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RAIN INNO

Placental transcriptome and miRNome in normal and complicated pregnancies

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Placental transcriptome and miRNome in normal and complicated pregnancies

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Institute of Molecular and Cell Biology, Institute of Genomics, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles:

1. Sõber, S., Reiman, M., Kikas, T., Rull, K., **Inno, R.,** Vaas, P., Teesalu, P., Marti, J.M.L., Mattila, P., Laan, M. 2015. Extensive shift in placental transcriptome profile in preeclampsia and placental origin of adverse pregnancy outcomes. Sci. Rep. 5, 13336.
2. Kikas, T., **Inno R.,** Ratnik, K., Rull, K., Laan M., 2020. C-allele of rs4769613 Near *FLT1* Represents a High-Confidence Placental Risk Factor for Preeclampsia. Hypertension, 76 (3), 884–891.
3. **Inno, R.,** Kikas, T., Lillepea, K., Laan, M. 2021. Coordinated Expressional Landscape of the Human Placental miRNome and Transcriptome. Front. Cell Dev. Biol. 9, 697947.

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

Study 1. One of the two researchers responsible for the design and conduct of the experimental validation of placental differential expression of 45 genes in term pregnancy complications, and the subsequent data analysis and interpretation. Contributed to the interpretation of the whole study data, critical reading and commenting, and final approval of the manuscript.

Study 2. One of the two persons responsible for the design, experimental, and analytical conduct of the genetic association study. Contributed to the interpretation of the whole study data, critical reading and commenting, and final approval of the manuscript.

Study 3. The main contributor to the study design and the experimental conduct. Designed the bioinformatics approach, performed the data analysis, and interpreted the outcomes. Wrote the first draft of the manuscript, contributed to the critical reading and commenting, and final approval of the manuscript.

LIST OF ABBREVIATIONS

ADAM12	ADAM metallopeptidase domain 12
ADM	Adrenomedullin
ANO9	anoctamin 9
BMI	Body mass index
C14MC	chromosome 14 microRNA cluster
C19MC	chromosome 19 microRNA cluster
CDR2L	cerebellar degeneration related protein 2 like
CGA	glycoprotein hormones, alpha polypeptide
CNV	copy number variation
CPM	counts per million
CS	cesarean section
CSH1	chorionic somatomammotropin hormone 1
CSH2	chorionic somatomammotropin hormone 2
CYP19A1	cytochrome P450 family 19 subfamily A member 1
DNAJC3	DnaJ heat shock protein family (Hsp40) member C3
DBP	Diastolic blood pressure
DEmiR	differentially expressed microRNA
DOT1L	DOT1 like histone lysine methyltransferase
DLX4	Distal-Less Homeobox 4
EO	early onset
EV	extracellular vesicle
eQTL	expression quantitative trait loci
FAM65B	family with sequence similarity 65, member B
FC	fold change
FDR	false discovery rate
FLT1	fms related receptor tyrosine kinase 1
g.day	gestational days
GD	Gestational diabetes
GDPD5	glycerophosphodiester phosphodiesterase domain containing 5
GH	gestational hypertension
GRCh38	Genome Reference Consortium Human Build 38
GTEx	The Genotype-Tissue Expression project
GTT	glucose tolerance test
g. week	gestational week
GWAS	genome-wide association study
GWG	gestational weight gain

HAPPY PREGNANCY	Development of novel non-invasive biomarkers for fertility and healthy pregnancy" study, supported by Archimedes Foundation
HSD17B1	17β-Hydroxysteroid dehydrogenase 1
HWE	Hardy-Weinberg equilibrium
IGHA1	immunoglobulin heavy constant alpha 1
IUGR	intrauterine growth restriction
KISS1	KiSS-1 metastasis suppressor
KLHL3	kelch like family member 3
LEP	leptin
LGA	Large for gestational age
LGWG	Low gestational weight gain
LO	late onset
MAF	minor allele frequency
MC1R	melanocortin 1 receptor
miRNome	sum total of all the microRNAs expressed in a tissue or organism
NBW	normal body weight
NGS	next generation sequencing
NORM	uncomplicated pregnancies with newborn birth weight >10th and <90th percentile
PAPPA	encoding pregnancy-associated plasma protein A
PBS	phosphate-buffered saline
PCA	principal component analysis
PE	preeclampsia
PTB	preterm birth
PTDSS2	phosphatidylserine synthase 2
PSG3	placenta-specific glycoprotein 3
REPROMETA	REPROgrammed fetal and/or maternal METAbolism, supported by Estonian Science Foundation
RELL2	RELT like 2
RISC	RNA-induce silencing complex
Rho	Spearman's correlation coefficient
RM	recurrent miscarriage
RNF17	ring finger protein 17
RPL	recurrent pregnancy loss
RT-qPCR	real time polymerase chain reaction
sFlt-1	soluble FLT1
SBP	Systolic blood pressure

SGA	Small for gestational age
SNX11	sorting nexin 11
STS	steroid sulfatase
TET3	tet methylcytosine dioxygenase 3
TMEM74B	transmembrane protein 74B
TFPI2	tissue factor pathway inhibitor 2
TRBP	transactivation response element RNA-binding protein
UBC	ubiquitin C
ZNF469	zinc finger protein 469
ZNF525	zinc finger protein 525

1. INTRODUCTION

Every mother wishes to have an uncomplicated pregnancy and a healthy newborn. As a unique organ, the placenta is the most important link between the mother and the developing fetus. Placenta regulates nutrient delivery and waste elimination, maintaining a supportive and healthy environment for the fetus. Early placental growth and cellular differentiation are 'pre-programmed' as during the first stages of pregnancy, the placental structure and functional capacity have to develop rapidly to be able to support the growing fetus throughout gestation. Furthermore, it is necessary for the placenta to constantly adjust its function based on the stimuli from the fetus or the mother. Placenta is to be considered a key communication hub between the mother and the fetus. Its maldevelopment and malfunction, or inability to achieve a proper utero-placenta perfusion, may lead to insufficient support for fetal nutrient and oxygen requirements and consequently to either maternal and/or fetal gestational complications.

As fetal and placental requirements depend on the stage of the pregnancy, placental gene expression and its regulators also must adjust correspondingly. The transcript levels of placental genes entering the translation process are co-regulated by different transcription factors and post-transcriptional modifiers of mRNA quantities and fate, called microRNAs. As transcription factors represent general or cell type-specific gene expression regulators, a defined set of micro-RNAs act *in consort* in fine-tuning and monitoring the levels if each specific transcript.

MicroRNAs are small RNA molecules, 18–24 nucleotides in length, that regulate gene expression levels by halting the translational activity of mRNA. Limiting the number of mRNAs entering translation allows faster changes in gene expression dynamics if needed or alerted by the changing cellular or organismal environment. Importantly, microRNAs are small, and some are secreted to the circulation to be used as a trans-signaling molecules. Several placental microRNAs are known to function locally and to be secreted into the maternal circulation system with possible roles in modulating maternal physiology during pregnancy.

This doctoral study aimed to profile the (co)dynamics of placental transcriptome and miRNome in healthy gestations, and in term pregnancy complications. The level, distribution, and gestational changes of microRNA expression during the three trimesters of pregnancy were analyzed, and the modulatory role of gestational disturbances and genetic variation on placental miRNome was evaluated.

2. LITERATURE REVIEW

2.1. Placenta

2.1.1. Evolution and function of the placenta

The placenta is a mammalian-specific organ, existing only for a relatively short period (**Figure 1**). Placenta also represents an endocrine organ managing information exchange between the mother and the fetus (Bowman et al., 2020). The main functions of the placenta are to provide sufficient nutrients and oxygen for the developing fetus, to support fetal programming (e.g. as a source of stem cells or signaling molecules), maternal-fetal communication and modulation of the maternal physiology during pregnancy, as well as to remove the fetal 'waste' (Turco and Moffett, 2019).

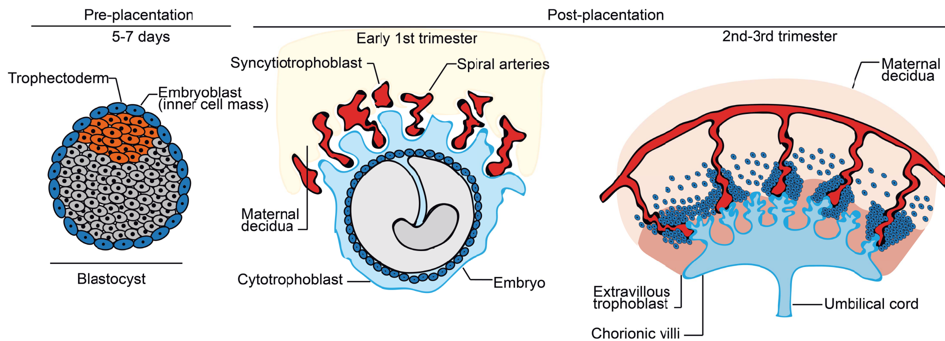

Figure 1. Development of the human placenta.
The fertilized egg develops into a blastocyst before implantation. Blastocyst trophoblast cells communicate with the maternal endometrial decidua to enable the invasion into the maternal uterine wall. By the third week of gestation, the definitive human placenta is formed and is composed of villous trees. At this stage of pregnancy, the placenta is not yet directly connected with maternal blood. Cytotrophoblast cells invade the maternal spiral arteries and replace maternal endothelium, enabling high blood flow. The surface of the villi is covered by the syncytiotrophoblast layer, which directly contacts the maternal blood and facilitates the transport of nutrients, gases, and waste across the placental barrier. Adapted from (Ander et al., 2019)

Studies of the placenta's functions have several challenges. The human placental research has ethical and clinical restrictions limited to measures that do not harm the mother or the fetus. The most common approach is to measure blood metabolites or investigate placenta samples after delivery. Using model organisms has some benefits, as it allows more flexibility to study different gestational time points. However, the most common research models have been mice and rats with different placental structures and key set of genes regulating placental development and functions compared to humans (Schmidt et al., 2015; Serman and Serman, 2011). In humans, only one layer of trophoblast separates mothers' and

fetuses' blood, compared to mice and rats, who have three layers of trophoblasts (Carter et al., 2020). This invasive nature of the placenta should trigger an immune response, yet the maternal immune system does not reject it. Placenta functions as an endocrine organ to secrete hormones for maternal circulation and adapt maternal physiology to adapt for required changes (Napso et al., 2018).

2.1.2. Placental pathologies

Placental malfunction may lead to pregnancy-related pathologies affecting the mother and/or the fetus. Problems with implantation (Murata et al., 2022), early placental development, or insufficient functional capacities in later pregnancy due to the growing demands of the fetus will increase the risk of gestational complications (Kosińska-Kaczyńska, 2022). The most common 'reflections' of placental stress during the second half of pregnancy are preeclampsia (PE) in the mother and intrauterine growth restriction (IUGR) in the fetus (**Table 1**).

PE is a hypertensive disorder, and it is defined by high blood pressure and elevated protein levels in the urine (Staff, 2019). PE is diagnosed after the 20th g. week and may also present a range of comorbidities, such as liver and kidney damage and swelling in the hands and feet. In extreme cases, it may lead to maternal and/or fetal death (Hogan et al., 2010). A possible cause of PE is insufficient placental infusion into the uterine wall, causing limiting blood flow to the placenta, which has been suggested (Rubin et al., 2022). The early onset PE (before 34th g. week) is characterized by endothelial damage that leads to maternal hypertension and organ damage due to vasoconstriction and micro thrombosis (Redman et al., 2014). Over time fetal blood supply progressively worsens due to placental insufficiency that may lead to IUGR. Late-onset PE (34th g. week or later) is associated with high cardiac output, hypertension, and weakened endothelial dysfunction as the placenta ages prematurely and reaches its potential functional limit before the time of expected delivery (Staff, 2019).

In IUGR fetuses, the inability to reach their full potential is suppressed (Valenzuela et al., 2022). The leading cause of IUGR is a critical restriction of the blood flow to the placenta and the fetus (Sharma et al., 2016). Newborn small-for-gestational-age (SGA) are small for their gestational age, less than 10th percentile or two standard deviations below average for their gestational age. IUGR reflects fetal distress, compared to SGA, that only provides a measure of the size and not a direct measure of antenatal growth quality (Sacchi et al., 2020).

A contrasting phenotype to SGA and IURG is fetal macrosomia, a large-for-gestational-age (LGA) newborn. These newborns have a higher risk for obesity in their childhood and adolescence (Bammann et al., 2014). In countries with a growing trend for obesities, the number of children born with LGA is rising (Hildén et al., 2020)

Table 1. Characteristics of common placental pathologies

Complication (abbreviation)	Characteristics	Prev	Major risk factors	Ref
Gestational hypertension (GH)	Newly onset hypertension (SBP >/= 140 mmHg or DBP >/= 90 mmHg) after 20 g.w., no features of PE	1.5% Estonia[a]	Pre-existing hypertension, kidney disease, diabetes, pregnant with multiples, aged < 20 or > 40 yrs	(Brown et al., 2018)
Preeclampsia (PE)	Newly onset hypertension after 20 g.w. and organ dysfunction, e.g. proteinuria, renal or liver dysfunction, coagulopathy, or IUGR.	1.13% Estonia[b]	Nulliparity, pre-pregnancy BMI, prior PE, chronic hypertension, multiple pregnancies, maternal young age, new partner, genetic risks	(Metoki et al., 2022)
Intrauterine growth restriction (IUGR)	Birthweight <10th percentile for gestational age, insufficient blood supply through the umbilical cord	10% world-wide[c]	Prior IUGR or being born as SGA, low maternal BMI, smoking, alcohol use, multiple pregnancies, genetic risks	(Galan and Grobman, 2019)
Small-for-gestational-age (SGA) newborn	Birthweight <10th percentile for gestational age	10% Estonia[d]	High blood pressure, kidney disease, diabetes, malnutrition, infection, alcohol use, smoking	(Mishima et al., 2023)
Large-for-gestational-age (LGA) newborn	Birthweight >90th percentile for gestational age	10% Estonia[d]	Maternal diabetes or high BMI, excessive gestational weight gain, age >35 yrs	(Beta et al., 2019)
Gestational diabetes (GD)	Chronic hyperglycemia during gestation	11.8% Estonia[a]	Maternal high BMI or age, excessive gestational weight gain, diet, family history of diabetes, or LGA or from GD pregnancy, genetic risks	(Plows et al., 2018)
Preterm birth (PTB)	Delivery of the infant prior to 37the g.w.	6.1% Estonia[b]	Previous preterm labor, multiple pregnancies, smoking, maternal age	(Khandre et al., 2022)
Recurrent pregnancy loss (RPL)	Loss of pregnancy <22 weeks	13.1% Estonia[b]	Age >35 yrs, previous miscarriages, smoking, alcohol, very low/high BMI	(Relph et al., 2023)

[a] Data from 2019 Estonian Health Statistics and Health Research Database (statistika.tai.ee); [b] Data from 2021 Estonian Health Statistics and Health Research Database (statistika.tai.ee); [c] (Armengaud et al., 2021); [d] based on Sildver et al., 2015.
BMI, body mass index; DBP, diastole blood pressure; GD, gestational diabetes; GH. Gestation hypertension; g.w., gestational weeks; IUGR, intra uterine growth restriction; LGA, large for gestational age; SBP, systolic blood pressure; SGA, small for gestational age; PE, preeclampsia; prev, prevalence; yrs, years;

Gestational diabetes (GD) is a condition of the mother when there is chronic hyperglycemia during gestation. GD is itself a risk factor for the birth of a LGA newborn. GD is alleviated after delivery but has been linked to cardiovascular diseases and metabolic syndromes (Zakaria et al., 2023).

The premature ending of pregnancy could be at any time during gestation. In case it happens before 22 g.w., it is considered as a pregnancy loss with a non-viable fetus (Sildver et al., 2015). Typically couples with three or more miscarriages are considered as recurrent pregnancy loss (RPL) (Kasak et al., 2019). Spontaneous premature termination of pregnancy at 22 g.w. or later is referred to as a preterm birth (PTB), and all available clinical measures are used to guarantee the survival of the newborn (Sildver et al., 2015). However, PTB newborns may have lifelong health complications and consequences (Dauengauer-Kirlienė et al., 2023).

Gestational diabetes and large-for-gestational-age newborn births are the most influenced by mother's lifestyle and behavior. Usage of alcohol and drugs significantly impacts the RPL and PTB, but placental malfunction can also cause these conditions. Insufficient nutrient supply for the fetus may lead to IUGR, SGA, or even PE.

2.2. Placental transcriptome

Many pregnancy complications could be described based on phenotypic and placental transcriptome changes. Placental transcriptome can be used to predict the fetus's and the placenta's health (Cox et al., 2015). Hypothesis-free methods to measure gene expression have shown added value in profiling placental transcriptome across gestation and in pregnancy complications, helping to find new regulating mechanisms. When analyzing placenta transcriptome data, multiple factors must be considered, such as the clinical details of each recruited pregnancy and the collection and processing of placental samples after delivery. Also, factors like sex, labor status, and mode of delivery could influence sequencing results (Gonzalez et al., 2018; Sood et al., 2006; Tsang et al., 2017).

Placental transcriptome studies can be broadly divided based on their primary focus, either aiming to bring novel insights to healthy pregnancy progression, analysis of placental samples of pregnancy complications, or investigating modulatory factors shaping the placental transcriptome (**Table 2**). Transcriptome studies of the normal placenta have enhanced understanding ofits formation and which regulatory mechanisms are required for its normal function. Comparison of humans with model organisms like mice has shown that first half of gestation, there are gene clusters with distinct co-expression patterns (Soncin et al., 2018). Studies focusing only on human samples have shown a distinct dynamic gene expression change between trimesters (Mikheev et al., 2008; Uusküla et al., 2012). Notably, the placental transcriptome is enriched in transcripts from organ-specific imprinted genes that are expressed only from the maternally or paternally derived gene copy and have targeted tasks in regulating placental development and function in different trimesters (Pilvar et al., 2019).

Table 2. Placental transcriptome studies.

Article	Complication	Method	Main result
A			
(Uusküla et al., 2012)	PE, GD, SGA, LGA	Microarray	Comparison of early and mid-gestation samples identified expression change for 154 genes. Investigation of expression levels of term normal samples compared to complicated cases showed an expression shift in complications.
(Nagirnaja et al., 2014)	RM	Microarray	A duplication at 5p13.3 increases the risk for RM. CNV disrupts *PDZD2* and *DOLPH3* genes expression in the placenta.
(Sõber et al., 2016)	RPL	NGS	The study identified 51 down and 138 up-regulated transcripts. RPL samples had decreased transcript levels of histones, regulatory RNAs, and genes involved in telomere, spliceosome, ribosomal, mitochondrial, and intracellular signaling functions.
(Pilvar et al., 2019)	Genomic imprinting	NGS, microarray	In total, 11 genes were imprinted in placental tissue, and 14 exhibited statistically biased expression from one parental allele.
(Kikas et al., 2019)	eQTL	NGS, microarray	The study confirmed 50 robust placental eQTLs in at least two studies.
B			
(Kim et al., 2012)	Normal term pregnancy	NGS	Transcriptome profile of placental amnion, chorion, and decidua was sequenced. Comparison of the placenta with other tissues revealed a novel set of splicing.
(Saben et al., 2014)	Normal term pregnancy	NGS	The study described the placental transcriptome of 20 healthy pregnancies. Highlighting the highest expressed genes:
(Bukowski et al., 2017)	PTB	Microarray	The study suggests pregnancy is maintained by the downregulation of chemokines at the maternal-fetal interface.
(Majewska et al., 2019)	IUGR	NGS	RNA-Seq data was used to profile protein-coding genes, and detect alternative splicing events, single nucleotide variants, in IUGR-affected placental transcriptome. Identifying 28 differentially-expressed genes in IUGR.
(Gong et al., 2021)	PE, FGR	NGS	Constructed a large-scale RNA-Seq dataset for 302 human placenta samples.

CNV, copy number variation; eQTL, expression quantitative loci; FGR, fetal growth restriction; GD, gestational diabetes; LGA, large for gestational age; NGS, next generation sequencing; PE, preeclampsia; PTB, preterm birth; RM, recurrent miscarriage; RPL, recurrent pregnancy loss; SGA, small for gestational age

Studies on placental pathologies have mainly focused on the analyses of differential placental expression in complicated and healthy pregnancies. The 'favourite' condition studied so far is PE. Most frequently, differentially expressed genes in preeclamptic pregnancies have been associated with oxidative stress, insufficient placental implantation, and spiral artery formation (Aplin et al., 2020; Eide et al., 2008; Zhang et al., 2023). Overlap between studies of differentially expressed genes in PE is modest, with about one-third of detected genes (Moslehi et al., 2013; Van Uitert et al., 2015).

Abnormalities in the number of chromosomes or changes in gene dose can lead to recurrent pregnancy loss (Kasak et al., 2021; Li et al., 2021; Sõber et al., 2016).

2.3. MicroRNAs

2.3.1 microRNAs as the modulators of gene expression levels

MicroRNAs are small 18–24 nucleotides in length RNA molecules. These molecules exist in different organisms, and orthologues exist between species (Berezikov, 2011). MicroRNAs are encoded by genes located between protein-coding genes or in their intronic region. RNA polymerase II transcribes pri-microRNA, a long stem-loop structure (**Figure 2**).

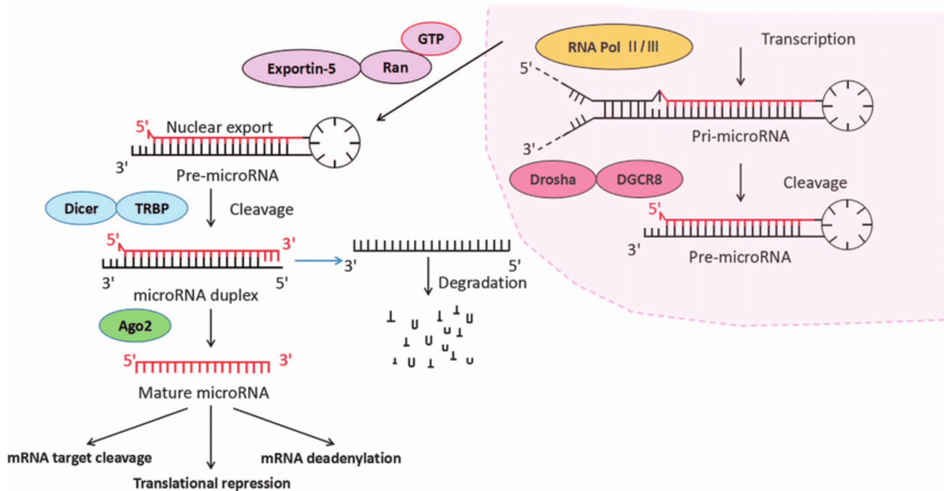

Figure 2. microRNAs as transcriptome regulators
Adapted from (Wu et al., 2018). MicroRNAs are transcribed from a microRNA gene. The maturation starts from the production of the primary microRNA transcript (pri-microRNA) by RNA polymerase II or III and cleavage of the pri-microRNA by the microprocessor complex Drosha-DGCR8 (Pasha) in the nucleus. Then the pre-miRNA hairpin is exported from the nucleus by Exportin-5-Ran-GTP into the cytoplasm. The RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The mature microRNA's functional strand is loaded with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), which guides the RISC to silence target mRNAs through mRNA cleavage, translational repression, or deadenylation.

Drosha trims the stem-loop ends to produce pre-microRNA, which is exported by exportin-5 to the cytoplasm. Dicer processes pre-microRNA into microRNA duplex. Duplex is unwound, producing two strands, indicated with –5p or –3p suffix (Winter et al., 2009). Strands can be incorporated into the RNA-induced silencing complex (RISC) that contains Argonaute (Ago) proteins at its core. The miRNA-RISC complex binds specific sites in the 3'-untranslated region (3'UTR) of target mRNAs, disabling them through destabilization and translational repression. Notably, recent studies have demonstrated extensive crosstalk between signaling pathways and miRNA processing, suggesting that microRNA biogenesis is under tight signaling control and has been an important part of the large regulatory networks (Komatsu et al., 2023).

2.3.2. microRNAs in health and disease

MicroRNA expression can change for multiple reasons, like the circadian rhythm (Anna and Kannan, 2021) and an illness (**Table 3**). Because microRNA's primary ability is to bind onto different mRNAs, microRNAs can regulate a large portion of the transcriptome. In cases of asthma and rheumatoid arthritis, it has been shown that microRNAs are differentially expressed and influence inflammation processes. A common microRNA is miR-155, a known microRNA associated with multiple autoimmune diseases (Xu et al., 2022). Because microRNA can regulate multiple genes, their effect may be broad, like miR-1 (Safa et al., 2020). They are known for developmental processes in muscle tissue, regulating immune cells.

Table 3. Disease-associated with microRNAs

Selection of associated microRNAs	Disease/ Function	Effect	Tissue	References
miR-17-5p, miR-24, miR-25, miR-29a, miR-30a, miR-96, miR-132, miR-143-3p, miR-192, miR-219, miR-494	Circadian rhythm	Circadian regulation	Cell-lines	(Anna and Kannan, 2021)
miR-21, miR-30e, miR-144, miR-155, miR-215, miR-582-3p	Asthma	Inflammation of the human lung	Lung	(Albano et al., 2023)
miR-16, miR-21, miR-132, miR-146a, miR-155	Rheumatoid arthritis	Inflammatory and immune processes	Cartilage	(Balchin et al., 2023)
miR-9, miR-15b, miR-16, miR-126, miR-155, miR-505	Hypertension	Angiogenesis and vascular integrity	Cardiac endothelium	(Caria et al., 2018)
miR-1, miR-99a, miR-100, miR-133a	Skeletal muscle metabolism	Insulin processing	Skeletal muscle	(Sjögren et al., 2018)

2.3.3. Placental miRNome

All of the expressed microRNAs (miRNome) in the placenta regulate many aspects of placental development and function, such as trophoblast invasion proliferation, differentiation, apoptosis, and cellular metabolism of trophoblast cell populations (Doridot et al., 2013; Ren et al., 2023). Investigating placental microRNA expression by sequencing all the available microRNAs has opened up new possibilities for microRNA research. Previous assay-based methods like qPCR and microassays have had a limited set of microRNAs detected. Next-generation sequencing (NGS) based approaches have allowed a more detailed and comprehensive description of the entire placental miRNome, defined as the total of all the microRNAs expressed in a tissue (**Table 4**). The number of microRNAs included per study has ranged from 601–2817, depending on the stringency of inclusion criteria.

Table 4. Studies of placental miRNome

Article	Pathology (sample set size)	analyzed miRNAs (n)	References
A Comprehensive Survey of miRNA Repertoire and 3′ Addition Events in the Placentas of Patients with Preeclampsia from High-Throughput Sequencing	Term, norm (n=1) Term, PE (n=2)	–	(Guo et al., 2011)
Hydroxysteroid (17-β) dehydrogenase one is dysregulated by miR-210 and miR-518c that are aberrantly expressed in preeclamptic placentas	Term, norm (n=10) Term, PE (n=8)	601	(Ishibashi et al., 2012)
Characterization of placenta-specific microRNAs in fetal growth restriction pregnancy	Term, norm (n=2)	–	(Higashijima et al., 2013)
Deregulated microRNA species in the plasma and placenta of patients with preeclampsia	Term, norm (n=1) Term, PE (n=4)	905	(Yang et al., 2015)
Placental expression of microRNAs in infants born small for gestational age	Term, SGA + LGWG (n=13) Term, SGA + NGWG (n=9) Term, NBW + LGWG (n=20) Term, NBW + NGWG (n=26)	1870	(Östling et al., 2019)
Placental microRNAs in pregnancies with early-onset intrauterine growth restriction and preeclampsia: potential impact on gene expression and pathophysiology	Term, norm (n=21) Term, EO-PE (n=20) Term, EO-IUGR (n=18) Term, EO-PE+IUGR (n=20)	–	(Awamleh et al., 2019)
Whole transcriptome expression profiles in placenta samples from women with gestational diabetes mellitus	Term, norm (n=3) Term, GD (n=3)	2817	(Tang et al., 2020)
High-throughput miRNA sequencing of the human placenta: expression throughout gestation	First trimester, norm (n=113) Term, norm (n=47)	801	(Gonzalez et al., 2021)
Profiling the small non-coding RNA transcriptome of the human placenta	First trimester, (n=5) Second trimester, (n=16) Term, (n=9)	654	(Martinez et al., 2021)
Global microRNA and protein expression in human-term placenta	Term, norm (n=19)	895	(Östling et al., 2022)
Sex differences in microRNA expression in first and third-trimester human placenta	First trimester, norm (113) Term, norm (n=47)	986	(Flowers et al., 2022)
Variation in placental microRNA expression associates with maternal family history of cardiovascular disease	Term, (n=230)	802	(Tehrani et al., 2023)

EO, early onset; GD, gestational diabetes; GWG, gestational weight gain; IUGR, intrauterine growth restriction; LGWG, low gestational weight gain; NBW, normal birth weight; NGWG, normal gestational weight gain; SGA, small for gestational age; PE, preeclampsia.

2.3.3.1. Placental-specific microRNA clusters

Two major microRNA clusters (at Chr 14 and Chr 19) are predominately expressed in the placenta in the parent-of-origin dependent manner (Morales-Prieto et al., 2012; Pilvar et al., 2019) (**Figure 3**). These clusters facilitate the investigation of the specific roles of placenta-specific microRNA during gestation. As these microRNAs are excreted from the placenta, they can also be detected in the maternal system. Maternally expressed Chromosome 14 microRNA cluster (C14MC) spanning 250 kb (14q32.31, GRCh38) is eutherian-specific, containing 52 microRNA genes and encoding 94 mature microRNAs. It is predominately expressed in the placental tissue but has been shown to have an aberrant expression in cancers (McCarthy and Dwyer, 2021).

Primate-specific paternally expressed Chromosome 19 microRNA cluster (C19MC) spans 100 kb (19q13.42, GRCh38), and contains 46 tandem repeating microRNA genes that encode 67 mature microRNAs. These microRNAs are exclusively expressed in the placenta; low levels are found in embryonic stem cells, testes, and some tumors (Augello et al., 2018; Kobayashi et al., 2022).

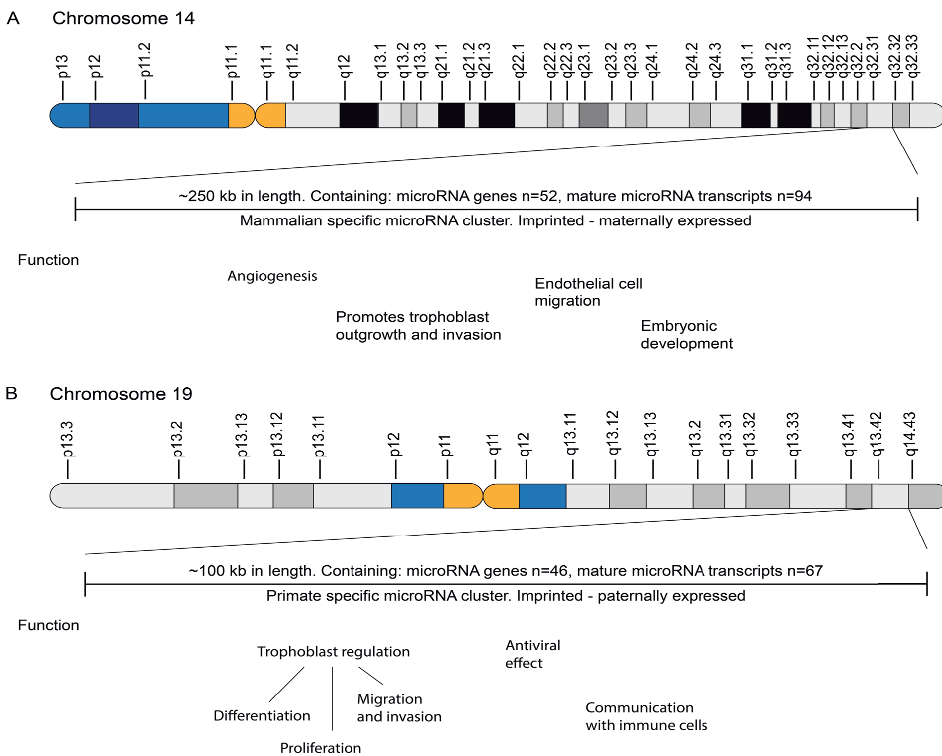

Figure 3. Chromosome 14 and 19 placenta-specific microRNA clusters C14MC and C19MC. Location, composition, and known functions in pregnancy. (A) Chromosome 14 microRNA cluster; (B) Chromosome 19 microRNA cluster.

2.3.4. microRNAs as biomarkers for diagnostics and disease monitoring

As microRNAs are secreted from tissues to the circulatory system, they can be detected from body liquids and could represent candidate biomarkers for organ pathologies. Extensive research is ongoing to identify specific microRNAs in liquid biopsy samples to serve as biomarkers for health conditions (Jain et al., 2023). As most of the microRNAs are broadly expressed in multiple tissues, it makes it difficult to validate the source tissue or organ. In cases where microRNAs are secreted directly into biofluid (Kondracka et al., 2023) or are almost exclusively expressed in a specific tissue (Miura et al., 2015), we can determine the tissue of origin. As microRNAs are transported out of the tissue in extracellular vesicles (EVs), EVs could be used for epigenetic programming, influencing various organs (Floris et al., 2016).

MicroRNA expression correlation between tissue and biofluid hints that biofluids are good candidates for biomarker medium. Comparison of microRNA expression in serum, plasma, and urine samples from 40 different healthy human tissues showed a moderate correlation (Rho > 0.48) (Cui and Cui, 2020).

2.4. Quantitative trait loci – QTLs

Variations in the DNA sequence near or in the transcription start site can influence gene or microRNA expression levels (Cheung et al., 2003; Flynn and Lappalainen, 2022). These single nucleotide variations are called expression quantitative trait loci (eQTL) (Nica and Dermitzakis, 2013). The transcription of a mRNA or microRNA gene can be co-modulated by multiple eQTLs (**Figure 4**).

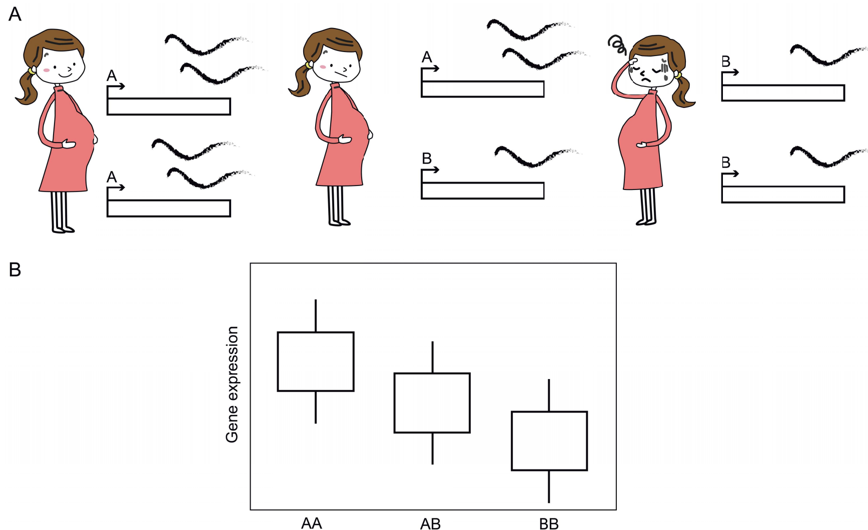

Figure 4. Location and function of gene eQTL
Schematic of an expression quantitative trait locus (eQTL). (A) Three individuals with different genotypes for a variant associated with altered gene expression. (B) Gene expression levels for individual eQTL variant genotype.

2.4.1. Placental eQTLs

The profile of placental eQTLs has been only recently investigated (Apicella et al., 2023; Delahaye et al., 2018; Kikas et al., 2019; Peng et al., 2017). As not the same eQTLs are effective in all tissues, it is important to determine eQTLs that are functional in the placenta in order to understand the role of genetic variation in modulating the risks for pregnancy pathologies. Previous studies have focused on genes associated with pregnancy complications or other pregnancy-related characteristics. Genome-wide association studies (GWAS) have identified 417 confident placental genes whose expression is modulated by eQTL and supported by at least two independent studies (Kikas et al., 2021). Placenta eQTLs, compared to other tissues, show around 1–3% overlap with other reported GWAS loci for adult disorders.

A promising placenta eQTL is rs4769613 near *FLT1* gene. *FLT1* is a well-acknowledged gene associated with preeclampsia, as high blood pressure increases the sFlt1 circulation (Biwer et al., 2023; Srinivas et al., 2010). SNV rs4769613 T/C within the enhancer element of *FLT1* has been previously identified as a risk factor for preeclampsia in the genome-wide association study (GWAS) targeting placental genotypes (McGinnis et al., 2017).

2.5. Summary of the literature review

Placenta is a unique organ, only being present for a relatively short time at the beginning of our lives as the bridge between the mother and the developing fetus. Placenta has multiple functions, including nutrient and oxygen delivery, contribution to fetal programming and modulation of maternal physiology, and elimination of waste generated by the fetus. It also functions as a hub for maternal-fetal communication and as an endocrine organ, producing and secreting hormones and other signaling molecules. Alterations in placental function may lead to maternal or fetal complications during the pregnancy.

Placenta function is complex and changes during gestation, a well-established baseline for gene and microRNA expression is needed to characterize differential gene expression in case of pregnancy complication. Multiple genes have been linked to a variety of complications, PE, GD, IUGR. Differential gene expression could be caused by gene expression regulators, one of which are microRNAs. These gene expression regulators are easily detected from the maternal system and have a great potential to be used to describe the wellbeing of the placenta. Some of these microRNAs are placenta specific and therefor their expression origin could be easily tracked. Knowing how transcriptome and miRNome interact, could give us a new insight how placenta function is regulated.

3. AIMS OF THE PRESENT STUDY

The present thesis aimed to characterize the landscape of placental miRNome in normal and complicated pregnancies and to investigate its correlation with placental transcriptome and genetic variation.

The specific aims were:

1. to investigate placental differential gene expression in term pregnancy pathologies – preeclampsia, gestational diabetes mellitus, small- and -large for gestational age newborns

2. to explore genetic variants near the *FLT1* gene as eQTL for placental gene expression and as risk factors for late-onset preeclampsia

3. to profile placental miRNome throughout gestation and describe microRNA expression variations caused by eQTLs and by term pregnancy pathologies – preeclampsia, gestational diabetes mellitus, small- and -large for gestational age newborns

4. to characterize the expression correlation of placental miRNome and transcriptome

5. to investigate placenta-specific microRNA clusters function and expression in our sample sets.

4. MATERIAL AND METHODS

4.1. Study design

To fulfill these thesis aims, samples from REPROMETA (full study name "REPROgrammed fetal and/or maternal METAbolism"; recruitment 2006–2011) and the HAPPY PREGNANCY study ("Development of novel non-invasive biomarkers for fertility and healthy pregnancy"; 2013–2015) were used (**Tables 5–6**). These datasets included first, second-trimester, and term placenta samples from pregnancies that ended with preeclampsia, gestational diabetes, small or large for gestational age diagnosis, or were without complications (**Figure 5**).

Gene expression was measured using RNA-Seq and validated with RT-qPCR. Genotypic variations were detected using microarray or qPCR. MicroRNA expression was detected using miR-Seq.

From this data, we expected to identify genes differentially expressed in pregnancy complications and to validate how much, if any, overlap between complications.

Independently verify the eQTL near *FLT1* gene in our dataset and evaluate its role in preeclampsia development.

Measure microRNAs expression during gestation and report the microRNAs with dynamical expression as microRNAs expression may change based on pregnancy complications and genomic variants. Aimed to report how microRNA expression changes based on these factors.

Give a comprehensive overview of how microRNA and gene expression is correlated in term placenta samples. Describe the expression dynamics of placenta-specific microRNAs.

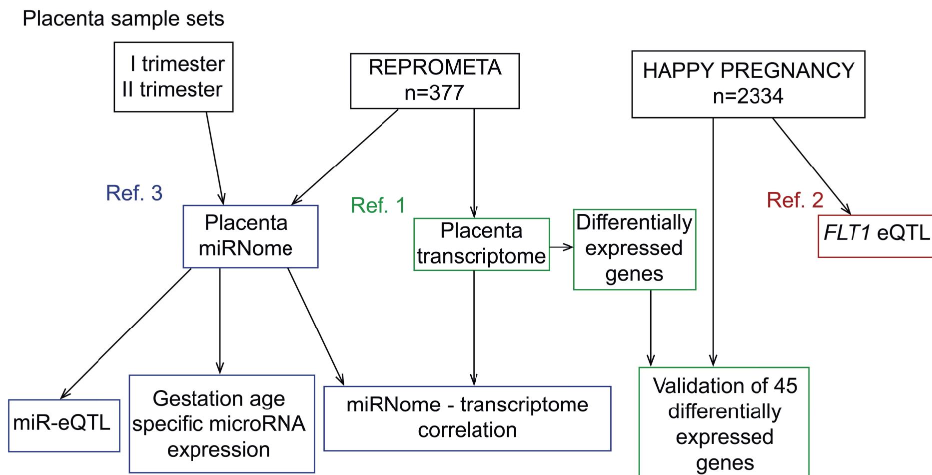

Figure 5. Study design
eQTL, expression quantitative loci; HAPPY PREGNANCY, study of Development of novel non-invasive biomarkers for fertility and healthy pregnancy**;** miR, microRNA; REPROMETA, study of REPROgrammed fetal and/or maternal METAbolism

4.2. Ethics

The study subjects were recruited, and clinical samples were collected during the REPROMETA (full study name "REPROgrammed fetal and/or maternal METAbolism"; recruitment 2006–2011, supported by Estonian Science Foundation) and the HAPPY PREGNANCY study ("Development of novel non-invasive biomarkers for fertility and healthy pregnancy"; 2013–2015, supported by Archimedes Foundation) at the Women's Clinic of Tartu University Hospital, Estonia. Written informed consent to participate in the study was obtained from each individual before recruitment. The studies were approved by the Research Ethics Committee of the University of Tartu, Estonia (permission 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). The study was carried out in compliance with the Helsinki Declaration, and all methods were in accordance with approved guidelines.

4.3. Study subjects

4.3.1. REPROMETA and HAPPY PREGNANCY pregnancy cohorts

The REPROMETA study (n=377) focused on the recruitment of extreme cases of selected term pregnancy complications – PE (n=53), GDM (n=50), SGA (n=72), and (LGA, n=97), and normal pregnancies (n=105). Epidemiological data, reproductive history, and parental lifestyle were obtained from self-reported questionnaires filled out shortly after recruitment by both parents, and the pregnancy outcome data were acquired from the medical records. Placental samples were available for 366 cases. For the transcriptome and miRNome sequencing in the current study, 40 placentas were selected (n=8 per clinical subgroup; **Table 5;** Ref 1, Ref 3). Experimental validation of differential expression in PE placentas by TaqMan RT-qPCR (Ref. 1) was carried out using an extended sample set of 24 PE, 24 SGA, and 24 normal pregnancy cases from the REPROMETA study (**Table 6**).

In the genetic association study (Ref. 2) placental samples were divided to PE cases and pregnancies with any other type of course. The study included 329 REPROMETA (PE, n=52 and non-PE, 277) and 1768 HAPPY PREGNANCY cases (PE, n=44 and non-PE, n=1724). All cases represented singleton pregnancies with placental DNA available during genotyping **(Table 6)**. HAPPY PREGNANCY cohort of 2334 pregnant women had been recruited prospectively during their first antenatal visit at the Women's Clinic. The patients were asked to fill out three questionnaires throughout their pregnancy concerning epidemiological data, reproductive history, parental lifestyle, and additional pregnancy course and outcome data collected from the medical records.

Table 5. Clinical characteristics of REPROMETA study pregnancies for the placental RNA-Seq and miR-Seq discovery studies.

	Early pregnancy						
Pregnancy-related parameters (units)/	I trimester	II trimester	Normal	PE	GD	SGA	LGA
Sample size (n)	5	7	8	8	8	8	8
Maternal age (years)	24 (19–33)	24 (15–39)	33 (18–37)	27 (19–39)	33 (22–36)	25 (20–32)	30 (18–39)
Maternal height (cm)	161 (160–165)	170 (160–173)	165 (158–175)	170 (163–173)	167 (158–175)	166 (153–172)	167 (160–179)
Pre-pregnancy BMI (kg/m^2)	21 (20–26)	22 (17–25)	24 (17–30)	26 (20–34)	26 (18–43)	21 (17–24)	24 (19–31)
Nulliparity (n, %)	1 (20%)	5 (65.5%)	3 (37.5%)	6 (75%)	3 (37.5%)	7 (87.5%)	2 (25%)
Gestational age at birth/abortion (days)	60 (51–81)	121 (108–140)	284 (260–291)	266 (260–271)	276 (268–284)	271 (264–289)	281 (275–288)
Vaginal/CS delivery	n.a	n.a	5/3	2/6	3/5	6/2	3/5
Fetal sex (M/F)	2/3	4/3	5/3	4/4	3/5	3/5	4/4
Birth weight (g)	n.a	n.a	3756 (3102–4220)	2803 (2170–3570)	4284 (3940–4680)	2517 (2004–2698)	4744 (4420–4986)
Birth length (cm)	n.a	n.a	51 (49–55)	48 (45–49)	53 (51–54)	46 (45–48)	53 (52–55)
Birth head circum-ference (cm)	n.a	n.a	36 (33–36)	34 (32–36)	36 (34–38)	32 (32–34)	38 (37–38)
Birth chest circum-ference (cm)	n.a	n.a	35 (33.5–38)	31 (28.5–35)	36 (34–38)	31 (28–34)	37 (36–39)
Placental weight (g)	n.a	n.a	575 (420–770)	463 (340–720)	588 (500–1060)	420 (200–470)	818 (610–970)
Utilized in study	Ref. 3		Ref. 1, Ref. 3				

Clinical characteristics values are given as median (range).CS, caesarian section; BMI, body mass index; GD, gestational diabetes; LGA, large for gestational age; PE, preeclampsia; SGA, small for gestational age.

Table 6. Clinical characteristics of pregnancies for the locus-specific gene expression and eQTL validation studies.

Parameter	REPROMETA Study			REPROMETA Study		HAPPY PREGNANCY Cohort Study	
Diagnosis	PE	SGA	Normal	PE	Non-PE	PE	Non-PE
Sample size (n)	24	24	24	52	277	44	1724
Maternal age (years)	31 (18–40)	25 (20–40)	26 (19–39)	27 (16–41)	28 (17–43)	26 (20–42)	29 (16–48)
Pre-pregnancy BMI (kg/m^2)	22.9 (17.0–30.0)	21.4 (16.5–24.9)	23.7 (17.1–33.5)	23.7 (16.8–38.1)	23.1 (16.5–45.8)	26.8 (18–45.7)	22.4 (14.5–53.3)
Nulliparity (n, %)	9 (37.5%)	14 (58.3%)	17 (70.8%)	33 (63.5%)	111 (40.1%)	27 (61.4%)	574 (33.3%)
Gestational age at birth/abortion (days)	277.5 (255–291)	270 (259–294)	264 (218–287)	251 (184–288)	279 (216–296)	265 (198–296)	281 (167–300)
Vaginal/CS delivery	17/7	15/9	6/18	10/42	170/106	22/22	1459/265
Birth weight (g)	3629 (2553–4220)	2462 (1585–2750)	2740 (1094–4250)	2240 (650–4250)	3880 (1170–5850)	2837 (842–4346)	3584 (560–5354)
Birth length (cm)	51 (48–55)	46 (42–49)	48 (35–51)	46 (32–53)	51 (37–57)	49 (34–55)	51 (29.5–64)
Placental weight (g)	550 (390–800)	420 (200–585)	450 (230–770)	400 (170–770)	600 (200–1122)	510 (230–920)	590 (190–1132)
Utilized in study		Ref. 1			Ref. 2		

Clinical characteristics values are given as median (range). CS, caesarian section; BMI, body mass index; PE, preeclampsia; SGA, small for gestational age.

4.3.1.1. Inclusion and exclusion criteria of analyzed REPROMETA pregnancy cases

The normal group was defined as uncomplicated pregnancies without previously mentioned conditions with a newborn between the 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 <10th or over 90th percentile newborns, 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 subdivided into early-onset (symptoms before 34th gestational weeks) and late-onset PE (after 34th gestational weeks). GD was diagnosed when a 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 one hour and two hours later plasma glucose level of ≥10.0 mmol/l and ≥8.5 mmol/l glucose, respectively (Metzger, 2012). Pregnancies with birth before the 37th gestational week were considered preterm.

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

4.3.2. Placental sampling and extraction of nucleic acids.

Placental sampling in REPROMETA and HAPPY PREGNANCY studies were 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 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 (Thermo Fisher Scientific, Waltham, Massachusetts, United States). 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.

4.4. Utilized placental 'Omics' datasets

4.4.1. Placental RNA-Seq dataset

The REPROMETA placental RNA sequencing dataset was first published by Sõber et al., 2015 (Ref 1). 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 (Qiagen, Germantown, Maryland, USA) according to the manufacturer's protocol. NanoDrop ND-1000 UV-Vis spectrophotometer (Applied Biosystems, Foster City, California, 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, Santa Clara, California, USA). Total RNA with high purity was used for rRNA depletion (Ribo-Zero rRNA Removal Kit, Illumina, San Diego, California, USA) and library preparation with Nextera Technology (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 37 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 the DESeq package for R. Only non-mitochondrial genes with sufficient expression levels (>100 normalized read count) were considered in the analysis (n=11,733).

4.4.2. Placental miR-Seq dataset from the placenta

The placental miRNome dataset generated in the current study consisted of 52 placental samples collected from first, second-trimesters, and term pregnancy cases (n = 5, 7, and 40, respectively).

Initial small-RNA libraries were prepared from 1 μg total RNA (TruSeq Small RNA kit, Illumina), followed by miRNA enrichment (Caliper LabChipXT, PerkinElmer, Waltham, Massachusetts, United States) according to the manufacturer's protocols. Small RNA-Seq libraries were sequenced on Illumina HiSeq 2000. Library preparation and sequencing were conducted in FIMM Sequencing Laboratory, University of Helsinki, Finland. Quality control of the raw reads was performed using FastQC (ver. 0.11.7) and MultiQC (ver. 1.7) (Ewels et al., 2016). Trimmomatic (ver. 0.38) was implemented to remove adapters and trim the quality of reads with the following settings – ILLUMINACLIP:2:30:9, LEADING:3, CROP:50, TRAILING:3, SLIDINGWINDOW:4:20, MINLEN:16. Reads were aligned to human genome reference GRCh38 using bowtie (ver. 1.2.2, settings: -n 1 -l 20 -q -m 40 -k 1 -t --best --strata) (Langmead et al., 2009). miRNA quantification was performed using featureCounts from the Rsubread package (ver. 1.20.6) (Liao et al., 2019) for R with miRNA annotations from miRBase 22.1 as reference (Kozomara et al., 2019).

4.4.3. Placental whole genome genotyping dataset

The same 40 term placental samples from the REPROMETA study with available RNA-Seq data from Ref.1 also underwent whole-genome genotyping (Kasak et al., 2015). The DNA of the placental samples was extracted using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. 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 of >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.

4.5. Single locus based experiments

4.5.1. TaqMan RT-qPCR

Gene expression was quantitated by singleplex reverse transcription-qPCR (RT-qPCR) of the target gene sequence using premade TaqMan Gene Expression Assays (Applied Biosystems, Life Technologies Waltham, Massachusetts, United States; Ref. 1 Supplementary Table 5). In all experiments, a housekeeping gene Ubiquitin C (UBC), was used as the reference gene. All qPCR reactions were performed in triplicate in 384 micro-well plates in ABI 7900HT Real-time PCR system (Applied Biosystems) using HOT FIREPol® Probe qPCR Mix (Solis BioDyne, Tartu, Estonia).

4.5.2. eQTL genotyping with TaqMan

Placental eQTL of rs4769613 T/C was tested using premade TaqMan genotyping using manufacturer's protocol (Applied Biosystems, Assay ID: C__32231378_10).

4.6. Bioinformatics and statistics

4.6.1. Differential gene expression analysis

Differential expression in RNA-Seq data was tested using DESeq, and DESeq2 packages for R. Read counts from htseq-count were used as input, and built-in normalization algorithms of DESeq and DESeq2 were used. Outlier detection and handling were performed using the default method in DESeq. In DESeq2, outliers were replaced using the replace Outliers With Trimmed Mean function with default Cook's distance cutoff. Statistical testing indicated that the two software

packages, DESeq and DESeq2 differ substantially in their sensitivity and robustness in the assessment of differential expression. Compared to the seminal DESeq package, analysis with the more recently developed DESeq2 program produced more significant results for all conducted differential expression tests with our data (Supplementary Table S1 in Ref. 1). More stringent level of significance was imposed on the test results of DESeq2. A gene was considered as differentially expressed when the statistical tests simultaneously satisfied the following empirically set thresholds: FDR < 0.1 for DESeq and FDR <0.05 for DESeq2. Genes with mean normalized expression <50 reads in all samples (n = 39425 DESeq; n = 39345 DESeq2) were considered as transcriptional noise and filtered out from the analysis. No covariates were automatically included in the tested models. Instead, potential confounders (delivery mode, initiated labor activity, gestational age, gender, placental weight, birth weight/height, maternal pre-pregnancy BMI, weight gain, age, and parity) were tested independently for the differential expression effect on all genes included into the analysis.

4.6.2. Single locus based data analysis

Statistical analyses for RT-qPCR results were performed using the statistical package STATA version 13.1. The Wilcoxon test assessed the significance of RT-qPCR measurements among the study groups. FDR was calculated according to Benjamini and Hochberg (additional information in Ref 1. Supplementary Methods). Association testing with placental *FLT1* eQTL variants was performed using PLINK 1.9 (www.cog-genomics.org/plink/1.9/). Nominal P<0.05 were considered suggestive of association.

4.6.3. Placental miR-QTL analysis (Placental whole-genome genotyping dataset)

To avoid the potential confounding effect of gestational expression dynamics, the discovery analysis of placental miR-eQTLs included only term placental samples (n = 40). SNV genotypes were derived from the genome-wide genotyping dataset of the same placental samples [Illumina HumanOmniExpress-12-v1 BeadChip (>733,000 SNVs; median spacing 2.1 kb)] (Kasak et al., 2015; Pilvar et al., 2019). The analysis was targeted to a ±100 kb window extending to both directions from the start and end of miRNA genes, annotated based on miRBase (ver. 22.1). The genomic regions flanking the analyzed 417 miRNAs included 6,274 common SNVs (MAF > 0.1). 17,302 linear regression association tests were carried out between SNV genotypes and miRNA expression levels, quantified as normalized miRNA read counts. All tests with miR-eQTLs were implemented in PLINK v1.07 using fetal sex and gestational age as cofactors (Purcell et al., 2007). The results were corrected for multiple testing using the Benjamini–Hochberg method, with a cutoff FDR < 0.05. All of the miR-eQTLs were tested for Hardy–Weinberg equilibrium.

4.6.4. Correlation analysis of miRNA and mRNA expression in placenta

Analysis of inter-relatedness between the expression of miRNAs and mRNA/lincRNA genes in 40 term placentas also utilized the above-mentioned published RNA-Seq data. The expressional correlation of miRNA/mRNA transcripts was evaluated using Spearman's correlation coefficient (parameter rho). Correlation analysis included 66 miRNAs showing differential expression in PE in the miR-Seq dataset and 16,567 genes with raw median read counts >50 in the RNA-Seq dataset. Spearman's rho values for 1,093,422 miRNA-gene pairs were estimated in R and visualized as a heatmap using the R package heatmap.2 (Gregory et al., 2015). Lists of genes showing confident expressional correlation with miRNA hierarchical cluster groups G1-G5 were formed using the following criteria: median Spearman's rho across 40 term placentas <−0.3 and for individual samples <−0.1 (negatively correlated genes); or median rho > 0.3 and for individual samples higher than rho > 0.1 (positively correlated genes). These gene lists were used as input for the gene enrichment analysis for in silico functional profiling.

4.6.5. Functional profiling of placental microRNAs

In order to evaluate potential microRNA enrichment in specific functional pathways or pathologies, TAM 2.0 computational tool was implemented (Li et al., 2018). TAM 2.0 microRNA dataset consists manually curated literature overview of selected microRNA-association pairs: microRNA family, cluster, tissue specificity, disease, function, and transcription factor. The intrinsic part of the microRNA enrichment analysis is the used set of background microRNAs. In silico functional query included only microRNAs expressed in the placental samples analyzed in this study. As TAM 2.0 platform is manually curated and may be prone to biases, we used the option to mask cancer-related and non-standard sets of microRNAs to exclude off-target in silico predictions and biologically and physiologically irrelevant interpretations. Investigating serum microRNAs target genes expression correlation miRTarBase database was used. The analysis used only confident target genes to assemble the list of experimentally validated target genes (Huang et al., 2020).

Statistical differences between subgroups were assessed using either Chi-Squared or Fisher's exact test. MicroRNA gestational dynamic expression was evaluated by using REPROMETA samples from first, second, and term samples miRNome expression data, calculating Z-scores. MicroRNA and target gene expression correlation was calculated using Kendall coefficient (parameter Tau).

5. RESULTS

5.1. Placental differential gene expression in complicated term pregnancy (Ref. 1)

This substudy aimed to comprehensively and systematically analyze the placental transcriptome in normal and complicated term pregnancies. To achieve this, an RNA-Seq dataset of placental transcriptomes of 40 samples over a broad range of pregnancy outcomes was utilized for differential expression profiling. Additionally, preeclampsia (PE) risk factors that alter gene expression were explored, including a recently proposed genetic variant near the *FLT1* gene.

5.1.1. Profile of differential gene expression in complicated term pregnancy

The study profiled placental differential gene expression profiles in prevalent adverse pregnancy outcomes at term, focusing on maternal late-onset PE (LO-PE), GD, and pregnancies ending with the birth of either SGA or LGA newborns. A large number of preeclamptic placentas genes had a prominent expression shift compared to the placentas of normal pregnancies and other term pregnancy complications. Whereas the change in placental gene expression in cases of SGA, LGA, and GD was less prominent than in PE, the overall differential expression profiles overlapped among pregnancy complications (**Figure 6**).

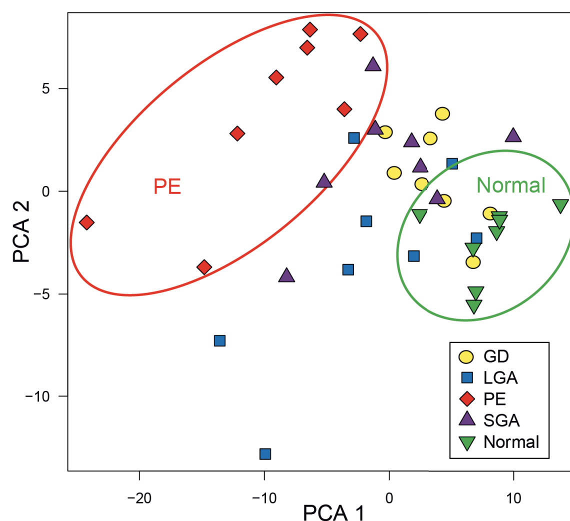

Figure 6. Principal component (PCA) analysis of RNA-Seq sample data.
LO-PE is distinctly separated from the without complications normal group. GD, SGA, and LGA are mixed in with the normal group. **Adapted from Ref.1.**

PE had the most prominent alteration of gene expression profile compared to the other investigated groups. Compared to normal pregnancies, LO-PE placentas exhibited differential expression of 215 genes. Notably, 80% (n = 173) of the differentially expressed genes showed significantly lower transcript levels than controls (Figure 2 and Supplementary Data S3 in Ref 1.). Among the highest expressed differentially expressed genes was *LEP*, needed for proper pregnancy function, and dysregulation is associated with fetal growth and PE.

5.1.2. Locus-specific validation of differential gene expression in preeclampsia

Locus-based experimental validation of 45 differentially expressed genes predicted by the RNA-Seq data analysis was performed using TaqMan RT-qPCR and analysis of an extended placental sample set (PE, n = 24; normal, n =24) (**Table 5**). The differences in gene expression in PE compared to normal placentas estimated from the RNA-Seq and TaqMan RT-qPCR showed high correlation, R^2=0.75 (linear regression, $P = 2.08 \times 10^{-14}$). Concordant effect direction was observed for 42 of 45 assessed genes (Ref. 1 Supplementary Table S3). The estimated log2(fold change) in transcript levels significantly correlated with the RNA-Seq dataset ($R^2 = 0.78$; $P = 1.22 \times 10^{-15}$) (**Figure 7**). Among these genes were *FLT1, HSD17B1, DLX4, ADM*, associated with PE. Rest of the validated genes refer to altered regulation of epigenetic (*DOT1L*, *TET3*), transcriptional (*ZNF469*), and apoptotic (*RELL2*) mechanisms as well as disturbances in the immune (*IGHA1*) and endocrine-metabolic systems (*HSD17B1*, *ADM*, *GDPD5*, *MC1R*). The functions of these validated genes could describe the broader changes that have taken place in placental tissue in the case of PE.

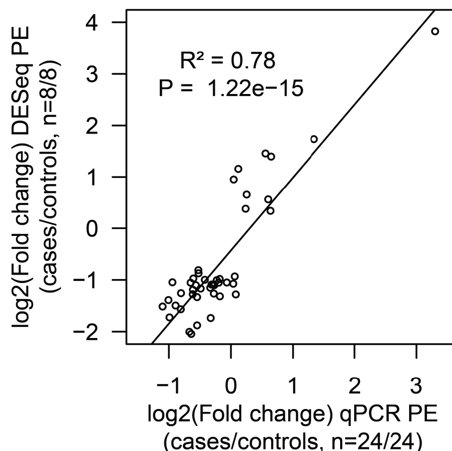

Figure 7. Estimated correlation of the 45 tested placental genes in PE placentas.
Estimated gene expression log2(fold change) of the 45 tested placental genes in preeclamptic placentas between the RNA-Seq and TaqMan RT-qPCR datasets for the combined data of discovery and follow-up placental samples (PE, n = 24; normal, n = 24). Adapted from Ref.1.

5.1.3. Locus-specific validation of differential expression in other term pregnancy complications

Only a few transcripts exhibited statistically significant placental differential expression in other complications: GD (*STS*, *FAM65B*, *ZNF525*, *DNAJC3*), SGA (*RNF17*, *RP11-333A23.3*) and LGA (*MIR205HG*). Principal component (PCA) analysis separated LO-PE from NORM placental samples (**Figure 6**), whereas the cluster of GD placentas overlapped with the NORM group. The placental gene expression profile in the SGA and LGA cases represented a more scattered profile partially overlapping with the PE and GD groups.

As PE and SGA placentas have been suggested to share common pathophysiology, comparison of RT-qPCR for the 45 PE-related genes for extended samples (SGA, n = 24; normal, n = 24; **Table 5**). For 78% of genes (n = 35), the direction of expression alteration was concordant between the PE and SGA placentas (Ref. 1, Supplementary Table S3). Only three genes, *TMEM74B*, *FLT1*, *CDR2L* had statistically significant differential expression in PE and SGA. As PE placentas exhibited a more major change in transcript levels, the effects in the PE and SGA groups were highly correlated (R^2 =0.68, linear regression $P = 3.80 \times 10^{-12}$; **Figure 8**). Although *LEP* after multiple testing correction differentially expressed was not statistically significant, the fold change (FC) in PE and SGA cases was the largest (FC 10 vs. 3, respectively). The altered gene expression level of validated 45 genes in complicated pregnancies indicates potentially altered molecular mechanisms of cellular development and differentiation compared to normal pregnancies.

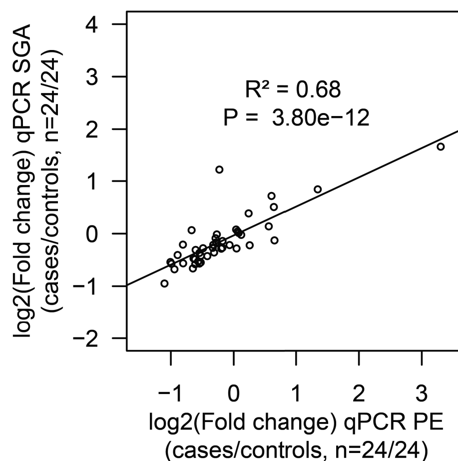

Figure 8. Correlation of the 45 placental genes subjected to TaqMan RT-qPCR in PE and SGA group.
Estimate correlation of the gene expression log2(fold change) of the 45 placental genes subjected to TaqMan RT-qPCR in small-for-gestational-age (SGA, n = 24; Y-axis) cases compared to normal gestation (NORM, n = 24) is correlated with gene expression shifts in PE placentas (n = 24; X-axis). Adapted from Sõber et al., 2015.

5.2. *FLT1* variant as a high-confidence genetic risk factor for preeclampsia (Ref. 2)

Single nucleotide variants have been shown to affect gene expression levels. This substudy aimed to independently replicate the reported GWAS finding that the placental genetic variant upstream of the *FLT1* gene, rs4769613, is associated with the risk of LO-PE (McGinnis et al., 2017). In cohort-based analysis, both independently recruited Estonian sample sets HAPPY PREGNANCY (prospective study; n=1768, **Table 5**) and REPROMETA (retrospective study; n=329) exhibited a suggestive association between the rs4769613[C] variant (**Figure 9**). Conducting a meta-analysis across two datasets (96/2001) replicated the genome-wide association study outcome (Bonferroni corrected $P=4\times10^{-3}$; odds ratio, 1.75 [95% CI, 1.23–2.49]).

When placental rs4769613 genotypes combined placental *FLT1* gene expression and maternal serum sFlt-1 measurements, significantly higher transcript and biomarker levels were detected in preeclampsia versus non-preeclampsia cases in the CC- and CT- (Student t-test, P≤0.02) subgroups. It was concluded that eQTL rs4769613 represents a conditional eQTL, whereby only the enhancer with the C-allele reacts to promote the *FLT1* expression in unfavorable placental conditions, highlighting the placental *FLT1* rs4769613 C-allele is a preeclampsia-specific risk factor.

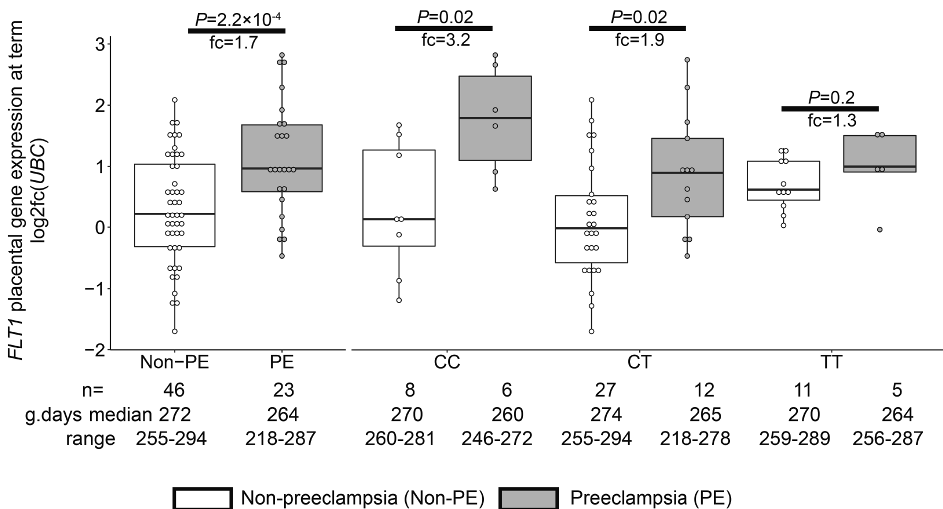

Figure 9. Effect of rs4769613 genotype on placental *FLT1* gene expression in preeclamptic (PE) and nonpreeclamptic third-trimester pregnancies.

Placental gene expression of *FLT1*, stratified by PE diagnosis and placental rs4769613 genotype. Each sample's relative gene expression level (shown in log2fc(*UBC*) scale) was estimated by normalizing RT-qPCR measurements relative to the *UBC* gene as an endogenous control. The median expression level across all placental samples from non-PE pregnancies was used as the baseline value. Fold change of the median *FLT1* expression in PE compared to non-PE samples is provided according to the estimates in linear scale. Adapted from Kikas et al., 2020.

5.3. Placental miRNome and its modulators

5.3.1. Gestational dynamics of placental microRNAs expression

As microRNAs are needed for fast-acting gene expression regulations, it was hypothesized that their expression dynamics evolves and depends on gestational age. MicroRNA levels in three gestational time points were assessed in 20 samples, representing the first trimester [n=5, median 60 (51–81) gestational days, g.days], second trimester [n=7, 121 (108–140) g.days], and term pregnancy [n=8, 284 (260–291) g.days] cases. MiRNome sequencing resulted in 2656 mature miRNAs transcripts across all 20 samples, whereas 417 microRNAs had median raw read counts over 50 across all analyzed samples (Ref. 3. Methods). A broad variability in expression ranges of individual placental microRNAs was detected in all trimesters (**Figure 10A** and Supplementary Figure 1 in Ref. 3). The highest expressed microRNAs demonstrated different gestational dynamics, miR-143-3p expression level increased throughout gestation (**Figure 10B**). Unlike miR-30d-5p, miR-92a-3p and miR-512-3p had the highest expression in the first trimester.

The majority, 319 of 417 (76.5%) of tested miRNAs, exhibited significant gestational expression dynamics (**Table 7**, Supplementary Tables 8, 9 in Ref. 3). In total, 227 (54.4%) miRNAs were differentially expressed between first and second trimester [FDR < 0.05; log2(FC) from −4.91 to 2.84; 125 down- and 102 upregulated], and 211 miRNAs (50.1%) between second trimester and term pregnancy placental samples [FDR < 0.05; log2(FC) from −2.41 to 2.52; 110 down- and 101 upregulated]. More than a quarter of tested miRNAs (n = 119/417; 28.5%) represented differentially expressed miRNAs (DEmiRs) in both comparisons, indicating their potential critical contribution in fine-tuning placental transcriptome profile in gestational age-dependent manner until term (Supplementary Table 10 in Ref. 3).

Table 7. Expressional patterns from first to second trimester – from the second trimester to term pregnancy

Category	All miRNAs (miRNA mature transcripts: *n*, %)[a,b]
Down – Down	30 (7.2%)
Down – No change	67 **(16.1%)**
Down – Up	28 (6.7%)
Up – Up	35 (8.4%)
Up – No change	41 (9.8%)
Up – Down	26 (6.2%)
No change – Down	54 **(13.0%)**
No change – Up	38 (9.1%)
No change – No change	98 **(23.5%)**

[a] Median raw read counts over 50 across all analyzed samples; empirically determined transcript level for robust differential expression testing. [b]Major patterns of expression dynamics are highlighted in bold; the expected proportion given an equal representation of each pattern is ∼11%.

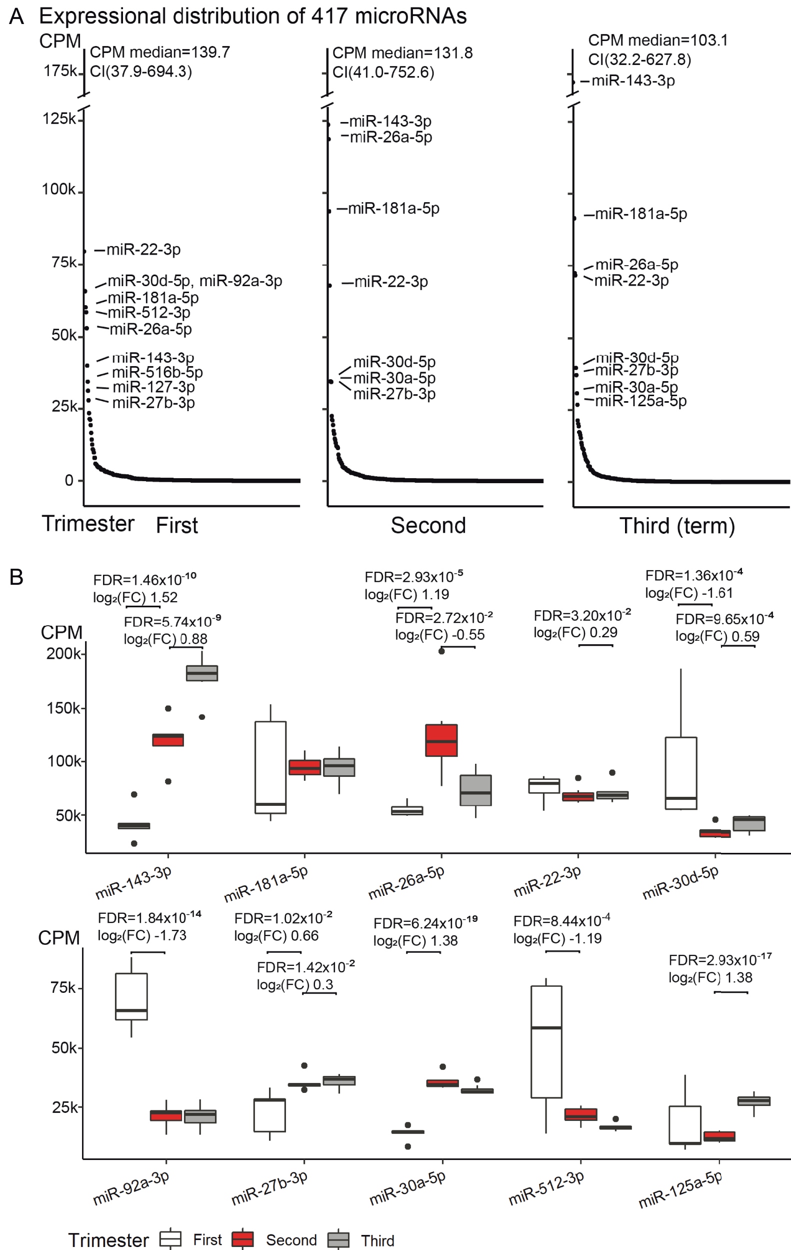

Figure 10. Distribution of placental miRNome during gestation.
(A) Transcript levels of the analyzed 417 miRNAs in the first (median 60; range 51–81 g.days) and second trimester (121; 108–140 g.days) and term placental samples (284; 260–291 g.days). microRNA expression was quantified in counts per million reads mapped (CPM). Highly expressed miRNAs (CPM > 25,000) are indicated. Full details are provided in Ref. 3 Supplementary Table 1. (B) Trimester-specific expression levels of placental miRNAs with the highest transcript levels. Differential expression testing between the three trimesters of pregnancy was implemented in DESeq2 (ver. 1.22.2) (Love et al., 2014) package for R with default settings. Log2(FC), log2 fold change in CPM; FDR, false discovery rate, calculated based on Benjamini–Hochberg method. Adapted from Inno et al., 2021.

The tested 417 placental microRNAs were assigned to one of nine subgroups representing their temporal expression dynamics pattern across three trimesters of pregnancy (**Table 7**, Supplementary Figure 2 in Ref. 3). The most general expression dynamics pattern represented microRNAs exhibiting specifically high transcript levels in early pregnancy (n = 67 miRNAs, ~16%). The second frequent pattern reflected microRNAs downregulated only at term (n = 54, ~13%). High microRNA expression restricted to the second trimester was the rarest observed expressional pattern (n = 26, ~6%). A stable expressional window from early pregnancy to term was identified for 98 miRNAs (23.5%).

With placental role changing during pregnancy, microRNAs are suitable for fine-tuning evolving requirements. These changes could also transpire in case of complications where placental function changes compared to a normal placenta.

5.3.2. Expression variation of PE associated microRNAs

Placental miRNomes representing term cases of late-onset preeclampsia (LO-PE), gestational diabetes (GD), and small- and large-for-gestational-age newborns (SGA, LGA) were tested for differential expression in comparison to normal pregnancies (n = 8 in each group; all cases after 37th g.week). Only PE placentas demonstrated a major shift in their miRNome profile that affected 66 of 417 (15.8%) microRNAs (FDR < 0.05; **Figure 11** and Supplementary Tables 21, 22 in Ref. 3).

Seven significantly upregulated microRNAs overlapped with the placental DEmiRs reported in early-onset PE cases (EO-PE, before 34th g.week) (Awamleh et al., 2019). Several of these showed large changes in their expression level: miRNAs miR-210-3p (FC = 2.64), miR-193b-3p (2.53), miR-193b-5p (2.29), miR-365b-3p (1.93), miR-365a-3p (1.92), miR-520a-3p (1.82) (Supplementary Table 21 in Ref. 3).

Differentially expressed miRNome in PE was comprised of both dynamic and stable miRNAs. No specific pattern of normal gestational dynamics was preferentially altered (**Figure 12**). Several miRNAs normally downregulated at term were characterized by increased transcript levels in PE placentas corresponding to their typical mid-gestation values (e.g., miR-210-3p, miR-31-5p, miR-96-5p, miR-193a-3p, miR-519a/b-5p; Figure 11). On other occasions, microRNA expression in PE placentas was significantly downregulated compared to other analyzed samples (e.g., miR-196b-5p, miR-411-3p, miR-1247-5p). PE miRNome also showed aberrant upregulation of several microRNAs typically stably expressed across gestation (e.g., miR-365a/b-3p).

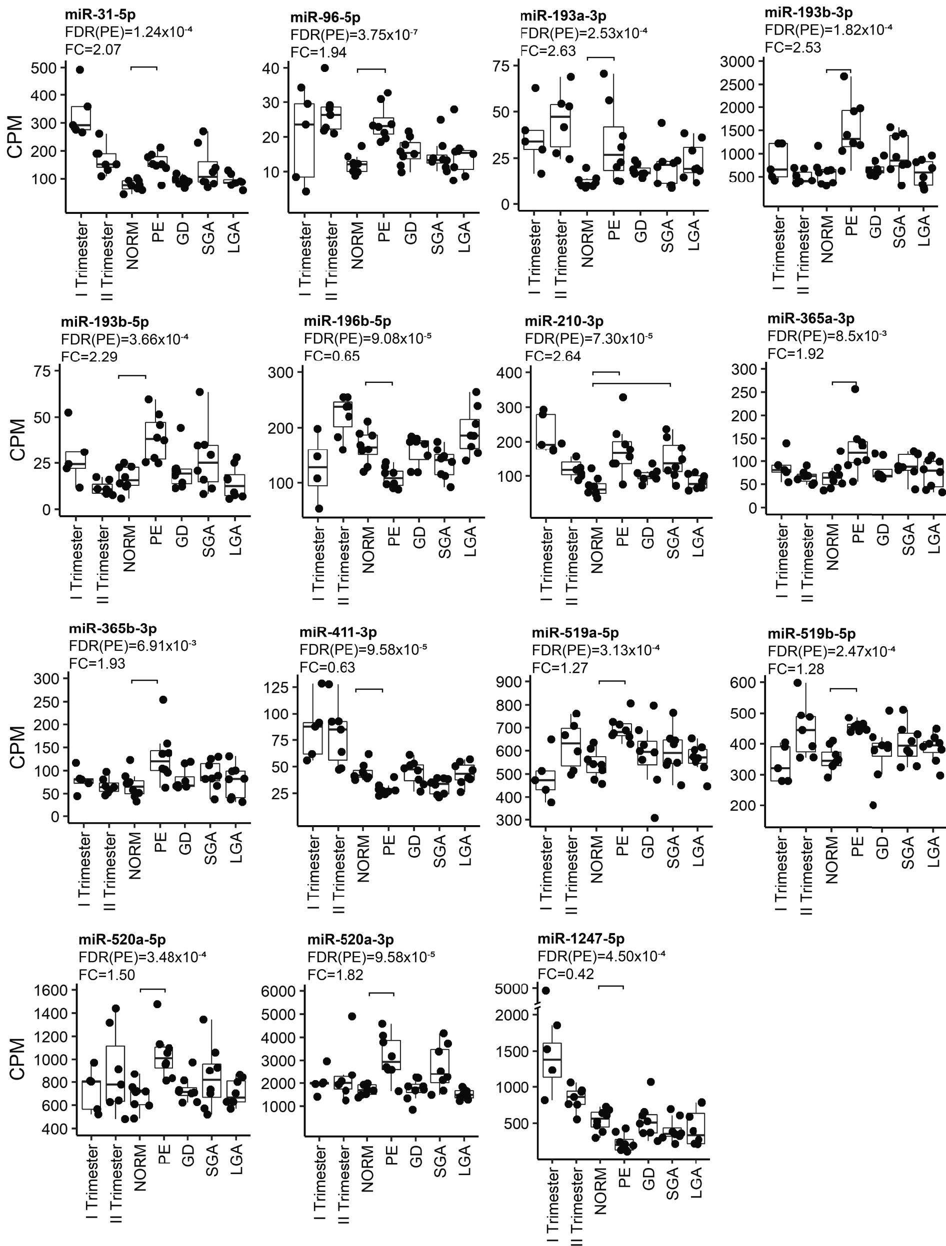

Figure 11. Examples of the most significant differentially expressed microRNAs in PE. microRNA expression was quantified in counts per million mapped reads (CPM). FDR, false discovery rate, calculated based on Benjamini–Hochberg method; GD, gestational diabetes; NORM, normal term pregnancy; PE, preeclampsia; LGA, large-for-gestational-age; FC, fold change in CPM; SGA, small-for-gestational-age. Adapted from Inno et al., 2021.

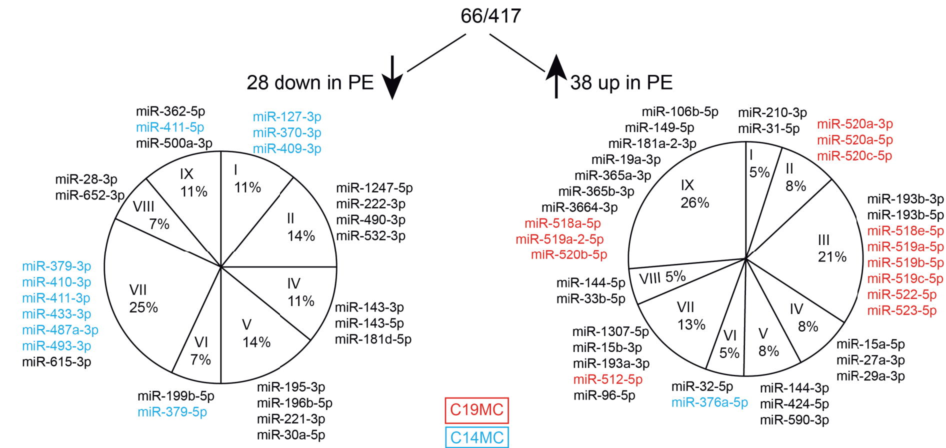

Figure 12. Differentially expressed microRNAs in preeclampsia (PE).
Distribution of differentially expressed microRNAs in PE placentas according to their gestational dynamics patterns. microRNAs transcribed from placental-specific C14MC and C19MC clusters are highlighted in blue and red, respectively. Adapted from Inno et al., 2021.

5.3.3. microRNA eQTL regulating microRNA expression

The effect of genetic variation on the placental miRNome was analyzed. Placental eQTLs for 417 miRNAs were assessed through genetic association testing between their transcript levels in 40 term placental samples (**Table 4**) and genotypes of 6,274 common SNVs located ±100 kb from the microRNA genes. In total, 66 miR-eQTLs for 16 microRNAs were detected (FDR < 0.05; 3.8% of tested miRNAs; **Figure 13**, Supplementary Table 33 and Supplementary Data 2 in Ref. 3).

Four of 16 placental microRNAs modulated by eQTLs were also identified as DEmiRs in PE (miR-30a-5p, miR-210-3p, miR-490-3p, miR-518-5p). Despite the limited sample size, the effect of miR-eQTL on some microRNAs was observed in all three trimesters of pregnancy (e.g., pairs rs447001 and miR-130b-3p/5p, rs2427554 and miR-941, rs12642661 and miR-1269a). The most extreme SNV-miRNA identified pair was rs7046565 (A/G) and miR-3927-3p. The major allele AA-homozygosity completely suppressed the expression of miR-3927-3p. This effect was also detected in second-trimester placental samples (**Figure 13**). Among 66 identified placental miR-eQTLs, 18 eQTLs were unique to placental microRNAs, and 48 have also been reported in the GTEx database as expressional modulators of 53 coding genes (Aguet et al., 2020).

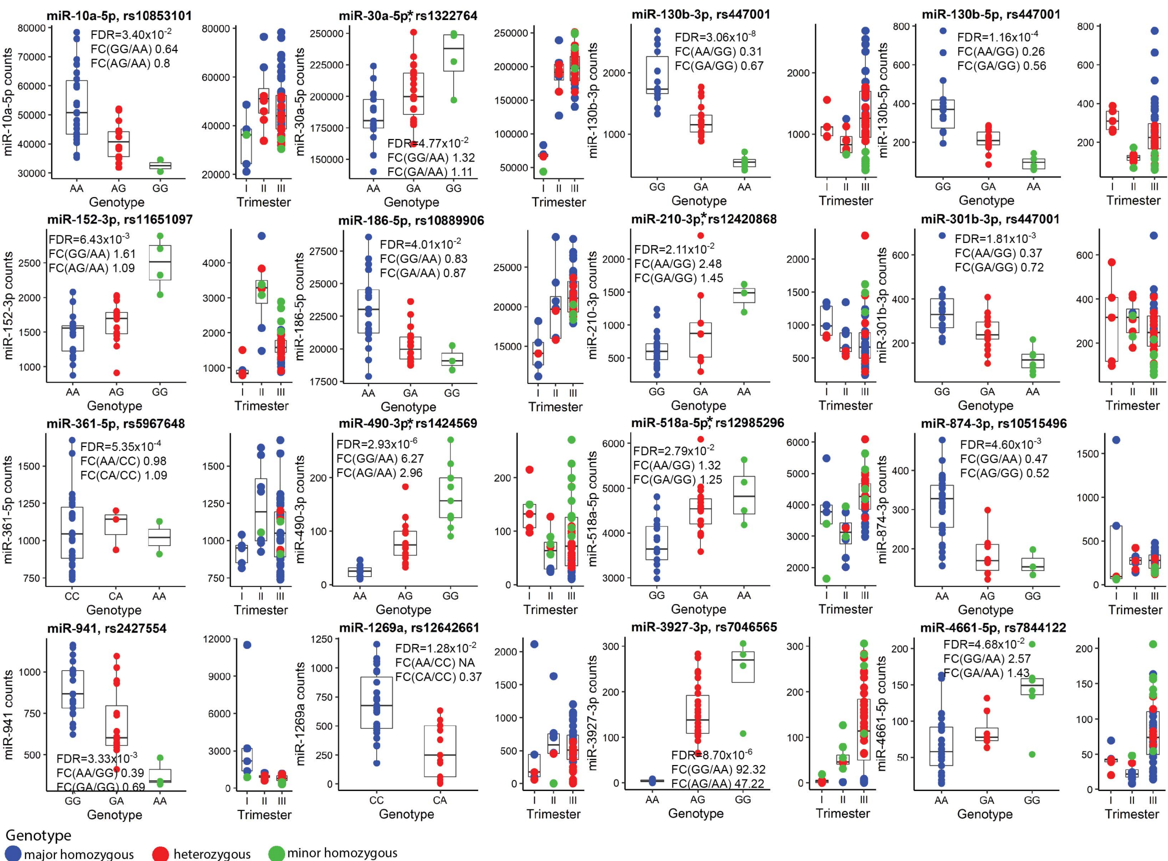

Figure 13. Placental miRNA expression Quantitative Trait Loci (miR-eQTLs).
Discovery analysis of single nucleotide variants (SNVs) modulating the expression of flanking microRNAs was carried out in 40 term placental samples, and the most significant miR-eQTLs are presented. In each subgraph, miRNA normalized read counts (y-axis) are shown for samples stratified based on SNV genotypes (x-axis). Blue, red, and green colors represent placentas homozygous for the major allele, heterozygous or homozygous for the minor allele of SNV. Additionally, the distribution of genotype-stratified samples in the trimesters is shown. microRNAs that are also differentially expressed in the term preeclampsia are indicated with an asterisk (*). Further details are presented in Supplementary Tables 1, 21, 33, and Supplementary Data 1 in Ref. 3. FDR, false discovery rate; FC, fold change. Adapted from Inno et al., 2021.

In our placental RNA-Seq dataset, 32 of them were expressed. Statistically significant associations (FDR < 0.05) were detected with transcript levels of the *KLHL3* (rs10515496), *SNX11* (rs11651097), *ANO9*, and *PTDSS2* (rs12420868) genes (Supplementary Table 34 in Ref 3). However, these statistical associations were weaker than the initially detected effect on the adjacent miR-874-3p, miR-152-3p, and miR-210-3p.

Three miR-eQTLs (SNVs: rs12420868, rs12985296, rs7046565) showed nominal associations in the discovery dataset (n = 40, Supplementary Table 35 in Ref. 3). These variants were used in replication testing with pregnancy traits in the REPROMETA (n = 326) and HAPPY PREGNANCY (n = 1,772) pregnancy-related cohorts (Supplementary Table 3 and Supplementary Methods in Ref. 3). No statistically significant associations were identified with the height, weight, head and chest circumference of newborns, placental weight and PE or GD incidence in independent cohorts or their meta-analyses (all tests, FDR > 0.05; Supplementary Table 36 in Ref. 3). A non-significant trend was detected between rs12420868 (eQTL for miR-210-3p) and newborns' head circumference (meta-analysis: nominal P < 0.05; Supplementary Figure 3 in Ref. 3).

5.4. Expression of placental miRNome is correlated with the transcriptome

MicroRNAs, as the gene expression regulator, have regulatory influence over multiple genes. Correlation analysis between the expression levels of 66 placental DEmiRs identified in PE and placental transcriptome was performed using the corresponding miR-Seq and RNA-Seq datasets of 40 term pregnancy samples. Hierarchical clustering based on the expressional correlation with the transcript levels of 16,567 genes assigned the tested microRNAs into five groups, G1-G5, each containing 6–22 miRNAs (**Figure 14**, Supplementary Data 1 in Ref. 2). In these groups, there was a highly non-random distribution of microRNAs from C19MC (G1:10 miRNAs, G5:3) and C14MC (G4:9, G3:2, G5:1) clusters ($\chi 2$-test, $p = 1.5 \times 10^{-5}$), providing further support to their distinct roles in the modulating placental transcriptome. The C14MC cluster outlier microRNA that did not correctly fit in either groups G4 or G5 was miR-376a-5p. Furthermore, this microRNA already stood out in the differential expression analysis with an opposite behavior compared to the rest of the C14MC microRNAs (**Figure 14**).

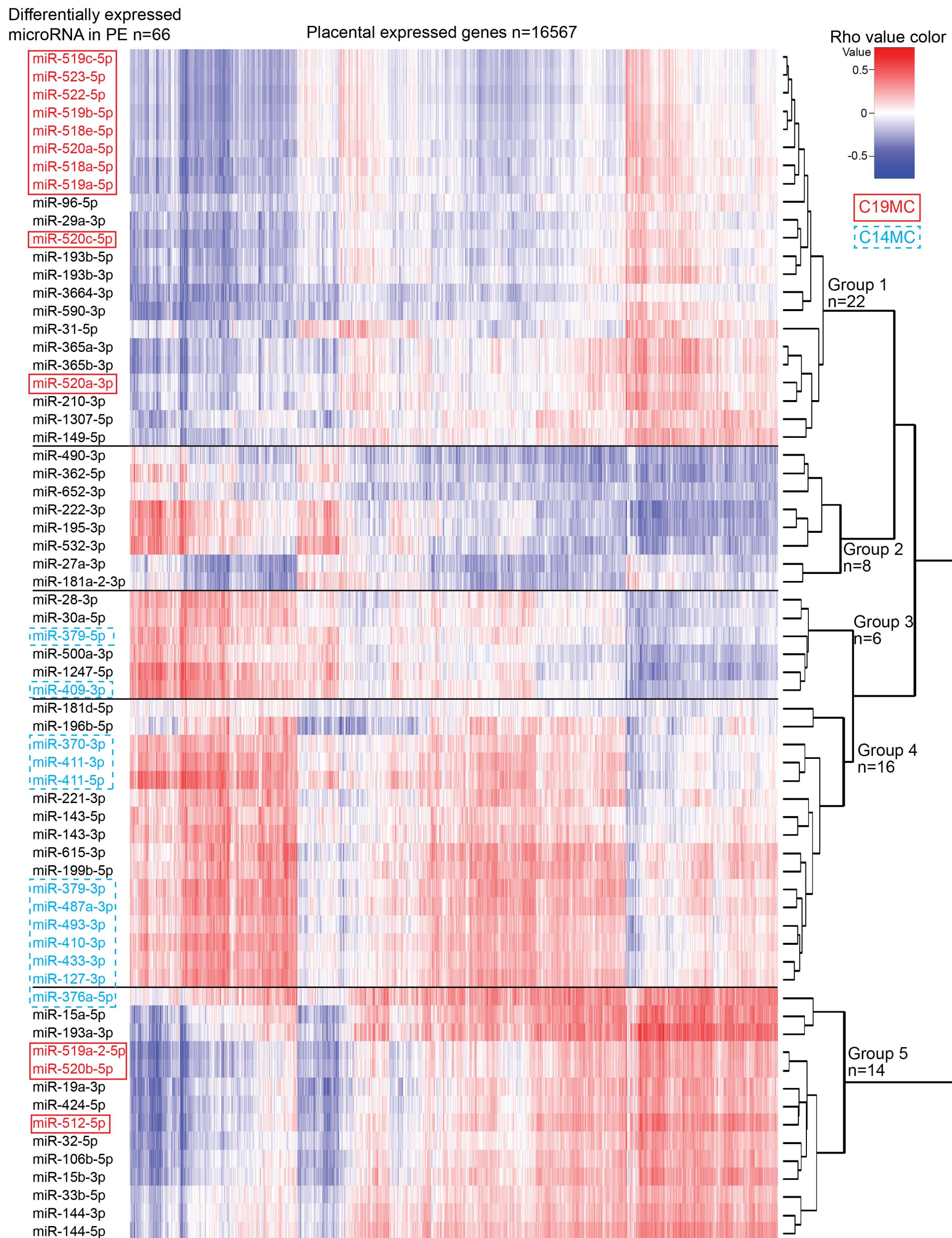

Figure 14. Correlation analysis between microRNAs altered in preeclampsia and the whole transcriptome of 40 term placental samples. The heatmap shows the hierarchical clustering of microRNAs based on the expressional correlation with mRNA transcripts of coding/lincRNA genes. Each row represents one microRNA, and each column one gene. Expressional correlation is presented from –1 (maximum negative correlation, blue color) to 1 (maximum positive correlation, red). The value 0 indicates no correlation. microRNAs groups G1-G5 were formed based on their clustering. Adapted from Inno et al., 2021.

5.5. Dynamics of placenta-specific C14MC and C19MC in normal and aberrant pregnancy

In the placental miRNome dataset, a notably high fraction, 125 out of 417 (~30%) expressed microRNAs, belonged to the primate-specific microRNA cluster C19MC (detected mature placental microRNAs, n = 65; 15.6%) or to the eutherian-specific clusters C14MC (n = 58; 13.9%) (**Table 8**, Table 2A and Supplementary Table 1 in Ref. 3).

Table 8. Placenta-specific microRNA cluster C14MC and C19MC description

Category	miRNA categories	
	C19MC[a] chr19q13.42	C14MC[b] chr14q32.31
Comparative general profile of miRNA categories		
Gene cluster size (kb)	~100kb	~250kb
Placenta-specific	All	All
Parent of origin expression	Paternal	Maternal
All miRNA genes[c] (n)	46	52
All mature miRNA transcripts[c] (n)	67	94
All identified placental mature microRNA transcripts in Ref. 3 (n)	67	93
Placental mature miRNA transcripts with adequate expression level for confident statistical testing (n)[d]	65	58

[a] Primate-specific miRNA cluster; [b] Eutherian-specific miRNA cluster; [c] Data from miRBase version 22.1 (Kozomara et al., 2019); [d] median raw read counts over 50 across all analyzed samples; empirically determined transcript level for robust differential expression testing; C14MC, chromosome 14 microRNA cluster; C19MC, chromosome 19 microRNA cluster

These clusters showed markedly different patterns of gestational expression dynamics. About ~2/3 of C19MC cluster microRNAs are specifically highly transcribed in early pregnancy, with a significant drop in the second trimester and a slight increase at term (**Figure 15** and Table 2B, Supplementary Tables 8, 9 in Ref. 3). The C14MC cluster showed diverse expression in the first trimester, but more coordinated transcript levels in later gestational ages. The majority of C14MC microRNAs showed high expression in the second trimester and significant downregulation before term. Only five C14MC but 22 C19MC microRNAs exhibited stable expression levels from early pregnancy until delivery.

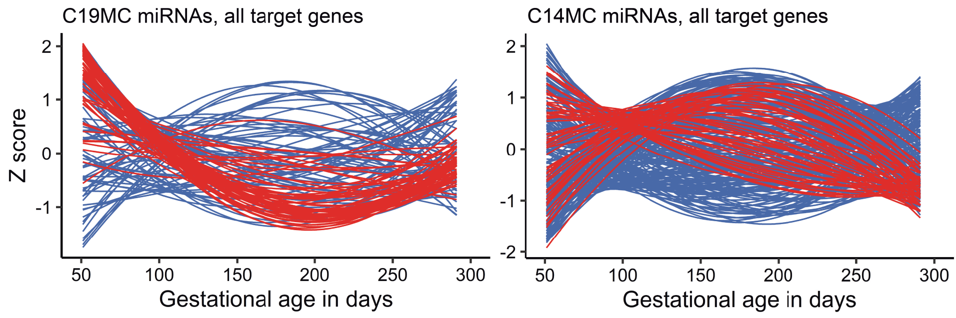

Figure 15. C19MC and C14MC microRNA expression compared with target genes trough out gestation.
Expression dynamics of C19MC and C14MC microRNA clusters across gestation compared to the transcript levels of their miRTarBase target genes. Placental transcript levels were confidently quantified for 63/76 and 215/262 predicted target genes of C19MC and C14MC, respectively. microRNA and gene expression levels during pregnancy are presented in Z-scores; expression data for microRNAs are shown in red, and for target genes in blue. Adapted from Inno et al., 2021.

6. DISCUSSION

6.1. Preeclamptic placenta shows a substantial shift in gene and microRNA expression

Placenta is an irreplaceable organ for mammalian pregnancy (Turco and Moffett, 2019). This thesis investigated gene expression changes in pregnancies with complications (Ref 1.), eQTL as a preeclampsia risk factor, and set forward to provide a comprehensive overview of the placental miRNome, its expression dynamics across gestation and in term pregnancy complications, and the correlation of placental microRNA levels with the transcriptome and genetic variations.

The development of a pregnancy complication is a complex process, encompassing multiple biological pathways regulating fetal development, placental function, and maternal physiology. To enhance the understanding of the occurrence and underlying causes of pregnancy complications, this study employed a placental multi-omics research approach, leading to novel insights. Statistically significant differential expression of 215 genes and 66 microRNAs was identified in LO-PE placental transcriptomes and miRNomes, respectively (Ref. 1 and Ref. 3). This shift of the placental transcriptome was only observed in PE, but not in the placentas of other term pregnancy complications. When comparing published placental transcriptome-based studies of PE cases, only around half of detected differentially expressed genes overlap between studies (Van Uitert et al., 2015). Therefore, it can be concluded that PE placentas represent a diverse set of functional errors and are not confined to specific molecular pathways or biological functional categories. A broader shift in gene expression in PE placentas observed in this study (Ref. 1) has also been reported by others (Kondoh et al., 2022).

Opposed to that, we identified little or no transcriptome shift in gestational diabetes (GD) or cases with a too-large or too-small newborn for their gestational age (LGA and SGA, respectively)(Ref. 1). Interestingly, SGA placentas showed a moderate change in the expression of the similar set of genes. This data contributes to increasing knowledge of the different origin and etiologies of pregnancy pathologies.

microRNAs are among the main transcriptome regulators, and multiple factors such as gestation, altered biological need, and genetic variations possibly modulate their placental expression levels. This study brings essential added value to understanding the dynamics and function of miRNome in normal and complicated pregnancy. In total, 66 differentially expressed microRNAs in PE were detected (Ref. 3). Circovic and colleagues conducted a meta-analysis for studies investigating microRNA expression in PE cases compared to healthy individuals (Cirkovic et al., 2021). Meta-analysis was performed for the most common 14 microRNAs in those datasets, and seven of them were upregulated in the case of PE. In comparison with Ref 3. only miR-210 overlapped in our studies. The minimum overlap between different studies could be caused by how samples are collected, cases and controls matched, or additional variables in the study design

and methods. Importantly, as microRNAs regulate more than one gene at a time and multiple microRNAs regulate one gene, the contribution of a single microRNA to pathology is considered modest (Komatsu et al., 2023). Notably, 23 out of 66 differentially expressed microRNAs in PE have been previously described in the context of pregnancy complications, representing potential non-invasive biomarkers when analyzed from maternal biofluids.

6.2. Genetic variations modulate placental gene and microRNA expression

Both genes and microRNAs are modulated by expression quantitative loci (eQTLs) (Xi et al., 2022). It has been shown that some of the eQTL are tissue- or even condition-specific (Zhang and Zhao, 2023). Placental eQTLs are analyzed in only a few studies (Kikas et al., 2021). Most studies have used a candidate gene-based approach for a known gene associated with pregnancy complications. Four studies have used a hypothesis-free approach for detecting placental eQTLs (Apicella et al., 2023; Delahaye et al., 2018; Kikas et al., 2019; Peng et al., 2017). Due to differences in the study design (number and nature of included pregnancies, different analysis methods), the profile of reported placental eQTLs has differed between studies, and the number of overlapping eGenes (regulated by eQTLs) is limited to less than 20.

This study robustly replicated the genetic association between LO-PE and SNP rs4769613 (T/C) in the expression regulatory region of the *FLT1* gene (Ref. 2). Importantly, this variant was shown to act as a conditional eQTL in LO-PE placentas whereby C-allele was associated with significantly higher *FLT1* transcript levels. Recently, it was also shown that the placental *FLT1* rs4769613 (T/C) genotype could be incorporated into the prognostic models and clinical and serum biomarker data to estimate the risk of developing PE (Ratnik et al., 2022).

For the first time, the study set forward to identify placental miR-eQTLs, SNPs modulating placental microRNA levels (Ref. 3). Previous microRNA eQTL studies have focused on candidate microRNA analysis (Konwar et al., 2019; Lu et al., 2021). This study used a hypothesis-free approach to detect microRNA eQTLs located in ±100 kb from the miRNA genes in placental tissue and identified 66 miR-eQTLs for 16 microRNAs. It has been discussed that miR-eQTLs may influence the expression of multiple genes due to the broad number of microRNAs target genes (Sonehara et al., 2022). However, even though miR-eQTLs could drastically affect single microRNA levels, other microRNAs could compensate for this change to guarantee required mRNA levels. Therefore, detectable changes are minuscule compared to eQTLs affecting protein-coding gene expression with potentially a more meaningful phenotypic effect. Also, in this study, miR-eQTL effects did not transfer to an apparent clinical phenotype – only a trend between one miR-eQTL and newborn head circumference was detected.

6.3. Placenta gene and microRNA expressions are interconnected

This study demonstrated that microRNAs have dynamic expression during gestation and can be subgrouped based on their expression patterns (Ref. 3). These dynamic expression changes represent placenta requirements that are changing during gestation. Pathways regulated by microRNAs are overlapped, as one microRNA can regulate more than one gene at a time, allowing a group of microRNAs to have a significant role in gene expression (Berezikov, 2011). The placenta needs cohesive and timely regulated gene expression (Suryawanshi et al., 2022). As microRNAs bind to mRNA and halt its translational activity, allowing higher accuracy in a shorter time (Winter et al., 2009).

When studying placenta transcriptome and miRNome, it would be prudent to consider their joint action in guiding the roles of the placental tissues. Studies that have been focused only on either transcriptome (Kaartokallio et al., 2016; Kim et al., 2012) or miRNome (Awamleh et al., 2019; Guo et al., 2011; Östling et al., 2019) have added substantial knowledge in advancing the understanding on the placental function. However, combining different omics datasets in this study allowed us more precise insight into the regulation of placental gene expression. This approach was previously also used by (Biró et al., 2016), incorporating available microRNA and gene expression microarray datasets to create a network of microRNA interactions. One of the limitations faced in this past study was the unavailable transcriptome and miRNome datasets generated from the same biological samples and insufficiently characterized clinical cases. Our datasets overcome these limitations, being generated from the same samples with a detailed clinical profile facilitating interpretation and drawing several novel conclusions, such as tight co-dependency between the placental transcriptome and microRNA expression profiles across pregnancy. Gong and fellows used a broader approach and aimed to describe the whole placental RNA landscape (Gong et al., 2021). They described different types of RNAs in the placenta, finding that the most common types were mRNA (81.4%) and microRNA (86.2%) based on total RNA-Seq and small RNA-Seq, respectively.

One challenge in analyzing gene-microRNA interactions is correctly interpreting the nature of the observed expression correlations. Altered gene and microRNA expression in term samples could reflect the compensation mechanism for the attempt to maintain functional homeostasis of the placenta (Torres-Torres et al., 2023). In addition to the expected negative expression correlation (Stavast and Erkeland, 2019), significant positive correlations between expression levels of a high number of genes and microRNAs were detected in this study. Two alternative scenarios could explain this. Firstly, high microRNA expression levels may reflect their rising concentrations before the actual effect on inhibition of the mRNA quantities. Secondly, these observations may also represent molecularly unlinked genes and microRNA. High levels of both are important to guarantee the required cellular transcriptome at each timepoint – inhibition of mRNA levels of some target genes by highly expressed microRNAs may indirectly enhance the transcripts levels of other genes.

6.4. Placenta-specific microRNAs clusters C14MC and C19MC have distinct functions in gestation

Two microRNA clusters, C14MC and C19MC, are predominantly expressed in placental tissue (Malnou et al., 2018). C14MC is mammalian-specific and maternally expressed. It is believed to regulate normal placental development (Morales-Prieto et al., 2013). In our study dataset, C14MC microRNA expression stayed high during most of the gestation and lowered at the end. As C14MC function is associated with fetal growth and neurological development, these systems develop at earlier pregnancy stages (Labialle et al., 2014). This fits with the expression pattern detected in our study.

C19MC is primate-specific and needed for more precise regulations of placental development (Malnou et al., 2018). The primate placenta has complex structures, and more regulatory elements are needed for cell invasion and the end of the pregnancy. C19MC has a vital role at the beginning and the end of gestation. This functional pattern is also detected in Ref. 3, where C19MC microRNA expression is highest in our first trimester and term samples. C19MC has previously been associated with the development of preeclampsia (Hromadnikova et al., 2015). As pregnancy complications development varies based on the time of onset, microRNA gestational expression could be an important factor for monitoring the progression of placental development (Jaszczuk et al., 2022).

C14MC and C19MC microRNAs have been of great interest in pregnancy research as biomarkers for gestational complications. Multiple studies have used serum or plasma samples to detect differential microRNA expression in pregnancy pathologies (Aplin et al., 2020). Knowing the microRNA expression level and its dynamical change in the placenta could increase the accuracy of tests based on microRNA expression levels (Sørensen et al., 2022).

7. CONCLUSIONS

In framework of current doctoral thesis placental whole transcriptome and miR-Nome dynamics during gestation and late pregnancy complications was profiled. The results can be summarized as follows:

1. In RNA-Seq based analysis, preeclamptic (PE) compared to normal placentas showed differential expression of 215 genes. In locus-based experimental validation using TaqMan RT-qPCR, 42 out of 45 tested differentially expressed genes exhibited concordant expressional change with the original dataset. As a large set of genes are differentially expressed in PE, the reason for these could be present in an earlier stage of pregnancy. Knowing the changes in genes expression could be used to focus on diagnostic studies.

2. In unfavorable placental conditions, single nucleotide variant rs4769613 near the *FLT1* genes was validated in combined datasets of the REPROMETA and HAPPY PREGNANCY to be a conditional eQTL. This risk factor could be integrated into a prognostic test for more precise risk evaluation.

3. Placental microRNA expression can change based on gestation progression, genetic variants near microRNA genes, or pregnancy complications influencing placental function can alter microRNA expression. In future studies, it is crucial to match study samples for gestational age and identify potential variants causing the change in microRNA expression.

4. A comparison of transcriptome and miRNome shows a significant correlation between certain microRNA subgroups and functionally linked sets of the placenta genes, potentially indicating a co-dependent expression regulation. Grouping microRNAs with genes that share functional pathways may help to find new gene expression regulators among microRNAs not detected by the microRNA target gene prediction approach.

5. The gestational expression profile of placenta-specific microRNA clusters C14MC and C19MC refer to their critical roles in different gestational stages. C14MC microRNAs have a broader range of expression at the beginning of pregnancy and with gradually reduced expression towards the delivery, whereas C19MC microRNAs exhibit high transcript levels in early and late pregnancy, with a slope in the mid-gestation. The unique role of these microRNA clusters makes them potential candidates for biomarkers in different stages of pregnancy.

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SUMMARY IN ESTONIAN

Platsenta transkriptoom ja miRNoom tervete ja komplikatsioonidega raseduste korral

Probleemideta rasedus on oluline igale emale, selle eelduseks on korrektne platsenta töö.

Platsenta omab raseduse ajal tähtsat rolli, olles oluline toit- ja jääkainete vahendaja ema ja loote vahel. Lisaks sellele täidab platsenta ka endokrinoloogilist funktsiooni tootes raseduse kestvuseks vajalikke hormoone ja teisi signaalmolekule, mis sekreteeritakse ema organism.

Platsenta funktsioneerimist mõjutavad mitmed tegurid, nii raseda elustiili harjumused kui erinevad sisemised ja välimised stiimulid. Selleks, et platsenta suudaks oma rolli täita, peab ta olema emaka seinaga põimunud ning saavutanud korrektse verevarustuse ema organismiga.

Kuna platsenta eksisteerib ainult lühikese ajaperioodi jooksul võrreldes teiste organitega, on tema areng samuti kiire. Esimese trimestri lõpuks peab platsenta olema välja arenenud ja võimeline toetama loote arengut. Platsenta kiire areng nõuab ajaliselt reguleeritud geenide ekspressiooni, platsenta transkriptoomi, regulatsiooni. Rasedusega suureneb mitmete kasvuhormoonide ja rasedusega seotud molekulide tase ema organismis. Nende geenide ekspressiooni kiireks reguleerimiseks on mitmeid erinevaid viise, milleks üks on mikroRNAd. Platsentas ekspresseeruvate mikroRNAde kogum, miRNoom, on üheks osaliseks geenide ekspressiooni tasemete regulatsioonis. Inimese organismis eksisteerib kaks raseduse spetsiifilist mikroRNA klastrit. Üks asub kromosoomil 14 ja teine kromosoomil 19. Lisaks nende asukohale genoomis, eristab neid ka nende spetsiifilisus. Kromosoomil 14 paiknev mikroRNAde klaster on imetajate spetsiifiline ning omab ortolooge teistes liikides. Kromosoomil 19 paiknev kromosoomide klaster on primaatide spetsiifiline ning on evolutsiooniliselt palju noorem.

Üks sagedasemad raseduse komplikatsioone on seotud vastsündinu sünnikaaluga, olles kas liiga suur või väikegestatsiooniaja kohta. Kui naisel esineb suurenenud rasedusaegne kaalutõus või on varasemalt olnud probleeme diabeediga, võib raseduse jooksul välja kujuneda gestatsioonidiabeet. Selle tagajärjel on häiritud loote ainevahetus ning on soositud liigne üsasisene kaalutõus. Osadel juhtudel on häiritud platsenta võime tagada lootele sobilik üsasisene elukeskkond ning varustada loodet vajalike toitainetega. Selline olukord toob kaasa suurenenud stressi ema organismile, väljendudes kõrgenenud vererõhu ning häirunud neerude funktsiooniga. Sellises olukorras võib välja areneda preeklampsia, mille üheks tunnuseks on ema uriinist tuvastatav normist suurem kogus valku. Ema organismile suurenenud stress võib viia ka tõsisemate sümptomite tekkeni ning ainuke ravivõimalus on sünnituse esilekutsumine. Peale raseduse lõppu ja platsenta eemaldamist sümptomid taanduvad.

Parimaks meetodiks platsentas esinevate kõrvalekallete analüüsimiseks on järgmise põlvkonna sekveneerimine (NGS). NGS võimaldab hüpoteesi vabalt hinnata platsentas eksisteerivate geenide ja mikroRNAde tasemeid. Hüpoteesi

vaba lähenemise eelis varasemate meetodite üle seisneb saadavas informatsiooni hulgas. Kui varasemate meetodite kasutamisega oli piiravaks faktoriks uuringusse kaasatud geenide ja mikroRNAde hulk, siis NGS-il piirang puudub, võimaldades tuvastada ja analüüsida kõigi proovis esinevate geenide ja mikroRNAde lugemit.

Antud doktoritöö põhieesmärk oli kirjeldata platsenta transkriptoomi ja miRNoomi, ning nende muutumist raseduskomplikatsioonide korral.

Püstitatud eesmärgid:

1. Leida platsentas raseduskomplikatsioonide korral diferentsiaalselt ekspresseerunud geenid.
2. Kirjeldata mikroRNAde ekspressiooni profiili muutust raseduse kulgemise jooksul ning leida diferentsiaalselt ekspresseerunud mikroRNAd raseduskomplikatsioonide korral.
3. Hinnata genoomis eksisteerivate ühenukleotiidsete variatsioonide mõju preeklampsia riskile ning mikroRNAde ekspressiooni tasemele.

Doktoritöö peamised tulemused on:

1. Võrdlesime raseduskomplikatsioonideta platsentade transkriptoomi preeklampsia (PE), gestatsioonidiabeedi (GD), ning liiga suure või liiga väikese sünnikaalu diagnoosiga raseduste (LGA ja SGA) platsentade trasnskriptoomiga. Leidsime, et preeklampsia erines selgelt teistest gruppidest, omades kõige enam diferentsiaalselt ekspresseerunud gene (n=215). Ülejäänud gruppide transkriptoomid olid üldiselt väga sarnased komplikatsioonideta platsentade transkriptoomidega, mis omakorda näitab platsenta olulisust PE puhul.
2. Valideerides 45 PE korral differentsiaalselt ekspresseerunud geeni ekspressiooni taset RT-qPCRiga, tuvastasime 42 geeni, mis omasid geenide sekveneerimisandmestikuga samasuunalist ekspressiooni taseme muutust. Lisaks tuvastasime 35 geeni, mis omasid samasuunalist ekspressiooni muutust nii PE kui ka SGA grupis, viidates PE ja SGA sarnasusele kuid siiski omades erineva suurusega transkriptoomi ekspressiooni kõrvalekallet.
3. Tuvastasime, et *FLT1* geeni lähedal paiknev ekspressiooni mõjutav lookuse rs4769613 [C] alleel reguleerib geeni ekspressiooni taset platsentas ebasobivate tingimuste korral. Antud riskifaktori hindamise kaasamine võimaldab parandada diagnostiliste meetmete võimekust.
4. Platsentas ekspresseeruvate mikroRNAde tasemed muutuvad raseduse jooksul. mikroRNAde ekspressiooni tasemete hindamise juures on oluline arvesse võtta proovi võtmise aega, vältimaks vale diferentsiaalse ekspressiooni tuvastamist.

5. PE korral on mikroRNAde ekspressiooni tase kõige enam normist kõrvale kaldunud (66 mikroRNAd), võrreldes teiste uuringusse kaasatud raseduskomplikatsioonidega (GD, LGA, SGA).
6. Transkriptoomi ja miRNoomi omavahelises võrdluses tuvastasime seose mikroRNAde ja platsentas ekspresseeruvate geenide vahel, moodustades kindla funktsiooniga gruppe.

Käesoleva doktoritööga on antud märgatav panus platsenta transkriptoomi ja miRNoomi paremaks mõistmiseks nelja erineva raseduskomkplikatsiooni, gestatsiooni aja ning DNA variatsioonide mõju kontekstis. Raseduskomplikatsioonide korral asetleidvate geenide ja mikroRNAde ekspressiooni kõrvalekallete avastamine annab võimaluse potentsiaalselt uute diagnostiliste lähenemiste loomiseks.

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Supervised dissertations

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Juhendatud väitekirjad

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DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

1. **Toivo Maimets**. Studies of human oncoprotein p53. Tartu, 1991, 96 p.
2. **Enn K. Seppet**. Thyroid state control over energy metabolism, ion transport and contractile functions in rat heart. Tartu, 1991, 135 p.
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