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Causes and Genomic Approaches to Female Reproductive Failure

Summary of the 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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Abbreviations

aCGH	Array comparative genomic hybridization
ACMG	American College of Medical Genetics
ADO	Allelic drop out
ART	Assisted reproductive technologies
BMI	Body mass index
CL	Cervical length
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDS	Ehlers-Danlos syndrome
EPL	Early pregnancy loss
eSET	Elective single embryo transfer
FFPE	Formalin fixed paraffin embedded
fPCR	Fluorescent polymerase chain reaction
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
GWAS	Genome wide association study
hCG	Human chorionic gonadotropin
HGNC	HUGO gene nomenclature
HPO	Human phenotype ontology
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
LDO	Locus drop out
LH	Luteinizing hormone
LHCGR	Luteinizing hormone chorionic gonadotropin recepto
MCC	Maternal cell contamination
MDA	Multiple displacement amplification
NGS	Next generation sequencing

nt	Nucleotide
OMIM	Online Mendelian inheritance in man
PCR	Polymerase chain reaction
PGD	Preimplantation genetic diagnosis
PGT	Preimplantation genetic testing
PGT-A	Preimplantation genetic testing for aneuploidies
PGT-M	Preimplantation genetic testing for monogenic disorders
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
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variation
STR	Short tandem repeat
VCF	Variant called file
VUS	Variant of unknown significance
WES	Whole exome sequencing
WGA	Whole genome amplification

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 (Singh, 2004; Maddirevula et al., 2020; Inhorn and Patrizio, 2014). 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". In this thesis, I use the term "female infertility" as an inability to conceive and a broader term "female reproductive failure" as an inability to conceive and/or carry pregnancy until term.

Genetic causes are recognized as important contributors to female reproductive failure at the level of all main constituents of successful conception and pregnancy progress starting with embryonic, maternal (e.g., endometrial) and common – placental factor. 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, 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. Naturally, genetic tests are becoming increasingly demanded in the frame of diagnosing and managing female reproductive failure as well. 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

1.1 Genetic causes of female reproductive failure

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) and luteinizing hormone (LH) 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 the 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).

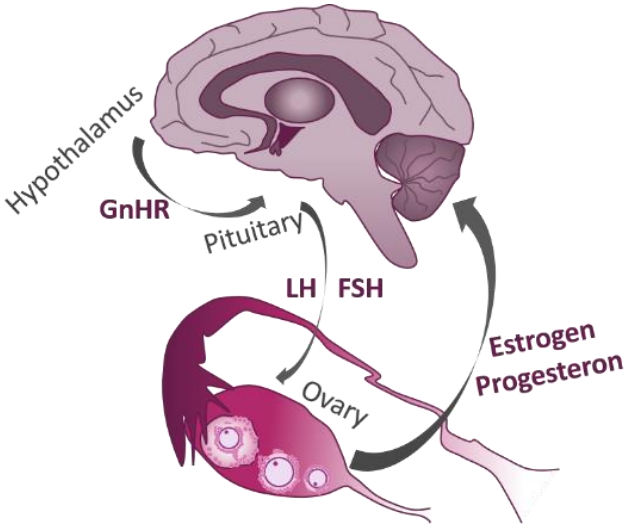

Figure 1.1 **Schematic representation of the hypothalamic-pituitary-gonadal axis functioning in female** (scheme by L. Voložonoka)

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.

When female manifests 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 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. 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). 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). 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). Table 1.1 summarizes genes implicated in the development of female reproductive failure caused by the pre-gonadal factors, at the level of gonads, as well as eugonadal phenotypes.

Table 1.1

Genes implicated in the development of female reproductive failure

Phenotype	Description	Gene	Reference
Hypogonadotropic hypogonadism	Functioning of the hypothalamic-pituitary-gonadal axis is disrupted due to malfunctioning of the hypothalamus or pituitary.	*GNRHR* [HGNC:4421] *FSHB* [HGNC:3964] *LHB* [HGNC:6584]	(Beau et al., 1998; Layman et al., 1997; de Roux et al., 1997).
Premature ovarian failure	The depletion or dysfunction of ovarian follicles with cessation of menses before the age of 40.	*STAG3* [HGNC:11356] *FMR1* [HGNC:3775] *SYCE1* [HGNC:28852] *MCM9* [HGNC:21484] *MCM8* [HGNC:16147] *GDF9* [HGNC:4224] *BMP15* [HGNC:1068]	(Le Quesne Stabej et al., 2016; Wood-Trageser et al., 2014; AlAsiri et al., 2015; Y. X. Zhang et al., 2020; Tenenbaum-Rakover et al., 2015; França et al., 2018; Laissue et al., 2006; Kovanci et al., 2007; Norling et al., 2014).
Empty follicle syndrome	A condition characterized by a lack of response to a repeated administration of human chorionic gonadotropin beta during ART resulting in empty oocytes.	*LHCGR* [HGNC:6585]	(Coulam, Bustillo and Schulman, 1986; Yariz et al., 2011; Awonuga et al., 1998; Yuan et al., 2017).
Absent zona pellucida	ART failure due to the absence of *zona pellucida* in oocytes.	*ZP1* [HGNC:13187] *ZP2* [HGNC:13188] *ZP3* [HGNC:13189] *ZP4* [HGNC:15770]	(H.-L. Huang et al., 2014). (T. Chen et al., 2017; P. Yang et al., 2017). (Sun et al., 2019; Dai et al., 2019).

Table 1.1 continued

Phenotype	Description	Gene	Reference
Oocyte maturation arrest	Oocyte maturation arrest occurring at the stage of germinal vesicle, meiosis I and II leading to ART failure.	*TUBB8* [HGNC:20773] *PATL2* [HGNC:33630]	(B. Chen et al., 2017; Z. Liu et al., 2020; L. Wu et al., 2019; A. C. Wang et al., 2018; L. Huang et al., 2017; Feng et al., 2016).
Fertilization failure	Failure of fertilization despite the intracytoplasmic injection of apparently healthy sperms in apparently healthy eggs resulting in zero-pronucleus (PN) zygotes, 3PN zygotes, or zygote degradation.	*TLE6* [HGNC:30788] *WEE2* [HGNC:19684] *REC114* [HGNC:25065]	(Sang et al., 2018; Zhao et al., 2019; X. Yang et al., 2019; Alazami et al., 2015; Lin et al., 2020).
Early embryonic arrest	Preimplantation embryo lethality and failure of an embryo to form blastocyst.	*PADI6* [HGNC:20449] *NLRP2* [HGNC:22948] *NLRP5* [HGNC:21269]	(Mu et al., 2019; Xu et al., 2016).
Hydatidiform mole	Development of the conceptus into a hydatidiform mole results from the overgrowth of the extraembryonic trophoblast whereas the embryo itself suffers early demise.	*NLRP7* [HGNC:22947] *KHDC3L* [HGNC:33699] *MEI1* [HGNC:28613] *C11orf80* [HGNC:26197] *REC114* [HGNC:25065]	(Parry et al., 2011; Rezaei et al., 2016; van den Veyver and Al-Hussaini, 2006; Murdoch et al., 2006; Nguyen et al., 2018).
Polycystic ovary syndrome	A hormonal disorder characterized by irregular menses, multiple cysts in the ovaries. Only syndromic forms of the condition have genetic associations.	*CYP11A1* [HGNC:2590] *CYP21A2* [HGNC:2600] *CYP11B1* [HGNC:2591]	(Unluturk et al., 2007; Gaasenbeek et al., 2004; Witchel and Aston, 2000; Reichman et al., 2014).

Table 1.1 continued

Phenotype	Description	Gene	Reference
Leiomyomata	Benign smooth muscle tumours of the uterus. Only syndromic forms of the conditions are associated with certain gene variants.	*FH* [HGNC:3700] *COL4A5* [HGNC:2207] *COL4A6* [HGNC:2208]	(Zhou et al., 1993; Tomlinson et al., 2002).
Spontaneous ovarian hyperstimulation syndrome	The syndrome occurs spontaneously during the first trimester of pregnancy.	*FSHR* [HGNC:3969]	(Smits et al., 2003; Vasseur et al., 2003; Montanelli et al., 2004).
Mullerian aplasia	Congenital absence or aplasia of the uterus and / or vagina.	*WNT4* [HGNC:12783] *HNF1B* [HGNC:11630] *LHX1* [HGNC:6593]	(Williams et al., 2017; Philibert et al., 2008).

1.2 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 the 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 task especially applicable to human reproduction – optimize usage of the assisted reproductive technologies (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 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, D'Argenio, and Tomaiuolo 2019). 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. 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 reproductive failure (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*.

1.2.1 Preimplantation embryo genetic testing

The per cent 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 as 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 the hope of increasing live birth rates per transfer (PGT-A) (Theobald, SenGupta, and Harper, 2020).

1.2.2 Genetic testing of products of conception

A number of approaches and methodologies are used for POC genetic testing including 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., a prerequisite for a successful karyotyping is the 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, certain success has been achieved during the last decades in deciphering the molecular and genetic basis of female reproductive failure. Nevertheless, usage of the existing knowledge in clinical practice is still fragmented and cumbersome (Cariati, D'Argenio and Tomaiuolo, 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 to address certain phenotypes / stages of female reproduction and / or overcoming shortcomings of these technologies, and demonstrating their suitable application to 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 that address 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 Introduction

Preimplantation genetic testing 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. With time, PGT has undergone significant methodological and approach changes, starting from polar body testing and blastomere analysis to the currently adapted trophectoderm biopsy with subsequent blastocyst freezing (Renwick et al., 2006). Despite

technological improvements, the development of PGT protocols is challenging and prone to amplification failure, DNA contamination and allelic dropout (ADO) – a phenomenon common to all single-cell based PCR tests, thus affecting the reliability of the test. ADO's incidence varies, but in extreme cases, up to 20% of amplifications were affected in the past leading to several misdiagnoses (Capalbo et al., 2016).

Choosing the type of WGA is also challenging due to difficulties in the 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 (Telenius et al., 1992) or primer extension PCR technology (L. Zhang et al., 1992). Leading positions are taken by OmniPlex linear WGA (Uda et al., 2007; S. U. Chen et al., 2008) technology developed by Rubicon Genomics and multiple displacement isothermal synthesis by Phi-29 polymerase approach (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 multiple displacement amplification (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). 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).

Despite numerous advances, 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 single gene disorder 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 (Vanneste, Voet, Melotte et al., 2009; Kieffer et al., 2016). Early results show that combined PGD and PGS increase the patient chance of healthy childbirth (Marshall et al., 2015; Sermon, 2017).

Taking into consideration the aforementioned information, the aim of our study was to develop an effective and robust individualized multifactor embryo testing protocol and to show the performance comparison of two WGA techniques in four different downstream applications – short tandem repeat (STR) sizing, Sanger sequencing, aCGH and SNaPshot technology.

2.2 Materials and methods

2.2.1 Pre-clinical work-up

Nine couples with a confirmed particular single gene disease transmitted in their family underwent counselling regarding the PGT procedure, ovarian stimulation, oocyte aspiration and IVF. Before processing a clinical case, a workup was carried out to prepare each PGT case. Linked microsatellites adjacent to the gene of interest were located through the University of California Santa Cruz genome browser (https://genome-preview.ucsc.edu/index.html). For all loci, semi-nested primers for two round multiplex fPCR were designed using the "Primer-BLAST" to ensure specificity (Ye et al., 2012).

DNA obtained from the peripheral venous blood of a couple seeking PGD and other family members was isolated using a standard procedure (Qiagen). Family haplotypes flanking loci of interest were assessed. When PCR linkage analysis was performed for a family, 6–13 (8.1 ± 2.5) informative or semi-informative STR markers (Table 2.1) were included in the following PGT cycle for embryo analysis. STR marker informativeness rate was 53%. 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.

2.2.2 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), for the rest it was carried out by OmniPlex linear WGA technology (SurePlex, Illumina, USA) (Table 2.2). Embryo haplogroup analysis was carried out, assessing informative markers found in a linkage step. 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 according to manufacturers' (24Sure, Illumina, USA) protocol for aCGH.

Table 2.1

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
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

Table 2.1. 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-case1	21/11	0.52	9	4; 1.5	1; 0.11	4; 0.44	Performed for all embryos	5 euploid, 4 aneuploid	eSET -> healthy carrier baby born, 2nd eSET-> failed implantation
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

Table 2.2

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
WGA material donated for research	–	11 OmniPlex	–	–	11 OmniPlex	11 OmniPlex

Table 2.2. continued

Case	Sanger sequencing		SnapShot analysis		STR analysis		STR ADO* (%)	aCGH analysis		Total embryos analysed
MDA:	32	1x ADO for TPP-case. Partial ADO detected.	20	Consistent results	39	Clear Electropherograms	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	Electropherograms 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.

2.3 Results

2.3.1 Embryo PGT analysis

For all 62 embryo biopsies, WGA amplification performed either by SureMDA or SurePlex kit was successful and eventually with a conclusive result. 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.

Overall ADO rate was 4.74% (Table 2.2), exceeding 5% cut-off only in Duchenne Muscular Dystrophy (*DMD*) case-1. Direct mutation testing was done for all cases processed except for *DMD*-case1, in all cases, the direct disease-causative variant testing complemented and matched haplotyping results.

2.3.2 Comparison of two different WGA techniques

Both types of WGA were subjected to all four downstream applications – Sanger sequencing, STR amplification and aCGH (Table 2.2). Our results show that both WGA methodologies result in partial ADO when Sanger sequencing is performed (Figure 2.1). Poor amplification of disease-causative allele can be distinguishable as low-level electropherograms in otherwise clear profiles. One *TPP1*-case sample resulted in complete disease-causative allele ADO even despite the hemi-nested amplification approach.

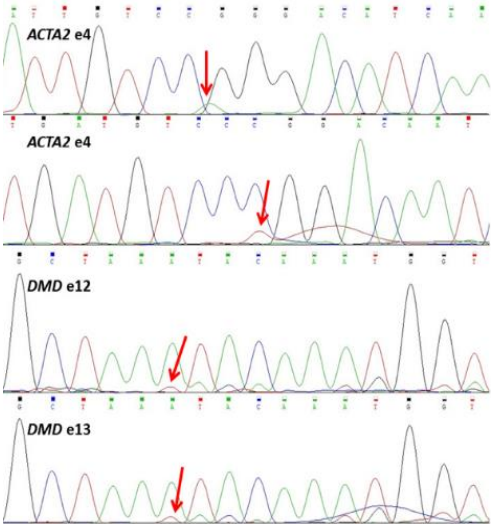

Figure 2.1 **Sanger sequencing profiles of different WGA technologies**

WGA for ACTA2-case embryo four (e4) performed by SurePlex amplification. The upper panel electropherogram represents sequence gained by the forward primer; lower panel represents the reverse primer sequence. Red arrows mark the partial loss (partial allelic dropout, ADO) 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 electropherograms represent sequences gained by the forward primer. Red arrows mark partial ADO of mutated allele – one nucleotide deletion. 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 2.2); the MDA product resulted in comparable results in 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.

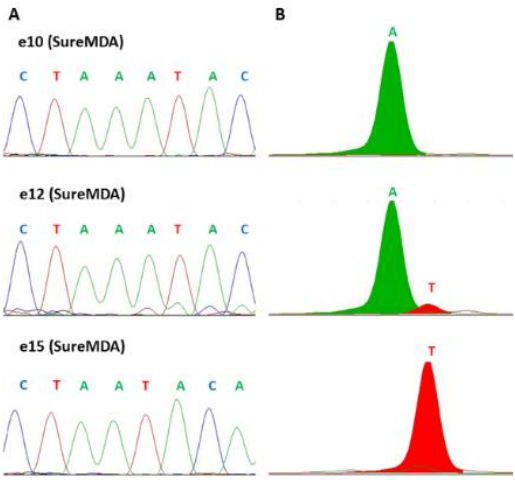

Figure 2.2 **Comparison of STR sizing (A) and SNaPshot (B)**

Results are shown for DMD case-two embryos variant locus (one nucleotide deletion). Whole-genome amplification performed by multiple displacement amplification (MDA) technique. Profiles completely match between 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 2.3). 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.

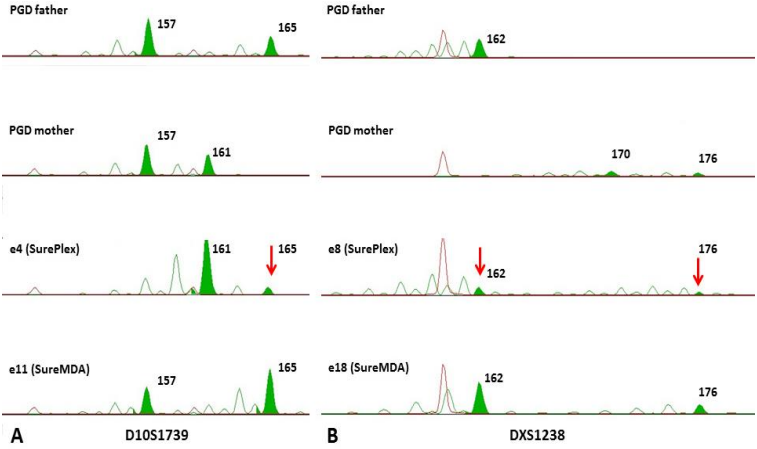

Figure 2.3 **Embryo haplotype analysis**

(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.

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 signed forms from all the co-authors to use this manuscript for my thesis (Supplement 4).

3.1 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, Horvath and Castaño, 2017; Romero et al., 2015).

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.

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. A bias towards an increased number of normal female karyotype reports in comparison to normal male karyotype reports has been noted (Lathi et al., 2014; Bell et al., 1999; Jarrett et al., 2001). However, not all laboratories fully address this important issue and its etiology (Nikitina et al., 2005).

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, Quillen and Yamane, 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 (Lathi et al., 2014; Romero et al., 2015; Jarrett et al., 2001), 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.

3.2 Materials and Methods

3.2.1 Patients and POC chromosome analysis

Patients experiencing miscarriage or spontaneous abortion before the 13th week of gestation were recruited to the study. In total, 86 POC samples were included in the study. 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. Visual appearance of the POC 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. Tissue sampling was performed for all the samples. Chromosome analysis was performed by aCGH for all the POC samples following the manufacturer’s protocol [24sure; Illumina, USA].

3.2.2 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. 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.

3.3 Results

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. 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. 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.

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.

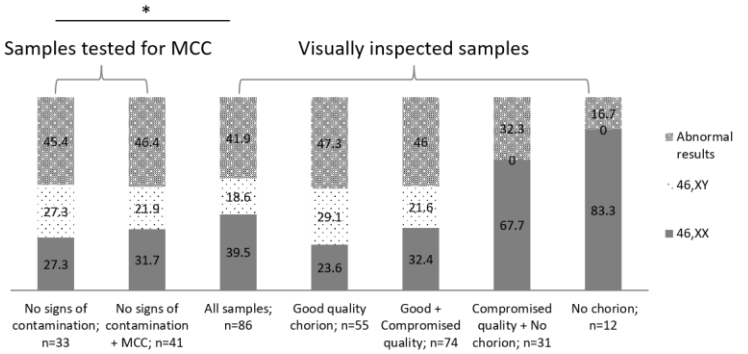

Figure 3.1 **Distribution of karyotype results across different POC evaluation groups**

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 (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.

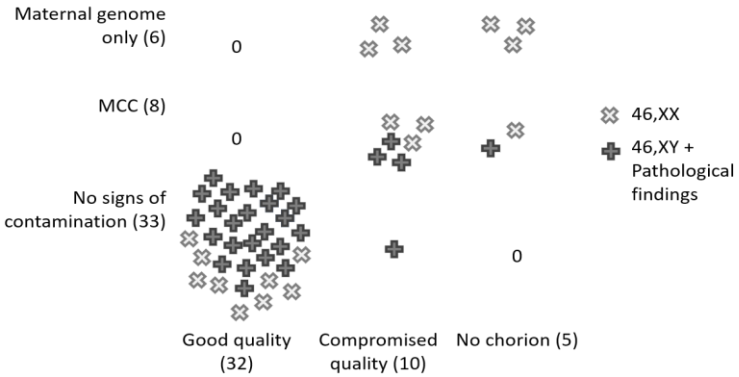

Figure 3.2 **Distribution of MCC-high and -low risk karyotypes across different product of conception evaluation groups**

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.

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 signed forms from all the co-authors to use this manuscript for my thesis (Supplement 5).

4.1 Introduction

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). 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. 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 (S. W. Wang et al., 2016; Mingione et al., 2003). 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 is the best predictive factor for spontaneous preterm birth (PTB) (Di Renzo, 2015). Epidemiological data show that fetuses/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).

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 (Strauss et al., 2018; Bezold et al., 2013). 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. 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?

4.2 Materials and Methods

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

We conducted a literature search according to the PRISMA guidelines (Moher et al., 2009). 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. 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.

4.2.2 Next-generation sequencing of patients with cervical insufficiency

Subjects

The study recruited 21 females of Caucasian ethnicity with presentation of painless cervical dilatation in the ongoing pregnancy and / or a positive anamnesis of pregnancy loss and / or preterm delivery due to cervical insufficiency without contractions in singleton pregnancies (Table 4.1).

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, bioinformatics analysis and variant filtering

NGS analysis was carried out using Illumina's TruSight One Sequencing Panel Capture Kit (USA). 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]. 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. The second filtering step retained variants covered with at least 10 reads, with a variant allele frequency of at least 25%, and excluded "benign" and "likely benign" variants of known clinical significance.

Variant classification, prioritization, and gene set enrichment analysis

Genetic variants were filtered using the three gene lists created by means of the systematic literature analysis. 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). To obtain unbiased information on pathway enrichments across the genes having rare and deleterious variants in our cohort, we annotated genes using the ConsensusPathDB interaction database (Kamburov et al., 2013).

4.3 Results

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

Altogether, only 12 genes were primarily identified in relation to cervical insufficiency (Table 4.2.), 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 human phenotype ontology (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).

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.

4.3.2 Patient NGS data analysis

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.). Fourteen variants were found in 10 genes known to cause EDS, osteogenesis imperfecta, or Bethlem myopathy. Ultimately, based on

a comprehensive curation of the variants' pathogenicity, including known gene-disease / gene-phenotype associations, gene expression patterns within cervical tissues, and mechanisms of diseases of particular genes, etc., 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.

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)	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)	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)	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)	VUS (PP3; PM1)	Pathogenic variants in the gene cause EDS;VUS previously found in EDS patients; Likely recessive type of inheritance.	Further investigation needed

Table 4.3 continued

Sample	Gene	Genotype (effect on protein if known)	ACMG Manual (Criteria)	Comments	Contribution to the phenotype of cervical insufficiency
Case3	*COL1A2*	NM_000089:c.[1808C > T];[=] (NP_000080.2:p.Thr603Ile)	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
Case4	*COL1A1 (rs778463556)*	NM_000088:c.[1663C > T];[=] (NP_000079.2:p.Pro555Ser)	LP (PP3; PM5; PP2; PM2)	Pathogenic variants in the gene cause EDS; Strong expression within cervix; Likely dominant type of inheritance.	Further investigation needed
Case5	*COL12A1 (rs201988277)*	NM_004370.6:[c.7853C > T];[=] (NP_004361.3:p.Thr1454Met)	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

Table 4.3 continued

Sample	Gene	Genotype (effect on protein if known)	ACMG Manual (Criteria)	Comments	Contribution to the phenotype of cervical insufficiency
Case5	*COL1A1 (rs537060488)*	NM_000088:c.[529G > A];[=] (NP_000079.2:p.Val177Met)	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)	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)	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)	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)	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)	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)	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)	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)	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)	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

Table 4.3 continued

Sample	Gene	Genotype (effect on protein if known)	ACMG Manual (Criteria)	Comments	Contribution to the phenotype of cervical insufficiency
Case11	*P3H1 (rs371232413)*	NM_001146289:c.[1720 + 4G > A];[=]	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
Case12	*P3H1 (rs371232413)*	NM_001146289:c.[1720 + 4G > A];[=]	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)	VUS (PP3; BS1)	Associated with EDS, but gene-disease association is dubious (one missense variant reported in publication); Likely recessive type of inheritance.	Unlikely

Table 4.3 continued

Sample	Gene	Genotype (effect on protein if known)	ACMG Manual (Criteria)	Comments	Contribution to the phenotype of cervical insufficiency
Case12	*MYO1F (rs747756979)*	NM_001348355:c.[1170 + 4C > T];[=]	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];[=]	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.

4.3.3 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, 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.

5 General Discussion

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 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 delivered healthy kids, which was confirmed postnatally. Only in 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 (Theobald, SenGupta and Harper, 2020; Butler et al., 2019). The 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 a 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 applications and choose the most suitable one. Our results reaffirm the known fact that the MDA amplifier is suitable for locus-specific applications, as we demonstrate – regardless of the downstream application technology, and SurePlex fully meets the criteria for 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 a more versatile PGT experience since chromosome microarray analysis in the 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 the 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 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 intention 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 the 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 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 a low-resource setting in addition to any existing POC testing protocol that has 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 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 it is quite expensive and laborious, and the cytogenetic testing, which leaves a significant proportion of samples without an answer due to 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, until now GWAS failed to identify common alleles as reliable markers for PTB. The condition causing PTB with an 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 the 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 have 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’s 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 a rare variant 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 (Levy et al., 2007; Polychronakos, 2008).

After 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. The 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 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 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 symptoms 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

1. In order to robustly link the currently identified genes to female reproductive failure phenotypes and use those as diagnostic markers in a clinical setting, a standardized clinical validity assessment of gene-disease relationships has to be performed.
2. In order to facilitate the development of the field of female reproduction and stimulate personalized treatment application, best practice guidelines on genetic testing in female reproductive failure have to be updated.
3. All genetic testing and preferably any ART procedure should be accompanied by genetic counselling to allow for the informed reproductive decision making and avoid adverse reproductive outcomes for the patients and their progeny.
4. Thorough patient phenotyping should be performed in research and clinics to separate patients with highly expected genetic defect from the ones whose phenotype is attributable to the external factors thus increasing the likelihood to identify certain genetic marker.
5. Targeted NGS assays including well characterized genes implementation into clinical practice will facilitate genetic cause identification of female reproductive failure minimizing unnecessary investigations and manipulations and thus accelerating turnaround time to the proper reproductive solution.

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 every 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 work – 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. I believe that *de novo* variants are responsible for the development of a proportion of female reproduction phenotypes in the outbred populations, 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 the data probably points to 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 a part of complex.

A variety of phenotypes and their genetic origins are to be discovered which now are hindered from our eyes. For example, thirty per cent of pregnancies are lost between implantation and the sixth week of pregnancy (Nybo Andersen et al., 2000; Jeve and Davies, 2014), 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. MDA 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. Perminovs, D., **Voložonoka, L**., Korņejeva, L., Jokste-Pīrmane, E., Blumberga, A., Krasucka, S., Seimuškina, N., Kovaļova, I. and Fodina, V., 2017. First preimplantation genetic testing case for monogenic disease in Latvia. *Gynecological Endocrinology*, 33(sup1), 47–49.

2. **Voložonoka, L**., Perminovs, D., Korņejeva, L., Alkšere, B., Novikova, N., Pīmane, E. J., Blumberga, A., Kempa, I., Miskova, A., Gailīte, L. and Fodina, V., 2018. Performance comparison of two whole genome amplification techniques in frame of multifactor preimplantation genetic testing. *Journal of assisted reproduction and genetics*, 35(8), 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. **Voložonoka, L**., Rots, D., Kempa, I., Kornete, A., Rezeberga, D., Gailīte, L. and Miskova, A., 2020. Genetic landscape of preterm birth due to cervical insufficiency: Comprehensive gene analysis and patient next-generation sequencing data interpretation. *PloS one*, 15(3), e0230771.

5. **Voložonoka, L**., Gailīte, L., Perminovs, D., Kornejeva, L., Fodina, V., Kempa, I. and Miskova, A., 2020. Reducing misdiagnosis caused by maternal cell contamination in genetic testing for early pregnancy loss. *Systems Biology in Reproductive Medicine*, 66(6), 410–420.

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". *Rīga Stradiņš 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**., Perminovs, 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. **Vološonoka, 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 Rīga Stradiņš University scientific conference. March 26–27, 2015.

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6. **Vološonoka, 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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Supplement

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

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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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Atzinums par pētījumu
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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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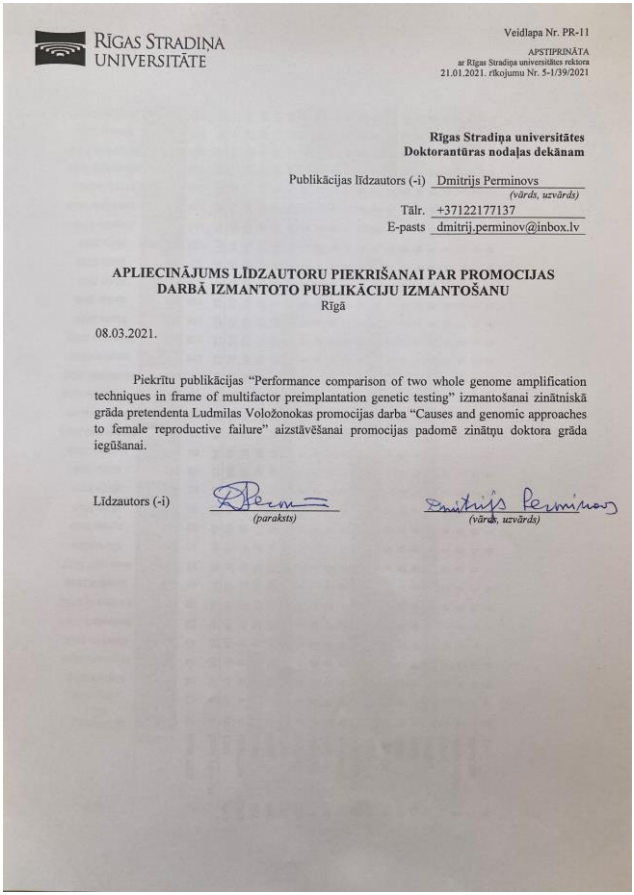
Agreement of co-authors (in order as appear in the publication) of the manuscript "Performance comparison of two whole genome amplification techniques in frame of multifactor preimplantation genetic testing" to use this manuscript as a part of the PhD thesis of Ludmila Vološonoka



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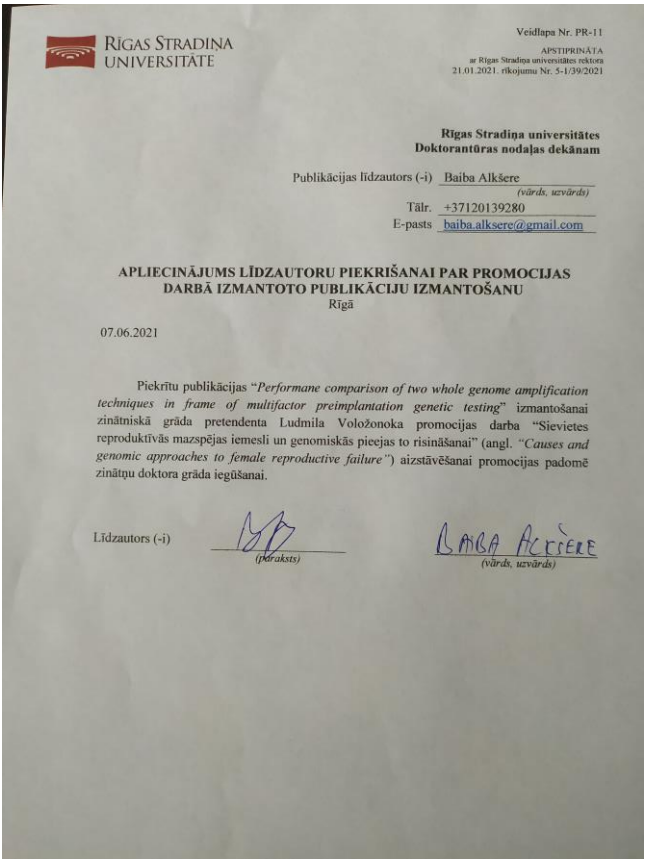

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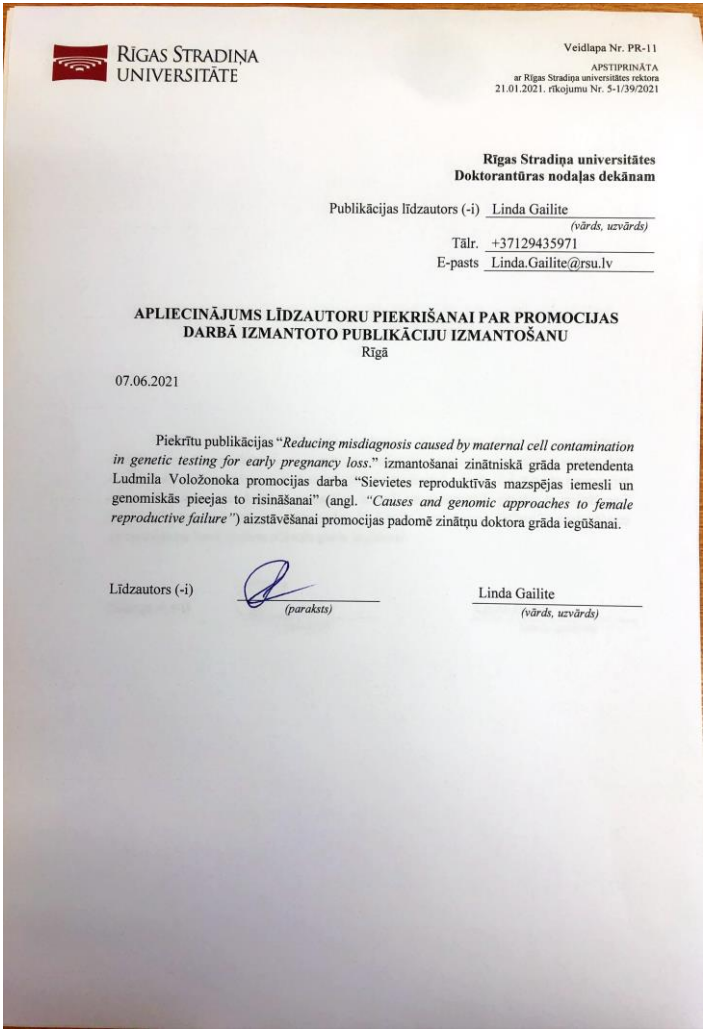

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