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LAURA KASAK

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# Genome structural variation modulating the placenta and pregnancy maintenance

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

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# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles and a book chapter referred to in the text by their Roman numerals:

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II **Kasak, L**; Rull, K; Vaas, P; Teesalu, P; Laan, M. 2015. Extensive load of somatic CNVs in the human placenta. *Scientific Reports*, 5 (8342), 1–10.

III **Kasak, L**; Rull, K; Sõber, S; Laan, M. 2017. Copy number variation profile in the placental and parental genomes of recurrent pregnancy loss families. *Scientific Reports*, 7 (45327), 1–12.

IV **Kasak, L**; Rull, K; Laan, M. Genetics and Genomics of Recurrent Pregnancy Loss. In: Leung, P and Qiao, J, editors. Human Reproductive and Prenatal Genetics. Elsevier Inc.; November 2018 (Invited book chapter, in press).



**Author's contribution**

Ref. I – carried out majority of the experiments, participated in data analysis and interpretation and contributed to manuscript preparation

Ref. II, III – contributed as the leading researcher, participated in experimental design, data analysis and interpretation, wrote the first draft of the paper

Ref. IV – contributed to the preparation of the book chapter

# LIST OF ABBREVIATIONS

| | |
|---|---|
| *ACAP3* | ArfGAP with coiled-coil, ankyrin repeat and PH domains 3 |
| aCGH | array comparative genomic hybridization |
| *ALDH1L1* | aldehyde dehydrogenase 1 family member L1 |
| aPL | antiphospholipid antibody |
| APS | antiphospholipid antibody syndrome |
| BAF | B-allele frequency |
| bp | base pair |
| BMI | body mass index |
| *BP* | breakpoint |
| *C4A* | complement C4A (Rodgers blood group) |
| *C4B* | complement C4B (Chido blood group) |
| *CDH19* | cadherin 19 |
| CEA | carcinoembryonic antigen |
| *CGB* | chorionic gonadotropin beta subunit |
| *GCH1* | GTP cyclohydrolase 1 |
| *CLEC* | C-type lectin domain family |
| CN | copy number |
| CNV | copy number variation |
| CNVR | copy number variation region |
| *COL11A1* | collagen type XI alpha 1 chain |
| *CPSF3L* alias *INTS11* | integrator complex subunit 11 |
| *CPXM2* | carboxypeptidase X, M14 family member 2 |
| *CSMD3* | CUB and Sushi multiple domains 3 |
| *CTNNA3* | catenin alpha 3 |
| CTS | cathepsins |
| *CYP* | cytochrome P450 family |
| DECIPHER | DatabasE of genomiC varIation and Phenotype in Humans using Ensembl Resources |
| DGV | Database of Genomic Variants |
| *DKK2* | Dickkopf 2 homolog |
| DNA | deoxyribonucleic acid |
| *DPYD* | dihydropyrimidine dehydrogenase |
| *DVL1* | dishevelled segment polarity protein 1 |
| E2F | transcription factor E2F family |
| *EPHA7* | ephrin receptor A7 |
| ESHRE | European Society of Human Reproduction and Embryology |
| *FII* | coagulation factor II, thrombin |
| *FV* | coagulation factor V |
| FFPE | formalin-fixed paraffin-embedded |
| *FGFRL1* | fibroblast growth factor receptor like 1 |

| | |
|---|---|
| FISH | fluorescence *in situ* hybridization |
| GDM | gestational diabetes mellitus |
| *GKLF* alias *KLF4* | Kruppel like factor 4 |
| *GLTPD1* | ceramide-1-phosphate transfer protein |
| *GOLPH3* | golgi phosphoprotein 3 |
| *GRIK2* | glutamate ionotropic receptor kainate type subunit 2 |
| GWAS | genome-wide association study |
| HLA | human leukocyte antigen |
| HOXA | homeobox A cluster |
| HOXC | homeobox C cluster |
| ICM | inner cell mass |
| *IFNG* | interferon gamma |
| *IGF2* | insulin like growth factor 2 |
| *IGF2R* | insulin like growth factor 2 receptor |
| IGH | immunoglobulin heavy locus |
| *IGHA2* | immunoglobulin heavy constant alpha 2 |
| *IGHE* | immunoglobulin heavy constant epsilon |
| *IGHG* | immunoglobulin gamma heavy chain |
| *IGKV* | immunoglobulin kappa variable cluster |
| *IL* | interleukin |
| IUGR | intrauterine growth restriction |
| IVF | *in vitro* fertilization |
| kb | kilobase |
| *KCNQ1* | potassium voltage-gated channel subfamily Q member1 |
| *KCNQ1OT1* | KCNQ1 opposite strand/antisense transcript 1 |
| *LEP* | leptin |
| LGA | large-for-gestational age |
| *LHB* | luteinizing hormone beta polypeptide |
| *LRP2* | low-density-lipoprotein-receptor-related protein 2 |
| LRR | log R ratio |
| Mb | mega base |
| MLPA | multiplex ligation-dependent probe amplification |
| mRNA | messenger ribonucleic acid |
| *MSR1* | macrophage scavenger receptor 1 |
| *MTHFR* | methylenetetrahydrofolate reductase |
| mTOR | mammalian target of rapamycin |
| *MTRR* | methionine synthase reductase |
| NGS | next generation sequencing |
| NK | natural killer |
| *NOS3* | nitric oxide synthase 3 |
| *NTM* | neurotrimin |
| OR | odds ratio |
| *PDZD2* | PDZ domain containing 2 |
| PE | preeclampsia |
| *PEG1* alias *MEST* | mesoderm specific transcript |

| | |
|---|---|
| *PEX10* | peroxisomal biogenesis factor 10 |
| PGD | preimplantation genetic diagnosis |
| *PHLDA2* | pleckstrin homology like domain family A member 2 |
| *PKP3* | plakophilin 3 |
| *PRDM16* | PR/SET domain 16 |
| *PSG* | pregnancy specific glycoprotein |
| *PUSL1* | pseudouridylate synthase-like 1 |
| qPCR | quantitative polymerase chain reaction |
| RPL | recurrent pregnancy loss |
| SAM | S-Adenosyl methionine |
| *SCNN1D* | sodium channel epithelial 1 delta subunit |
| *SEPT14* | septin 14 |
| sFlt-1 | soluble fms-like tyrosine kinase 1 |
| SGA | small-for-gestational age |
| *SLC22A3* | solute carrier family 22 member 3 |
| SNP | single nucleotide polymorphism |
| SNV | single nucleotide variant |
| *SREBP-2* | sterol regulatory element binding transcription factor 2 |
| *SOHLH1* | spermatogenesis and oogenesis specific basic helix-loop-helix 1 |
| *TAS1R3* | taste 1 receptor member 3 |
| *TFAP2A* | transcription factor AP-2 alpha |
| TGC | trophoblast gigant cell |
| *TIMP2* | TIMP metallopeptidase inhibitor 2 |
| *TMEM203* | transmembrane protein 203 |
| *TNF* | tumor necrosis factor |
| *TP* | tumor protein |
| *VEGFA* | vascular endothelial growth factor A |
| *VWF* | von Willebrand factor |
| *WDR27* | WD repeat domain 27 |
| WES | whole-exome sequencing |
| WGS | whole-genome sequencing |
| *ZF5* alias *ZBTB14* | zinc finger and BTB domain containing 14 |

# INTRODUCTION

The establishment and maintenance of pregnancy relies on the well-coordinated crosstalk between the mother and the embryo. Human reproduction has very low efficiency with 30% of all conceptions failing already before implantation. Another 40% result in miscarriage, the most prevalent human gestational complication. Recurrent pregnancy loss (RPL), defined as ≥3 consecutive losses before gestational week 24, affects 1–2% of couples trying to conceive. RPL is known for its multifactorial etiology; however, 25–50% of all cases are still reported as idiopathic.

Pregnancy is an enormous physiological challenge a woman can experience and can temporarily expose subclinical diseases that will reoccur later in life. For some women, the physiological adaptations during pregnancy cause late gestational complications, including preeclampsia (PE), gestational diabetes mellitus (GDM) and fetal growth abnormalities, which can lead to chronic physiological outcomes for the mother. Additionally, children born from complicated pregnancies may also have an increased risk of different diseases throughout life. As these gestational complications affecting women and their offspring all over the world are often associated with poor trophoblast invasion and placental function, there is an urgent need for better understanding of placental biology, development and function. Placenta, the only transient organ in the body, mediates all interactions between the mother and the baby, but often remains under-investigated due to complex sample collection procedures (sampling, storage, accompanying clinical information) and ethical restrictions. Placental gene expression levels and dynamics regulate the function of placenta and therefore pregnancy maintenance. Transcriptome of the human placenta is modulated by genetic variation of which single nucleotide variants (SNVs) have been investigated the most.

DNA copy number variations (CNVs) are defined as genomic segments of >50 bp present at a variable copy number in comparison to a reference genome. CNVs are estimated to contribute to 10% of the human genome and have been implicated in various human diseases. CNVs may affect the expression of genes critical for (early) pregnancy maintenance by direct (deletion or duplication of entire genes) or indirect (rearrangement of regulatory elements) ways. Studies of CNVs are lacking in reproductive disorders; however, the few published reports have indicated that genomic rearrangements may play a role in pregnancy complications. Understanding the architecture of placental and parental genomes of complicated as well as normal gestations may reveal biomarkers that will help to predict pregnancy outcome.

In the current thesis, the literature review gives an up to date overview of the placental development and function, early (RPL) and late pregnancy complications of the mother (PE, GDM) and the newborn (growth abnormalities). A major part of the overview focuses on CNVs and their role in pregnancy complications.

The experimental part of this doctoral thesis explores the role of CNVs in the parental and placental genomes during normal pregnancies and gestational complications. First, a genome-wide analysis of CNVs was performed in couples with unexplained recurrent pregnancy loss and fertile controls to identify common rearrangements conferring risk for RPL. Second, a genome-wide profile of subchromosomal rearrangements was studied for the first time in the human placental genome. Third, based on the results of the previous study, an analysis of trio (mother-father-placenta) and duo (mother-placenta) CNV profiles of patients with idiopathic RPL was conducted. The role of CNVs in the placental genome in particular and in the etiology and clinical management of pregnancy complications is discussed in the light of the outcomes of this thesis.

# 1. REVIEW OF LITERATURE

## 1.1 Biology of pregnancy maintenance

The biological processes leading to pregnancy establishment, maintenance, and ultimately to birth are extremely complex and dependent on the well-coordinated crosstalk between the mother and the semi-allogenic embryo. The establishment of pregnancy requires maternal recognition of pregnancy and implantation. The success of each stage (apposition, adhesion, and invasion) is vital in order to reach the next step. Once fertilization has taken place, the presence of an embryo does not guarantee a healthy fetus and a live offspring. Human female meiosis is full of segregation errors; young women (<30 years) produce 3–61% oocytes with an incorrect number of chromosomes (Webster and Schuh, 2017). If fertilized, these oocytes result in aneuploid embryos that mostly fail to develop into functional blastocysts. Pregnancy loss has been recognized in all mammals; however, human reproduction is particularly inefficient with only ~30% of all fertilized oocytes resulting in a live birth (**Figure 1**) (Macklon et al., 2002).

The maintenance of pregnancy relies on the interactions between the conceptus and the maternal endometrium to establish immune tolerance, regulate trophoblast invasion, and remodel the uterine spiral arteries.

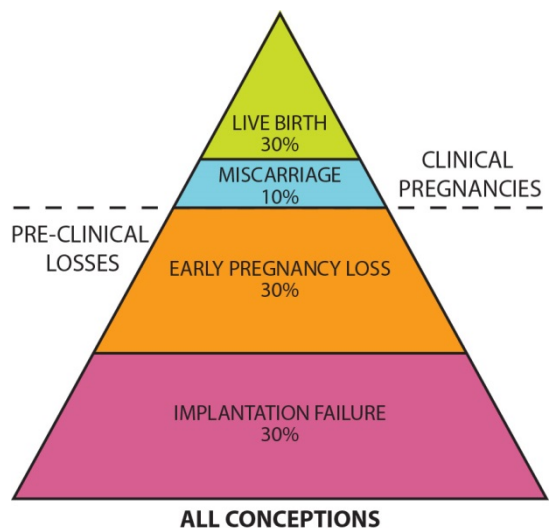


**Figure 1.** The iceberg of pregnancy loss. Roughly 70% of all spontaneous conceptions fail to succeed in a live birth (based on Macklon et al., 2002).

### 1.1.1 Implantation in human

Implantation is one of the most critical stages of pregnancy establishment, depending not only on the quality of the embryo, but also on the receptivity of the endometrium as well as the communication between the two.

Implantation involves the apposition, adhesion, and invasion of the blastocyst into the uterus (Cross et al., 1994; McGowen et al., 2014). The uterus has to undergo enormous structural and molecular changes named 'decidualization' to become receptive during the mid-secretory phase (days 19–23) of the menstrual cycle, known as the 'window of implantation' (Lessey 2011). The blastocyst has to break through the epithelial lining of the uterus and damage the endometrial tissue to invade (**Figure 2**). Next, trophoblast cells need to remodel maternal spiral arteries in order to secure a sufficient placental-fetal blood supply. All these events produce a battlefield of invading, dying, and repairing cells. An inflammatory reaction is vital for implantation in order to provide the repair of the uterine epithelium and the removal of cellular debris. After implantation, the endometrium switches to an anti-inflammatory stage, which is required to prevent the rejection of the fetus (Mor et al., 2011).

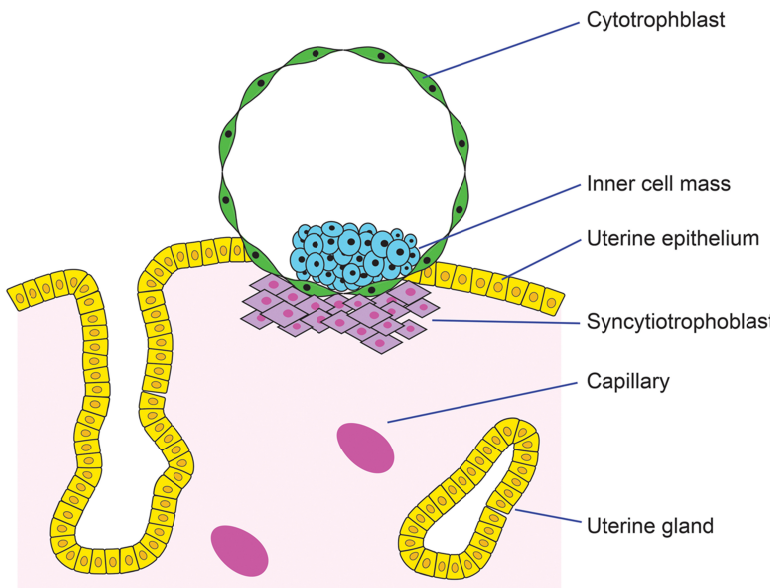


**Figure 2.** Implantation of blastocyst in the maternal endometrium. Embryo enters the uterus as a blastocyst ~5 days after fertilization, when the uterus is in the secretory phase. Implantation begins with receptors on cells of the outer embryonic trophoblast binding glycoprotein ligands on the endometrial epithelium. The syncytiotrophoblast invades the maternal endometrium to create a cavity into which the blastocyst can embed (modified from Giakoumelou et al., 2016).

The trophectoderm differentiates into two separate trophoblast cell types immediately prior to invasion into the decidua, while the differentiation of the inner cell mass, giving rise to the embryo proper, does not begin until the first placental structure is formed (Cross et al., 1994; Fitzgerald et al., 2008). The syncytiotrophoblast (non-mitotic mass of multinucleated cytoplasm) invades the maternal endometrium to create a cavity into which the blastocyst can embed (Baines and Renaud, 2017). Next, cytotrophoblasts (a layer of mitotically active trophoblast stem cells) provide a supply of invasive trophoblasts (extravillous cytotrophoblasts), while the syncytiotrophoblast acquires a more endocrinological task. Trophoblast cells drive the stages of implantation, modulating the maternal endocrine system to establish the hormonal milieu that guides changes in the uterus crucial for pregnancy maintenance (Cross et al., 1994; Mescher, 2013).

Defective decidualization can lead to adverse pregnancy phenotypes including defects in placentation, spontaneous abortion, intrauterine growth restriction, preterm birth and fetal death (Cha et al., 2012; Garrido-Gomez et al., 2017). For example, one of the causes of preeclampsia is considered to be shallow implantation due to poor trophoblast invasion into the decidua (Roberts and Gammill, 2005). On the other hand, if the placenta invades too deep it may cause placenta accreta (deep attachment to the uterine wall) or choriocarcinoma. All of these unfavorable outcomes of pregnancy compromise the lifelong health of the offspring as well as the mother.

## 1.2 Placenta as a key organ for pregnancy success

### 1.2.1 Placental development and function at the fetal maternal interface

Placental development is precocious, as it has to be ready and perform the role of all the major organs while these develop and mature in the fetus. Trophoblasts secrete over 100 peptide and steroid hormones that control the physiology of the mother, so that the fetus is supplied with oxygen and necessary nutrients needed for successful growth (Burton and Jauniaux, 2015). In addition, the placenta protects the fetus from maternal immunologic attack and removes waste products.

Trophoblasts, forming the external layer of the blastocyst, are the first cell lineage of the human placenta. Following successful implantation and initiation of placentation, undifferentiated cytotrophoblasts undergo extensive proliferation and differentiate through fusion (villous) or invasive (extravillous) lineage. The fusion lineage yields syncytiotrophoblast cells that form the outer epithelial layer of the chorionic villi, where the majority of the maternal-fetal exchange takes place (Gude et al., 2004). The invasive lineage gives rise to interstitial cytotrophoblasts. The cytotrophoblast stem cell first differentiates into extravillous cytotrophoblast intermediate and then into an interstitial cytotrophoblast, which in turn may differentiate further into endovascular cyto-

trophoblast or form a syncytium. Endovascular and interstitial cytotrophoblasts possess migratory and invasive properties, being able to recognize and modify the activity of other cell types at the feto-maternal interface (Silva and Serakides, 2016). The purpose of these cells is to invade and replace vascular endothelial cells in order to remodel uterine arteries to maximize blood flow and fulfil fetal oxygen and nutrient demands (**Figure 3**).

Fetal development and growth during the first trimester of pregnancy takes place in a physiologically low oxygen microenvironment that is stimulated by secretions from the endometrial glands that supply nutrients and growth factors (histiotrophic nutrition). By the end of the first trimester, onset of the maternal arterial circulation produces a three-fold rise in the intra-placental oxygen concentration (Burton et al., 2010). The maternal blood circulates in the intervillous space and bathes the villi, enabling exchanges with the fetal blood, but no direct contact between fetal and maternal blood occurs. The final structure of the human placenta is discoid, which is 2–3 cm thick at its center, up to 25 cm in diameter, and weighs 400–500 g (Lewis et al., 2013).

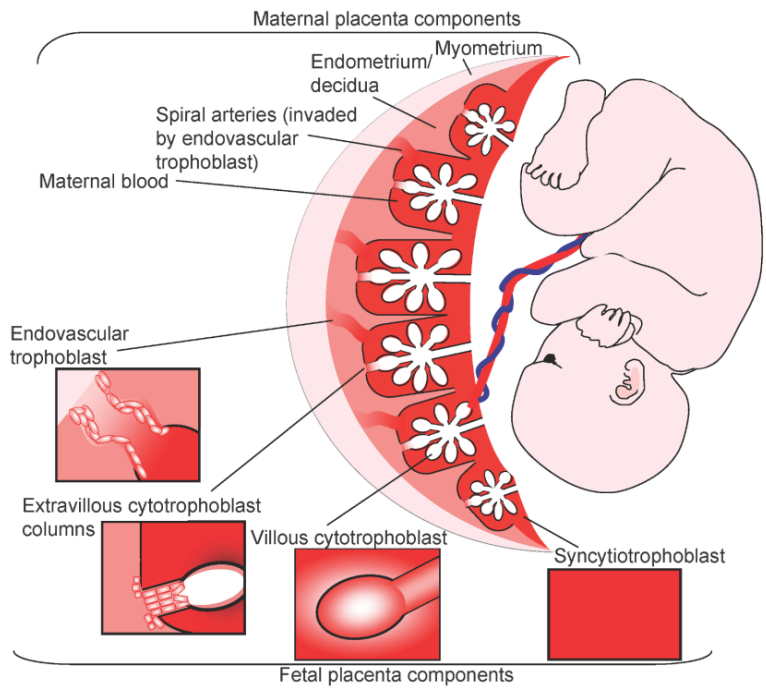


**Figure 3.** The human placenta and fetus. Villous trophoblasts of the human placenta grow as a branched structure in order to maximize exchange with maternal blood. Endovascular cytotrophoblasts arise from extravillous trophoblasts that invade into the maternal endometrium. Endovascular cytotrophoblasts colonize maternal spiral arteries to maximize blood flow through the placenta (modified from Frost and Moore, 2010).

### 1.2.2 Placenta as a unique mammalian organ

Placenta is one of the least understood organs, as it has evolved multiple times in different species in various shapes, structures, and even cell types while having the same basic role (Griffith and Wagner, 2017). Fundamentally, the trophoblast cells of the placenta perform two distinct tasks in all mammals – create a large surface area for nutrient exchange and interact with the uterus by producing hormones, growth factors, and cytokines that model maternal physiological systems for increased blood flow and nutrient delivery to the fetus and the placenta (Cross et al., 2003).

The evolution of the placenta in mice and humans has employed two unique evolutionary mechanisms. Ancient genes participating in growth and metabolic processes were co-opted for use in the course of early embryonic development, while recently duplicated genes are used during later stages of fetal development, taking on a specialized species specific placental function (Knox and Baker, 2008). For example, the mouse placenta is enriched for expression of rodent-specific genes, e.g. prolactin-related proteins (23 members), pregnancy-specific glycoproteins (17 genes), and carcinoembryonic antigen (CEA) family of glycoproteins (31 genes), whereas the human placenta is enriched for expression of primate-specific genes, e.g. CEA genes and pseudogenes (23 members), growth hormones (5 members), pregnancy-specific glycoproteins (10 genes), and gonadotropic glycoproteins (*LHB/CGB* cluster, 7 homologous genes) (Hallast and Laan, 2009; Carter, 2012; Roberts et al., 2016). These genes have enabled deep invasion of the endometrium and direct contact of the maternal blood with the trophoblast surface (hemochorial placentation) characteristic only for primates and rodents.

### 1.2.3 Mosaicism in the human placenta

Mosaicism, the existence of two or more distinct cell lines, is very common during the cleavage stage in human preimplantation development, occurring in ~15–90% of embryos (Taylor et al., 2014). It is also prevalent at the blastocyst stage; however, research has shown that 1–2% of viable pregnancies present with a chromosomally abnormal placenta but a normal fetus (Ledbetter et al., 1992; Grati et al., 2017). This suggests a selection mechanism against mosaicism in later stages of development. The fetus is derived from only a small subset (3 out of 64) of the blastocyst progenitor cells, the rest of the cells give rise to extraembryonic membranes (Bianchi et al., 1993). In addition, studies have shown that euploid cells proliferate at a higher rate than abnormal cells, which can be repelled from the fetal lineage (Taylor et al., 2014).

Nevertheless, chromosomal mosaicism is a challenging factor in prenatal diagnosis due to the complex prediction of fetal involvement. The clinical outcomes of mosaicism depend on several factors, including when exactly the error arises during development and whether it is confined to a particular area. Placental mosaicism occurs in normal pregnancies, but has been associated also

with adverse pregnancy outcomes including preeclampsia, intrauterine growth restriction, stillbirth, and recurrent pregnancy loss (Kalousek and Barrett, 1994; Yong et al., 2006; Warren and Silver, 2008; Robinson et al., 2010).

## 1.3 Recurrent pregnancy loss

Recurrent pregnancy loss (RPL) is defined as ≥2 consecutive losses before 24 weeks' gestation according to the latest European guideline (ESHRE, 2017). The definition used to be ≥3 consecutive losses, but the new guideline suggests that defining RPL as two or more pregnancy losses will facilitate research and psychological support to couples. The American Society for Reproductive Medicine already updated the definition to two or more consecutive pregnancy losses six years ago (ASRM, 2012).

Up to 15% of all clinically recognized pregnancies result in spontaneous abortion. Due to chance alone, RPL would occur in 0.34% of couples with three losses, whereas it is seen in 1–2% of couples trying to conceive (Bashiri et al., 2016). This discrepancy suggests a specific clinical cause for RPL.

What makes it extremely stressful for the couple is the fact that each subsequent pregnancy loss leads to higher risk of experiencing further losses, as the live birth rate in patients with three pregnancy losses is 70% vs. 50% in couples with 5–6 losses (Sugiura-Ogasawara et al., 2014). Maternal age is one of the most important factors determining prognosis for live birth, whereas the number of pregnancy losses often increases due to the fact that the following pregnancies occur at an advanced maternal age (Nybo Andersen et al., 2000; Sauer, 2015).

### 1.3.1 Known causes of RPL

Although a wide range of factors are known to increase the risk of RPL, including anatomic and endocrine factors, immune system dysfunction and chromosomal abnormalities, it is often difficult to determine a definite cause of this condition. Until today, the underlying cause remains undiagnosed in nearly 50% of cases based on the current clinical guidelines (Ford and Schust, 2009; Bashiri et al., 2016; Popescu et al., 2018).

Unlike spontaneous abortions that are mostly caused by chromosomal abnormalities, negative correlation has been detected between the occurrence of aneuploidies and the number of pregnancy losses (Ogasawara et al., 2000). However, abnormal embryonic karyotype was shown in 41% of cases with unknown cause of pregnancy loss in a recent study and it was suggested that the percentage of patients with RPL of truly unknown cause may actually not exceed 24.5% (**Figure 4**) (Sugiura-Ogasawara et al., 2012). Microarray testing of miscarriage tissue is currently not recommended by the American and European RPL guidelines, though a very recent prospective cohort study has shown that >90% of patients with RPL will have a cause identified when genetic testing on

pregnancy loss tissue is combined with standard evaluation (Popescu et al., 2018).

Antiphospholipid antibody syndrome (APS) and uterine anomalies are established non-genetic causes of RPL (Bashiri et al., 2016). Presence of antiphospholipid antibodies (aPL) has been associated with RPL for a long time. The prevalence of APS is expected to be 5–20% (El Hachem et al., 2017). aPL induce thrombosis, inhibit differentiation and invasion of the trophoblast, and may cause inflammation and immune response at the fetal-maternal interface as well as disrupt spiral artery remodeling (Di Simone et al., 2001; Bashiri et al., 2016). Errors in the endocrine system and anatomical abnormalities affect 10–20% of women with RPL. Occurrence of congenital as well as acquired uterine anomalies in RPL cases is about threefold higher compared to the general population (ASRM, 2012).

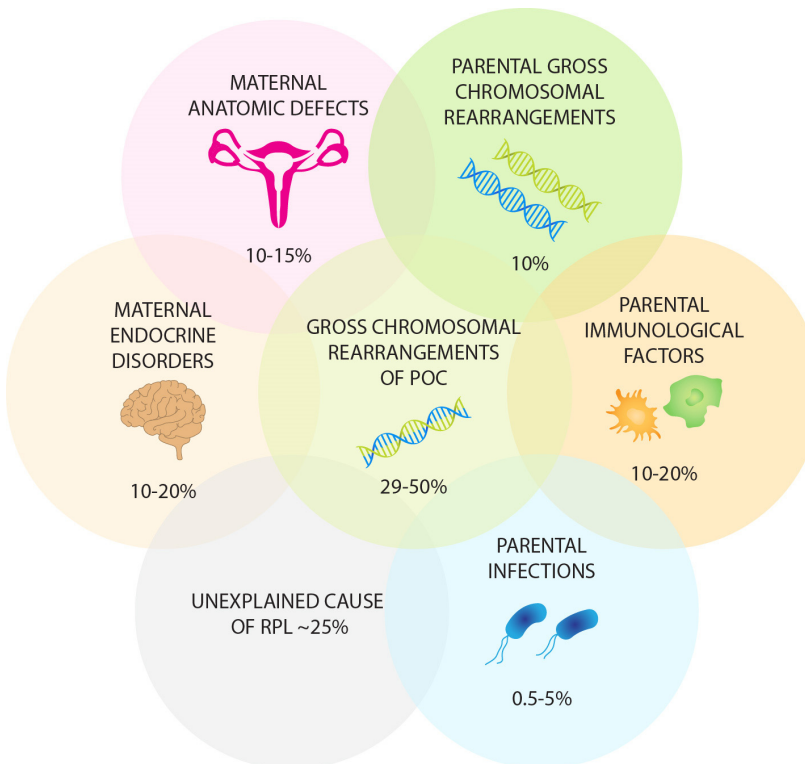


**Figure 4.** The etiology of recurrent pregnancy loss. Known parental causative factors for RPL include gross chromosomal rearrangements (aneuploidy, microdeletions/ duplications, translocations, extensive CNV load etc), infections and immunological factors in either of the partners, maternal endocrine disturbances and anatomical defects. An RPL couple is often diagnosed with multiple potential parental contributing factors and the sole causal factor cannot be assigned. Several parental risk factors may also predispose to the chromosomal disturbances of the POC. Of all RPL cases, approximately 25% remain truly unexplained (modified from Ref. IV).

It is not clear yet to what extent thrombophilia, infections and immune dysfunction as well as environmental factors and lifestyle contribute to RPL. The prevalence of risk factors among RPL couples varies to a great extent due to diagnostic criteria used in different clinics. In clinical practice, a typical RPL couple is diagnosed with various potential contributing factors simultaneously and the single causal factor cannot be identified. Popescu et al., 2018 reported 14% of women with two or more abnormal factors ascertained. Eventually, every etiology has its own prognosis and depends on whether it is treated or not.

### 1.3.2 Genetic factors causing RPL (also reviewed in Ref. IV)

The prevalence of RPL among first degree relatives of women with idiopathic RPL is sixfold higher than in controls, which points to the involvement of genetic factors (Christiansen et al., 1990). Kolte et al., 2011 have additionally shown a nearly twofold higher miscarriage rate among the siblings of patients with unexplained RPL compared to the general population.

Chromosomal abnormalities (mostly balanced translocations) account for 2–4% in either partner of an RPL couple (Ford and Schust, 2009). Although over two-thirds of the products of these couples' conceptions have abnormal karyotype (unbalanced translocations), the cumulative live birth rate exceeds 60% (Franssen et al., 2006). Preimplantation genetic diagnosis (PGD) may help to decrease time to live birth in a fraction of these RPL couples.

Hundreds of hypothesis based candidate gene studies regarding RPL have been carried out to pinpoint causative genes. Already more than 100 genes and nearly 500 variants have been reported in the scientific literature (Pereza et al., 2017; Rull et al., 2012; Shi et al., 2017). Most of the genes analyzed are involved in immune dysfunction and inflammation (e.g. *HLA, IFNG, TNF,* and *IL* genes), blood coagulation (e.g. *MTHFR, FII, FV*), placental development, and function (e.g. *NOS3, TP53, VEGFA*). However, none of the studied single nucleotide variants (SNV) have displayed sufficient diagnostic and prognostic value in the clinical management of couples with RPL. In addition, the recent RPL guideline does not suggest to screen for hereditary thrombophilia unless in women with additional risk factors for thrombophilia (ESHRE, 2017). Taking into account the multifactorial background of RPL, it can be assumed that no single gene/variant can be responsible for this condition.

The key to success in association studies is proper design. Most of the research has been done in women only, whereas embryonic development is a delicate and precise interplay between the maternal and fetal/placental genome as well as the paternal genetic component. Genetic variants have been investigated in male partners of RPL women in only about 12% of all studies (Pereza et al., 2017). In addition, there are differences in the definition of RPL and selection criteria for patients/controls between studies that may often produce controversial results (Rull et al., 2012).

## 1.4 Late pregnancy complications

Pregnancy is a stress test for the woman's body that may reveal a number of disorders that can lead to acute as well as chronic physiological consequences. Also, fetal development may be disturbed and, in case the neonate survives, the child may have an increased risk of different diseases throughout life. Late pregnancy complications can thus be divided into gestational complications of the mother (preeclampsia and gestational diabetes mellitus) or the newborn (growth abnormalities).

### 1.4.1 Preeclampsia

Preeclampsia (PE) is a uniquely human pregnancy-specific condition of placental origin affecting 2% of pregnancies in Estonia (according to Estonian Medical Birth Registry data in 2016) and 2–8% worldwide (Jeyabalan, 2013). It is traditionally diagnosed by maternal blood pressure greater than 140/90 mmHg and proteinuria in the second half of pregnancy (after 20 weeks' gestation) (Brown et al., 2001). All guidelines include gestational hypertension as part of the definition of PE; however, three out of eight national guidelines do not include proteinuria as a mandatory requirement in the definition of PE anymore (Bro Schmidt et al., 2017). Many factors have been associated with the risk to preeclampsia: chronic hypertension, renal diseases, obesity and insulin resistance, diabetes mellitus, pre-existing thrombophilia, family history of preeclampsia and smoking (Mol et al., 2016).

The only effective treatment of preeclampsia is delivery of the fetus and placenta which often leads to preterm and low birth weight babies. The initiation of early onset preeclampsia (<34 weeks) is associated with inappropriate vascular remodeling of the uterine spiral arteries due to shallow cytotrophoblast migration, which takes place during very early stages of pregnancy (**Figure 5**) (Roberts and Gammill, 2005), and has worse perinatal and maternal outcomes (Myatt and Roberts, 2015). While early onset PE is often complicated by intrauterine growth restriction and placental pathology, late onset PE is associated with predisposing maternal factors rather than placental origin (Steegers et al., 2010).

Despite the known risk factors and amount of research in this field, the etiology of preeclampsia is still poorly understood. Similar to RPL, daughters and sisters of preeclamptic women have a higher risk for developing hypertension during pregnancy. Although, heritability of preeclampsia has been estimated to be 30–55% (Boyd et al., 2013; Williams and Broughton Pipkin, 2011), knowledge about the exact genetic architecture is still limited. Genome-wide association studies (GWAS) have not found any maternal sequence variants of genome-wide significance replicated in independent samples (Johnson et al., 2012; Zhao et al., 2013a). However, a recent GWAS of offspring from PE pregnancies discovered the first genome-wide significant susceptibility locus near *FLT1* gene in 4,380 cases and >300,000 controls (McGinnis et al., 2017). The

strongest association was found in late-onset preeclampsia (LO-PE), whereas placental isoform of this protein (sFlt-1) is an established marker of early-onset PE (Staff et al., 2013). Various studies of placental RNA expression have also been conducted to find novel genes and possible biomarkers for PE (Brew et al., 2016; Kaartokallio et al., 2015; Tsang et al., 2017). The RNA-sequencing study on 40 human placentas from our group found that the transcriptome profile of late-onset PE placentas is distinct from normal pregnancies and other gestational complications, supporting the placental origin of this complex disease (Sõber et al., 2015).

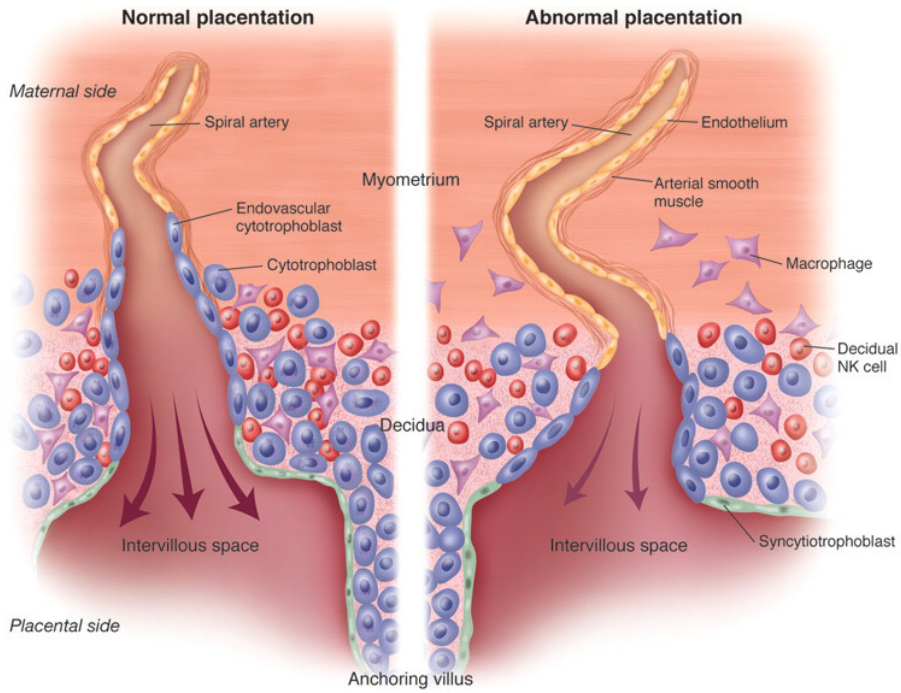


**Figure 5.** Normal and abnormal placentation (preeclampsia). The placenta is linked to the maternal decidua by anchoring villi. In normal placentation, cytotrophoblasts (blue) invade the maternal decidua and adjacent spiral arteries. They penetrate the walls of the arteries and replace part of the maternal endothelium (yellow). During normal pregnancy, immune cells facilitate deep invasion and promote extensive spiral artery remodeling. In the preclinical stage of preeclampsia, invasion is restricted with impaired arterial remodeling and lowered blood supply to the fetus (modified from Redman and Sargent, 2005).

### 1.4.2 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is the onset of glucose intolerance during pregnancy. It is a result of reduced maternal insulin sensitivity occurring during pregnancy that leads to higher amounts of blood glucose to pass through the placenta into the fetal circulation (Lain and Catalano, 2007). The prevalence of GDM in Estonia was 6.5% in 2016 (Estonian Medical Birth Registry) and varies from 1–16% worldwide due to numerous diagnostic criteria used in different countries (Kirss et al., 2015). Despite the criteria applied, GDM is increasing in prevalence across the world in parallel with the growing number of people with obesity and type 2 diabetes (Coustan, 2013).

GDM is diagnosed by glucose testing early in pregnancy to reveal diabetes, and again with a glucose tolerance test at 24–28 weeks' gestation (IADPSG, 2010). Major risk factors for developing GDM are a BMI >30 kg/m², a previous macrosomic baby (≥4.5 kg), previous gestational diabetes, a family history of diabetes, and a family minority ethnic origin with a high prevalence of diabetes (NICE Guideline, 2015). It is associated with adverse pregnancy outcomes, including fetal macrosomia as well as intrauterine growth restriction and stillbirth (Coustan, 2013).

Elevated delivery of maternal glucose may release signal molecules, e.g. elevated leptin levels, from the fetus and initiate methylation changes in the placenta (Moen et al., 2017). Several studies have suggested transgenerational transmission of gestational diabetes as well as other cardio-metabolic diseases later in life is due to epigenetic mechanisms, explaining the enormous increase in the prevalence of type 2 diabetes worldwide (Claesson et al., 2007; Vrachnis et al., 2012). As an example of fetal genotype impact on maternal metabolism, variation in paternal allele of the imprinted gene *IGF2* has been associated with increased IGF2 protein content in the placenta and maternal glucose levels (Petry et al., 2011). Several genetic association studies have been based on the assumption that the genetic architecture of GDM and type 2 diabetes is similar, but not many significant associations have been detected for GDM, mostly due to lack of power (Lowe et al., 2016). Future studies of larger sample sets are needed.

### 1.4.3 Fetal growth abnormalities

Normal fetal growth is a crucial component of a successful pregnancy and impacts the long term health of the offspring and the mother. There are two main types of fetal growth abnormalities: intrauterine growth restriction (IUGR) and macrosomia. Defined cut-offs of fetal growth below the $10^{th}$ and above the $90^{th}$ centiles or two standard deviations below/above the population norms on the growth charts are commonly used to define small-for-gestational age (SGA) and large-for-gestational age (LGA) fetuses (Anderson, 2005; Sildver et al., 2015). Diagnosis of fetal macrosomia is made if the fetus grows beyond a specific weight, usually 4.5 kg, irrespective of the gestational age (ACOG, 2000). IUGR

is defined on the basis of clinical features of malnutrition and *in utero* growth restriction, regardless of the birth weight percentile (Sharma et al., 2016).

The prevalence of IUGR and macrosomia in developed countries is 5–10% and 5–20% of all births, respectively (Lenoir-Wijnkoop et al., 2015; Tang et al., 2017). IUGR is the result of maternal, fetal, as well as placental and genetic factors (Sharma et al., 2016). Almost 50% of pregnancies complicated by maternal (gestational) diabetes result in LGA or macrosomic babies (Najafian and Cheraghi, 2012). Several risk factors for macrosomia, such as advanced maternal age and obesity, are also conversely associated with fetal growth restriction (Walsh and McAuliffe, 2012).

Currently, there are no methods or biomarkers to distinguish healthy SGA and LGA newborns from neonates who have growth disturbances as a result of pathological processes. It is known that imprinted genes have growth-related functions in mammals. Imprinted genes with enriched expression in the placenta (e.g. *IGF2*, *PEG1*, *PHLDA2*) have been shown to play a role in distributing maternal resources to the fetus (Tang et al., 2017). Differential placental gene expression in IUGR has been associated with nervous system development and cardiovascular system development and function, while in macrosomic infants the strongest association has been detected with nutritional disease, behavior, digestive system development, and lipid metabolism (Sabri et al., 2014). Four genes (*LEP*, *GCH1*, *LRP2*, and *CPXM2*) showed differential expression levels in both FGR and macrosomia. Higher expression of *LEP* was also detected in a recent transcriptome study of IUGR placental samples (Madeleneau et al., 2015). The genetic background is still obscure in these late pregnancy complications, thus genetic studies are needed in the field of fetal growth extremes.

## 1.5 Structural variation in the human genome

The DNA sequence of the human genome is constantly changing, enabling us to evolve and adapt. The genome sequence of two individuals has been found to differ about 1.6% (Pang et al., 2010). Over ten years ago, due to the advancement in technology, scientists started to identify an intermediate class of variation termed as structural variation (SV) (Iafrate et al., 2004; Sebat et al., 2004). This includes deletions, duplications, inversions, insertions, translocations, and complex genomic rearrangements. Every child is born with up to 9.2 *de novo* SVs (Acuna-Hidalgo et al., 2016). Structural variation has a larger impact on the function of the genome than single nucleotide variants (SNVs). It has been reported that while 14.6% of *de novo* SVs affect exons, only ~1.3% of *de novo* SNVs do (Kloosterman et al., 2015). However, the effect depends on the size, location, penetrance, and origin of the rearrangement, as well as number of disrupted genes critical to a certain phenotype.

### 1.5.1 DNA copy number variation

DNA copy number variation (CNV), involving submicroscopic deletions and duplications, accounts for the largest component of structural variation (**Figure 6**). CNVs are now defined as genomic segments of >50 bp present at a variable copy number in comparison to a reference genome.

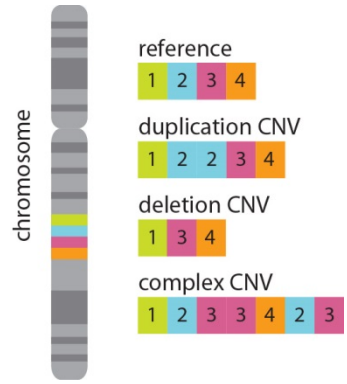


**Figure 6.** Different types of copy number variation (CNV). Relative duplication and deletion of the '2' locus compared with the reference genome is illustrated. Complex rearrangement of '2' and '3' loci is also shown.

A recently constructed CNV map estimated that 4.8–9.5% of the human genome contributes to CNV (Zarrei et al., 2015). The exons of all genes were reported to be more variable than the genome average, with exons of non-coding genes having the highest number of CNVs. However, exons of constrained genes, especially cancer-related and DECIPHER genes, were under strong negative selection. Deletions encompassing genes have been shown to be significantly rarer than intergenic deletions, whereas genic duplications show no such skew (Sudmant et al., 2015). In addition, deletions have been found to decrease in frequency as a function of size, suggesting that during human evolution selection has formed deletions and duplications in a different way (Sudmant et al., 2015; Zarrei et al., 2015).

CNVs can have no apparent phenotypic consequence in individuals or result in adaptive advantages. For example, the copy number of human salivary amylase gene is increased in populations that have higher starch content in their diets (Perry et al., 2007). A major challenge in clinical practice as well as scientific research is to differentiate benign CNVs from pathogenic CNVs contributing to diseases. Pathogenic rearrangements are associated with altered gene dosage and have been linked with numerous disorders, including developmental diseases (Lee and Scherer, 2010), autism (Pinto et al., 2010), schizophrenia (The International Schizophrenia Consortium, 2008; Sekar et al., 2016), diabetes (WTCC et al., 2010), autoimmune disorders (Olsson and Holmdahl,

2012), and cancer (Krepischi et al., 2012; Zhang et al., 2016). However, the consequence of a large number of rearrangements still remains unknown.

Besides existing in the germline, CNVs can arise *de novo* in certain cells and tissues, referred to as somatic genome rearrangements. For example, mammalian liver is a mixture of cells with gains and losses, helping it to adapt to metabolic and toxic challenges (Tang and Amon, 2013). Rather than being randomly present in the genome, CNVs are preferentially found in regions that are rich in low-copy repeats (segmental duplications), heterochromatic areas, and replication origins (Zarrei et al., 2015). They can arise by various mechanisms, including non-allelic homologous recombination, non-homologous end joining, or defects in DNA replication and repair (Hastings et al., 2009).

### 1.5.2 CNV detection methods

There are two approaches to detect CNVs: genome-wide and locus-specific. Each detection method has its strengths and shortcomings, and is typically used in certain applications. Comparison of different detection methods is given in **Table 1**.

Microarray is the main approach used to detect CNVs at the genome-wide scale. This is represented by aCGH (array comparative genomic hybridization) and SNP microarrays. aCGH uses two dyes to compare the DNA copy number between a target sample and a reference sample that are hybridized together on the same array (Pinkel et al., 1998). The signal intensity ratio (log ratio) is used as a proxy for copy number. On the other hand, SNP arrays hybridize only one sample to each array and compare the data *in silico* to a reference dataset. The ratio between the total signal and the reference signal produces copy number information similar to the intensity ratio from aCGH. The ratio between the two allele intensities yields a value for the genotype of the SNPs (**Figure 7**) (Alkan et al., 2011; Vandeweyer and Kooy, 2013). Next generation sequencing (NGS) has several advantages over microarrays by detecting very small rearrangements and defining exact breakpoints. However, despite the high resolution of new array-based and NGS methods, karyotyping still remains the primary method to detect balanced chromosomal rearrangements (translocations, inversions) (Vandeweyer and Kooy, 2013).

Locus-specific methods are commonly used to confirm CNVs that were detected by genome-wide methods. The simplest and most used method is quantitative real-time PCR (qPCR) that measures products generated during each PCR cycle, which are proportional to the amount of original template at the start of the PCR. Two basic chemistries are mostly used: hydrolysis probes (TaqMan®) and double-stranded DNA-intercalating dye SYBR® Green (Arya et al., 2005). Irrespective of the chemistry used, the increase in fluorescence emission during the PCR process is detected in real time by a thermocycler.

**Table 1.** Copy number variation detection methods[a]

| Method (Resolution) | Application | Advantages | Disadvantages |
|---|---|---|---|
| *Genome-wide* | | | |
| Karyotyping Microscope (>3Mb) | Genome-wide detection | Gold standard for aneuploidies<br>High specificity | Low resolution<br>High cost<br>Time-consuming<br>Chance of culture failure |
| aCGH (500 bp) | Genome-wide detection<br>Validation | High resolution<br>Quick results | Balanced rearrangements not detectable<br>Lack of probes in certain regions<br>Overestimation of breakpoints |
| SNP microarray (500 bp) | Genome-wide detection<br>Validation | High resolution<br>Quick results | Balanced rearrangements not detectable<br>Lack of probes in certain regions<br>Overestimation of breakpoints<br>Biased detection of deletions |
| WES, WGS (1 bp) | Genome-wide detection | Highest resolution<br>Detects mosaicism<br>Very low false positive rate<br>Accurate breakpoint estimation | Biased detection of deletions<br>High cost<br>Difficulty in detecting the low coverage copy number |
| *Locus-specific* | | | |
| FISH Microscope (200 kb) | Validation | Detects polyploidy and aneuploidy<br>Quick results | Limited resolution<br>Detects aberrations only where probes are designed |
| qPCR (200 bp) | Validation<br>Copy number quantification | Quick results<br>Simultaneous analysis of a large number of samples | Triplicates are required for each run<br>Challenging to design qPCR primers for some regions |
| MLPA (200 bp) | Validation<br>Copy number quantification | Low cost<br>Quick results<br>Simultaneous analysis of a large number of samples | Challenging to design primers for some regions |

[a]based on (Massaia and Xue, 2017; Zarrei et al., 2015; van den Berg et al., 2012; Vandeweyer and Kooy, 2013)

aCGH, Array comparative genomic hybridization; FISH, Fluorescence in situ hybridization; MLPA, Multiplex ligation-dependent probe amplification; qPCR, Quantitative real-time PCR; WES, Whole Exome Sequencing; WGS, Whole Genome Sequencing

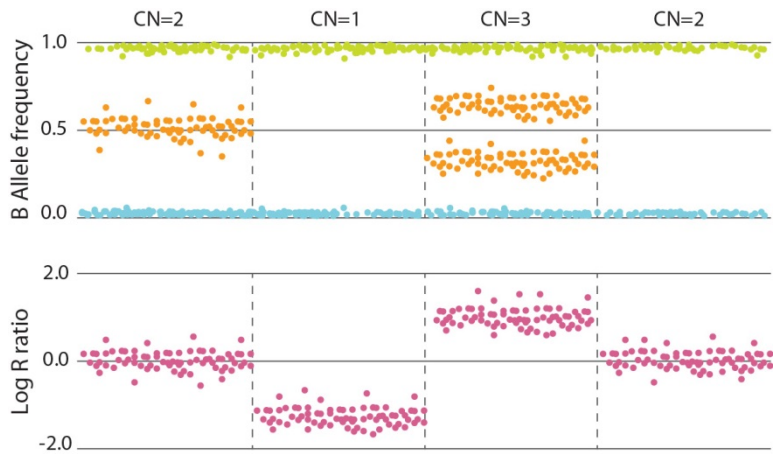


**Figure 7.** Examples of log R ratio (LRR) (lower panel) and B Allele Frequency (BAF) values (upper panel). Negative LRR (total probe intensity of a given SNP) indicates a region of DNA copy number loss (CN=1) and positive LRR shows a region of DNA copy number gain (CN=3). BAF is a normalized measure of the allelic intensity ratio of two alleles. A BAF of 0 represents the genotype (A/A or A/–), 0.5 represents (A/B) and 1 represents (B/B or B/–). The BAF also enables the detection of copy-neutral events, which result when a segment of one chromosome is replaced by the other allele without a change in copy number (LRR=0, BAF shows loss of heterozygotes).

At the moment there is no universal method to detect all structural variation in the human genome, as the detection depends largely on the platforms and also the various algorithms used, therefore multiple approaches are typically applied. Although, third generation sequencing technologies (e.g. PacBio single molecule sequencing) and novel algorithms have already been developed that may enable stand-alone assessment of CNVs (Kerkhof et al., 2017; Zhao et al., 2013b).

### 1.5.3 CNVs in pregnancy complications

The literature regarding the genome-wide profile of submicroscopic structural rearrangements in early as well as late pregnancy complications is limited. Only a few studies have been carried out in the past decade (**Table 2**).

Regarding the CNV profile of miscarried conceptuses from RPL cases, two low-resolution aCGH studies have been published. Rajcan-Separovic et al., 2010 identified 11 unique inherited rearrangements in the placental samples of RPL couples and highlighted two maternally imprinted genes (*CTNNA3* and *TIMP2*) disrupted by CNVs. A follow-up study showed that the duplication involving the *TIMP2* gene directly reduces its placental expression levels (Wen et al., 2015). A recent genomic analysis of 44 RPL patients (16 couples and 12

mothers) reported a positive correlation between the number of CNVs and increasing number of miscarriages (Karim et al., 2017).

A genome-wide study of structural rearrangements in a group of children born small-for-gestational age (SGA, n=51) identified 18 rare CNVs, out of which 8 were classified as pathogenic or probably pathogenic (Canton et al., 2014). These were all *de novo*, gene rich and of significant size, involving genes that function in cell growth, developmental process and cell cycle pathways. A parallel study by Wit et al., 2014 discovered 6 pathogenic or potentially pathogenic CNVs in 49 cases born SGA. A 2.5 Mb 22q11.2 deletion, known to be associated with short stature, was detected in both of these studies.

Concerning preeclampsia (PE), two genome-wide association studies have been published by one group (Zhao et al., 2013a; Zhao et al., 2012). In the first study, three rare recurrent CNVs, including a functionally relevant deletion in the *PSG11* gene, were discovered in 169 PE cases. Unfortunately, these deletions were not detected in the following study. Several candidate CNV regions were identified in three ethnic groups in the next study, but none overlapped among them. However, one candidate CNV (12p11.21) in European mothers and two (5p13.3 and 7q11.23) in Hispanic mothers were detected in their previous CNV study samples.

The first genome-wide study in placental samples of unexplained stillbirth (n=29) discovered 24 novel CNVs, out of which 8 were also confirmed in fetal samples (Harris et al., 2011). A 2.9 Mb heterozygous deletion on chromosome 9, resulting in the loss of 25 genes, was suggested as causative in one stillbirth. Another study that used FFPE (formalin-fixed paraffin-embedded) umbilical cord samples of stillborn and control placentas did not find stillbirth to be associated with the presence of CNVs (Ernst et al., 2015). Nevertheless, chronic placental inflammation was linked with the carrier status of case-specific CNVs (Ernst et al., 2015).

In conclusion, most of the studies have individually discovered causative or (potentially) pathogenic CNVs in early and late pregnancy complications, but not much overlap has been detected. This may be attributable to heterogeneity of the phenotypes, but also to geographic differences between the study samples, variable microarray probe coverage, different algorithms used, and absence of an appropriate control group. The detected CNVs are mostly rare events, large in size and heterozygous. Common recurrent (homozygous) rearrangements that confer risk for multiple pregnancy complications are still to be discovered.

**Table 2.** Published genome-wide CNV studies in pregnancy complications, not including the material handled in the current thesis

| Complication | Study subjects | No of cases/ controls | Platform | Main outcome | Reference |
|---|---|---|---|---|---|
| Preeclampsia | White mothers | 169/114 | Affymetrics Genome-Wide Human SNP Array 6.0 | Three rare recurrent deletions were found that may confer risk for PE, including a functionally relevant deletion in the *PSG11* gene. | (Zhao et al., 2012) |
| Preeclampsia | European ancestry mothers | 50/1,152 | Illumina Human610-Quad | Several candidate CNV regions were discovered in each of the three ethnic groups, but none were shared among them. | (Zhao et al., 2013a) |
| | Afro-Caribbean mothers | 21/962 | Illumina Human1M-Duo | | |
| | Hispanic mothers | 60/645 | Illumina Human1M-Duo | | |
| RPL | Placental samples | 27/DGV[a] | Agilent 105 K aCGH | Two rare inherited CNVs involving imprinted genes *TIMP2* and *CTNNA3* were associated with RPL. | (Rajcan-Separovic et al., 2010) |
| RPL | Couples, mothers | 44/DGV[a] | Agilent 400 K aCGH | Immune system related pathways were enriched in RPL patients. | (Karim et al., 2017) |
| SGA | Children | 51/400, DGV[a] | Agilent 60 K aCGH | Several rare CNVs were detected that involve genes, which may be essential for growth regulation. | (Canton et al., 2014) |
| SGA | Children | 49/DGV[a] | Affymetrix GeneChip | A known cause of short stature with prenatal onset was found in five cases. | (Wit et al., 2014) |
| Stillbirth | Placental samples | 29/10, DGV[a] | Illumina CNV370-Duo | In at least one case, a potentially causative deletion was detected. | (Harris et al., 2011) |
| Stillbirth | FFPE umbilical cord samples | 86/8, DGV[a] | Illumina HumanOmniExpress | Validation of the usability of FFPE umbilical cord samples for genome-wide CNV analysis. | (Ernst et al., 2015) |

[a] Database of Genomic Variants (DGV) was applied as a reference dataset in addition to control samples if available
[b] Only deletions were prioritized
aCGH, Array comparative genomic hybridization; RPL, Recurrent pregnancy loss; SGA, Small-for-gestational age; FFPE, Formalin-fixed paraffin-embedded

# 2. AIMS OF THE STUDY

The general aim of the present thesis was to elucidate the role of DNA copy number variants (CNVs) in human placental and parental genomes of normal gestations, early and late pregnancy complications. The specific aims of my thesis were:

I. to address the role of parental CNVs in predisposing to recurrent pregnancy loss (RPL) and to identify new loci and functional pathways implicated in early pregnancy maintenance;

II. to compare the CNV profiles of placental samples to respective parental genomes across normal gestation and cases of complicated pregnancies at term;

III. to compare the profile of submicroscopic genomic rearrangements in placental chorionic villi of RPL cases to healthy first trimester gestations.

# 3. RESULTS AND DISCUSSION

## 3.1 DNA copy number variation in recurrent pregnancy loss parental genomes (Ref. I, III)

At the beginning of this project, only one study had been published that investigated the role of copy number variants (CNVs) in miscarriage samples of couples with recurrent pregnancy loss (RPL). Thus, these are the first high resolution genome-wide studies of CNVs in RPL parental genomes.

### 3.1.1 Study design

Ref. I is a case-control study that included altogether 558 idiopathic RPL patients with ≥3 consecutive pregnancy losses from Estonia (80 female and 39 male partners, recruited by K. Rull at the Women's Clinic of Tartu University Hospital) and Denmark (229 female and 210 male; recruited by O. B. Christiansen at the Fertility Clinics, Rigshospitalet, Copenhagen University Hospital). For all the recruited cases known clinical risk factors had been excluded. The control group consisted of 205 fertile women (90 from Estonia and 115 from Denmark) with ≥3 live births and no history of miscarriages before recruitment. In the discovery phase, a subset of Estonian cases (n=43) and controls (n=27) were genotyped with Illumina Human370CNV-Quad array (>370,000 markers). CNV calling was performed in parallel with two algorithms, QuantiSNP and PennCNV (Colella et al., 2007; Wang et al., 2007). TaqMan qPCR was used for experimental validation of prioritized CNVRs and replication using the extended Estonian and Danish sample sets (**Figure 8**; Figure 1 in Ref. I).

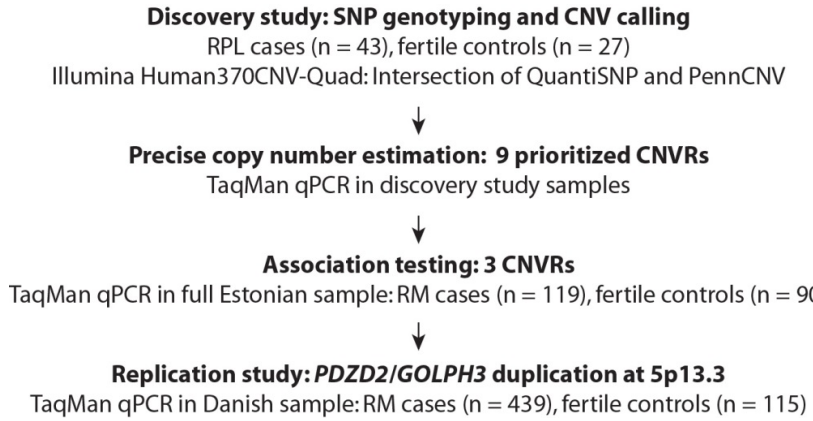


**Figure 8.** Study design. Initial CNV discovery was performed in a subsample of Estonian subjects. Association testing of three CNVRs and replication study of one region was conducted in extended Estonian and Danish sample sets.

Ref. III is an RPL family-based study (mother-father-placenta trios or mother-placenta duos) that included 25 patients with idiopathic RPL (9 couples and 7 female patients) and 13 placental samples (including 10 pregnancy losses and 3 live births). Control datasets representing normal 1st trimester (8 females and 9 placentas) and term pregnancies (8 mother-father-placenta trios) were derived from Ref. II. Study subjects were recruited by K. Rull at the Women's Clinic of Tartu University Hospital. All samples were genotyped with Illumina Human-OmniExpress Beadchips (>700,000 markers) and three algorithms [QuantiSNP (Colella et al., 2007), GADA (Pique-Regi et al., 2010) and CNstream (Alonso et al., 2010)] were applied for CNV calling. CNVs called by at least two algorithms for the same individual in the same genomic region were included in the subsequent analysis.

### 3.1.2 Increased burden of CNVs in the genomes of RPL patients

In order to define the genome-wide profile of CNVs in a subset of Estonian patients with RPL (n=43) and fertile controls (n=27), genome-wide SNP arrays were applied (Ref. I). The discovery patient group included 16 male and 27 female idiopathic RPL patients. A total of 423 non-overlapping CNV regions (CNVRs) were detected in RPL patients and controls (Suppl. Table S3 in Ref. I). RPL cases carried an average of 13.3 CNVs per individual compared to 12.6 in controls.

As RPL is known for its multifactorial etiology (Ref. IV), a case-by-case CNV profile analysis was applied to assess the individual-specific risk. This revealed two outlier cases with more than fivefold increased cumulative span of CNVs (6.1 Mb and 5.4 Mb vs. median of 1.0 Mb in the rest of RPL cases and 1.2 Mb in controls) (**Figure 9A**). In addition, a load of large (≥100 kb) deletions involving numerous genes was detected in RPL patients compared to fertile women (Fisher's exact test, OR=1.60, *P*=0.0266) with four patients having heterozygous loss of over 70 genes (**Figure 9B**). The average number of large deletions per individual was 2.2 for the RPL cases, compared to 1.4 for the fertile controls, and the mean number of disrupted genes in these CNV regions was 18.2 and 6.2, respectively. The genes disrupted by CNVs in the outlier cases are involved in immune function (*IGH* gene cluster at 14q32.33), male reproductive function (*SOHLH1*, *TMEM203*) and some have previously been associated with RPL (*C4A*, *C4B*, *IGF2*). Functional pathway analysis of genes affected by CNVs in all RPL cases (excluding the two outlier cases with high CNV burden) and fertile controls revealed enrichment of immune signaling pathways specifically among RPL patients (Table 1 in Ref. I).

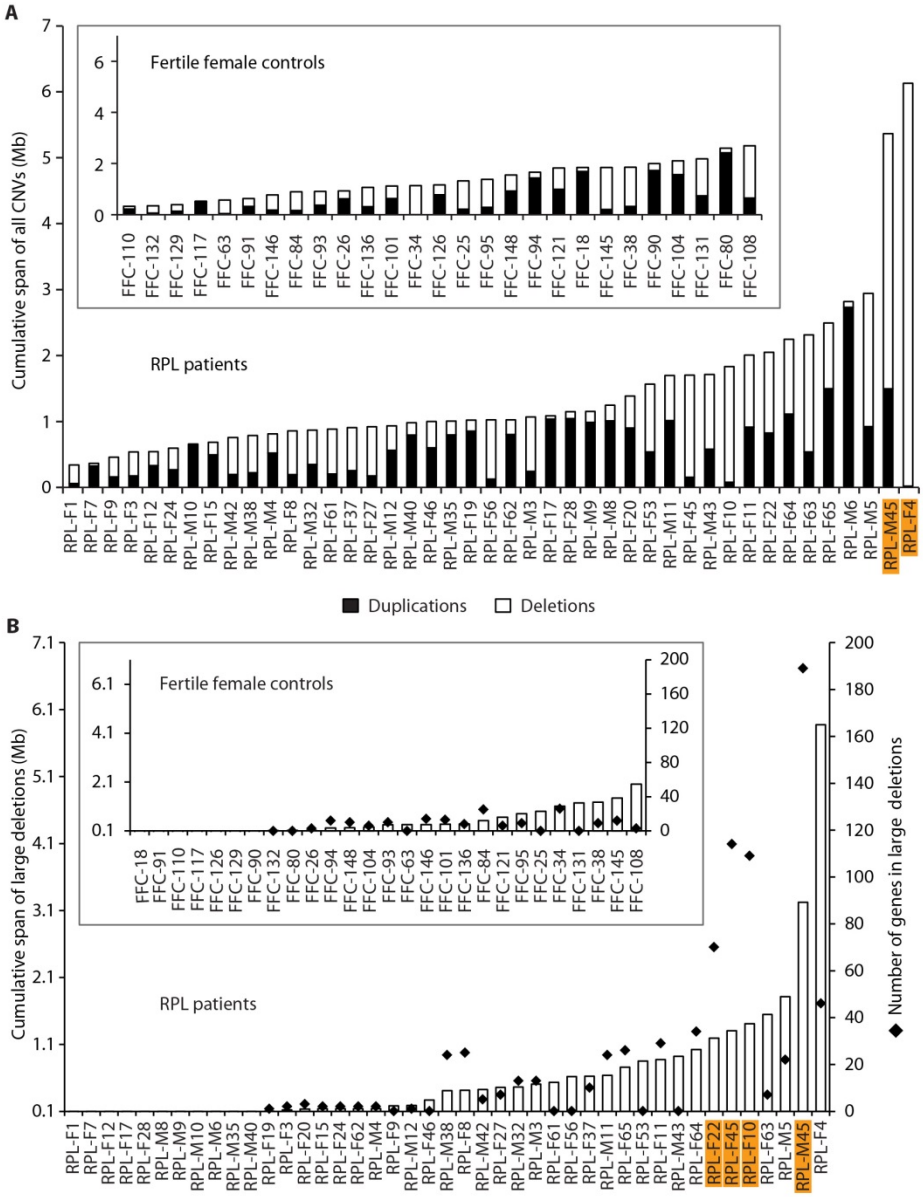


**Figure 9.** Genomic burden of all CNVs and the subgroup of large (≥100 kb) deletions in the Estonian discovery sample set. (A) Cumulative span of deletions and duplications per individual among the discovery phase Estonian RPL cases (n=43) and fertile controls (n=27). Outlier cases with increased cumulative burden of CNVs are shown in orange. (B) Cumulative span of large deletions and the number of disrupted genes per individual. Outlier cases with increased number of genes disrupted by large deletions are shown in orange. Female and male patients with identical number-codes represent couples (e.g. RPL-F45 and RPL-M45). FFC, fertile female control; RPL-F, female RPL patient; RPL-M, male RPL patient.

Our following genome-wide profiling of CNVs in 25 idiopathic RPL patients and 24 controls (Ref. III) also revealed a 1.3-fold higher number of >100 kb long CNVs per genome in RPL cases compared to controls (**Table 3**). However, higher excess of even larger rearrangements (>300 kb) was detected in RPL cases by comparison with the previous study. Two-thirds of these >300 kb CNVs were pericentromeric or subtelomeric rearrangements (Table 4 and Suppl. Figure S3 in Ref. III). One male patient carried a 0.5 Mb pericentromeric microduplication at 15q11.2, between recurrent breakpoints (BP1-BP2) for chromosomal rearrangements. This family showed varying reproductive history including numerous miscarriages, one healthy child, and one offspring with severe developmental delay and intellectual disability. This CNV shows incomplete penetrance as other phenotypically normal carriers have been identified; however, 40% of the 15q11.2 BP1-BP2 microduplication carriers suffer from delayed development and speech, neuro-behavioral problems, and autism (Burnside et al., 2011; Chaste et al., 2014).

**Table 3.** Comparison of the number of large CNVs between RPL cases and controls in Ref. I and Ref. III

| CNVs per genome | Case/Control ratio Ref. I | Case/Control ratio Ref. III |
|---|---|---|
| All | 1.06 | 0.97 |
| >100 kb | **1.31** | **1.35** |
| >300 kb | **1.44** | **2.00** |

A subgroup of patients may display an increased risk of RPL attributable to excessive genomic burden of CNVs. Large rearrangements, especially in pericentromeric and subtelomeric regions, may affect correct chromosome pairing in mitosis and meiosis, leading to overall genomic instability which prevents establishment of a successful pregnancy. In addition to failed pregnancies, such parental genomes ('unfavorable genomes') may cause developmental delay or future health problems in their live born offspring (Khoury and Erickson, 1993). CNVs rearranging the repertoire of presented antigens may affect the immune tolerance at the fetomaternal interface, as has been also reported in a later study of RPL patients (Karim et al., 2017). If anything goes wrong with the immune response, there is a higher risk of (recurrent) pregnancy loss (Grimstad and Krieg, 2016; Ref. IV).

### 3.1.3 Experimental validation and replication of recurrent CNVs

In order to identify common CNVs that increase the risk to RPL, nine CNVRs (**Table 4**; Suppl. Table S4 in Ref. I) were prioritized from the genome-wide screening results under the following criteria: CNVR i) is present in >1 individual, ii) is only represented in RPL cases or overrepresented in patients with

odds ratio (OR) ≥1.5, iii) overlaps or is located in the vicinity (up to 200 kb) of biologically relevant candidate genes. TaqMan qPCR method was used for validation of the microarray results in the discovery sample set (n=70). Three CNVRs (*IGKV* at 2p11.2, *DKK2* at 4q25 and *PDZD2:GOLPH3* at 5p13.3) that showed precise results with TaqMan copy number assays were tested in the full Estonian RPL case-control sample set (119 cases and 90 controls). *SEPT14* CNVR was not included in the next stage due to restricted testis-specific expression of this gene (The Human Protein Atlas). Five selected regions were validated by TaqMan qPCR as copy number variable (1p36.33, 6p21.33, 8p22, 12p13.31, 14q32.33), but the precise copy number estimation per each patient was hampered due to complex genomic architecture.

**Table 4.** Prioritized CNVRs subjected to experimental validation using TaqMan qPCR in the discovery sample set. Association testing in the full Estonian sample set was performed with CNVRs in bold.

| Chr | Start; End (hg38) | Length (kb) | Type | Genes in the CNV region | Carriers | |
|---|---|---|---|---|---|---|
| | | | | | Cases n=43 | Controls n=27 |
| 1p36.33 | 1284756, 1334343 | 49.6 | Del | *SCNN1D*, *ACAP3*, *PUSL1*, *CPSF3L*, *GLTPD1*, *TAS1R3* | 4 | 0 |
| **2p11.2** | **90055411, 90081309** | **25.9** | **Del/ Dup** | ***IGKV* region** | **5** | **1** |
| **4q25** | **107144581, 107152053** | **7.5** | **Del** | ***DKK2*** | **4** | **0** |
| **5p13.3** | **32106978, 32159411** | **52.4** | **Dup** | ***PDZD2, GOLPH3*** | **3** | **0** |
| 6p21.33 | 32038826, 32039119 | 0.3 | Del/ Dup | *CYP21A2* | 4 | 1 |
| 7p11.2 | 55798750, 55838203 | 39.5 | Del/ Dup | *SEPT14* | 2 | 0 |
| 8p22 | 16405000, 16415383 | 10.4 | Del | Intergenic; 212 kb 5'of *MSR1* | 5 | 2 |
| 12p13.31 | 6134743, 6138398 | 3.7 | Del | Intergenic; 10 kb 5' of *VWF* | 3 | 1 |
| 14q32.33 | 105562232, 105646379 | 84.2 | Del | *IGHA2, IGHE, IGHG4, IGHG4* | 3 | 0 |

Chr, chromosome; Del, deletion; Dupl, duplication

The strongest effect in the full Estonian RPL sample set was observed for *PDZD2:GOLPH3* duplication (OR=7.28) with an increased prevalence among RPL patients compared to controls (7.6% vs. 1.1%, respectively) (Suppl. Table S7 in Ref. I) and was further selected for a replication study in Danish RPL cases and fertile controls (n=554). The other two CNVRs (*IGKV* locus, del/dupl and *DKK2* locus, del) were not studied further as they exhibited minimal

differences in carrier frequencies in the full Estonian case-control sample set, having minor if any effect on the RPL phenotype in our study.

The results of the replication study confirmed the higher duplication carrier frequency in RPL patients compared to controls (4.3% vs. 1.7%, respectively). Notably, comparison with fertile women revealed a stronger effect in Danish female patients with a similar prevalence as in Estonian cases (6.6% vs. 7.6%, respectively) (**Table 5, Figure 10**). Statistically significant association of the *PDZD2:GOLPH3* duplication with an increased risk for maternal RPL (OR=4.82, *P*=0.012) was detected in the meta-analysis combining the results of the Estonian and Danish female patient-control samples (in total, 309 cases and 205 controls). The difference in the duplication prevalence in Danish compared to Estonian men could be caused by the difference in sample size (249 vs. 39 men, respectively).

**Table 5.** Association analysis of the *PDZD2:GOLPH3* CNV at 5p13.3 with recurrent pregnancy loss in Estonia and Denmark. Statistically significant association is marked in bold.

| Controls | No of subjects/ carriers (%) | Cases | No of subjects/ carriers (%) | *P*-value |
|---|---|---|---|---|
| *Full Estonian sample set* | | | | |
| Fertile women | 90/1 (1.1) | All | 119/9 (7.6) | 0.062 |
| | | Men | 39/3 (7.7) | 0.087 |
| | | Women | 80/6 (7.5) | 0.070 |
| *Replication study: Danish RPL case-control sample* | | | | |
| Fertile women | 115/2 (1.7) | All | 439/19 (4.3) | 0.211 |
| | | Men | 210/4 (1.9) | 0.916 |
| | | Women | 229/15 (6.6) | 0.071 |
| *Meta-analysis: Estonian and Danish RPL cases and fertile controls* | | | | |
| Fertile women | 205/3 (1.5) | All | 558/27 (4.8) | **0.036** |
| | | Men | 249/7 (2.8) | 0.268 |
| | | Women | 309/21 (6.8) | **0.012** |

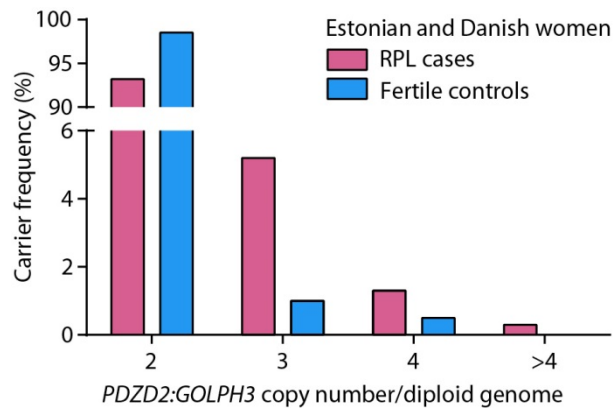


**Figure 10.** Copy number distribution and carrier frequency of the *PDZD2:GOLPH3* duplication at 5p13.3 among Estonian and Danish female RPL patients (n=309) compared to fertile controls (n=205). CNV carriers have 3 to >4 copies per genome.

Supporting this finding, a genome-wide association study of maternal CNVs in preeclampsia (PE) was published shortly before (Zhao et al., 2013a), identifying a statistically significant association with a 50.4 kb *PDZD2*:*GOLPH3* duplication and risk for PE (OR=4.80, *P*=0.011). The carrier frequencies of this duplication were similar to the current RPL case-control study (**Table 5**) with 8.3% in PE patients (n=60) compared to 1.6–1.9% in controls (n=645). Preeclampsia and recurrent pregnancy loss have been shown to share similar etiological factors, and have been linked with disturbed early placental development (Germain et al., 2007; Trogstad et al., 2009). In addition, the combination of fetal and maternal genotypes that increases the risk of PE (Hiby et al., 2004) has been shown to increase the risk of RPL as well (Hiby et al., 2008).

### 3.1.4 Genomic context of the *PDZD2:GOLPH3* duplication and expression profile of the disrupted genes

Experimental fine-scale mapping of the 5p13.3 duplication confirmed the extent of the CNV from the last intron of the *PDZD2* to the first intron of the *GOLPH3* gene, adjusting the size to 61.6 kb (compared to 52.4 kb based on the microarray estimation) (**Figure 11A**). The flanking breakpoints of the CNV were characterized by various repetitive elements; however, there was no extensive DNA sequence similarity between the proximal and distal repeat elements (Suppl. Figure S7 in Ref. I). Only a microhomology of one nucleotide was detected at the junction of the duplication endpoints, suggesting a CNV formation mechanism other than nonallelic homologous recombination (Hastings et al., 2009).

Expression analysis of *PDZD2* and *GOLPH3* in human tissue cDNA panels revealed the highest expression in placenta for both genes (**Figure 11B**). *GOLPH3* was expressed in all tissues with high levels also in prostate and ovary. On the other hand, the level of *PDZD2* transcripts was very low in most tissues. These results highlight the importance of these genes in human placental/reproductive function.

Interestingly, a homozygous missense variant in *PDZD2* has been recently associated with caudal regression syndrome, which is attributed to abnormal early fetal development of the lower spine (Porsch et al., 2016). Amplification and overexpression of *PDZD2* as well as *GOLPH3* has been reported in diverse human cancers (Gamwell et al., 2013; Scott et al., 2009; Zeng et al., 2012; Tan et al., 2014; Xi et al., 2016). Importantly, statistically significant correlation has been shown between *GOLPH3* expression level and copy number status (Scott et al., 2009). GOLPH3 activates the mammalian target of rapamycin (mTOR), which controls cell growth, proliferation, and survival through various signaling pathways (Wullschleger et al., 2006). Disturbances in mTOR signaling have been linked with several female as well as male reproductive disorders in mice and human, including RPL (Jesus et al., 2017; Roos et al., 2007; Rosario et al., 2017; Zhang et al., 2017; Vatin et al., 2012; Wen et al., 2005; Xiang et al.,

2015), therefore providing the potential functional link between the duplication of *GOLPH3* and development of RPL.

Although the *PDZD2:GOLPH3* CNV does not duplicate the complete coding regions of these genes, the local genomic architecture is modified and may physically impair the access of important transcription factors. It may result in alternative transcripts and lead to changes in the expression of the involved genes as well as genes located in their vicinity (Gamazon and Stranger, 2015; Henrichsen et al., 2009). This needs to be clarified in further functional studies.

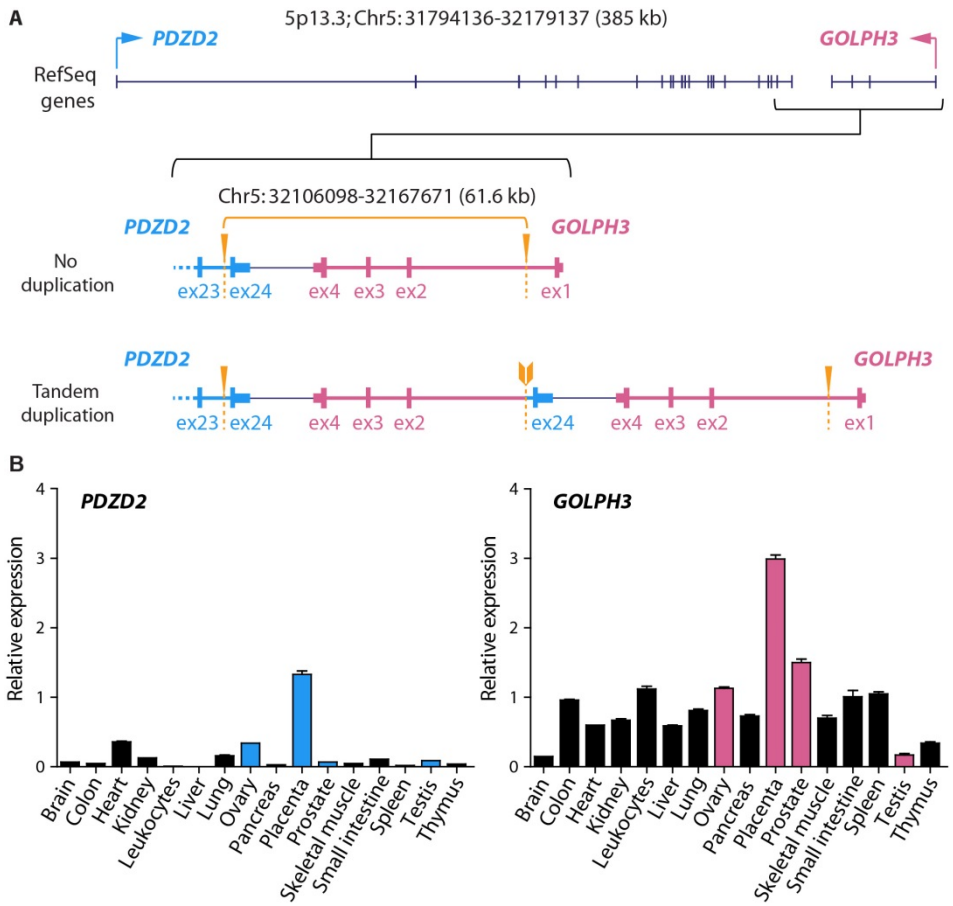


**Figure 11.** (A) Schematic illustration of 5p13.3 CNV locus with or without the tandem duplication (coordinates according to hg38). The opposite transcription of the *PDZD2* and *GOLPH3* genes is indicated with blue and pink arrows, respectively. Experimentally confirmed duplication endpoints are indicated with orange arrowheads and dotted lines. The breakpoint junction of the tandem duplication is marked with orange arrow tail. (B) Gene expression profile of *PDZD2* and *GOLPH3* in the human cDNA tissue panels. Expression level is provided relative to the reference gene *HPRT* and as an average of three amplification reactions ± SEM. Gene expression levels in reproductive tissues are highlighted with colored bars.

### 3.1.5 Major contribution of this study to the field of RPL research

1. This is the first high-resolution genome-wide study of CNVs in women with RPL and their partners.
2. Functional enrichment analysis of genes disrupted by CNVs in RPL cases compared to controls confirmed the importance of immune tolerance in early pregnancy maintenance.
3. Large cumulative burden of CNVs in specific individuals and excess of large deletions may confer risk to RPL due to the disruption of key genes essential in early development, but also increasing predisposition to further genomic rearrangements during gametogenesis and division of cells in the early embryo.
4. As a major outcome, a common duplication was discovered at 5p13.3 as a risk factor for RPL in two North European populations, Estonia and Denmark. The CNV involves two genes, *PDZD2* and *GOLPH3*, highly expressed in the placenta and associated with pregnancy loss for the first time. Importantly, this duplication may be a pleiotropic risk factor in the genetic etiology of multiple pregnancy complications.

## 3.2 Submicroscopic genomic rearrangements in the placental and parental genomes of normal and complicated pregnancies (Ref. II)

### 3.2.1 Study design

Genome-wide analysis of autosomal submicroscopic rearrangements was conducted in 38 family trios comprised of placental and parental blood DNA, 17 placenta-mother duos and two singleton placental samples (Figure 1 and Table 1 in Ref. II). The placental samples included all three trimesters of normal gestations and term cases of late pregnancy complications [preeclampsia (PE), gestational diabetes mellitus (GDM), small-for-gestational age (SGA), and large-for-gestational age newborns (LGA)]. CNV calling was performed by three algorithms [QuantiSNP (Colella et al., 2007), GADA (Pique-Regi et al., 2010) and CNstream (Alonso et al., 2010)] based on the genome-wide genotyping dataset (Illumina HumanOmniExpress). CNVs called by at least two algorithms for the same individual in the same genomic region were included in the subsequent analysis. Functional enrichment analysis of somatic and inherited placental CNVs was performed with g:Profiler software (Reimand et al., 2007).

### 3.2.2 Extensive load of CNVs in the placental genomes

All placental samples regardless of the trimester or complication group exhibited enrichment in the number of CNVs compared to the respective parental samples (Figure 2A and Suppl. Tables 1–4 in Ref. II). A threefold excess in the number of CNVs (mean no of CNVs per genome: 28.5 vs. 9.5, $P<2.2 \times 10^{-16}$) and nearly sixfold higher amount of duplications (15.3 vs. 2.7) was detected in the placental genomes compared to the parental blood DNA (**Table 6**). The greatest difference in the number of CNVs, especially duplications, occurs between the 1st and 2nd trimesters of normal pregnancy (mean no of duplications/placenta: 10.9 vs. 27.9, respectively; $P$=0.002) (**Figure 12**; Suppl. Table 1 in Ref. II).

Interestingly, placental genome duplications were shorter (mean 130.8 vs. 183.8 kb, respectively; $P>0.05$) and deletions longer (117.6 vs. 49.8 kb, $P<2.2 \times 10^{-16}$) compared to the parental genome. The average loss/gain ratio in the placental samples was 0.9 which is a strong deviation from the expected 2–3-fold excess of deletions compared to duplications based on previous reports on other human tissues (Conrad et al., 2010; Redon et al., 2006; Ref. I).

**Table 6.** Comparative profile of placental and parental CNVs.

| | | Placental DNA (n=57) | Parental blood DNA (n=93) | Placental/ Parental genome ratio |
|---|---|---|---|---|
| Number of CNVs per sample | All | 28.5 (23.0) | 9.5 (9.0) | 3.0 (2.6) |
| | Dupl | 15.3 (12.0) | 2.7 (3.0) | 5.7 (4.0) |
| | Del | 13.2 (9.0) | 6.8 (7.0) | 1.9 (1.3) |
| Cumulative span of all CNVs per sample, Mb | All | 3.6 (2.4) | 0.8 (0.6) | 4.5 (4.0) |
| | Dupl | 2.0 (1.4) | 0.5 (0.3) | 4.0 (4.7) |
| | Del | 1.5 (0.7) | 0.3 (0.3) | 5.0 (2.3) |
| CNV length across samples, kb | All | 124.7 (70.4) | 87.8 (23.0) | 1.4 (3.1) |
| | Dupl | 130.8 (74.9) | 183.8 (68.3) | 0.7 (1.1) |
| | Del | 117.6 (63.8) | 49.8 (16.5) | 2.4 (3.9) |
| Ratio (loss/gain) | | 0.9 (0.8) | 2.5 (2.3) | 0.4 (0.3) |

Data are given as means (medians). All, pooled duplication and deletion CNVs.

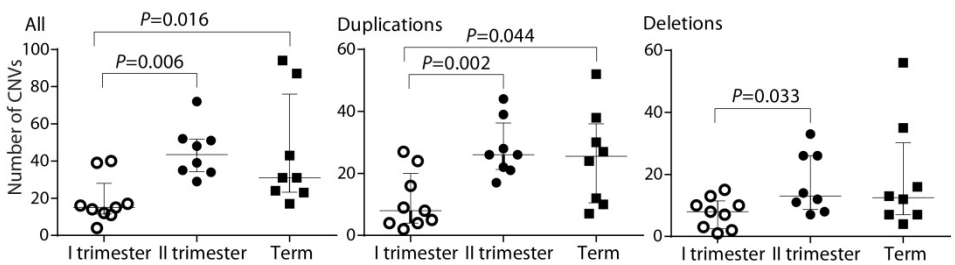


**Figure 12.** Gradient of increasing number of CNVs from the 1st trimester towards term pregnancy. *P*-values were calculated by Welch two-sample t-test/Wilcoxon rank sum test. Error bars show medians with interquartile range.

To confirm the excessive amount of CNVs in the placental genomes, two placental DNA samples were sent to a commercial service provider (Atlas Biolabs GmbH; Berlin, Germany) for external validation using an alternative array comparative genomic hybridization (aCGH) platform. aCGH confirmed the high load of genomic rearrangements in these samples and validated 56% (53/94) and 49% (28/57) of all CNVs identified by the SNP array (Suppl. Figure 2 in Ref. II). The dominant factor affecting array performance is probe distribution (Haraksingh et al., 2011) (in our study 715,000 vs. 400,000 markers; 2.1 vs. 5.3 kb spacing) and implication of stringent criteria for the CNV calling in our study (intersection of three independent algorithms) might have further reduced the proportion of CNVs to be validated using aCGH. In addition, different from SNP-array based analysis, CNV detection by aCGH is based on the comparison of the investigated sample with the reference DNA. In order to avoid any bias and to confirm robustness, the utilized reference genomic DNA in our study was an anonymous DNA sample provided by the company. However, aCGH is unable to detect CNVs carried by the reference DNA. Also somatic mosaicism may have affected the validation rate.

The limitation of the current study was that the investigated whole-placental material did not allow equivocal determination; whether the existence of somatic CNVs is a common feature to the entire organ or it is specific to only certain placental cell types. However, as the analyzed first-trimester samples (n=8) in this study represented purified chorionic villi containing cyto- and syncytiotrophoblasts, the data confirms the presence of somatic genomic rearrangements in these, solely placenta-specific cell types.

The CNV profile of maternal (n=55) and paternal (n=38) DNA samples was similar in the mean number (9.5 and 9.3, respectively), cumulative span (both 0.8 Mb), and mean loss/gain ratio (2.9, 2.1) of CNVs per genome (Suppl. Table 4 in Ref. II).

### 3.2.3 Profile of inherited and somatic placental CNVs

The CNV dataset for 38 family trios including term placental and maternal-paternal blood DNA samples enabled dissection of CNVs into inherited and somatic (not identified in either of the parental genomes) placental genome rearrangements. Majority of the placental CNVs were classified as somatic (n=944, 88.9%) and the rest had been equally transmitted from the mother and the father (n=118, 11.1%; **Figure 13A**). The inherited CNV profile was similar to the parental genome rearrangements (**Figure 13B**); however, the somatic deletions were significantly longer (mean 144.6 vs. 63.9 kb; $P=3.81 \times 10^{-6}$) and duplications shorter (mean 125.0 vs. 194.1 kb; $P=4.81 \times 10^{-5}$) compared to the size distribution of inherited CNVs (**Figure 13C**). These results are controversial to published data in other human tissues, where large duplications are better tolerated in the human genome than deletions (Itsara et al., 2009; Zarrei et al., 2015).

CNV regions (CNVRs) were distributed all over the genome (Suppl. Figure 3 in Ref. II); however, there were differences in the fraction of somatic and inherited CNVRs among chromosomes (**Figure 14**). The highest proportion of variable sequence (up to 5.26%) was found in chromosomes 17 (hotspot for deletions) and 14 (hotspot for duplications) on the somatic CNV map and chromosomes 11 and 19 (up to 0.91%) on the inherited CNV map. The lowest/null proportion was in chromosome 21 for both somatic and inherited maps, chromosome 20 was also void of inherited structural variation. For duplications, chromosomes 21 and 17 showed the lowest proportion of variability (0.17%) in the somatic map. On the inherited map of duplications, the highest proportion was found in chromosomes 18, 10 and 4, while chromosomes 1, 14, 16, 19–22 showed no variation. For deletions, chromosomes 11 and 19 showed the highest proportion of variable sequence (up to 0.9%) in the inherited map. Zarrei *et al*., 2015 also reported the highest proportion in chromosome 19 for losses.

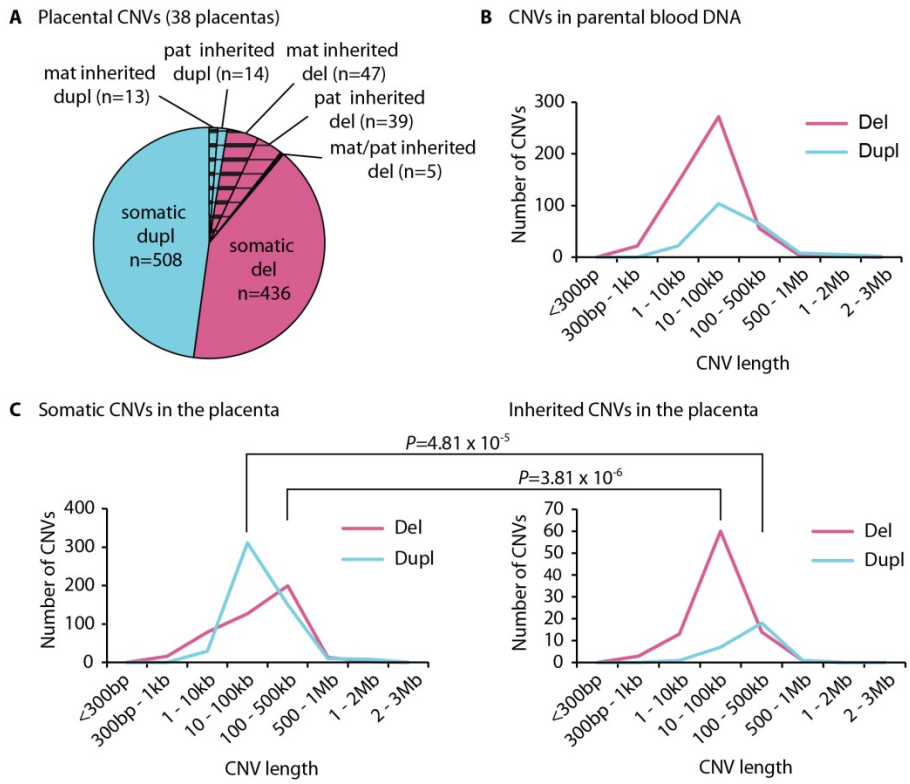


**Figure 13.** Distribution of placental and parental CNVs. (A) Distribution of somatic and inherited CNVs in the placental genome. (B) Length distribution of parental blood CNVs. (C) Length distribution of placental CNVs. *P*-values were calculated by Wilcoxon rank sum test. Dupl, duplications; del, deletions; mat, maternal; pat, paternal.

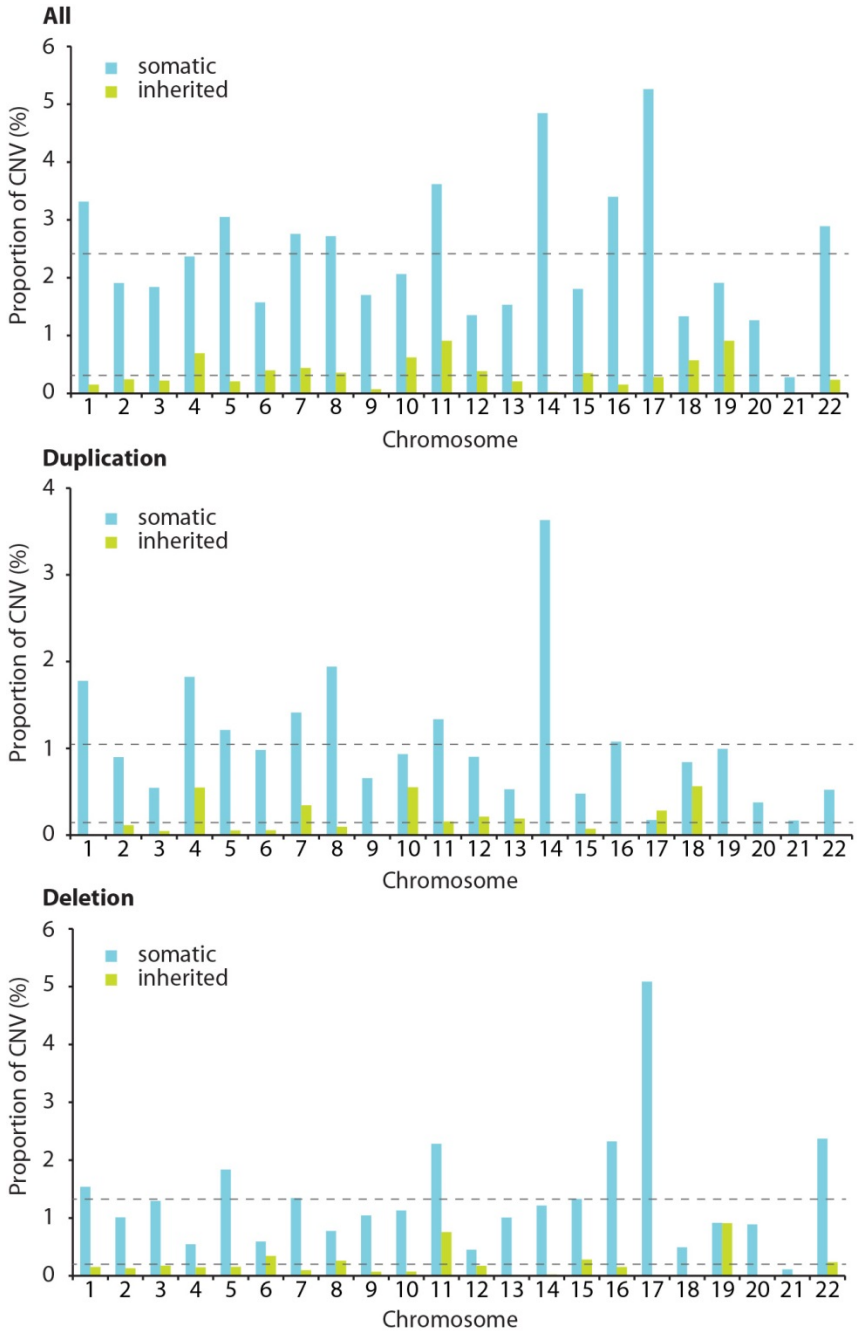


**Figure 14.** Proportion of each chromosome that is copy number variable based on the somatic and inherited placental CNV maps. The horizontal dashed lines mark the genome average for the somatic (upper) and inherited (lower) CNVRs. The y axes denote the percentage of nucleotides of each chromosome that are involved in CNVRs.

Functional enrichment analysis of somatic and inherited placental CNVs was performed in order to find out whether the rearrangements are random or cluster in specific biological pathways. Among the somatic duplications, the results highlighted enrichment of genes involved in biologically relevant pathways for fetal development (Table 3 and Suppl. Table 5 in Ref. II), including 'anterior/ posterior pattern specification' (GO:0009952, 10.4% of genes rearranged in the pathway, $P$=1.34 × $10^{-5}$), 'homophilic cell adhesion' (GO:0007156, 12.2% of genes, $P$=3.48 × $10^{-5}$), and 'embryonic skeletal system development' (GO:0048706, 12.3% of genes, $P$=2.33 × $10^{-4}$). Additionally, enrichment was detected for genes targeted by microRNA mir-210 (34 genes, $P$=3.90 × $10^{-3}$), upregulated in preeclamptic placentas (Pineles et al., 2007; Zhu et al., 2009). miR-210 has been associated with hypoxia, angiogenesis, trophoblast migration, and invasion, as well as immune response (reviewed by Bounds et al., 2017).

Somatic deletions were enriched for pathways related to cellular processes, such as 'cell cycle process' (GO:0022402, 5.2% of genes, $P$=3.49 × $10^{-5}$), 'microtubule-based process' (GO:0007017, 6.4% of genes, $P$=1.87 × $10^{-3}$), and 'mitosis' (GO:0007067, 7.1% of genes, $P$=3.04 × $10^{-3}$). Sher *et al*. have described reduced expression of mitotic genes in murine trophoblast giant cells compared to diploid control embryonic cells (Sher et al., 2013). Deletions disrupting genes involved in cell cycle could repress mitosis in certain cell types of the human placenta and promote endocycle. Endoreduplication, a cell cycle consisting only of synthesis and gap phases, to the ploidy level of 4–8 copies of DNA has been reported in extravillous trophoblasts enabling rapid growth and differentiation (Biron-Shental et al., 2012).

On the other hand, the inherited placental CNVs and parental blood CNVs represented mostly common polymorphic loci involved in 'olfactory receptor activity', 'amylase activity', and 'female pregnancy' (Table 3 and Suppl. Table 5 in Ref. II).

### 3.2.4 Enrichment of imprinted genes in somatic duplications

By screening the Geneimprint database (http://www.geneimprint.com) and a published list of imprinted loci (Court et al., 2014), I detected a significant enrichment of imprinted genes in somatic placental duplications (21/1,180 duplicated genes vs. 214/57,952 Ensembl genes; $P$=2.67 × $10^{-13}$). The majority of these disrupted genes are maternally expressed and essential in placental and embryonic development (**Table 7**). As an example, *CTNNA3* (duplicated in five placental samples) controls the proliferative versus invasive state of trophoblasts in the course of placentation (Tyberghein et al., 2012). Further, *PRDM16* gene, which is a regulator of postnatal growth and maintenance of brown adipose tissue (Harms et al., 2014), was disrupted in two LGA placental samples.

Placenta is known for its unique epigenetic profile, which is strikingly similar to cancer genomes (Smith et al., 2017), including the overall lower level of DNA methylation compared to embryonic tissues (Hamada et al., 2016).

Aberrant imprinting in the human placenta has been linked with disturbed embryonic development leading to pregnancy loss, preeclampsia, intrauterine growth restriction, gestational diabetes, and imprinting disorders (Jacob et al., 2013; Moen et al., 2017; Robinson and Price, 2015). However, the possible role of CNVs in the regulation of imprinted genes in the placenta is still unknown. I propose that evolution towards parent-of-origin-specific gene expression in the placenta may have emerged through silencing of one parental copy or by generating extra gene copies through somatic duplication on the other parental chromosome.

**Table 7.** Imprinted genes disrupted by placental CNVs. Data modified from http://www.geneimprint.com/; * from Court et al. 2014.

| Gene | Location | Status | Expressed Allele | No of carriers | Groups |
|---|---|---|---|---|---|
| *Genes disrupted by somatic duplication CNVs* | | | | | |
| *ALDH1L1* | 3q21.3 *AS* | Predicted | Maternal | 3 | SGA, GD, norm |
| *CTNNA3* | 10q22.2 *AS* | Provisional Data | Maternal | 5 | 2 GD, 1 PE, SGA, norm |
| *CYP1B1* | 2p21 *AS* | Predicted | Paternal | 3 | 2 PE, 1 GD |
| *DVL1* | 1p36 *AS* | Predicted | Maternal | 1 | LGA |
| *FGFRL1* | 4p16 | Predicted | Maternal | 1 | norm |
| *HOXA11* | 7p15-p14 *AS* | Predicted | Maternal | 1 | norm |
| *HOXA2* | 7p15-p14 *AS* | Predicted | Maternal | 1 | norm |
| *HOXA3* | 7p15-p14 *AS* | Predicted | Maternal | 1 | norm |
| *HOXA4* | 7p15-p14 *AS* | Predicted | Maternal | 1 | norm |
| *HOXA5* | 7p15-p14 *AS* | Predicted | Maternal | 1 | norm |
| *HOXC4* | 12q13.3 | Predicted | Maternal | 1 | norm |
| *HOXC9* | 12q13.3 | Predicted | Maternal | 1 | norm |
| *KCNQ1* | 11p15.5 | Imprinted | Maternal | 1 | norm |
| *KCNQ1OT1* | 11p15 | Imprinted | Paternal | 1 | norm |
| *NTM* | 11q25 | Imprinted | Maternal | 1 | SGA |
| *PEX10* | 1p36.32 *AS* | Predicted | Maternal | 2 | LGA |
| *PKP3* | 11p15 | Predicted | Maternal | 1 | norm |
| *PRDM16* | 1p36.23-p33 | Predicted | Paternal | 2 | LGA |
| *SLC22A3* | 6q26-q27 | Imprinted | Maternal | 6 | 2 PE, norm, 1 LGA, GD |
| *TP73* | 1p36.3 | Imprinted | Maternal | 2 | 1 LGA, norm |
| *WDR27* | 6q27 | Provisional Data* | Paternal | 1 | SGA |

SGA, small-for-gestational age; GD, gestational diabetes; norm, normal term; PE, preeclampsia; LGA, large-for-gestational age.

### 3.2.5 Reduced amount of somatic duplications in late pregnancy complications

Placental genomes from normal term pregnancies carried on average 25.0 duplications per sample, whereas placentas representing fetal or maternal pregnancy complications exhibited a significant reduction, having on average only 11.0 amplifications per genome ($P$=7.83 × $10^{-3}$, Figures 2, 4a in Ref. II). SGA group had the lowest mean number and cumulative span of CNVs (mean number of CNVs/span per genome: 18.3/1.5 Mb vs. 43.6/5.7 Mb in normal term). There was no statistical difference in the number of deletions between normal and complicated term pregnancies.

Functional enrichment analysis of group specific somatic placental CNVs was performed to see whether there are any biological pathways enriched in each term pregnancy group. Placental samples from uncomplicated pregnancies confirmed significant enrichment of duplicated genes in biological pathways involved in normal embryonic development (**Figure 15**; Suppl. Table 5C in Ref. II). Enrichment of duplicated genes regulated by mir-615-5p was detected in the LGA group (Suppl. Table 5D in Ref. II). Supportive to our finding, a recent study showed downregulation of mir-615-5p in livers from offspring from high-calorie diet fed dams (Zheng et al., 2016). In addition, two groups have suggested this miRNA to be involved in the regulation of lipid and energy metabolism (Miyamoto et al., 2014; Siengdee et al., 2015). Maternal high-calorie diet predisposes offspring to a larger body weight, insulin resistance, and impaired glucose homeostasis (Parlee and MacDougald, 2014). In our study, mean BMI of mothers in the LGA group was barely in the normal range (24.8) and mean paternal BMI was barely within the overweight range (29.9).

Genes in somatic duplications specific to the GDM group clustered in the functional category ‘low-density lipoprotein receptor activity’ (GO:0005041, $P$=2.87 × $10^{-2}$) (**Figure 15**; Suppl. Table 5 in Ref. II). One of the disrupted genes, *CD36*, was lately shown to be upregulated in the placentas of women with high pre-pregnancy BMI as well as GDM (Segura et al., 2017). The methylation level of the second duplicated gene in this pathway, *LRP1B*, has been demonstrated to be decreased in cord blood and in the placenta in response to maternal glycaemia (Houde et al., 2015). Differential expression of the genes involved in this functional category seems to play an important role in the pathogenesis of gestational diabetes.

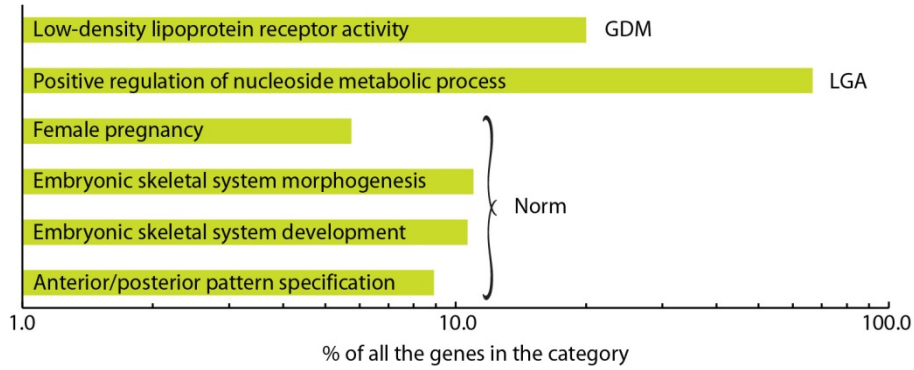


**Figure 15.** Functional categories displaying significant (FDR<0.05) enrichment among the genes underlying the placental somatic duplications and involving >5% of genes in the pathway (shown in log scale). GDM, gestational diabetes mellitus; LGA, large-for-gestational age; norm, normal term.

### 3.2.6 Older mothers have reduced amount of placental CNVs

The number of CNVs was assessed in normal term placentas (n=8) in connection with maternal factors. Although the numbers are small, some interesting correlations were found that are worth mentioning. I found negative correlation between the number of placental CNVs and maternal age at delivery (Spearman's rank correlation, rho=-0.86, *P*=0.007) (**Figure 16**). Young women (<20 years old) had around 100 CNVs per term placental genome, whereas middle-aged women (30–40 years old) had 3–4 fold lower numbers of CNVs. The correlation of maternal age with the number of duplications was enhanced (rho=-0.87, *P*=0.005), whereas there was no significant effect on the number of deletions. A similar significant trend was observed when the number of CNVs was tested in correlation with the number of successful pregnancies (rho=-0.92, *P*=0.001) (**Figure 16**). However, as the middle-age women also tend to have had higher number of deliveries, the current dataset does not make it possible to conclude whether the reduced generation of somatic placental duplications is due to aging per se, or due to previously remodeled uterine tissue in experienced pregnancies that enables effective trophoblast invasion without the support of extensive somatic duplications of respective genes.

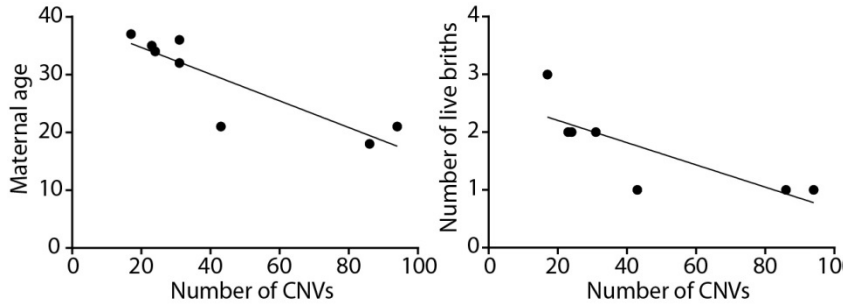


**Figure 16.** Correlation with maternal factors between the number of CNVs and (A) maternal age at birth in normal term pregnancy; (B) maternal lifetime cumulative number of live births (including index pregnancy).

### 3.2.7 Major contribution of this study to the field of placental research

1. This is the first report on genome-wide CNV profiles of human placental samples representing three trimesters of normal pregnancies and late pregnancy complications, including mother-father-placenta trios.
2. The major finding is the load of somatic placental CNVs, especially duplications, in normal pregnancies that are enriched for genes involved in pathways relevant to embryonic development.
3. Late gestational complications may arise during the burst of somatic genome rearrangements due to sporadic unfavorable rearrangements involving genes critical in gestational metabolism.
4. Enrichment of imprinted genes in somatic placental duplications suggests an additional level of gene expression regulation that needs to be clarified in future studies.

## 3.3 DNA copy number variation in recurrent pregnancy loss placental genomes (Ref. III)

The CNV map of placental genomes from late pregnancy complications differed from normal term gestations in the number of CNVs, foremost duplications (Ref. II). Thus, I proceeded to perform a genome-wide analysis of submicroscopic genomic rearrangements in recurrent pregnancy loss (RPL) placental samples under the hypothesis that their CNV profile is even further altered compared to normal pregnancies. The study design is described in chapter 3.1.1.

### 3.3.1 Reduced amount of rearrangements and low fraction of shared CNVs in the placental samples of RPL cases compared to normal pregnancy

Placental genomes of RPL cases showed >45% reduction in the mean number of CNVs compared to normal 1st trimester gestations (mean number of CNVs/genome: 10.0 vs. 18.6; Wilcoxon rank sum test, $P$=0.02; Figure 1A and Table 1 in Ref. III). The substantial difference in the cumulative span of CNVs was caused by 3–10-fold decrease in the number of duplications in the RPL placental samples compared to controls. As mentioned above, among term complications the lowest mean number of placental CNVs was identified in the small-for-gestational age group; however, in the RPL placentas it was almost twofold further reduced (18.3 vs. 10.0 CNVs, respectively; **Figure 17**). These results point to a common aspect of all pregnancy complications – lack of somatic structural variation in the placental genome that may impair early trophoblast development. Surprisingly, three live birth placental samples from RPL couples also carried a limited amount of CNVs like pregnancy loss samples (**Figure 17**).

Placental genomic rearrangements were clustered into CNV regions (CNVRs, at least two rearrangements of the same type (deletion or duplication) overlapping with 40% coverage) and RPL samples were compared with normal 1st trimester and term pregnancy groups. Normal 1st trimester placental samples shared 35.1% (n=46/131) of CNVRs with term placentas, while only 5.3% (n=7/131) of rearrangements coincided with RPL samples (Fisher's exact test, $P$=1.1 × $10^{-9}$; Figure 1B in Ref. III).

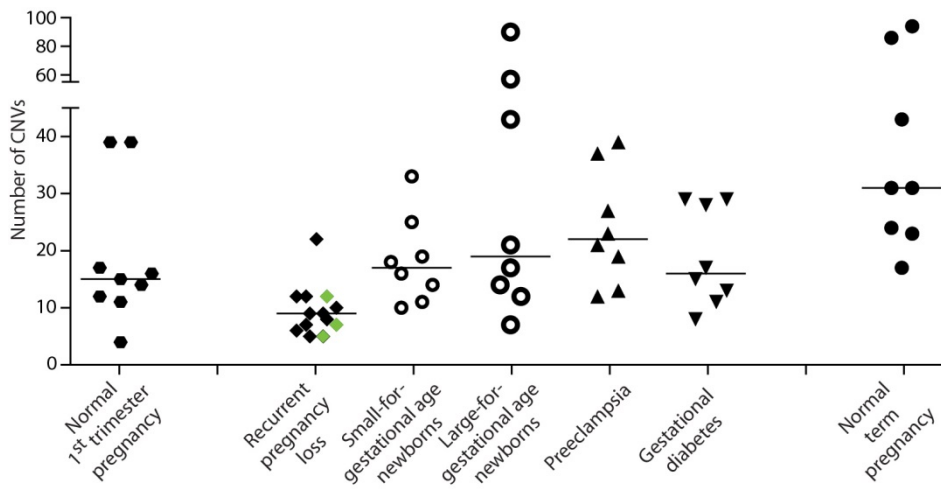


**Figure 17.** Number of all autosomal CNVs in the placental samples of RPL cases compared to controls representing normal 1st trimester, normal term pregnancy and late pregnancy complications. Placentas from RPL couples eventually succeeding in a term live birth after a successful pregnancy are indicated with green diamonds.

Interestingly, 8 CNVRs that were shared or encompassed the same gene between RPL and control samples were alternatively rearranged in pregnancy loss placentas (Table 2 in Ref. III). For example, two control placentas carried duplications involving the whole *MTRR* (methionine synthase reductase) gene, but the duplication in RPL placenta disrupted the gene (Figure 2 in Ref. III). MTRR is essential for utilization of methyl groups from the folate cycle to activate methionine synthase, necessary for methionine synthesis (Shane and Stokstad, 1985) and further generation of S-adenosylmethionine (SAM). SAM acts as a methyl donor for loads of cellular substrates, including DNA, RNA and proteins (Kusakabe et al., 2015). *Mtrr* deficiency has been shown to have transgenerational effect through epigenetic markers, causing growth defects and congenital malformations in mice (Padmanabhan et al., 2013). The folate-methionine cycle certainly has an important role in early embryonic development. Three control placental genomes carried CNVs in *CTNNA3* gene, which were distinct from the maternally inherited deletion identified in the RPL placenta (Table 2 in Ref. III). As described above, a maternally transmitted deletion at the same locus was detected in an independent study of recurrent miscarriage samples (Figure 2 in Ref. III) (Rajcan-Separovic et al., 2010). In our previous study of late pregnancy complications, I detected no deletions, but somatic duplications in two GDM, one PE, and one SGA placenta (Ref. II, **Table 7**). Further confirming its role in early fetal development, a fetus with syndromic malformations was recently reported to carry 180 kb microdeletion in the *CTNNA3* gene (Cancemi et al., 2016). *CTNNA3* is highly expressed in the placenta during the first trimester and shows cell-type specific genomic imprinting with biallelic expression in extravillous trophoblast and monoallelic expression of the maternal allele in villous trophoblast (van Dijk et al., 2004).

### 3.3.2 Functional pathway analysis of placental CNVRs specific to RPL cases and controls

Functional enrichment analysis of genes located within the CNVRs unique to RPL placental samples revealed enrichment of only polymorphic olfactory receptor loci (Suppl. Table S3 in Ref. III). However, analysis of the CNVRs specific to controls highlighted enrichment of binding sites for multiple transcription factors (TF) relevant to placental and fetal development (**Figure 18**). Over 70% of the query genes (n=630) showed binding motifs for ZF5 and E2F transcription factors. Supportive to this finding, another study from our group found that almost two-thirds of differentially expressed genes in RPL placental samples compared to controls have binding sites for E2F transcription factors (Sõber et al., 2016).

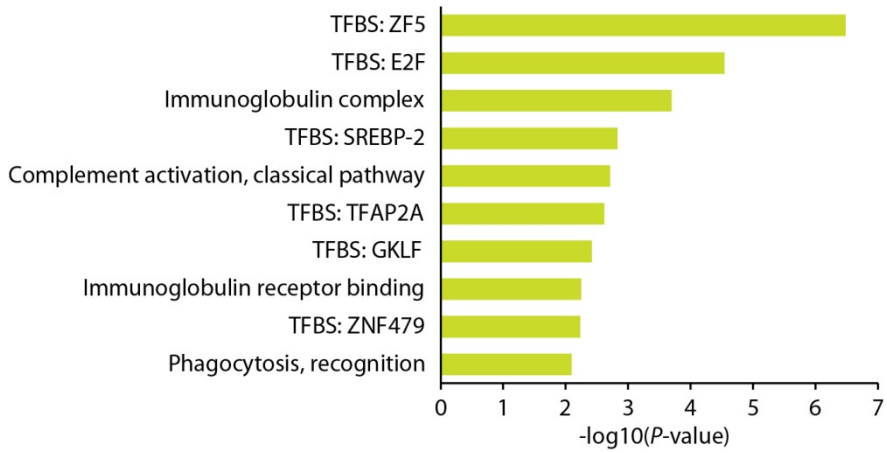


**Figure 18.** Functional enrichment analysis of genes within the CNVRs exclusively detected in the placental genomes of normal 1st trimester and term pregnancies. TFBS, transcription factor binding site.

Not much is known about the function of ZF5, but E2F activator and repressor proteins orchestrate the mammalian endoreplication process which refers to multiple genome amplifications in the absence of cell division/cytokinesis (Chen et al., 2012). Mice embryos deficient of *E2f7/E2f8* have been shown to display overall smaller placentas and severely compromised tissue architecture, and also fetal growth retardation and midgestation lethality (Ouseph et al., 2012). Furthermore, loss of atypical E2Fs (*E2F7/8*) causes severe vascular defects during embryonic development in zebrafish and mice via transcriptional regulation of *VEGFA* (Weijts et al., 2012), indicating an important role in angiogenesis. Strikingly, the same group discovered that loss of these E2Fs in cancer cells actually promotes angiogenesis and branching of tumor blood vessels (Weijts et al., 2017). *TFAP2A*, the regulator of placental *CGB* expression (Glodek et al., 2014), has been recently proposed as one of the earliest drivers of trophoblast specification in human embryonic stem cells (Krendl et al., 2017). Additionally, GKLF has been reported as a potential regulator of Pregnancy-specific glycoproteins (PSGs) (Blanchon et al., 2001), the most abundant placental proteins in maternal blood during human pregnancy (Moore and Dveksler, 2014). Rearrangements of genes containing binding sites for critical TFs involved in the regulation of intrauterine development could represent an alternative mechanism to activate or repress genes at specific time points.

Most of the genetic studies regarding RPL have focused on maternal genomes; however, an ideal study would include the analysis of both parents as well as miscarried conceptuses (placental samples) and liveborn children. Our seminal study of CNVs in recurrent pregnancy loss included only parental genomes (Ref. I), but the availability of placental samples from different stages

of normal gestations and pregnancy loss in this study gave us an excellent resource to discover possible novel biomarkers and pathways important in early *in utero* development.

### 3.3.3 A closer look at the parental and placental CNV profiles of the three live birth RPL cases

The number of CNVs in RPL live birth placentas was as low as in pregnancy loss samples; however, the rearranged regions involved genes also duplicated in normal term placentas. A 42% overlap in respective gene content was detected, compared to only 25% with miscarriage samples. This may have supported the successful maintenance of these index pregnancies. Additionally, in two of the three families, either one (RPL3) or both or the parents (RPL11) carried large pericentromeric CNVs (0.3–0.6 Mb/genome; Suppl. Table S5 in Ref. III), which were not transmitted to the successful pregnancy placentas.

In one of the families (RPL3), two successful pregnancies preceded the index live birth. As described above, the number of live births showed negative correlation with the number of placental CNVs in normal term pregnancies. Interestingly, the index babies born to the other two families were firstborn and large-for-gestational age (LGA; RPL11: male 4,488 g, RPL12: female 5,060 g; Suppl. Data S1 in Ref. III). Moreover, both couples had primary RPL (no live births before three consecutive pregnancy losses), had further losses after the index case and another successful pregnancy resulting again in LGA newborns (RPL11: male 4,990 g, RPL12: female 4,590 g). I propose that there are compensatory mechanisms that allow for the overcoming of early pregnancy failure, but also contribute to complications such as fetal overgrowth in late gestation.

### 3.3.4 Major contribution of this study to the field of placental and RPL research

1. This study confirmed our hypothesis that RPL placental genomes carry a reduced amount of CNVs, possibly affecting normal trophoblast development and pregnancy success.
2. Alternative rearrangements of certain genes (e.g. *CTNNA3*, *MTRR*) were detected in RPL placentas compared to normal pregnancies, which may confer risk to pregnancy loss.
3. Uncovering the role of specific transcription factors (E2F, AP2, KLF4 and ZF5) involved in the process of placental and fetal development in forthcoming studies may lead to new targets of treatment and management for RPL.

# 4. EXTENDED DISCUSSION AND FUTURE PERSPECTIVES

## 4.1 Load of structural variants in the placenta – harm or benefit?

During normal cell division, the genome is completely duplicated and after that segregated to two daughter cells. However, in some tissues the cells duplicate their genome without disassembling cellular and nuclear membranes, resulting in polyploidy. Polyploidy can be achieved by endoreplication, which is comprised of DNA synthesis and gap phases without mitosis (Fox and Duronio, 2013). As an example, around 50% of human hepatocytes are normally polyploid, suggesting that endoreplication is necessary for normal liver function to manage stress and injuries (Duncan, 2013). Gene amplification can be triggered by developmental signals. Polyploid *Drosophila* follicle cells, for example, require several copies of the chorion genes in order to produce enough protein for normal eggshell formation (Calvi and Spradling, 1999). Murine trophoblast giant cells (TGC) are also known to be polyploid and exhibit repressed mitotic machinery similar to *Drosophila* endocycling cells (Sher et al., 2013).

In our seminal study (Ref. II) of CNVs in the human placental genome, an extensive load of duplications was found characteristic for normal pregnancy placental samples. Hannibal *et al.* have thoroughly demonstrated the variable nature and flexibility of murine placental genome in two reports (Hannibal and Baker, 2016; Hannibal et al., 2014). Their seminal study showed that specific regions of the TGC genome are under-replicated and enriched for genes involved in cell adhesion and neurogenesis. I compared the somatic placental CNVs (Ref. II) with their data and found five recurrent CNV regions in the human placenta overlapping with respective syntenic regions in murine placental polyploid TGCs described with decreased copy number, involving the genes *EPHA7*, *CSMD3*, *COL11A1*, *DPYD* and *GRIK2* (Suppl. Table 8 in Ref. II). In their latest article, Hannibal et al., 2016 reported selective amplification of prolactins, serpins, cathepsins, and the NK/CLEC complex in the mouse placenta. All these gene families encode genes that are crucial for murine early pregnancy. Rodents have undergone extensive tandem amplifications of the cathepsin and prolactin gene families and these regions represent a hotspot for additional rearrangements. Humans have only one prolactin gene and several scattered serpin, cathepsin, and CLEC genes located all over the genome. I did not discover any duplications in these genes, although I did find amplifications of genes that interact with several SERPIN, cathepsin (CTS) and CLEC proteins. In humans, most of these genes are not placenta-specific but are expressed in various tissues. These observations suggest that while the somatic rearrangements seem to be a characteristic feature of placental genomes in mammals, the respective hotspots and involved genes may differ due to local genomic structure or the ‘necessity’ to promote amplification of lineage-specific placental genes.

Functional pathway analysis of CNVRs detected exclusively in normal pregnancy placentas compared to recurrent pregnancy loss samples revealed enrichment for genes that have binding sites for E2F transcription factors. E2Fs control the expression of genes required for DNA synthesis and cell cycle (Fox and Duronio, 2013). Conversely, the embryonic development and cell cycle related pathways were enriched in the copy number stable map (most dosage-sensitive regions) of the human genome (Zarrei et al., 2015). While most human tissues may be void of rearrangements in these regions, because there is no need for such rapid and precocious development as in the case of placenta, functions related to cell cycle, growth, and adhesion are preferably reorganized also in cancer genomes (Koutsogiannouli et al., 2013).

A recent report on the genomic profile of choriocarcinoma (gestational trophoblastic disease) did not find major overlap with our CNV data in normal placentas; however, loss of 18q22.1 involving *CDH19* gene was detected in four gestational choriocarcinoma samples (Mello et al., 2017). On the contrary, I found duplications in the same gene in three 2$^{nd}$ trimester normal pregnancy samples and one SGA placenta. Thus, differential rearrangement and possibly expression of this gene may play a role in both normal placental development as well as cancer progression. A recent study has shed light on how copy gain can be induced and that it is not always permanent. Black et al. demonstrated that hypoxia stimulates transient site-specific copy number alterations (also seen in hypoxic primary tumors) dependent on chromatin modulation (Black et al., 2015). Hypoxia is a common phenomenon in a majority of malignant tumors besides early placental development (Fajersztajn and Veras, 2017; Muz et al., 2015). There is a definite need for interdisciplinary studies in research areas regarding placental and cancer development in order to find out the exact mechanisms behind CNV formation and function.

## 4.2 Placental CNVs and non-invasive prenatal testing

Noninvasive prenatal testing (NIPT) by maternal plasma DNA sequencing has been in use since 2011 to screen for common fetal aneuploidies. The circulating cell-free DNA reflects maternal (~85–90%) and fetoplacental DNA (Wang et al., 2013). The specificity and sensitivity of NIPT has become high over the years; however, discordant results still remain (Hartwig et al., 2017). These could be due to confined placental mosaicism, true fetal mosaicism, maternal pathology or presence of a deceased twin. Recently, maternal incidental findings (e.g. B12 deficiency, autoimmune disease, tumors) have been suggested as the main cause of false-positive and false-negative sequencing results (Bianchi, 2017). Based on this review, before invasive fetal diagnostic procedure is performed, maternal peripheral blood karyotype should be analyzed. If maternal abnormalities can be ruled out, invasive diagnostic (chorionic villus sampling or amniocentesis) testing is still needed to verify true fetal abnormality.

Most of the present NIPT methods provide information only about trisomies 13, 18 and 21. However, some centers also apply genome-wide algorithms and look for subchromosomal abnormalities besides aneuploidies (Brison et al., 2017; Lefkowitz et al., 2016). Brison et al. reported recurrent CNVs (<500 kb) associated with developmental disorders and adult-onset conditions in ~0.4% of samples. Such approaches may provide more clinically important results, but on the other hand lead to the identification of fetal/maternal variants of unknown significance that complicates counselling. As the methods opt for higher resolution and detect smaller rearrangements, it is important to consider the extensive somatic genomic rearrangements and mosaicism in the placenta that may disturb reliable detection of the fetal CNV profile, and increase discordant results.

## 4.3 Importance of CNV profiling in the clinical management of pregnancy complications

Firstly, the current thesis has demonstrated that large genomic rearrangements in the parental genomes predispose towards poor pregnancy outcomes. In addition to classical karyotyping, chromosomal microarray analysis should be recommended for couples in order to identify large submicroscopic and pericentromeric CNVs, and overall load of CNVs as risk factors for RPL. Parental genetic testing in couples with RPL is currently not recommended by ESHRE; however, it may help in the early detection of cases at risk and provide close monitoring in their subsequent pregnancies, as well as suggest available prenatal testing options.

Secondly, the ultimate goal of the analysis of structural variants would be the discovery of specific diagnostic CNVs leading to the possibility of targeting gene products deleted/duplicated in particular pregnancy complications. Although cancer is a different phenotype, an excellent example of a trackable biomarker in cell-free DNA from blood was recently published (Goh et al., 2017). We have discovered a common duplication at 5p13.3 that could be a pleiotropic risk factor for recurrent pregnancy loss as well as preeclampsia; however, it is not very likely to find recurrent CNVs that cause gestational complications; rather every case carries its own (combination of) risk variants. The detection of specific submicroscopic rearrangements as a diagnostic method in pregnancy complications requires a great deal of further investigation. At the moment, family-based studies are justified in research to identify the cause of a specific pregnancy loss event as an example, and to define segregation of the discovered causal CNV in the given family.

Thirdly, microarray-based CNV analysis of products of conception (POC) should be recommended to couples with RPL. A 24-chromosome microarray evaluation of miscarriage tissue has recently proven to be very cost-effective and time-efficient (Popescu et al., 2018). This may provide a definitive cause for the specific miscarriage in a majority of cases, prognosis for subsequent pregnancy success, and estimation and/or prevention of having a child with birth defects. It would lead to better clinical diagnoses, improve counselling of couples, and thereby exclude unnecessary analyses and save costs to the health-care system.

# 5. CONCLUSIONS

The current doctoral thesis project unravelled a small fraction of the enormous puzzle of the profile and role of CNVs in human gestational complications and placental genomes of normal and disturbed pregnancies. The results of this thesis can be summarized as follows:

1. Genome-wide CNV profiling of parental genomes of couples with recurrent pregnancy loss confirmed the role of structural variation in modulating the genetic susceptibility to RPL, and may provide new biomarkers for multiple gestational complications.
2. Extensive load of CNVs was detected as a hallmark of normal placental development. Early as well as late pregnancy complications showed reduced capacity to promote somatic genomic rearrangements in the placenta.
3. CNVs detected exclusively in normal pregnancy placentas compared to RPL samples were shown to be enriched in genes regulated by transcription factors critical in early embryonic development, and the regulation of basic cellular processes, such as E2F, ZF5, KLF4, AP2, and SREBP. In perspective, these pathways represent a potential source for new biomarkers for the evaluation of fetal health and early detection of pregnancy complications.

The material presented here serves as a great resource of CNV data for future studies in reproductive medicine.

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# SUMMARY IN ESTONIAN

## Genoomi struktuursed varieeruvused platsenta ja raseduse mõjutajatena

Ema ja loote vaheline suhtlus loob aluse raseduse edukaks kulgemiseks, sünnituseks ning ka sünnitusjärgseks ajaks. Rasedus on naise jaoks füsioloogiline väljakutse, mida kinnitab fakt, et inimese viljakuse efektiivsus on madal, ligikaudu 30% kõigist viljastatud munarakkudest ei pesastu emakasse. Spontaanne raseduse katkemine on kõige sagedasem rasedusaegne tüsistus. 40% rasedustest hakkavad emaka sees küll arenema, kuid nendest 30% juhtudel toimub katkemine väga varajases etapis (nn biokeemiline rasedus) ja 10% juhtudest on tegemist kliinilise raseduse katkemisega (ultraheliuuringuga tuvastatav). Korduv raseduse katkemine (KRK), defineeritud kui ≥3 järjestikust raseduse katkemist enne 24-ndat gestatsiooninädalat, esineb 1–2% kõigist last saada üritavatest paaridest. KRK-d võivad põhjustada anatoomilised, infektsioossed, endokriinsed, immuunsüsteemiga seotud põhjused ning geneetilised faktorid, kuid 25–50% juhtudest on siiski põhjus selgusetu.

Rasedusaegsed füsioloogilised muutused põhjustavad osadel naistel hiliseid rasedustüsistusi nagu preeklampsia, gestatsioonidiabeet ning raseduskestuse kohta väike ja suur sünnikaal, mis võivad omakorda naise organismis paljastada eelsoodumuse mitmetele kroonilistele haigustele (diabeet, südame-veresoonkonna haigused). Lisaks on häired üsasiseses arengus riskifaktoriks erinevatele haigustele ka lapse tulevases elus. Eelmainitud gestatsiooniaegseid komplikatsioone, mis mõjutavad naisi ja lapsi üle kogu maailma, on sageli seostatud puuduliku trofoblasti invasiooni ning platsenta funktsiooniga. Seetõttu on äärmiselt oluline täielik arusaam platsenta bioloogiast, arengust ja funktsioonist. Platsenta, ainus ‘lühiajaline’ organ, vahendab kõiki interaktsioone ema ja loote vahel, kuid paraku ei ole seda veel piisavalt põhjalikult uuritud nii eetilistel kui ka metodoloogilistel põhjustel. Platsenta geenide avaldumise tase ja dünaamika reguleerib platsenta funktsiooni ja seeläbi raseduse kulgemist. Inimese platsenta transkriptoomi moduleerib geneetiline varieeruvus, millest kõige enam on uuritud ühenukleotiidseid muutusi.

DNA koopiaarvu varieeruvused (*copy number variation*, CNV) on >50 bp ümberkorraldused, mis moodustavad ligikaudu 10% inimese genoomist. CNV-d võivad olla tingitud üht või mitut geeni või DNA piirkonda hõlmavatest deletsioonidest ja duplikatsioonidest. Need struktuursed variandid annavad oma osa genoomi normaalsele varieeruvusele, kuid samas soodustavad mõned ümberkorraldused erinevate haiguste teket. CNV-d võivad mõjutada (varajases) embrüonaalses arengus oluliste geenide doosi otseselt (kogu geeni hõlmav duplikatsioon või deletsioon) või kaudselt (regulatoorsete elementide ümberkorraldused). CNV-de seost reproduktiivhäiretega on siiani vähe uuritud. Platsenta ja vanemate genoomi struktuuri mõistmine nii normaalse raseduse kui ka rasedusaegsete tüsistuste puhul võimaldab tuvastada biomarkereid, mille eesmärk oleks ennustada raseduse kulgu/lõpptulemust.

Käesoleva doktoritöö kirjanduse ülevaates on tutvustatud platsenta arengut ja funktsiooni, varaseid (KRK) ja hiliseid (preeklampsia, gestatsioonidiabeet, raseduskestuse kohta väike/suur sünnikaal) rasedusaegseid komplikatsioone, nende riskitegureid ja geneetilist tausta. Lisaks on kirjeldatud DNA koopiaarvu varieeruvusi, nende tuvastamise meetodeid ning võetud kokku tänaseks avaldatud info koopiaarvu varieeruvuste rollist rasedustüsistuste kujunemisel.

Doktoritöö eksperimentaalses osas on püstitatud järgmised eesmärgid:

I. Tuvastada korduva raseduse katkemisega paaridel üldlevinud CNV-sid kui uusi KRK geneetilisi markereid ning leida uusi lookuseid ja bioloogilisi radasid, mis mõjutavad raseduse kulgu.

II. Võrrelda kogu genoomi CNV-de profiili vanemate verest eraldatud ja platsentast eraldatud DNA-s nii normaalse raseduse erinevatel trimestritel kui rasedustüsistuste korral.

III. Kirjeldada võrdlevalt submikroskoopiliste genoomsete ümberkorralduste profiili samas raseduse kestuses KRK-ga platsentades ja normaalselt kulgenud raseduse platsentades.

Doktoritöö peamised tulemused on:

1. KRK paaridel esinevad CNV-d hõlmavad oluliselt enam geene, mis pärinevad immuunvastuse väljakujunemisega seotud bioloogilistest radadest. See leid kinnitab immuunsüsteemi olulist rolli varajase raseduse säilitamisel.

2. KRK patsientidel on CNV-de koguulatus ning pikkade deletsioonide osakaal genoomi kohta suurem võrreldes viljakate kontrollidega. Laialdane CNV-de esinemine genoomis võib hõlmata olulisi geene varajaseks arenguks ning soodustada omakorda ümberkorralduste teket gametogeneesis ning rakkude jagunemisel varajases embrüonaalses arengus. Lisaks leidub KRK vanemate genoomides enam pikki peritsentromeerseid ja subtelomeerseid CNV-sid, mis soosivad vigu kromosoomide normaalsel jagunemisel.

3. Üldlevinud CNV piirkondade uuring Eesti ja Taani valimites tuvastas duplikatsiooni 5p13.3 kromosoomi piirkonnas, mis suurendab KRK riski naistel. Antud CNV hõlmab kahte platsentas kõrgelt avaldunud geeni (*PDZD2* ja *GOLPH3*), mida pole varasemalt rasedusega seostatud.

4. CNV-de genoomne kaardistamine duo/trio uuringus tuvastas submikroskoopiliste ümberkorralduste suure hulga platsentas võrreldes vanemate genoomidega. Eelkõige oli CNV-de rohkus tingitud duplikatsioonide arvust. Nendes esinevate geenide funktsionaalse rikastatuse analüüsist ilmnes, et ümberkorraldused asuvad geenides, mis on olulised just embrüonaalses arengus.

5. Nii varaste kui hiliste rasedustüsistuste puhul esines CNV-sid platsentas oluliselt vähem kui normaalselt kulgenud raseduste proovides, viidates CNV-de kriitilisele rollile raseduse edukal kulgemisel.

6. Somaatilistes (ainult platsenta koes esinevates, mitte vanematelt päritud) duplikatsioonides tuvastati vermitud geenide rikastatus. Selle puhul võib tegu olla geenide avaldumise regulatsiooni lisatasandiga, mis tuleks välja selgitada edasistes uuringutes.
7. KRK ja kontrollide platsentas esinevate CNV-de võrdlusest tuvastati alternatiivsed ümberkorraldused, mis hõlmasid mõlemas grupis küll samu geene (näiteks *CTNNA3*, *MTRR*), kuid erinesid genoomse ümberkorralduse ulatuse või asukoha suhtes. Ühe geeni eri piirkondi hõlmavad CNV-d võivad omada erisugust efekti ning mõjutada KRK riski.
8. Üksnes normaalse I ja III trimestri platsentades esinevate CNV-de (võrreldes KRK platsenta genoomi CNV profiiliga) geenide funktsionaalse rikastatuse analüüs näitas, et genoomsed ümberkorraldused mõjutavad geene, mis on reguleeritud teatud transkriptsioonifaktorite (näiteks E2F, AP2, KLF4) poolt. Edasised uuringud näitavad, kas tuvastatud faktoreid oleks võimalik tulevikus kasutada rasedustüsistuste varajasel prognoosimisel ja/või ravis.

Doktoritöös analüüsiti esmakordselt kogu genoomi CNV-de profiili KRK paaride genoomis. Hüpoteesi-vaba lähenemine võimaldas leida uusi lookusi seoses KRK-ga ning kinnitas struktuursete varieeruvuste rolli raseduse katkemise etioloogias.

Lisaks viidi läbi ka esimene duo/trio uuring (platsenta-ema-isa), et kirjeldada genoomi struktuursete ümberkorralduste profiili normaalse raseduse kolmel trimestril ning hiliste rasedusaegsete tüsistuste korral. Töö üheks olulisemaks sõnumiks on see, et normaalse platsenta arenguks on vajalik oluliselt suurem CNV-de hulk kui seda on muudes mittemaliigsetes rakkudes.

Kuna platsenta funktsioon mõjutab loote ja ema tervist oluliselt nii raseduse vältel kui ka edasises elus, siis on platsenta genoomi uurimine äärmiselt oluline, paljastamaks täpsed mehhanismid, misläbi oleks tagatud edukas rasedusaegne ja -järgne periood. Käesoleva uurimistöö materjal on suurepäraseks CNV andmete allikaks edasisteks uuringuteks reproduktiivmeditsiinis.

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# PUBLICATIONS

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| Since 2012 | University of Tartu, Institute of Molecular and Cell Biology, Chair of Biotechnology, PhD studies in Gene Technology |
| 2010–2012 | University of Tartu, Institute of Molecular and Cell Biology, Chair of Biotechnology, MSc in Gene Technology |
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**Additional studies**

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| 06.2017 | Estimated (disease) risk for coronary atherosclerosis using lifestyle factors and genetic markers. ePerMed project; Tartu, Estonia |
| 05.2017 | "Bioinformatics for advanced analysis in human genetics", Fulbright Specialist (USA) programme; Tartu, Estonia |
| 04.2017 | "Bioinformatics for genomic medicine", Fulbright Specialist (USA) programme; Tartu, Estonia |
| 11.2016 | "Introduction to programming", University of Tartu; online course |
| 04.2015 | "Pre- and perinatal determinant of health in child- and adulthood", HAPPY PREGNANCY project; Tartu, Estonia |
| 07.2014 | "Placental Biology Course", Centre for Trophoblast Research, University of Cambridge; Cambridge, United Kingdom |
| 01.2011 | Sequenom iPLEX and CNV/QGE training; Hamburg, Germany/Tartu, Estonia |

**Main Fields of Research**

The contribution of DNA copy number variation in modifying the genetic predisposition to early/late pregnancy complications, and genetic determinants of male infertility.

**Conference presentations**

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| 30.08–02.09.2017 | IFPA 2017 ("Placenta: the journey of a lifetime"); oral presentation "Copy Number Variation is an Essential Part of Human Placental Genome"; Manchester, United Kingdom |
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| 19–22.01.2017 | 2nd World Congress on Recurrent Pregnancy Loss; oral presentation "Deficiency of placental copy number variations in recurrent pregnancy loss patients"; Cannes, France |
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| 10–12.04.2013 | Genomic Disorders 2013: From 60 Years of DNA to human genomes in the clinic; poster presentation "A duplication CNV encompassing *SLC35F2* in recurrent miscarriage"; Cambridge, United Kingdom |
| 28–31.05.2011 | European Human Genetics Conference 2011; poster presentation "Locus copy number variation (CNV) in recurrent miscarriage"; Amsterdam, The Netherlands |

**Publications**

Kasak, L; Rull, K; Laan, M. Genetics and Genomics of Recurrent Pregnancy Loss. In: Leung, P and Qiao, J, editors. Human Reproductive and Prenatal Genetics. Elsevier Inc.; November 2018 (Invited book chapter, in press).

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Kasak, L; Rull, K; Sõber, S; Laan, M. 2017. Copy number variation profile in the placental and parental genomes of recurrent pregnancy loss families. *Scientific Reports*, 7 (45327), 1−12.

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Kasak, L; Rull, K; Vaas, P; Teesalu, P; Laan, M. 2015. Extensive load of somatic CNVs in the human placenta. *Scientific Reports*, 5 (8342), 1−10.

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**Scholarships and Awards**

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2016 Kristjan Jaak Scholarship, "Understanding the Function of Human Genome Variation" conference attendance
2016 Keystone Symposia fellowship to attend "Understanding the Function of Human Genome Variation" conference (Uppsala, 31.05–05.06.2016)
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2011 Kristjan Jaak Scholarship, ESHG 2011 conference attendance

**Supervised dissertations**

Since 2016 Marion Villemson, BSc studies, Institute of Molecular and Cell Biology
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DNA koopiaarvu varieeruvuste roll varaste/hiliste rasedusaegsete komplikatsioonide tekkes ja meeste viljatuse geneetilise tausta uurimine.

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| 27–30.05.2017 | European Human Genetics Conference 2017; suuline ettekanne (Best Posters session), E-poster and stendiettekanne “Copy number variation profile in the placental and parental genomes of recurrent pregnancy loss families”; Kopenhaagen, Taani |
| 19–22.01.2017 | 2nd World Congress on Recurrent Pregnancy Loss; suuline ettekanne “Deficiency of placental copy number variations in recurrent pregnancy loss patients”; Cannes, Prantsusmaa |
| 31.05–04.06.2016 | Keystone Symposium “Understanding the Function of Human Genome Variation”; suuline- ja stendiettekanne “Somatic CNVs in the placental genome and link to pregnancy success”; Uppsala, Rootsi |
| 22–24.05.2015 | Happy Pregnancy Project Workshop of 2015 „Reproductive health: the trends of clinical, diagnostical and biomedicine studies in Estonia“; suuline ettekanne “Extensive load of somatic CNVs in the human placenta”; Tartu, Eesti |
| 10–12.04.2013 | Genomic Disorders 2013: From 60 Years of DNA to human genomes in the clinic; stendiettekanne “A duplication CNV encompassing *SLC35F2* in recurrent miscarriage”; Cambridge, Suurbritannia |
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Kasak, L; Rull, K; Laan, M. Genetics and Genomics of Recurrent Pregnancy Loss. In: Leung, P and Qiao, J, editors. Human Reproductive and Prenatal Genetics. Elsevier Inc.; November 2018 (Kutsutud raamatu peatükk, trükis).

Kasak, L; Rull, K; Laan, M. Genetic causes of Recurrent Pregnancy Loss: overview. In: Arora, M, editor. Recurrent Pregnancy Loss. 3rd ed. Jaypee Brothers Medical Publishers Pvt. Ltd.; 2018 (Kutsutud raamatu peatükk, trükis).

Kasak, L; Rull, K; Sõber, S; Laan, M. 2017. Copy number variation profile in the placental and parental genomes of recurrent pregnancy loss families. *Scientific Reports*, 7 (45327), 1−12.

Laura Kasak, Kristiina Rull, Pille Vaas, Pille Teesalu, Maris Laan (2015). Geneetilise materjali ümberkorralduste rohkus platsentas. *Naistearstide Seltsi Teataja*, 29−32.

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