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Rīga Stradiņš University

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Causes and Genomic Approaches to Female Reproductive Failure

Doctoral Thesis for obtaining a doctoral degree (*Ph.D.*)

Sector – the Basic Sciences of Medicine, Including Pharmacy
Sub-Sector – Medical Genetics

Riga, 2021

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Abstract

Clinical recognition of the genetic causes of female reproductive failure using increasingly advancing genetic technologies to preserve patient safety and move towards personalized treatment application is a major challenge of reproductive medicine in the 21st century. The aim of this thesis was to demonstrate a reliable application of advanced genomic techniques in different stages of female reproductive failure in real-life clinical or research scenarios. Several genetic approaches were exploited – starting from the multifactor genetic testing of preimplantation embryos to select the ones free of inherited monogenic conditions and chromosomal aberrations, then following with the analysis of fetal material in case of early pregnancy loss using array comparative genomic hybridization and short tandem repeat analysis to exclude maternal cell contamination, and finally using next generation sequencing technology to analyze genetic landscape leading to preterm delivery in women with cervical insufficiency. The practical work described here was published as three scientific articles now forming three chapters of this thesis.

Array comparative genomic hybridization combined with loci-specific genetic testing techniques allowed for a versatile and reliable analysis of preimplantation embryos to select the ones free of genetic conditions analyzed, and in combination with microsatellite analysis it also allowed to access the chromosomal causes of early pregnancy loss while reducing the misdiagnosis caused by maternal cell contamination. Next generation sequencing application allowed to identify the disruptive variants potentially contributive to the development of non-syndromic cervical insufficiency. Pathway enrichment analysis of variant genes from our cohort revealed an increased variation burden in genes playing roles in tissue mechanical and biomechanical properties. Literature analysis allowed to conclude that number of genes can be reliably attributed to female reproductive failure and an increasing number of genes form a pool of good candidates. In order to develop diagnostic gene panels and facilitate genetic advancement inclusion in the clinical practice of female reproduction, a standardized clinical gene-disease validity assessment of the identified genes has to be performed and best practice guidelines have to be composed.

Keywords: genetic etiology, genomic technology, next generation sequencing, female reproduction, preimplantation embryo, missed abortion, cervical insufficiency, preterm birth.

Anotācija

Sievietes reproduktīvās mazspējas iemesli un genomiskās pieejas to risināšanai

Sievietes reproduktīvās mazspējas ģenētisko cēloņu identificēšana, izmantojot mūsdienīgas ģenētiskās tehnoloģijas, vienlaikus saglabājot pacientu drošību un virzoties uz personalizētas ārstēšanas lietošanu, ir reproduktīvās medicīnas izaicinājums 21. gadsimtā. Darba mērķis bija demonstrēt genomisko tehnoloģiju lietojumu dažādos sievietes reproduktīvās mazspējas etapos reālos klīniskajos vai pētījumu apstākļos. Tika lietotas vairākas genomiskās metodoloģijas – pirmsimplantācijas embriju testēšanai, lai atlasītu embrijus bez iedzimtas monogēnas patoloģijas un hromosomālajām aberācijām, salīdzinošā genoma hibridizācija uz mikročipiem un mikrosatelītu analīze augļa hromosomu analīzei pārtraukušās grūtniecības materiālā, nosakot mātes šūnu kontaminācijas klātbūtni paraugā, un, visbeidzot, nākamās paaudzes sekvencēšana dzemdes kakla nepietiekamības izraisītu priekšlaicīgu dzemdību ģenētiskās etioloģijas raksturošanai. Paveiktais praktiskais darbs tika publicēts trijos zinātniskajos rakstos, kas veido trīs darba sadaļas. Salīdzinošā genoma hibridizācija uz mikročipiem apvienojumā ar lokusa ģenētiskās testēšanas metodēm deva iespēju veikt pirmsimplantācijas analīzi, lai atlasītu embrijus bez testētās patoloģijas, savukārt kombinācijā ar mikrosatelītu analīzi ļāva noteikt hromosomālās patoloģijas izraisošas agrīnas grūtniecības pārtraukšanos, vienlaikus mazinot mātes šūnu kontaminācijas izraisītas kļūdainas diagnozes iespēju. Lietojot nākamās paaudzes sekvencēšanu, tika identificēti gēnu varianti, kas potenciāli veicina nesindromiskas dzemdes kakla nepietiekamības attīstību. Gēnu ceļu bagātināšanas analīze atklāja palielinātu gēnu variācijas slogu gēnos, kas nodrošina audu mehānisko un biomehānisko izturību. Literatūras analīze ļauj secināt, ka gēnu skaits ar zināmu ietekmi uz sievietes reproduktīvās mazspējas attīstību nepārtraukti aug un aizvien lielāks gēnu skaits veido labu kandidātu kopu, kas gaida replikācijas pētījumus. Diagnostisko gēnu paneļu klīniskajai izveidei un lietošanai un ģenētisko sasniegumu iekļaušanai sievietes reprodukcijas klīniskajā praksē nepieciešama standartizēta identificēto gēnu klīniskās validitātes novērtēšana un labās prakses vadlīniju izstrāde.

Atslēgvārdi: ģenētiskā etioloģija, genomiskās tehnoloģijas, nākamās paaudzes sekvencēšana, sievietes reprodukcija, pirmsimplantācijas embrijs, pārtraukusies grūtniecība, dzemdes kakla nepietiekamība, priekšlaicīgas dzemdības.

Table of contents

Abbreviations

aCGH	Array comparative genomic hybridization
ACMG	American College of Medical Genetics
AD	Autosomal dominant
ADO	Allelic drop out
AF	Allelic frequency
AR	Autosomal recessive
ART	Assisted reproductive technologies
ASRM	American Society for Reproductive Medicine
β-hCG	Human chorionic gonadotropin beta
BMI	Body mass index
CL	Cervical length
CNV	Copy number variation
COC	Oocyte cumulus complexes
cRNA	Complementary ribonucleic acid
DNA	Deoxyribonucleic acid
DOP	Degenerate oligonucleotide primer
ECM	Extracellular matrix
EDS	Ehlers-Danlos syndrome
EFS	Empty follicle syndrome
EPL	Early pregnancy loss
ERA	Endometrial Receptivity Analysis
eSET	Elective single embryo transfer
ESHRE	European Society of Human Reproduction and Embryology
FFPE	Formalin fixed paraffin embedded
FISH	Fluorescence in situ hybridization
fPCR	Fluorescent polymerase chain reaction
FSH	Follicle stimulating hormone
GHR	Genetics home reference
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GV	Germinal vesicle
GWAS	Genome wide association study
hCG	Human chorionic gonadotropin
HGNC	HUGO gene nomenclature
HLA	Human leukocyte antigen

HP	Human phenotype
HPO	Human phenotype ontology
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
LDO	Locus drop out
LH	Luteinizing hormone
LHCGR	Luteinizing hormone chorionic gonadotropin receptor
MCC	Maternal cell contamination
MDA	Multiple displacement amplification
MI	Meiosis I
MII	Meiosis II
MLPA	Multiple ligation probe amplification
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
nt	Nucleotide
OHSS	Ovarian hyperstimulation syndrome
OMIM	Online Mendelian inheritance in man
PBS	Phosphate buffer saline
PCCR	Precocious cervical ripening
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PEP-PCR	Primer extension polymerase chain reaction
PGD	Preimplantation genetic diagnostic
PGDIS	Preimplantation Genetic Diagnosis International Society
PGT	Preimplantation genetic testing
PGT-A	Preimplantation genetic testing for aneuploidies
PGT-M	Preimplantation genetic testing for monogenic disorders
pH	Potential of hydrogen
PN	Pronucleus
POC	Products of conception
POF	Premature ovarian failure
POI	Premature ovarian insufficiency
POP	Pelvic organs prolapse
PPROM	Premature prelabor rupture of fetal membranes
PTB	Preterm birth
QF-PCR	Quantitative fluorescent polymerase chain reaction

RIF	Recurrent implantation failure
RPL	Recurrent pregnancy loss
SD	Standard deviation
SET	Single embryo transfer
SGD	Single gene disorder
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
STR	Short tandem repeat
VCF	Variant called file
VTE	Venous thromboembolism
VUS	Variant of unknown significance
WES	Whole exome sequencing
WGA	Whole genome amplification
ZP	Zona pellucida

Introduction

Female reproductive failure is an ongoing global challenge having significant medical, social, and financial implications, being estimated to affect as many as 16.2% of women in certain countries (Inhorn and Patrizio, 2014; Maddirevula et al., 2020; Singh, 2004). There are numerous definitions of infertility, e.g., WHO defines infertility as "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse". Wider definitions of the condition characterizes infertility as an inability of those of reproductive age (15–49 years) to become or remain pregnant within five years (Mascarenhas et al., 2012; Rutstein and Shah, 2004). In this thesis, I use term "female infertility" as an inability to conceive and a broader term "female reproductive failure" as inability to conceive and/or carry pregnancy until term.

In order to carry a successful term pregnancy, different organs such as the uterus, cervix, placenta, and amniotic membranes as well as the fetus itself must cohesively interact and create a healthy symbiotic relationship with each other and the rest of the female body (Vink and Myers, 2018). Genetic causes are recognized as important contributors to female reproductive failure at the level of all main constituents of successful conception and pregnancy progress. Identification of genetic causes began in the late fifties of the 20th century when Turner syndrome was discovered by the means of karyotyping (Ford et al., 1959), and continues to this day aided by the development of novel molecular techniques and technological advancements. Nonetheless, still relatively little is known about the genetic background of most cases of reduced female fecundity, and unfortunately even less is translated into clinical practice, preventing the progress of personalized treatment entry into reproductive medicine.

Without doubt, the 21st century for clinical medicine and especially research can be addressed as the century of genomics since major breakthrough was possible due to the underpinning of molecular mechanisms for the majority of the diseases. Consequently, for some disorders (e.g., cancer, inherited metabolic disorders) genetic tests are on the frontline before any further interventions can be undertaken. Naturally, genetic tests are becoming increasingly demanded in the frame of diagnosing and managing female reproductive failure as well, and should be carried out for three main purposes: to identify the cause of reproductive failure, identify genetic diseases transmissible to offspring, and provide direction towards the most appropriate treatment. Unfortunately, often there is a lag in the understanding of the data resulting from the state-of-art technologies and its clinical applicability due to a variety of peculiarities associated with each technique and insufficient knowledge of molecular and genetic pathophysiology of impaired female reproductive capability. In the context

of missing and/or insufficient guidelines regulating the field, this all results in the increased risks of mismanagement, psychological burden and excess costs for the patients, their family members, and offspring.

Aim of the study

To demonstrate a reliable application of advanced genomic techniques in different stages of female reproductive failure in real-life clinical or research scenarios in order to increase couple's chances to conceive a healthy child, improve the reliability of genetic testing in early pregnancy loss, and unravel the underlying genetic cause of cervical insufficiency.

Objectives of the study

1. To develop preimplantation genetic testing protocols and to compare the performance of two different whole genome amplification techniques for multiple downstream applications in preimplantation embryo genetic testing.
2. To develop a protocol for maternal cell contamination assessment in the genetic testing of products of conception in early pregnancy loss.
3. To perform a systematic analysis of the genes implicated in uterine cervix functioning to assist next-generation sequencing data interpretation from patients with cervical insufficiency.
4. Through the application of NGS to a patient cohort with preterm delivery due to cervical insufficiency, to characterize the genetic landscape of the condition and to identify the gene variants that increase the likelihood of cervical insufficiency development.

Hypothesis of the study

Advanced genetic technologies could be successfully used in various stages of female reproductive failure to reliably assess several classes of genetic variations perturbing female reproductive potential, while the lack of best practice guidelines on genetic testing and gene-disease clinical validity assessment prevents clinical applicability of the existing genetic knowledge in the field of female reproduction.

Scientific novelty of the study

The work described in Chapter 2 represents the development of individualized preimplantation embryo genetic testing protocols and the introduction of such testing in Latvia, it also depicts a comparison of two widely used whole genome application techniques, which is something done for the first time. Chapter 3 is devoted to the known problem of maternal cell contamination in the genetic testing of products of conception and offers a remastered low-resource setting protocol of visual and technological material evaluation and handling capable to successfully address the issue. Chapter 4 not only describes the results of next generation sequencing applied to the cohort of patients with isolated cervical insufficiency, which was done for the first time, but also contains a comprehensive and systematic work on literature and gene analysis on this topic, which also was done for the first time.

Ethics

The work done during this study is in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Central Medical Ethics committee of Latvia (please see Supplement 1 and 2 at the very end of this work).

1 Literature Review

Successful human reproduction requires orchestrated functioning of processes of spermatogenesis, oogenesis, fertilization, embryonic development as well as the intrauterine development of the fetus. Discoordination in any of these sophisticated steps caused by genetic abnormalities, mitochondrial and epigenetic disturbances, hormonal disorders, and exposures to environmental toxins/endocrine disruptors often underlie impaired fertility in both genders (Tarín et al., 2014) or inability to carry pregnancy until term.

Male infertility has been widely reported, and its underlying genetic basis has been extensively studied (Oud et al., 2019). However, the genetic contribution to female reproductive failure – predicted to be caused by chromosomal and single-gene defects – has been relatively poorly investigated (Sang et al., 2019).

This literature review intends a) to describe the latest advances on genetic causes of non-syndromic female reproductive failure – a term broadly covering impaired oogenesis, fertilization, and early embryo development, as well as female's contribution to preterm delivery and pregnancy loss, and b) to provide insight into genetic testing approaches used to study and diagnose female reproductive failure in clinics and research.

1.1 Genetic causes of female reproductive failure

Due to the enormous complexity of physiological processes and the number of organs/organ systems ensuring normal reproduction in female, the classification of female reproductive phenotypes is burdensome. The situation is especially complex for ovarian and / or follicle disorders (e.g., ovarian failure/insufficiency, ovulation disorders, empty follicle syndrome etc.). This chapter consecutively describes problems in natural path of female reproduction caused by certain genetic defects starting with impaired oogenesis, accompanied by the findings assessable only through the usage of ART, and also covers reproductive failure due to an embryo/fetal demise and preterm birth (PTB). Where possible only genetic defects with existing and/or predicted causative roles in female reproduction are included, not covering abundant genetic risk factors without an assessable genotype-phenotype correlation.

1.1.1 Female gonadal disorders

The development and functioning of female gonads and thus major aspects of female reproduction are mostly dictated by the proper performance of the hypothalamic-pituitary-gonadal axis. The activity of the pituitary gland is stimulated by the gonadotropin-releasing hormone (GnRH) produced by neurons in the hypothalamus. The gonadotropins – follicle

stimulating hormone (FSH, FSH subunit beta is encoded by the *FSHB*, HGNC:3964) and luteinizing hormone (LH, LH subunit beta is encoded by the *LHB*, HGNC:6584) secreted by gonadotroph cells located in the anterior pituitary gland have a central role in folliculogenesis and regulation of ovulation. FSH is required for the monthly recruitment and growth of cohorts of developing follicles while LH activity mediates the final stages of follicle maturation and induces a cascade of events leading to ovulation (McGee and Hsueh, 2000). During the late stages of follicular development, *granulosa* cells within the follicle acquire LH / chorionic gonadotropin receptors (LHCGR) and become responsive to the presence of the ligand (Mitri et al., 2014). Eventually sex steroids then exert negative feedback at both the hypothalamus and pituitary to control the gonadotropin stimulus (Figure 1.1). Various genetic defects in genes ensuring activity of the hypothalamic-pituitary-gonadal axis that result in gonadal dysfunction and infertility have been described (Beau et al., 1998; Layman, 2013).

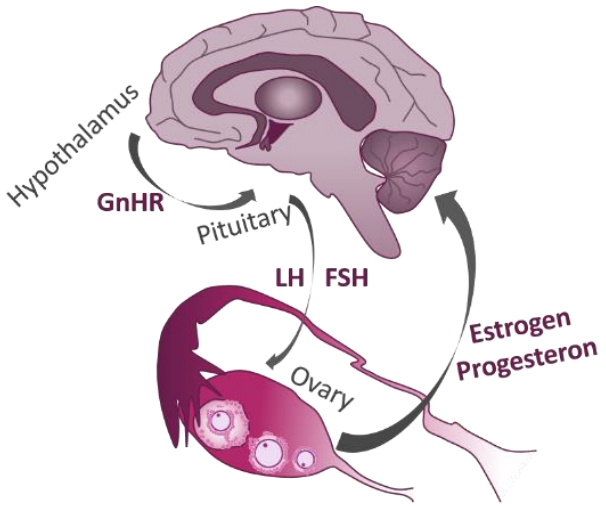

Figure 1.1 **Schematic representation of the hypothalamic-pituitary-gonadal axis functioning in female** (scheme by L. Voložonoka)

The hypothalamus releases gonadotropin releasing hormone (GnRH), which stimulates the anterior pituitary gland to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH), which target the ovary to produce estrogen and progesterone; the latter provides negative feedback back to the hypothalamus and pituitary.

Hypogonadotropic hypogonadism

When female manifest symptoms of estrogen deficiency, such as absent breast development or hypoestrogenic amenorrhea, there is a lack of negative feedback to the hypothalamus and pituitary gland. Serum gonadotropin levels in these patients are low (or inappropriately normal), indicating that the defect is in the hypothalamus or pituitary. These patients usually have GnRH (encoded by the *GNRH1*, HGNC:4419) deficiency. If the sense of smell is normal, this disorder is termed normosmic hypogonadotropic hypogonadism; when an impaired sense of smell accompanies hypogonadotropic hypogonadism, Kallmann

syndrome is present. Smell disturbance occurs because during embryogenesis the GnRH neurons originate outside the brain in the nasal region and migrate alongside olfactory nerves into the hypothalamus (Tobet and Schwarting, 2006).

In the 1990s, two groups independently identified a genetic cause involved in normosmic hypogonadotropic hypogonadism / Kallmann syndrome development in women. Compound heterozygous variants were found in the GnRH receptor gene *GNRHR* [HGNC:4421] (de Roux et al., 1997; Layman et al., 1998). Of clinical interest was the observation that the phenotypes caused by the defects in *GNRHR* could range from very severe – a complete lack of puberty – to partial pubertal development or constitutional delay of puberty (Kim et al., 2010). To date, *GNRHR* pathogenic variants are known to cause normosmic hypogonadotropic hypogonadism without other additional somatic anomalies, but they only account for 4% of normosmic hypogonadotropic hypogonadism patients (Bhagavath et al., 2005).

At the level of the pituitary, variants of *FSHB* or *LHB* genes have been detected to cause hypogonadothropic hypogonadism (Beau et al., 1998; Layman et al., 1997). Notably, infertility caused by the isolated deficiency of FSH due to biallelic *FSHB* variants (OMIM:229070) is reversible by treatment with gonadotropin, which induces ovulation and allows pregnancy to occur (C. Matthews and Chatterjee, 1997; C. H. Matthews et al., 1993; D. Rabinowitz et al., 1979). This is possible because early follicular development is not dependent on signaling through the FSH pathway (K Aittomäki et al., 1996; Kumar et al., 1997). Similarly, women with hypogonadotropic hypogonadism caused by *LHB* variants (OMIM:228300) present with oligo-amenorrhea and infertility, may be successfully treated with human chorionic gonadotropin (hCG) (Arnhold et al., 2009).

Intriguingly, in comparison to genes ensuring proper gonadal axis functioning, which cause infertility in both genders, no females with biallelic variants in X-linked *ANOS1* / *KAL1* [HGNC:6211] gene, which causes Kallman syndrome in males, have been reported in the medical literature (Bhagavath et al., 2007; Genetics Home Reference, 2020). Until now variants in an array of genes are well described to cause hypogonadotropic hypogonadism with or without anosmia, most of these genes affect the hypothalamus; please refer to a comprehensive summary of Layman (Layman, 2013).

Hypergonadotropic hypogonadism

In contrast to hypogonadotropic hypogonadism, if serum FSH and LH remain elevated on several occasions, hypergonadotropic hypogonadism is expected, indicating that the defect is at the level of the gonads (i.e., ovaries in females). There are several gonadal disorders with

a clinical presentation of hypergonadotropic hypogonadism and overlapping phenotypical features, e.g., premature ovarian failure / insufficiency, and empty follicle syndrome (EFS).

When a patient has hypergonadotropic hypogonadism, it is always important to think about Turner syndrome (pure monosomy X – 45,X or mosaic forms) (ICD-10 Q96.9) (Layman, 2013). In patients with primary amenorrhea without breast development, but present vagina and uterus, the differential diagnosis should also consider Swyer syndrome – disorder of sex development with an incidence of approximately 1:80000 characterized by pure 46,XY karyotype and complete gonadal dysgenesis i.e. streak gonads [ICD-10 Q99.1] (Da Silva Rios et al., 2015). The female phenotype in these patients is caused by several gene defects, with variants in the *SRY* accounting for about 15% of cases (Sim et al., 2008). Few other genes are related to Swyer syndrome, including, but not limited to *MAP3K1* [HGNC:6848], *DHH* [HGNC:2865], *NR5A1* [HGNC:7983], *NR0B1* [HGNC:7960] – also associated with sex development disorders of various phenotypes (Bashamboo et al., 2010; Gazizova et al., 2020; Paliwal et al., 2011).

The first gene associated with isolated hypergonadotropic hypogonadism [ICD-11 GA30.6] characterized by primary amenorrhea and streak or hypoplastic ovaries in females with normal karyotype, was *FSHR* [HGNC:3969], coding the FSH receptor. Using genetic linkage approach, Aittomaki with colleagues identified variant *FSHR* (NM_000145.4):c.566C > T (rs121909658) segregating with the condition in six Finnish families. Histological examination of the patients' ovaries demonstrated follicular development blocked at the stage of primary follicles (Kristiina Aittomäki et al., 1995; Beau et al., 1998).

Premature ovarian failure

The most common phenotype of hypergonadotropic hypogonadism present in clinical practice is premature ovarian insufficiency, also known as premature ovarian failure (POI / POF). The condition is characterized by the loss of ovarian activity before the age of 40, its incidence increases with age, eventually affecting about 1% of women under 40 (Goswami and Conway, 2005; Laissue et al., 2008). Women affected by POF have females' sex chromosome set (in this case defined by the absence of chromosome Y), nevertheless, X chromosome abnormalities, namely aneuploidies and rearrangements, represent the majority of POF cases (Cordts et al., 2011). Thus, genes situated in these regions are obvious candidates for POF (Laissue et al., 2008). The condition is observed as a syndromic form as well (Caburet et al., 2014; Laissue et al., 2008), e.g., in the case of Turner's syndrome, autoimmune polyglandular syndrome type 1 caused by variants in *AIRE* [HGNC:360] or Blepharophimosis epicanthus inversus and ptosis syndrome (OMIM:110100) caused by variants in *FOXL2*

[HGNC:1092]. Other syndromic associations of POF are extensively summarized in studies of Furtuno and Rosseti (Fortuño and Labarta, 2014; R. Rossetti et al., 2017).

The most common and clinically important cause of non-syndromic POF is CGG trinucleotide / triplet expansion in *FMR1* [HGNC:3775] – the so-called premutation allele. Full mutation in *FMR1* causes fragile X syndrome – an X-linked disorder associated with intellectual disability in hemizygous males. Careful genetic counselling should be offered to patients-ART candidates carrying the *FMR1* premutation, as premutation allele is predisposing to further expansion of the repeat in the germ line and a high risk of delivering a male child with mental retardation. Preimplantation genetic testing could be considered in these cases (Foresta et al., 2002).

More recently, usage of massive parallel sequencing in non-syndromic POF kindreds has revealed variations in a handful of autosomal genes playing a role in the development of ovarian demise. Using an unbiased approach of whole exome sequencing (WES) combined with linkage analysis of a large consanguineous family with inherited POF, a group of Caburet identified a homozygous 1-bp deletion in *STAG3* [HGNC:11356] – a gene encoding a meiosis-specific protein ensuring correct sister chromatid cohesion. It was also demonstrated that female mice devoid of *Stag3* are sterile with their fetal oocytes being arrested at early prophase I (Caburet et al., 2014). Two years later another group, exploiting an analogous approach, strengthened the implication of *STAG3* in POF by identifying a truncating gene variant in consanguineous Lebanese family with two affected sisters presenting primary amenorrhea and an absence of any pubertal development (Le Quesne Stabej et al., 2016). Thus, it was also demonstrated that WES combined with linkage analysis offers a powerful tool to efficiently find novel genetic causes of POF.

At the same time, using WES alone a nonsense homozygous variant was identified in the *SYCE1* [HGNC:28852] as causative for POF in two sisters (De Vries et al., 2014). The implication of the biallelic disruption of the gene in POF was later confirmed by another group, who using WES, revealed a large homozygous deletion in two sisters from a large consanguineous Han Chinese family (Zhe et al., 2020). Simultaneously, a humanized mouse model represented a proof that impaired expression of *SYCE1* disturbs homologous chromosome synapsis during meiosis (Hernández-López et al., 2020). Importantly, the gene is also a candidate for non-obstructive male azoospermia (Maor-Sagie et al., 2015; Pashaei et al., 2020).

The next significant finding in the genetic etiology of POF was *MCM9* [HGNC:21484]. Disruptive gene variants were identified in two unrelated consanguineous families by the means of homozygosity mapping and WES. Individuals

homozygous for the *MCM9* (NM_017696.3):c.1732 + 2T > C (rs587777871) and *MCM9* (NM_017696.3):c.394C > T (rs587777872) pathogenic variants shared a Turner-like phenotype characterized by POF, short stature and low weight (Wood-Trageser et al., 2014). Further, another group provided the first confirmation of the *MCM9* implication in POF by finding a truncating homozygous *MCM9* variant in a consanguineous family (Fauchereau et al., 2016). Paralogue of *MCM9* – *MCM8*, which together form a functioning unit participating in DNA repair by homologous replication, – was also alleged a causality for POF. Biallelic *MCM8* [HGNC:16147] variants were found to segregate with the POF in several unrelated families using an unbiased approach of WES (AlAsiri et al., 2015; Tenenbaum-Rakover et al., 2015; Ya Xin Zhang et al., 2020).

Another gene with the alleged function in the ovaries, *GDF9* [HGNC:4224], has long been assigned a role in POF in various case control studies, where only heterozygous gene alterations were identified (Kovanci et al., 2007; Laissue et al., 2006; Norling et al., 2014). Recently identified homozygous truncating variant strengthened the involvement potential of *GDF9* in the development of POF (M. M. França et al., 2018).

Similarly, it was suggested that *BMP15* [HGNC:1068] variations may predispose to POF (OMIM:300510) and contribute in association with other gene alterations to generate the ovarian defect (Raffaella Rossetti et al., 2009). Initially, only heterozygous gene variants were found (Di Pasquale et al., 2004). Eventually, a homozygous variant inherited from both parents was identified, suggesting that heterozygosity for the *BMP15* is insufficient to cause the phenotype (W. Zhang et al., 2018).

Few other strong POF candidate genes were found using WES, e.g. *HFM1* [HGNC:20193] encoding a protein necessary for homologous recombination (J. Wang et al., 2014) and *NOBOX* [HGNC:22448], playing a critical role in early folliculogenesis with several variants reported in POF patients (Bouilly et al., 2011; Monica M. França et al., 2017; Lin Li et al., 2017). Overall, numerous POF candidate genes also discussed elsewhere await functional and replication studies (Fortuño and Labarta, 2014). As seen from the existing studies, the etiology of POF is vastly heterogenous with WES being the most suitable method to unravel its genetic landscape.

1.1.2 Empty follicle syndrome

With the development of *in vitro* fertilization (IVF) techniques and the burgeoning increase of its application worldwide, the processes of oocyte development, fertilization, and early embryonic development can now be accurately evaluated and investigated, facilitating

the discovery of new phenotypes and genes responsible for female reproductive failure (Sang et al., 2019).

The next phenotype overlapping with POF is EFS – a condition observed after ART application when no oocytes are retrieved from the mature follicles after controlled ovarian stimulation and characterized by a lack of response of these patients to a repeated administration of human chorionic gonadotropin beta (β-hCG). The condition was first described in 1986, its frequency is estimated to range from < 1–7% among patients presenting for IVF treatment (Awonuga et al., 1998; Coulam et al., 1986; Yariz et al., 2011). However, genuine EFS occurs only around 0.016% (Revelli et al., 2017), in contrast to false EFS which is associated with circulating β-hCG below a critical threshold and flaws in ART performance.

The first insight into the genetic etiology of genuine EFS came from the work of Yariz with colleagues (Yariz et al., 2011), who performed unbiased WES of two affected sisters described earlier (Önalan et al., 2003). They identified a homozygous variant in *LHCGR* [HGNC:6585] that cosegregated with EFS in the family and was not present in 500 ancestry-matched controls. The variant impairing the function of *LHCGR* was considered compatible with the lack of response in these patients to a repeated administration of β-hCG (Yariz et al., 2011). Females with defects in this gene showed normal secondary sex characteristics but were infertile due to LH resistance – clinical presentation also typical for hypergonadotropic hypogonadism. Following the success of Yariz, few other groups found evidence for *LHCGR* variation as a cause for EFS, e.g. group of Yuan using Sanger sequencing found gain-of-function biallelic variant *LHCGR* (NM_000233.4):c.1345G > A (rs763889232) in a patient with genuine EFS (Yuan et al., 2017). Notably, the biallelic variants in *LHCGR* are well known to cause Leydig cell hypoplasia in males resulting in various phenotypes of sex development disorders (OMIM:238320).

The spectrum of phenotypic expressions related to *LHCGR* are summarized in Yuan study (Yuan et al., 2017). Reproductive pathogenesis in females with *LHCGR* variations lies in the fact that the receptor cannot be expressed in *theca* or *granulosa* cells, therefore cannot bind LH and β-hCG, eventually leading to low estrogen production and consequently – impaired folliculogenesis (C. Chen et al., 2018).

Recently, a completely different mechanism of action was described leading to a similar clinical representation of EFS observed during IVF. In a number of patients from consanguineous families, WES application led to an identification of pathogenic biallelic variants in genes coding the oocyte's zona pellucida proteins, respectively *ZP1* [HGNC:13187], *ZP2* [HGNC:13188], and *ZP3* [HGNC:13189] (Dai, Chen, et al., 2019; Dai, Hu, et al., 2019; Q. Xu et al., 2020; Z. Zhou et al., 2019). All patients presenting primary infertility due to EFS

and / or having few abnormal oocytes or small ooplasm-like fragments following the aspiration of mature follicles. In contrast to the EFS caused by *LHCGR*, these patients exhibit normal ovarian reserves, regular menstrual cycles, and their basal sex hormone levels and other infertility-related examinations do not reveal any abnormalities. As seen, the infertility in these patients no longer lies within the action of the hypothalamic-pituitary-gonadal axis, however, the question, whether it should be considered hypogonadism, remains open.

Intriguingly, the variants in *ZP1*, *ZP2*, and *ZP3* have been also linked to the reproductive failure characterized by oocyte degeneration, absent *zona*, or failed fertilization (also discussed below).

1.1.3 Absent zona pellucida

The initial study, focusing on identifying a genetic cause of ART failure due to the absence of *zona pellucida* (ZP) in oocytes, performed Sanger sequencing of *ZP1*, *ZP2*, *ZP3*, and *ZP4* [HGNC:15770] in a consanguineous Han Chinese family. They identified a homozygous truncating variant in *ZP1* in six family members segregating with the disease. (H.-L. Huang et al., 2014). Further two groups identified only heterozygous variants in ZP genes in several families with multiple infertile women, who presented only (degenerated) oocytes with absent ZP, cumulus-oocyte complexes lacking oocyte (corresponds to EFS) or only small fragments of ooplasm upon oocyte retrieval after ovarian stimulation (T. Chen et al., 2017; P. Yang et al., 2017). Soon, causality of biallelic disruption of ZP genes was proved by independent studies via the usage of WES and cosegregation analysis in consanguineous families. Functional studies demonstrated that the absence of any of the ZP proteins disrupts interaction among ZP1, ZP2, and ZP3, preventing the formation of functional ZP. Defective and thin ZP forming in certain cases was defective for sperm-binding (Dai, Hu, et al., 2019; Sun et al., 2019).

1.1.4 Oocyte maturation arrest

The maturation of an oocyte reflects its intrinsic developmental competence and is a key factor for successful fertilization, the growth and development of an embryo, as well as infertility treatment (P. Yang et al., 2017). Oocyte maturation arrest occurs at different stages, including the germinal vesicle (GV), meiosis I and II (MI, MII) leading to reproductive failure (Beall et al., 2010; Dean, 2016). At present, in order to conceive, patients with oocyte maturation arrest need to receive donor eggs (A. C. Wang et al., 2018).

Studies have suggested that some human oocyte maturation arrest and early embryonic arrest cases follow a Mendelian inheritance pattern (B. Chen et al., 2017). Although the first case of human oocyte maturation arrest was reported in 1990 (Rudak et al., 1990), genetic factors related to these phenotypes have been poorly investigated, remaining largely unknown (Okutman et al., 2020; A. C. Wang et al., 2018). However, despite the relatively recent implication with the condition, phenotype-genotype correlation is well established for the two genes, *TUBB8* [HGNC:20773] responsible for ~33% of the oocyte maturation arrest cases, and *PATL2* [HGNC:33630] (OMIM:616780 and 617743, respectively) (A. C. Wang et al., 2018).

The protein encoded by *TUBB8* represents the primary beta-tubulin subunit of oocytes and the early embryo. Pathogenic gene variants affect folding and assembly of the α / β-tubulin heterodimer, eventually causing spindle assembly defects that result in oocytes arrested at the GV and/or MI stages, polar body I oocytes that cannot be fertilized, and embryos arrested at an early stage (A. C. Wang et al., 2018).

Of note, *TUBB8* is one of the rare genes with the proposed autosomal dominant inheritance pattern causing infertility in human. In affected females, heterozygous *TUBB8* variants are inherited from the unaffected fathers and exhibit a dominant-negative effect, indicating that TUBB8 has a specific pathophysiological role in oogenesis but not in spermatogenesis. However, homozygous individuals are also described (Feng et al., 2016; L. Huang et al., 2017). Intriguingly, one group showed that exogenous wild-type *TUBB8* supplementation could effectively improve spindle assembly, allowing for the subsequent embryo development and production of live offspring in mice (Jia et al., 2020).

Numerous individuals, mostly from consanguineous families, suffering from the same issue of oocyte arrest at different stages and also early embryonic arrest, were reported as having pathogenic biallelic *PATL2* variants (B. Chen et al., 2017; Z. Liu et al., 2020; L. Wu et al., 2019). Functional studies demonstrated that *PATL2* was more highly and specifically expressed in the human germinal vesicle, meiosis I oocytes and polar body I oocytes than in various kinds of somatic tissues (B. Chen et al., 2017).

Important is the fact that in order to conceive, patients with oocyte maturation arrest need to receive donor eggs (A. C. Wang et al., 2018). The genetic diagnosis of the oocyte maturation failure could save a couple from the financial and emotional hardship of multiple failed cycles using own oocytes (Beall et al., 2010).

1.1.5 Fertilization failure

Two of the most important processes of sexual reproduction are, first, meiosis resulting in specialized gametes that contain half the normal number of chromosomes, and, second, fertilization, when two gametes recreate a genetically distinct organism and restore the normal number of chromosomes (Bianchi and Wright, 2016). During fertilization, an ordered schedule of cellular recognition events ensures a successful fusion of the sperm and egg. Despite the fundamental role of fertilization, the basic mechanisms involved have remained poorly understood (Bianchi and Wright, 2016). ART provides a unique real-time insight into these otherwise inaccessible events.

Failure of IVF can be caused by a multitude of factors. However, failure of fertilization despite an intracytoplasmic injection of apparently healthy sperms in apparently healthy eggs is highly unusual. The observation of this phenotype only in female members of certain families suggested that this phenotype is female-limited, perhaps due to a recessive variant of a maternal effect gene (Alazami et al., 2015). Two independent groups studying patients with recurrent failure of IVF / intracytoplasmic sperm injection (ICSI), displaying an abnormal fertilization with zero-pronucleus (PN) zygotes, 3PN zygotes, or zygote degradation identified biallelic *TLE6* [HGNC:30788] variants (Alazami et al., 2015; J. Lin et al., 2020) as a cause of earliest known human embryonic lethality. Pronuclei formation is a critical process during fertilization. Normally, there are two pronuclei (2PN), one paternal and one maternal, indicating a successful fertilization (Payne et al., 1997). *TLE6* exhibit its function within a subcortical maternal complex that assembles during the oocyte growth and is essential for zygote to progress beyond the first embryonic cell divisions in mice (Lei Li et al., 2008).

The next gene implicated with recurrent pronucleus formation failure and female reproductive failure was *WEE2* [HGNC:19684] (Sang et al., 2018; X. Yang et al., 2019; Zhao et al., 2019), biallelic variants in which resulted in the phenotype. Another candidate gene for the fertilization failure due to multiple pronuclei formation is *REC114* [HGNC:25065], which is involved in DNA double-strand break formation during meiosis.

1.1.6 Early embryonic arrest

Normal embryonic development is the key to establishing a successful pregnancy. Recent evidence has raised the possibility that a part of idiopathic reproductive failure cases may be caused by very early forms of embryonic lethality (Alazami et al., 2015). Without ART application, the phenotype of fertilization failure or early embryonic arrest could be credited as idiopathic infertility in man or women. Embryo cultivation after fertilization, especially with

the usage of time-lapse imaging, granted access to the cause of infertility in these couples. Early embryonic arrest is referred to as preimplantation embryo lethality and failure of an embryo to form a blastocyst (OMIM:616814).

Although the zygote is formed by the fusion of the maternal and paternal pronuclei, the earliest phases of embryo development are uncoupled from new transcription and rely exclusively on the maternal macromolecules deposited in the ooplasm during the oocyte growth. Disruption of several genes with the alleged role within the subcortical maternal complex – a multiprotein complex uniquely expressed in mammalian oocytes and early embryos essential for human early embryonic development (Bebbere et al., 2016) – has recently been linked to the early embryonic arrest. This also includes the earlier-mentioned *TLE6*, which also causes fertilization failure (Y. Xu et al. 2016).

In 2016, Xu with colleagues attempted to analyze whole exomes of three sisters from a consanguineous family experiencing repeated early embryo arrest. The only variant segregating with the phenotype was a homozygous nonsense change NM_207421.4:c.1141C > T (rs1057517681) in *PADI6* [HGNC:20449]. These findings were later replicated, and a functional study revealed that *PADI6* was highly expressed in oocytes, but weakly – in sperm or somatic tissues (Y. Xu et al., 2016).

Later sequence changes in two other subcortical maternal complex genes were found in patients from consanguineous families with embryos failing to reach the cleavage stage / develop into blastocysts. Five independent individuals carried biallelic variants in *NLRP2* [HGNC:22948]. Three individuals from two families carried biallelic variants in *NLRP5* [HGNC:21269] (Mu et al., 2019). Of note is finding that a gene with a similar function to *NLRP2*/*5*, *NLRP7* [HGNC:22947] has been associated with the development of hydatidiform moles as a rare and specific cause of failed pregnancy (a closer discussion below).

It has been shown in mice that knockout of any of the subcortical maternal complex genes leads to infertility or subfertility caused by embryonic arrest (Mu et al., 2019; Yurttas et al., 2008), since the complex is necessary for embryonic progression past the 2-cell stage (Lei Li et al., 2010).

1.1.7 Failed embryo implantation

Following the early embryonic arrest, failed embryo implantation is the next phenotype leading to infertility assessable only during ART application. The term implantation failure refers to the cases when after embryo transfer patient does not show quantifiable signs of implantation, such as increased levels of hCG, or who have increased hCG production without later ultrasound evidence of a gestational sac (Coughlan et al., 2014). The failure

of embryo to implant can be a consequence of female, male, or embryo factors, or the specific type of IVF protocol (Bashiri et al., 2018). The primary cause of failed embryo implantation in ART is considered aneuploidy (Toft et al., 2020).

The frequency of losses in human preimplantation embryos is known to be very high. Of morphologically normal embryos about 50–80% show numerical chromosomal abnormalities, depending upon maternal age. These data were initially based on observation of couples undergoing preimplantation genetic testing for monogenic disorders (PGT-M) (Munne et al., 1996), whose embryos were studied using fluorescent *in situ* hybridization with five to seven chromosome-specific probes. Using comprehensive chromosomal screening methods, rates of aneuploidy are as high as 85–100% in women aged 43 and above (M. Rabinowitz et al., 2012). Earlier, our group has also demonstrated that 57% of embryos are aneuploid in patients with complicated reproductive history (L Volozonoka et al., 2015). However, no single gene alterations leading to a (recurrent) embryo implantation failure can be found in the scientific literature.

1.1.8 Miscarriage

Up to 15% of all clinically recognized pregnancies end in miscarriage (Everett, 1997). Approximately 5% of women experience recurrent pregnancy loss (RPL) if two or more pregnancies miscarry, whereas three or more first trimester pregnancy losses may affect as many as 1–2% of women of reproductive age (Clifford 1994; Cook 1995; Stirrat 1990).

Common causes of RPL are immunological, endocrine, anatomic, or genetic factors. About 50% of RPL cases still remain unexplained. In this group of patients, fetal chromosomal abnormalities have been reported to be the most common cause of RPL, similarly, as it is with the implantation failure, leaving the remainder truly unexplained (Jeve and Davies, 2014; Sugiura-Ogasawara et al., 2012). The first evidence demonstrating that RPL couples have an increased number of chromosomally abnormal embryos (ranging from 50–80%) was shown (Simón et al., 1998) and confirmed by different groups (Garrisi et al., 2009; RubIo et al., 2009). The prevalence of fetal chromosome abnormalities in women facing a single sporadic miscarriage is around 45% (van den Berg et al., 2012).

The next common and well-described cause of miscarriage and unbalanced chromosomal aberrations in fetus is parental balanced chromosomal rearrangements (Kochhar and Ghosh, 2013; Stern et al., 1999). Other genetic abnormalities in the conceptus leading to pregnancy loss are skewed X inactivation and inherited or *de novo* single gene disruptions (Blue et al., 2019). Increased lethal single-gene disorders leading to pregnancy loss or

intrauterine fetal death have been indirectly demonstrated in consanguineous couples, in whom the aneuploidy rates are significantly lower than in outbred populations (Najafi et al., 2019).

Although RPL is considered a multifactorial disorder, there is a hypothesis that it can develop in a single-gene fashion (Bolor et al., 2009). Unfortunately, the collected evidence for this so far is poor and contradictive leaving the true genetic etiology of miscarriage still *terra incognita.*

Hydatidiform mole

In contrast to miscarriage, a certain success has been reached in the genetic cause identification of a specific type of pregnancy loss / abnormal embryo formation called hydatidiform mole. Development of the conceptus into a hydatidiform mole results from the overgrowth of the extraembryonic trophoblast, whereas the embryo itself suffers an early demise. In most complete hydatidiform moles, the conceptus is wholly androgenetic, i.e. paternal, in origin (Parry et al., 2011). Notably, in the familial form of the disorder [OMIM: 231090], the molar tissues are not androgenetic but show a normal pattern of biparental diploid inheritance.

The familial biparental hydatidiform mole was shown to display a pure maternal-effect autosomal recessive inheritance (Van den Veyver and Al-Hussaini, 2006). Biallelic variants in *NLRP7* were identified (Murdoch et al., 2006) in patients suffering from this condition, and subsequent reports have confirmed the findings suggesting that pathological *NLRP7* variants are found in the majority of families with familial biparental hydatidiform mole (Hayward et al., 2009). Soon, a biallelic disruption of the second gene, *KHDC3L* [HGNC:33699], was implicated in the condition (Parry et al., 2011; Rezaei et al., 2016). Overall, about 55% of the cases are caused by variants in *NLRP7* and about 5% – in *KHDC3L* (Hammouda, 1964). Notably, both genes are members of the subcortical maternal complex, mentioned earlier.

Later, androgenetic complete hydatidiform moles with all chromosomes originating from a haploid sperm and no maternal chromosomes were associated with biallelic deleterious variants in *MEI1* [HGNC:28613], *C11orf80* [HGNC:26197], and *REC114*. All three genes play key roles in the formation of double-strand DNA breaks, which is essential for homologous chromosome synapsis and recombination during meiosis I (Nguyen et al., 2018). Strikingly, in one family biallelic *MEI1* variants were found in a male with azoospermia inherited from both parents (Nguyen et al., 2018). It transpires that there are more reports linking male infertility with the gene (Ben Khelifa et al., 2018; Krausz et al., 2020). This study also demonstrated that *Mei1*$^{-/-}$ mice's oocytes extruded all the chromosomes into the polar body, explaining the fully androgenetic genome of the mole. The overall great work of Nguyen and

colleagues successfully unravels the genetic etiology of hydatidiform moles and miscarriage formation (Nguyen et al., 2018), and the further replication and functional studies are awaited.

If etiology of the hydatidiform mole remains unknown, the risk of recurrent molar pregnancy is around 1.5% after one molar pregnancy and around 25% after two molar pregnancies. Women presented with pathogenic variants in the *NLRP7, KHDC3L* or *PADI6* are unlikely to obtain normal pregnancies, with a major risk of reproductive failure (Cozette et al., 2020); and oocyte donation may be the option of choice.

1.1.9 Preterm birth

Birth at less than 37 weeks of gestation – important but poorly understood outcome of pregnancy – represents a major public health problem in the United States and worldwide (Chaudhari et al., 2008; Frey and Klebanoff, 2016). Determining the genotypes associated with PTB has been especially difficult due to the multifactorial contributors, heterogeneous phenotypes, and the involvement of two genomes (Chaudhari et al., 2008; Plunkett and Muglia, 2008).

Studies in humans of both familial aggregations and racial disparities in PTB have contributed to the understanding that heritability is evident in prematurity, a significant portion of which is due to polygenic causes with few monogenic contributions (Chaudhari et al., 2008; Porter et al., 1997; Winkvist et al., 1998). For example, Ehlers-Danlos Syndrome is a genetically heterogeneous connective tissue disorder associated with an increased risk of PTB and other pregnancy complications (Lind and Wallenburg, 2002; Murray et al., 2014; Sorokin et al., 1994). Although the overall contribution of the syndrome to the incidence of preterm birth is minimal, the existence of this predominantly autosomal dominant disorder demonstrates that genetic effects can significantly increase the risk of PTB (Chaudhari et al., 2008).

Few studies have found common DNA variations to be associated with PTB, but meta-analyses indicate that these are at best weak or population-specific (Pereza et al., 2017; H. Wu et al., 2017). Although common variants detectable by genome wide association studies (GWAS) typically explain only a minor proportion of the heritability of complex diseases (Asimit and Zeggini, 2010), GWAS can indicate genes having a substantial biological value in understanding the PTB (Brubaker et al., 2016).

Certain success in elucidating the genetic context of idiopathic non-syndromic PTB has been reached using genome wide association studies. Thus, genotyping a large cohort of preterm infants (< 36 weeks of gestation) of Finnish origin has revealed an association with the *SLIT2* [HGNC:11086] gene. Both SLIT2 and its receptor ROBO1 were shown to locate in villous and decidual trophoblasts of embryonic origin, and SLIT2-ROBO1 signaling was

linked with regulation of genes involved in inflammation, pregnancy-specific glycoproteins, decidualization and fetal growth (Tiensuu et al., 2019). Unfortunately, even highly significant single nucleotide variants (SNVs) associated with prematurity often fail to replicate in independent cohorts (Brubaker et al., 2016), but usage of extremely large cohorts with the aid of the platforms like 23andMe can help (G. Zhang et al., 2017).

As seen, identification of genetic causes of PTB is rather burdensome and new scientific approaches are needed. Although the largest number of studies has focused on idiopathic PTB, this phenotype should be considered with caution, since PTB often encompasses cervical insufficiency, premature rupture of membranes, placental abruption, uterine overdistension, or a combination of these (Manuck, 2016). Therefore, extensive phenotyping and subgrouping of the patients based on their phenotype should precede any genetic study in order to identify more specific genetic markers.

1.1.10 Eugonadal disorders

Female reproductive system disorder classification based on serum gonadotropin levels and functional activity of the gonads implies two main groups: the hypogonadal disorders reviewed earlier, and much more common eugonadal disorders characterized by a normal estrogen state (Trofimova et al., 2017). Genetic association studies have shown certain common alleles that are linked to eugonadal disorders (e.g. endometriosis (Painter et al., 2011; Uno et al., 2010)), but these findings do not signify causation. The causation requires demonstration of gene variants impairing normal function, segregation with the disease phenotype, and *in vitro* evidence showing biological plausibility (Trofimova et al., 2017). Nevertheless, some genotype-phenotype correlations deserve to be acknowledged in connection with eugonadal conditions.

Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is one of the most common female endocrine disorders and a leading cause of female subfertility found in about 7% of women of reproductive age, and in about 40% of women who are struggling to conceive (Knochenhauer et al., 1998). The genetic basis of the condition is not well understood, but familial cases have been recognized. The most common biochemical abnormality associated with PCOS is hyperandrogenemia. Therefore, researchers have long been trying to analyze the number of genes involved in the androgen biosynthetic pathway for association or linkage with PCOS (Unluturk et al., 2007). Thus, in one study a linkage evidence was established with *FST* gene,

which encodes the protein inhibiting FSH release (Urbanek et al., 1999). Unfortunately, subsequent comprehensive studies conducted by the same authors did not replicate the initial findings (Urbanek et al., 2000).

Similarly, *CYP11A1* [HGNC:2590] and number of other cytochrome P450 family genes remain potential candidates for PCOS pathogenesis. Notably, few other genes from the same family e.g., *CYP21A2* [HGNC:2600] (OMIM: 201910), *CYP11B1* [HGNC:2591] (OMIM: 202010) are known to cause syndromic forms of female infertility due to congenital adrenal hyperplasia clinically associated with PCOS (Reichman et al., 2014). But further investigations are required to link these steroid biosynthesis gene variants to PCOS (Gaasenbeek et al., 2004; Unluturk et al., 2007; Witchel and Aston, 2000).

As infertility is one of the primary manifestations of PCOS, it is the most important factor limiting the availability of large pedigrees for linkage studies resulting in more negative than positive findings (please see the great review of Unluturk et al., 2007). Recently, a large-scale meta-analysis of GWAS covering more than 10'000 PCOS cases (with the 23andMe platform contributing to the study) demonstrated 14 genetic susceptibility loci associated with PCOS, 11 of which were replicated from previous studies being implicated in neuroendocrine, metabolic, and reproductive pathways and three completely new ones (SNVs next to *PLGRKT* [HGNC:23633], *ZBTB16* [HGNC:12930] and *MAPRE1* [HGNC:6890]) (Day et al., 2018). At this point, findings from such large genetic studies help to elucidate the pathophysiology of PCOS but cannot be used as diagnostic / prognostic markers for the disease.

Leiomyomata

Leiomyomata – benign smooth muscle tumors of the uterus (also known as fibroids) – is one of the most common diseases in gynecological practice. These tumors are clonal and somatic in origin with uncertain etiology. When fibroids occur in unusual locations in association with other phenotypic expressions, at least two Mendelian forms should be considered. Firstly, hereditary leiomyomatosis associated with renal cell cancer can be caused by autosomal dominant variants in *FH* [HGNC:3700] (Tomlinson et al., 2002). Secondly, Alport syndrome, caused by changes in genes coding the type IV collagen, characterized by glomerulonephritis, hearing loss, eye disease, and diffuse leiomyomatosis (Hansen et al., 2020; J. Zhou et al., 1993). However, *COL4A5* [HGNC:2207] and *COL4A6* [HGNC:2208] deletions were also implicated in the development of non-syndromic forms of leiomyosarcoma (B.J. et al., 2017). Additionally, it was shown that 75% of women with uterine fibroids had variants in the *MED12* (Mäkinen et al., 2011). Notably, germline variants in *MED12* [HGNC:11957]

results in severe childhood X-linked recessive syndromes (OMIM:309520, 300895, 305450) without predisposition to fibroids.

Spontaneous ovarian hyperstimulation syndrome

Normally, ovarian hyperstimulation syndrome (OHSS) is an iatrogenic disorder resulting from the administration of exogenous gonadotropins for infertility treatment (Siegel et al., 2013). Although inactivating variants in *FSHR* are implicated in hypergonadotropic hypogonadism (Aittomaki et al., 1995), activating gene variants are now a well-described cause of the spontaneous familial forms of OHSS occurring during the first trimester of pregnancy (Montanelli et al., 2004; Smits et al., 2003; Vasseur et al., 2003). Impaired receptor displays promiscuous activation by both hCG and thyroid stimulating hormone explaining the clinical presentation of the condition.

Mullerian aplasia

Mullerian aplasia, also known as Mayer–Rokitansky–Küster–Hauser syndrome – the most severe uterine anomaly – presents with congenital absence of uterus and vagina and occurs in ~ 1:5000 women and in ~10% of women with primary amenorrhea (Reindollar et al., 1986). These patients display normal female karyotype and typically normal ovarian function with normal development of breasts and external genitalia. The condition is often syndromic, however, the genetic basis of it in human is largely unknown because the genetic transmission is difficult to ascertain, since affected families are often small and affected individuals are unable to have children unless they undergo surrogacy (Petrozza et al., 1997).

A strong candidate gene, most often implicated with the condition – *WNT4* [HGNC:12783], initially was found to account for ~10% of the cases (Philibert et al., 2008). However, the authors only performed targeted sequencing of the gene and did not perform parental DNA analysis to establish the inheritance pattern of the identified variant. Surrogacy results strongly suggest that congenital absence of the uterus and vagina is not inherited in a dominant fashion as shown by 17 born females without congenital anomalies from the oocytes of females with Mullerian duct aplasia (Petrozza et al., 1997).

A later replication study sequenced 100 probands with Mullerian aplasia and failed to reveal the evidence of deletions, small indels, or likely pathogenic variants in *WNT4*, as well as two other candidate genes, *HNF1B* [HGNC:11630] and *LHX1* [HGNC:6593] (Williams et al., 2017). In turn, the same group identified large CNVs in 19% of the patients, including 17q12 deletion – one of the most commonly identified CNVs in Mullerian aplasia, suggesting

that genes within this region play some role. Overall, the repetitive identification of few CNVs suggests their involvement, however, further work is needed to prove causation (Williams et al., 2017).

1.2 Genomic approaches to female reproductive failure

1.2.1 Historical insight

The oldest studies with an implication into the genetics of infertility in the scientific literature could be spotted already about a century ago. For example, X-rays were exploited to generate chromosomal aberrations and study the heritability of the so-called semi-sterility |in mice (Snell, 1933, 1941). Despite experiments using radiation on different animals to actually induce infertility, a group of clinicians applied X-rays to the pituitary and / or ovaries of more than 700 women with the intention to actually cure infertility (Kaplan, 1954). This study received extensive criticism by the geneticists, stating that such controversial application of radiation by a "genetically untrained obstetrician" would result in irreparable chromosomal and gene variants that would eventually induce hereditary changes, referring to the work of Nobel Prize geneticist H. J. Muller as follows "each newly mutated gene, no matter how small the detriment it occasions, eventually takes its toll in the form of making a major contribution to the extinction of the line of descent" (Rugh, 1955). In turn, this criticism was called a "genetic speculation" eventually resulting in hot debates in the "The Journal of obstetrics and gynaecology" (KAPLAN, 1956).

Notwithstanding the thorny path of genetics into different medical fields, major concepts of female infertility genetics compatible with the ones we know today, were formulated already in the middle of the twentieth century. It was known that reduced fertility may be the result of a faulty genetic constitution in the patient or the products of conception (zygote, embryo, or fetus) of a normal patient (J. R. Miller, 1965). Great investigations have been carried out to unravel the fertility issues caused by various errors in sex chromosomes, including Turner syndrome, chromosomal lesions and mosaicism (Harnden and Jacobs, 1961; McKusick, 1962). Spontaneous aneuploidies (Carr, 1963) or inherited from translocation carrier parents unbalanced chromosomal rearrangements in a fetus were uncovered to lead to a miscarriage (Boué et al., 1975). Even though certain genes were not known back then, the genetic origins of reduced fitness and fertility of syndromic and isolated nature caused by single gene defects and of various types of inheritance were also described (J. R. Miller, 1965).

1.2.2 Research based studies

Genetic linkage and association studies

Before the advent of unravelling the human genome and the development of high throughput technologies, family-based linkage studies allowed the identification of a locus of interest based on cosegregation of a chromosomal region with a certain trait within one or preferably several families (Monangi et al., 2015). Traditionally, individuals in extended family pedigrees would be genotyped with a set of genome-wide markers that would narrow the region of interest to approximately mega base size chromosomal region (Ott and Wang, 2011).

When the function of the majority of protein-coding genes and their pathway relationships were known, testing selected candidate genes for linkage and especially genetic associations became incredibly popular. For instance, Urbanek with colleagues tested 37 candidate gene variations in families with PCOS for linkage and associations, found a significant linkage with follistatin gene (Urbanek et al., 1999). Similarly, *LH* variants were associated with disturbed pituitary-gonadal function, menstrual disorders and female infertility (Liao et al., 1998; Ramanujam et al., 1998).

With the advancing of the genetic techniques, large GWAS, typically with a case-control design, were utilized to study complex diseases. GWAS approach involves rapid scanning of markers across the complete sets of DNA or even genomes of many people thus allowing to identify the genetic variations associated with a particular disease without initial hypothesis, and therefore offers the advantage to overcome difficulties imposed by the incomplete understanding of the disease pathophysiology (Monangi et al., 2015). For example, a GWAS using a high-density SNP-array in seven large Northern Finnish non-consanguineous families, complemented with segregation analysis and supported by a follow-up case-control study, identified a locus on 15q26.3 containing the *IGF1R* [HGNC:5465] gene as a susceptibility marker for PTB (Haataja et al., 2011).

In general, the linkage approach had proven useful with single gene disorders, but not complex traits (Altmüller et al., 2001). In addition to limited resolution, GWAS approach is very sensitive to locus heterogeneity – a major drawback with complex traits. Genetic implications revealed through association studies are typically very weak, but while they may not explain much of the risk, they can provide a significant insight into genes and pathways that can be important. Additionally, the interpretation of association findings in pregnancy related phenotypes is particularly precarious – the correlation between fetal and maternal genotypes makes it difficult to ascribe findings to either source unless both the mother and the infant are genotyped (Hill et al., 2011).

Gene expression profiling

Another general approach for identifying genes involved in a certain phenotype is gene expression profiling, which involves searching for differences between the gene expression in the patients' tissue of interest and control tissue. Resembling the natural path of DNA studies, candidate gene approach was also used to study gene expression. The early studies involved immunohistochemistry, allowing to assess gene expression at the protein level. Further expression differences on mRNA level were largely exploited. For example, dysregulation of anti-inflammatory cytokines in the human cervix was found to be involved in the pathogenesis of PTB (Dubicke et al., 2010).

While target gene approach is subjected to a bias, genome wide expression profiling promises to reveal objective transcriptomic landscape. Makieva with colleagues exploited genome wide RNA expression arrays to analyze cervical transcriptome signature in women experiencing preterm labor alone in comparison to women with premature prelabor rupture of fetal membranes (PPROM). They identified four novel proteins acting through modulation of gelatinases MMP2 and MMP9, which degrade collagens type I and III (the main constituents of the cervical extracellular matrix) and in this way potentially affect cervical remodeling eventually leading to PPROM (Makieva et al., 2017). Similarly, transcriptomic analyses revealed a unique expression signature for idiopathic spontaneous PTB distinct from controls who delivered prematurely due to infection. This finding included the upregulation of IGF binding proteins, supporting the role of aberrant IGF signaling in spontaneous PTB (Brockway et al., 2019). Another group were first to develop the transcriptome-wide approach for assessing embryo implantation competence based on low-input RNA sequencing, thus establishing the foundation for RNA-based diagnostic in IVF in the future (Groff et al., 2019).

In general, gene expression studies dissect the genetic and molecular mechanisms underlying various reproductive phenotypes and inspire further investigations toward the development of better diagnostic tactics and possibly targeted therapies.

Modern designs

The fact that some early methods have outlived themselves, also remaining gaps in the knowledge of the genetics of particular traits, has prompted more sophisticated and complex study designs success, which was mainly dictated by the following factors: precisely phenotyped patient cohort; thoroughly selected methodological package; usage of state-of-art bioinformatic tools and pipelines.

In such a way even association studies acquire absolutely different sense. Example of such non-straightforward design is the research conducted by McElroy with colleagues while studying PTB. Initially, using WES, they identified novel variants shared between mother-daughter pairs experiencing PTB. Further, these genes were investigated for pathway aggregations. Genes from the enriched pathways were then surveyed for association in a larger number of nuclear families. As a result, they found statistically significant associations in three *CR1* [HGNC:2334] SNVs and revealed over-representation of rare variants in the complement / coagulation factor cascade (McElroy et al., 2013).

One successful and relatively simple approach in terms of identifying causative disease variants is based on genotyping consanguineous families. Consanguineous marriages significantly contribute to the manifestation of rare autosomal recessive disorders (J. R. Miller, 1965), including severe cases of reproductive phenotypes like sterility or repeated miscarriages. In cases of consanguinity, it is likely that disease-causing variant will lie within homozygosity stretches of the family members. Now, when the NGS approach has been widely available, these studies resulted in the discovery of a significant number of genes implicated in female infertility and are broadly described in this literature review.

1.2.3 Genetic testing in a diagnostic setting

Whilst research is free in terms of choosing approaches and methodologies, drafting conclusions and being responsible in front of a patient, the main tasks of genetic testing in a diagnostic setting should follow quite a steady path, respectively, elucidate the true genetic cause / risk of the disease clearly defining testing limitations; identify relatives having an increased risk of developing the condition; identify genetic diseases transmissible to offspring; identify specific subtypes of the condition suitable for tailored management if such exists; and the one especially applicable to human reproduction – optimize the usage of the ART.

Several recommendations have been established for genetic preconception carrier screening, including the ones in the frame of ART (Edwards et al., 2015), however, no guidelines or committee opinions are released regarding the genetic testing in female reproductive failure. Consequently, very few specific tests are routinely recommended to investigate the presence of chromosomal disorders or single-gene defects related to their clinical phenotypes (Cariati et al., 2019).

There is one publication addressing the common guidelines for the appropriate use of genetic tests in infertile couples. The committee has recommended karyotype analysis during the diagnostic workup of infertile women presenting with primary ovarian dysfunction or recurrent fetal loss; *FMR1* expansion testing during the diagnostic workup prior to the ART

of women with oligomenorrhea caused by POF or poor response to ovarian stimulation in ART cycles; *ANOS1* analysis in women with hypogonadotropic hypogonadism, and *CFTR* [HGNC:1884] screening since the prevalence of pathogenic *CFTR* variants in the general population is high (Foresta et al., 2002). While the authors worthily emphasize that genetic diagnosis and genetic counselling should always be part of an extensive evaluation of these patients, and basic clinical analysis should precede any genetic analysis, the recommendation for assessing karyotype and only three genes seems outdated in the light of an acquired knowledge during the last two decades after the initial work has been released, and there is an urgent need in establishing up to date guidelines for genetic testing in female infertility.

Despite the lack of attention to this important subject matter, there are some initiatives to establish genetic testing panels for conditions affecting female reproduction to be used in a clinical setting. For example, one group has developed a targeted NGS panel consisting of 87 genes related to both male and female infertility (Patel et al., 2018). The authors have covered conditions related to female reproductive failure like POF, PCOS, OHSS and thrombophilia-related pregnancy loss. Unfortunately, the gene selection process and especially gene-phenotype correlation assessment of the included genes is unavailable, but as seen in the gene list, the included genes cover syndromic and non-syndromic forms of infertility and for some of them causality link has not been unequivocally established.

Similarly, Strom with colleagues has established a female infertility test called Fertilome, which is available for purchase online. Authors claim that they have applied principles of clinical validity classification framework to rank the evidence linking the genes with the reproductive conditions, eventually coming up with a set of 46 variants across 32 genes increasing the risk of one or more of the following reproductive phenotypes – endometriosis, PCOS, POI, RPL, idiopathic infertility, and recurrent implantation failure (Strom, Northrop, and Beim 2019; Sartor 2019, date accessed 18.05.2020.). Again, how they got to the final list of genes and variants is not easily accessible information. Thus, it is difficult to evaluate the clinical utility of the panel, especially given the fact that the covered conditions are known to have complex etiologies.

While the authors state that from a clinical care perspective, such NGS tests have the ability to influence the key decisions in patient management (Patel et al., 2018), evidence for that is largely limited. Currently, decisions like early oocyte cryopreservation in case of POF or selection of a specific ovarian stimulation protocol in case of PCOS are mostly influenced by the clinical presentation of the condition.

In contrast, Centogene, a reputable genetic testing company offers female infertility NGS based test consisting of eight genes, all with an established clinical relevance to infertility phenotypes ("Female Infertility Panel", date accessed 18.05.2020.): *BMP15*, *CYP21A2*, *FSHR*, *LHB*, *LHCGR*, *TUBB8*, *ZP1*, and *FMR1*.

Notwithstanding the unsettled state of genetic testing in female reproduction, there are several tests, which conquered its niche and are routinely applied during the female reproductive workup in a diagnostic setting. For example, karyotyping is used to assess chromosomal changes like Turner syndrome, or to distinguish Swyer syndrome in phenotypical females with 46,XY chromosomal composition, caused by gene defects and considered a disorder of sex development. Karyotyping currently is the only methodology applied to diagnose balanced karyotype changes, as structural autosomal aberrations may be found in about 5% of females with non-syndromic infertility (Gekas et al., 2001).

The next well-known test with an established position in female infertility is the assessment of CGG repeat expansion in *FMR1*. Triplet expansions are considered one of the most difficult genetic regions to analyze. Currently, within the diagnostic setting the *FMR1* CGG expansions are analyzed, using fragment size analysis function of capillary electrophoresis exploiting few commercially available kits or Southern blots for large expansions. However, technological advances are now pushed forward to allow triplet expansions to be analyzed, using NGS by adding special bioinformatic pipelines (Bahlo et al., 2018), thus allowing all-in-one test implementation in the future.

In many instances, there are female infertility cases associated with a variety of multi-system genetic syndromes e.g. galactosemia, mucopolysaccharidoses, myotonic dystrophy, aromatase deficiency, cystic fibrosis, hemochromatosis (Foresta et al., 2002). Normally, genetic testing in these patients is done primarily due to major expressions associated with the particular syndrome often in the pre-reproductive age (Harper et al., 2018). Syndromic forms of female infertility are beyond this work and are comprehensively summarized elsewhere (for reference please see Cariati et al., 2019).

Extended carrier screening

A separate niche in reproductive medicine is extended carrier screening, also known as preconception screening. The primary task of these tests is identification of genetic diseases transmissible to the offspring, thus allowing the couples who are planning to conceive to know their reproductive risk *a priori* (Cariati et al., 2019), and subsequently to change or adapt the ART algorithm. Furthermore, carrier screening can be applied to gamete donors. Abulí with colleagues developed an NGS-based panel covering 368 severe disorders in 200 genes.

The implementation of the screening in the clinical context has allowed to identify 56.3% carriers of at least one (likely) pathogenic variant in a gene responsible for Mendelian disease. Testing led to the exclusion of 1.7% of oocyte donors due to X-linked conditions and identification of 3% of preassigned donor-recipient matches with a high reproductive risk for transmitting a severe autosomal-recessive genetic condition to their offspring (Abulí et al., 2016), thus offering oocyte donation in a personalized manner. This perspective is becoming increasingly important, as a high number of women want to conceive in their thirties and forties (R. Rossetti et al., 2017), inevitably leading to a more frequent usage of the oocyte donation cycles. According to the European Society of Human Reproduction and Embryology (ESHRE) report, oocyte donation is used in up to 22% of ART cycles in some European countries (Kupka et al. 2016).

Preimplantation embryo genetic testing

The percent of women aged 15–49 who have ever used infertility services in the United States of America is 12.7%. Although the use of ART is still relatively rare compared to the potential demand, its use has almost doubled over the past decade – approximately 1.9% of all infants born in the US every year are conceived using ART (Singh, 2004). Since the birth of Louise Brown, the world's first 'test-tube baby' in 1978 (Steptoe and Edwards, 1978), ART have undergone significant technological and methodological improvement. Live birth rates using IVF alone range from 27% to 55%, depending on the patient age group and methodology used (Dahdouh et al., 2015). Unsuccessful treatment of infertility is one of the pitfalls in clinical reproduction.

One of the major breakthroughs in ART – preimplantation embryo genetic testing (PGT) is now routinely used to investigate the genetic make-up of embryos produced by IVF. Originally PGT was introduced to analyze embryos from the known carriers of monogenic disorders (PGT-M), but later evolved to screen a whole set of chromosomes as an embryo selection tool in hopes of increasing live birth rates per transfer (PGT-A) (Theobald et al., 2020).

Initially, PGT-A was performed, using fluorescent *in situ* hybridization on single blastomeres of a day-three embryo that allowed to assess a limited number of chromosomes, but eventually the predictive value of this test was acknowledged unacceptably low (P. N. Scriven and Bossuyt, 2010; Paul N. Scriven et al., 2010). Soon after, array comparative genomic hybridization allowing for a comprehensive chromosome screening was recognized as the gold standard of PGT-A (Greco et al., 2014; Z. Yang et al., 2014), while now this testing

is replaced by a widely accepted NGS technology (Fiorentino, Biricik, et al., 2014; Fiorentino, Bono, et al., 2014).

Personalized embryo transfer

Although embryonic aneuploidy is likely a major contributor to human implantation failure, the proportion of euploid embryos failing to implant is approximately 30% (Forman et al., 2013; Z. Yang et al., 2012), leaving this percentage as a black matter in regards of the solid genetic or other etiology. For the successful implantation, a genetically competent embryo has to be transferred to the utero (assessed by the PGT), and a competent and receptive endometrium has to allow for an embryo to implant. A number of research groups addressed the molecular signature of the competent endometrium. A study of Koot and colleagues, after analyzing women experiencing recurrent implantation failure (RIF) and healthy controls, claimed that endometrial gene expression signature can accurately predict implantation failure after IVF (Koot et al., 2016). Eventually, a commercially available test was developed to evaluate endometrial receptivity – the Endometrial Receptivity Analysis (ERA) patented by Igenomix.

Our group in the pilot study on embryo implantation failure also demonstrated that half of the patients with failed euploid embryo transfer *post-factum* showed nonreceptive endometrium assessed by the ERA test (Vološonoka et al., 2016). However, not all evidence confirms these positive findings, e.g. Cozzolino with colleagues failed to confirm that the ERA test can benefit RIF patients (Cozzolino et al., 2020).

The disadvantage of such testing is their invasive nature required to obtain endometrial tissue sample, precluding its use in a treatment cycle. Nevertheless, patients would benefit from a test of endometrial receptivity as a part of their initial investigations, in order to identify whether there is a significant endometrial factor, potentially affecting their chances of conceiving either spontaneously or by IVF (Koot et al., 2016).

Inherited susceptibility for venous thromboembolism

The incidence of venous thromboembolism (VTE) during pregnancy is approximately 0.6–1.7:1000 deliveries (Bates et al., 2012). A body of evidence has implicated inherited thrombophilia in adverse obstetrical events like intrauterine growth restriction, (recurrent) miscarriage, severe pre-eclampsia, and placental abruption (de Jong et al., 2014; Kupferminc et al., 1999; Middeldorp, 2011). Genetic testing of common gene variants associated with

inherited VTE is widely performed across reproduction specialists despite being controversial (McNamee et al., 2012).

A meta-analysis completed by Ziakas with colleagues demonstrated that factor V [HGNC:3542] "Leiden" variant F5(NM_000130.4):c.1601G > A (rs6025) and prothrombin [HGNC:3535] variant F2(NM_000506.5):c.*97G > A (rs1799963, commonly known as G20210A) were associated with certain risks of VTE in pregnancy (odds ratio 7.28 and 5.43 respectively) (Ziakas et al., 2015), suggesting that on clinical grounds, these findings imply that variant carriers may need to receive anticoagulation prophylaxis. Other case-control studies have shown only a modest association (odds ratios of 2 to 3), also demonstrating that the association is stronger for fetal deaths, such as stillbirths after 20 weeks of gestation, than for early losses (summarized in (Jeve and Davies, 2014)).

A consensus statement on general thrombophilia genetic screening claims that clinical management of VTE in infertility and obstetrics is not altered in patients with or without an inherited hypercoagulable state, therefore routine thrombophilia screening is not indicated (Ashraf et al., 2019). Especially because of a disadvantage associated with the high costs of testing (Jeve and Davies, 2014). The risk of VTE in pregnancy seems to be significantly elevated only in patients with a personal or family history of VTE (0.5–1.5% without history, 17% with history) (Rambaldi et al., 2014).

Similarly, the data is conflicting on hyperhomocysteinaemia as a risk factor for recurrent miscarriage. There is insufficient evidence to advise *MTHFR* [HGNC:7436] testing in reproduction, since the variant NM_005957.5:c.665C > T (rs1801133) had no association with VTE risk in pregnancy (Jeve and Davies, 2014; Ziakas et al., 2015). Importantly, a practice guideline released by American College of Medical Genetics states that there is lack of evidence for *MTHFR* polymorphism testing (Hickey et al., 2013).

Genetic testing of products of conception

Numerous approaches and methodologies are used for POC genetic testing, including the classical cytogenetic techniques (karyotyping, fluorescent *in situ* hybridization), PCR based methods and genomic techniques like array comparative genomic hybridization (aCGH) and NGS. All of them have limitations, e.g., prerequisite for a successful karyotyping is presence of viable choroidal tissues in the primary biological material (Lomax et al., 2000), but most importantly all methods can give misleading results when maternal cell contamination (MCC) in the sample is overlooked. MCC problem in POC testing is recognized in the laboratory practice (Jarrett et al., 2001; Shen et al., 2016), however, it still places a burden on analysis

interpretation and reporting, and specific protocols allowing to acknowledge and surpass the issue for all POC cases are not widely adopted as seen in the scientific literature.

1.3 Literature review: summary

As seen from the comprehensive overview of the genes involved in the development of female reproductive failure and methodologies used to address genetic causes, certain success has been achieved during the last decades in the deciphering molecular and genetic basis of this matter. Nevertheless, the usage of the existing knowledge in a clinical practice is still fragmented and cumbersome (Cariati et al., 2019). This perhaps could be explained with i) the explicitly broad and sometimes overlapping spectrum of reproductive phenotypes and their heterogeneity, ii) an array of existing genomic technologies and testing approaches, each of which is associated with different limitations and peculiarities. Therefore, the aim of this study included the development of reliable protocols exploiting advanced genomic technologies capable addressing certain phenotypes / stages of female reproduction and / or overcoming shortcomings of these technologies, and demonstration of their suitable application to a real-life clinical or research scenarios.

Thus, part of this work described in **Chapter 2** was devoted to the development of multifactor preimplantation embryo testing protocol where a performance comparison of two whole genome amplification techniques for different downstream applications was demonstrated. Furthermore, we addressed the existing problem of MCC in genetic testing of products of conception – **Chapter 3** of this thesis was devoted to the development of MCC assessment protocol and to the formulation of recommendations addressing the entire workflow of POC samples handling from preanalytical, through the analytical stages.

Lastly, in **Chapter 4** we attempted to comprehensively elucidate the genetic landscape of non-syndromic cervical insufficiency using NGS since the etiology of this complex phenotype is largely missing.

2 Performance comparison of two whole genome amplification techniques in frame of multifactor preimplantation genetic testing

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Personal input:

My personal input into this work includes the design of the embryo testing protocols, selection of the methodologies to be used for the testing, hands-on testing of three families out of nine, data interpretation, comparison of the two whole genome amplification techniques, writing the original draft of the manuscript. Please see supplementary files for the signed forms from all the co-authors to use this manuscript for my thesis (Supplement 3).

2.1 Abstract

Purpose

To compare multiple displacement amplification and OmniPlex whole genome amplification techniques performance during aCGH, Sanger Sequencing, SNaPshot and fragment size analysis downstream applications in the frame of multifactor embryo preimplantation genetic testing

Methods

Pre-clinical workup included linked short tandem repeats (STR) marker selection and primer design for loci of interest. It was followed by family haplotyping, after which *in vitro* fertilization preimplantation genetic testing (IVF-PGT) cycle was carried out. A total of 62 embryos were retrieved from nine couples with confirmed single gene disorder being transmitted in their family with various inheritance traits – autosomal dominant (genes – *ACTA2, HTT, KRT14*), autosomal recessive (genes – *ALOX12B, TPP1, GLB1*) and X-linked (genes – *MTM1, DMD*). Whole genome amplification (WGA) for the day five embryo trophectoderm single biopsies was carried out by multiple displacement amplification (MDA) or polymerase chain reaction (PCR) based technology OmniPlex and was used for direct (Sanger sequencing, fragment size analysis, SNaPshot) and indirect mutation assessment (STR marker haplotyping), and embryo aneuploidy testing by array comparative genome hybridisation (aCGH).

Results

Family haplotyping revealed informative / semi-informative microsatellite markers for all clinical cases and all types of inheritance. Indirect testing gave a persuasive conclusion for all embryos assessed, which was confirmed through direct testing. Overall allele dropout (ADO) rate was higher for PCR based WGA; MDA showed better genomic recovery scale. Five euploid embryos were subjected to elective single embryo transfer (eSET), which resulted in four clinical pregnancies and a birth of two healthy children, proved free of disease causative variants running in family postnatally.

Conclusions

The developed multifactor PGT protocol can be adapted and applied to virtually any genetic condition and is capable of improving single gene disorder preimplantation genetic testing in patient tailored manner, thus increasing pregnancy rates, saving costs and patient reliability.

Keywords

Embryo, preimplantation genetic testing, single gene disorder, aneuploidy, whole genome amplification

2.2 Introduction

Preimplantation genetic testing (PGT) is an alternative to prenatal testing for couples being at risk of transmitting a genetic disorder to their offspring. PGT allows exclusion of affected embryos before a clinical pregnancy has been established thus avoiding invasive prenatal testing and elective termination of pregnancy due to a prenatally confirmed diagnosis. The material for PGT can be collected from day three or day five of a developing embryo before its transfer to the uterus. The process initially requires ovarian controlled hyperstimulation, oocyte retrieval and subsequent oocyte in vitro fertilization (IVF), most commonly by intracytoplasmic sperm injection (ICSI), followed by embryo cultivation until the desired stage of development and a biopsy procedure (Bick and Lau, 2006). Depending on a protocol, PGT can be done with or without embryo vitrification for the time of testing. Only the embryos proved free of the disease-causing variant under consideration are subsequently transferred into the uterine cavity.

The success of the whole procedure depends mostly on competence and appropriate collaboration of a multidisciplinary team consisting of clinical geneticist, reproductologist, gynaecologist, embryologist and molecular geneticist, and are achieved through safety and accuracy, improving genetic and reproductive medicine practices (Dahdouh et al., 2015). PGT is currently performed for single gene disorders (SGD), late onset disorders with genetic predisposition, chromosomal disorders, including aneuploidy and structural rearrangements, and HLA (human leukocyte antigen) typing to improve the access to HLA matched stem cell transplantation (Samuel et al., 2009).

The history of PGT backs to 1989, when A. Handyside performed the first preimplantation genetic diagnostic (PGD) cases, detecting Y chromosome specific region with PCR in case of X-linked adrenoleukodystrophy and X-linked mental retardation (A. H. Handyside et al., 1991). Now defining embryo gender is known as sexing and can complement the genetic testing of monogenic disorders linked to the sex chromosomes.

With time, PGT underwent significant methodological and approach changes, starting from polar body testing and blastomere analysis until now adapted trophectoderm biopsy with subsequent blastocyst freezing (Renwick et al., 2006). The analysis of more than a single cell leads to a more robust downstream molecular investigation, which sets among the reasons the blastocyst stage biopsy strategy (Cimadomo et al., 2016). Molecular genetic testing developed from the single loci direct PCR to the sophisticated single cell whole genome amplification (Fiorentino, 2012). Embryo haplotyping offers a more generic approach to preimplantation diagnosis, and is especially useful for diseases with a wide spectrum of causative variants, such as cystic fibrosis and Duchenne muscular dystrophy (Renwick et al., 2006).

Despite technological improvements, the development of PGT protocols is challenging and prone to amplification failure, DNA contamination and ADO – a phenomenon common to all single-cell based PCR tests, thus affecting the reliability of the test. The ADO can be defined as amplification failure affecting only one of the parental alleles. ADO's incidence varies, but in extreme cases has affected up to 20% of amplifications and in the past has led to several misdiagnoses (Capalbo et at. 2016). The causes of misdiagnosis include a swap of samples, transfer of the wrong embryo, maternal or paternal contamination, ADO, use of inappropriate probes or primers, probe or primer failure and chromosomal mosaicism (Harton et al., 2011).

ADO rates should be as low as possible, preferably, less than 10%. Higher ADO rates can be tolerated when dealing with WGA-based protocols and autosomal recessive diseases, compared to autosomal dominant or compound heterozygous cases. However, in such cases, an increased number of linked markers have to be used (Harton et al., 2011).

Choosing the WGA type is also challenging due to difficulties in interpretation of downstream applications like short tandem repeat (STR) marker sizing with fluorescent polymerase chain reaction (fPCR) or array comparative genomic hybridization (Rechitsky et al., 2015). At the moment, several WGA technologies exist (Zheng et al., 2011) e.g. PCR based approaches like degenerate oligonucleotide primer (DOP) (Telenius et al., 1992) or primer extension (PEP) PCR technology (L. Zhang et al., 1992). Leading positions are taken by OmniPlex linear WGA (S. U. Chen et al., 2008; Uda et al., 2007) technology developed by Rubicon Genomics and multiple displacement isothermal synthesis by Phi-29 polymerase approach (Alan H. Handyside et al., 2004). Both of them have advantages and disadvantages. The use of *Taq* DNA polymerase in PCR based approaches limits the fragment lengths to 3 kb. Phi-29 polymerase used for MDA generates DNA fragments up to 100 kb and has a 3'→5' exonuclease proofreading activity. Often it is not clear which technology could be prioritized in custom designed protocols (Zheng et al., 2011).

Better PGT results are now achieved through combining direct and indirect testing, using platforms like Karyomapping (Alan H. Handyside et al., 2010) for genome wide linkage analysis or turning to next generation sequencing (NGS) protocols (Fiorentino, Biricik, et al., 2014). However, current studies still highlight clinically important limitations in the reliability of the technologies, e.g. using Karyomapping ~14% of embryos are expected to remain without a conclusive result (Konstantinidis et al., 2015). NGS has the potential power to increase throughput and evaluate multiple genetic loci in parallel, but it is also well known for sequencing artefacts, which may complicate its application to PGD (Treff et al., 2013). As well as costs are still quite high especially for a limited sample amount.

Regardless of the fact that PGT is recognized for its benefits, it is still relatively unregulated and lacks standardization compared with other forms of diagnostic testing (Harton et al., 2011). It is partially because PGD lies at the intersection of two technologies with a confusing regulatory status: assisted reproduction and genetic testing (Neri-Castracane, 2015). It is admitted that robust PGT test should be able not only to distinguish between a normal and affected embryo, but also to highlight all the unexpected events that may happen during meiosis, fertilization or PGD experimental procedure, and thus it should detect recombination, monosomy or trisomy and therefore diagnose abnormal embryos, detect ADO and contamination (Kieffer et al., 2016). In case of adverse misdiagnosis, lessons can be very painful to patients and staff (Hellani et al., 2004; Wilton et al., 2009).

Despite the numerous advances, assisted reproductive technology (ART) live birth rates are still low, ranging from 27% to 55%, depending on the patient age group and methodology used (Dahdouh et al., 2015). Another step in reaching considerably good results for SGD-PGT is embryo aneuploidy exclusion since it is well known that preimplantation human embryos are prone to chromosome instability (Vanneste, Voet, Le Caignec, et al., 2009) and high aneuploidy rates (Kieffer et al., 2016; Vanneste, Voet, Melotte, et al., 2009). Early results show that combined PGD and PGS increase the patient chance of healthy childbirth (Marshall et al., 2015; Sermon, 2017).

Taking into account the aforementioned information, the aim of our study was to develop individualized effective and robust multifactor embryo testing protocol and to show the performance comparison of two WGA techniques in four different downstream applications – STR sizing, Sanger sequencing, aCGH and SNaPshot technology. We present our PGT experience for single gene diseases of autosomal dominant (genes: *ACTA2*, *HTT*, *KRT14*), autosomal recessive (genes: *ALOX12B*, *TPP1, GLB1*) and X-linked (genes: *MTM1*, *DMD*) types of inheritance.

2.3 Materials and methods

2.3.1 Cases processed

Nine couples (Table 2.1) with a confirmed particular single gene disease being transmitted in their family underwent counselling regarding the PGT procedure, ovarian stimulation, oocyte aspiration and IVF at the single centre of infertility and reproductive genetics, where all biological material samples were collected and processed.

Table 2.1

Processed PGT cases description

PGD case	Disease	Gene	Type of inheritance	Mutation(s) assessed	Female partner age	Male partner age	Family members analyzed to establish linkage
HTT-case	Huntington Disease (HD)	*HTT*	AD	CAG repeat expansion	34	29	Carrier female partner, her affected father and healthy mother, a healthy male partner
ACTA2-case	Familial Thoracic Aortic Aneurysm and Dissection	*ACTA2*	AD	c.635G > A	32	32	Affected male partner, his affected mother and brother, healthy father, healthy female partner
KRT14-case	Epidermolysis Bullosa Simplex	*KRT14*	AD	c.374G > A	30	31	Affected male partner, healthy female partner, their affected child
TPP1-case	Classic Late Infantile Neuronal Ceroid Lipofuscinosis	*TPP1*	AR	c.622C > T	38	37	Carrier female and male partners, their affected child
ALOX12B-case	Nonbullous Congenital Ichthyosiform Erythroderma	*ALOX12B*	AR	c.883G > C; c.1790C > A	35	32	Carrier female and male partners, their affected child
DMD-case1	Duchenne Muscular Dystrophy (family 1)	*DMD*	X-linked	Duplication of exons 45–47 and 51–52	39	35	Carrier female partner, healthy male partner, their affected son, and carrier daughter
DMD-case2	Duchenne Muscular Dystrophy (family 2)	*DMD*	X-linked	c.6420del	33	31	Carrier female partner, her affected and healthy brothers, a healthy male partner

Table 2.1 continued

PGD case	Disease	Gene	Type of inheritance	Mutation(s) assessed	Female partner age	Male partner age	Family members analyzed to establish linkage
MTM1-case	Myotubular Myopathy	*MTM1*	X-linked	c.70C > T	34	35	Carrier female partner, her healthy sister, her carrier mother and healthy father, healthy male partner, their affected child
GLB1-case	GM1 Gangliosidosis	*GLB1*	AR	c.1768C > T; c.833delG	36	38	Carrier female and male partners, their affected child
–	–	–	–	Average	34.4 ± 2.8	32.8 ± 2.5	–

2.3.2 Compliance with Ethical Standards

The study is in accordance with the Declaration of Helsinki ethical principles. All patients considered for PGT underwent genetic counselling. Procedures and manipulations needed for the embryo genetic testing were explained in detail and signed informed consent was obtained, study protocol was approved by local Ethical Community. PGD is recognized as "an established procedure with specific and expanding applications for standard clinical practice" by the Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology (2006). No research was conducted on the embryos. All genetic conditions, for which PGT was performed, are approved by HFEA (Human Fertilization and Embryology Act) as suitable for genetic testing in preimplantation embryos.

2.3.3 Pre-clinical work-up

Before processing a clinical case, a work-up was carried out to prepare each PGT case. Linked microsatellites adjacent to the gene of interest (within ~2Mb upstream and downstream from mutation locus) were located through the University of California Santa Cruz (UCSC) genome browser (https://genome-preview.ucsc.edu/index.html). For all loci (disease causative variant site and STR markers), semi-nested primers for two round multiplex fPCR (inner primer was fluorescently tagged with 6-FAM of HEX fluorophores at 5'end) were designed using the "Primer-BLAST" to ensure specificity (Ye et al., 2012), following good practice guidelines (Hellani et al., 2004). Primer dimers and primer-amplicon secondary structure formation was checked using OligoAnalyzer 3.1 online software tool (Owczarzy et al., 2008).

DNA obtained from the peripheral venous blood of a couple seeking PGD and other family members (usually three to five individuals) was isolated using a standard procedure (Qiagen). Family haplotypes flanking the locus of interest were assessed. STR marker informativeness was evaluated as follows: fully informative (three or four different paternal and maternal alleles, depending on the type of inheritance, both disease causative and healthy, are distinguishable). Semi-informative (one or two different alleles can be distinguished and assigned to the normal or disease causative haplotype), and not-informative (origin of the allele or their assignment to the haplotype cannot be distinguished) (Harton et al., 2011).

When PCR linkage analysis was performed for a family, 6–13 (8.1 ± 2.5) informative or semi-informative STR markers (Table 2.2.) were included in the following PGT cycle for embryo analysis. STR marker informativeness rate was 53%. For autosomal recessive disorders, a significantly higher STR amount contributed to overall assay informativeness rate:

13/15 and 10/15 compared to autosomal dominant or X – linked conditions i.e., 7/17, 6/13 informative markers (please refer to Table 2.2). Disease causative variant confirmation in family members was carried out via Sanger sequencing for single nucleotide variation (SNV) or by fragment size analysis for trinucleotide repeat expansion.

Table 2.2

PGD results

Case	STS markers tested / informative markers	STR informativeness rate	Embryos analyzed	Affected embryo amount	Carrier embryo amount	Mutation free embryo amount	aCGH performed	aCHG result	Overall PGT result
HTT-case	13/6	0.46	7	1; 0.14	NA	6; 0.85	Performed for 2 embryos scored highest according to ES algorithm	1 aneuploid, 1 euploid	eSET - > healthy non-carrier baby born (post-natal confirmatory analysis)
ACTA2-case	18/7	0.39	9	4; 0.44	NA	5; 0.55	Performed for all mutation free embryos	4 euploid, 1 aneuploid	eSET - > clinical pregnancy
TPP1-case	15/10	0.67	3	1; 0.33	1; 0.33	1; 0.33	Performed for only one mutation free embryo	Euploid	eSET - > clinical pregnancy
ALOX12B-case	15/13	0.87	12	3; 0.25	7; 0.58	2; 0.17	Performed for one embryo scored highest according to ES algorithm	Euploid	eSET - > healthy non-carrier baby born (post-natal confirmatory analysis)
DMD-case1	21/11	0.52	9	4; 1.5	1; 0.11	4; 0.44	PGD by aCGH performed initially when the molecular analysis was unavailable, later all stored WGA were subjected to haplotype analysis	5 euploid, 4 aneuploid	eSET - > healthy carrier baby born (post-natal indirect linkage confirmatory analysis), second eSET- > failed implantation.

Table 2.2 continued

Case	STS markers tested / informative markers	STR informativeness rate	Embryos analyzed	Affected embryo amount	Carrier embryo amount	Mutation free embryo amount	aCGH performed	aCHG result	Overall PGT result
DMD-case2	16/7	0.44	17	3; 0.18	5; 0.29	9; 0.52	Performed for 9 mutation free embryos	6 euploid, 3 aneuploid	Waiting for eSET
GLB1-case	15/8	0.53	5	0; 0	4; 0.8	1; 0.2	Performed for all 5 embryos	2 euploid, 3 aneuploid	Waiting for eSET (carrier embryo)
MTM1-case	14/7	0.50	No oocytes were successfully fertilized						
KRT14-case	17/7	0.41	Ovary stimulation still to be performed						
–	Average:	0.53	9.5						

* ES – EmbryoScope time-lapse incubator. eSET – elective single embryo transfer.

2.3.4 IVF and embryo biopsy

Oocyte cumulus complexes (COC) were retrieved by a needle transvaginal aspiration procedure. All oocytes were fertilized through intracytoplasmic sperm injection (ICSI) and placed in a time-lapse incubator (Embryoscope, Vitrolife, UK). Fertilization was acknowledged as successful if two pronuclei (PN) were observed on the next day after ICSI. Embryos were incubated until the day-five blastocyst stage. Embryo development rate was scored based on a time-lapse system monitoring algorithm (Milewski et al., 2015). Through natural selection, the average 5^{th} day survival rate was 70%. In total 62 embryos were subjected to PGT (Table 2.3). Embryo biopsies were made using laser assisted micromanipulator (Narishige, Japan). From each embryo 1–8 trophectodermal cells were taken from the outer layer of the blastocyst. Biopsied cells were washed in 1x phosphate buffer saline (PBS) buffer (Cell Signaling Technologies, USA) drops to reduce the risk of contamination, subsequently placed in 0.2 ml tubes within a 2.0 µl of 1% polyvinylpyrrolidone (FertiPro, Belgium) 1× PBS buffer, and frozen immediately, each blastocyst culture media contamination control was collected as well. Biopsied embryos were vitrified.

2.3.5 Performance of clinical cases

As the first step for all embryo biopsies, WGA was carried out. For one part of the embryos, WGA was done by MDA technology (SureMDA, Illumina, USA), the rest were carried out by OmniPlex linear WGA technology (SurePlex, Illumina, USA) (Table 2.4). An aliquot of WGA product from each sample was used to carry out different downstream tests.

Table 2.3

Embryological data of processed cases

Case	COC retrieved	Performed ICSI	Oocytes fertilized (day 1 assessment)	Successfully fertilized oocytes*	Fertilization rate**	Embryos biopsied	5th day survival rate***
HTT-case	16 COC (3 GV, 1 MI, 12 MII)	13	1 deg, 2×0PN, 10×2PN	10	0.77	7×2PN	0.70
ACTA2-case	18 (1 empty ZP, 1 MI, 16 MII)	17	4 deg, 2×0PN, 11×2PN	11	0.65	9×2PN	0.82
TPP1-case	8 COC (2 GV, 6 MII)	6	1 deg, 1×1PN, 1×3PN, 3×2PN	4	0.67	3×2PN	0.75
ALOX12B-case (two stimulations)	15 COC (1 empty ZP, 2 atretic, 3 GV, 1 MI, 8 MII)	9	2 deg, 1×0PN, 2×3PN, 3×2PN	5	0.56	2×2PN, 1×3PN	0.6
	16 COC (2 empty ZP, 2 GV, 2 MI, 10 MII)	12	2 deg, 1×1PN, 9×2PN	9	0.75	9×2PN	1.0
DMD-case1	18 COC (1 empty ZP, 1 MI, 16 MII)	17	4 deg, 2×0PN, 11×2PN	11	0.65	9×2PN	0.82
DMD-case2	22 COC (2 empty ZP, 2 GV, 18 MII)	18	1×1PN, 17×2PN	17	0.94	16×2PN, 1×1PN	1.00
MTM1-case (two stimulations)	5 COC (1 GV, 4 MII)	4	3×0PN, 1×1PN	0	0.00	NA	NA
	10 COC (2 ZP, 1 MI, 7 MII)	8	2 deg, 4×0PN, 2×3PN	2	0.25	0	0.00
GLB1-case	15 COC (1 ZP, 14MII)	14	2×0PN, 2×1PN, 1×3PN, 9×2PN	10	0.71	5×2PN	0.5
–	Average	11.6 ± 5.1	–	7.67 ± 5.34	0.58 ± 0.29	7.13 ± 5.25	0.71 ± 0.32
–	–	–	–	–	Total:	57.0	–

* COC – cumulus oocyte complex; ZP – zona pellucida, GV – germinal vesicle stage oocyte; MI – meiosis I stage oocyte; MII – meiosis II stage oocyte; deg – degraded oocyte. *Successfully fertilized oocytes are the ones having two or three pronuclei. **Fertilization rate is calculated dividing day one embryos showing two or three pronuclei (PN) with the total amount of ICSI performed. ***Fifth day survival rate is calculated dividing day five embryos by successfully fertilized embryos.

Table 2.4

WGA techniques comparison

Case	Sanger sequencing		SnapShot analysis		STR analysis		STR ADO* (%)	aCGH analysis		Total embryos analysed
HTT-case	–		–		7 MDA		4,5	2 MDA		7 MDA
ACTA2-case	4 MDA; 5 OmniPlex		–		4 MDA; 5 OmniPlex		1,5	3 MDA; 2 OmniPlex		4 MDA; 5 OmniPlex
TPP1-case	3 MDA		–		3 MDA		1,4	1 MDA		3 MDA
ALOX12B-case	12 MDA		12 MDA		12 MDA		2,8	2 MDA		12 MDA
DMD-case1	–		–		9 OmniPlex		13,3	9 OmniPlex		9 OmniPlex
DMD-case2	8 MDA; 9 OmniPlex		8 MDA; 9 OmniPlex		8 MDA; 9 OmniPlex		4,7	3 MDA; 5 OmniPlex		8 MDA; 9 OmniPlex
GLB1-case	5 MDA		–		5 MDA		2.0	5 MDA		5 MDA
Donated WGA material	–		11 OmniPlex		–		–	11 OmniPlex		11 OmniPlex
MDA:	32	1x ADO for TPP-case. Partial ADO detected.	20	Consistent results	39	Clear Electropherogramms	2.98	17	Noisy profiles, resolution: full chromosomes, ~30% of samples have to be reanalysed	39
OmniPlex:	14	Partial ADO detected.	20	Failed reaction or inconsistent result for > 60% of cases	23	Electropherogramms overrepresented with stutter peaks	6.5	27	Clear profiles, resolution: ~5Mb	34
TOTAL:	46	–	40	–	62	–	Average 4.74	44	–	73

* *STR ADO rate was calculated by dividing homozygous genotypes when heterozygous (Hz) was expected to all expected Hz loci. Calculations were made for individual cases and separately for both WGA types.

Embryo haplogroup analysis was carried out assessing the informative markers found in the linkage step. The following two round (nested) PCR conditions were used: 8.2µl of Type IT master-mix (Qiagen, USA), 0.32µl of 0.2 µM forward (outer-forward for first stage of hemi-nested PCR and inner-forward primer for second round of PCR; synthesized by Bioneer, China) and 0.2 µM reverse primer (same for both PCR steps), 6.8µl of ddH2O and 0.62µl of WGA product. Cycling conditions: initial denaturation 5min in 95°C, followed by 28 (1st round PCR) or 22 (2nd round PCR) cycles of 30s in 95°C, 1min30s in 60°C, 30s in 72°C, and final extension 10min in 72°C. Amplified products were run on agarose gel electrophoresis to detect PCR product. Amplicon detection was performed by capillary electrophoresis (ABI Prism 3500 DNA Analyzer; Applied Biosystems, USA). Allele sizing was carried out using GeneMapper v.4.0 software (Applied Biosystems).

Direct mutation analysis for SNVs was carried out by standard Sanger sequencing protocol (Sambrook and W Russell, 2001) or SNaPshot technology (Applied Biosystems, USA). *HTT* gene (OMIM# 613004) CAG repeat expansion (RCV000030659, HGVS nomenclature – NM_002111.6(HTT):c.53_55[(41_?)] (p.Gln40(41_?)) was detected by capillary electrophoresis using the same protocol as for STR marker loci amplification.

Embryo chromosome analysis was performed following the manufacturers' (24Sure, Illumina, USA) protocol for aCGH, shortly: WGA product was fluorescently labelled by nick-translation method with Cy3 and Cy5 fluorophores, the sample and reference DNA was hybridized on BAC array microchips, microchip glasses were washed and scanned with InnoScan (Inopsys, France) scanner. Tiff images were imported into the BlueFuse Multi V4.0 software (standard settings), the resulting copy number karyotypes were assessed. The given methodology detects unbalanced chromosomal material changes and polyploidy if sex chromosomes are represented by at least one X and Y chromosomes.

2.4 Results

2.4.1 Embryo PGT analysis

For all 62 embryo biopsies WGA amplification performed either by SureMDA or SurePlex kit (Table 2.4) was successful and eventually with a conclusive result (Table 2.2, Figure 2.1 and Supplementary Figures 2.1–2.6 for the pedigrees). Additional 11 OmniPlex samples were donated for research. In the case of *MTM1* gene testing after two stimulation cycles, none of the oocytes underwent successful fertilization. *KRT14*-case family underwent only linkage analysis and now are preparing for the follicular stimulation.

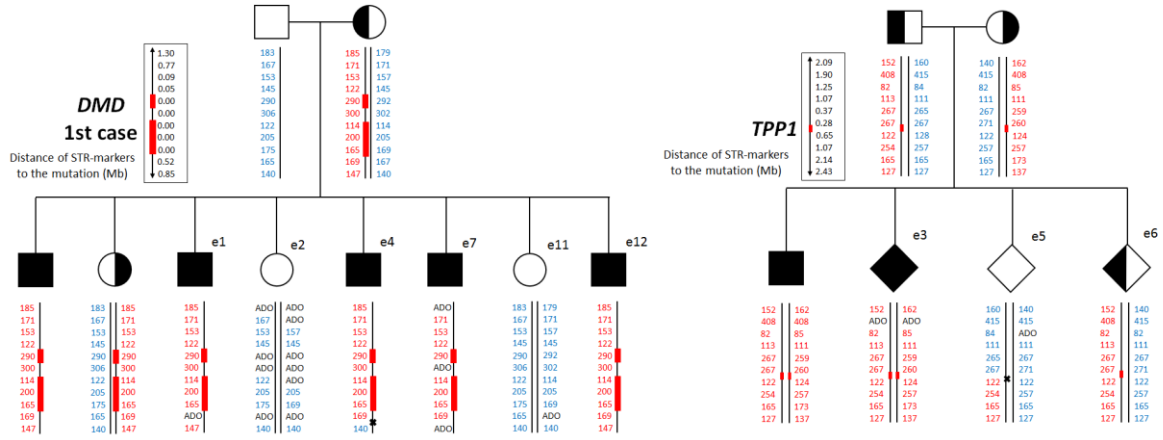


Figure 2.1 **Pedigrees**

TPP1-case and DMD-case-1 pedigrees showing family members participating in haplotype establishment and all embryos analyzed, gender is shown only for X-linked condition. Red bars represent disease variant loci. Black crosses indicate recombination events. In blue variant free haplotypes are indicated. In red variant haplotypes are indicated. ADO – allelic drop out.

A portion of each embryo WGA product was used for haplotyping of informative or semi-informative markers detected by initial family linkage analysis. The overall ADO rate was 4.74% (Table 2.4), exceeding the 5% cut-off only in Duchenne Muscular Dystrophy (*DMD*) case-1, where WGA product generated by OmniPlex had lower quality due to the long-time storage and repeated freeze-thaw events. An aCGH for this *DMD*-case1 was performed firstly when haplotyping was unavailable (please refer to Table 2.2 and the corresponding pedigree).

In one case maternal uniparental disomy of tested locus was observed (*GLB1* e4, please see the pedigree). In three embryos, crossover events were detected through haplotyping. For *TPP*-case e5, the analysis was encumbered due to the proximity of the crossover site to the mutation site, making it impossible to exclude direct testing ADO and possible heterozygous embryo genotype. In all cases, crossover occurred next to the mutation locus, which complicates particular embryo analysis, but direct mutation analysis complemented the haplotyping results.

Direct mutation testing was done for all cases processed except for *DMD*-case1. For *HTT* gene's CAG triplet repeat sizing was performed by fPCR. Sanger sequencing and / or SNaPshot analysis was applied for SNV analysis, and in all cases direct disease causative variant testing complemented and matched the haplotyping results. In all cases, at least one embryo free of tested disease causative variant was detected.

In most cases, a portion of WGA product from mutation free embryos was subjected to aCGH analysis to exclude chromosomal aneuploidies. For *HTT*-case and *ALOX12B*-case, only some of the tested disease causative variant free embryos were subjected to a chromosome analysis due to financial reasons, in these cases only embryos, showing the best development scores according to the EmbryScope algorithm, were taken to analysis. In all cases, at least one

euploid embryo was available (Table 2.2). Only embryos free of the disease causative variant assessed and euploid were rated as transferable.

Elective single euploid embryo transfers (eSET) in two cases resulted in the birth of healthy babies. Transfer of *TPP1* and *ACTA2* variant-free embryos resulted in progressing clinical pregnancies. For the first Duchene muscular dystrophy case initially only sexing for PGD by aCGH was performed and 46,XX embryo transfer resulted in a healthy carrier baby birth, only later all their embryos were haplotyped and second eSET resulted in failed embryo implantation. Another DMD family is preparing for eSET procedure. Three babies born after PGT underwent postnatal mutation assessment and preimplantation genetic testing results were confirmed.

2.4.2 Comparison of two different WGA techniques

To compare the two WGA methods one part of the biopsies was subjected to the MDA technique and the rest were amplified by OmniPex reagent kit (please refer to Table 2.4 for a detailed view). Typical MDA product pattern (smear) on 1,5% agarose gel is observable as bands at about 6–12 kb. On the contrary PCR based WGA results in much shorter products visible as smear appearing between 1kb and 100bp with the most prominent bands at around 500pb (Figure 2.2). Both types of WGA were subjected to all four downstream applications – Sanger sequencing, STR amplification and aCGH (Table 2.4).

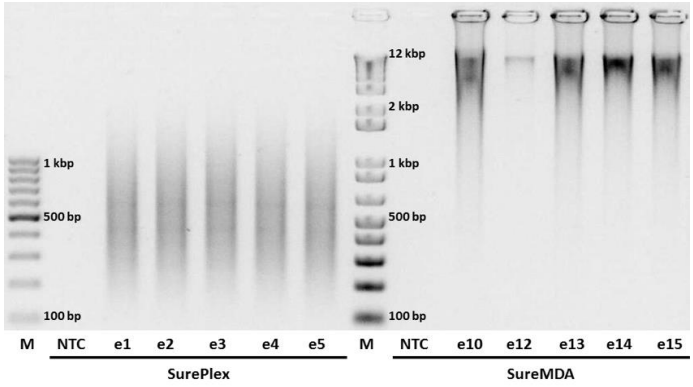


Figure 2.2 **WGA product agarose gel electrophoresis**

Embryo trophectoderm biopsies WGA products are shown for DMD-case-2 embryos. The left panel represents WGA by OmniPlex kit; the right panel represents WGA amplification by MDA technology. NTC was amplified by STR and sequencing primers, no contamination was detected. Poor amplification of e12 did not affect haplotyping results. M – allelic ladder, NTC – no template control.

Our results show that both WGA methodologies result in partial ADO when Sanger sequencing is performed (Figure 2.3). Poor amplification of disease causative allele can be distinguishable as a low-level electropherogram in otherwise clear profiles. One *TPP1*-case sample resulted in complete disease causative allele ADO even despite the hemi-nested amplification approach.

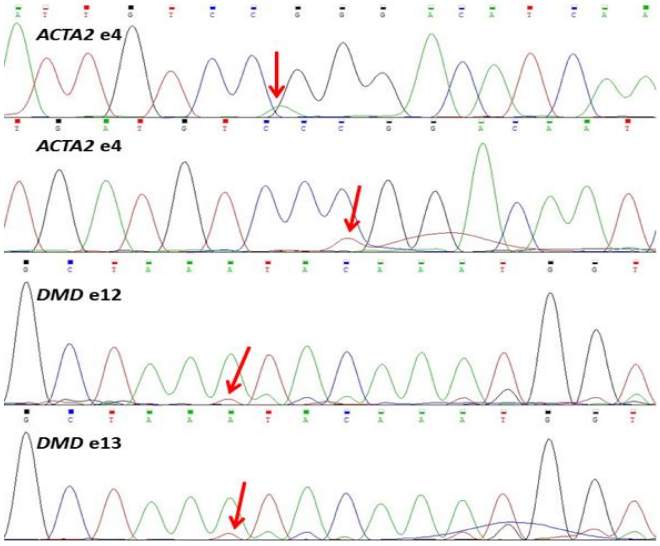


Figure 2.3 **Sanger sequencing profiles of different WGA technologies**

WGA for ACTA2-case embryo four (e4) performed by SurePlex amplification. Upper electropherogramm represents sequence gained by forward primer; lower panel represents reverse primer sequence. Red arrows mark partial allelic drop out of a disease-causing allele. Haplotype analysis of given embryo corresponds to heterozygous genotype. WGA for DMD case-two embryos (e12 and e13) performed by SureMDA amplification. Given electropherogramms represent sequences gained by forward primer. Red arrows mark partial ADO of mutated allele – one nucleotide deletion. The mutated allele is only detectable as weak background profile similar no noise. Haplotype analysis of given embryo corresponds to heterozygous genotype.

We were also interested in comparing both WGA when subjected to SNaPshot genotyping technology (Figure 2.4, supplementary figure 2.8), the MDA product resulted in comparable results to haplotyping and Sanger sequencing, all the genotypes matched, whereas OmniPlex product repeatedly did not produce any reliable profiles (not shown) in more than 60% of samples.

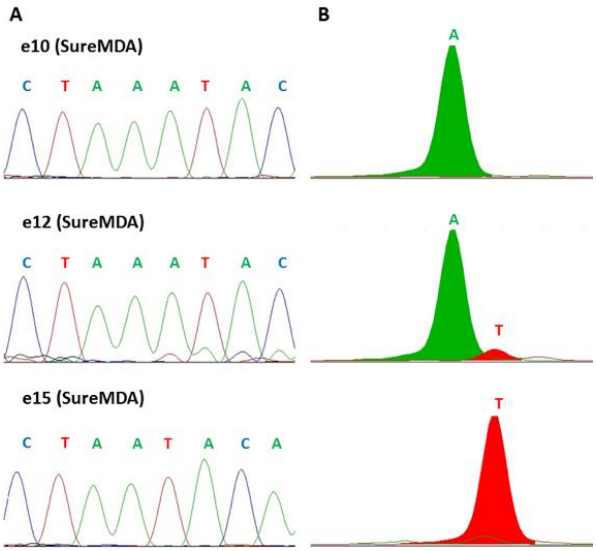


Figure 2.4 **Comparison of STR sizing (A) and SNaPshot (B)**

Results are shown for DMD case-two embryos variant locus (one nucleotide deletion). Whole genome amplification performed by the multiple displacement amplification (MDA) technique. Profiles completely match between the two technologies, partial allelic drop out is visible on both profiles for the heterozygous embryo (e12).

Due to the nature of two WGA types, they arise in completely different downstream STR amplification product sizing patterns performed on capillary electrophoresis (Figure 2.5, 6). Prominent false peaks arise due to polymerase slippage during OmniPlex amplification and subsequent preferential amplification of particular PCR products, making it possible to distinguish the true alleles from the false ones only by comparing them to parental genomic DNA samples run in parallel.

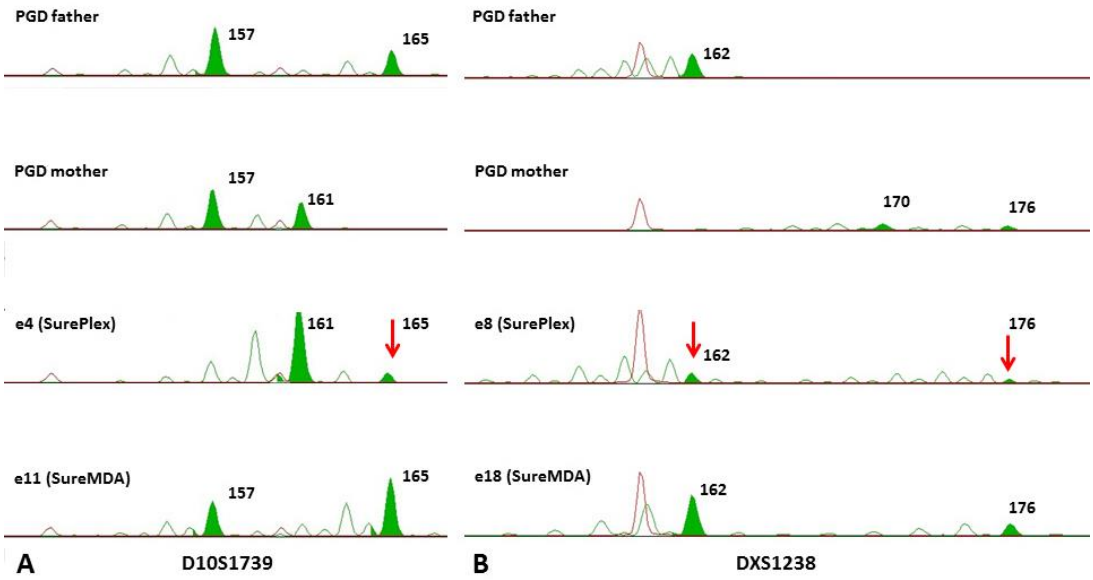


Figure 2.5 **Embryo haplotype analysis**

(A) D10S17390 STR marker sizing by capillary electrophoresis. Potential ADO in DXS1238 marker is indicated by arrow for SurePlex performed WGA. (B) Arrow indicates true maternal allele for SurePlex performed WGA whereas most prominent peaks are of artificial nature.

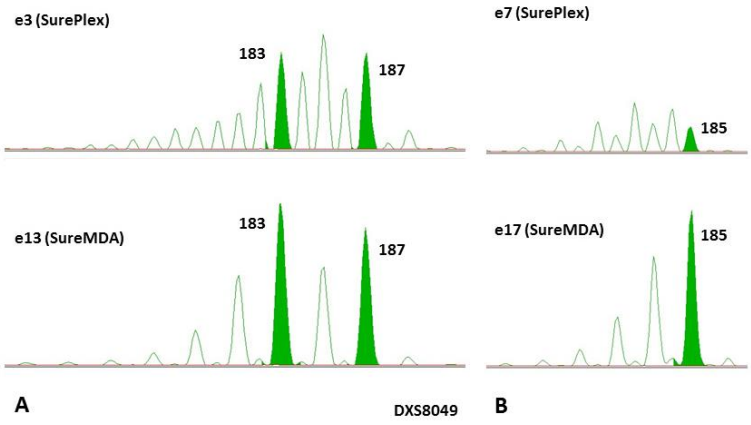


Figure 2.6 **Comparison of two WGA techniques in downstream DXS8049 marker sizing**

Rubicon Genomics technology has been proven to perform well in single cell aCGH amplifications resulting in clear flat profiles, which is true in our study as well. Whereas the MDA aCGH profile results in increased noise compared to OmniPlex WGA (Figure 2.7), therefore chromosome microarray analysis in such case is possible only for the whole chromosomes, but not the partial copy number variations.

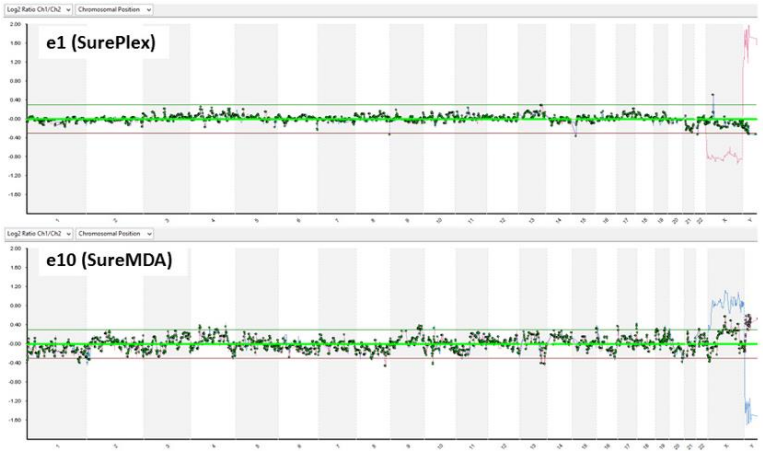


Figure 2.7 **Comparison of two WGA techniques in downstream aCGH analysis**

2.5 Discussion

It is widely recognized that the development of preimplantation genetic testing protocols is time consuming, costly and laborious, because of the wide spectrum of technical complications and biology driven obstacles (Capalbo et al., 2016; Wilton et al., 2009). It is essential to remember that the interpretation of results may influence not only particular family wealth but in the long-term even wellbeing of the whole society, since PGT is a potential tool to cease out at least some genetic conditions.

Performance and outcome requirements for our approach were subjected to the following measures: the possibility to combine several technologies in order to distinguish a normal embryo from a carrier and an affected one; to distinguish possible contamination, loci / allelic drop out events and to perform embryo chromosome screening. It was relevant to get a conclusion on all embryos subjected to biopsy and avoid any additional embryo manipulations like repeated thawing and rebiopsy. Such a wide spectrum of requirements was set carefully taking into consideration all the previous historical obstacles of PGT. We aimed, first, to meet the highest safety standards and, secondly, to prioritize the purpose of achieving desired pregnancy in a personalized and customized manner saving patients' time and expenses, which was shown through a comprehensive comparison of two different WGA techniques.

In general, obtaining micrograms of DNA through WGA of day-five embryo biopsies allowed us to perform embryo haplotype analysis, aneuploidy screening by aCGH and direct mutation testing through SNaPshot, Sanger sequencing or fragment size analysis. As shown before direct variant locus testing boldly complements the indirect one (Kieffer et al., 2016; Mascarenhas et al., 2012), since crossover events cannot be completely ruled out and ignored, (Gueye et al., 2014) especially in the case of ADO, which was also true in our cohort. We

designed hemi-nested primers for all assessed loci following the PGDIS guidelines for good practice in PGD (Hellani et al., 2004; Piyamongkol et al., 2003). We conclude that having as large as possible number of semi-informative and / or informative linked markers within a reasonable distance upstream and downstream from a gene is the best way to minimize the risk of misdiagnosis or no conclusive diagnosis for a particular embryo.

To our knowledge, our work is the first attempt in evaluating PicoPlex and MDA amplifiers performance across different downstream applications in the frame of embryo preimplantation genetic testing. Provided figures give insight in the understanding of both WGA methodologies applicability in different molecular techniques and assist in choosing the one when customizing particular SGD-PGT depending on the mutation type and the technical equipment of the laboratory.

Currently single / few cell WGA might be done with a wide array of amplification strategies (Börgstrom et al., 2017). We conclude that methodology choice depends on multiple factors like desired downstream application techniques as well as embryo amount. STR analysis efficacy including the possible ADO event detection depends mostly on particular genomic region nucleotide composition and can be improved through the PCR reaction condition optimization. MDA WGA product comparing to OmniPlex produces more heavy DNA strings thus exhibiting properties closer to genomic DNA and therefore electropherograms are much clearer. Our results are consistent with other group findings that per base error rate for MDA is at least two times lower comparing to PCR based approaches. In general MDA shows better genome recovery sensitivity as also concluded before (Hou et al., 2015) while allowing for more convenient genotyping. However, MDA results in significant amplification bias (De Bourcy et al., 2014), which contributed to the observed high aCGH noise levels. For a full-fledged analysis, we recommend the usage of both WGA techniques dividing embryo cohort if embryo amount is big enough. If the number of (semi)informative markers is low, it is favorable to use the MDA technique since this will result in a more robust SGD analysis. If STR marker informativeness is high enough, ADO will not drastically affect the result when detecting possible crossing over events, one might consider using OmniPlex since it gives more reliable aCGH profiles.

It is known that embryo aneuploidy and implantation potential is highly correlated with the biopsy stage. Cleavage stage embryo blastomere biopsy still represents the most commonly used method in Europe, although this approach has been shown to have a negative impact on embryo viability and implantation rates (Cimadomo et al., 2016; R. T. Scott et al., 2013). Therefore, day five biopsy is highly favorable. In our study trophectoderm biopsy performance was additionally complemented with the usage of time-lapse embryo imaging system, which

aims not only in biopsy timing, but also can give a clue for the best choice of developing embryo for transfer through the assessment of embryo rating by time-lapse system algorithm when multiple embryos are SGD free and euploid.

Our experience with the preimplantation testing began with a lot of goals and aims that were expected from the clinical and molecular point of view. We tried to set up a diagnostic algorithm that would suit every case and be foolproof. It became apparent already with our first cases that the approach should be more patient tailored than universal, more based on close communication between the patient, clinical geneticist, reproductologist, embryologist, and molecular geneticists than on pure data analysis. Proper genetic counselling before planning a PGT case is crucial as the patient has to be acquainted with any potential pitfalls to give a fully informed consent for testing. The final strategy of molecular testing should be made after taking into consideration the available embryo amount and morphology, the type of disorder and family specifics and preferences. Although the main goal during monogenic disease preimplantation testing would always be the disease causative variant free embryo selection, we found it expedient to use aneuploidy testing besides the morphological embryo evaluation to determine the most suitable embryo for eSET, thus increasing the chance for a successful embryo implantation and development, saving extra efforts and costs. The final result will always depend on a lot of different factors – even after all embryo testing is done there is a possibility of failed implantation due to maternal age factor, endometrial receptivity problems and many more – this is why a multidisciplinary approach is a key to success for each family and thus community altogether.

2.6 Conclusions

Single blastocyst biopsy whole genome amplification ensures the possibility of multifactor preimplantation genetic testing without compromising embryo viability and in general a chance of achieving healthy pregnancy. A semi-nested direct and indirect testing system minimizes embryo misdiagnosis risk due to allelic drop out, non-specific amplification or contamination. 24-chromosome aneuploidy screening when performed concurrently with single gene disorder preimplantation embryo testing provides valuable information for embryo selection excluding leading failed embryo implantation cause and notably improving single embryo transfer rates thus saving time and money leading to higher pregnancy rates. The developed protocol can be further applied to customize PGT protocols for families seeking alternatives for prenatal testing.

2.7 Acknowledgements

The authors would like to thank Olga Sterna for the validation of the *HTT* gene expansion in embryo biopsies at Children's Clinical University Hospital, Clinic of Medical Genetics and Prenatal Diagnosis, and the "IVF Riga" clinic practitioners and nurses for the accomplishment of the reproductive part of the patient care.

2.8 Authors' Roles

LV and DP designed the research, performed the experiments, analysed the data, and wrote the paper; LK designed the research, analysed the data and wrote the paper; BA and NN performed the experiments and analysed the data; EJP and AB performed embryo cultivation and biopsies, AM, LP and IK discussed and revised the paper; VF revised the paper.

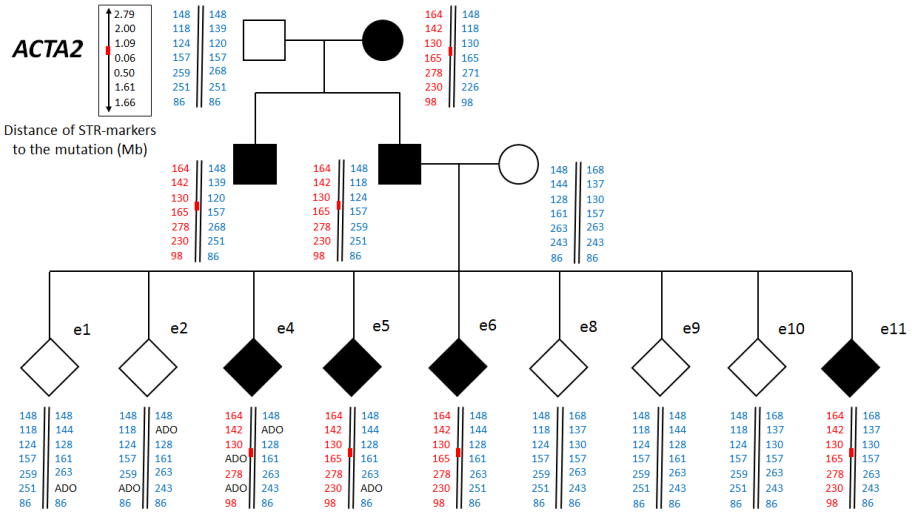
2.9 Funding

No external funding was either sought or obtained for this study.

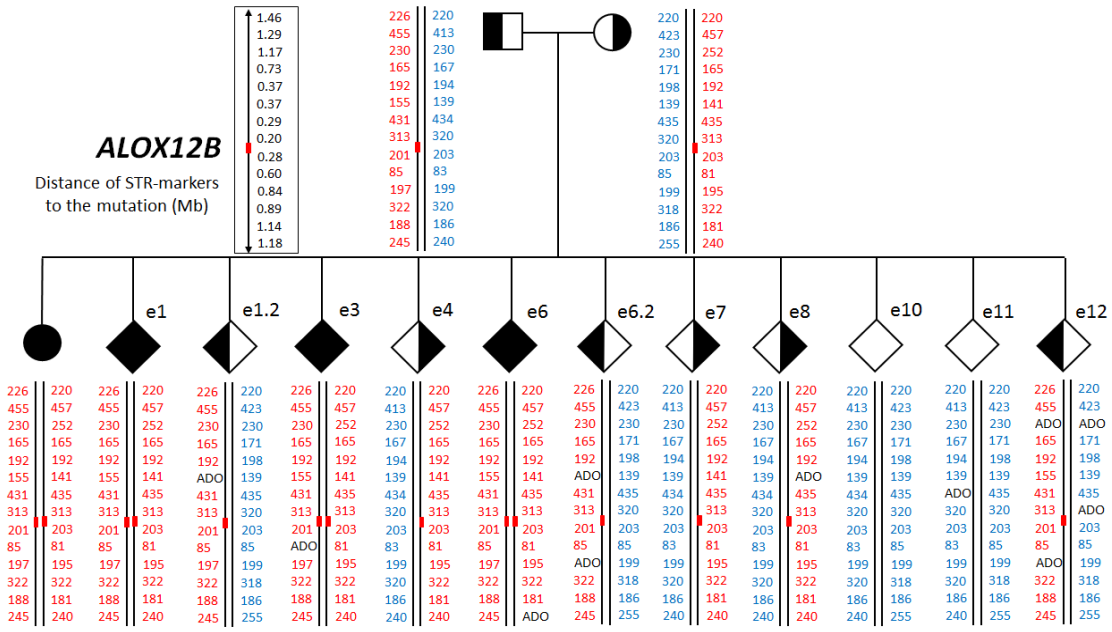
2.10 Conflict of Interest

None declared.

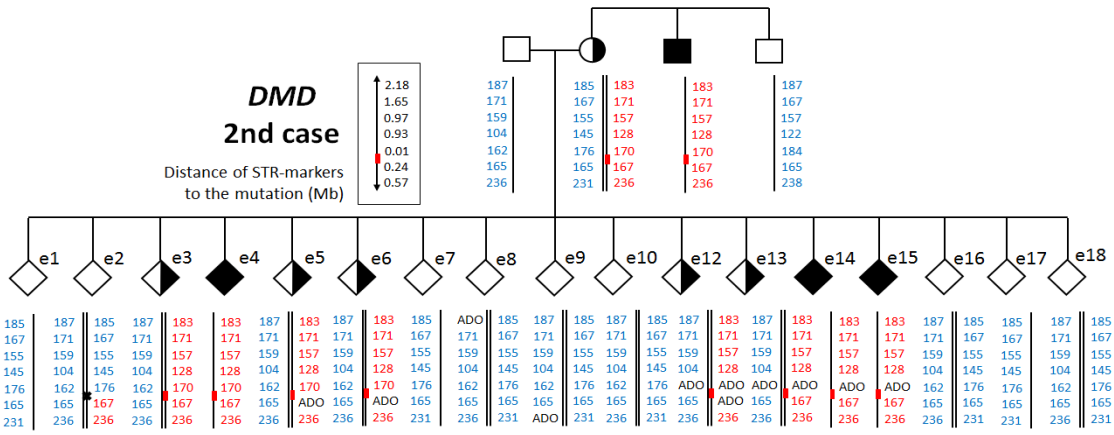
2.11 Supplementary files



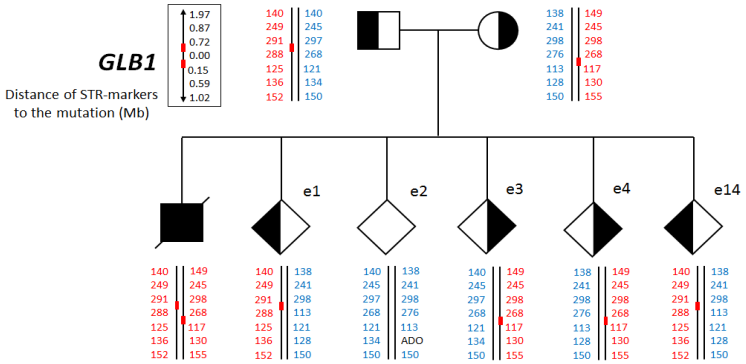
Supplementary figure 2.1 **ACTA2 case**



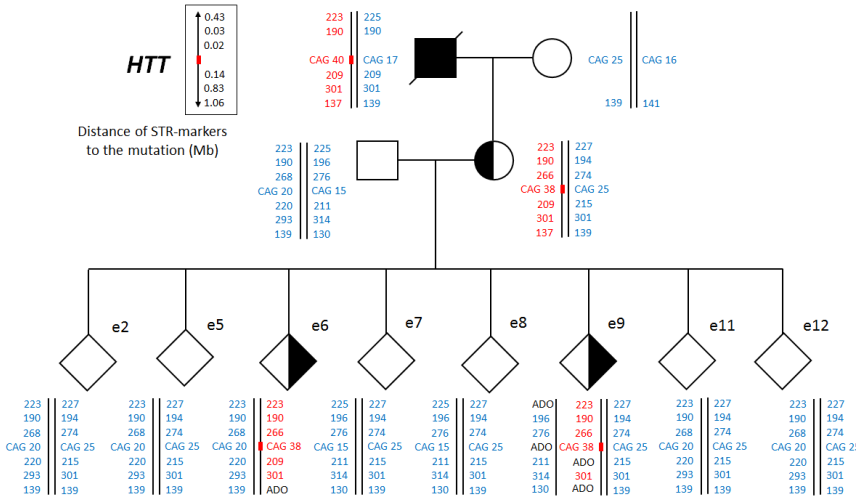
Supplementary figure 2.2 **ALOX12B case**



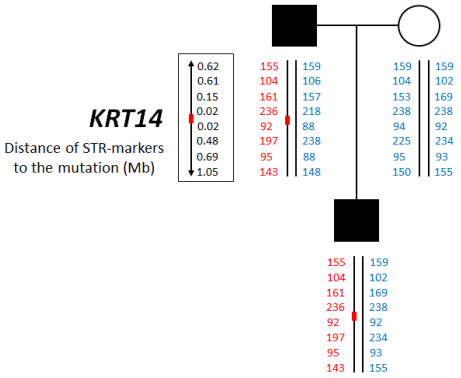
Supplementary figure 2.3 **DMD case**



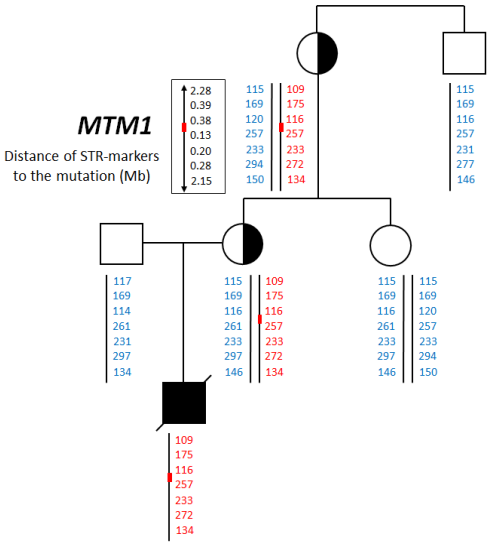
Supplementary figure 2.4 **GLB1 case**



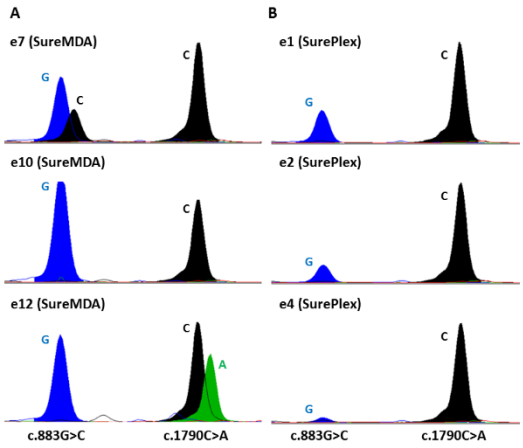
Supplementary figure 2.5 **HTT case**



Supplementary figure 2.6 **KRT14 case**



Supplementary figure 2.7 **MTM1 case**



Supplementary figure 2.8 **ALOX12B case SNaPshot electropherogram**

3 Reducing misdiagnosis caused by maternal cell contamination in genetic testing for early pregnancy loss

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Personal input:

My personal input into this work includes study design, hands-on testing of all samples, data interpretation, writing the original draft of the manuscript. Please see supplementary files for the the signed forms from all the co-authors to use this manuscript for my thesis (Supplement 4).

3.1 Abstract

The analysis of products of conception (POC) is clinically important to establish the cause of early pregnancy loss. Data from such analyses can lead to specific interventions in subsequent natural or assisted conceptions**.** The techniques available to examine the chromosomal composition of POC have limitations and can give misleading results when maternal cell contamination (MCC) is overlooked. The aim of this study was to develop a protocol for MCC assessment and to formulate POC material handling, testing, and reporting recommendations. Using array comparative genomic hybridization, we tested 86 POC samples, of which 47 sample pairs (DNA extracted from the POC sample and maternal DNA) were assessed for the presence of MCC. MCC was evaluated using an approach we developed, which exploited the genotyping of 14 STR, *AMEL*, and *SRY* loci. POC samples showing the clear presence of villi (63.9%) did not contain any signs of the maternal genome and can therefore be reliably tested using conventional methods. The proportion of 46,XX karyotype

in the unselected sample batch was 0.39, which fell to 0.23 in visually good samples and was 0.27 in samples having no signs of contamination upon MCC testing. MCC assessment can rescue visually poor samples from being discarded or wrongly genotyped. We demonstrate here that classification based on visual POC material evaluation and MCC testing leads to predictable and reliable POC genetic testing outcomes. Our formulated recommendations covering POC material collection, transportation, primary and secondary processing, as well as the array of pertinent considerations discussed here, can be implemented by laboratories to improve their POC genetic testing practices. We anticipate our protocol for MCC assessment and recommendations will help reduce the misconception regarding the etiology of miscarried fetuses and foster informed decision-making by clinicians and patients dealing with early pregnancy loss.

Keywords

Miscarriage, maternal cell contamination, product of conception, genetic testing, aneuploidy, early pregnancy loss.

3.2 Introduction

Miscarriage is a traumatizing experience for the patient and places a burden on the practice of obstetrics and gynecology. The magnitude of miscarriage appears to be increasing, particularly in developed countries where the population is aging fairly rapidly and consequently the age of mothers at childbirth is rising (Heazell et al., 2018). Fetal chromosomal aberrations play the biggest role in the etiology of miscarriage. The majority of embryos with an aberrant karyotype decease during the first weeks of pregnancy (Davis et al., 2017; S. T. Romero et al., 2015). Specifically, 40–75% of fetuses aborted between 6 and 7 weeks of gestation have an abnormal karyotype, 20–25% aborted between 12 and 17 weeks, and only 2–7% aborted between 17 and 28 weeks (Agarkova, 2010).

The analysis of products of conception (POC) is clinically important to establish the cause of early pregnancy loss and choose specific interventions in subsequent natural or assisted conceptions. Various techniques are currently used to detect chromosomal aneuploidies and structural rearrangements in POC, including but not limited to karyotyping, quantitative fluorescent polymerase chain reaction, multiple ligation probe amplification, array comparative genomic hybridization (aCGH), single nucleotide polymorphism / variation (SNP) arrays, and next-generation sequencing.

G-banding of chorionic villi metaphases is considered to be the gold standard in routine POC testing. However, its application is limited due to the quality and viability of chorion cells in the primary biological material. Molecular testing has overcome some of the disadvantages inherent to conventional cytogenetic techniques and can be used as a rescue molecular karyotyping (Lomax et al., 2000) if culture failure occurs or POC material is degraded.

All of the techniques examining POC chromosomal composition have drawbacks and especially can give misleading results when maternal DNA contamination (commonly referred to as maternal cell contamination (MCC)) is overlooked. POC degradation is not unusual given the fact that a missed abortion can go unnoticed for several days during which cell maceration has already started (De La Rochebrochard and Thonneau, 2002).

A bias towards an increased number of normal female karyotype reports in comparison to normal male karyotype reports has been noted (Bell et al., 1999; Jarrett et al., 2001; Lathi et al., 2014). However, not all laboratories fully address this important issue and its etiology (Nikitina et al., 2005). There are cases of possible misconception of a higher incidence of normal female karyotype results with the 69,XXX karyotype (Shen et al., 2016) when maternal DNA contamination was not considered.

While there are several factors possibly influencing the differing sex ratio in spontaneous abortions (Jarrett et al., 2001) like unrecognized 46,XX molar samples, maternal age at gestation, X-linked lethal mutations acting *in utero*, and sex chromosome-specific failure of chromosome preparation (Eiben et al., 1990; Hassold et al., 1983), studies have demonstrated that up to 59% of normal female karyotypes reported in POC testing are in fact cases of MCC, when contamination completely obscures the fetal material. The overall MCC rates of POC samples across different laboratories vary, but can occur in as high as in 89.7% of cases (Jarrett et al., 2001; Lathi et al., 2014; S. T. Romero et al., 2015), thus indicating different sample management and demonstrating that the general awareness of MCC in this context is limited and needs to be improved.

The recorded bias towards a higher number of 46,XX karyotype reports together with the existing problem of MCC in POC testing points to a limited awareness of the technical limitations and critical aspects of methodologies used for POC analysis. Crucially, this failing needs to be acknowledged by laboratory specialists and consulting physicians. Therefore, the aim of this study was to develop a protocol for MCC assessment and to formulate POC material handling, testing, and reporting recommendations.

3.3 Results and Discussion

3.3.1 Visual POC inspection and MCC genetic testing

POCs specimens are considered ones not containing any identifiable material from the *fetus proper* (e.g. cord, amnion), but rather consisting of villi, membranous material (Jarrett et al., 2001) and other tissues of unspecified origin. Visual inspection of the primary POC material (n = 86) resulted in the following observations: 55 were good quality samples, 19 compromised quality POCs with signs of tissue maceration, and 12 samples where no tissue with typical villous morphology could be detected – marked "no chorion". Four formalin fixed paraffin embedded (FFPE) samples were marked as compromised and one had no signs of villi.

Forty-seven sample pairs (POC and maternal genomic DNA) subjected to polymorphic microsatellite (short tandem repeat, STR) loci genotyping based MCC detection protocol developed by us revealed that in 33 (70.2%) of the POC samples maternal genome was not detected; of those, one was marked as compromised quality, while the remainder demonstrated good quality chorions. Eight samples (17.0%) showed the presence of MCC; of those, six were classified as compromised quality chorions and two showed no villi upon visual inspection; one sample with MCC was positive for the *SRY* region. Six POC samples (12.8%) showed only maternal genome; three were of poor quality and three showed no visual presence of villi.

Data from forty-one sample pairs (excluding samples showing only maternal genome) was used to calculate allelic frequencies (AF) of the STR loci included in the assay and is presented in S1. Each STR locus revealed 9.7 ± 1.5 alleles (average ± standard deviation (SD)). Out of 14 loci 8.5 ± 2.0 (average ± SD) were informative in distinguishing fetal and maternal genotypes. Theoretical probability of the assay being not-informative i.e., giving false perception that fetal sample contains only maternal genome, was calculated to be 1.9E-08. Thus, it can be assumed that MCC detection system provides reliable results and can be used with high confidence.

3.3.2 Chromosomal microarray analysis

All POC samples (n = 86) were subjected to aCGH analysis irrespective of biological material quality and MCC testing. In total, 34 samples corresponded to normal female karyotype and 16 to normal male karyotype (sex ratio 2.1:1). The remaining 36 (41.9%) samples exhibited some kind of chromosomal abnormality, out of those 12 contained an XX sex chromosome set, 11 contained XY (sex ratio 1:0.9), and 13 were associated with sex chromosome copy number variations (Table 3.1). The majority of chromosomal imbalances were autosomal trisomies, followed by pure monosomy X (four cases). Of seven cases showing

some kind of sex chromosome discrepancy, four indicated a mosaic form of X monosomy – arrmos(X)x1, while three cases were unable to be resolved using aCGH analysis alone. Lastly, following structural aberrations were detected: loss of 8p23.2p11.21, gain of 22q13.2q13.33, and combined gain of 11p15.5p15.2 and 15q26.1q26.3 in one sample.

Table 3.1

Chromosomal pathology distribution across positive samples (n=36)

Chromosomal pathology	Cases
Trisomy 2	1
Trisomy 13	3
Trisomy 15	4
Trisomy 16	4
Trisomy 17	1
Trisomy 18	1
Trisomy 19	2
Mosaic trisomy 19	1
Trisomy 20	1
Trisomy 21	1
Trisomy 22	1
Monosomy X	4
Triploidy (69,XXY)	2
Sex chromosome discrepancies	7
Structural aberrations	3

3.3.3 Analysis of POC with high risk of MCC

A result indicative of the 46,XX karyotype should be treated with caution since it might arise from the analysis of maternal cells, especially in samples of unsatisfactory visual quality. As seen from the Figure 3.1., the poorer the quality of the samples included in the analysis (visually inspected), the higher the proportion of 46,XX samples and the lower the fraction of 46,XY and chromosomally abnormal samples. This was also true for samples tested for MCC if samples with partial MCC were included in the calculations. A significant difference (p-value 0.02) in the observed genotypes distribution was seen between the group having no signs of contamination upon MCC testing and all samples group. 46,XY samples were completely absent in the groups “Compromised quality + No chorion” and “No chorion”. The “Only maternal genome” group was not included since it contained solely 46,XX results upon aCGH testing as expected.

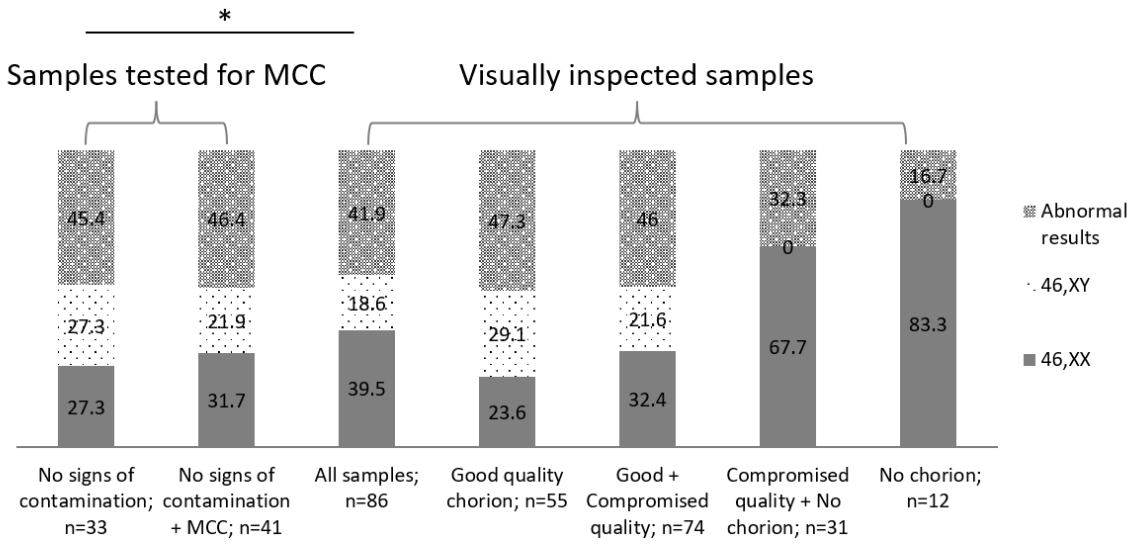

Figure 3.1 **Distribution of karyotype results across different POC evaluation groups**

Samples were grouped based on the MCC testing results (no signs of contamination; MCC; maternal genome only – not included in the figure since those contains only 46,XX results as expected) and visual sample evaluation (good quality chorion; compromised quality; no chorion). *Significant difference (p-value 0.02) in the observed genotypes distribution was seen between the groups "No signs of contamination" and "All samples". MCC – maternal cell contamination.

The origin of cells / tissue having the 46,XY karyotype or any chromosomal abnormality (n = 28) is indubitable (i.e. fetal). Figure 3.2 visually represents that vast majority of these cases (82.1%) concentrated among samples showing good visual quality and no signs of contamination upon MCC investigation. Nevertheless, four cases (14.3%) were found amongst compromised quality POC, three of them having a certain amount of MCC. One case indicative of a sex chromosome discrepancy upon aCGH analysis was localized in the "no chorion" group and also displayed MCC but was positive for the SRY region. Based on standard criteria samples of compromised visual quality would have been discarded (S. T. Romero et al., 2015), because having viable cells or POC with identifiable villi was crucial for cytogenetic and molecular cytogenetic techniques, e.g. FFPE samples typically required pathologist conclusion on fetal cells presence prior to DNA extraction. Here we demonstrate that simple and quick step of MCC evaluation can rescue some percentage of poor primary biological samples and increase number of correct diagnoses.

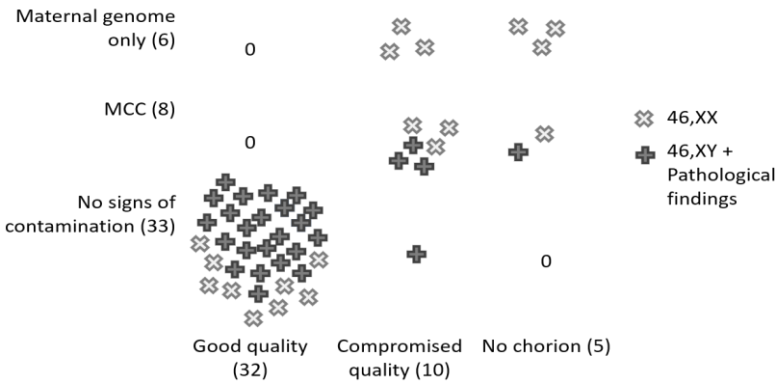

Figure 3.2 **Distribution of MCC-high and -low risk karyotypes across different product of conception evaluation groups**

Y-axis depicts sample evaluation based on MCC genetic testing using STR genotyping. X-axis depicts visual examination of primary biological sample. MCC-high risk are samples corresponding to "46,XX" karyotype (depicted as white X's) can arose from analysis of fetal cells or maternal cells thus masking any genuine fetal karyotype. MCC-low risk samples are the ones showing 46,XY karyotype or any chromosomal pathology (depicted as grey crosses). MCC – maternal cell contamination.

3.3.4 Recommendations to reduce MCC in POC testing

The identification of a chromosomal abnormality in POC material not only provides an explanation for the miscarriage but also removes the need for further investigations. Current American Society for Reproductive Medicine (ASRM) recommendations state that chromosome testing of miscarriage specimens may be of psychological benefit to the patient and may aid treatment decisions in the setting of treatment of recurrent pregnancy loss ("Evaluation and Treatment of Recurrent Pregnancy Loss: A Committee Opinion," 2012). The main reasons for unsuccessful POC testing are loss of cell viability (applies to cytogenetic techniques) and MCC (applies to a majority of methodologies) (Table 3.2). Therefore, the ASRM recommends that in the case of a 46,XX result, maternal blood should be obtained for the differentiation of maternal from fetal source of the euploid result by the means of microsatellite analysis ("Evaluation and Treatment of Recurrent Pregnancy Loss: A Committee Opinion", 2012). Solutions to MCC assessment are well documented for cases of prenatal genetic testing when the material for testing is obtained by the means of amniocentesis or chorionic villi sampling of ongoing pregnancy. Authorities in the field of medical genetics (American College of Medical Genetics, Association for Molecular Pathology) agree and emphasize that the presence of MCC that may interfere with the interpretation of fetal results must be excluded, using STR testing or exploiting technology capable of distinguishing MCC e.g. SNP-arrays (American College of Medical Genetics (ACMG), 2009; Monaghan et al., 2020; Nagan et al., 2011).

Table 3.2

Comparison of different methodologies used for POC analysis

	POC testing methodology	Advantages	Disadvantages / Limitations	Reference
Cytogenetics	Direct karyotyping of non-cultivated cytotrophoblast / syncytiotrophoblast	Relatively inexpensive. Assessment of the full set of chromosomes, structural / balanced aberrations, and ploidy in spontaneous mitoses.	Laborious. Degradation of primary biological material. Limited number of trophoblast cells within a sample. Limited number of metaphases (critical in case of mosaicism). Limited resolution. MCC.	(Schlesinger et al., 1990; Simoni et al., 1983)
	Karyotyping of cultivated cytotrophoblast / syncytiotrophoblast	Relatively inexpensive. Assessment of the full set of chromosomes, structural / balanced aberrations, and ploidy detection.	More laborious than direct karyotyping. Degradation of primary biological material. Culture failure. Maternal cell overgrowth. Limited number of metaphases. Limited resolution. MCC.	(Bell et al., 1999; Y. X. Zhang et al., 2009)
	FISH	Relatively quick, simple, and inexpensive. Possibility to study non-cultured cells and FFPE samples. Analysis of metaphase (mFISH) and interphase cells (iFISH). Low-grade mosaicism, ploidy and sex discrepancies assessment.	Analysis of limited number of chromosomes. Can miss structural rearrangements. Structural or micro-rearrangements could be analyzed only on demand. Analysis can be compromised due to quality of primary biological material (mFISH). MCC.	(Dória et al., 2010; Fejgin et al., 2005; Russo et al., 2016; Shearer et al., 2011)
Molecular cytogenetics	aCGH	Assessment of the full set of chromosomes. Broad resolutions available (possibility to detect submicroscopic changes). Analysis does not depend on the quality of the primary biological material. Possibility to analyze FFPE samples.	Relatively laborious and expensive. Ploidy can be assessed only in certain cases – if chromosome Y is present, some polyploidies will be missed (e.g. 69,XXX). Cannot distinguish balanced aberrations and low-level mosaicism. Burdened interpretations of structural VUSs in case of high-resolution arrays. Analysis of parental samples may be necessary for correct interpretation of the results. MCC.	(Ballif et al., 2006; Dong et al., 2016; S. Bin Lin et al., 2015; Sahoo et al., 2017; Schaeffer et al., 2004; Y. X. Zhang et al., 2009)

Table 3.2 continued

	POC testing methodology	Advantages	Disadvantages / Limitations	Reference
Molecular cytogenetics	SNP arrays	High resolution assessment of the full set of chromosomes. Analysis does not depend on the quality of the primary biological material. Possibility to analyze FFPE samples. Assessment of copy neutral loss of heterozygosity, uniparental disomy (heterodisomy), and triploidy (diandric, digynic). Detects chromosomal aberration's parent of origin. Mosaicism (up to ~10–20%) and MCC detection.	Relatively laborious and expensive. Cannot distinguish balanced aberrations. Cannot detect tetraploidy caused by the failure of cytoplasmic cleavage at the first division in the zygote. Burdened interpretations of small structural VUSs – analysis of parental samples may be necessary for correct interpretation of the results.	(Lathi et al., 2012; B. Levy et al., 2014; S. A. Scott et al., 2010; Huimin Zhang et al., 2016)
Molecular	QF-PCR	Fast, simple, inexpensive copy number assessment of critical chromosomes. Analysis does not depend on the quality of the primary biological material. Possibility to analyze FFPE samples.	Limited number of chromosomes. Normally needs confirmatory analysis. Discrepancies in copy number call due to mosaicism and structural chromosome aberrations. MCC.	(Ari et al., 2018; Donaghue et al., 2010)
	MLPA	Relatively inexpensive. Analysis of the whole set of chromosomes (copy number assessment of subtelomeric regions). Ploidy detection. Analysis does not depend on the quality of the primary biological material. Possibility to analyze FFPE samples	Normally needs confirmatory analysis. Cannot distinguish balanced aberrations. Analysis of parental samples may be necessary for the correct interpretation of the results. Indicate only combined copy number of X and Y. Discrepancies in copy number call due to mosaicism and structural chromosome aberrations. MCC.	(Ari et al., 2018; Bruno et al., 2006; Saxena et al., 2016)
	NGS	Assessment of the whole set of chromosomes with broad possibility of resolution adjustment. Analysis does not depend on the quality of the primary biological material. Possibility to analyze FFPE samples. Possibility to assess copy neutral loss of heterozygosity.	Expensive, laborious, not routinely used for the POC analysis. Cannot reliably detect all types of polyploidy but can detect embryos with unbalanced sex chromosome ratios. Complicated interpretation. Maternal cell contamination (pipeline can be adjusted to detect MCC).	(S. Liu et al., 2015; Shen et al., 2016)

Table 3.2 continued

	POC testing methodology	Advantages	Disadvantages / Limitations	Reference
Other	Flow cytometry	Rapid, relatively inexpensive. Ploidy analysis. Possibility to analyze FFPE samples.	Only used to complement conventional methods with ploidy status. Analyzer routinely unavailable at genetic laboratories (cytology laboratory).	(Berezowsky et al., 1995; Hedley et al., 1983; Lomax et al., 2000)

* POC – products of conception; MCC – maternal cell contamination; FISH – fluorescence in situ hybridization; aCGH – array comparative genomic hybridization; FFPE – formalin-fixed paraffin-embedded; SNP – single nucleotide polymorphism / variation; QF-PCR – quantitative fluorescent polymerase chain reaction; MLPA – multiple ligation probe amplification; NGS – next-generation sequencing.

Assessment of STR loci is widely used in human genetic identification (e.g. CODIS core loci (Karantzali et al., 2019)), because STRs are highly variable and evenly distributed throughout the genome; and while there are various ways to assess MCC in POC (Bell et al., 1999; Hassold et al., 1983), STR genotyping using commercially available or in-house tests is an easy and reliable way to perform MCC testing (American College of Medical Genetics, 2011; Jarrett et al., 2001; Schrijver et al., 2007).

Although MCC testing of prenatal samples is recommended in guidelines, only 60% of surveyed laboratories across US performed it without exception (Schrijver et al., 2007). Unfortunately, the exact data for the POC testing is unknown. However, as can be seen from the issues raised in literature the numbers might be similar. While the problem of MCC is universal to POC and prenatal samples, there are major differences in processing the both types of samples. Therefore, due to lack of comprehensive practice guidelines, we aimed to formulate recommendations addressing the entire workflow of POC samples handling from preanalytical, through the analytical stages.

As a starting point, clinicians carrying out the collection of primary material after curettage of a missed abortion need to be trained to visually inspect the tissues and collect the correct ones. It is not unusual that the transportation medium is unsuitable, e.g., distilled water or formalin. To reduce number of rejected samples, it is the responsibility of the laboratory to provide clinicians with unequivocal sampling guidelines and collection containers with the appropriate medium.

In comparison to curettage medically induced abortion is considered safer procedure for the female (Behnamfar et al., 2013; Kovavisarach and Jamnansiri, 2005; Niinimäki et al., 2006). This allows for the patient to collect the miscarried material herself in a stationary or home setting – the same also applies to the situations when the spontaneous abortion is clinically expected. While the laboratory cannot control the quality of the collected material, it can provide these patients with the instructions and a suitable container with the proper medium. Alternatively, if access to the collection medium is not possible, transportation without medium is possible. In all cases, the POC sample has to be transferred to the laboratory as quickly as possible, preferably within 24 hours, in order to preserve the viability of cells and ensure appropriate sample evaluation and processing prior to genetic testing. Sampling of maternal material (blood / buccal swab) concurrently with POC sampling / sample delivery to the laboratory is a beneficial practice to implement to avoid repeated visits to the clinic.

The next important step is the visual inspection of primary biological material by trained laboratory personnel. We did not find it necessary to perform POC dissection under a microscope since typical villous morphology can be clearly visualized with the naked eye.

Chorion tissues need to be carefully separated and thoroughly washed with an appropriate buffer (saline, PBS) to remove any maternal decidua, blood, mucus (Figure 3.3).

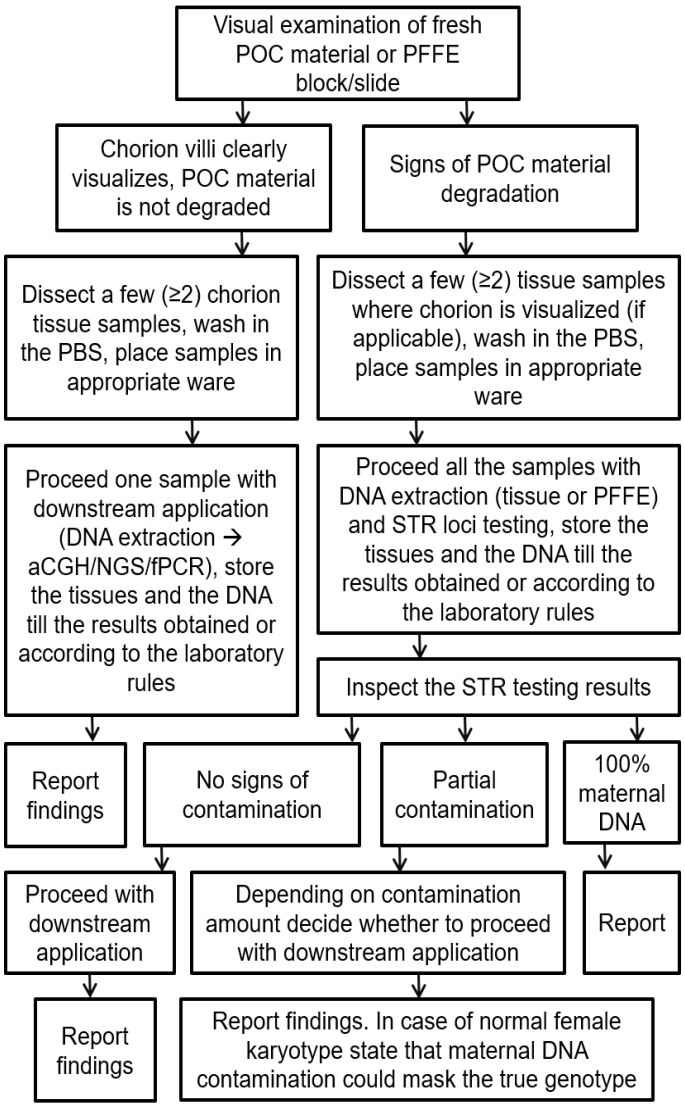

Figure 3.3 **POC handling and testing workflow**

Recommendations addressing the entire workflow of POC samples handling from preanalytical, through the analytical stages suitable for molecular / molecular cytogenetic techniques. POC – product of conception. FFPE – formalin fixed paraffin embedded samples; PBS – phosphate buffer saline; STR – short tandem repeats; aCGH – array comparative genomic hybridization; NGS – next generation sequencing, fPCR – fluorescent polymerase chain reaction.

From our investigations, we advise to avoid selection of poor specimens i.e., grossly necrotic tissues with no signs of typical villous morphology, if no MCC testing is performed, as they rarely contain fetal genome. It was suggested before that POC samples where only villi or a combination of villi and membranous material was identified and which resulted in normal female karyotype should be viewed with suspicion (Rodgers et al., 1996). In contrast, our experience shows that DNA extracted from samples displaying clear villous morphology do not have any signs of maternal genome and consequently is able to be processed without MCC

testing. Sampling of multiple (two or three) pieces and written witnessing of the sampled tissue quality are beneficial for downstream application results interpretation and proper reporting.

Depending on the POC testing methodology used, the impact of an arbitrary amount of maternal genome on the result can range from absolutely no effect to complete obscuration of the genuine fetal genome. Therefore, it is recommended that MCC systems capable of quantifying the amount of MCC are developed and also that the extents to which certain methodologies can tolerate the presence of MCC are validated. We exploited our MCC detection protocol solely for the qualitative evaluation of MCC. However, based on the peak height observed in our fluorescent PCR assay, it was possible to detect as low as approximately 5% of maternal genome in the POC sample, although such an amount is unlikely to obscure results obtained by molecular cytogenetic techniques and can be considered negligible.

It is the responsibility of the laboratory to evaluate the practicability and validity of any particular methodology before its application in clinical practice. Reporting should follow specific diagnostic reporting practices and all reports must be complemented with adequate counseling.

3.3.5 Further considerations to improve POC testing practice

In the era of genetic testing diversity, it is essential to choose the most precise, detailed and reliable techniques adding to cost-efficiency and overall utility for POC analysis e.g. aCGH which has demonstrated certain advantages over classic cytogenetic methodologies (Hyde and Schust, 2015; Lomax et al., 2000). A great tool for MCC confirmation without the need for additional MCC testing is SNP microarrays which also yields higher diagnostic return (Lathi et al., 2014; B. Levy et al., 2014). Additionally, more sophisticated primary biological material sampling practices (if applicable) could be adopted, e.g. precise chorion or embryo proper sampling *in utero* using hysteroscopic embryoscopy (Robberecht et al., 2012).

If, for any reason, MCC testing is unavailable, it is essential to estimate the probability of obtaining an unreliable 46,XX result. To demonstrate how biased POC reporting could be if a laboratory adopted a procedure of testing all samples without visual evaluation and MCC testing, we applied a suitable mathematical model developed by Nikitina and colleagues (Nikitina et al., 2005) to our results. The proposed model exploits samples having XX or XY sex chromosomes set excluding all cases with sex chromosome discrepancies (e.g., XO and XX / XY) and is largely based on the initially obtained MCC coefficient (k). Since we have performed MCC testing, we were able to calculate actual k (Table 3.3). The model demonstrated that the percentage of samples obscured by MCC could be as high as 21.24%

(16 samples in our scenario), which significantly (p-value 0.0006) changes expected distribution of the genotypes across the samples.

Table 3.3

Observed and expected genotypes distribution when corrected for maternal cell contamination based on the approach described in (Nikitina et al., 2005)

Parameter	Value	Formula
Maternal cell contamination coefficient, k	0.32	Maternal genome only samples (n = 6) / XX samples (n = 19)
Actual genotypes distribution excluding XO and XX / XY cases (n = 73)		
46,XX normal (A)	34	–
46,XX abnormal (B)	12	–
46,XY normal (C)	16	–
46,XY abnormal (D)	11	–
Number and distribution of genotypes obscured by MCC		
Female normal A(fn)	18.49	A(1-k)-(ABk / C+D)
Female abnormal A(fa)	4.77	ABk / C + D
Male normal A(mn)	6.36	ACk / C + D
Male abnormal A(ma)	4.37	ADk / C + D
Percentage of wrongly genotyped samples	21.24%	(Afa + Amn + Ama) / N
Expected genotype distribution		
A expected	18	A = A(fn)
B expected	17	B + A(fa)
C expected	22	C + A(mn)
D expected	15	D + A(ma)
Chi-square (observed genotypes vs expected), p-value	0.0006	–

Samples with the 46,XX karyotype would be expected to fall from 0.47 to 0.25 when corrected for MCC, which is similar to our value of 0.27 obtained from samples having no signs of contamination upon MCC testing. Despite the theoretical nature of the model, it may be useful for estimating the MCC value in an established culture regimen in any laboratory, thus serving as a valuable tool for quality control (Nikitina et al., 2005).

We have demonstrated how different approaches of visual POC material evaluation complemented with MCC testing lead to predictable and reliable POC genetic testing outcomes. While our formulated recommendations covering all steps of POC material processing cannot influence the quality of primary biological samples, those once implemented by laboratories can actually improve their POC genetic testing practices, acknowledge, and diminish problem of MCC. Ultimately, through a combination of different techniques and the development of an up-to-date approaches to POC handling and testing, from the perspective of reproductive counseling a significant advancement could be achieved with the elucidation of the cause of miscarriage for most cases.

3.4 Materials and Methods

3.4.1 Patients

Patients experiencing miscarriage or spontaneous abortion before the 13th week of gestation were recruited to the study. POC material was either obtained by uterine curettage performed by a gynecologist or collected by patients themselves and transferred in 0.9% sodium phosphate buffer or without medium to the laboratory within one to two days after collection. Cases of *fetus proper* (n = 2) were excluded. Five samples were FFPE tissues. In total, 86 POC samples were included in the study. The gestational age at the time of early pregnancy loss was 8.6 ± 2.2 (average ± SD) weeks and the patient age was 32.7 ± 5.5 (average ± SD) years. Peripheral blood samples were obtained only from 47 women for genetic analysis of MCC, unfortunately we could not obtain blood specimens from rest of the patients.

3.4.2 Primary biological material handling

Upon receipt, each POC sample was visually inspected by trained laboratory personnel. Visual appearance of the sample was recorded as follows: "good quality chorion" – if presenting typical villous morphology; "poor quality chorion" – if presenting tissue maceration and only a few villi could be dissected; or "no chorion visualized" – if no tissues with typical villous morphology could be localized. Even in the absence of typical villous morphology, tissue sampling was performed for all the samples. Tissues were washed in 1x PBS [Thermo Fisher Scientific, UK] and three pieces of each sample were placed in separate tubes for subsequent nucleic acid extraction. DNA was extracted from fresh POC, FFPE tissues, and peripheral blood following the manufacturer's standard protocol [Qiagen, Germany].

3.4.3 POC chromosome analysis

Chromosome analysis was performed by aCGH for all the POC samples following the manufacturer's protocol [24sure; Illumina, USA]. Microchip slides were scanned with an InnoScan scanner [Innopsys, France]. Images in Tiff format were imported into BlueFuse Multi v.4.0 [Illumina] and the resulting copy number karyotypes were assessed. The given methodology detects unbalanced chromosomal material changes > 5Mb and can detect polyploidy if sex chromosomes are represented by at least one X and Y chromosomes.

3.4.4 MCC testing system design

To test for MCC signs in samples where maternal DNA was available (n = 47), we designed a detection system employing fluorescent PCR with visualization by capillary electrophoresis. The system encompassed 14 microsatellite (STR) loci, the AMEL region giving different amplicon lengths on X and Y, and the *SRY* region for the more precise genotyping of chromosome Y. In order to detect MCC, maternal DNA and DNA extracted from the POC sample need to be tested in parallel.

All loci of interest were located through the University of California Santa Cruz's genome browser (https://genome-preview.ucsc.edu/index.html). For all genomic regions, fluorescently tagged (5′ end 6-FAM or HEX) primers for fluorescent PCR were located manually and specificity was confirmed using IDT's primer tool (https://eu.idtdna.com/ calc / analyzer) and Primer-BLAST (Ye et al., 2012). Primer pairs were multiplexed into four mixes, each containing four loci (S2).

The PCR mixture for each multiplex was as follows: 3.6 μl of ddH_2O, 7.5 μl of Type-it Master Mix [Qiagen], 0.32 μl of each primer (0.2 μM, eight primers for one multiplex), and 1.5 μl of DNA (~50 ng / μl) in a total reaction volume of 15.16 μl. The cycling conditions were: initial denaturation for 5 min at 95°C, followed by 28 cycles of 30 s at 95°C, 1 min 30 s at 60°C, and 30 s at 72°C; final extension for 10 min at 72°C. For the initial PCR product visualization as a quality control step, the amplified products were run on 2% agarose gels. Amplicon separation and sizing were carried out using capillary electrophoresis [ABI Prism 3500 DNA Analyzer; Applied Biosystems, USA] and GeneMapper v.4.0 software [Applied Biosystems], respectively.

STR marker is considered not-informative when the paternal allele transmitted to the conceptus being the same size as the non-inherited maternal allele – such situation can result in the false consistency of MCC. The probability of this event decreases with the usage of reasonable number of highly polymorphic STR markers, increasing overall informativeness of the assay (Nagan et al., 2011). A proposed minimum number of STR markers to be analyzed is four to six (Jarrett et al., 2001). Fourteen STR markers exploited here have previously been used for linkage analysis in preimplantation genetic testing and therefore are known to be biallelic and polymorphic, and the individual primer pair performance has already been checked (Ludmila Volozonoka et al., 2018). Since population AF of the STR markers used is unavailable, those were calculated from the genotyping data of POC and maternal genomic DNA sample pairs (n = 47); allele shared between mother and fetus was excluded from AF counting. Highest allelic frequencies of each locus were multiplied, thus estimating probability of the whole assay being not informative.

MCC testing results were classified as follows: “MCC” – in case of informative STR marker characterized by three alleles visible on electropherogram, two of which match the alleles of the mother (or two alleles, if mother was homozygous); “maternal genome only” – characterized by the complete allelic match of the two samples across all loci; “no signs of contamination” – characterized by the second allele in a fetus distinguishable from the mothers’ alleles across informative markers. The developed STR testing system only allows for the qualitative not the quantitative evaluation of MCC based on STR loci differences between the genomes being compared.

3.4.5 Statistical analysis

Data were described with average values and SD if appropriate. Genotypes across different sample categories were compared using the Chi-square test. Statistical significance was assumed at $p < 0.05$. Statistical analyses used the Microsoft Excel 2019, Version 16.0.

3.4.6 Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki’s ethical principles and its protocol was approved by the Central Medical Ethics Committee (14.04.2016. Nr.1/16-04-14). Patients considered for genetic testing were counseled and the testing principles were clearly explained. All the recruited patients signed an informed consent. Patients acknowledged that they could not be identified from the article as personal data were fully anonymized.

3.5 Declaration of interest statement

The authors report no conflict of interest.

3.6 Author contributions

Conceptualization: LV, DP, LG, LK, AM; Data curation: LV, LG, LK, AM; Methodology: LV, DP, LG, LK, AM; Software: LV, DP; Visualization: LV; Writing - original draft: LV; Writing – review and editing: LV, DP, LK, IK, VF, AM; Funding acquisition: LG, IK, VF; Project administration: LG, IK, VF, AM; Resources: LG, IK, VF, AM; Supervision: LG, IK, AM.

3.7 Appendices

S1. STR loci used for maternal cell contamination assessment in products of conception (POC). Allelic frequencies of each allele were obtained from genotyping POC and maternal genomic DNA sample pairs (n = 47). Highest allelic frequencies of each locus are marked in grey.

D4S2936		D17S1791		D4S3023		D17S647		D17S960		DXS8086		D17S906	
1	0.0265	1	0.0083	1	0.2301	1	0.0261	1	0.0690	1	0.0085	1	0.0450
2	0.0177	2	0.1167	2	0.3451	2	0.4174	2	0.0776	2	0.1453	2	0.0450
3	0.3097	3	0.0083	3	0.1504	3	0.1043	3	0.0259	3	0.0171	3	0.2342
4	0.2655	4	0.0583	4	0.0088	4	0.0435	4	0.0086	4	0.1795	4	0.1351
5	0.1239	5	0.0500	5	0.1239	5	0.0087	5	0.0259	5	0.2051	5	0.0811
6	0.1150	6	0.0250	6	0.0177	6	0.0174	6	0.1810	6	0.3077	6	0.0360
7	0.0442	7	0.0167	7	0.0885	7	0.1913	7	0.1724	7	0.0256	7	0.0360
8	0.0177	8	0.0583	8	0.0177	8	0.1652	8	0.0776	8	0.0342	8	0.3423
9	0.0354	9	0.0167	9	0.0088	9	0.0087	9	0.2586	9	0.0513	9	0.0360
10	0.0088	10	0.1500	10	0.0088	10	0.0174	10	0.1034	10	0.0256	10	0.0090
11	0.0177	11	0.0750	–	–	–	–	–	–	–	–	–	–
12	0.0177	12	0.1083	–	–	–	–	–	–	–	–	–	–
–	–	13	0.2333	–	–	–	–	–	–	–	–	–	–

DXS1215		DXS1200		D4S126		D4S127		DXS8011		DXS8377		D4S412	
1	0.0420	1	0.0336	1	0.0179	1	0.0086	1	0.0085	1	0.1167	1	0.0164
2	0.0336	2	0.2185	2	0.0089	2	0.0517	2	0.0169	2	0.1583	2	0.2623
3	0.3109	3	0.2689	3	0.0357	3	0.1983	3	0.2034	3	0.0417	3	0.1967
4	0.1681	4	0.1933	4	0.1071	4	0.1810	4	0.0339	4	0.0750	4	0.0410
5	0.1765	5	0.2101	5	0.2054	5	0.0948	5	0.1695	5	0.2750	5	0.0246
6	0.1092	6	0.0588	6	0.2679	6	0.1983	6	0.1610	6	0.0833	6	0.0738
7	0.1345	7	0.0168	7	0.1696	7	0.0776	7	0.2119	7	0.0500	7	0.2377
8	0.0252	–	–	8	0.1161	8	0.1034	8	0.1186	8	0.1750	8	0.1230
–	–	–	–	9	0.0268	9	0.0862	9	0.0763	9	0.0250	9	0.0246
–	–	–	–	10	0.0446	–	–	–	–	–	–	–	–

S2. Primers used for MCC testing

Locus	Repeat	Forward Primer	Reverse Primer	Length (bp)	Mix
SRY	NA	HEX-TCGCACTCTCCTTGTTTTTGA	ATCATCGCTGTTGAATACGCT	71	1
AMEL	NA	HEX-CTCACATTCTCAGGCTATCAATG	GCATGCCTAATATTTTCAGGGA	219(X) 222(Y)	1
D4S127	CA repeat	HEX-TACGGAAAGGAGAAGGGCAT	CTCTGAAGAGGTGGTTCGC	187	1
DXS8011	CA repeat	FAM-CAAAACCCTGTCTACACGC	TGAATCACCCAATTTTCTCAAAC	341	1
DXS1200	CA repeat	HEX-CCACTCTTAGCAATTGACAGC	TGTCGTTGGGTGGAATGATG	142	2
D4S412	CA repeat	FAM-AGGGATAAAGGAAATGTGCGA	GCACTGGTAAGCACCAATCT	218	2
DXS8377	Complex repeat	FAM-CAGCCTACATCTACCACTTCA	CCCACTGATACAGCAGGAAAG	310	2
D17S906	Complex repeat	FAM-TGAGCCTGGGGAGGTCG	CGTTCTAGCAGAGTGAAACTGT	418	2
D17S960	CA repeat	FAM-AGGCCTGTTTGGGGTTG	CTCCTGGCTACGTGGTGAT	163	3
D17S1791	CA repeat	FAM-ATGCTTGCAGCAAGAAGGC	GGGAGCTTTTGGTCAACCT	235	3
DXS8086	CA repeat	HEX-CCATTGGCTTTCTTGGGTTTC	TTGTGTATTAGCCAGGGTTCTC	271	3
D4S2936	CA repeat	FAM-TAAAAACACGCACACACAGG	AACCATCGCCGGAGAAATAAT	433	3
D4S3023	CA repeat	FAM-CGGTCTAGATGCCTCAGAAAC	CTCACTGGAAACTAAATGGGAC	133	4
D17S647	Complex repeat	FAM-CAAGGCTCCCATCGAGATT	GCCTGGAGAAGAGAAGATGAA	165	4
DXS1215	CA repeat	FAM-ACTAGCAGATGTGTAAGCTTGG	CCTTCCTTTCCTTGACTCTACA	220	4
D4S126	CA repeat	HEX-CCAAAGTTAGAGGTCAGAAAGAC	GGTAGCCGAGATCCTGTCA	266	4

4 Genetic landscape of preterm birth due to cervical insufficiency: Comprehensive gene analysis and patient next-generation sequencing data interpretation

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Personal input:

My personal input into this work includes study design, systematic literature and gene analysis, NGS data interpretation, writing the original draft of the manuscript. Please see supplementary files for the the signed forms from all the co-authors to use this manuscript for my thesis (Supplement 5).

4.1 Abstract

Preterm delivery is both a traumatizing experience for the patient and a burden on the healthcare system. A condition distinguishable by its phenotype in prematurity is cervical insufficiency, where certain cases exhibit a strong genetic component. Despite genomic advancements, little is known about the genetics of human cervix remodeling during pregnancy. Using selected gene approaches, a few studies have demonstrated an association of common gene variants with cervical insufficiency. However, until now, no study has employed comprehensive methods to investigate this important subject matter. In this study, we asked: i) are there genes reliably linked to cervical insufficiency and, if so, what are their roles? and ii) what is the proportion of cases of non-syndromic cervical insufficiency attributable to these genetic variations? We performed next-generation sequencing on 21 patients with a clinical presentation of cervical insufficiency. To assist the sequencing data interpretation, we retrieved all known genes implicated in cervical functioning through a systematic literature analysis and additional gene searches. These genes were then classified according to their relation to the questions being posed by the study. Patients' sequence variants were filtered for pathogenicity and assigned a likelihood of being contributive to phenotype development. Gene

extraction and analysis revealed 12 genes primarily linked to cervical insufficiency, the majority of which are known to cause collagenopathies. Ten patients carried disruptive variants potentially contributive to the development of non-syndromic cervical insufficiency. Pathway enrichment analysis of variant genes from our cohort revealed an increased variation burden in genes playing roles in tissue mechanical and biomechanical properties, i.e., collagen biosynthesis and cell-extracellular matrix communications. Consequently, the proposed idea of cervical insufficiency being a subtle form of collagenopathy, now strengthened by our genetic findings, might open up new opportunities for improved patient evaluation and management.

Keywords: human uterine cervix, cervical insufficiency, precocious cervical ripening, preterm birth, preterm delivery, pregnancy loss, genetics, genetic etiology, collagenopathy, systematic literature analysis, gene.

4.2 Introduction

In order to carry a successful term pregnancy, different organs such as the uterus, cervix, placenta, and amniotic membranes as well as the fetus itself must cohesively interact and create a healthy symbiotic relationship with each other and the rest of the female body (Vink and Myers, 2018). However, preterm birth (PTB) remains the leading cause of perinatal morbidity, mortality, and hospitalization in the first year of life in the developed world. Approximately 5–12% of newborns worldwide are born preterm (< 37 weeks of gestation) (Chawanpaiboon et al., 2019). Prematurity is a tremendous burden on the healthcare system as outcomes are associated with disability-specific lifetime medical, special education, and lost productivity costs (Frey and Klebanoff, 2016).

A common phenotype of spontaneous PTB is primarily characterized by progressive cervical effacement, after which preterm premature rupture of membranes (PPROM), persistent uterine contractions, prolapsed fetal membranes, or uterine bleeding may be the reason for acute care seeking.

4.2.1 Isolated cervical insufficiency

A distinguishable medical condition in obstetrics in which the cervix spontaneously starts to dilate (open) and efface (become thinner) in the absence of the signs and symptoms of labor is cervical insufficiency. The cervix, a collagen-rich organ, must remain closed during pregnancy yet simultaneously undergo a progressive physiological remodeling to prepare for the birth. Physiological cervical remodeling along with uterine contractile activation are the two

key events facilitating the birth of a child (Word et al., 2007). This remodeling can be loosely divided into four overlapping phases: 1) softening beginning in early pregnancy, 2) ripening shortly before the birth, 3) dilation starting with the onset of regular uterine contractions and resulting in cervical opening to allow passage of the term fetus, and 4) postpartum repair (Danforth, 1983; Timmons and Mahendroo, 2007; Word et al., 2007). In cases of cervical insufficiency, dilation of the cervix occurs without painful uterine contractions, leading to inability of the cervix to retain a term pregnancy. Repeating in consecutive pregnancies, cervical insufficiency is one of the causes of recurrent pregnancy loss (Vink and Feltovich, 2016) and can be a serious obstacle to the birth of a healthy child and complication-free postpartum period for the mother and newborn. In contrast, failure of the cervix to dilate would result in unsuccessful parturition (Banõs et al., 2015).

Clinically relevant isolated cervical insufficiency occurs in about 1–2% of all pregnancies, but is associated with as much as 5–15% of pregnancy losses in the second trimester (Mingione et al., 2003; S. W. Wang et al., 2016). However, as one of the factors in a complex PTB context, the condition is found much more frequently. In 2011, routine recording of cervical ripening was recommended by the Global Alliance to Prevent Prematurity and Stillbirth (Goldenberg et al., 2012), since a short cervix (defined as a transvaginal sonographic cervical length ≤ 25 mm in the mid-trimester of pregnancy) is the best predictive factor for spontaneous PTB < 34 weeks of gestation in both singletons and twins (Di Renzo, 2015). The shorter the cervix, the higher the risk; cervical insufficiency is likely at the extreme of this continuum (Manuck, 2016).

Multiple factors such as age, inflammation, stress, nutrition, physical activity, socio-economic status, vaginal microbiome, and uterine anomalies affect PTB (Bezold et al., 2013; Dunlop et al., 2015; R. Romero et al., 2014). Mid-trimester cervical weakness may be associated with a variety of events, e.g. cervical ablation (cryo, laser, or electro) or excision (knife, laser, or loop-electrosurgical), cervical intraepithelial neoplasia *per se*, cervical hypoplasia after diethylstilbestrol, or intrauterine infections (Kyrgiou et al., 2017).

PTB is currently perceived as a frequent complex medical condition which corresponds to the concept of a multifactorial disorder (Plunkett and Muglia, 2008) – analogous to, e.g., cardiovascular disease – the development of which depends on a number of interacting factors including environmental and genetic. Familial aggregation is evident in prematurity (Porter et al., 1997; Winkvist et al., 1998), including cases of cervical insufficiency, with up to 27% of patients having a first-degree relative with the same diagnosis on the mother's side (Raffi and Anumba, 2007; Warren et al., 2007). By contrast, the risk appears to be unaffected by a history of prematurity in the partner's family (Boyd et al., 2009). Epidemiological data show that

foetuses / neonates with Ehlers-Danlos syndrome (EDS), osteogenesis imperfecta, and restrictive dermopathy are at an increased risk of adverse pregnancy outcomes including PTB, PPROM, and cervical insufficiency (Anum et al., 2009; Young et al., 2007). A few studies have demonstrated a positive association of common gene variants in the mother's genome with cervical insufficiency (Sundtoft et al., 2016; Warren et al., 2007, 2009).

4.2.2 Current understanding of genetics of cervical remodeling during pregnancy is limited

Prior to the era of '-omics', the majority of studies investigating the role of genetics in prematurity targeted candidate genes with known biological roles potentially related to processes occurring during pregnancy (Plunkett and Muglia, 2008; Ribeiro de Andrade Ramos and da Silva, 2018). For example, common allelic variants / polymorphisms in *TNF*, *IL1B* and *IL6* genes have most consistently been associated with PTB (Varner and Esplin, 2005), underlining the role of inflammation in the pathogenesis of prematurity.

More recent studies on the genetics of PTB in humans can be roughly divided into two major categories. The first group is comprised of a small number of large-scale genomic studies investigating possible genetic risk factors for preterm delivery (Huusko et al., 2018; J. Li et al., 2017; McElroy et al., 2013; Heping Zhang et al., 2015). Unfortunately, none of these studies has addressed PTB as a result of cervical insufficiency. The second group consists of transcriptomic studies evaluating differential gene expression during different stages of gestation / parturition in eventless gestations (Bukowski et al., 2006; Hassan et al., 2009, 2010) and ones of particular phenotypes, e.g. cases of PTB (HP:0001622) or PPROM (HP:0001788; OMIM:610504) (Makieva et al., 2017; H. Xu et al., 2008). For a comprehensive evaluation, please refer to the excellent systematic review and meta-analysis of (Eidem et al., 2015).

Although the largest number of studies has focused on idiopathic PTB, this phenotype should be considered with caution since preterm delivery often encompasses cervical insufficiency, PPROM, placental abruption, uterine overdistension, or a combination of these complications (Manuck, 2016). This idea is further supported by a meta-analysis of gene expression studies across distinct gestational tissues and clinical phenotypes which demonstrated a limited overlap of genes identified as differentially expressed across the studies (Eidem et al., 2015). This suggests possible different physiological mechanisms underlying each phenotype and also indicates that large gaps still exist in the design of transcriptomic studies in prematurity.

Furthermore, in order to attain the true transcriptomic signature of a certain phenotype, the tissue of study should be chosen wisely. Considering the known heterogeneity of certain tissues, even the biopsy site may have an impact on the results. For example, a recent study has highlighted that it remains to be resolved whether a PPROM signature can be determined in the cervix as the gene expression patterns in cervical biopsies of PPROM in comparison to preterm labor samples did not share cluster membership, suggesting a distinct genetic signature specific to PPROM pathology (Makieva et al., 2017). Nonetheless, the authors proposed the notion that the rupture of membranes might be accelerated through PPROM-specific remodeling events within the cervix (Makieva et al., 2017). Of note, cervical insufficiency often results in unscheduled PPROM as well. Moreover, similar to the cervix, the mechanical strength of the fetal membranes is mainly ensured by the collagen network (Strauss, 2013; Uldbjerg et al., 1983). Indeed, collagen types I, III, IV, V, and VI, to name but a few, have been localized in both cervical (derived from the two paramesonephric ducts during embryogenesis) and fetal membrane (derived from the outer trophoblast layer of the implanting blastocyst) tissue (Malak and Bell, 1994; Minamoto et al., 1987).

Without doubt, our current understanding of human cervix remodeling in pregnancy is limited (Vink and Myers, 2018). This may be the reason for the bias of studied genes in relation to cervical insufficiency and the surprisingly little information that presently exists on the genetics of pathological cervical remodeling during pregnancy.

Since common variants detectable by genome-wide association studies (GWAS) typically explain only a minor proportion of the heritability of complex diseases (Asimit and Zeggini, 2010), there is a hypothesis that the rare variants in multiple genes implicated in PTB may cumulatively contribute to the predisposition of delivering preterm (Bezold et al., 2013; Strauss et al., 2018). We decided to test this hypothesis by performing next-generation sequencing (NGS) of the DNA of females with a positive anamnesis of isolated non-syndromic cervical insufficiency.

Due to the lack of knowledge of genes implicated in cervix functioning, we also conducted a systematic literature analysis to derive all possible studies on the genetics of the cervix. We subsequently composed a list of genes that play a role in the normal and pathological biology of the cervix in relation to pregnancy and prematurity and used this obtained knowledge to assist our NGS data interpretation. Given the described heritability of cervical insufficiency, the main questions we addressed in this study were: i) are there genes reliably linked to cervical insufficiency and, if so, what are their roles? and ii) how many cases of isolated non-syndromic cervical insufficiency are attributable to these genetic variations?

4.3 Materials and Methods

4.3.1 Identification of genes playing a role in the biology of the cervix

Systematic literature analysis: search strategy and study selection

We conducted a literature search according to the PRISMA guidelines (Moher et al., 2009) (Figure 4.1). The screening strategy aimed to retrieve studies focusing on genetic research of defective uterine cervix functioning leading to cervical insufficiency, preterm delivery, or pregnancy loss, as well as records on functional studies addressing the differential expression of genes within the cervix during different stages of normal / compromised pregnancy / parturition.

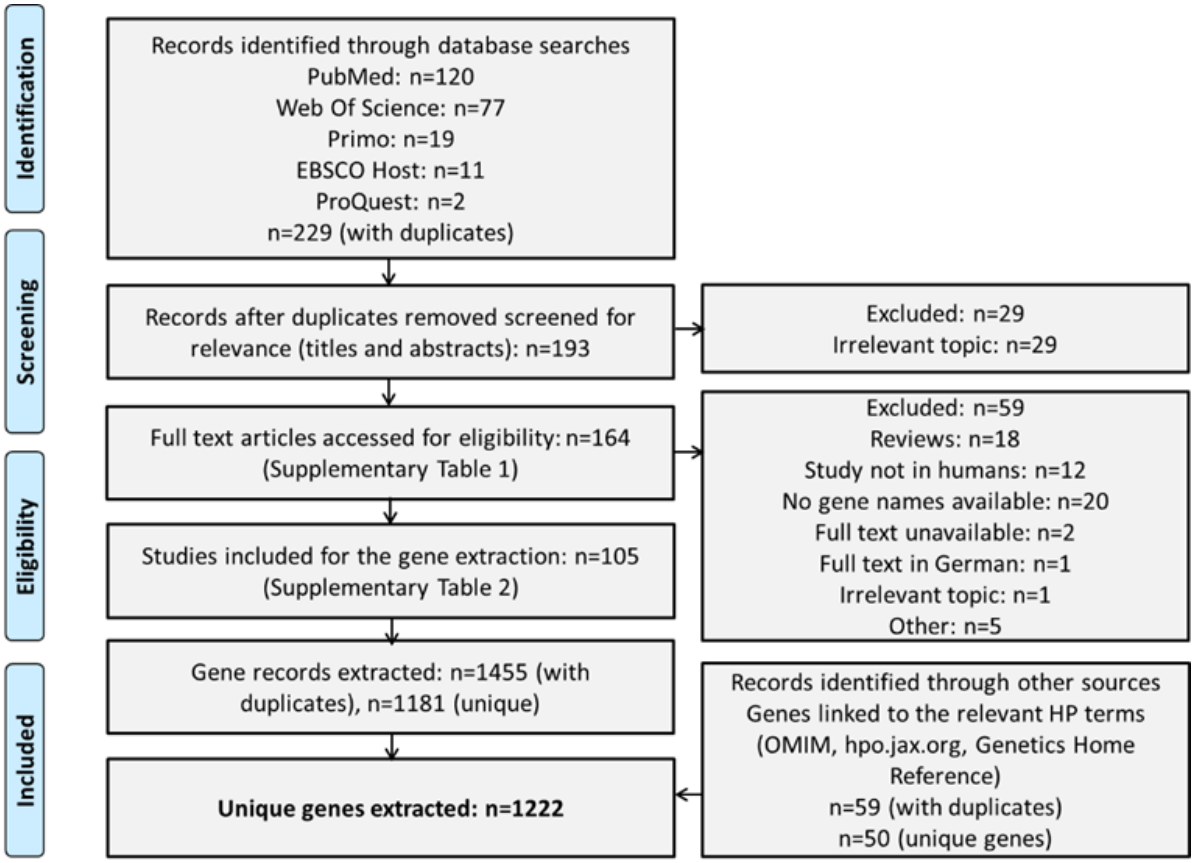


Figure 4.1 **PRISMA flowchart depicting the literature search and gene extraction**

The search was performed using the MeSH term “Uterine Cervical Incompetence” (D002581, which is indexed under two higher categories in the MeSH hierarchy: “Pregnancy Complications” and “Female Urogenital Diseases”) OR the following keywords: “precocious cervical ripening”, “cervical weakness”, “cervical insufficiency”, “istmocervical insufficiency”, “cervical incompetence”, “uterine cervix” AND “gene”, “genetics”, “gene expression”, “gene transcription”, “transcriptome” AND Humans [Mesh] NOT “cancer”, “microbiome”, “papilloma”. The search was performed in PubMed, EBSCO Host database,

Web of Science, ProQuest database, and Primo database. The search was performed in parallel by two reviewers on December 16, 2019, without further restrictions on the publication date.

Inclusion criteria: Study published in a peer-reviewed journal; Study presents original data; Study concentrates on finding a genetic cause of cervical insufficiency and / or preterm delivery; Study concentrates on functional gene analysis of physiological cervical ripening, cervical insufficiency, and / or preterm delivery as a source using cervical tissues. Only human studies were included. Exclusion criteria: Study concentrates on miscarriage and / or the first trimester of pregnancy; Study concentrates on microRNA, lncRNA, cell-free DNA, ribosomal DNA, cervico-vaginal microbiome, cancer analysis; Study is not in humans; Study is not available in English (S1 Table).

From the eligible papers presenting original data (S2 Table), we extracted the following: gene symbols in a HUGO Gene Nomenclature Committee-approved manner; recorded patient phenotypes, i.e. condition, or relevancy to our study's questions if healthy individuals were analyzed; biological material used for the analysis (DNA, cervical biopsy, other); type of study, e.g. functional, association, or other; information on the gene selection approach (unbiased genome-wide or selected gene approach). Screening of all the reviews (n = 18) for original references did not yield any additional articles to those in the original search list.

Additional gene identification

Further, we searched the most relevant Human Phenotype Ontology (HPO) terms for association with certain genes as well as all EDS-, osteogenesis imperfecta-, and restrictive dermopathy-related genes through Online Mendelian Inheritance in Man (OMIM), Genetics Home Reference (GHR), and https://hpo.jax.org as they previously showed clinical association with cervical insufficiency. The following HP terms were screened: Premature birth following premature rupture of fetal membranes (HP:0005100); Premature rupture of membranes (HP:0001788); Premature birth (HP:0001622); Premature delivery because of cervical insufficiency or membrane fragility (HP:0005267); Uterine rupture (HP:0100718); Uterine prolapse (HP:0000139); and Cervical insufficiency (HP:0030009).

4.3.2 Gene analysis

Based on the data obtained from all the eligible studies and additional syndromic gene searches, we composed three different lists of genes according to their relation to the genetics of the cervix. The first list encompassed genes primarily linked to cervical insufficiency and were either: i) studied directly in relation to cervical insufficiency alone or with any other

obstetrical condition; ii) shown to have an association with cervical insufficiency in cases of any genetic syndrome; or iii) shown to have an established gene-phenotype relationship with cervical insufficiency (HP:0030009; HP:0005267) as identified through the HPO / OMIM searches.

The second list contained genes with less evidence for cervical insufficiency than those in the first list and / or alleged collagen-related associations and were either: i) studied in relation to PPROM alone or with any other obstetrical condition; ii) shown to have an established gene-phenotype relationship with obstetrical complications (HP:0005100; HP:0001788; HP:0100718; HP:0000139; HP:0000140; HP:0001622 – not alone) as identified through the HPO / OMIM searches; iii) known to cause a genetic syndrome clinically associated with cervical insufficiency; or iv) studied in relation to cervical insufficiency but having no association as shown from case-control studies.

The third list consisted of genes demonstrating a function within the uterine cervix as shown from functional gene studies of physiological cervical ripening / pregnancy / parturition using either cervical biopsies / swabs from females without obstetrical complications or tissue cultures.

Additionally, to identify any differences in the biological information of genes studied exploiting selected gene approaches in comparison to those studied using unbiased approaches (i.e. genome-wide studies), we functionally annotated genes from both groups using the ConsensusPathDB interaction database (Kamburov et al., 2013) gene set analysis function ‘over-representation analysis’ and looked for ‘Pathway-based sets’ in all built-in pathway databases and ‘Gene ontology categories’ (level 2 categories) with a p-value cut-off of 0.01.

4.3.3 Next-generation sequencing of patients with cervical insufficiency

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki’s ethical principles. The study protocol was approved by the governmental Central Medical Ethics Committee (Nr.2/18-03-21). Patients considered for genetic testing were counseled and the testing principles were explained. All the patients recruited signed an informed consent.

Subjects

The study recruited 21 females of Caucasian ethnicity (attending Riga Maternity Hospital between 2017 and 2019) with presentation of painless cervical dilatation in the ongoing pregnancy (as identified during a standard cervical length measurement using

transvaginal ultrasound between the 18th and 22nd week of gestation) and / or a positive anamnesis of pregnancy loss and / or preterm delivery due to cervical insufficiency without contractions in singleton pregnancies, and the absence of diagnosed genetic conditions. Vaginal infection (exclusion criterion) was ruled out by a pH assessment, where pH > 4.4 indicated the presence of infection.

Among the recruited females, the total number of pregnancies excluding legal abortion, indicated medical abortion, and extra-uterine pregnancies was 3.5 ± 2.2 (TP-OP group, Table 4.1.). Out of those, 52% resulted in late pregnancy loss (LPL, > 12 weeks < 22 weeks) or PTB (< 37 weeks) – a group most likely associated with cervical insufficiency. Early pregnancy losses (EPL, < 12 weeks) were separated as they are mostly related to fetal chromosomal abnormalities. Six out of the 21 patients experienced PPROM in one of their pregnancies. Table 4.1 was completed after the outcome of each patient's ongoing pregnancy was resolved.

Table 4.1

Baseline demographic and clinical characteristics of participants

Age, years	35 ± 4.8
Weight, kg	73.2 ± 16.7
Height, m	1.7 ± 0.05
BMI, kg / m^2	26 ± 5.5
TP	4.5 ± 2.5
OP	1.0 ± 1.1
TP-OP	3.5 ± 2.2
EPL	0.5 ± 1.0
LPL + PTB	1.9 ± 1.7
CL, cm	1.53 ± 0.5

* BMI – Body Mass Index; TP – Total Pregnancies; OP – Other Pregnancies including legal abortion, indicated medical abortion and extra-uterine pregnancies; TP-OP – Total Pregnancies excluding OP; EPL – Early Pregnancy Loss (< 12 weeks); LPL + PTB – Late Pregnancy Loss (> 12 weeks < 22 weeks) and Preterm Birth (< 37 weeks); CL – Cervical Length.

Next-generation sequencing analysis

Genomic DNA was isolated from whole peripheral blood using an adapted phenol-chloroform extraction. NGS analysis was carried out using Illumina's TruSight One Sequencing Panel Capture Kit (USA) covering all genes currently reviewed in the clinical research setting (4810 genes) and generating indexed paired end (2 × 75) reads. Template DNA fragmentation and indexing (tagmentation) was followed by target capture and enrichment. Reads were dual indexed by Nextera i7 and i5 primers. Libraries were prepared for subsequent cluster generation and sequencing on Illumina's NextSeq 500 platform (USA) using

a 150-cycle output flow cell (V2 reagents). Samples were run at an envisioned depth of 100 × per sample.

Bioinformatics analysis

Read mapping and variant calling were performed using Sentieon's DNAseq (Freed et al., 2017; Kendig et al., 2019) FASTQ to VCF pipeline implemented on the DNAnexus cloud [USA]. Briefly, sequence reads were aligned to the GRCh38 reference genome using the BWA-MEM algorithm (H. Li, 2013). Duplicate reads were removed from further analysis. Base Quality Score Recalibration and indel realignment was performed on the mapped reads. Sentieon Haplotyper was exploited to call the variants and produce GVCF files. Sentieon GVCFtyper produced the final variant calling output as a VCF file. Only variants passing standard Sentieon filter criteria were used in the further analysis. Functional annotation of the variants was made using VarAFT v.2.151 (Desvignes et al., 2018) – a comprehensive annotation system for contextualizing variants and examining their functional consequences supported by multiple layers of disease phenotype-related databases.

Variant filtering

The first filtering step retained non-synonymous exonic variants or variants affecting splice donor / acceptor sites (±10nt) of canonical (longest) transcripts. Minor allele frequency (MAF) cut-off < 1% was applied to 1000 Genomes, ExAC, and gnomAD genomic databases. Since only female samples were analyzed, autosomes and X chromosome variants were assessed identically. The second filtering step retained variants covered with at least 10 reads, with a variant allele frequency of at least 25%, and the following deleteriousness scores: Phred scaled CADD score (Kircher et al., 2014) score >10, DANN (Quang et al., 2015) score > 0.9, GERP (Cooper et al., 2005) score > 4, and excluded "benign" and "likely benign" variants of known clinical significance. Variants not assigned a particular score were also included in the further analyses.

Variant classification, prioritization, and gene set enrichment analysis

Each patient's gene variants retained after the second filtering step were pooled together to generate a single file containing rare deleterious variants. The list consisted of 1258 variants in total from 691 genes, 60 variants on average for each sample. Further, the pooled genetic variants were filtered using the three gene lists created by means of the systematic literature

analysis (please refer to the 'Systematic literature analysis' section above for the detailed methodology).

The variants identified in genes from the first and second lists were considered to be of great interest and were consequently investigated more closely to discern the ones most likely to be contributive to the patients' phenotype. The pathogenicity of each variant from this list was assessed manually by three independent evaluators according to the American College of Medical Genetic (ACMG) guidelines (Richards et al., 2015) using MetaDome (Wiel et al., 2019) and automatically using the online tool VarSome (Kopanos et al., 2019). *In silico*-predicted mode of inheritance was assessed using the DOMINO tool (https://wwwfbm.unil.ch/domino/index.html). Gene expression patterns were assessed through a consensus dataset available at https://www.proteinatlas.org.

Variants located in splicing regions were analyzed using four splice prediction tools: SSF, MaxEntScan, NNSPLICE, and GeneSplicer (Pertea et al., 2001; Reese, 1997; Shapiro and Senapathy, 1987; Yeo and Burge, 2004) implemented on Alamut Visual v2.13. A variant was considered to have an effect on splicing if at least two of the four tools showed a > 2% difference between the predicted splice scores of the wild-type and variant alleles, as described previously (Sangermano et al., 2019).

Further, to obtain unbiased information on pathway enrichments across the genes having rare and deleterious variants in our cohort, we annotated genes from the pooled list of variants (n = 1258) using the ConsensusPathDB interaction database (Kamburov et al., 2013) over-representation analysis and looked for 'Pathway-based sets' with a p-value cut-off of 0.01. As a background, we used the TruSight One gene list to exclude any bias from the target genes present in the kit.

4.4 Results

4.4.1 Systematic literature analysis

Publication data

We conducted a systematic literature analysis according to the PRISMA guidelines to cumulate published information on all genes assigned to cervical insufficiency and / or playing a role in the biology of the cervix during normal / compromised pregnancy / parturition.

Out of 105 eligible studies selected for the gene extraction (S1 Fig; S2 Table), 51 were solely association studies with the majority focusing on the analysis of common genetic variants in a limited number of candidate genes (selected gene approach). Of those, five studies exploited array genotyping of 206–1536 single nucleotide polymorphisms (SNPs)

in 9–190 genes. One study performed NGS analysis of 329 candidate genes, while only a single study employed a genome-wide association approach using genome-wide SNP arrays.

Four studies sought to find a direct genetic involvement in preterm delivery, cervical insufficiency, or PPROM. However, all the studied patients were syndromic and not isolated cases of the aforementioned obstetrical complications. Thirty-eight studies were functional gene analyses performed on different sites of cervical biopsy, supracervical fetal membranes, and a few other tissues; 22 assessed differential RNA expression, while the remainder exploited other types of functional analysis. Again, the vast majority of the functional studies (31 in total) used selected gene approaches, with only seven employing the unbiased approach of genome-wide RNA microarray analysis. The rest of the studies used a combined analysis or a more sophisticated unbiased data analysis, e.g., case-parent triad design.

Overall, only eight genetic studies addressed cervical insufficiency primarily or in connection with preterm delivery, PPROM, or an associated genetic disorder. One was a functional study, five were association studies linking the condition with common genetic variants, and two were syndromic studies. The majority of articles focused on preterm delivery (n = 66), whether primarily or in connection with PPROM. The rest of the studies were performed on healthy females at different times during pregnancy / parturition in order to study the physiological ripening of the uterine cervix and physiological pregnancy.

4.4.2 Gene analysis: genes linked to cervical insufficiency are mostly syndromic

The gene extraction from the 105 selected publications resulted in 1455 entities with duplicates. Duplicate removal yielded 1181 genes. Our phenotype-based gene search using HPO terms (OMIM, GHR, https://hpo.jax.org) related to prematurity yielded 50 unique genes. The addition of syndromic genes resulted in the final list of 1222 unique genes (1509 with duplicates; S3 Table). In total, 1024 genes (83.7%) were reported in the literature only once; the remainders were indexed at least twice. The most cited genes in relation to the genetics of the cervix were *IL6* (16 citations), *CXCL8* (12 citations), *IL1B* (10 citations), *TNF*, *PTGS2* (8 citations each), and *COL1A1*, *COL5A1* (7 citations each). Notably, *IL6* was mostly reported in publications focusing on selected gene approaches and was twice documented as being differentially expressed across seven genome-wide studies of RNA expression in cervical tissues.

Altogether, only 17 genes were primarily identified in relation to cervical insufficiency, with six being syndromic, i.e. *COL1A1* and *COL3A1* causing EDS; *FBN1* causing Marfan syndrome; *ZMPSTE24* and *LMNA* causing restrictive dermopathy; and *MATR3* causing myopathy. *COL3A1* was the only gene with an established gene-phenotype role as shown

through HPO term ‘Cervical insufficiency’ (HP:0030009) along with ‘Premature delivery because of cervical insufficiency or membrane fragility’ (HP:0005267), ‘Uterine rupture’ (HP:0100718), and ‘Uterine prolapse’ (HP:0000139), and is known to cause EDS, vascular type (OMIM:130050). The remaining genes were studied in association with cervical insufficiency using selected gene approaches. Five genes had no association (*ADRB2*, *IL1A*, *IL6R*, *LTA*, and *TNF*) as shown by case-control studies (Endres and Wang, 2003; R. Miller et al., 2015; Sundtoft et al., 2016). They were therefore excluded from the first list of genes primarily linked to cervical insufficiency (n = 12; summarized in Table 4) composed from the data of the literature analysis and additional gene searches. Lastly, only one functional study was conducted to analyze *HIF1A* gene expression in the amniotic fluid from patients with isolated cervical insufficiency and indicated cerclage (Song et al., 2019).

Table 4.2

Genes primarily linked to cervical insufficiency (first list of genes)

Gene	Associations from the literature and additional searches*
COL1A1	Ehlers-Danlos syndrome; Cervical insufficiency; Preterm delivery; PPROM; Physiological ripening of the uterine cervix; Physiological pregnancy
COL3A1	Ehlers-Danlos Syndrome; Cervical insufficiency HP:0030009 / Premature delivery because of cervical insufficiency or membrane fragility HP:0005267 / Uterine rupture HP:0100718 / Uterine prolapse HP:0000139; PPROM; Preterm delivery; Physiological ripening of the uterine cervix; Physiological pregnancy; Premature uterine contractions
FBN1	Marfan syndrome; Cervical insufficiency; PPROM; Premature uterine contractions
HIF1A	Cervical insufficiency; Physiological ripening of the uterine cervix; Physiological pregnancy
IL10	Cervical insufficiency; Preterm delivery
IL1B	Cervical insufficiency; Preterm delivery; Physiological ripening of the uterine cervix; Physiological pregnancy
IL6	Cervical insufficiency; Preterm delivery; Physiological ripening of the uterine cervix; Physiological pregnancy
LMNA	Restrictive Dermopathy; Premature delivery because of cervical insufficiency or membrane fragility HP:0005267; Premature rupture of membranes HP:0001788;
MATR3	Myopathy due to MATR3 mutations; Cervical insufficiency
MBL2	Cervical insufficiency; Preterm delivery
TGFB1	Cervical insufficiency; Preterm delivery; Physiological ripening of the uterine cervix; Physiological pregnancy
ZMPSTE24	Restrictive Dermopathy; Premature delivery because of cervical insufficiency or membrane fragility HP:0005267; PPROM; Preterm delivery

* HPO term indicated if reported in https://hpo.jax.org.

Based on the previously described clearly distinguishable clinical pattern of cervical insufficiency from classical idiopathic preterm labor and its relatedness to PPROM through the role of connective tissue – particularly the role of collagens – we composed a second list

of genes (genes potentially linked to cervical insufficiency; S4 Table) comprising 91 entities also containing syndromic genes identified through the HPO/OMIM searches.

The third list contained 812 genes having a function within the uterine cervix as shown by differential gene expression studies of the physiology of pregnancy, cervical ripening, and labor (S5 Table). Studies focusing solely on idiopathic preterm delivery (both functional and DNA analysis) were excluded. A Venn diagram was constructed from the three lists (Oliveros, 2007) (S2 Fig). Certain genes occurred in more than one list, indicating multiple lines of evidence. The creation of all three lists can be replicated through S5 Table column E.

Functional annotation of genes studied using genome-wide versus selected gene approaches

Lastly, to assess the bias in the existing knowledge on the genetics of cervical functioning, we analyzed differences in the biological information of genes reported in studies using unbiased genome-wide approaches in comparison to selected gene approach studies. In total, 816 genes emanated from seven genome-wide studies (excluding genome-wide studies subjected to gene filters). They were all gene expression studies exploiting genome-wide expression arrays. Specifically, 64 genes (7.8%) were denoted as being differentially expressed in more than one study (with a maximum of five studies). Eighty-seven genes emerged from 53 studies exploiting selected gene approaches, with 27 of them occurring multiple times (with a maximum of six studies). There were 27 genes occurring in both selected gene approach and genome-wide approach studies.

The gene lists were annotated using the ConsensusPathDB interaction database (Kamburov et al., 2013) for Gene Ontology (GO) and pathway enrichments. Annotation showed a large overlap among the strongest entities (as indicated by the p-value; S6 Table, S7 Table) and were enriched for GO terms primarily related to extracellular matrix (ECM) organization (e.g., GO:0031012; GO:0005201; GO:0007155) and a variety of cellular responses including immune (GO:0006955). The pathway analysis showed a high enrichment of immune-related pathways overlapping between both gene lists (a variety of interleukins). Separately, each list showed different pathways, but, again, they could be accommodated under common denominators related to elastic fiber / collagen formation as well as immunity (e.g., ‘ECM proteoglycans’; ‘Elastic fiber formation’; ‘Collagen formation’; etc.).

4.4.3 Patient NGS data analysis

Using Illumina's TruSight One NGS kit covering 4810 genes of known clinical significance, we sequenced the DNA from 21 patients presenting with isolated non-syndromic cervical insufficiency. The sequencing resulted in a median coverage depth of 135 ± 38 × of the target region, with 94.4% of target regions being covered at least 10 times and 89.7% being covered at least 20 times. Rare deleterious variants (filtered based on CADD, DANN, and GERP scores and known clinical significance; S8 Table) from all the patients were pooled and screened for the genetic variations in the three lists created from the literature analysis (Figure 4.2).

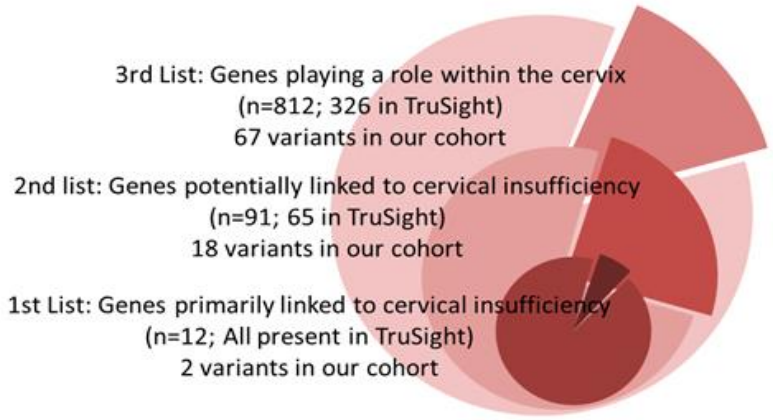


Figure 4.2 **Each circle represents one of the three gene lists generated in the study**

The smaller the list, the closer the association with cervical insufficiency. The number of genes covered in the TruSight NGS kit are mentioned, as well as the number of deleterious variants identified in our patients across each gene list.

Twenty heterozygous variants found in 14 of our patients (67%) and the first and second lists of genes were subjected to a closer analysis as they were considered most likely to contribute to the patients' phenotype based on existing knowledge (Table 4.3.). Nine patients had one variant, four patients had two variants, and one patient had three variants. Fourteen variants were found in 10 genes known to cause EDS, osteogenesis imperfecta, or Bethlem myopathy. According to manual classification following the ACMG guidelines, 14 variants were classified as variants of unknown significance (VUS, class 3) and two as likely pathogenic (class 4). The criteria were inapplicable to the remaining four variants (please see the full information about each variant in S9 Table)

Table 4.3

Closer analysis of variants most likely contributing to the development of cervical insufficiency in our patients

Sample	Gene	Genotype (effect on protein if known)	GnomAD v2.1.1.	CADD score	ACMG Manual (Criteria)	Comments	Contribution to the phenotype of cervical insufficiency
Case1	*MYO1F (rs200225777)*	NM_012335.4:c.[2461G > A]; [=] (NP_036467.2:p.Gly817Arg)	0.000106 (30/280708)	23.2	Not applicable[a] (PP3; BP6)	Mechanism of the disease is unknown and no phenotype for the gene is known. Likely recessive type of inheritance.	Unlikely
Case2	*FKBP14 (rs542254849)*	NM_017946:c.[496_498del];[=] (NP_060416.1:p.Lys166del)	0.001070 (279/260856)	15	VUS (PP3; PM4; PM1)	Pathogenic variants in the gene cause EDS, which is associated with cervical insufficiency. Likely recessive type of inheritance.	Further investigation needed
Case3	*B4GALT7 (rs142476892)*	NM_007255:c.[277C > T];[=] (NP_009186.1:p.His93Tyr)	0.001347 (379/278326)	26.7	VUS (PP3; PM1)	Pathogenic variants in the gene cause EDS; VUS previously found in EDS patients; Likely recessive type of inheritance.	Further investigation needed
	COL1A2	NM_000089:c.[1808C > T];[=] (NP_000080.2:p.Thr603Ile)	0	17	VUS (PP3;PM2)	Pathogenic variants in the gene cause EDS, which is associated with cervical insufficiency and PPROM; Strongest expression within the cervix. Likely dominant type of inheritance.	Further investigation needed

Table 4.3 continued

Sample	Gene	Genotype (effect on protein if known)	GnomAD v2.1.1.	CADD score	ACMG Manual (Criteria)	Comments	Contribution to the phenotype of cervical insufficiency
Case5	*COL12A1 (rs201988277)*	NM_004370.6:[c.7853C > T];[=] (NP_004361.3:p.Thr1454Met)	0.000311 (74/237906)	25.2	VUS (PP3)	Pathogenic variants in the gene cause Ehlers-Danlos / Bethlem-like myopathy overlap syndrome associated with both connective tissue abnormalities and muscle weakness. Likely dominant type of inheritance.	Further investigation needed
	COL1A1 (rs537060488)	NM_000088:c.[529G > A];[=] (NP_000079.2:p.Val177Met)	0.000014 (4/281442)	23.2	VUS (PP2)	Pathogenic variants in the gene cause EDS; Strong expression within cervix; Likely dominant type of inheritance.	Further investigation needed
Case6	*CHST14 (rs144629123)*	NM_130468:c.[635T > C];[=] (NP_569735.1:p.Val212Ala)	0.000513 (145/282534)	25	VUS (PP3; PM1; BS2)	Strong expression within cervix. Gene is associated with EDS. Likely dominant type of inheritance.	Further investigation needed
	GK (rs371481560)	NM_000167:c.[989G > A];[=] (NP_976325.1:p.Arg330His)	0.00001 (4/204700)	25.6	VUS (PP3; PM1)	Phenotype of the disease associated with GK gene does not overlap with the phenotype of interest; Poor expression with within cervix. X linked recessive.	Unlikely
Case7	*MYO1F (rs761308378)*	NM_001348355:c.[2270G > A];[=] (NP_036467.2:p.Arg757Gln)	0.000224 (63/280790)	34	Not applicable[a] (PP3)	Mechanism of the disease is unknown and no phenotype for the gene is known. Likely recessive type of inheritance.	Unlikely

Table 4.3 continued

Sample	Gene	Genotype (effect on protein if known)	GnomAD v2.1.1.	CADD score	ACMG Manual (Criteria)	Comments	Contribution to the phenotype of cervical insufficiency
Case7	*COL4A3 (rs765655100)*	NM_000091.4:c.[5010_*14del];[=] (NP_000082.2:p.His1670_ Ter1671delinsXaa)	0.000064 (16/249376)	40	LP (PP3; PM1; PM4; PM2)	Localized at the end of the gene (loss of stop-codon); Poor expression within cervix; Dominant or recessive.	Unlikely
Case8	*TNXB*	NM_001365276:c.[3793G > A];[=] (NP_001352205.1:p.Gly1265Arg)	0	25.9	VUS (PP3; PM2; BP1)	Gene is associated with EDS hypermobile type; Likely recessive type of inheritance.	Further investigation needed
Case9	*B4GALT7 (rs142476892)*	NM_007255:c.[277C > T];[=] (NP_009186.1:p.His93Tyr)	0.001347 (375/278326)	26.7	VUS (PP3; PM1)	Pathogenic variants in the gene cause EDS; VUS previously found in EDS patients; Likely recessive type of inheritance.	Further investigation needed
	TNXB (rs141190850)	NM_001365276:c.[2030A > G];[=] (NP_061978.6:p.Glu677Gly)	0.002140 (583/272438)	23.1	VUS (PP3; BP1)	Gene is associated with EDS hypermobile type; Likely recessive type of inheritance.	Further investigation needed
Case10	*PLOD1 (rs772861343)*	NM_000302:c.[475G > A];[=] (NP_001303249.1:p.Gly159Ser)	0.000035 (9/251374)	34	VUS (PP3; PM2)	Pathogenic variants in the gene cause EDS; VUS previously found in EDS patients; Strong expression within the cervix; Likely recessive type of inheritance.	Further investigation needed
Case11	*P3H1 (rs371232413)*	NM_001146289:c.[1720+4G > A];[=]	0.000213 (59/276822)	5.4	VUS	Pathogenic variants in the gene known to cause Osteogenesis imperfecta, which was clinically associated with cervical insufficiency; Effect on splicing is not clear.	Further investigation needed

Table 4.3 continued

Sample	Gene	Genotype (effect on protein if known)	GnomAD v2.1.1.	CADD score	ACMG Manual (Criteria)	Comments	Contribution to the phenotype of cervical insufficiency
Case12	*P3H1 (rs371232413)*	NM_001146289:c.[1720 + 4G > A];[=]	0.000213 (59/ 276822)	5.4	VUS	Pathogenic variants in the gene known to cause Osteogenesis imperfecta, which was clinically associated with cervical insufficiency; Effect on splicing is no clear; Likely recessive type of inheritance.	Further investigation needed
	C1S (rs148105120)	NM_001734:c.[100A > G];[=] (NP_001725.1:p.Ser34Gly)	0.000438 (124/ 282870)	22.4	VUS (PP3; BS1)	Associated with EDS, but gene-disease association is dubious (one missense variant reported in publication); Likely recessive type of inheritance.	Unlikely
	MYO1F (rs747756979)	NM_001348355:c.[1170 + 4C > T];[=]	0.000039 (11/280872)	0	Not applicable[a] (BP4)	Mechanism of the disease is unknown and no phenotype for the gene is known. Variant does not have a consistent impact on the splice site; Likely recessive type of inheritance.	Unlikely
Case13	*ADRB2 (rs753894727)*	NM_000024:c.[1072G > C];[=] (NP_000015.1:p.Gly358Arg)	0.000074 (28/282828)	12.5	Not applicable[a] (BP1; BP4)	Gene is not associated with phenotype; Study, from which information is extracted, looked only for SNP and did not find any association with cervical insufficiency; Likely dominant inheritance.	Unlikely

* [a] Not applicable – if mechanism of disease is not known, or phenotype is not known; VUS – variant of unknown significance, LP – likely pathogenic.

Two variants were located at non-canonical splicing sites (> 2nt from exon / intron junction). Based on splicing predictions, variant *P3H1*:c.1720 + 4G > A (found in two patients) strengthens the existing donor site and may have a mild effect on the creation of a new acceptor site at position c.1720 + 23. The variant *MYO1F*:c.1170 + 4C > T does not have a consistent impact on the splice site.

Ultimately, based on a comprehensive curation of the variants' pathogenicity, including known gene-disease / gene-phenotype associations, gene expression patterns within cervical tissues (S3 Fig), and mechanisms of diseases of particular genes, etc. (all criteria used for the curation can be found in S9 Table), we assigned a likelihood for contribution of the variant to the patient's phenotype (last column in Table 4.3.). A variant was *unlikely contributing* (n = 7) if classified as benign / likely benign according to the manual pathogenicity curation, did not show any or poor expression within the cervix, or known gene-disease / gene-phenotype associations did not correspond to the phenotype of interest. A variant *needs further investigation* (n = 13) if it showed a theoretical potential to increase susceptibility to the development of the phenotype of interest based on the criteria assessed, but more data are required to declare the variant as definitively contributive to the development of cervical insufficiency.

4.4.4 Gene pathway enrichment analysis

To determine whether the genes having rare deleterious variants identified in our highly selective patient cohort exhibited any phenotype-relevant pathway enrichment, we annotated all the genes (n = 694) using the ConsensusPathDB interaction database (Kamburov et al., 2013) with the TruSight One gene list (n = 4810) as background. As illustrated by the 20 most significant entities (Table 4.4), the analysis revealed a high overrepresentation of pathways related to tissue mechanical and biomechanical properties (collagens and proteoglycans, integrins). There was not only high enrichment of ECM pathways, but also of cell to ECM communication (e.g. hemidesmosomes, focal adhesion) and basal membrane components (laminins). Moreover, a number of the pathways identified here matched ones shown to be enriched with genes studied in relation to the genetics of the cervix as identified from our literature search (marked with an asterix, Table 4.4).

Table 4.4

Pathway enrichment analysis of genes having rare deleterious variants in patients with cervical insufficiency

Pathway name	Pathway set size (Number in the background gene list)	Genes contained in the analyzed list	p-value	q-value	Pathway Source
Collagen formation*	92(59)	24 (40.7%)	3.30E-07	4.71E-04	Reactome
ECM-receptor interaction – Homo sapiens (human)	82(60)	24 (40.0%)	4.80E-07	4.71E-04	KEGG
Extracellular matrix organization*	294(180)	47 (26.1%)	7.21E-06	4.33E-03	Reactome
Collagen biosynthesis and modifying enzymes*	68(40)	17 (42.5%)	8.82E-06	4.33E-03	Reactome
Type I hemidesmosome assembly	9(9)	7 (77.8%)	2.76E-05	9.10E-03	Reactome
Assembly of collagen fibrils and other multimeric structures	48(39)	16 (41.0%)	2.78E-05	9.10E-03	Reactome
Laminin interactions	23(19)	10 (52.6%)	7.26E-05	2.04E-02	Reactome
Axon guidance	358(160)	40 (25.0%)	1.02E-04	2.51E-02	Reactome
Collagen chain trimerization	44(28)	12 (42.9%)	1.73E-04	3.78E-02	Reactome
Human papillomavirus infection - Homo sapiens (human)	339(173)	41 (23.7%)	2.95E-04	5.79E-02	KEGG
Integrin*	124(75)	22 (29.3%)	3.71E-04	6.63E-02	INOH
Non-integrin membrane-ECM interactions*	42(31)	12 (38.7%)	5.39E-04	8.83E-02	Reactome
ECM proteoglycans*	57(41)	14 (34.1%)	8.36E-04	1.24E-01	Reactome
Beta1 integrin cell surface interactions*	66(55)	17 (30.9%)	8.85E-04	1.24E-01	PID
Protein-protein interactions at synapses	88(33)	12 (36.4%)	1.04E-03	1.36E-01	Reactome
Focal adhesion - Homo sapiens (human)	199(113)	28 (24.8%)	1.30E-03	1.59E-01	KEGG
Alpha6 beta4 integrin-ligand interactions	11(11)	6 (54.5%)	1.77E-03	2.05E-01	PID
Developmental Biology	620(262)	53 (20.2%)	2.40E-03	2.55E-01	Reactome
Interaction between L1 and Ankyrins	29(23)	9 (39.1%)	2.47E-03	2.55E-01	Reactome
Myo-inositol *de novo* biosynthesis	4(3)	3 (100.0%)	2.70E-03	2.65E-01	Human Cyc

* Pathways in bold are related to tissue mechanical and biomechanical properties. Pathways marked with an asterix were found to be enriched with genes studied in relation to the genetics of the cervix as identified from our literature search.

4.5 Discussion

An evidence-based, effective system of pregnancy and maternity care identifying risks and localizing problems in a timely manner is one of the fundamental public health elements for maintaining the well-being and demography of a population. However, obstetrics and gynecology practice is associated with a plethora of different complications, thus placing a burden on the healthcare and socio-economic systems and is a traumatizing experience for the patient. A distinct risk factor for pregnancy loss and preterm delivery is cervical insufficiency. Currently, cervical insufficiency is clinically distinguishable only in the ongoing pregnancy or based on a patient's anamnesis.

A strong genetic component is expected in certain cases of preterm delivery and cervical insufficiency (Raffi and Anumba, 2007; Warren et al., 2007). However, despite genomic advancements, we know surprisingly little about the genetics of prematurity. Indeed, until recently, there was not a single gene unequivocally linked to the specific phenotypes associated with cervical insufficiency or PPROM and PTB.

In this work, we aimed to comprehensively explicate the existing genetic studies on prematurity with cervical insufficiency as the focal point. To accomplish this, we performed a systematic literature analysis followed by a gene extraction and data analysis. We wanted to evaluate whether there is a bias in our understanding of the genetics of the cervix and estimate how many genes can be reliably linked to cervical insufficiency and explore their possible roles. We subsequently applied the obtained knowledge from the literature to the analysis of NGS data of 21 patients with an anamnesis of isolated cervical insufficiency. As evidenced in previously conducted research, it is still not clear whether the mother or the preterm-delivered infant should be considered the proband, and as a result, which individual's DNA should be examined (Plunkett and Muglia, 2008). Based on the available evidence, we hypothesized that issues relating to the cervix during pregnancy are most likely to be dictated by the maternal genome. Therefore, we analyzed the mother's genomic DNA.

4.5.1 Collagenopathic nature of cervical insufficiency

Our literature analysis revealed that at present there are only eight studies directly addressing the link between genetics and cervical insufficiency, and 12 genes are primarily linked to the condition. A few of them are known to cause syndromic forms of cervical insufficiency associated with collagen disorders such as EDS, Marfan syndrome, restrictive dermopathy, and myopathy due to *MATR3* mutations. Notably, no studies have been conducted to identify direct genetic implications in the non-syndromic form of cervical insufficiency,

highlighting the insufficient knowledge on the genetics of (patho)physiological cervical remodeling during pregnancy.

All the genes retrieved in this study were classified into three lists according to their relation to the question being posed. The second list contained genes (n = 91) procured based on knowledge of the importance of collagen for proper cervical morphophysiology. Not surprisingly, 32 of the genes in the list (approximately one third) have been shown to associate with collagenopathy syndromes. Consequently, we anticipated that a large number of these genes also play a crucial role in the development of cervical insufficiency. Therefore, we comprehensively evaluated our patients' NGS variants identified in genes classified in the first and second gene lists by adhering to the robust framework for variant interpretation in the research setting in order to identify ones potentially contributive to the development of cervical insufficiency.

Currently, only one gene – *COL3A1* – has an established gene-disease relationship with cervical insufficiency as shown through HPO term association (HP:0030009). This gene is also linked to 'Premature delivery because of cervical insufficiency or membrane fragility' (HP:0005267), as are the genes *ZMPSTE24* and *LMNA*. Further, *COL5A1* is the only gene associated with 'Premature birth following premature rupture of fetal membranes' (HP:0005100) and 'Premature rupture of membranes' (HP:0001788), with a few other genes (*PLOD1*, *ADAMTS2*, *SERPINH1*, *ZMPSTE24*, *LMNA*, and *ATP6V0A2*) associated with PPROM alone. Notably, we identified only one VUS in these genes in our patient cohort. The patient carrying the *PLOD1*:c.475G > A variant had two preterm deliveries, a cervical length of 1.8 cm as identified during her last pregnancy, and no history of PPROM. However, the variant's contribution to the patient's phenotype needs to be investigated further as the suggested inheritance is autosomal recessive and the described gene-phenotype correlation is more severe.

Another collagen gene – *COL1A1* – has already been implicated in the development of cervical insufficiency from the data of large case-control studies (Sundtoft et al., 2016; Warren et al., 2007) showing a positive genetic association (OR > 3) and pathogenic variants in which known to cause EDS similarly to *COL1A2* gene. We believe both genes, *COL1A1* and *COL1A2*, are good candidate genes for involvement in the development of isolated cervical insufficiency. Nonetheless, the variants identified in our cohort, *COL1A1*:c.1663C > T, *COL1A1*:c.529G > A, and *COL1A2*:c.1808C > T, require more in-depth investigation and replication to be reliably assigned as causative.

Further, we identified the same variant in the *B4GALT7* gene in two of our patients. This gene is known to cause EDS, autosomal recessive type. Similarly, two patients were found to carry the same variant in the *P3H1* gene causing autosomal recessive osteogenesis imperfecta. Notably, all the other likely pathogenic variants and VUSs, excluding ones classified as unlikely contributive, were identified in genes in which pathogenic variants / mutations might lead to the development of certain types of collagenopathy. Such a situation raises the question: is it the case that patients carrying variants likely causing certain connective tissue disorders, do not exhibit any other collagenopathic features? Indeed, no one, to our knowledge, has evaluated cervical insufficiency as an expression point in a phenotypic continuum of collagenopathies when no other symptoms or subtle symptoms are apparent – and neither did we. Therefore, our findings allow us to hypothesize further that cervical insufficiency could be expressed as one of the mild forms of collagenopathy, a condition which is known to range from mildly loose joints to life-threatening complications such as aortal rupture. In order to substantiate this, a rigorous phenotyping following a custom-developed assessment protocol would have to be conducted as at present the only existing and validated scale used worldwide for collagenopathies is the Beighton joint hypermobility score (Beighton and Horan, 1969), which does not address any other disease-related symptoms apart from hypermobility of joints.

In general, reports linking obstetrical complications with EDS date back to the 1990s with descriptions of patients with hypermobile joints, kyphoscoliosis, and hyper elastic skin having cervical insufficiency and PPROM (De Vos et al., 1999; Leduc and Wasserstrum, 1992). Additional complications in cases of EDS might include scoliosis (causing problems with anaesthesia), atonic uterus, vaginal and / or perineal tearing, pelvic organ prolapse, symphysiolysis, abdominal herniation, wound dehiscence, severe varicosities, and postpartum hemorrhage. Maternal mortality risk is heightened due to uterine rupture or rupture of large vessels (De Paepe et al., 1989; Rudd et al., 1983). It is also known that the coincidence of Marfan syndrome and pregnancy means a high risk for mother and child as it might be complicated by PPROM, premature uterine contractions, and cervical insufficiency (Tzialidou et al., 2007). Therefore, knowledge that the patient is suspected of having or indeed has a connective tissue disorder or a tendency towards connective tissue laxity (perhaps only in certain scenarios) would provide a unique opportunity for the multidisciplinary team members to more effectively support pregnant women through increased understanding and awareness (Pezaro et al., 2018).

4.5.2 Main pathways involved in cervical functioning

Candidate genes from a variety of pathways have been shown to be important in prematurity: hemostasis and coagulation, local inflammation, collagen metabolism, and matrix degradation in PPROM (Makieva et al., 2017); focal adhesion, cell communication, and ECM receptor interaction in spontaneous PTB (Myking et al., 2011). No functional gene studies with subsequent pathway enrichment analysis have been performed in relation to cervical insufficiency.

In this study, we wanted to evaluate whether there is a bias in the cervix-related genes studied exploiting selected gene approaches in contrast to unbiased genome-wide approach studies. Our pathway and GO enrichment analysis of both groups did not identify a large difference in the overrepresented GO terms / enriched biological pathways and showed a certain overlap with other phenotypes in prematurity. The genes studied in relation to cervical patho(physiology) during pregnancy / parturition were found to be mostly enriched for the two main functional categories – immunity / inflammation and connective tissue remodeling. This indicates that the genes chosen for the selected gene approach studies follow the existing understanding of the most well-known biological pathways in cervical remodeling.

Importantly, the process of parturition at both term and preterm is consistently associated with the induction of many proinflammatory mediators, suggesting that these components are central for the parturition cascade in humans (Keelan et al., 2003). In turn, the inflammatory infiltrate *per se* activates fibrinolysis (De Vos et al., 1999). As a consequence, true (transcript)omic signature comprising less known pathways of the specific phenotype might simply be masked by the massive expression of (pro)inflammatory agents followed by fibrinolysis. It is worth mentioning that the first two phases of cervical remodeling, namely, softening and ripening, are not dependent on inflammatory processes (Sakamoto et al., 2004), and so may dictate the timing of sampling in at least physiological pregnancy / parturition studies.

Despite study limitation of relatively small sample cohort, our unbiased *in silico* pathway enrichment analysis of genes having rare deleterious variants in our patients – specifically selected for isolated non-syndromic cervical insufficiency – identified an increased variant burden in genes involved in collagen and / or ECM production. These results not only strengthen our target gene variation findings that pathways involved in collagen biosynthesis play a major role in cervical insufficiency, but also imply that cell-ECM communication pathways, in which molecules such as integrins, laminins, keratins, and fibronectins participate, might be involved in the development of cervical insufficiency. For example, one

of the enriched pathways was ‘Type I hemidesmosome assembly’. Hemidesmosomes play a critical role in the maintenance of tissue integrity and are highly dynamic structures capable of disassembling quickly during cell division, differentiation, or migration (Litjens et al., 2006). Desmosomes present in the uterine cervix (Jordan et al., 2009) – the functioning of which might be compromised due to genetic variations – can also affect the integrity of the cervix. There is currently insufficient information on the genes of these pathways to reliably implicate them in the pathology of the cervix. Therefore, this could be a focus for future studies.

4.5.3 Overview and future perspectives

The findings of our systematic overview of the genes related to (patho)physiological cervical remodeling are a further step towards unraveling the complex genomics of prematurity. Specifically, the obtained data should help to fill in the gaps in our knowledge about cervical insufficiency, as there is still controversy surrounding this condition’s development and treatment due to a largely unclear pathophysiology (Warren et al., 2007). In the future, findings from molecular-based studies could potentially be translated into outcome changes for women at risk, as they may lead to the discovery of a particular metabolite’s deficit and consequently the development of screening tests. Since the biophysical properties of the cervix are mainly determined by collagen content (Uldbjerg et al., 1983), perhaps the basis of future screening tests lies in the observed collagen changes during cervical remodeling. It has been demonstrated that women with cervical insufficiency exhibited a markedly lower median cervical hydroxyproline (the most abundant amino acid in the collagen molecule) concentration, high collagen extractabilities and collagenolytic activities, and their biomechanically tested biopsy specimens had low strength and high extensibility (Rechberger et al., 1988), even in the non-pregnant state (Petersen and Uldbjerg, 1996). Unfortunately, our patients did not undergo such testing.

We have demonstrated for the first time that rare pathogenic allelic variants leading to collagenopathies might be responsible for the increased susceptibility of the development of isolated cervical insufficiency in non-syndromic patients. Nonetheless, the genetic landscape summarized within the scope of this work points to a wider genetic heterogeneity of the condition. At present, the majority of genes (particularly those listed in the third list of genes; S5 Table) cannot with certainty be implicated in the development of cervical malfunctioning. The third gene list contains data from functional studies and encompasses genes shown to be differentially expressed within the cervix during physiological pregnancy in healthy females. With the current knowledge, it is difficult to interpret the contribution of the deleterious variants identified in those genes (n = 67). However, the genes are still likely to play an important role

in cervical functioning and / or preterm delivery and further evidence might emerge as knowledge increases over time. Further studies with larger patient cohorts and perhaps better designs – specifically, precise tissue selection in combination with the timing of sampling during physiological / compromised pregnancy / labor, and most importantly precise phenotyping of patients – are necessary before any conclusions regarding thorough genetics of cervical insufficiency and clinical applicability of any genetic testing can be drafted.

A possible limitation of our extensive literature analysis could be an issue with multiple terms associated with cervical insufficiency. For example, the term PreCocious Cervical Ripening (PCCR) was initially coined by Papiernik *et al.* in 1986 (Papiernik et al., 1986) and was proposed as more appropriate and less confusing (Caritis and Simhan, 2012; Odibo, 2014) than others such as cervical weakness / incompetence, (istmo)cervical insufficiency, premature cervical shortening / remodelling / failure, or failing cervix. Although all of them are used, we encourage usage of 'cervical insufficiency' since this is adopted by the American College of Obstetricians and Gynecologists, HPO, and MeSH. We tried to include all known terms of the condition in our literature search, but specific forms may have been omitted.

Another limitation of our study is the usage of a limited NGS panel (4810 genes) to study the DNA of our patients. Nevertheless, we were able to analyze all the genes primarily linked to cervical insufficiency as revealed by our literature analysis results. Having a relatively small patient cohort, we wanted to elucidate the particular roles of rare pathogenic variants since they are thought to have greater effects on the development of complex human diseases in comparison to common genetic variants, testing of which exploits the idea 'common disease – common variation' (Bomba et al., 2017) and demands enormous patient cohorts. Ultimately, by means of the target gene variation analysis and pathway enrichment analysis, we were able to demonstrate that the development of isolated cervical insufficiency is likely influenced by rare variations in genes involved in ECM / collagen production and synthesis.

Despite the distinctive phenotypical pattern of cervical insufficiency in contrast to idiopathic preterm delivery, it still remains a multifactorial condition, development of which depends on a number of factors. Therefore, no marker will be 100% sensitive or specific. From the genetic point of view, the most likely scenario is that a combination of both multiple rare and common variants in a number of genes contributes to disease development risk.

However, before the era of genetic testing enters obstetrics and gynecology, there are measures that can be introduced now into clinical practice. For instance, routine cervical length screening is not always performed on low-risk women. This paucity of screening may lead to a clinically unrecognized short cervix being missed and ultimately preterm labor (Manuck, 2016). Additionally, a number of EDS types are poorly recognized, with symptoms / complaints

of generalized joint hypermobility and / or chronic musculoskeletal pain being wrongly attributed to rheumatologic disorders (De Vos et al., 1999) or other non-specific conditions. Therefore, awareness regarding the nature of cervical insufficiency needs to be raised among obstetrical and gynecological teams to, at best, avoid complications or, at least, successfully manage them.

4.6 Conclusions

Isolated cervical insufficiency is a distinct phenotype in prematurity with a heterogeneous etiology. Our current understanding of the genetic landscape of the (patho) biology of the cervix is incomplete. One of the causes of non-syndromic cervical insufficiency may be associated with pathogenic variants in genes involved in collagen synthesis and production, allelic variants in which are known to cause connective tissue disorders. The notion that cervical insufficiency is an expression point in a phenotypic continuum of collagenopathies should be investigated further using multiple approaches.

4.7 Funding

The study was supported by a Riga Stradins University Internal Study Grant (to LV).

4.8 Acknowledgments

We would like to express our special thanks of gratitude to Riga Stradins University librarian Natalija Kislakova for her the help in the literature search.

4.9 Supporting information

S1 Table. Full text articles assessed for eligibility according to the PRISMA guidelines. (Online version available).

S2 Table. Publication analysis. (Online version available).

S3 Table. Full list of genes. All genes with duplicates obtained from a literature analysis and additional gene searches. (Online version available).

S4 Table. The second list of genes. Containing genes selected for association with cervical insufficiency, PPROM, or other connective tissue-related issues. (Online version available).

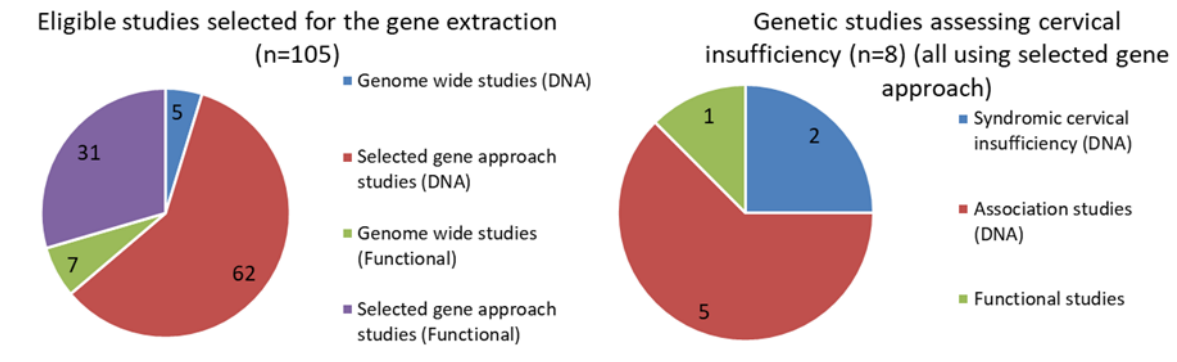
S5 Table. The third list of genes. Containing genes having a function within the cervix as identified from functional studies of physiological / compromised pregnancy / parturition. (Online version available).

S6 Table. GO analysis of genes identified from genome-wide and selected gene approaches. (Online version available).

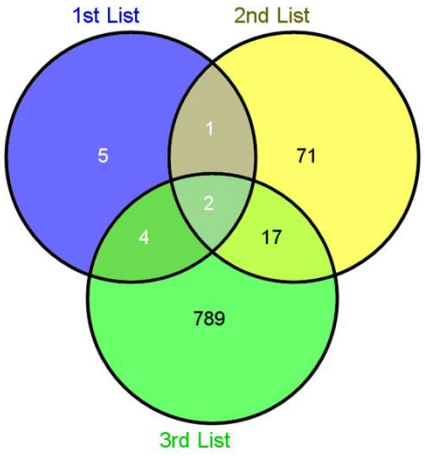
S7 Table. Pathway analysis of genes identified from genome-wide and selected gene approaches. (Online version available).

S8 Table. Sequence variants retained after each filtering step. (Online version available).

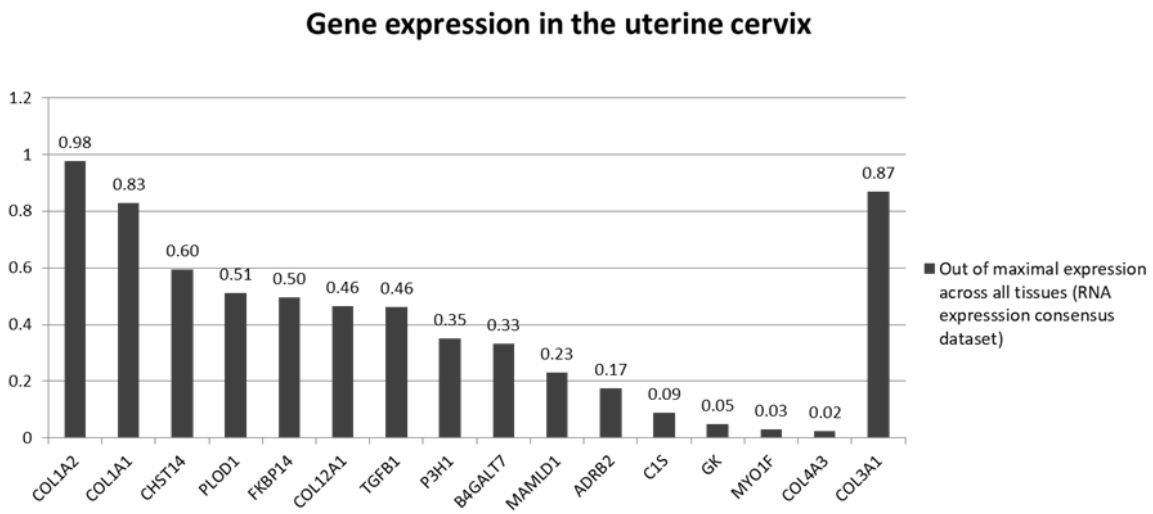
S9 Table. Full analysis of selected gene variants of interest. (Online version available).



S1 Fig**. Genetic studies included in the study. All studies included for the gene selection (left) and ones focusing primarily on cervical insufficiency (right)**



S2 Fig. **Venn diagram of the three gene lists created from publication data and additional gene searches.**



S3 Fig. **Gene expression in the uterine cervix. Genes found to be mutated in our patient cohort as shown through the 1st and 2nd gene list analysis. Data obtained through an RNA expression dataset available at https://www.proteinatlas.org. In our cohort, none of the rare or pathogenic variants were found in the COL3A1 gene; however, it is included as it is the only gene unequivocally linked to cervical insufficiency**

5 General Discussion

Comparing to other mammalian species, human fecundity is less efficient – the probability of achieving a pregnancy within one menstrual cycle is about 20–30%. For example, in baboons, this number reaches 80%, and is as high as 90% in rabbits (Chard, 1991; Foote and Carney, 1988; Stevens, 1997). There are several non-biological factors influencing reproduction in modern societies, such as the delayed first conception, new social models, environmental pollution, and chemical expositions. However, it all could mask the biological basis of reproductive failure (Vendrell, 2018).

The first success in the identification of monogenic forms of female reproductive failure using molecular techniques came in the nineties, when numerous researchers with an aim of Sanger sequencing and segregation analysis started to shed light on the phenotypes associated with disturbances in the hypothalamic-pituitary-gonadal axis. A further breakthrough was possible due to the technological evolution in genetics and broad usage of ARTs for couples experiencing difficulties to conceive – this in turn allowed for an array of previously unobserved phenotypes (or phenotypes collected under the denominator of idiopathic infertility) ultimately to reveal themselves. Thus, the number of genes implicated in female gonadal disorders (especially POF), oocyte maturation arrest and early embryonic arrest are constantly growing, which mirrors what has long been appreciated in animal models, i.e., many genes are required for normal fertility in females (Maddirevula et al., 2020). However, we still face a quite limited number of confident gene-disease relationships in female reproduction failure. The scarcity of reliable genetic markers unfortunately also results in poor allocation of the up-to-date genetic and genomic methodologies to female reproductive failure in clinics. Karyotyping was the first test employed to investigate the presence of genetic abnormalities in failed female reproduction and to this day remains the most widely used diagnostic test.

This work demonstrated application of advanced genomic techniques and / or adaptation of certain genomic methodology in three different stages of female reproduction: i) at the level of a preimplantation embryo to increase a couple’s chances to conceive a healthy child, ii) to improve the reliability of genetic testing in early pregnancy loss, aiding clinical decision making, iii) to unravel the underlying genetic cause in female genome of preterm delivery due to cervical insufficiency.

5.1 Selecting the best technology for multifactor preimplantation genetic testing

The first practical part of this thesis described in Chapter 2 addressed the preimplantation embryo analysis for the couples with an increased likelihood of delivering a child with monogenic disorder. Apart from aiming to meet the highest PGT safety standards, we prioritized the purpose of achieving desired pregnancy for every couple. Since no existing testing systems were available on the market, we designed the whole testing protocol from a scratch, at the very beginning facing the challenge of choosing the right tools i.e., reagents and methodologies. This is why we decided i) to compare the two most popular whole genome amplification techniques on a subset of downstream applications and ii) to share in detail our practical experience with those facing the same challenge.

Subsequently, we were satisfied with our performance as eight couples out of nine (unpublished data) delivered healthy kids, which was confirmed postnatally. Only for one case (*MTM1*), no oocytes were successfully fertilized perturbing the couple's opportunity to conceive. In addition, three embryo transfers resulted in a failed implantation making the overall birth rate per embryo transfer 72.7%, which is still above the average reported in the literature (Butler et al., 2019; Theobald et al., 2020). A small number of the cases processed makes it difficult to predict the trend of high pregnancy rates in the long run, but we associate those with the exclusion of embryonic factors of reproductive failure in all transferred embryos (aneuploid embryo rate 37.5%). However, it is highlighted that randomized controlled trials are needed to conclude the clinical effect of PGT-A for PGT-M (Toft et al., 2020).

Out of 73 embryos processed, 39 were amplified using MDA technology and 34 – using SurePlex to assess the performance of both whole genome amplifiers in the four different downstream application and choose the most suitable one. Our results reaffirm the known fact that MDA amplifier is suitable for locus specific applications, as we demonstrate – regardless of the downstream application technology, and SurePlex fully meets the criteria for the genomic applications like aCGH or NGS. Although in only two families we were able to use both WGA methods simultaneously, we found it practical and pragmatic as this allows for more versatile PGT experience since chromosome microarray analysis in case of MDA is possible only for approximately two thirds of the cases and only for the whole chromosomes, but not the partial copy number variations. As we cross validated performance of different applications, we can conclude that both amplifiers can be used for any downstream application with sensitivity good enough if best practice guidelines of PGT-M (Hellani et al., 2004; Piyamongkol et al., 2003) are followed. After all, our endeavors allow for the adaptation of the developed testing system for the virtually any single gene disorder.

5.2 Improving reliability of genetic testing in early pregnancy loss

The next practical work described in Chapter 3 – genetic testing of products of conception to exclude fetal chromosomal rearrangements – was initiated due to a clinical demand. Despite the controversial status of POC testing (Carp, 2007), there are scenarios where knowing the karyotype of a miscarried fetus can help in clinical management (Lathi et al., 2012), since any prognosis is empirical if the karyotype of the abortus is unknown. However, the known problem of MCC can jeopardize the whole intentions to provide the best management to these patients.

As aCGH was shown as a rescue karyotyping methodology (Kudesia et al., 2014), we selected it as the most suitable tool for the clinical application. However, soon after we faced an issue of an increased number of apparently normal female karyotypes. POC testing demonstrates that modern technology application can be disappointing if used without an understanding of the peculiarities of the certain methodology and / or the specifics of the particular biological material. This forced us to pursue the development of a foolproof protocol capable to acknowledge MCC in case of its presence for every sample. The work resulted in the development of an MCC detection protocol, which is low-resource setting addition to any existing POC testing protocol having a considerable implication in improving clinical management of the patients dealing with early pregnancy loss. Not only we offered a new set of polymorphic STR markers as reliable as the commercially available kits (e.g., Identifiler by Thermofisher), but this is also a low-cost solution, which can be an important consideration for certain countries. Our approach of aCHG combined with MCC testing is an alternative between the SNP-arrays able to detect MCC constitutionally (Lathi et al., 2014) but being quite expensive and laborious, and the cytogenetic testing which leaves a significant proportion of samples without an answer due to the lost viability of the cells. Our practical recommendations on how to reduce MCC in POC testing will be found useful by those only initiating POC testing.

5.3 Deciphering genetic etiology of cervical insufficiency

As described in Chapter 4, PTB is considered a multifactorial disorder. From the genetic epidemiology, it is known that a substantial part of the etiology of common diseases is a genetic risk behaving as a complex trait (Polychronakos, 2008). The identification of complex disease genes has largely relied on population-based approaches, e.g. GWAS, mainly owing to their unbiased and hypothesis-free nature (Agler and Divaris, 2020). Unfortunately, till now GWAS failed to identify common alleles as reliable markers for PTB.

A condition causing PTB with even less clear genetic background is cervical insufficiency. We attempted to address its genetic etiology using NGS in 21 well phenotyped patients. It is important to admit that the topic of this study arose from a prominent clinical need since possibilities to timely predict and prevent consequences of the condition in clinics currently are very limited due to its unclear nature (Artymuk et al., 2019). Since the gene number associated with cervical functioning at the beginning of our study was countable on one hand, severely limiting NGS analysis opportunities in our patients, we armed ourselves with the *a priori* knowledge by performing comprehensive and systematic gene analysis. In total, we identified 12 genes primarily linked to cervical insufficiency, six of which (*COL1A1*, *COL3A1, FBN1, LMNA, MATR3, ZMPSTE24*) were known to cause certain collagenopathies, while *MBL2* deficiency has been associated with susceptibility to autoimmune and infectious diseases, *IL6, IL1B, IL10* – are all mediators of the inflammatory process, *TGFB1* regulates cell proliferation and growth, and *HIF1A* is a transcription factor. Further, we identified 91 genes potentially linked to cervical insufficiency. Both gene lists subsequently were used for NGS data analysis. After careful variant filtering, exploiting ACMG best practice guidelines, we identified 13 deleterious variants of high interest in 10 patients. Being apprehensive with the variant interpretation, we called these variants "variants showing a theoretical potential to increase susceptibility to the development of the cervical insufficiency needing further investigation". Most importantly, 11 variants were in genes associated with EDS development and two in genes associated with Osteogenesis imperfecta.

While collagen role has long been implicated in the development of cervical insufficiency, direct evidence from clinical studies to this was largely missing. We were first to attempt and demonstrate rare variants involvement in this phenotype development since before only associations with common collagen gene variants were described. Importantly, such implication of rare variants not detectable by association studies into the biology of complex phenotypes was predicted already long ago (S. Levy et al., 2007; Polychronakos, 2008).

After of our manuscript publishing, another novel study aiming to identify the molecular signature through which cervix opening is being controlled under progesterone and interleukin IL-1β signaling (Kniss and Summerfield, 2020) came out indirectly supporting our findings.

Evidence of the therapeutic utility of progesterone for the prevention of preterm cervical ripening and preterm labor in women at-risk is well known (Conde-Agudelo and Romero, 2016), because progesterone receptor signaling underpins many of the physiological processes opposing untimely cervical dilation (Word et al., 2007). However, unanswered questions persist regarding the mechanisms through which progesterone acts. Authors of the study exploited a primary culture model of human cervical stromal fibroblasts treated with progesterone,

interleukin-1β or the combination of both. Results demonstrated that interleukin-1β induced differential expression of extracellular matrix proteins, ECM-degrading enzymes, and enzymes involved in glycosaminoglycan biosynthesis (particularly *COL3A1* [HGNC:2201] – the only gene with an established gene-phenotype role as shown through HPO term 'Cervical insufficiency', *ELN* [HGNC:3327], *COL4A1* [HGNC:2202], *HAS2* [HGNC:4819] – all included in our gene lists, as well as *B4GALT1* [HGNC:924], *CHST11* [HGNC:17422], *EXT1* [HGNC:3512], *FUT8* [HGNC:4019], and *HS3ST3B1* [HGNC:5198]) – all to a lesser or higher degree involved in extracellular matrix interactions, tissue mechanical and biomechanical strength (Kniss and Summerfield, 2020). These findings echo our pathway and GO enrichment analysis findings on the significance of the collagen-related pathways in the cervical remodeling, and also provide an insight into the control of these events by the progesterone signaling (Kniss and Summerfield, 2020).

Simultaneously with our manuscript an interesting case-control study by Ben-Zvi on the association of cervical insufficiency with pelvic organ prolapse (POP) and urinary symptoms was published (Ben-Zvi et al., 2020). The assessment demonstrated that women with a history of cervical incompetence experienced a higher rate of POP and urinary symptoms (odds ratio 12.8), demonstrating that both conditions have a similar pathophysiological mechanism (Ben-Zvi et al., 2020). Indeed, evidence exists that the integrity of the pelvic organs and their supportive tissue is mostly maintained by the fibrillar extracellular matrix components (Carley and Schaffer, 2000; X. Liu et al., 2006). Similarly, as weakened connective tissue leads to cervical insufficiency, it cannot properly support the organs resting on the pelvic floor leading to POP (Ben-Zvi et al., 2020). Since we were fascinated by the preliminary results of our pilot-study, it was decided to pursue a further investigation on the collagenopathic nature of the cervical insufficiency (FLPP Project Nr. 2020/1-0042, 2021-2023). Currently, a study design is under development, it also involves a comprehensive assessment of the collagen-related phenotypical features of the patients including POP and urinary symptom evaluation. We look forward to the results and opportunity to compare those with the findings of Ben-Zvi.

5.4 Recommendations for assessing genetics of female reproductive failure in research and clinics

It is possible to conclude that currently the field of genetics of female reproduction is still in its embryonic stage looking forward to new discoveries. In order to improve the current situation in female reproduction genetics, field specialists have to arm themselves with the best existing tools – state of the art methodologies and study designs. Below are few

recommendations, application of which should accelerate the progress in human reproduction genetics as well as its clinical implementation.

5.4.1 Standardized gene-disease association clinical validity assessment

Clinical testing of genes with an unclear role in disease is exceedingly difficult and could lead to incorrect diagnoses, mismanagement of the patients and prevent further evaluations of the gene role. In order to robustly link gene dysfunction to disease and use it as a diagnostic marker in clinics, a standardized clinical validity assessment considering multiple levels of evidence has to be performed. Gene curation is a fundamental process of gene inclusion into genetic testing panels for clinical application. An example of such framework for clinical validity assessment of gene-disease relationships is the one developed by the NIH-funded Clinical Genome Resource (ClinGen) (Strande et al., 2017) and its simplified version offered by Smith (Smith et al., 2017).

Highlighting this important matter, we have now attempted to characterize all genes ever described in relation to female reproductive failure. Our preliminary results show that at least 16 genes have definitive (the highest) association with the condition, 30 – strong and 17 – moderate link to the diseases of female reproductive failure, thus providing a very positive direction towards the soon establishment of testing panels and guidelines.

5.4.2 The need to update best practice guidelines on genetic testing for female reproductive failure

Very few tests are routinely recommended in clinics to investigate failed female reproduction (Cariati et al., 2019). No specific guidelines or committee opinions can be identified in the available resources specifically addressing this important matter. Only Foresta with colleagues in his “Guidelines for the appropriate use of genetic tests in infertile couples” in 2002 recommends karyotype, *FMR1* expansion, *KAL1*, and *CFTR* testing (Foresta et al., 2002). Even relatively recent works also highlight mainly well-known facts. For example, Harper is his “Recent developments in genetics and medically assisted reproduction: from research to clinical applications” says that chromosomal aberrations remain a major known cause of POI and recurrent miscarriages, and that a sizeable proportion of disorders of sexual development are caused by gonosomal (X and Y chromosomes) aberrations (Harper et al., 2018). The recommendation for assessing the karyotype and a handful of genes seems outdated and despite the existing shortcomings, there is enough reliable basis collected for the guideline update. Even more data exist to be stratified and systematized in order to compose guidelines suitable for the 21st century.

5.4.3 Importance of genetic counselling

It is important to bear in mind that infertility naturally prevents the transmission of gene variants causing the condition itself and possibly *de novo* genetic variations arising in the gametes of a patient. As we know ART might bypass this situation (J. Wu et al., 2021). Consequently, the frequency of infertility is likely to increase in future generations conceived through ART, especially given the fact that more and more children are being conceived using ART (Hyrapetian, Loucaides, and Sutcliffe, 2014). Therefore, efforts in the area of ART should focus on achieving not only a successful pregnancy, but most importantly – a healthy baby at home.

Moreover, there are series of genetic peculiarities, which can jeopardize almost any genetic testing results and, without a proper geneticist consultation, could lead to unpredictable outcomes. An example of such an issue is genetic mosaicism. Every human is mosaic. Genetic mosaicism can go unnoticed, underlie a genetic disease, and may be transmitted to the next generation. A wide array of Mendelian disorders have been observed in the mosaic state (e.g. Duchene muscular dystrophy, hemophilia, Marfan syndrome) (Erickson, 2010). The developmental stage and timing of de novo variants influences their phenotypic effects and the chance of transmission (Campbell et al., 2014). We know that variants arising after primordial germ cell migration to the gonadal ridge can result in germline mosaicism. Embryo mosaicism is a well characterized phenomenon having implications in the PGT and whole ART success.

In order to avoid "genetic speculation" and similar peripetias described in the historical context of genetic test development almost a century ago, all genetic testing and preferably any ART procedure should be accompanied by genetic counselling. Consequently, in the era of genetic testing, genetic counselling cannot be disregarded but should be treated as a cornerstone of reproductive medicine.

5.4.4 Importance of thorough patient phenotyping

"From a genetic point of view, there is a natural continuum from death in utero to sterile states in the adult – all result in failure to transmit genes to the succeeding generation. However, from the point of view of the physician, the divisions are meaningful since each poses different problems" (J. R. Miller, 1965). Impossible to disagree with the quotation of Miller, but in the scope of the current thesis I would like to replace the words "genetic point of view" with the words "evolutionary point of view" since in most of the cases those are exactly different underlying genetic mechanisms driving "different problems".

Female reproductive failure and female infertility are terms too broad to apply without classification and specification nor in clinics nor research. As was already highlighted before, precise patient phenotyping is extremely important for genetic studies. Idiopathic infertility often encloses series of distinct syndromes sometimes recognizable only under certain conditions, e.g., when using ART (in this sense ART serves not only as a therapeutic but diagnostic tool as well). Importantly, such pre-work of thorough phenotyping helps to separate patients with highly expected genetic defect from the ones whose phenotype is attributable to the external factors or at least – completely different genetic mechanism(s) thus increasing the likelihood for the whole study to identify certain genetic marker. Standardization of reproductive phenotypes reporting should also improve data storage, sharing, and comparison, as well as facilitate collaboration between the groups.

5.4.5 NGS – an effective method to identify genetic causes of female reproductive failure, and the power of gene panels

When the right cohort of patients is selected, the next important step to consider is the methodology. Despite the success of targeted Sanger sequencing that has led us to the causative gene identifications in the past, a fundamental issue with the candidate gene approach is its susceptibility to the winner's curse. Latter success was indeed achieved mostly due to the exploitation of NGS. Since its discovery, NGS revolutionized gene sequencing by overcoming many of the limitations of the Sanger technique (Goodwin et al., 2016) allowing to excel causative gene discovery mostly because of its unbiased approach and opportunity to screen very large cohorts of patients and controls (Boycott et al., 2013; Fernandez-Marmiesse et al., 2017).

Usage of one NGS assay in comparison to multiple diagnostic assays, each one addressing a separate class of DNA variants, not only leads to a potential reduction in cost but also decreases the turnaround time required to make a definitive diagnosis (Patel et al., 2018). However, like any other methodology, NGS has shortcomings. Special data analysis pipelines have to be established in order to detect certain classes of genetic variations, e.g., CNV, mosaicism, trinucleotide expansions. Often validation of the results may be required. To ensure optimal NGS performance, appropriate quality thresholds have to be established, which as shown leads to very high validation rates (> 99%) eventually removing the necessity for orthogonal validation (Beck et al., 2016) for at least some genetic variation classes.

Recent study of Maddirevula with colleagues demonstrates the success of NGS application to well phenotyped patients of female reproductive failure. WES revealed variants in the known genes in 43% of patients with infertility and 13% with RPL (Maddirevula et al., 2020).

Perhaps it is too early to apply exome analysis for female reproductive failure in a diagnostic setting, because of the field's current *modus operandi*, but targeted assays including well characterized genes could be easily implemented into clinical practice due to their competitive price, easy implementation, and scalability. It is not expected that diagnostic pickup rates will rise immediately. But even a slight improvement will be a big step towards patients' wellbeing, minimizing unnecessary investigations and manipulations, and accelerating the turnaround time to the proper reproductive solution. In different clinical fields, the use of gene panels has been the method of choice already for some time (Kamps et al., 2017), e.g., targeted NGS assays have proven themselves in diagnostics of inherited breast cancer (Neveling et al., 2017).

It is also feasible that in the nearest future WGS will be performed routinely for every diagnostic question as a generic test and particular genes of interest will be targeted ad-hoc *in silico* for each specific case. Such approach would also leave an opportunity to reanalyze the data later without the need to perform additional testing, since the whole data will be stored on the cloud.

5.5 Finalizing remarks

An umbrella denominator of female reproductive failure covers extremely diverse and distinct phenotypes, all of which might be influenced by the individual's genetic background. Genetic testing is becoming increasingly requested in almost each step of failed female reproduction, from the non-functioning ovaries through unsuccessful attempts to conceive, to a missed pregnancy. Some genomic technologies are suitable to meet the increasing demands of the field – each chapter of this thesis demonstrated a reliable application of a certain methodology to the certain reproductive issue. It is possible to conclude that the possessed hypothesis of the thesis – that advanced genetic technologies could be successfully used to reliably assess several classes of genetic variations perturbing female reproductive potential – is confirmed.

I anticipate that the number of genes discovered to date after an awaited systematic gene-disease clinical validity evaluation will form the basis for the targeted gene panels implementation in the nearest future. Together with updated best practice guidelines and proper

genetic counseling, this should increase the number of positive diagnoses and patient-tailored ARTs usage, bringing the overall wellbeing of reproductive medicine to a new level.

Disorders related to female reproduction, preventing natural propagation of the causative variants, are expected to be highly heterogenous (Laissue, 2015). In mice more than 500 genes have already been associated with female infertility, many more disease genes are waiting to be identified in humans in the coming years (Harper et al., 2018). To uncover this data, a variety of deliberative genomic approaches and sophisticated study designs in large patient cohorts, followed by functional validation studies, have to be exploited.

Importantly, different approaches have to be applied when studying inbred and outbred populations. In inbred populations the majority of the causative variants are biallelic gene disruptions, whereas in outbred populations a combination of different disease mechanisms can be expected. For example, the exciting initiative of Prof. Joris Veltman to explore the *de novo* variation hypothesis for male infertility has already resulted in the certain success (Stouffs et al., 2014). I believe that *de novo* variants are responsible for the development of a proportion of female reproduction phenotypes in the outbred populations as well, though this exciting hypothesis has to be adequately addressed. Moreover, there are some indirect hints to this hypothesis unraveled by the ExAC consortium data analysis. Respectively, in the human genome there are 3230 genes identified to be loss-of-function sensitive, with 72% of those having no associated human disease phenotype. These genes not necessarily are disease genes, but probably those are genes in which heterozygous loss of function has been reproductively disadvantageous over recent human history (Lek et al., 2016).

To date, the total number of genes with phenotype-causing mutation identified reaches more than 4000 (OMIM, 2020). Overall, there are more than 20000 genes in a human genome, meaning that more than 16000 genes without known clinical significance still have the potential to be involved in female reproduction as a single cause or in complex.

A variety of phenotypes and their genetic origins are to be discovered which now are hindered from our eyes. For example, thirty percent of pregnancies are lost between implantation and the sixth week of pregnancy (Jeve and Davies, 2014; Nybo Andersen et al., 2000), currently this time span is completely inaccessible for analysis, as is the moment of embryo-endometrial talk. Similarly, there are no studies focusing on phenotypic effects of mosaicism associated with human infertility. Could that explain the proportion of the POF / POI cases?

I believe, in the years to come, the number of novel genes described for female reproductive failure will increase rapidly. Molecular and genetic understanding of the patient's phenotype will provide unprecedented opportunity to establish new targets for the therapy or

prevention of certain conditions in female reproduction failure, bringing personalized medicine to the forefront of reproductive medicine.

Conclusions

1. Multiple displacement apmlification methodology performs better for single locus applications, while SurePlex technology suits genomic application needs, usage of both amplifiers simultaneously allows for a versatile and reliable analysis of embryos to select ones free of single-gene disorders and chromosomal aberrations facilitating healthy conception.
2. Fourteen STR loci-based protocol for the detection of maternal cell contamination in a combination with an array comparative genomic hybridization reduces misdiagnosis in genetic testing for early pregnancy loss and has implication to foster informed decision-making by clinicians and patients.
3. Systematic literature and gene analysis identified 11 genes primarily associated with cervical insufficiency with the majority causing collagenopathies, thus efficiently complementing patient NGS data analysis.
4. Pathway enrichment analysis and stringent filtering pipeline of genes and gene variants identified through NGS application discovered increased gene variation burden in pathways related to tissue mechanical and biomechanical strength and localized 13 sequence variants in genes causing collagenopathies that potentially increase the likelihood of cervical insufficiency development.

List of author's publications

Scientific publications in journals included in international databases

1. Dmitrijs Perminovs, **Ludmila Voložonoka**, Liene Korņejeva, Evija Jokste-Pīrmane, Arita Blumberga, Sandra Krasucka, Nellija Seimuškina, Irina Kovaļova and Violeta Fodina. "First preimplantation genetic testing case for monogenic disease in Latvia". *Gynecological Endocrinology* 33, no. sup1 (2017): 47–49.
2. **Ludmila Voložonoka**, Dmitrijs Perminovs, Liene Korņejeva, Baiba Alkšere, Natālija Novikova, Evija Jokste-Pīmane, Arita Blumberga, Inga Kempa, Anna Miskova, Linda Gailīte and Violeta Fodina. "Performance comparison of two whole genome amplification techniques in frame of multifactor preimplantation genetic testing". *Journal of assisted reproduction and genetics* 35, no. 8 (2018): 1457–1472.
3. Oud, Manon, **Ludmila Voložonoka**, Roos M. Smits, Lisenka ELM Vissers, Liliana Ramos and Joris A. Veltman. "A systematic review and standardized clinical validity assessment of male infertility genes". *Human Reproduction* 34, no. 5 (2019): 932–941.
4. **Ludmila Voložonoka**, Dmitrijs Rots, Inga Kempa, Anna Kornete, Dace Rezeberga, Linda Gailīte and Anna Miskova. "Genetic landscape of preterm birth due to cervical insufficiency: Comprehensive gene analysis and patient next-generation sequencing data interpretation". *PloS one* 15, no. 3 (2020): e0230771.
5. **Ludmila Voložonoka**, Linda Gailīte, Dmitrijs Perminovs, Liene Kornejeva, Violeta Fodina, Inga Kempa and Anna Miskova. "Reducing misdiagnosis caused by maternal cell contamination in genetic testing for early pregnancy loss". *Systems Biology in Reproductive Medicine* (2020): 1–11.
6. Oud Manon, **Ludmila Voložonoka**, Corinna Friedrich, Sabine Kliesch, Liina Nagirnaja, Christian Gilissen, Moira K. O'Bryan et al. "Lack of evidence for a role of PIWIL1 variants in human male infertility". *Cell* 184, no. 8 (2021): 1941–1942.
7. Oud Manon, Brendan Houston, **Ludmila Voložonoka**, Kumara Mastrorosa, Bialal Alobaidi, Petra deVries, Galuh Astuti, Liliana Ramos, Robert McLachlan, Moira O'Bryan, Joris Veltman, Hector Chemes, Harsh Sheth. "Exome sequencing reveals variants in known and novel candidate genes for severe sperm motility disorders". *Human Reproduction* (2021): deab099.
8. **Ludmila Voložonoka**, Anna Miskova, Liene Kornejeva, Inga Kempa, Veronika Bargatina, Linda Gailite. A systematic review and standartized clinical validity assessment of genes involved in female reproductive failure. Submitted to *Human Reproduction*.

Scientific articles published in peer-reviewed journals in Latvia

1. **Ludmila Voložonoka**, Liene Korņejeva, Anna Miskova, Violeta Fodina. "Preimplantation Genetic Screening – Summary of First Results in Patients with Complicated Reproductive History". *Riga Stradins University Scientific Proceedings.* (2015): 241–45.

Presentation at an international scientific conference with an oral report or thesis

1. **Voložonoka, L**., Korņejeva, L., Novikova, N., Fodina, V. Benefits of combined preimplantation genetic screening and endometrial receptivity assessment on IVF outcome for complicated reproductive history patients. Poster presentation at 49th European Human Genetics Conference. Barcelona, Spain. May 21–24, 2016.
2. **Voložonoka, L**., Fodina, V., Kornejeva, L. Molecular karyotyping of products of conception – evaluation of performance and capacity. Poster presentation at ESHRE 32nd Annual Meeting. Helsinki, Finland. 3–6 July 2016.

3. **Voložonoka, L**., Perminovs, D., Korņejeva, L., Kempa, I., Miskova, A., Šterna, O., Fodina, V. Preimplantation genetic testing of monogenic diseases and aneuploidies using single blastocyst biopsy approach. Poster presentation at the European Human Genetics Conference, May 27–30, 2017.
4. **Voložonoka, L**., Perminov, D., Korņejeva, L., Alkšere, B., Kempa, I., Miskova, A., Gailīte, L., Fodina, V. Development and application of individualized customizable preimplantation genetic testing algorithm. Balkan Journal of Medical Genetics, ICGEB Workshop Next generation diagnostics, Vol 21, 2018, Supplement 1:71.
5. **Voložonoka, L**., Kornete, A., Krūmiņa, Z., Rota, A., Gailīte, L., Kempa, I., Rezeberga, D., Miskova, A. Clinical Characterization and Collagenopathic Phenotyping in Preterm Birth due to Cervical Insufficiency. Knowledge for Use in Practice, Riga, Latvia, 24–26 March, 2021.

Presentation at a local scientific conference with an oral report or thesis

1. **Voložonoka, L**. Clinical application of embryo preimplantation genetic testing by comparative genomic hybridization on microchips in frame of reproductive medicine. Oral presentation at Latvian Obstetrics and Gynecology Specialist Congress. October 16–18, 2014.
2. **Voložonoka, L**., Miskova, A. Assessing molecular karyotyping applicability in recurrent early pregnancy loss etiology determination. Abstract for Latvian Obstetrics and Gynecology Specialist Congress. October 16–18, 2014.
3. **Voložonoka, L**., Korņejeva, L. Miskova, A. Fodina, V. Embryo preimplantation genetic screening effectiveness in ART cycles for the patients with burdened reproductive history – first results. Poster presentation at Riga Stradins University scientific conference. March 26–27, 2015.
4. **Voložonoka, L**. Array Comparative Genomic Hybridization application in reproductive genetics. Oral presentation at The Second Annual International Reproductive, Genetic and Biotechnology Conference. Riga, Latvia. 14 October 2016.
5. Novikova, N., Perminovs, D., **Voložonoka, L**., Korņejeva, L., Fodina, V. Non-invasive fetal rhesus factor genotyping assay development and validation. Poster presentation at the 75th Scientific Conference of the University of Latvia. Riga, Latvia. February 24, 2017.
6. **Voložonoka, L**., Gailīte, L., Miskova, A., Kempa, I. Next generation sequencing identifies pathogenic variants in genes involved in collagen production in patients with preterm birth due to precocious cervical ripening. Poster presentation at RSU Research Week 2019. Knowledge For Use In Practice. April 1–3, 2019.

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.

Supplements

Approval of the central medical ethics committee to perform the study "Investigation of genetic etiology of infertility"

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Rīgā

14.04.2016. Nr.1/16-04-14

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Zinātniskajai laboratorijai

Atzinums par pētījuma pieteikumu "Neauglības ģenētiskās etoloģijas iemeslu izpēte"

Centrālā medicīnas ētikas komiteja 2015.gada 22.septembrī izskatīja Rīgas Stradiņa Universitātes Molekulārās Ģenētikas Zinātniskās laboratorijas iesniegto pētījuma pieteikumu *"Neauglības ģenētiskās etoloģijas iemeslu izpēte"*.

Pamatojoties uz Centrālās medicīnas ētikas komitejas 2015.gada 22.septembra sēdes protokola Nr.6 punktu Nr.3 – pieteikuma projektu konceptuāli atbalstīt, bet, lai saņemtu apstiprinājumu, veikt precizējumus un labojumus projekta pieteikuma dokumentācijā – un iesniegtajiem pieteikuma projekta precizējumiem un labojumiem, tiek izsniegts atzinums, ka Rīgas Stradiņa Universitātes Molekulārās Ģenētikas Zinātniskās laboratorijas pētījuma pieteikums *"Neauglības ģenētiskās etoloģijas iemeslu izpēte"* nav pretrunā ar bioētikas normām.

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Approval of the central medical ethics committee to perform the study "Investigation of genetic etiology of cervical insufficiency"

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21.03.2018. Nr.2/18-03-21

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Atzinums par pētījumu
„Istmocervikālās nepietiekamības
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Centrālā medicīnas ētikas komiteja 2018.gada 18.janvārī ir izskatījusi Rīgas Stradiņa Universitātes Molekulārās Ģenētikas Zinātniskās laboratorijas iesniegto pētījumu *„Istmocervikālās nepietiekamības ģenētiskās etioloģijas iemeslu izpēte"*.

Pamatojoties uz Centrālās medicīnas ētikas komitejas 2018.gada 18.janvāra sēdes protokola Nr.2018-1 punktu Nr.5 un iesniegtajiem labojumiem, tiek izsniegts atzinums, ka Rīgas Stradiņa Universitātes Molekulārās Ģenētikas Zinātniskās laboratorijas pētījums *„Istmocervikālās nepietiekamības ģenētiskās etioloģijas iemeslu izpēte"* nav pretrunā ar bioētikas normām.

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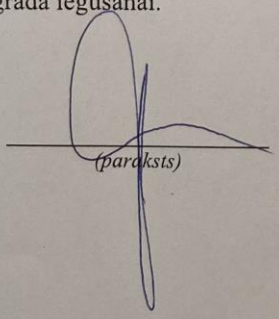

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