanism occurs by limiting the bioavailability of PlGF (Chau et al., 2017). Taken together, in case of PE the PlGF levels are lower compared to healthy pregnancies and this trend has been observed in all trimesters. On the contrary, the reactive sFlt-1 has an increased concentration in case of PE and especially expressed in late pregnancy. It is important to note that when measuring the maternal circulating

levels, the assay measurement is shadowed, and no true placental production is understood as the assay's quantification is disturbed as the epitope site might be blocked by sFlt-1 – PlGF native interaction. As demonstrated by Study 3, all above-described dynamics were observed also by using the novel 6PLEX assay when assessing HAPPY PREGNANCY samples. sENG is acting in concert with sFlt-1 in all evaluated time points in this study, though their active mechanisms are not the same, but both play a significant role in angiogenesis (Margioula-Siarkou et al., 2022). sENG-expressing transgenic mice study has shown that its overexpression resulted in placental alterations comparable to those caused by the poor remodelling of the spiral arteries characteristic of PE (Pérez-Roque et al., 2020). ADAM12, a metalloproteinase secreted by the placenta that cleave insulin-like growth factor binding proteins, is significantly decreased in severe and mild preeclampsia, and linked with severity of PE, maternal complications, and fetal outcome (El-Sherbiny et al., 2012). A recent study measured ADAM12 levels at 36th gestational week and confirmed its reduced levels in PE and highlighted its potential as a marker for SGA (Andres et al., 2022).

PTX3 and leptin are not placenta-specific biomarkers and that way correspond to maternal response to pregnancy course. The excessive inflammation in PE increases the levels of proinflammatory factors, which in turn results in the synthesis of PTX3 and the activation of vascular and placental endothelial cells, thus leading to endothelial dysfunction (Garcés et al., 2015). Also, PTX3 can promote the differentiation of T lymphocytes into type 1 T helper cells and inhibit the recognition function of dendritic cells on late apoptotic cells, which may lead to the imbalance of apoptosis, contributing to the development of PE. These facts are in line with our study finding, however, the argument of PTX3 having more dramatic increase in severe or EOPE did not gain confirmation in our results, likely due to sample size and selection (Xiong et al., 2020). A note from earlier study has stressed the PTX3 relation to PE but not to isolated FGR that could benefit its use in PE prediction (Cetin et al., 2009). As obesity has significant input to PE risk, the obvious reasons have been to look for biomarkers regulating the metabolism. Leptin, an adipokine and hormone that regulates lipid metabolism, has also been linked with inflammatory processes and trophoblast invasion (De Knegt et al., 2021). During gestation, leptin is also produced by placenta (Beneventi et al., 2020) and total leptin levels in PE pregnancies have been reported to be elevated compared to healthy cases based on meta-analysis (Veiga et al., 2022). In our results we did not see this dynamics.

6.2. New alternative PE prediction models based on 6PLEX assay exhibit promising performance

As the PE has remained a burden and one of the most severe pregnancy complications, new tools for both early prediction and isolation of the syndrome in late gestation are sought for. The most studied maternal biomarkers PlGF and

sFlt-1 are already incorporated into many PE prediction and management guidelines applicable either during the 1st trimester or 2nd half of pregnancy (ACOG, ISSHP, NICE, FMF), but still some uncertainties are present. As the PROGNOSIS study showed, the ratio of sFlt-1/PlGF performs well for ruling out disease development over the next week after blood collection in late pregnancy setting, but the real prediction value remained modest (Zeisseler et al., 2016). The very same performance was seen when using the B·R·A·H·M·S Kryptor assay on HAPPY PREGNANCY sample set. In Study 2 we show that when applied to asymptomatic pregnant women with gestational age ranging 206-257 days, general DR including all samplings was moderate, 58.3% with quite high FPR of 9.7%. MacDonald and her colleagues have also pointed to this in their review to challenge the sFlt-1/PlGF ratio approach by adding alternative biomarkers (MacDonalt et al., 2022). In Study 1 the generalized linear regression model approach based on 6PLEX measurements together with maternal characteristics was able to outperform the conventional approach PE prediction with extremely good DR 96.5%.

The use of FMF model (use of MAP, UtA-PI, PAPP-A and/or PlGF) for early PE prediction to offer prophylaxis has decreased the disease prevalence in many high-income countries that can afford the Doppler ultrasound examination. The DR of ~90% for women at 11 to 13 weeks' gestation to develop EOPE and ~75% for LOPE has been reported (Wright et al., 2020). Although a good test in antenatal care for monitoring fetal well-being, its limitations include not being scalable, needing high-cost instrumentation and a well-trained ultrasonoghrapher. In addition, the FMF models have been so far mostly evaluated in high-income societies whereas the cost-effectiveness in low to middle income settings may require a pragmatic approach to the implementation of multiparametric screening given limited resources (Malone et al., 2022). Also, suggested in the International Federation of Gynecology and Obstetrics (FIGO) guidance, just assessing the maternal characteristics and MAP should be achievable (Poon et al., 2019), with PE DR of 63% (Poon et al., 2011). The use of immunoassays falls between simple maternal characteristics evaluation and ultrasonography by the need of input cost and trained specialists. With the use of 6PLEX assay applying the proposed model 1A (comprised of PTX3, sFlt-1, and ADAM12 measurements, the subject's parity, and gestational age at sampling) between 70–98th g.days, this approach enabled final PE prediction accuracy of 88.2%.

Of note, the use of machine learning approaches is getting more attention in the field of PE prediction (Maric et al., 2020; Bertini et al., 2022). For example, the traditional factors such as white blood cell count, creatinine level, liver function, and urinary protein reported to be related to PE development were determined to be influential factors in PE prediction (Jhee et al., 2019). This is the benefit of step-wise selection of variables together with LOOCV approach where the final model validation is more specific.

6.3. Innovative incorporation of genetic variants into PE risk models

In Study 3, incorporation of the placental genetic risk variant for late PE (*FLT1* rs4769613 C/T) increased the disease prediction to 93.5% accuracy. *FLT1* rs4769613 was first shown in 2017 associated between preeclampsia and a variant upstream the respective gene (McGinnis et al., 2017). This result was robustly replicated in Study 4 included in the current thesis. A follow-up study analyzing placental samples from European and Central Asian origin with total of 6,775 PE cases and 375,372 controls revealed the most significant associated signal to gene *FLT1* locus on 13q12 for the sentinel variant rs4769612 (in allelic association with rs4769613) (Steinthorsdottir et al., 2020).

In clinical practice, direct placental or fetal material sampling during pregnancy for DNA extraction and genotyping is ethically not possible. One approach to obtain placental/fetal genetic data could be amniocentesis or chorionic villus sampling. However, as the costs of procedures are high and there exists a risk for miscarriage, these are not reasonable screening tools (Odibo & Acharya, 2020). Non-invasive prenatal testing (NIPT) brought along a revolution enabling placental DNA testing using cell-free fetal DNA (cff DNA) extracted from maternal blood (Badeau et al., 2017; Rafi et al., 2017). The current NIPT testing is targeted towards the detection of chromosomal abnormalities in the fetus, but deeper resolution methods enabling to analyze single nucleotide variants are under development and could be soon available for routine clinical practice (Zhang et al., 2019). Thus, using cffDNA to screen the placental *FLT1* rs4769613 T/C genotypes to be incorporated into PE prediction models may soon be a feasible approach. In this perspective, a rational solution could be incorporating this variant into gene panels developed for NIPS targeting fetal single gene defects.

6.4. Limitations of the study

Limitations of this study are moderate sample size (not covering the real-life variation of clinical settings and patient phenotypes in all its aspects) and lack of an independent validation of the current 6PLEX assay setup, ideally in a prospective clinical setting. PE risk models, currently built on small-scale data, will need further polishing with the biomarker measurements in a large pregnancy cohort. The data of the dynamics of PE-related biomarkers during pregnancy could also be extended with additional gestational time points, enabling to build more precise disease prediction models.

Criticism must be raised of the use of commercial antibodies in 6PLEX assay. Thus, the properties of the antibodies could not be selected for optimal epitopes, modification, etc. This limitation may have affected the technical performance of the 6PLEX assay.

7. CONCLUSIONS

The current doctoral thesis developed a novel Luminex® based 6PLEX assay for the measurement of multiple PE-linked biomarkers, described their dynamics between 70–275 g.days in healthy and affected pregnancies, and proposed several new PE prediction models applicable either in the 1st trimester or 2nd half of the pregnancy. The core outcome is summarized as follows:

1. The novel Luminex® based 6PLEX assay measured simultaneously maternal circulating levels of six PE related biomarkers ADAM12, PlGF, sENG, sFlt1, PTX3 and leptin with high precision and low intra- and interassay variation. The developed single-tube multiplex assay is the first of its kind in scientific literature, potentially offering shorter turn-over time to deliver the PE risk screening results without sarcificing the quality of assay performance.
2. 6PLEX assay based multicomponent disease risk modelling enabled PE prediction in late pregnancy (180–275 g. days) with 96.5% accuracy (2 false positive cases). This was superior performance compared to the currently used sFlt-1/PlGF ratio based approach (accuracy 73.7%; 2 false positive and 5 false negative cases). This outcome suggests the potential use of the novel test as an effective PE ruling-out method in late pregnancy. Of note, the sFlt-1/PlGF ratio test resulted in 8 inconclusive cases that in real-life setting would have needed a recall of the patients in 7 days for a follow-up measurement.
3. Applying the 6PLEX assay during 10-14 g.weeks allowed 88.2% precise PE prediction without any false negative cases and 80% specificity. Incorporation of the placental *FLT1* rs4769613 C/T variant genotype information further improved the prediction accuracy to 93.5%. This highlighted the potential of the novel test for early screening of PE enabling timely identification of high-risk pregnancies, application of preventive measures and targeted monitoring of patients.

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SUMMARY IN ESTONIAN

Preeklampsia riski ennustustesti ja -mudeli väljatöötamine

Rasedus on naise jaoks unikaalne aeg, mille käigus organismis toimub suur hulk muutusi. Rasedusaegsed füsioloogilised muutused on tingitud nii rasedusest endast, emapoolsetest kui ka välistest teguritest. Patoloogilised muutused võivad tuua endaga kaasa hiliseid rasedustüsistusi nagu preeklampsia (PE), gestatsioonidiabeet ning raseduskestuse kohta väike või suur sünnikaal.

Sõltuvalt maailmajaost ja riigis kättesaadavast tervishoiuteenuse kvaliteedist mõjutab PE 3–5% kõigist rasedustest. Eestis on PE osakaal kõigist elussündidest vähenemas – 1,9% 2016. aastal ja 1,3% 2020. aastal. PE peamisteks kliinilisteks sümptomiteks on raseduse teisel poolel arenev hüpertensioon, millele lisandub üks või mitu järgmistest sümptomitest: proteinuuria, neerufunktsiooni langus, maksakahjustuste markerite tõus seerumis, uteroplatsentaarne puudulikkus, neuroloogilised häired. Kõige sagedasem kaasus lootele on kasvupeetus, mida esineb *ca* 1/3 juhtudest. Ainus tõeline ravi PE suhtes on sünnitus. PE tekkepõhjused ei ole täielikult teada, küll aga on haigusel mitu riskitegurit: esmane rasedus, eelnev PE esinemine, rasvumine, ema vanus, krooniliste haiguste esinemine, etniline päritolu (Rull et al., 2021).

PE-d iseloomustav uteroplatsentaarne puudulikkus põhjustab raskendatud ainevahetust ema ja loote vahel, laguproduktide eemaldamist ja üldist regulatiivset pärsingut. Platsenta arenguhäiret uurides on leitud, et trofoblastide vahendatud müomeetriumi spiraalarterite invasioon on PE korral puudulik. Tulemuseks on raseduse kulgedes II ja III trimestril loote toitainete puudus ja muud peetused, nagu eespool kirjeldatud. Kliiniliselt eristatakse varajast, enne 34. rasedusnädalat avalduvat ja hilist, alates 34. rasedusnädalast avalduvat PE-d. Varajast PE-d esineb *ca* 25% juhtudel ja see on tõsisemate tagajärgedega. Tänapäeval peetakse oluliseks varajast PE ennustust aspiriinil põhineva profülaktika alustamiseks ning hilisemas raseduse faasis haiguse kinnitamist või välistamist. Mõlemad mõjutavad oluliselt raseduse jälgimise ja sünnitusabi kliinilist praktikat. I trimestril teostatav ennustus põhineb ema baasnäitajatel (eelnev rasedu-ste arv, vanus, varasem PE, rass), ultraheli uuringul ja vereseerumist määrataval PlGF või PAPP-A määramisel. Kombineeritud riskihinnang võimaldab tuvastada 90% varajastest PE juhtudest, hilise PE korral aga ainult 40%. PE riskihinnang sFlt-1/PlGF kaudu on raseduse teisel poolel efektiivne ainult varajase PE korral. Hilise PE korral on see lähenemine spetsiifiline ainult 75% juhtudel (Magee et al., 2022).

Käesoleva doktoritöö eesmärk oli luua uuenduslik multimarker-immuunuuring ja kombineerida selle abil saadud mõõtmiste andmestikust PE ennustusmudelid:

1. Töötada välja uudne Luminex® xMAP-il põhinev immuunuuring 6PLEX PE seoseliste seerumi biomarkerite määramiseks: ADAM12, sENG, leptiin, PlGF, sFlt-1 ja PTX3.
2. Koostada PE ennustusmudelid hilise raseduse korral (III trimester), määrates PE seoselisi biomarkereid ja võrrelda nende täpsust sFlt-1/PlGF meetodi suhtes.

3. Koostada PE ennustusmudelid varajase raseduse faasil kasutamiseks (I trimester).

Doktoritöö peamised tulemused:

1. Optimeerimiskatsete tulemusel saavutati kõrge määramise täpsuse, hea tundlikkuse ja väikese varieeruvusega uudne Luminex® xMAP-il põhinev 6PLEX-immuunuuring.
2. Kaubanduslikult ja meditsiinidiagnostikas kasutatava B·R·A·H·M·S Kryptor platvormiga võrdlevalt läbi viidud sFlt-1 ja PlGF mõõtmised kinnitasid 6PLEX-meetodi võrdväärset täpsust eristamaks PE ja mitte-PE juhtumeid.
3. PE ennustus oli III trimestril kogutud proovidest kõige parem, kui ennustusmudelisse kaasati viis biomarkerit (sFlt-1, PlGF, ADAM12, sENG, leptiin) koos ema lisa-faktoritega. Nimetatud mudeli PE ennustustäpsus oli 96,5%.
4. Väljatöötatud 6PLEX-protokolliga määrati PE seoseliste biomarkerite tase laiemas gestatsioonivahemikus, alates 70. raseduspäevast. Saadud tulemused kattusid kirjanduses avaldatud teabega uuritud biomarkerite dünaamika kohta raseduse kestel.
5. I trimestri PE ennustusmudeli väljatöötamisel kasutati masinõppel põhinevat algoritmi, mis tagas parima PE ennustusmudelina 88,2% täpsuse (kaasates ADAM12, PTX3, sFlt-1 ja emapoolsed faktorid).
6. Platsenta *FLT1* rs4769613 genotüübi kaasamisel paranes I trimestri PE ennustusmudeli täpsus 93,5%-ni.

Doktoritöö tulemusena töötasin välja kõrge tundlikkuse ja spetsiifilisusega innovaatilise multimarker-immuunuuringu 6PLEX, mis võimaldab ema vereproovi alusel kõrge täpsusega hinnata PE tekkeriski raseduse esimesel ja teisel poolel. Sellise uuringu eeliseks on kuluefektiivsus ja aja kokkuhoid, kuna huvipakkuvad biomarkerid määratakse samaaegselt ühest proovimaterjalist. I trimestri ennustustestiga on maailmas esmakordselt kombineeritud platsenta ehk loote geneetiline komponent ja on saavutatud väga hea PE ennustustäpsus. Kõnealune immuunuuring põhineb uudsel metodoloogilisel ja analüütilisel lähenemisel laborimeditsiinis ning ootab edasiarendust, saavutamaks vastavad kvaliteedinõuded EU IVD regulatsiooni 2017/746 kohaselt.

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PUBLICATIONS

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2. Invention: METHOD OF PROGNOSING PREECLAMPSIA; Owners: University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine; Authors: Maris Laan, Kaspar Ratnik, Kristiina Rull, Kalle Kisand, Ele Hanson; Priority number: GB2012830.2; Priority date: 17.08. 2020.
3. Invention: METHOD OF PROGNOSING AND DIAGNOSING PREECLAMPSIA; Owners: University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine; Authors: Maris Laan, Kaspar Ratnik, Kristiina Rull, Kalle Kisand, Ele Hanson; Priority number: GB1910133.6; Priority date: 15.07.2019.

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Tööstusomand:

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