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TRIIN KIKAS

Single nucleotide variants affecting placental gene expression and pregnancy outcome

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Single nucleotide variants affecting placental gene expression and pregnancy outcome

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Press

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

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Study 1. Participated in the experiments and interpretation of the data. Contributed to the critical reading of the manuscript.
Study 2. Participated in the design of the analyzes and experiments. Performed the experiments. Analyzed and interpreted the data. Prepared the figures and tables. Contributed to the writing of the manuscript.
Study 3. Participated in the design of the analyzes and experiments. Performed the experiments. Analyzed and interpreted the data. Prepared the figures and tables. Contributed to the writing of the manuscript.
Study 4. Participated in the collection and analysis of the data. Prepared the figures and tables and contributed to the writing of the manuscript.

ABBREVIATIONS

ABCG2	ATP binding cassette subfamily G member 2
aCA	acute ChorioAmnionitis
ADHD	attention deficit-hyperactivity disorder;
ALPG	alkaline phosphatase, germ cell
ALPP	alkaline phosphatase, placenta
ALSPAC	Avon Longitudinal Study of Parents and Children
AMD	age-related macular degeneration;
APS	antiphospholipid syndrome
AQP11	aquaporin 11
ART	assisted reproductive technology
ATG10	autophagy related 10
AV	arteriovenous
BeWo	human choriocarcinoma cell line
BMI	body mass index
BP	blood pressure
CEP72	centrosomal protein 72
CEU	Northern Europeans from Utah
cffDNA	cell-free fetal DNA
CNV	copy number variant
CT	threshold cycle
CTB	cytotrophoblast
CTRL	non-pathological pregnancies
CXCR3	C-X-C motif chemokine receptor 3
CYREN	cell cycle regulator of NHEJ
DBP	diastolic blood pressure
DCTN5	dynactin subunit 5
DDX11	DEAD/H-box helicase 11
Dec	decidium
DOHaD	Developmental Origins of Health and Disease
dQTL	dispersion quantitative trait locus
E	embryonic day (mouse)
ELISA	enzyme-linked immunosorbent assay
EOPE	early-onset preeclampsia
eQTL	expression Quantitative Trait Locus
ER	endoplasmic reticulum
ERAP1	endoplasmic reticulum aminopeptidase 1
ERAP2	endoplasmic reticulum aminopeptidase 2
eSNV	expression-modulating single nucleotide variant
EVT	extravillous trophoblast
FAM118A	family with sequence similarity 118 member A
FDR	false discovery rate
FGR	fetal growth restriction

FINNPEC	Finnish Genetics of Preeclampsia Consortium
FKBP5	FKBP prolyl isomerase 5
FLT1	fms related receptor tyrosine kinase 1
FO393415.1	Ribosomal protein L30 (RPL30) pseudogene
FTO	FTO alpha-ketoglutarate dependent dioxygenase
GD	gestational diabetes
GlyT	glycogen trophoblast
GO	gene ontology
GTEx	the Genotype-Tissue Expression Project
GTT	glucose tolerance test
GUCY1B2	guanylate cyclase 1 soluble subunit beta 2
GWAS	genome-wide association study
HAPO	the Hyperglycemia and Adverse Pregnancy Outcome study
HAPPY PREGNANCY	Development of novel non-invasive biomarkers for fertility and healthy pregnancy" study
hCG	human chorionic gonadotropin
HEATR5A	HEAT repeat containing 5A
HELLP	hemolysis, elevated liver enzymes, and a low platelet count
HIF1A	Hypoxia inducible factor 1 subunit alpha
HLA-G	Major histocompatibility complex, class I, G
hPL	human placental lactogen
HTR7P1	5-hydroxytryptamine receptor 7 pseudogene 1
HWE	Hardy-Weinberg equilibrium
IGF2	insulin-like growth factor 2
IGFBP-1	insulin-like growth factor binding protein-1
IL-10	interleukin 10
INHBB	inhibin subunit beta B
IP6K3	inositol hexakisphosphate kinase 3
iPSC	induced pluripotent stem cell
ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	intra-uterine growth restriction
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCL	lymphoblastoid cell lines
LD	linkage disequilibrium
LEPR	leptin receptor
LGA	large-for-gestational-age newborns
LNPEP	leucyl and cystinyl aminopeptidase
LOPE	late-onset preeclampsia
LR	linear regression
MAF	minor allele frequency
MW	Mann-Whitney *U* test
n.a.	not applicable
NICE	The National Institute for Health and Care Excellence

NIPT	non-invasive prenatal testing
NMRK1	nicotinamide riboside kinase 1
NNNS	Neonatal Intensive Care Unit Network Neurobehavioral Scales
NORM	uncomplicated pregnancies with newborn birth weight >10th and <90th percentile
NPE	non-preeclamptic pregnancies
NS	not significant
P21 alias *CDKN1*	Cyclin Dependent Kinase Inhibitor 1A
PAMG-1	placental alpha-microglobulin-1
PAPP-A	pregnancy-associated plasma protein A
PE	preeclampsia
PGH	placental growth hormone
PLEKHG1	pleckstrin homology and RhoGEF domain containing G1
PlGF	placental growth factor
PPIE	peptidylprolyl isomerase E
P-PROM	preterm prelabor rupture of membranes
PRRG4	proline rich and Gla domain 4
PSG1-9	pregnancy-specific glycoprotein family 1-9
PSMD5	proteasome 26S subunit, non-ATPase 5
PTB	preterm birth
RBPJ	Recombination signal binding protein for immuno-globulin kappa J region
REPROMETA	REPROgrammed fetal and/or maternal METAbolism
RPE	retinal pigment epithelium;
RPL9	ribosomal protein L9
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SERPINA3	serpin family A member 3
sFlt-1	soluble FLT1
SGA	small-for-gestational-age newborn
SLC25A43	solute carrier family 25 member 43
SMG1P5	SMG1 pseudogene 5
SPSB2	splA/ryanodine receptor domain and SOCS box containing 2
SpT	spongiotrophoblast
STB	syncytiotrophoblast
STC1	stanniocalcin 1
T2D	type II diabetes
TCIM	transcriptional and immune response regulator
TGCs	trophoblast giant cells
THUMPD2	THUMP domain containing 2
TLDC1	TBC/LysM-associated domain containing 1
TNF-a	tumor necrosis factor alpha

TPRN	taperin
Trim	trimester
TRIM66	tripartite motif-containing 66
UBC	ubiquitin C
VEGF-A	vascular endothelial growth factor A
WDR91	WD repeat domain 9
YBX1P6	Y-box binding protein 1 pseudogene 6
ZNF100	zinc finger protein 100
ZNF831	zinc finger protein 831
ZSCAN9	zinc finger and SCAN domain containing 9

1. INTRODUCTION

Pregnancy is something that most women will go through during their lives, so it potentially affects half of the world's population. The placenta mediates nutrients and waste products between the mother and the fetus during pregnancy. It is essential for a successful pregnancy outcome that the placental unit is working as intended, growing and adapting to fetal needs through pregnancy. The placenta expresses many different protein-coding genes with various purposes to function as required, some of which also reach the maternal bloodstream. It is still not fully known how the regulation of all these genes and proteins is carried out at the exact times it is needed. However, when the placental gene expression profiles have strayed from the norm, pregnancy complications can occur.

Early pregnancy complications such as ectopic pregnancies or miscarriages often result in the loss of pregnancy. Even though late pregnancy complications have better outcomes, there is still danger to maternal and fetal life. Many of these have also been linked to placental dysfunction, such as preeclampsia (PE) and fetal growth restriction. Preeclampsia occurs in up to 5% of pregnant women worldwide, claiming about 63,000 maternal lives each year in the middle- and low-income countries, where healthcare is not always available (Burton et al., 2019). Only palliative care is possible during the pregnancy as the removal of the placenta is the only known cure for PE. However, the sooner PE is diagnosed, the better the chances to keep the symptoms under control until birth. For this, high-risk women need to be identified as early as possible in the pregnancy. There are known risk factors for PE, such as nulliparity, body mass index, and previous PE history, that are considered when assessing the risk for PE. Genetic risk factors would have an added value as these are determined only once and retain the information for subsequent pregnancies. Not many robust genetic risk factors have been determined for PE so far.

Expression quantitative trait loci (eQTLs) are variants that modify the target gene expression. In the Genotype-Tissue Expression (GTEx) project (Aguet et al., 2020), analyzing 49 tissues revealed over 5 million variants that affect at least one gene in at least one tissue. Many eQTLs are also tissue-specific, making it necessary to study the variants in the tissue of interest. Even though eQTLs are well studied in readily available tissues such as blood, pregnancy-related tissues, such as the placenta, have only recently been started to be addressed. Identifying placental eQTLs could provide additional insight into placental gene expression regulation as well as provide a list of new candidate genes and variants for various pregnancy complications.

The main aim of the current thesis was to describe the regulatory effect of eQTLs on placental transcriptome and the link to pregnancy complications.

2. LITERATURE REVIEW

2.1. Human pregnancy

2.1.1. Pregnancy course

Human pregnancy lasts on average for about 40 weeks. The pregnancy is initiated by the fusion of an egg and a sperm in the fallopian tube. During the first two weeks, the resulting embryo moves from the tube into the uterus, where the embryonic trophoblast cells begin to invade into the maternal decidua (James et al., 2012). These invading trophoblasts create the basis for the placenta.

The pregnancy is divided into three trimesters, each focusing on different fetal development and growth stages. As the embryo develops, the conditions needed for optimal growth change. In the first trimester (up to 11th gestational week), the embryo is growing in a hypoxic environment until the remodeling of decidual spiral arteries occurs (Jauniaux et al., 2000; Rodesch et al., 1992). Until then, the primary source of nutrition for the embryo comes from the secretions of the endometrial glands (Burton et al., 2002). Therefore, during this period, maternal circulatory factors do not reach the embryo in large quantities. However, the substances secreted by the placenta and the embryo still reach the maternal bloodstream even at this time. (Huppertz et al., 2008; Michelsen et al., 2019) All fetal structures and major organs start their development already during the first trimester.

At the start of the second trimester, the placenta has developed into a fully functional organ, and maternal blood is used as the primary source of nutrition for the fetus (Aplin et al., 2020; Jauniaux et al., 2000). Other fetal organs continue their development in the second and third trimesters. In the third trimester, the fetus goes through rapid growth, gaining weight. In addition, the nervous system, lungs, and metabolism mature to adjust to life out of the uterus.

2.1.2. Placenta, a pregnancy-specific organ at the maternal-fetal interface

The placenta is a unique organ (**Table 1**). It is the only organ present only at a specific time point in human life – pregnancy. It is constantly changing to meet the needs of the growing embryo, reaching full-thickness by the fourth gestational month but continuing to grow in circumference through pregnancy. The central role of the placenta is to mediate maternal and fetal needs. Nutrients and oxygen are moved through the placenta, from the mother to the fetus, with waste products, and secreted proteins moved in the opposite direction (Aplin et al., 2020; Jauniaux et al., 2000; Michelsen et al., 2019).

Table 1. Examples of some notable features of the human placenta

Features	Reference
Evolutionarily new endocrine organ, present only in mammals	(Burton & Fowden, 2015; Cross et al., 2003)
It is a temporary organ	(Burton & Fowden, 2015; Fox & Sebire, 2007)
Contains new cell types a) syncytiotrophoblast that is the only true multinuclear syncytium b) extravillous trophoblasts that are capable of invading maternal decidua deeper than other mammals	(Burton & Fowden, 2015; Cross et al., 2003; Fox & Sebire, 2007)
Organ function-specific genes, proteins, and miRNA families	(Liang et al., 2007; Rawn & Cross, 2008)
General hypomethylation of the genome compared to postnatal somatic and fetal cells	(Ehrlich et al., 1982; Schroeder et al., 2013)
The placental genome is prone to changes, including somatic mutations, genomic chromosomal instability with confined placental mosaicism	(Coorens et al., 2021; Kalousek & Dill, 1983; Kasak et al., 2015, 2017)
The placental microbiome is nearly absent	(de Goffau et al., 2019; Leiby et al., 2018)

The primary cell type in the placenta that makes it functional is trophoblasts. During the invasion phase, cytotrophoblast (CTB) differentiates into two different types: multinuclear syncytiotrophoblast (STB) and mononuclear, highly invasive extravillous trophoblast (EVT). By the third gestational week, the placenta has vascularized villi bathed in the maternal blood in the intervillous space (**Figure 1.**) (Demir et al., 1989; Te Velde et al., 1997). Placental villi consist of layers of CTB and STB cells covering the fetal blood vessels (Fox & Sebire, 2007). Remodeling of the spiral arteries widens and relaxes them locally, so increased blood flow into the placenta is possible. Incomplete remodeling is thought to be one of the underlying causes of maternal hypertension, preeclampsia (PE), and fetal growth restriction (Staff, 2019).

The human placenta is hemochorial, which means the chorionic villi come into direct contact with maternal blood. Even among the same type of placentas among mammals, the human placenta has a profound trophoblast invasion, even up into a third of the maternal myometrium. A similar pattern has also been found in gorillas and chimpanzees, but not in Old World monkeys (Carter, 2011). The most commonly used model animal, the mouse, has reasonably different morphology from a human placenta despite being hemochorial (**Figure 2.**) (Hemberger et al., 2020). The absence of a perfect model is one of the reasons researching the placenta is complex.

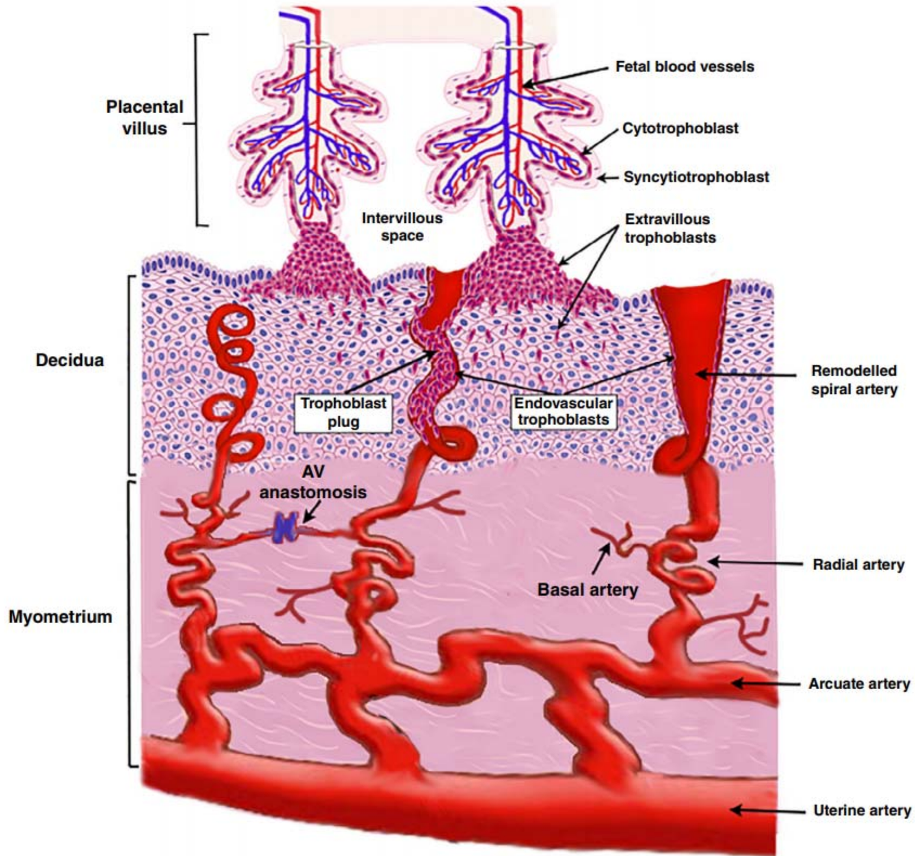

Figure 1. Structure of the human placental-maternal interface. Adapted from (A. R. Clark et al., 2020). AV, arteriovenous

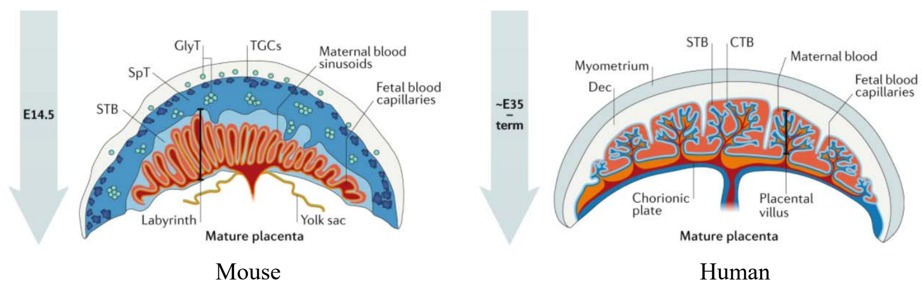

Figure 2. Comparison between the mouse and the human placenta. Adapted from (Hemberger et al., 2020). E, embryonic day; STB, syncytiotrophoblast; SpT, spongiotrophoblast; GlyT, glycogen trophoblast; TGCs, trophoblast giant cells; CTB, cytotrophoblast; Dec, decidium

The placenta releases different hormones, microRNAs (small single-stranded RNAs), cell-free DNA, extracellular vesicles, and other products into the maternal blood stream at specific time points during pregnancy. Different estrogens and progesterone are produced by the placenta, as well as some placenta-specific peptide hormones like human chorionic gonadotropin (hCG) (Bonduelle et al., 1988; Rull et al., 2008), human placental lactogen (hPL) (Kliman et al., 1986; Männik et al., 2010), human placental growth hormone (PGH) (Alsat et al., 1997), and others (Costa, 2016; Fox & Sebire, 2007). According to Human Protein Atlas (proteinatlas.org, (Uhlen et al., 2015)), there are 91 genes with expression enriched for the placenta (>4 times higher mRNA levels than in any other tissue). Among these are some well-known genes like pregnancy-specific glycoprotein family (*PSG1-9*), pappalysin 1 and 2 (*PAPPA1-2*), fms-like tyrosine kinase 1 (*FLT1*), placental alkaline phosphatase (*ALPP*), insulin-like growth factor 2 (*IGF2*), and others. Predominantly placenta-expressed genes are expressed mainly by trophoblast cells and are integral to trophoblast differentiation and function (Handwerger & Aronow, 2003; Szilagyi et al., 2020). The placental products can affect the maternal physiology and response to pregnancy and, therefore, potentially the pregnancy outcome. For example, placenta-derived soluble Flt-1 (sFlt-1) rises in the maternal serum about five weeks before developing PE (D. E. Clark et al., 1998; Levine et al., 2004).

2.2. Pregnancy complications

2.2.1. General overview

Pregnancy affects the whole maternal body causing anatomic, hormonal, and metabolic changes in several organs. When the maternal body is not able to adapt to the changes, complications can occur. Many obstetrical complications have been linked to the placenta, either by insufficient or excessive invasion (Brosens et al., 2011; Burton & Jauniaux, 2018; Jauniaux et al., 2018). Depending on the timing, these can be broadly divided into two groups – early and late pregnancy complications.

Early pregnancy complications such as ectopic pregnancy, miscarriage, and molar pregnancy are severe and incompatible with pregnancy continuation. If not removed, ectopic and molar pregnancies can become life-threatening for the mother (Rehman & Muzio, 2019). A brief overview of selected early pregnancy complications can be found in **Table 2**.

Even though late pregnancy complications manifest in the second half of the pregnancy, the cause can be already present in early pregnancy. For example, PE and fetal growth restriction are thought to be mainly caused by inadequate placental invasion (Burton & Jauniaux, 2018; Staff, 2019). In general, late pregnancy complications are less severe but can become life-threatening to the child and the mother in some cases.

Table 2. Characteristics of some of the most common pregnancy complications.

Complication	Characteristics	Prevalence[a]	Major risk factors	References
Early pregnancy complications				
Early pregnancy loss	Loss of pregnancy <13–20 weeks.	10–30% estimated, 7.7% Estonia[b]	Maternal age, previous early pregnancy loss, alcohol consumption, smoking, infections, chronic diseases	(Alves & Rapp, 2020; American College of Obstetricians and Gynecologists, 2018)
Recurrent pregnancy loss	Three consecutive early pregnancy losses (including biochemical) or two early clinical pregnancy losses	1–3%	Maternal age, parental chromosomal anomalies; maternal thrombophilic, anatomic, endocrine, and immunological disorders	(Homer, 2019)
Ectopic pregnancy	Implantation of the embryo outside of the uterus, mostly fallopian tube.	1–2% worldwide, 1.2% Estonia[c]	previous ectopic pregnancy, pelvic infection, or -surgery, ART, increased maternal age, smoking	(Graham et al., 2015; Hendriks et al., 2020)
Late pregnancy complications				
Preeclampsia (PE)	Hypertension with additional symptoms of maternal organ damage (classically proteinuria)/ uteroplacental dysfunction	2–5% worldwide, 1.7% Estonia[c]	Nulliparity, pre-pregnancy BMI, prior PE, chronic hypertension, multiple pregnancy, APS, maternal age, new partner, genetic risk factors	(Burton et al., 2019; Rana et al., 2019)
Small-for-gestational-age newborn (SGA)	Newborn weight <10th percentile on the population-specific sex-adjusted growth curves	10% Estonia[d]	Prior SGA or being born SGA, low BMI, APS, smoking, alcohol use, multiple pregnancy, genetic risk factors	(Beaumont et al., 2020; Finken et al., 2018; McCowan & Horgan, 2009)

Complication	Characteristics	Prevalence[a]	Major risk factors	References
Large-for-gestational-age newborn (LGA)	Newborn weight >90th percentile on the population-specific sex-adjusted growth curves	10% Estonia[d]	Prior LGA, obesity, advanced age, gestational weight gain, (gestational) diabetes, genetic risk factors	(Beaumont et al., 2020; Walsh & McAuliffe, 2012)
Gestational diabetes (GD)	Hyperglycemia in pregnancy that is not diabetes mellitus	7–14% worldwide, 11.9% Estonia[c]	Maternal BMI, excessive gestational weight gain, diet, familial history of diabetes, being born LGA or from GD pregnancy, maternal age, genetic risk factors	(Hod et al., 2015; Plows et al., 2018)
Preterm birth	Birth before the 37th gestational week	5–13% worldwide, 5.8% Estonia[c]	Prior preterm birth, multiple pregnancy, Infections, smoking, cervical surgery, maternal BMI, diet	(Koullali et al., 2016; Singh et al., 2020)

APS, antiphospholipid syndrome; ART, assisted reproductive technologies; BMI, body mass index. [a] prevalence is calculated among clinical pregnancies for early complications or births for late complications. [b] Data from 2017 Estonian Abortion Registry report (https://www.tai.ee/et/valjaanded/eesti-meditsiiniline-sunniregister-1992–2016-eesti-abordiregister-1996–2016); [c] Data from 2019 Estonian Health Statistics and Health Research Database (statistika.tai.ee/); [d] according to Estonian growth curves by Sildver et al., 2015

2.2.2. Preeclampsia – a placental disease in late pregnancy

Preeclampsia (PE) is a syndromic pregnancy complication characterized by *de novo* onset of hypertension accompanied by other maternal organ damage symptoms. No one symptom is specific for PE, but the collection of symptoms is used for the diagnosis. Current diagnostic criteria endorsed by the International Society for the Study of Hypertension in Pregnancy (ISSHP) are as follows:

Gestational hypertension (systolic BP ≥140 and/or diastolic BP ≥90 mmHg) with one or more new-onset conditions ≥20th gestational week:

a) Proteinuria
b) Other maternal organ dysfunction (acute kidney injury, elevated transaminases indicating liver damage, neurological complications, hematological complications)
c) Uteroplacental dysfunction (fetal growth restriction, abnormal Doppler analysis, stillbirth) (Brown et al., 2018)

Preeclampsia affects about 4.6% of pregnant women globally, but regional differences can be vast (Abalos et al., 2013). In Estonia, the incidence of PE in 2019 was about 1.7%, according to Estonian Health Statistics and Health Research Database (www.statistika.tai.ee/). Advances in health care have reduced PE-related mortality among mothers in high-income countries (0.03% in the UK) but remains an issue in the middle- and low-income countries accounting for 63 000 maternal deaths per year (Burton et al., 2019).

Currently, the clinical focus is finding the high-risk women, surveillance and managing the emerging symptoms, and delaying preterm delivery unless maternal health is at risk. Some at-risk women benefit from low-dose aspirin treatment started at 16–20th week of gestation to reduce the incidence of PE or the severity of the symptoms (Brown et al., 2018). When the low-dose aspirin treatment was started at 11–14th week for high-risk women, the reduction of preterm PE was over 60% (Rolnik et al., 2017). The end of pregnancy and departure of the placenta is currently the only definitive cure for PE.

Within PE, two distinct subtypes are differentiated according to the time of presentation of symptoms (Lisonkova & Joseph, 2013). The common consensus is that early-onset PE (EOPE) manifests from 20th to 34th gestational week and late-onset (LOPE) from 34th week onward (Tranquilli et al., 2013). EOPE tends to have more severe symptoms and results more often in preterm delivery. The two subtypes of PE are also thought to have differing pathologies. Whereas insufficient placental invasion is the leading cause of early PE, late-onset PE is believed to be caused by the aging of the placenta (Ness & Roberts, 1996, **Figure 3**).

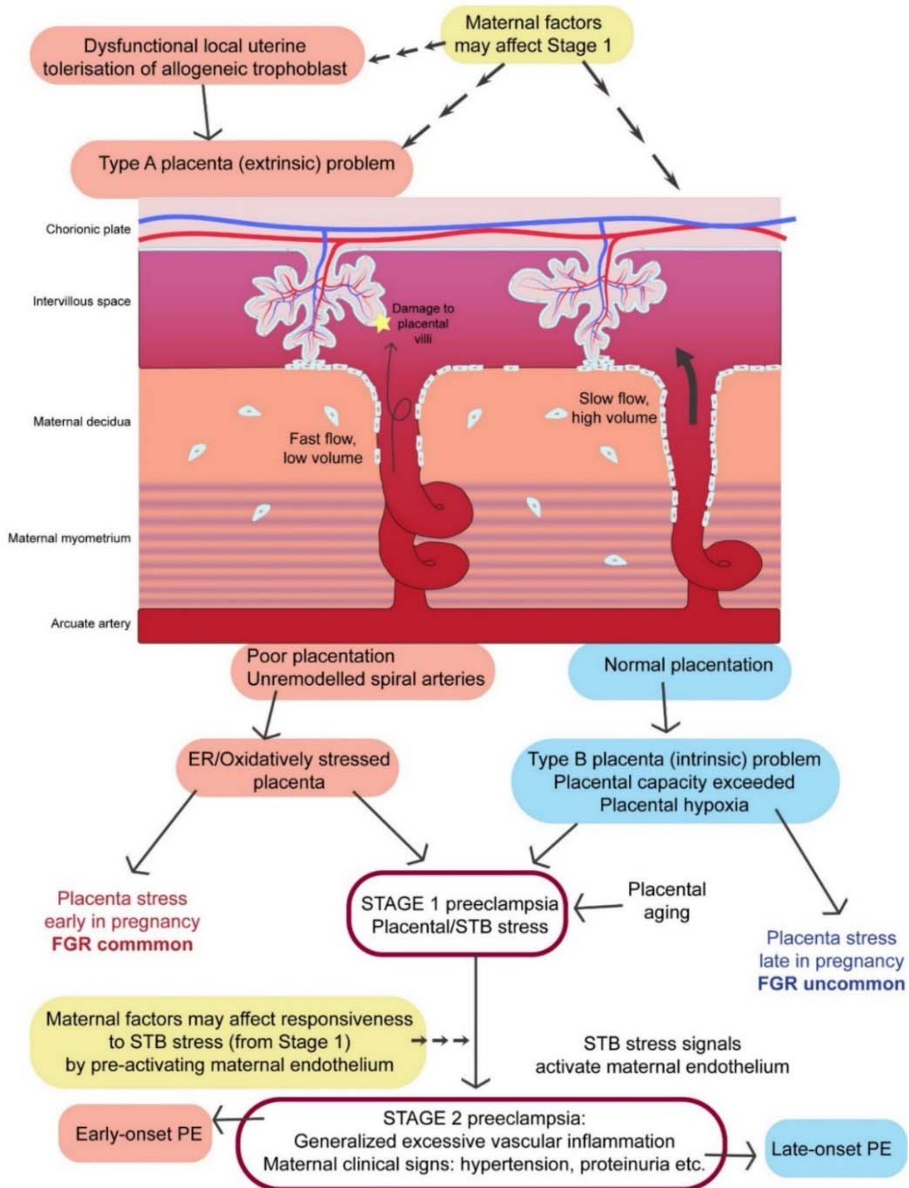

Figure 3. Pathways to the development of preeclampsia (PE). Adapted from (Aplin et al., 2020; Staff, 2019). ER, endoplasmic reticulum; FGR, fetal growth restriction; STB, syncytiotrophoblast

2.2.3. Diagnostic and prognostic biomarkers of pregnancy complications

In Estonia, screening for fetal chromosomal and gross anatomical abnormalities is carried out during the first trimester visits from the 11th to 14th gestational week. The risk for early PE can also be assessed during the first trimester by utilizing serum PAPP-A, placental growth factor (PlGF), and uterine artery Doppler combined with maternal characteristics and medical history in an algorithm by the Fetal Medicine Foundation (FMF) (O'Gorman et al., 2016; Tan et al., 2018). The FMF algorithm has been in use in Estonia since 2019.

Some less established biomarker screenings for late pregnancy have been proposed for a few pregnancy complications (**Table 3**). National Institute for Health and Care Excellence (NICE) guidance recommends additional use of commercial PlGF/sFlt-1 ratio or the Triage PlGF test for excluding the manifestation of both early and late PE up to four weeks in the second or third trimester (Herraiz et al., 2018; NICE, 2016). Fetal fibronectin can be used to assess immediate risk for preterm birth in the next 48 hours (NICE, 2015).

The clinical risk factors for gestational diabetes (GD) include high fasting blood sugar, glycosuria, excessive weight gain, pre-pregnancy body mass index (BMI) $\geq$25 kg/m^2, prior GD, familial diabetes, previous newborn >4500g, and polycystic ovary syndrome are associated with GD. The presence of any of these demand a glucose tolerance test (GTT) at 24–28 gestational weeks to detect abnormal carbohydrate metabolism and GD (Hod et al., 2015). Blood pressure measurement and urine test at every visit after 20th gestational weeks are used to discover gestational hypertension or PE. Small- (SGA) and large-for-gestational-age (LGA) newborns are diagnosed most commonly after birth according to sex-adjusted growth curves. Still, they can be predicted during pregnancy by estimating the fetal weight by fundal height measurements and/or during the ultrasound scan (Sildver et al., 2015) (**Table 3**).

2.3. Genetics of late pregnancy complications

2.3.1. Challenges in the genetic research of pregnancy complications

Researching pregnancy complications offers some unique challenges. To start, in most diseases, the influence of the individual's genetic makeup together with environmental factors is studied. In pregnancy, there is a third counterpart – the fetal genome. This also complicates the choice of study subjects; either the mother, fetus, or both should be studied (Rull et al., 2012).

Table 3. Prognostic and diagnostic biomarkers in use for different pregnancy complications

Pregnancy complication	Prognostic biomarkers	Availability	Trim.	Comment	Diagnostic biomarkers
Preeclampsia	PAPP-A, PlGF, uterine artery Doppler, and maternal characteristics combined by FMF algorithm	clinic	I	determines high-risk pregnancies	blood pressure, proteinuria, blood tests, ultrasound/ doppler
	PlGF, PlGF/sFlt-1	commercial	II–III	1–2 weeks rule-out	
Gestational diabetes	fasting glycose >5,1 mmo/l, random glucose >6,9mmol/l	clinic	I	determines high-risk pregnancies	GTT >24 gestational weeks: fasting glucose >5.1mmol/, 1h glucose >9.9mmol/L, 2h glucose >8.4mmol/L
Preterm birth	cervical length	clinic	II	determines high-risk pregnancies	Start of labor activity; IGFBP-1, PAMG-1 (for P-PROM)
	fetal fibronectin	commercial	III	48h rule-out (intact membranes)	
Small-for-gestational age	poor weight gain, low fundal height	clinic	II–III	determines high-risk pregnancies	fetal abdominal circumference, estimated fetal weight <10th centile

The table is compiled using the National Institute for Health and Care Excellence and Estonian Gynecologist Society Guidelines. IGFBP-1, insulin-like growth factor binding protein-1; PAMG-1, placental alpha-microglobulin-1; PAPP-A, pregnancy-associated plasma protein A; PlGF, placental growth factor; P-PROM, preterm prelabor rupture of membranes; sFlt-1, soluble fms related receptor tyrosine kinase 1; Trim., trimester.

Most pregnancy complications have been acknowledged to be multifactorial, affected by maternal and fetal genetics and lifestyle. However, the exact proportions have not been identified for all complications at this time. For PE, it has been estimated that maternal genetic factors account for 35–38% and placental 20–21% to a combined heritability of 55–59% (Cnattingius et al., 2004; Steinthorsdottir et al., 2020). The heritability of SGA and LGA is not established. However, combined genetic contributions have been proposed to account for up to 50% of the birth weight variation (Clausson et al., 2000).

An additional challenge is the approach of analysis. One option is to investigate candidate genes where a hypothesis of effect can be easily constructed from previous knowledge. However, the selection is limited by the current understanding of the disease pathophysiology. Genes may be selected based on signaling or biochemical pathways. The main drawback of this approach is that not all contributors to the disease pathophysiology might be known already. Previously, candidate gene based studies have had low reproducibility, possibly due to small sample sizes. Another method can be used to overcome this aspect – genome-wide association studies (GWAS). GWAS offers a hypothesis-free analysis of the whole genome, enabling the identification of novel candidate genes. However, a significant drawback of this method is the need for extensive sample sets to detect significant associations after correcting for multiple testing. Collecting large sample sets of pregnancy-related tissues such as placental samples has been more complex than simple blood samples.

2.3.2. Genetics of preeclampsia

As the disease, so is the genetics of PE complex. Hundreds of genes and variants have been associated with PE. However, only a small portion of the finds have been replicated in separate studies. The genetic background of PE might partly be shared with other traits such as elevated diastolic blood pressure (DBP) and high BMI (Gray et al., 2021). However, genetic risk scores for hypertension or DBP were not found to be predisposing PE, indicating still different underlying pathways (Smith et al., 2016).

Another complicating factor of PE genetics is the sub-types of PE. There is a consensus that EOPE and LOPE have different genetic backgrounds (Burton et al., 2019; Oudejans et al., 2007), but a few studies have proposed other genetically distinct PE subsets (Leavey et al., 2015, 2016). Using aggregate data from several gene expression microarray-based studies, Leavey et al., 2016 proposed three distinct PE subgroups – canonical", immunological", and maternal" PE. However, the gene expression profiles of immunological" and maternal" PE patients were not as distinct from controls as canonical" PE patients. This also illustrates the importance of group selection for conducting studies on PE, especially with smaller sample sizes. The collection of a more distinct PE group might reduce the noise and improve the results.

2.3.2.1. Candidate gene studies for preeclampsia

Traditionally, PE candidate genes have been selected from various affected pathways, including endothelial function (e.g., renin-angiotensin system, *FLT1*), the oxidative stress and thrombophilia pathways, hemodynamics, immune response (e.g., *IL-10*, *TNF-a*, *HLA-G*), and lipid metabolism (Thakoordeen et al., 2018; Williams & Broughton Pipkin, 2011). However, many of the candidate gene studies have conflicting results.

Most studies so far have been conducted on maternal samples. These are more readily available as maternal blood samples are taken routinely during pregnancy. Another reason to prefer maternal samples is that they are less invasive than placental or fetal samples that can be taken during pregnancy. Therefore, maternal samples have a higher potential for later clinical use.

A study using maternal samples focusing on approximately 2000 cardiovascular candidate genes (including 27 429 variants) was recently conducted (Gray et al., 2018). Only one variant (rs9478812) in the *PLEKHG1* gene reached statistical significance in the multiethnic meta-analysis (OR=1.40, 95% CI 1.23–1.60, $P=5.9\times10^{-7}$). The gene is expressed in most tissues and has been previously associated with blood pressure, body weight, and neurological disorders, but the exact function of *PLEKHG1* is still largely unknown.

Placental stanniocalcin-1 (*STC1*), a glycoprotein hormone that regulates calcium homeostasis in fish (Wagner et al., 1986), is a relatively new candidate gene for PE. It has been shown to exhibit trimester-specific dynamics and higher levels in term PE placentas. Maternal serum levels of STC1 have also been shown to have increased in post-PE pregnancy samples (Uusküla et al., 2012). The hormone expression increases in hypoxic conditions in the BeWo cell line, possibly to protect the placenta against low oxygen in PE (Abid et al., 2020). *STC1* expression has been shown to increase in the endometrium in the mid-secretory phase and is dysregulated in endometriosis and polycystic ovary syndrome (Aghajanova et al., 2016; Khatun et al., 2020).In animal models, several reproductive phenotypes have been linked to STC1, such as ovarian function (Deol et al., 2000), implantation (Song et al., 2009), gestation, and lactation (Deol et al., 2000; Varghese et al., 2002). STC1 seems to have a diverse functionality in mammals, and the evidence for importance in pregnancy is increasing.

FLT1, on the other hand, is one of the best-known candidate genes for PE. As the sFlt-1 inhibits angiogenesis in the placental vasculature through trapping VEGF-A, it is a clear candidate for PE pathophysiology (Shibuya, 2013). The soluble form of Flt-1 is not expressed in many other tissues besides the placenta, but for example, the avascularity of the cornea is maintained by sFlt-1 (Ambati et al., 2006). Placenta-derived sFlt-1 is known to increase in maternal serum slightly before and during a presentation of PE symptoms (Levine et al., 2004; Maynard et al., 2003). The gene expression levels in the placenta have also been shown to rise in PE (Uusküla et al., 2012). The genetics behind *FLT1* isoform splicing are still poorly known. One region in *FLT1* intron 13 has been shown to

regulate the alternative splicing of the gene but only had a modest effect on sFlt-1 isoform abundance (Thomas et al., 2010).

2.3.2.2. Linkage studies for preeclampsia

The first genome-wide scan for susceptibility loci in PE was conducted using linkage analysis of 15 Australian families (Harrison et al., 1997). The study identified a region on chromosome 4q that was associated with PE. Four more linkage studies have been conducted on family sets from Iceland (Arngrímsson et al., 1999), Australia/New Zealand (Moses et al., 2000), Netherlands (Lachmeijer et al., 2001), and Finland (Laivuori et al., 2003). Arngrimsson et al. and Moses et al. identified both a region on chromosome 2 associated with PE. Moses et al. and Laivuori also detected nominal significance for the 4q region identified by Harrison et al. However, other regions among these studies did not replicate.

2.3.2.3. Genome-wide studies for preeclampsia

A handful of GWA studies have been conducted for PE in maternal samples (**Table 4**); however, very few variants have reached significance, and none have been replicated in independent datasets (Burton et al., 2019). A HAPO study (137 cases, 2986 controls) (Zhao et al., 2013), as well as an earlier study (177/116) (Zhao et al., 2012), did not identify any significant single nucleotide variants (SNVs) after correction for multiple testing. Another GWAS (538/540) identified a single independent signal near the *INHBB* gene (Johnson et al., 2012). Most recent maternal GWAS applying 12,150 PE cases and 164,098 controls implicated two variants near *ZNF831* and *FTO* genes. Both variants have been previously implicated with blood pressure levels, and the *FTO* variant also with several other metabolic traits (Steinthorsdottir et al., 2020).

As genome-wide studies in maternal samples have not brought expected success, more studies have been conducted in placental samples (**Table 4**). Recently, the first PE GWAS was conducted in placental tissue (4,380/310,238), identifying only one variant (rs4769613) near the *FLT1* gene with genome-wide significance despite the large sample size (McGinnis et al., 2017). Another recent GWAS included Central Asian placentas in addition to European samples and confirmed the previously detected association between late-onset PE and *FLT1* variant rs4769613 (linkage disequilibrium (LD) SNP rs4769612 was used in the study) (Steinthorsdottir et al., 2020). *FLT1* is a known PE candidate gene as the soluble form of the encoded protein rises sharply in maternal serum in response to PE. The genetic background for this increase is still not thoroughly known.

Table 4. Genome-wide association studies conducted for preeclampsia

Population	Sample size (PE/controls)	Analyzed variants	Identified risk variants	Nearest gene	Reference
Maternal					
American	177/116	705,969	No variants with genome-wide significance	n.a.	(Zhao et al., 2012)
Australian	538/540	648,175	rs7579169, rs12711941[a]	*INHBB*	(Johnson et al., 2012)
American, Australia, Barbados, Canada, Thailand, United Kingdom	Afro-Caribbean 23/1075; European 52/1250; Hispanic 62/661	979,693, 541,023, 964,533	No variants with genome-wide significance	n.a.	(Zhao et al., 2013)
Finnish	1352/699/6119 population reference	259,919	No variants with genome-wide significance	n.a.	(Kaartokallio et al., 2016)
Denmark, Finnish, Icelandic, Kazakhstan, Norwegian, United Kingdom, Uzbekistan	European 9262/161,678; Central Asian 2888/2420	11,796,347	rs1421085, rs259983	*FTO, ZNF831*	(Steinthorsdottir et al., 2020)
Fetal					
Finnish, Icelandic, Norwegian. United Kingdom	4,380/310,238	7,476,169	rs4769613	*FLT1*	(McGinnis et al., 2017)
Denmark, Finnish, Icelandic, Kazakhstan, Norwegian, United Kingdom, Uzbekistan	European 5210/374,105; Central Asian 2597/2388	12,130,433	rs4769612[b]	*FLT1*	(Steinthorsdottir et al., 2020)

Full gene names are found in abbreviations. n.a., not applicable; [a] in high LD with rs7579169 (r^2=0.92); [b] in high LD with rs4769613 (r^2 = 0.99)

Without using extensive datasets, it is clear that it is difficult to identify any clear associations with PE as the syndrome is so varied. However, large datasets for placental tissues are not common, so defining a distinct subgroup could help analyze candidate genes also in smaller sample sets

2.4. Direct effect of genetic variation on gene expression levels

2.4.1. Expression quantitative trait loci – eQTLs

Single nucleotide variants in the genome that affect the expression of specific genes are historically called expression quantitative trait loci (eQTLs, Farrall, 2004) and just eSNVs or eVariants. The first study to assess the genetic variants affecting gene expression genome-wide was done in yeast only 20 years ago (Brem et al., 2002) and in mammals a year later (Schadt et al., 2003). The largest project to map such variants to date in humans is The Genotype-Tissue Expression (GTEx) Project. The GTEx project has mapped over 4 million eQTLs affecting gene expression in at least one of the 49 tested tissues (Aguet et al., 2020). These variants have been shown to modulate the expression of 94.7% of all protein-coding genes.

eQTLs can be divided into two subtypes based on their distance from the affected gene (**Figure 4**). Nearby variants (cis-eQTLs) are located up to 1Mb from the gene; however, the definition can vary among different studies. Trans-eQTLs, on the other hand, can be located even on another chromosome, the effect on gene regulation mediated by three-dimensional links or intermediate genes. Most cis-eQTLs tend to be less tissue-specific than trans-eQTLs; among both, the specificity follows a U-curve with a higher percentage of both tissue-specific and highly universal variants (Aguet et al., 2020).

Other, more specific types of eQTLs have also been proposed. Dynamic eQTLs exhibit temporal influence on gene expression, only regulating the mRNA levels in specific time periods. During differentiation from induced pluripotent stem cell (iPSC) to cardiomyocytes, a linear dynamic eQTL effect was found for 550 genes (Strober et al., 2019). Additionally, 693 genes were suggested to exhibit nonlinear eQTL effects, with 28 having the highest effect in the middle. Similarly, during iPSC differentiation to endoderm, about 30% of identified eQTLs were stage-specific (Cuomo et al., 2020). However, dynamic eQTL analysis has been limited primarily to *in vitro* studies due to the inaccessibility of *in vivo* tissue samples. Such dynamic *cis*-eQTLs in early development might appear to act in trans later in life (Umans et al., 2020). Another subtype of dynamic eQTLs is a response or conditional eQTLs that only have an effect in certain conditions, usually to a response to an extrinsic stimulus. Change in the set of identifiable eQTLs has been shown in monocytes after stimulation, imitating innate immune response (Fairfax et al., 2014).

Variants having an allele-specific effect on gene expression variance instead of the mean value have been proposed (dispersion QTLs, dQTLs), possibly affecting disease penetrance (Sarkar et al., 2019). However, the detection of such variants needs massive datasets due to the considerable influence of non-genetic background.

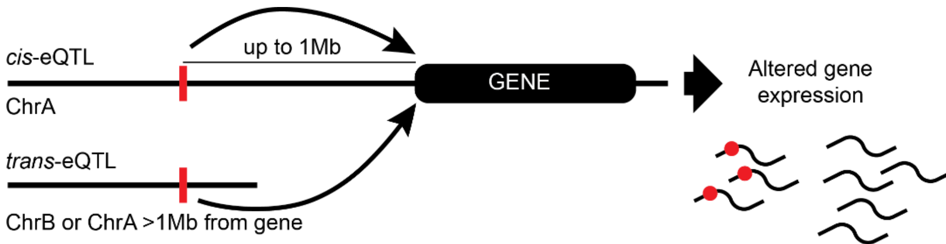

Figure 4. Both cis- and trans-eQTLs can affect gene expression.

2.4.2. eQTLs and human disease

The first eQTL screen done in a mouse also identified that some eQTL loci are linked to murine obesity subtypes (Schadt et al., 2003). The authors also proposed that combining gene expression, genotype, and clinical data could help identify underlying pathways. Since then, combining GWAS and eQTL data from appropriate tissue has become a common practice (Cookson et al., 2009, **Table 5**). As most variants identified in GWAS are intragenic, it can be challenging to determine the functional relevance to the disease (Maurano et al., 2012). As more and more eQTL studies are conducted in various tissues, these results are integrated with GWAS data. Without additional information on the variants, usually closest genes to the GWAS variant were prioritized. However, a study demonstrated that among 104 identified functionally relevant genes after eQTL data integration for five GWAS traits, even as much as two-thirds were not the nearest gene (Z. Zhu et al., 2016).

Several studies have demonstrated enrichment of eQTLs among variants identified in GWAS, both in the whole catalog and in specific diseases or traits (**Table 5**)(Aguet et al., 2020; Peng et al., 2017; Viñuela et al., 2020). Overall, the GTEx project identified cis-eQTLs have a 1.46-fold enrichment among GWAS catalog variants. Some studies have also used eQTL data to prioritize candidate genes from GWAS data to further investigate diseases such as osteoarthritis and Crohn's disease (Marigorta et al., 2017; Tachmazidou et al., 2019). Whether the co-localization is due to pleiotropic effects or mediated by gene expression changes is unclear in many cases. Defining the cause is made more challenging by difficulty determining the causative variant among many in high LD.

Table 5. Examples of studies integrating eQTL data with GWAS variants

Study	eQTL analysis tissue	Individuals (n)	eQTLs (n)	Enrichment fold among GWAS loci	Co-localized with GWAS (n, % among GWAS loci)	GWAS phenotype
Studies utilizing the whole GWAS catalog						
(Nicolae et al., 2010)	LCL	87[a]	345,249	$p<0.05$[b]	625/1,598 (39%)[c]	GWAS catalog[d]
(Westra et al., 2013)	blood	8,086	664,097 *cis*, 1,513 *trans*	$p<0.05$[b]	254/2,082 (12%) *trans*	GWAS catalog
(Peng et al., 2017)	placenta	159	3,218	1.68	835/16,439 (5%)	GWAS catalog
(Gong et al., 2018)	33 cancer tissues	36–1092	5,606,570	not reported	337,131/1,167,961 (29%)[e]	GWAS catalog
Studies utilizing specific disease-related GWAS datasets						
(Smoller et al., 2013)[f]	prefrontal cortex cortex cortex liver skin LCL	269 193 364 427 57 270	1,628 3,240 1,571 1,268 6,250 7,015	NS $p<0.05$[b] $p<0.05$[b] NS NS NS	not reported	autism spectrum disorder, ADHD, bipolar disorder, major depressive disorder, schizophrenia
(Walker et al., 2019)	mid-gestational prenatal brain	201	7,962	$p<0.05$[b]	not reported	schizophrenia
(Aguet et al., 2020)	49 tissues	70–706	4,278,636	1.46	2,315/5,385 (43%)	87 complex traits
(Viñuela et al., 2020)	pancreatic islet	420	7,741	1.09–1.27	46/459 (10%)	T2D, glycemic traits
(Orozco et al., 2020)	RPE/choroid, retina	129	75,666	not reported	15/35 (43%)	AMD

[a] CEU individuals only; [b] enrichment fold not reported; [c] p-value threshold 10^{-4}; [d] entire GWAS catalog available at the time of the study (www.ebi.ac.uk/gwas); [e] including linkage disequilibrium variants; [f] study utilized various previously published eQTL datasets, including two separate datasets from a cortex. ADHD, attention deficit-hyperactivity disorder; AMD, age-related macular degeneration; GWAS, genome-wide association study; LCL, lymphoblastoid cell lines; NS, not significant; RPE, retinal pigment epithelium; T2D, type II diabetes

2.5. Summary of the literature review

The placenta is a unique, transient organ in human pregnancy delivering oxygen and nutrients to the fetus. In addition, the placenta acts as an endocrine organ, synthesizing macromolecules that modify the course of gestation. It has been shown that in several pregnancy complications, such as fetal growth restriction and PE, the placental transcriptome profile is significantly changed. PE is a serious complication that affects up to 5% of pregnant women, posing a risk for maternal and fetal health. Many candidate genes have been identified for PE, but few have been replicated in independent sample sets. *FLT1* is a well-known PE candidate gene that codes sFlt-1, a soluble molecule that reaches maternal serum rising few weeks before PE symptoms. Even for *FLT1*, the exact genetic causes behind the gene expression changes are not well known.

Genetic variation associated with specific gene expression changes is one of the proposed processes behind the precise placental expressional dynamics. Such variants, commonly called expression quantitative trait loci or eQTLs, have been identified in many tissues. Many of the variants are tissue-specific and the data cannot be transferred to other tissues. Previously, it has been noted that many eQTLs co-localize with GWAS loci, suggesting their role in risk for disease development. Identifying placental eQTLs could provide further insight into pregnancy gene expression dynamics as well as pregnancy complications. However, only a few studies have conducted placental eQTL analyses so far.

3. AIMS OF THE PRESENT STUDY

The general aim of this thesis was to explore and describe the landscape of placental genetic regulation of gene expression through expression quantitative trait loci (eQTLs) and their role in the development of pregnancy complications.

The specific aims of this thesis were:

1. To investigate eQTLs of candidate genes for preeclampsia, *STC1* and *FLT1*.

2. To identify and characterize robust placental eQTLs in the placental tissue.

3. To compile the current knowledge of placental eQTLs identified for candidate genes and in transcriptome-wide association studies.

4. MATERIAL AND METHODS

4.1. Ethics

The protocols of REPROMETA (full study name: REPROgrammed fetal and/or maternal METAbolism) and HAPPY PREGNANCY (full study name: Development of novel non-invasive biomarkers for fertility and healthy pregnancy) studies were reviewed and accepted by the Ethics Review Committee of Human Research of the University of Tartu, Estonia (permissions no 146/18, 27.02.2006; 150/33, 18.06.2006; 158/80, 26.03.2007; 221/T-6, 17.12.2012; 286/M-18, 15.10.2018).

Ethical approval for the Avon Longitudinal Study of Parents and Children (ALSPAC) was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. Consent for biological samples was collected in accordance with the Human Tissue Act (2004). Informed consent for the use of data collected via questionnaires and clinics was obtained from participants following the recommendations of the ALSPAC Ethics and Law Committee.

The Finnish Genetics of Preeclampsia Consortium (FINNPEC) study protocol was approved by the coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa. The Southern Finnish participant study was approved by the local ethical review committee at the Helsinki University Hospital.

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

4.2.1. Recruitment and characteristics of REPROMETA and HAPPY PREGNANCY sample sets

Participants of both REPROMETA (supported by HHMI#55005617 and ETF9030 grants) and HAPPY PREGNANCY (supported by SLOMR12214T grant) studies were recruited at the Women's Clinic of Tartu University Hospital, Estonia (2006–2011 and 2013–2015, respectively). All participants were of Caucasian ancestry.

The REPROMETA study (n=377) focused on recruitment of extreme cases of selected pregnancy complications (preeclampsia (PE, n=53), gestational diabetes (GD, n=50), small- (SGA, n=72) and large-for-gestational-age newborns (LGA, n=97)) and uncomplicated pregnancies (NORM, n=105). The family trios or duos were recruited before or shortly after delivery at the Women's Clinic. Epidemiological data, reproductive history, and parental lifestyle were obtained from self-reported questionnaires filled shortly after recruitment by both parents. Pregnancy outcome data was acquired from the medical records. Placental samples were

available for 366 cases. REPROMETA infant growth data was collected from questionnaires filled by the parent or the general practitioner at 6 (n=233) and 12 months (n=216).

Individuals in the HAPPY PREGNANCY cohort study (n=2334) were recruited prospectively during their first antenatal visit at the Women's Clinic. The patients were asked to fill three questionnaires throughout their pregnancy concerning epidemiological data, reproductive history, parental lifestyle, and additional pregnancy course and outcome data collected from the medical records. Placental samples were available for 1768 singleton cases.

HAPPY PREGNANCY consisted of PE (n=44), SGA (n=129), LGA (n=141), GD (n=102), preterm (n=58), and NORM (n=1294) pregnancies.

The exact number of individuals utilized from each sample set has been noted in each study (Juhanson et al., 2016; Kikas et al., 2019, 2020) (**Table 6**). Some individuals were excluded from the studies due to lack of DNA or other targeted materials or genotyping failure.

Table 6. Sample sets used in the studies

Sample set	**Ref. 1 Juhanson et al., 2016**	**Ref. 2 Kikas et al., 2020**	**Ref. 3 Kikas et al., 2019**
Estonian cohorts			
REPROMETA (REPROgrammed fetal and/or maternal METAbolism)			
Maternal DNA	50 PE/316 NPE	40 PE/253 NPE	n.a.
Maternal plasma	50 PE/316 NPE	n.a.	n.a.
Paternal DNA	n.a.	42 PE/227 NPE	n.a.
Placental DNA	50 PE/316 NPE	52 PE/227 NPE	336
Placental RNA-seq	n.a.	n.a.	40
Placental genotyping	n.a.	n.a.	40
HAPPY PREGNANCY (Development of novel non-invasive biomarkers for fertility and healthy pregnancy)			
Placental DNA	n.a.	44 PE/1724 NPE	408
Maternal serum	n.a.	18 PE/135 NPE	n.a.
Collaboratory cohorts			
FINNPEC (Finnish Genetics of Preeclampsia Consortium)			
Maternal DNA	547 PE/513 NPE	n.a.	n.a.
Umbilical cord DNA	378 PE/496 NPE	n.a.	n.a.
ALSPAC (Avon Longitudinal Study of Parents and Children)			
Placental DNA	n.a.	n.a.	7669

C, controls; n.a., not applicable; NPE, non-preeclampsia; PE, preeclampsia

4.2.1.1. Clinical subgrouping in REPROMETA and HAPPY PREGNANCY sample sets

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

NORM group was defined as pregnancies uncomplicated by previously mentioned conditions with a newborn between 10th and 90th percentile on the growth curves calculated based on data from Estonian Medical Birth Registry growth standards (Sildver et al., 2015). SGA and LGA pregnancies had a newborn either $<10^{th}$ or over 90th percentile, respectively, on the growth curves. PE cases were defined as hypertensive (systolic blood pressure ≥160mmHg and/or diastolic blood pressure ≥110mmHg) and had proteinuria of ≥5g in 24 hours or neurological symptoms (Brown et al., 2018). PE was further subdivided into early-onset (symptoms before 34th gestational weeks) and late-onset PE (after 34th gestational weeks). GD was diagnosed when 75g oral glucose tolerance test (GTT) performed at 24–28 weeks of gestation indicated either a fasting venous plasma glucose level of ≥5.1 mmol/l and/or at 1h and 2h later plasma glucose level of ≥10.0 mmol/l and ≥8.5 mmol/l glucose, respectively (International Association of Diabetes and Pregnancy Study Groups Consensus Panel, 2010). Pregnancies with birth before the 37th gestational week were considered preterm.

4.2.2. Collaborative replication sample sets – ALSPAC, FINNPEC

The Finnish Genetics of Preeclampsia Consortium (FINNPEC) study recruited preeclamptic and control participants in 5 university centers in Finland (Helsinki, Turku, Tampere, Kuopio, and Oulu) in 2008–2011 (Jääskeläinen et al., 2016). The controls were recruited for each PE patient by inviting the next available patient to give birth at the same hospital with no PE. Only individuals meeting the REPROMETA diagnostic criteria for PE and NORM groups were included in the analyses. PE patients were further divided into EO-PE (n=165) and LO-PE (n=382) subgroups.

In total, 547 PE patients and 513 controls were included in the study. For fetal samples (n=EO-PE, n=88; LO-PE, n=290; NORM, n=496), the DNA was extracted from cord blood.

The Avon Longitudinal Study of Parents and Children (ALSPAC) initially recruited 14,541 pregnant women residents in Avon, United Kingdom, with expected delivery dates from April 1, 1991, to December 31, 1992 (Boyd et al., 2013; Fraser et al., 2013) (http://www.alspac.bris.ac.uk). For all recruited cases, medical data from obstetric and perinatal records were documented. From the initial pregnancies, 14,062 resulted in live births. Gestational age at the delivery was recorded the nearest gestational week. The study analyzed 7,669 newborns with available genotype data. Please note that the ALSPAC study website contains details of all the available data through a fully searchable data dictionary and variable search tool: http://www.bristol.ac.uk/alspac/researchers/our-data/.

4.2.3. Placental and blood sampling in REPROMETA and HAPPY PREGNANCY studies

Placental sampling in REPROMETA and HAPPY PREGNANCY studies was conducted within one hour after cesarean section or vaginal delivery by trained nurses following the same protocol. In the meanwhile, placentas were kept at +4 °C. A full-thickness block of 2 cm was taken from the middle region of each placenta, avoiding the umbilical cord insertion site, large vessels, and any visible or palpable infarction, hematoma, or damage. In the HAPPY PREGNANCY study, this step was repeated for each quadrant of the placenta. Placental samples were washed with 1x PBS to remove maternal blood and subsequently divided into sections for DNA and RNA extraction. Tissue for RNA extraction (1 g or 100 mg in REPROMETA or HAPPY PREGNANCY study, respectively) was placed into 10 ml or 1 ml RNAlater (AM7021; Thermo Fisher Scientific). Samples were kept in RNAlater for 1–3 days at +4 °C and then stored at –80 °C until RNA extraction. The rest of the tissue sample was placed into a dry tube and stored at –80°C until DNA extraction.

Maternal blood sampling in the REPROMETA study was conducted on the day of the delivery. The blood sample was aliquoted and stored at –80 °C immediately after collection. Maternal plasma was extracted from the same sample during aliquoting.

Maternal serum samples from HAPPY PREGNANCY individuals were collected during routine blood tests throughout pregnancy and stored at –80 °C.

4.3. Utilized resource: placental whole-genome datasets

4.3.1. Placental RNA-Seq dataset

The REPROMETA placental RNA sequencing dataset was first published by Sõber et al., 2015. The dataset included 40 term placentas from various pregnancy outcomes (PE, SGA, LGA, GD, NORM, n=8 each).

RNA from the placental sample (200–300mg) was extracted using the Trizol protocol and purified with RNeasy MinElute columns (74204; Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. NanoDrop ND-1000 UV-Vis spectrophotometer (Applied Biosystems, Foster City, USA) was used to determine the purity and concentration of isolated total RNA. RIN (RNA integrity number) was estimated by Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

Total RNA with high purity was used for rRNA depletion (Ribo-Zero rRNA Removal Kit, MRZH11124; Illumina, San Diego, CA, USA) and library preparation with Nextera Technology (FC-121-1030; Illumina). Total RNA sequencing was conducted in Finland Institute for Molecular Medicine (FIMM) Sequencing Core Laboratory on Illumina Hiseq2000 using 46 bp paired-end reads. Initial data refinement was performed with RNA-Seq pipeline v.2.4 (FIMM; Helsinki, Finland). Human genome assembly (GRCh37.p7/hg19) from Ensembl v67 was

used as a reference. The initial dataset included gene expression data for 53,893 genes. Gene expression was quantified by HTSeq analysis (as raw read counts) and later normalized for read depth using DESeq package for R. Only non-mitochondrial genes with sufficient expression levels (>100 normalized read count) were considered in the analysis (n=11,733).

4.3.2. Placental whole-genome genotyping dataset

The same 40 samples with available RNA sequencing data also underwent whole-genome genotyping (Kasak et al., 2015). The DNA of the placental samples was extracted using a NucleoSpin Tissue kit (Macherey-Nagel, Germany) according to the manufacturer's instruction. The genotyping was conducted with Illumina HumanOmniExpress-12-v1 BeadChip at the institutional genotyping core facility (Estonian Genome Center; http://www.geenivaramu.ee/en). The array included >733,000 SNPs with a median spacing of 2.1 kb. Samples were genotyped with an average overall call rate >99% per individual per genotype.

Variants deviating from Hardy-Weinberg Equilibrium (HWE; $P<1\times10^{-6}$) or with no minor alleles in our dataset were excluded from subsequent analyses. In total, 661,354 SNVs were included in the eQTL analysis.

4.4. Locus-based genetic analysis methods

The study designs in Refs 1–3 included varied methods for genotyping genetic variants and quantifying gene and protein levels. A quick overview of different targeted methods used is given in **Table 7**.

4.4.1. Genotyping

To determine variants possibly affecting *STC1*, 13 tag-SNVs (**Table 7**) from the genic (12,893 bp) and promoter (2,672 bp, 5′ upstream) region were selected that captured additional 22 variants ($r^2>0.9$). The variants were identified based on genotyping data from the 1000 Genomes Project (http://www.international genome.org/) for UTAH residents with Northern and Western European ancestry (CEU) as input for Haploview Tagger software (version 4.2) (Barrett et al., 2005).

The 13-plex primers (Table S3 in Ref. 1) for the PCR and the extension were designed using MassARRAY® Assay Design 3.1 software. Positive and negative control samples and duplicate samples were included in every assay plate to assess the genotyping quality. The genotyping call rate was determined to be >95%, and all identified genotypes were in HWE (p>0.05). All 13 variants were genotyped for 366 REPROMETA maternal samples. REPROMETA placental samples (n=366) and FINNPEC maternal (n=1060) and fetal samples (n=874) were genotyped for three variants (rs12678447, rs3758089, rs3758086).

Table 7. Summary of utilized methods for locus-based analyses in the current thesis

Analysis target	Sample set	Samples, n	Method	Study
rs11135775, rs9969426, rs12681669, rs11779426, rs423432, rs76369571, rs12678447, rs578094, rs1438453, rs3758089, rs1369836, rs3758087, rs3758086	REPROMETA maternal DNA	366	multiplex geno-typing with Sequenom MassArray® assays	Ref. 1
rs12678447, rs3758089, rs3758086	REPROMETA placental DNA/ FINNPEC maternal DNA/ FINNPEC umbilical cord DNA	366/1060/874	multiplex geno-typing with Sequenom MassArray® assays	Ref. 1
STC1	REPROMETA maternal plasma	366	ELISA	Ref. 1
STC1	REPROMETA placental RNA	120	TaqMan® Gene Expression Assay	Ref. 1
rs4769613, rs12050029	REPROMETA placental DNA/ HAPPY PREGNANCY placental DNA/ REPROMETA maternal and paternal DNA	329/1768/562	TaqMan® SNP Genotyping Assay	Ref. 2
sFlt-1	HAPPY PREGNANCY maternal serum	153	B·R·A·H·M·S™ sFlt-1 Kryptor™ assay	Ref. 2
FLT1	REPROMETA placental RNA	69	TaqMan® Gene Expression Assay	Ref. 2
rs1150707, rs10044354, rs11678251	REPROMETA placental DNA	366	multiplex geno-typing with Sequenom MassArray® assays	Ref. 3
ZSCAN9, UBC, ERAP2, ALPG, LNPEP, ALPP	REPROMETA placental RNA	24/gene	TaqMan® Gene Expression Assay	Ref. 3
rs11678251	HAPPY PREGNANCY placental DNA	408	TaqMan® SNP Genotyping Assay	Ref. 3

Three variants [rs1150707 (*ZSCAN9* c.568+1990 C>T); rs10044354 (*ERAP2* g.96984791 C>T) and rs11678251 (*ALPG* c.-318 G>A)] were genotyped using Sequenom MassArray® platform in a REPROMETA placental dataset (n=366) to validate the eQTL associations (**Table 7**). The variants were chosen based on the low false discovery rate (FDR), larger than two-fold expression difference between the heterozygote and major homozygote carriers, and link to protein-coding gene. The primers for the 3-plex were similarly designed using the provided software.

All multiplex genotyping was conducted using Sequenom MassArray® platform (Sequenom, San Diego, CA, USA) according to the manufacturer's protocol in all cases.

Singleplex genotyping of selected variants was conducted using pre-made Taqman assays.

The rs11678251 (*ALPG* c.-318 G>A) variant was genotyped in the HAPPY PREGNANCY placental samples (n=408) using a Taqman assay (ID C__27838320_10, Applied Biosystems, Foster City, CA, United States) (**Table 7**).

Two variants (rs4769613 T/C, rs12050029 A/G) previously associated with PE were genotyped using Taqman assays (Applied Biosystems, Foster City, USA; Assay ID: C__32231378_10, C___1445411_10) in combined REPROMETA and HAPPY PREGNANCY placental sample set (n=2,097) and REPROMETA parental sample set (n=562) (**Table 7**).

4.4.2 Taqman-assay based expression quantification

The expression levels of *ZSCAN9* (Hs00196838_m1), *ERAP2* (Hs01073631_m1) and *ALPG* (Hs00741068_g1), as well as the neighboring genes ALPP (Hs03046558_s1), ERAP1 (Hs00429970_m1), and LNPEP (Hs00893646_m1), were determined using Taqman RT-qPCR gene expression assays according to manufacturer's protocol (**Table 7**). The housekeeping gene Ubiquitin C (*UBC*, Hs00824723_m1) was used as a reference gene. Twenty-four individuals were chosen for each gene to represent genotypes for previously identified variants (rs1150707, rs10044354, rs11678251) equally when possible.

4.5. Biomarker analysis from maternal blood

Maternal plasma levels of STC1 protein were determined using enzyme-linked immunosorbent assay (ELISA) implemented by DuoSet ELISA kit (DY2958; R&D Systems) according to the manufacturer's protocol (**Table 7**). STC1 levels were measured in 366 samples. All the measurements were performed in duplicate with each plate, including a reference sample for variability assessment. The estimated average intra-assay variability for processed ELISA plates (n=10) was 4%, and respective inter-assay variability 7%. The details of the ELISA protocol have been discussed in Uusküla et al., 2012.

A set of commercially measured sFlt-1 levels in HAPPY PREGNANCY maternal plasma samples (n=153) was utilized for analyses (**Table 7**). Serum concentrations were retrospectively measured with a commercial immunoassay, B·R·A·H·M·S sFlt-1 Kryptor assay (Thermo Fisher Scientific) by the service provider Synlab Germany (Leinfelden, Germany). The measurement was done in immunofluorescent automated sandwich assays implemented on the KRYPTOR compact PLUS platform (#BM0106172) and using B·R·A·H·M·S reagents for the sFlt-1 assay (#845.075). Only samples drawn before term (<37th gestational week) and >1 week prior delivery were included in the analysis as sFlt-1 levels sharply increase before delivery (Palm et al., 2011)

4.6. Bioinformatics and statistics

4.6.1. Whole transcriptome *cis*-eQTL analysis and validation

The analysis focused on proximal cis-eQTLs (±100 kbp from the gene start/end coordinates) as this region is likely to contain significant and functionally relevant eQTL hits (Veyrieras et al., 2008). Variant and gene coordinates were extracted using BioMart (Ensembl v54). Association analysis for eQTL discovery was conducted in Matrix eQTL package for R (Shabalin, 2012) using linear regression. The analysis was adjusted by the pregnancy outcome group (NORM, PE, SGA, LGA, GD), labor activity, and newborn sex. The test included 353,599 variants for a total of 659,826 tests. Nominal P-values were corrected for multiple testing using a built-in Benjamini and Hochberg method in Matrix eQTL. A statistically significant eVariant-eGene association was defined as FDR<0.05. For each identified eQTL, the proportion of gene expression variability explained by the variant was calculated (R^2).

In the RT-qPCR validation of selected variants, the analysis was conducted similarly with an additional covariate of gestational age.

4.6.2. Genetic association testing

In brief, all genetic association testing was carried out in PLINK, version 1.07 or 1.9 (Purcell et al., 2007) either using an additive model with linear (newborn, maternal, or birth parameters) or logistic regression (case-control analyses). For each analysis, appropriate cofactors were included. All meta-analyses were combined under a fixed-effects model in PLINK or R.

Mann-Whitney U test (MW) was used parallel with linear regression for testing the difference of maternal STC1 hormone and placental *STC1* expression levels between minor allele carriers and major homozygote carriers of *STC1* variants (Ref. 1).

Association testing in REPROMETA with newborn parameters and three eQTLs (rs1150707, rs10044354, rs11678251) was implemented using both

additive and recessive models. Testing with infant postnatal growth was conducted only under the recessive model. (n=7,669) cohorts. For the ALSPAC cohort, a proxy variant rs744873 was used in the analysis, obtained from the genome-wide array dataset (Boyd et al., 2013) (Ref. 3).

Analysis of preference in allele transmission from the rs4769613 T/C heterozygous parents was conducted using the χ^2 test (Ref. 2).

Analysis of rs4769613 and placental *FLT1* expression utilized previously available RT-qPCR data (*FLT1* and *UBC* levels) for 23 PE and 46 non-PE REPROMETA samples (Sõber et al., 2015). Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. *FLT1* mRNA expression and sFlt-1 serum levels were transformed using log2 to resemble a normal distribution better. The difference in median *FLT1* or sFlt-1 between PE and non-PE individuals was assessed using the Student t-test (Ref. 2).

4.6.3. Functional profiling of placental eGenes

Functional profiling analysis of eGenes identified in at least two of the three studies was conducted in the g:Profiler web server (version e100_eg47_p14_7733820 (Reimand et al., 2016)). Terms were considered statistically significant at an adjusted P-value <0.05.

4.7. Literature search on placental expression-modifying variants

A literature search was conducted in PubMed using query: human and (placenta*) and (gene expression" or transcriptome") and (SNP" or SNV" or polymorphism" or eQTL") not review and (y_10 [Filter])" (time of accession 31.08.2020). Articles that were not relevant (non-human subjects, no reported association testing between genetic variants and gene expression) were excluded. Only journal articles written in English were included.

5. RESULTS

5.1. Candidate gene studies of genetic variants modulating placental gene expression and their link to pregnancy complications

Two genes were selected for further investigation to determine the genetic variants coding for expression changes in PE. *STC1* is a novel candidate gene for preeclampsia with no known expression-modulating variants. The second gene, *FLT1*, is a known gene implicated in PE with only recent insight into potential variants affecting its expression.

5.1.1. The effect of genetic variants in the *Stanniocalcin-1* locus (Ref. 1)

The first study (Juhanson et al. 2016) aimed to comprehensively characterize STC1 hormone and *STC1* gene expression in human pregnancies, including in pregnancies complicated with PE.

5.1.1.1. Placental and maternal variants affect gene and protein expression

Three placental genetic variants (rs126788447, rs3758089, rs3758086) were tested for correlation with *STC1* gene expression. One variant, rs126788447, significantly affected the placental gene expression working as an eQTL (linear regression adjusted to the delivery mode and pregnancy complication group, P=0.014, **Figure 5B**). Another variant, rs3758089, showed lower *STC1* expression in TT individuals, but the link was not statistically significant (P=0.067). Placental *STC1* gene expression in REPROMETA samples (n=120) was not affected by maternal lifestyle (e.g., maternal age, gestational weight gain, etc.) or birth parameters (e.g., gestational age, newborn weight, etc.) (Supplementary Figure 4 in Ref. 1).

Maternal STC1 hormone levels in REPROMETA maternal plasma samples (n=366) were similarly investigated with respective maternal variants. Variant rs3758089 (linear regression (LR) P=0.014, Mann-Whitney *U* (MW) test P=0.027) was significantly associated with maternal serum levels of STC1, but rs126788447 had a significant association only with the MW test (P=0.034) (**Figure 5A**). As maternal STC1 levels were additionally affected by PE (MW P<0.01), delivery mode (MW P=0.01), smoking status (MW P=0.02), maternal age in non-PE individuals (LR P=0.05), and pre-pregnancy BMI (LR P<0.01) in PE individuals, these were included in the analysis as cofactors.

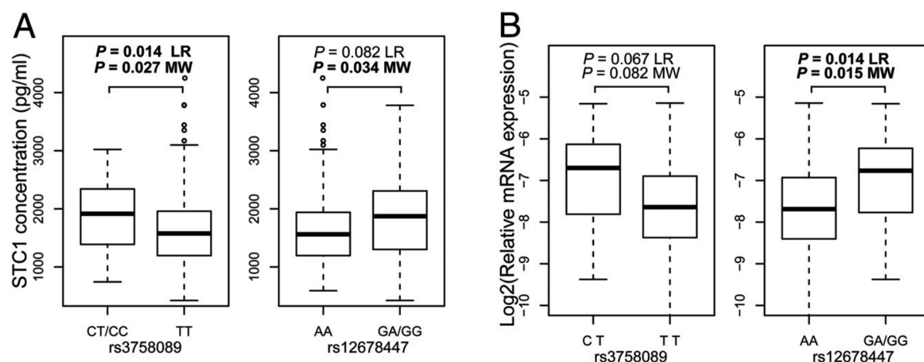

Figure 5. Maternal (A) and placental (B) variants in the *STC1* gene affect the serum hormone levels and placental gene expression, respectively. Linear regression testing association between genetic variants and maternal STC1 levels were adjusted for the presence of preeclampsia, delivery mode, maternal age, smoking status, and pre-pregnancy BMI. Tests for gene expression were adjusted for the study group and delivery mode. LR, linear regression; MW, Mann-Whitney *U* test

5.1.1.2. STC1 levels and genetic variants affect PE risk

Maternal STC1 hormone level was significantly affected by PE status even after adjusting for other variables (delivery mode, smoking status, maternal age, and pre-pregnancy BMI, $P=1.8\times10^{-6}$). In contrast, placental mRNA levels were not significantly different in PE or non-PE individuals (P=0.49, Ref. 1 Figure S4A). However, it is not certain if maternal circulating STC1 is a reaction to PE or is a part of PE pathophysiology.

As the genetic variants regulated STC1 protein and gene expression levels, it was also investigated if the genetic variants might affect the risk for developing PE. Maternal variants rs12678447 and rs3758089 were associated with PE risk in REPROMETA mothers (P=0.05, OR=2.8, 95% CI 0.99–7.85; P=0.01, OR=4.7, 95% CI 1.39–15.96, respectively) (**Table 8**). To further investigate the association, the PE individuals were further divided into subgroups based on the time of the PE diagnosis, early-onset PE (<34th gestational weeks, EOPE), and late-onset PE (>34th gestational week, LOPE). Only LOPE risk was associated with rs3758089 remained significant (P=0.04, OR=4.5, 95% CI 1.06–19.41, **Table 8**). Additional samples from the FINNPEC sample set (n=1220) were included in the meta-analysis with the REPROMETA samples under the fixed effects model. However, neither genetic variants reached statistical significance in the expanded dataset (P<0.1, **Table 8**).

Table 8. Maternal variants in *STC1* and their effect on the risk for preeclampsia

Phenotype n (case/control)	rs12678447 (A/G)			rs3758089 (T/C)		
	MAF (%) case/control	P-value	OR (95% CI)	MAF (%) case/control	P-value	OR (95% CI)
REPROMETA mothers						
PE 50/110	9.0/6.4	0.051	2.8 (0.99–7.85)	7.0/3.6	0.013	4.7 (1.39–15.96)
EOPE 25/110	8.3/6.4	0.242	2.24 (0.58–8.63)	6.0/3.6	0.09	4.0 (0.80–19.88)
LOPE 25/110	10.0/6.4	0.105	2.86 (0.80–10.20)	8.0/3.6	0.041	4.54 (1.06–19.41)
FINNPEC mothers						
PE 547/513	9.0/8.8	0.429	1.14 (0.82–1.56)	5.5/5.0	0.387	1.19 (0.80–1.80)
EOPE 165/513	7.5/8.8	0.667	0.9 (0.55–1.46)	5.0/5.0	0.734	1.11 (0.61–2.00)
LOPE 382/513	10.0/8.8	0.129	1.31 (0.92–1.85)	6.0/5.0	0.265	1.28 (0.82–2.01)
Meta-analysis						
PE 597/623	n.a.	0.182	1.23 (0.90–1.67)	n.a.	0.108	1.37 (0.93–2.02)
EOPE 190/623	n.a.	0.992	0.99 (0.63–1.57)	n.a.	0.364	1.3 (0.74–2.25)
LOPE 407/623	n.a.	0.059	1.38 (0.98–1.93)	n.a.	0.096	1.43 (0.93–2.19)

Association testing was conducted using logistic regression adjusted for maternal age, BMI, and parity in both studies. The recruitment center was included as an additional cofactor in analyses with the FINNPEC dataset. P values for meta-analysis were combined under the fixed effects model. MAF, minor allele frequency; n.a., not applicable

5.1.2. The effect of genetic variants in the *fms related receptor tyrosine kinase 1* locus (Ref. 2)

The second study (Kikas et al., 2020) aimed to replicate the identified association and further investigate the variant effect on placental gene expression, maternal serum expression, and birth parameters.

5.1.2.1. Variant rs4769613 near *FLT1* as a risk factor for PE

The C-allele of placental variant rs4769613 was significantly overrepresented in preeclamptic (n=96, 52.1%) compared to non-preeclamptic placentas (n=2001, 43.6%, *P*=0.02). The CC homozygous genotype showed a similar tendency (26.0% vs. 18.5%, *P*=0.06) (Table 2 in Ref. 2).

Analysis between variant alleles and PE risk identified an association in both REPROMETA (*P*=0.02, OR=1.91) and HAPPY PREGNANCY (*P*=0.04, OR=1.64) sample sets which was enhanced in the meta-analysis of the two datasets (*P*=0.002, OR=1.75) (**Figure 6**). Additional meta-analysis was carried out with previously reported data from five cohorts included in the published GWAS study (McGinnis et al., 2017). The combined meta-analysis improved the previously reported result (P=3.8×10^{-12}, OR=1.22) (**Figure 6**). However, parental genotypes of rs4769613 did not show any association with PE risk in REPROMETA samples.

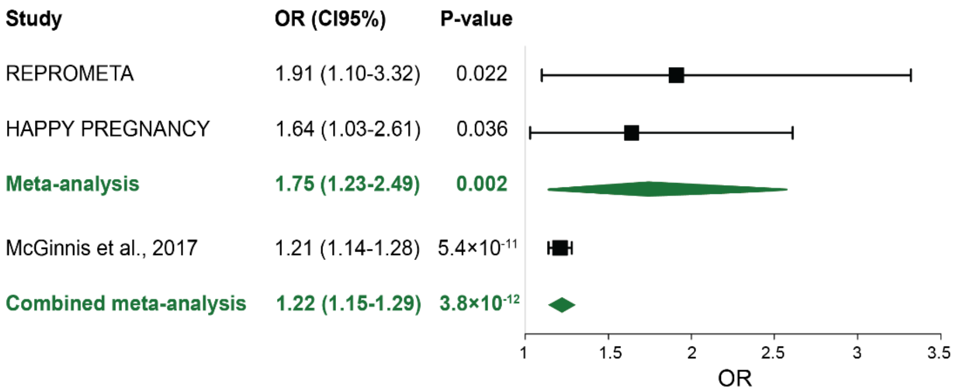

Figure 6. Association of rs4769613 placental genotypes with risk for developing PE. Meta-analysis with Estonian sample sets REPROgrammed fetal and/or maternal METAbolism (REPROMETA), and Development of novel non-invasive biomarkers for fertility and healthy pregnancy (HAPPY PREGNANCY) included 96 preeclampsia (PE) cases and 2001 non-PE pregnancies. The combined meta-analysis included 5 European cohorts from the published genome-wide association study meta-analysis (PE cases, n=4476; non-PE, pregnancies, n=312239, Table S4 in Ref. 2) in addition to the Estonian sample sets. Association testing was carried out using logistic regression adjusted to newborn sex and gestational age.

Notably, the placental risk variant is specific to PE and is not associated with other tested late pregnancy complications (gestational diabetes, small- and large-for-gestational-age newborns) neither in REPROMETA nor HAPPY PREGNANCY sample sets. Overall, the variant was not associated with birth and newborn parameters such as weight, length, placental weight, and gestational age. However, pregnancies with CC-genotype placentas developed PE about two weeks later than CT- and TT-genotype carriers (P<0.05).

5.1.2.2. Variant rs4769613 near *FLT1* represents a potential conditional eQTL

To further investigate the effect of the placental risk variant on phenotype, the placental *FLT1* gene expression and maternal serum levels of sFlt-1 were targeted. Even though no linear association was found with either, the *FLT1* expression was significantly higher in PE placentas with the CC-genotype compared to CT/TT-genotype (P=0.05) (**Figure 7**, Figure 2 in Ref. 2). The same trend was not present in non-PE placentas, indicating possible conditional gene expression regulation in preeclamptic pregnancies.

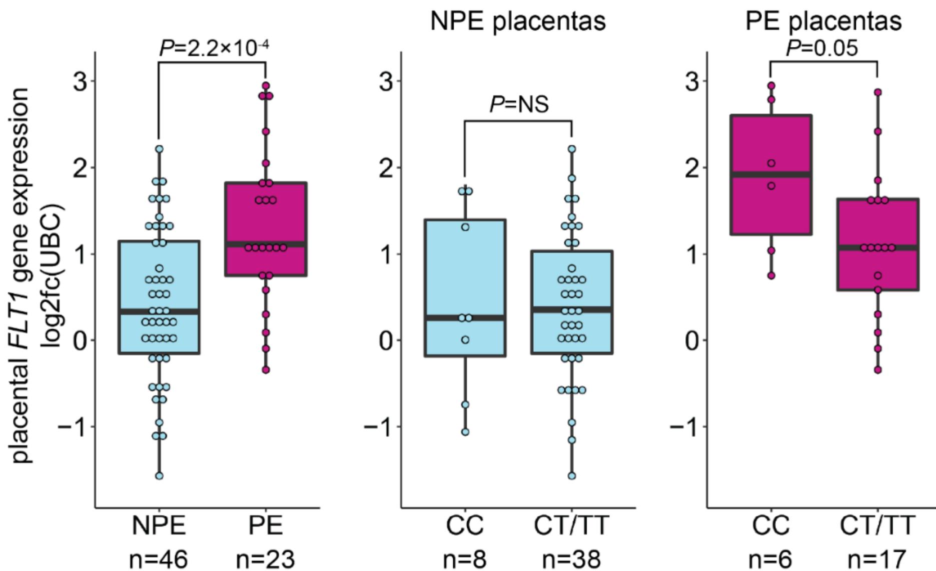

Figure 7. Placental gene expression of FLT1 is upregulated in preeclampsia placentas (PE) compared to non-preeclampsia placentas (NPE), especially in PE placentas with CC-genotype. The changes in median levels of *FLT1* between groups were tested with the Student t-test.

5.1.3. Review of placental eQTLs in candidate gene based studies (Ref. 4)

As a part of the doctoral thesis, a systematic analysis was conducted of placental eQTLs identified in candidate gene analyses. The search query and filtering principles have been detailed in the Methods. It was found that in the last decade, only ten studies have been carried out that met the criteria (**Table 9**). However, the design of these studies for eQTL analysis is quite diverse, and some included placental samples also from complicated pregnancies (most commonly PE). The tissue samples themselves also vary by site, either including full-thickness placenta or more specific regions, making it hard to conclude the universality of these results in the placenta. Almost all these associations represent cis-eQTLs, either in genic (eGenes *ABCG2*, *FKBP5*, *CXCR3*, *STC1*, *HIF1A*, *P21*, *LEPR*) or nearby regulatory regions (*SERPINA3*, *FLT1*) with only one variant located on another chromosome (*miR-518b*). No variants have been identified as eQTLs in other tissues except for *SERPINA3* and *LEPR* eVariants, according to the GTEx database. However, rs1360780 and rs9436746 also regulate the mRNA splicing of *FKBP5* and *LEPR*, respectively.

5.1.4. Take home message from candidate genes studies of genetic variants modulating placental gene expression and their link to pregnancy complications

For the first time, the maternal and fetal modulators of maternal plasma STC1 and placental *STC1* expression were characterized in pregnancy. The link between STC1 levels and PE was confirmed, and two new genetic PE risk variants, rs12678447 (A/G) and rs3758089 (T/C), in the *STC1* gene were identified.

Placental variant rs4769613 near the *FLT1* gene is the only genetic PE-specific risk factor validated in several independent cohorts. No association with PE risk was found with maternal or paternal rs4769613. The variant possibly represents a conditional eQTL, affecting enhancer response due to unfavorable placental conditions. However, no effect was seen on maternal serum levels of sFlt-1.

The two studies provided excellent in-depth analyses of the candidate genes and provided novel placental eQTLs. As the number of candidate gene studies addressing expression-modulating variants is limited, these were a needed addition to the knowledge base.

Table 9. Candidate gene based studies that have addressed eQTL effects, based on Ref. 4 (Kikas et al., 2021).

Gene – SNV ID Alleles (MAF[a])	**Location**	**Expression (genotype)**	**Tissue**	**Sample size (eQTL analysis/association analysis) (n)**	**Phenotypes associated with variant (additionally tested)**	**Gestational age**	**Reference**
SERPINA3 – rs1884082 G>T (47.8%)	promoter	decreased (GT, GG)	chorionic villi	36/PE 150, IUGR 38, PE+IUGR 26, PE+HELLP 185, CTRL 242	IUGR (PE, HELLP)	27–39 w.	(Chelbi et al., 2012)
ABCG2 – rs2231137 G>A (11.0%)	exon	decreased (GA, AA)	full thickness	46/CTRL 202, cases 206	isolated septal defects	38–41 w.	(C. Wang et al., 2014)
FKBP5 – rs1360780 C>T (31.4%)	intron	increased (TT)	parenchyma	61/CTRL 275, SGA 102, LGA 132	stress-abstinence (NNNS outcomes[b])	39±1 w.[c]	(Paquette et al., 2014)
CXCR3 – rs2280964 C>T (24.9%)	intron	decreased (AA)	basal plate, fetal membranes	48/PTB 389, CTRL 1090	spontaneous PTB, recurrent PTB	25–35 w.	(Karjalainen et al., 2015)
STC1 – rs12678447 A>G (5.8%)	intron	decreased (AA)	full thickness	120/PE 428, non-PE 606	None (PE)	non-PE 278±12 d., PE 245±23 d.[c]	(Juhanson et al., 2016)
HIF1A – rs11549465 C>T (8.6%)/ rs11549467 G>A (0.9%)	exon	increased (TT/GG, TT/GA)	not specified	170/PE 203, CTRL 202	PE	non-PE 272±17 d., PE 252±23 d.[c]	(Harati-Sadegh et al., 2018)
P21 – rs1801270 C>A (15.3%)	exon	decreased (AA)	not specified	221/PE 109, non-PE 112	PE	non-PE 273 ± 18 d., PE 254 ± 24 d.[c]	(Harati-Sadegh et al., 2019)

Gene – SNV ID Alleles (MAF[a])	Location	Expression (genotype)	Tissue	Sample size (eQTL analysis/association analysis) (n)	Phenotypes associated with variant (additionally tested)	Gestational age	Reference
miR-518b – rs1800796 G>C (10.3%)	trans[d]	decreased (GC, CC)	chorionic villi	48/72 aCA, 197 non-aCA	aCA	18–41 w.[c]	(Konwar, Del Gobbo, et al., 2019; Konwar, Manokhina, et al., 2019)
FLT1 – rs4769613 C>T (41.2%)	enhancer	decreased in PE (CT,TT)	full thickness	69/PE 96, non-PE 2001	PE	non-PE 36–42 w., PE 31–41 w.	(Kikas et al., 2020)
LEPR – rs9436301 T>C (27.5%), rs9436746 A>C (48.0%)	intron	Increased (TC, CC), decreased (AC, CC)	full thickness	854/not applicable	None	40 ± 1 w.	(Vlahos et al., 2020)

Full gene names are available in abbreviations. aCA, acute chorioamnionitis; CTRL, non-pathological pregnancies; d., days; HELLP, hemolysis, elevated liver enzymes, and a low platelet count; IUGR, intrauterine growth restriction; LGA, large-for-gestational-age newborn; MAF, minor allele frequency; NNNS, Neonatal Intensive Care Unit Network Neurobehavioral Scales; PE, preeclampsia; PTB, preterm birth; SGA, small-for-gestational-age newborn; w., week.
[a] total MAF from gnomAD (https://gnomad.broadinstitute.org/, (Karczewski et al., 2020)); [b] habituation, attention, stress-abstinence, quality of movement, handling, and arousal; [c] all samples, including individuals not used in the analysis; [d] located on another chromosome.

5.2. Whole-genome screening of placental eQTLs (Ref. 3)

I aimed to map placental variants that affect gene expression and potentially pregnancy course. To investigate robust eQTLs in the placenta, we included samples from different pregnancy outcomes (PE, SGA, LGA, GD, NORM, n=8 each group) from available datasets (Kasak et al., 2015; Sõber et al., 2015) and focused on nearby variants, i.e., *cis*-eQTLs. The region of interest for *cis*-eQTLs was limited to 100 kbp from the start or end of the gene to exclude possible sporadic associations, including the intragenic region, only genes with a median expression >100 normalized read counts were included and associations with FDR<5% were considered significant. Details of the study design have been discussed in paragraph 5.2.4. and in **Table 11**. The final analysis included 11,733 genes and 353,599 variants.

5.2.1. Distribution of eQTLs in the placenta

The study identified 199 eSNV-eGene associations, including 88 independent variants (LD $r^2<0.8$) affecting 63 placental genes. Variants were detected across the genome, excluding only four smaller chromosomes (chr18, 20, 21, and Y, **Figure 8A**, Figure S2 in Ref. 3). On average, the proportion of gene expression explained by the eQTLs was 0.52 (R^2 range 0.45–0.77, **Figure 8B**).

ZSCAN9 gene had the most eQTLs (seven independent signals) associated with its expression levels. This gene was also the top two result among protein coding eGenes (FDR $P=9.7\times10^{-7}$), only marginally surpassed by *RPL9* (FDR $P=9.7\times10^{-7}$) (Table 3 in Ref. 3). Three top protein coding eGenes with expression change >2 fold (*ZSCAN9*, *ERAP2*, *ALPG*) were selected for further validation in an independent sample set (Figure 3 in Ref. 3). Only *ALPG* did not reach the statistical significance level during validation, possibly due to lower minor allele frequency.

Placental eGenes were had primarily moderate to high gene expression in the placenta (**Figure 8C**), but only two (*PSG7*, *ALPG*) had placenta enriched protein expression according to the Human Protein Atlas (proteinatlas.org) (Figure 1 in Ref. 3). Identified eGenes were associated with a wide array of functional categories, most commonly with cellular transport (n=10) and cell structure (n=10) (Table 5 in Ref. 3). Almost 10% of eGenes were linked to immunity-related functions, known to be important in pregnancy maintenance.

Among the identified eGenes, 13 (10 protein coding, 3 pseudogenes) were previously undetected by other genome-wide investigations into placental eQTLs (**Table 10**). In addition to *ZSCAN9*, novel eGene *TLDC1* was also among the top protein coding eGenes (FDR $P=4.1\times10^{-4}$). The GTEx database did not report four genes (*RBPJ, TCIM, TPRN, THUMPD2*) out of 13 as eQTLs in any other tissue.

Table 10. Characteristics of 13 novel placental eGenes identified in Ref. 3.

Lead *cis*-eSNP	MAF	eGene (number of *cis*-eSNPs)	*P* (FDR)[a]	R^{2} [d]	Biological function	GTEx[f]
rs1150707 T<C	0.30	ZSCAN9 (23)	9.68×10^{-7}	0.77	Transcription factor[g]	Y
rs1132812 A<G	0.45	SMG1P5 (2)	1.02×10^{-4}	0.67	Pseudogene	Y
rs567637 A<G	0.48	TLDC1 (5)	4.07×10^{-4}	0.64	Cell proliferation (Nguyen et al., 2018)	Y
rs9320475 T<C	0.21	FO393415.1 (1)	1.26×10^{-3}	0.61	Pseudogene	Y
rs3810756 T<C	0.05	SLC25A43 (2)	4.48×10^{-3}	0.56	Mitochondrial transporter (Gabrielson et al., 2016)	Y
rs10767971 T<C	0.35	PRRG4 (4)	6.45×10^{-3}	0.55	Neuronal regulation (Justice et al., 2017)	Y
rs1053846 G<T	0.21	PPIE (3)	1.24×10^{-2}	0.52	Splicing (Chanarat & Sträßer, 2013)	Y
rs2871198 C<T	0.33	RBPJ (1)	1.68×10^{-2}	0.51	Transcriptional regulation (H. Han et al., 2002)	N
rs7046565 C<T	0.43	YBX1P6 (1)	3.22×10^{-2}	0.48	Pseudogene	Y
rs10869496 A<C	0.36	NMRK1 (1)	3.52×10^{-2}	0.47	NAD metabolism (Bieganowski & Brenner, 2004)	Y
rs4370521 G<A	0.33	TCIM (1)	3.62×10^{-2}	0.47	Cell cycle regulator (Jung et al., 2006)	N
rs7850758 G<A	0.06	TPRN (1)	4.69×10^{-2}	0.45	Hearing (Y. Li et al., 2010)	N
rs11692913 A<G	0.35	THUMPD2 (1)	4.77×10^{-2}	0.45	tRNA metabolism[g]	N

eQTLs were tested using linear regression adjusted by the pregnancy outcome (NORM, PE, GD, SGA, LGA pregnancy), labor activity, and newborn sex. Modified from Ref. 3. Full gene names are available in abbreviations. FDR, false discovery rate; GTEx, the Genotype-Tissue Expression Project; MAF, minor allele frequency. [a] False discovery rate was calculated according to Benjamini and Hochberg method; [c] fraction of gene expression variation explained by the *cis*-eSNP genotypes; [e] eQTL identified in GTEx for at least one tissue; [f] predicted annotation from UniprotKB.

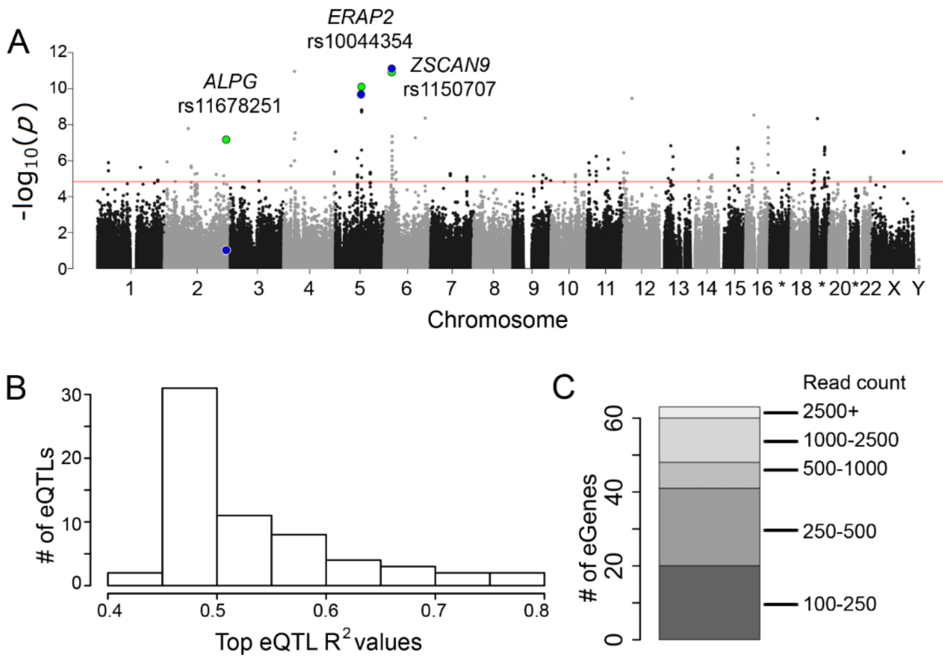

Figure 8. Genome-wide placental eQTL analysis outcomes. A) Manhattan plot of identified P-values from the discovery analysis. The P-values of selected eSNPs-eGene pairs are shown in green (discovery) and blue (validation analysis). The red line indicates the chosen statistical significance threshold (FDR 5%). B) The proportion of eGene expression (R^2) explained by the detected eSNPs. C) The expression levels of identified placental eGenes (in read counts). Modified from Ref. 3.

5.2.2. Associations with pregnancy complications and newborn parameters

All identified placental eQTLs were tested for association with pregnancy complications, but none reached statistical significance following multiple testing correction. Top variants selected for validation were also analyzed for a link to newborn and birth characteristics in REPROMETA samples. *ERAP2* and *ZSCAN9* variants were not associated with any parameter, excluding nominal connection of ZSCAN9 variant rs1150707 and placental weight with the recessive model (*P*=0.04, Table 4 in Ref. 3). In contrast, *ALPG* variant rs11678251 was associated with several parameters, including birth weight, placental weight, chest circumference, and postnatal child weight and height at 6 months (**Figure 9**). These links were further explored in two additional cohorts – HAPPY PREGNANCY and ALSPAC. However, the result was not replicated in the other sample sets, and only head and chest circumference reached nominal significance in the meta-analysis.

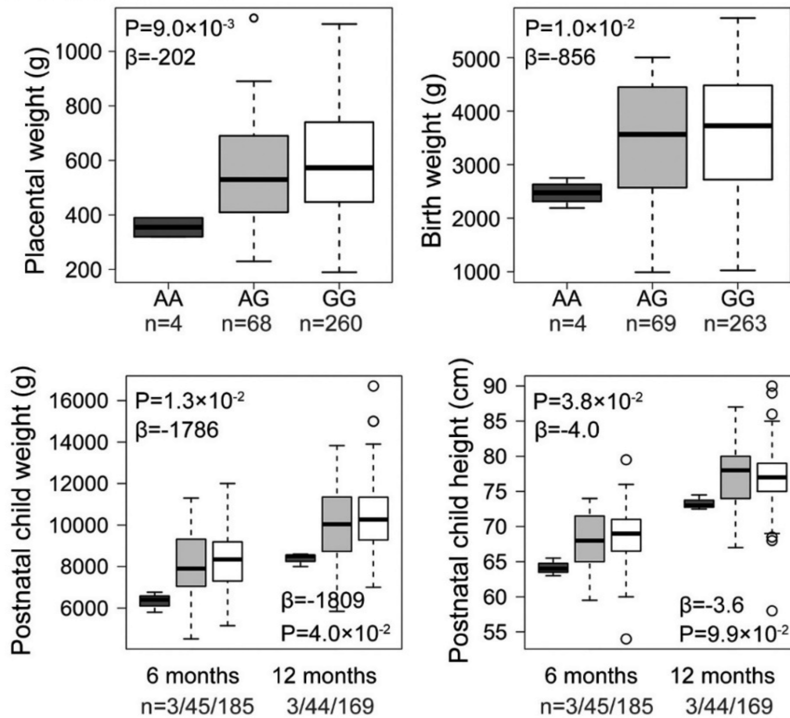

Figure 9. Association of *ALPG* rs11678251 with newborn and child growth parameters.

5.2.3. Comparison of published genome-wide placental eQTL association studies (Ref. 4)

Three hypothesis-free genome-wide eQTL mappings have been conducted to date (Delahaye et al., 2018; Peng et al., 2017; Kikas et al., 2019). These studies detected 3218, 985, and 199 cis-eVariant-gene links with 3218, 615, and 63 genes affected (eGenes), respectively. As the study design differs in each analysis, the raw results cannot be compared robustly. In addition, Peng et al. only reported one cis-eQTL per gene. However, the number of identified eQTL associations reflected the stringency of the study design and the sample size (**Table 11**).

Only 18 eGenes were detected in all three studies (**Table 12**), and additional 367 eGenes overlapped in at least two of the studies. These robust eGenes were enriched for several gene ontology (GO) terms, including "transmembrane transporter", "ATPase activity", and immunity-related Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, such as "allograft rejection" or "graft-versus-host disease" (Figure 2C in Ref. 4).

Table 11. Study designs for identifying placental variants modulating gene expression in *cis* using a genome-wide approach, based on Ref. 4 (Kikas et al., 2021).

Study characteristic	**Peng et al., 2017**	**Delahaye et al., 2018**	**Kikas et al., 2019**
Sample set			
Ethnicity	Mixed (USA)	Mixed (USA)	Estonian
Sample type	Placental parenchyma	Placental parenchyma	Full-thickness placenta
Mean gestational age in days (range)	273.7 (259–287)[a]	276.4 (254–299)[b]	274.8 (260–291)
Sample size (n)	159	80	40
Pregnancy outcomes	Normal pregnancies, SGA, LGA	Normal pregnancies	Normal pregnancies, PE, GD, SGA, LGA
Analysis design			
Utilized DNA genotyping array	Illumina MegaEx SNP array	Illumina HumanOmni2.5 Beadchip	Illumina HumanOmniExpress-12-v1 BeadChip
Imputation	Applied	Not used	Not used
Analyzed variants, after quality control	5,748,854	1,374,581	661,354
Analyzed genes	Not reported	23,003	11,733
eVariant distance from gene	500 kb	1 Mb	100 kb
Covariates in the analysis	Newborn sex, top 10 transcriptome-derived principal componentsc, top 3 genotype-derived principal components	Top 2 transcriptome-derived principal components	Pregnancy outcome, labor activity, and newborn sex
FDR significance level	10%	5%	5%
Cis-eQTL analysis outcome			
eGenes	3243	615	63
eVariants[d]	3165[e] individual SNVs/ 3218 eQTL effects	608 individual SNVs/ 985 eQTL effects	198 individual SNVs/ 199 eQTL effects

[a] calculated from weeks; [b] included individuals not used in the eQTL analysis (n=223); [c] principal components are calculated variables explaining the maximum amount of variance of the initial variables; [d] total number of SNVs identified that modulated expression of adjacent genes/total number of associations between identified SNVs and their neighboring genes (one SNV may represent cis-eQTL to several loci); [e] one *cis*-eQTL reported per gene.

Table 12. An overview of placental eGenes identified in all three genome-wide eQTL studies, based on Ref 4 (Kikas et al., 2021)

Gene	Phenotypes associated with the gene variants and/or function	Refs
AQP11	Chronic kidney disease	(Choma et al., 2016; B. Han et al., 2019)
	Obesity	(Frühbeck et al., 2020)
	Survival in cancers	(Chetry et al., 2018; Thapa et al., 2018; L. Zhu et al., 2019)
	Sperm function	(Laforenza et al., 2017)
ATG10	Cancer treatment efficacy	(Sun et al., 2020)
	Cancers	(Shen & Lin, 2019)
	age of menopause	(Bae et al., 2019)
	Amyotrophic lateral sclerosis	(Vats et al., 2018)
CYREN	Immune response to smallpox vaccine	(Kennedy et al., 2012)
CEP72	Cancer	(X. D. Li et al., 2019)
	Drug response	(Geng et al., 2018; Stock et al., 2017)
DCTN5	Favorable prognosis in melanoma	(Q. Wang et al., 2018)
DDX11	Warsaw Breakage Syndrome	(van der Lelij et al., 2010)
	Cancers	(J. Li et al., 2019; Park et al., 2020)
ERAP2	Cancers	(C. Li et al., 2020; Wu et al., 2020)
	Arterial stiffness, hypertension	(Logan et al., 2020; Zee et al., 2018)
	Immune-related diseases	(Cortes et al., 2013; Kuiper et al., 2014; Yin et al., 2015)
	Preeclampsia	(Seamon et al., 2020)
	Susceptibility to infectious diseases	(Saulle et al., 2020)
FAM118A	Ankylosing spondylitis	(Robinson et al., 2016)
	Glioblastoma	(Stangeland et al., 2015)
GUCY1B2	Bipolar disorder	(Djurovic et al., 2010)
HEATR5A	Preeclampsia	(Jacobo-Baca et al., 2020)
HTR7P1	NA	
IP6K3	Inflammatory bowel disease	(Frenkel et al., 2019)
	Hashimoto's thyroiditis	(Brčić et al., 2019)
	Late-onset Alzheimer's disease	(Crocco et al., 2016)
PSG7	Trophoblast differentiation	(Camolotto et al., 2010)
PSMD5	Colorectal tumor progression	(Levin et al., 2018)
SPSB2	Indicator of oocyte aneuploidy	(Fragouli et al., 2012)
ZNF100	Ovarian cancer survival	(Glubb et al., 2017)
TRIM66	Cancers	(Cao et al., 2020; Chen et al., 2015; Zhan et al., 2015)
WDR91	Neuronal development in mice	(K. Liu et al., 2017)

Full gene names are available in Abbreviations.

Peng et al. demonstrated enrichment of placental eQTLs among GWAS loci for birth weight, childhood obesity, and adult diseases in general. The highest enrichment was detected among GWAS loci linked to metabolic traits (e.g., total cholesterol), neurological (e.g., Alzheimer's disease), and immunological diseases (e.g., asthma). This enrichment was not replicated in the other studies, but a fraction of placental eQTLs (1–3%) were listed as GWAS loci for adult disorders in similar categories. Variants near *ERAP2*, an eGene detected in all the studies, have been previously linked to various diseases, such as PE, hypertension, and immune-related diseases (**Table 12**).

5.2.4. Take home message from genome-wide eQTL screening in the placenta

My study was one of the first genome-wide studies to address placental eQTLs, identifying almost 200 robust variants in the near vicinity of genes expressed in the placenta. Placenta-specific eQTLs are of interest as these could be potential candidates for further research in pregnancy complications. For example, *ALPG* eQTL exhibited a potential effect on newborn and child weight in the initial analysis. Placental eQTLs could also have implications for adult diseases and traits, as all placental genome-wide eQTL studies so far have demonstrated co-localization of placental variants with GWAS loci.

A list of about 400 robust placental eGenes was enriched for several GO terms, including allograft rejection" that could be linked to maternal tolerance of the fetus. However, the designs of the three studies varied greatly, and therefore some eGenes could have been overlooked.

6. DISCUSSION

6.1. Expression quantitative trait loci as unrecognized genetic risk factors for human disease

Historically, considerable focus in genetic studies on human rare diseases has been on exonic variants. As these can result in a change in protein structure, the link to the phenotype is easily predicted and possible to experimentally validate. Reasons behind associations with variants in non-coding regions are more challenging to pin down and are often excluded from the analysis from the start. However, the coding variants make up only about 0.5% of all short sequence variants mapped in the human genome (Marian, 2020). With the emergence and widespread use of genome-wide association studies (GWAS), more intergenic variants have been linked with various diseases and traits. About 80% of associated variants in complex diseases are located in non-coding regions (Suzuki et al., 2019). In parallel, more information is becoming known about these regions enabling more accurate variant effect prediction.

Disease-linked variants in non-coding regions are thought to exert their effect through either enhancers/suppressors or causing instability in the mRNA. Several studies have shown an enrichment of expression quantitative trait loci (eQTLs) among GWAS loci (**Table 5**), providing the missing link between single nucleotide variants and associated phenotypes. As many eQTLs are tissue- or even cell-type specific, the data could also provide additional information about the cell types and tissues central to the pathophysiology of the disease. For example, a study applying eQTL effect directions derived from analyses conducted in fractioned immune cell populations, identified an involved cytokine pathway significantly activated in CD4+ T cells in rheumatoid arthritis data set (Ishigaki et al., 2017). Identifying eQTLs in the tissue of interest is crucial because of tissue-specificity, using data from other tissues will not produce an accurate result.

Even though more studies have been published in recent years focusing on placental variants affecting gene expression, the subject is still underexplored. Placental function has been previously implicated in adult health through fetal programming in utero (Bonnin et al., 2011; Longtine & Nelson, 2011), a concept known as the Developmental Origins of Health and Disease (DOHaD) (Barker & Osmond, 1986). It has been also demonstrated that genes specifically expressed in the second trimester are associated with adult health outcomes (Uusküla et al., 2012). Placental eQTLs have a part in regulating the uterine environment through placental gene expression and potentially affecting fetal programming. Many of the eGenes identified in all genome-wide placental eQTL studies were associated with adult diseases, most commonly cancer (n=10/18) but also several complex diseases such as hypertension (*ERAP2*) and obesity (*AQP11*). Also, GWAS loci for adult traits as well as birth weight and childhood obesity are enriched among placental eQTLs (Peng et al., 2017, 2018). Still, it is unlikely that a single eQTL

could strongly affect fetal growth as the trait is highly multifactorial and dependent on non-genetic factors such as maternal nutrition (Warrington et al., 2019).

Apart from affecting the newborn's future health, placental eQTLs also can affect the risk for pregnancy complications. I identified few nominal associations among placental eQTLs with risk to gestational diabetes, small-for-gestational-age newborns, and large-for-gestational-age newborns. The lack of robust associations may be due to the eQTL analysis design. As different phenotypes included had known differences in gene expression profiles, the pregnancy complication group was used as a cofactor. This could have led to filtering out variants with stronger links to any pregnancy complication. Conducting further analysis would be warranted to investigate these links between eQTLs and pregnancy complications. Some identified placental eQTLs have already been previously associated with pregnancy complication risk. Placental eQTL rs2549782, a missense variant in *ERAP2*, has been associated with PE in Australian and African American populations (Hill et al., 2011; Johnson et al., 2009). Additionally, variant rs9478812 that was strongly linked to PE in a recent study (K. J. Gray et al., 2021) is located in the intron of a robust placental eGene *PLEKHG1*. However, the variant itself is only in mild linkage disequilibrium with identified eVariants (rs7738394 (Kikas et al., 2019), rs55646755 (Peng et al., 2017) $r^2=0.1$ in CEU population, Ensembl v103 http://www.ensembl.org/). Several studies concentrating on candidate genes have identified additional placental eQTLs, including for *STC1* (Ref. 1) and *FLT1* (Ref. 2). The *FLT1* variant rs4769613 had a robust link to PE risk.

6.2. Limiting factors in current placental eQTL studies and further studies

Genome-wide placental eQTL analyses have provided a list of potential candidate genes and variants that can be focused on in future research of pregnancy complications. However, the current list of robust eGenes is relatively short due to different study designs and small sample sizes. Moreover, the identified variants for most eGenes were unique among the studies. This is most likely due to small statistical power as about 400 individuals would be needed for adequate power for genome-wide cis-eQTL analysis (power 0.8 for variants with MAF≥5% and effect size ≥standard deviation) (Dong et al., 2021, https://bwhbioinfo.shinyapps.io/powerEQTL/). A meta-analysis of all three current placental eQTL studies, and any future ones, could increase the analysis power and the list of robust results. Currently, the genome-wide eQTL studies were unable to replicate the variants identified in placental candidate gene studies. This suggests there is still a need for fine-mapping of candidate gene genetic modulators as these types of studies usually have more power to detect more minor effects.

In addition, as demonstrated by Ref. 2, not all eVariants are detectable in all sample sets if the effect is brought on by some trigger (i.e., conditional eQTLs)

or a particular timepoint in development (i.e., dynamic eQTLs). Future analyses focusing on certain clinical sample sets or placental samples from the first and second trimester of pregnancy could provide additional insight into placental regulation in normal and complicated pregnancies.

As described previously, the placenta consists of various cell types already in early pregnancy (Y. Liu et al., 2018; Vento-Tormo et al., 2018). When using a whole-thickness sample, it cannot be separated which cells are the original source of the signal. Opposite effects on gene expression in different cell types might complicate the detection of associations. This limitation could be tackled in two ways in future studies. First, separating cells by type, followed by DNA and RNA extraction and analysis. However, this approach is work- and funding-intensive. The second option would be to take the known proportions of placental cell types into account when conducting the analysis using available tools (Jew et al., 2020; Newman et al., 2015). Recently, methylation profiles for five main placental cell types were generated to be used as reference for deconvolution (Yuan et al., 2021), but no reference of placental cell composition based on gene expression has been published to date.

Another complicating factor is confined placental mosaicism. Placental tissue includes many clonal populations of trophoblast cells due to fast proliferation in placental development. It has been shown that the placenta can include chromosomal aberrations, copy number variants (CNV), and single nucleotide substitutions that are not present in the fetus (Coorens et al., 2021; Kasak et al., 2015). The placental genomic landscape was comparable to childhood cancer regarding mutation burden (Coorens et al., 2021). Each bulk sample taken from a single placenta can represent a clonal expansion. On average, placental samples included 145 substitutions, and almost half of the samples included a CNV. The effect of placental somatic changes on the transcriptome is still unexplored. As even gross chromosomal changes present do not change placental gene expression linearly (Bianco et al., 2016; Lim et al., 2017; Rozovski et al., 2007), comprehensive studies are needed to address the topic.

6.3. Clinical implications of the study – the potential of applying novel genetic risk factors to screen and identify high-risk pregnancies

Many genes have been associated with PE, either by gene or protein expression changes. However, the genetic reasons behind the alterations are often still unknown. Identifying the cause can provide insight into the pathology of PE and potentially provide targets for treatment.

The assessment of FLT1 variant rs4769613, first identified by McGinnis et al. and replicated in the Estonian cohort in the current doctoral project, could be a valuable addition to the current risk assessment protocol for PE. The *FLT1* variant is the first PE-associated genetic marker that has been consistently associated

with PE in several independent sample sets from different populations (Kikas et al., 2020; McGinnis et al., 2017; Steinthorsdottir et al., 2020). The C-allele of the variant increases the odds of developing PE 1.2 times. Notably, the variant is linked to late-onset PE in particular and does not affect the risk of other late pregnancy complications, making the interpretation more straightforward. Currently, the main obstacle to adding fetal variants as biomarkers is obtaining fetal genomic material.

Until recently, fetal DNA was only obtainable through amniocentesis or chorionic villus sampling. These are invasive procedures that carry a 0.1–0.2% risk of fetal loss (Odibo & Acharya, 2020). For this reason, these are only used if either first-trimester screening shows a high risk for aneuploidy or after an abnormal ultrasound. In recent years, non-invasive prenatal testing (NIPT) has been incorporated into a clinical routine in Estonia that allows testing the fetal genome from maternal blood. The method is based on detecting cell-free fetal DNA (cffDNA) that originates from the placenta. CffDNA was first detected in the maternal serum in 1997 (Dennis Lo et al., 1997). Currently, it is used in clinics to detect common chromosomal abnormalities (trisomies 21, 13, 18) and micro-deletion syndromes (e.g., 22q11.2 and 1p36 deletions). However, it has been shown to be able to determine fetal single nucleotide variants for monogenic diseases (L. S. Chitty et al., 2011; Lyn S. Chitty et al., 2013; Zhang et al., 2019). As the methods for cffDNA extraction constantly improve, in future, genotyping fetal genetic risk variants could be done during routine blood draw.

Most biomarkers used in current clinical practice for PE risk assessment are reactionary to some fault in pregnancy, such as hypertension or sFlt-1 rise in PE. Both of these are present only slightly before the onset of PE. On the other hand, fetal genetic risk could be already determined in the first trimester with NIPT. Using placental and maternal factors in a combined manner would enable more precise classification of high-risk pregnancies as early as possible.

7. CONCLUSIONS

The current doctoral thesis investigated placental genetic variants that modify gene expression and could be potential risk factors for pregnancy complications. The results can be summarized as follows:

1. Systematic assessment of preeclampsia-associated hormone stanniocalcin-1 determined that STC1 levels were modified by several maternal characteristics, e.g., maternal smoking and delivery mode. Fetal variant rs12678447 was identified as an eQTL, modifying placental *STC1* mRNA expression. However, only maternal rs12678447 was associated with late-onset preeclampsia in a meta-analysis combining Estonian REPROMETA and Finnish FINNPEC sample sets.

2. Placental variant rs4769613 located upstream of *FLT1* was confirmed to increase preeclampsia risk during pregnancy. The *FLT1* variant was not associated with any other late pregnancy complication or newborn parameters. In preeclamptic placentas, rs4769613 CC homozygotes had significantly increased *FLT1* gene expression than CT/TT genotype carriers. The variant did not have any effect on the levels of sFlt-1 in the maternal serum.

3. Third ever placental eQTL screen was conducted focusing on variants in the immediate vicinity of genes. Utilizing several filters to exclude spurious results, 199 variant-gene associations were detected. These affected the expression of 63 eGenes, of which 13 were previously unknown for the placenta. Among the identified placental eVariants, few had a suggestive association with either pregnancy complication or newborn parameters.

4. Robust eGenes that were identified in at least two studies (n=417) were enriched for several GO terms such as "transmembrane transporter", "organelle membrane" but also "allograft rejection". This illustrates that the identified eGenes are not random. The current data is a good starting point for further research into placental eQTLs and their effect on pregnancy and prenatal developmental programming.

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SUMMARY IN ESTONIAN

Platsenta geeniekspressiooni ning raseduse kulgu mõjutavad ühenukleotiidsed variandid

Platsenta on unikaalne organ, mis eksisteerib inimkehas ainult raseduse ajal. Platsental on oluline roll ema ja loote vahelise toit- ja jääkainete vahetuses, lisaks toodab platsenta mitmesuguseid makromolekule, mis mõjutavad raseduse kulgu. Platsenta tekib juba esimesel trimestril, kui tsütotrofoblasti rakud tungivad ema endomeetriumi, ning saab küpseks pärast 11. rasedusnädalat. Platsenta arenguhäired võivad tekitada erinevaid tüsistusi – liiga sügava invasiooni korral tekib *placenta accreta,* mille korral platsenta ei eraldu pärast lapse sündi ning sünnitus tüsistub tugeva verejooksuga. Liiga vähese invasiooni korral tekib loote kasvupeetus, preeklampsia ning suureneb risk platsenta enneaegseks irdumiseks. Platsenta kasv ja areng peab olema täpselt reguleeritud, et vältida tüsistuste teket raseduse jooksul.

Preeklampsia (PE) avaldub raseduse teisel poolel uustekkelise arteriaalse vererõhu tõusuna, millele lisanduvad ema organite kahjustuse või platsentaarse puudulikkuse sümptomid. Paljudel juhtudel lisandub proteinuuria, kuid võib esineda ka maksakahjustus, neuroloogilised sümptomid, loote kasvupeetus ja teised sümptomid. PE esineb Eestis alla 2% (1.7% 2019. aastal), kuid mitmel pool maailmas on esinemissagedus kuni 5% rasedate naiste hulgast. Tänu tervishoiu arengule on arenenud maades PE suremus madal, kuid see on jätkuvalt kõrge arengumaades, kus igal aastal sureb PE tõttu üle 60 000 naise. Preeklampsia tekkeks on väga oluline platsenta roll. On teada, et platsenta geeniekspressiooni profiil muutub suurel määral PE korral. Platsenta eemaldamine on ainuke lõplik ravi PE puhul ning rasketel juhtudel tuleb esile kutsuda enneaegne sünnitus, millega kaasnevad riskid lapsele.

Üheks platsenta geeniekspressiooni võimalikuks regulaatoriks on geeniekspressiooni mõjutavad geneetilised variandid (*expression quantitative trait loci*, eQTL, eVariant). Selliste variantide geeniekspressiooni mõjutavat efekti on näidatud paljudes inimese kudedes. Suurimaks sellekohaseks uurimuseks on GTEx projekt, mis sisaldab andmeid umbes 5 miljoni variandi kohta 49 koes. Platsenta koes on eQTL-sid ülegenoomselt uuritud vaid kahe teadustöö raames. Kuna aga suur osa variante mõjutavad ekspressiooni vaid kindlates kudedes, on oluline eQTL-sid uurida just huvipakkuvas koes. Usaldusväärsete platsenta eGeenide tuvastamine võimaldab paremini mõista platsenta dünaamika regulatsiooni ning pakub uusi kandidaatgeene rasedustüsistuste uuringuteks.

Käesolevas doktoritöös on antud lühiülevaade inimese rasedusest, platsentast ning sagedasematest rasedustüsistustest. Pikemalt on käsitletud preeklampsia geneetikast ning koondatud on praegused teadmised eQTL-idest platsentas haiguste kontekstis.

Antud doktoritöö põhieesmärk oli kirjeldada platsenta geneetiliste variantide mõju geeniekspressiooni regulatsioonile ning rasedustüsistuste tekke riskile. Selleks sõnastasime kolm spetsiifilist eesmärki:

1. Uurida preeklampsia kandidaatgeenide, *STC*1 ja *FLT1*, ekspressiooni mõjutavaid geneetilisi variante.
2. Leida ja iseloomustada ülegenoomsel uuringul eQTL-d platsenta koes.
3. Koostada terviklik ülevaade kirjanduses tuvastatud platsenta eQTL-dest nii kandidaatgeenide kui ülegenoomsetes uuringutes.

Uuritavad ja meetodid

Esimese kahe eesmärgi täitmiseks kasutati emadelt ja platsentadest võetud proove eesti REPROMETA (*REPROgrammed fetal and/or maternal METAbolism*) ja HAPPY PREGNANCY (*Development of novel non-invasive biomarkers for fertility and healthy pregnancy*) valimitest. Mõlemad valimid koguti koostöös Tartu Ülikooli Kliinikumi naistekliinikuga vastavalt 2006–2011 ning 2013–2015. REPROMETA uuringu puhul kaasati indiviidid uuringusse rasedustüsistuse avaldumise (preeklampsia, PE; gestatsiooniea kohta suured ja väiksed vastsündinud, LGA, SGA; gestatsiooni diabeet, GD; või ilma kaasuva tüsistuseta kontrollid, NORM) ajal vahetult enne või pärast sünnitust. HAPPY PREGNANCY uuringusse kaasati naised, kes pöördusid rasedusaegsele jälgimisele esimesel visiidil ning kelle raseduse kulgu jälgiti kuni sünnituseni. Doktoritöös teostatud uuringu jaoks jagati indiviidid HAPPY PREGNANCY valimis sarnaste põhimõtete järgi alagruppideks kui REPROMETA valimis.

PE diagnoositi juhtudel, kui süstoolne vererõhk ≥140 mmHg ja/või diastoolne vererõhk ≥90 mmHg, millele lisandus proteinuuria ≥3g 24 tunni uriinis või neuroloogilised sümptomid. PE-ga indiviidid jagati varase (<34. gestatsiooninädalat) ja hilise algusega PE (>34. gestatsiooninädalat) alagruppidesse vastavalt sümptomite tekke ajale. GD diagnoositi kui glükoosi taluvustestil oli vereplasma glükoosi tase enne glükoosi manustamist >5.1mmol/l ja/või >10.0mmol/l ja /või >8.5mmol/l vastavalt 1ja 2 tundi pärast 75g glükoosi manustamist. LGA ja SGA diagnoositi kui vastsündinu kaal oli vastavalt >90 või <10 protsentiili lapse soole ja raseduskestusele kohandatud kasvukõveral (Sildver *et al*., 2015). NORM juhud defineeriti kui rasedused, kus ei esinenud eelnevalt mainitud rasedustüsistusi.

Uuringud oli eelnevalt heaks kiitnud Tartu Ülikooli Inimuuringute Eetikakomitee (protokollid 146/18, 27.02.2006; 150/33, 18.06.2006; 158/80, 26.03.2007; 221/T-6, 17.12.2012; 286/M-18, 15.10.2018)

Lisaks Eesti valimitele oli kaasatud doktoritöö raames tehtud uuringutesse FINNPEC (kogutud 2008–2011 Soomes, Jääskeläinen *et al*., 2016) ning ALSPAC (kogutud 1991–1992 Ühendkuningriigis, (Boyd *et al*., 2013; Fraser *et al*., 2013)) valimid.

Statistiliseks analüüsiks kasutati programmi PLINK (Purcell *et al*., 2007) ja R paketti Matrix eQTL (Shabalin, 2012).

Uurimistöö tulemused ja järeldused

1. STC1 hormooni tase ema veres seostus sünnitusviisi ja suitsetamisega nii PE kui ka ilma PEta mittetüsistunud raseduste korral. Ema vanus seostus hormooni tasemega ilma PE-ta raseduste korral ning raseduseelne KMI ainult PEga tüsistunud raseduste korral. *STC1* geeni ekspressiooni tase seostus loote variandiga rs12678447. Nii Eesti REPROMETA kui Soome FINNPEC valimite meta-analüüsis seostus ainult emapoolne rs12678447 preeklampsia riski tõusuga (šansside suhe 1.38).
2. Uuringu tulemusena kinnitus *FLT1* geenist ülesvoolu asuva variandi rs4769613 seos PE riskiga. Teiste testitud rasedustüsistuse ega lapse sünniparameetritega eelnimetatud variant ei seostunud. *FLT1* ekspressiooni tase oli oluliselt kõrgem neis PE tüsistunud rasedustest pärinevatest platsentades, mis kandsid rs4769613 CC genotüüpi võrreldes CT ja TT genotüübi kandjatega. FLT1 variandi määramine võib olla abistavaks markeriks kliinikus kasutatavates preeklampsia riski ennustamise mudelites.
3. Doktoritöö projekti raames läbiviidud ülegenoomsel platsenta eQTL-de uuringus tuvastasin 199 usaldusväärset geenilähedast varianti, mis mõjutavad 63 geeni ekspressiooni platsentas. Neist 13 olid kirjeldatud platsentas eGeenina esmakordselt. Mõned platsenta eQTL-dest seostusid pilootvalimis ka rasedustüsistuse või lapse sünniparameetritega.
4. Kolme seni läbiviidud platsenta eQTL-de uuringu alusel on 16 valku kodeerivat geeni, mis on tuvastatud läbivalt kõigis kolmes uuringus. Vähemalt kahes uuringus tuvastatud eGeene oli 417 ning nende funktsionaalsete radade analüüsil ilmnes rikastatus mitmete GO radadega, näiteks „transmembraanne transporter”, „organelli membraan” ning „transplantaadi äratõukereaktsioon”. Doktoritöö raames koostatud ülevaade on hea lähtepunkt tulevastele uurimustöödele platsenta eQTL-dest, nende mõjust rasedusele ning üsasisesele programmeerimisele.

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