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**REASONS FOR FAILED IMPLANTATION IN IVF CYCLES  
METHODS TO IMPROVE IVF OUTCOMES**

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## ACRONYMS AND ABBREVIATIONS

2-COX 2	Cyclooxygenase	DPP4	Dipeptidyl peptidase-4
aCGH	array comparative genomic hybridization	E2	oestradiol
aCLs	anticardiolipin antibodies	EDN3	gene that encodes Endothelin 3 protein
AFC	antral follicle count	EM	Effector memory
AMH	anti-müllerian hormone	EPL	early pregnancy loss
ANXA4	Annexin A4	ERA	Endometrial Receptivity Array
aPLs	Antiphospholipid Antibodies	ESHRE	European Society of Human Reproduction and Embryology
Apo A-I	Apolipoprotein A-I	ET	embryo transfer
APS	Antiphospholipid syndrome	EU	European Union
ART	assisted reproductive technologies	FET	frozen embryo transfer
ASRM	American Society of Reproductive medicine	FISH	Fluorescence in situ hybridization
aβ2GPI	anti-β2glycoprotein I antibodies	FOXP3	forkhead box P3, also known as scurfin
BCL6	B-cell lymphoma 6 protein	GADD45A	gene that encodes Growth arrest and DNA-damage-inducible protein
BMI	Body mass index	GAST	Gonadotropin-releasing Hormone Agonist Test
BRCA1	Breast cancer gene 1	GLI1	Glioma-associated oncogene protein
Ca <sup>2+</sup>	Calcium	GPX3	glutathione Peroxidase 3 protein coding gene
CAM	cellular adhesion molecules	hCG	Human chorionic gonadotropin
CD138	Syndecan-1, marker of plasmocytic differentiation	hCG+7	7 <sup>th</sup> day of hCG triggering or 168 hours of hCG administration
CD16	a molecule of the Ig superfamily known to be involved in antibody-dependent cellular cytotoxicity	HGExERdb	Human Gene Expression Endometrial Receptivity database
CD25	α-chain of the IL-2 receptor	HOXA-10	Homeobox A10 protein
CD3	Glycoprotein that serves as a co-receptor for the T-cell receptor	HRT	hormone replacement therapy
CD4	Glycoprotein that serves as a co-receptor for the T-cell receptor	ICSI	Intracytoplasmic sperm injection
CD56	archetypal phenotypic marker of natural killer cells	IFN- γ	Interferon-γ
CLDN4	Protein coding gene	IGF-1	Insulin-like growth factor-binding protein
CNV	Copy number variations	IgG	Immunoglobulin G
COS	controlled ovarian stimulation	IL-1	Interleukin 1
cPLA2a	cytosolic phospholipase A2	IL-10	Interleukin 10
CRABP2	Cellular retinoic acid-binding protein 2	IL-11R	Interleukin 11 Receptor
CST	community state types	IL-12	Interleukin 12
DET	deferred embryo transfer	IL-13	Interleukin 13
DFI	DNA fragmentation index of sperm	IL-15	Interleukin 15
DNA	deoxyribonucleic acid		

IL-18	Interleukin 18	pH	acidity, potential of hydrogen
IL-2	Interleukin 2	POR	Poor ovarian response
IL-21	Interleukin 21	qPCR	quantitative PCR
IL-23	Interleukin 23	RAGs	receptivity Associated Genes
IL-4	Interleukin 4	rDNA	ribosomal DNA
IL-5	Interleukin 5	REM	Recurrent early miscarriage
IL-6	Interleukin 6	rFSH	recombinant follicle stimulating hormone
IVF	in vitro fertilization		
LA	lupus anticoagulant	RIF	recurrent implantation failure
LH	Luteinizing hormone	RNA	ribonucleic acid
LIF	Leukaemia inhibitory factor	RNA-seq	RNA sequencing
LPA3	lysophosphatidic acid receptor 3	ROBs	Robertsonian translocations
MAOA	Monoamine Oxidase A protein coding gene	RPL	Recurrent pregnancy loss
mIU/ml	milli-international units per millilitre	RRA	Robust rank aggregation
MMP7	matrix metalloproteinase-7	RSA	Recurrent spontaneous abortion
mRNA	messenger RNA	SART	Society for Assisted Reproductive Technology
MUC-1	mucin 1 protein coding gene	SCD test	Sperm chromatin dispersion test
NGS	next-generation sequencing	SET	selective single embryo transfer
NK	Natural Killers	SFRP4	Secreted frizzled-related protein 4 coding gene
NKp46 <sup>+</sup>	novel triggering receptor expressed by all human NK cells that is involved in natural cytotoxicity	SIGs	Special Interest Groups
OLFM1	Olfactomedin 1 protein coding gene	SIRT1	Histone deacetylase protein
P+3	3 <sup>rd</sup> day of progesterone administration or 72 hours	SNP	single nucleotide polymorphism
P+5	5 <sup>th</sup> day of progesterone administration or 120 hours	sPLA2-IIA	secretory phospholipase A2
P+7	7 <sup>th</sup> day of progesterone administration or 160 hours	SPP1	Secreted phosphoprotein 1 protein coding gene
p300	Histone acetyltransferase p300	TGF- $\beta$	Transforming growth factor beta
P4	Progesterone	TGF- $\beta$ 1	Transforming growth factor beta 1
p53	Tumour protein P53	Th1	Type 1 helper T cells
PAEP	Progesterone associated endometrial protein coding gene	Th17	subset of T helper cells producing interleukin 17
PCR	Polymerase chain reaction	Th2	Type 2 helper T cells
pET	personalised embryo transfer	TLM	time-lapse microscopy
PG	Prostaglandins	TNF- $\alpha$	Tumour necrosis factor $\alpha$
PGD	preimplantation genetic diagnostic	Treg	Regulatory T cells
PGT	preimplantation genetic testing	TVUS	transvaginal ultrasound
PGT-A	preimplantation genetic testing for aneuploidies	UC/min	uterine contractions per minute
PGT-M	preimplantation genetic testing for monogenetic diseases	uNK	uterine Natural Killers
		UP	uterine peristalsis
		WGA	whole genome amplification
		WHO	World Health Organisation
		WOI	window of implantation
		X3	Homeobox protein

## ABSTRACT

Infertility is a widespread medical and social problem affecting approximately 17.5% of couples worldwide (WHO 2021). Severe cases of infertility require *in-vitro fertilization* (IVF) treatment, and the proportion of IVF procedures performed to achieve a pregnancy is continuously growing. The main challenges facing couples and physicians during IVF treatments are recurrent implantation failure (RIF) and early pregnancy loss (EPL). There is still a lack of diagnostic and therapeutic methods to facilitate improved prognosis and to reduce the incidence of RIF and ELP.

This work was done to assess the ability of different methods and approaches to improve the outcome of IVF treatment, and to decrease RIF and EPL. Both non-invasive and invasive IVF work-up methods were assessed to reach this goal.

During the first part of the study, we assessed the impact of the non-invasive method of time-lapse microscopy (TLM) on IVF outcomes. The sample comprised 897 embryos analyzed using TLM in 146 embryo transfers. The control group included 1723 embryos cultivated and selected conventionally using the traditional static observation method which preceded 386 embryo transfers. The total EPL in the TLM group (both biochemical and clinical) was reduced statistically significantly (TLM 28.4% versus control 41.8%,  $\chi^2$ ,  $p = 0.045$ ). The EPL rate per embryo transfer was also significantly reduced in the TLM group (TLM 9.1% versus control 19.1%,  $p = 0.043$ ).

During the second part of the study, the performance of two invasive methods was assessed, – preimplantation genetic testing for aneuploidies (PGT-A) of embryos and endometrial receptivity array (ERA) to improve the outcome in RIF patients. In total, 253 ICSI cycles were included in the study. They were further divided into 4 groups: Group I – frozen embryo transfers without any additional tests or procedures (FET),  $n = 72$  cycles; Group II – FET with PGT-A,  $n = 67$ ; Group III – FET with PGT-A and ERA,  $n = 72$  and Group IV – FET with ERA,  $n = 22$ . When clinical outcomes of biochemical pregnancy, clinical pregnancy and pregnancy loss were compared to the end-point of “no pregnancy”, Group II (FET+PGT-A) showed a statistically significant higher chance of achieving both biochemical pregnancy ( $p = 0.01$ , OR = 5.5) and clinical pregnancy ( $p = 0.049$ , OR = 2.3), as compared to Group I. The Group II (FET+PGT-A) and Group III (FET+PGT-A+ERA) biochemical pregnancy results showed that ERA test significantly decreases biochemical pregnancy loss. The clinical pregnancy was statistically significant in the Group III (FET+PGT-A+ERA) comparing to the Group II (FET+PGT-A).

In addition, the efficacy of PGT-A in IVF cycles was tested for a specific group of 10 patients who were carriers of balanced translocations in their karyotypes. Three of them had the most frequent type of Robertsonian translocation and 7 had various reciprocal translocations. 180 oocytes were collected and 48 embryos were analyzed with PGT-A.

In 18 embryos where a female was a carrier of a balanced translocation, and 30 embryos where a male was a carrier of a balanced translocation, the percentages of unbalanced translocation rates in embryos were 89% and 73%, respectively. The highest euploidy rate was in the male carrier group – 27%. The unbalanced translocation rate in embryos was highest in the female carrier group – 78%. The prevalence of aneuploidy rates related to translocations was 4.5 times higher in the balanced reciprocal translocation carrier group, when compared with the balanced Robertsonian translocation carrier group.

Thus, the main finding, the application of the TLM selection model increased biochemical, clinical and ongoing pregnancy rates, and decreased EPL rates. Patients with RIF can further benefit from the embryo aneuploidy testing using the PGT-A method in combination with ERA test. The proportion of total embryo aneuploidy rates was higher in the specific group of 10 patients having translocations, when compared with the patients having normal karyotypes. Therefore, in this specific group of 10 patients, PGT-A is of utmost importance.

In conclusion, these data showed that a combination of TLM and PGT-A with the ERA test is an effective approach for improving the outcome of IVF treatment, decreasing implantation failure and early pregnancy loss in patients with normal karyotypes and application of the PGT-A in patients with balanced translocations provides positive results in IVF treatment.

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# INTRODUCTION

## Actuality of the work

1978 was the year when the first IVF (*in vitro fertilization*) baby Louise Brown was born. Progress in reproductive technology grew exponentially from that time along with the expertise of reproductologist and the number of scientists working in the field. In the next years following the birth of Louise Brown, initial implantation rates were <5% per embryo (Niederberger et al. 2018). As assisted reproduction techniques (ART) progressed, many clinics replaced cleavage-stage embryos to blastocyst-stage embryos and switched from multiple embryo transfers to double- or single-embryo transfers. Those achievements led to the point reproductology has reached today.

According to European Policy Audit on Fertility, in 2017 (*Fertility Statistics - Statistics Explained*, n.d.) one of six people worldwide experience one of the forms of infertility during their reproductive years. In Europe 25 million citizens have been diagnosed as being infertile. Despite the lack of updated comparative data on Infertility and ART on wider regions, in 2021, the follow-up analysis (*Fertility Statistics – Statistics Explained*, n.d.) showed that across EU countries surveyed, nine with the highest fertility rates (France – 1.84, Czech Republic – 1.83, Iceland – 1.82, Romania – 1.81, Ireland – 1.78, Montenegro – 1.76, Denmark – 1.72, Slovenia – 1.64 and Slovakia – 1.63) remained below the replacement rate of 2.1 live births per woman. During the last 5 years the necessity for ART continued to increase.

RPL is a disorder defined by the American Society of Reproductive medicine (ASRM) as the loss of two or more consecutive clinical pregnancies prior to 20 weeks of gestation (Bashiri et al. 2018). Statistical data show that around 5% of all women experience two consecutive pregnancy losses and 75% of these are implantation failures (Comins Boo et al. 2016). There is a lack of consensus on the definition of RIF, resulting from rapidly changing ART practices and methods of infertility treatment. Today, the definition of RIF is still not clearly specified. The operational definition of RIF used in this study is: “the persisting failure of pregnancy after several attempts of IVF and embryo transfer”(Margalioth et al. 2006). This definition applied only to patients undergoing ART. The problems with definition start when we try to clarify what are exact criteria to include or correctly identify implantation failure. Through the history of ART clinics, many factors have changed, like the number of transferred embryos, the stage in which they are transferred, the percentage of collective implantation failure and others.

Lukasz T Polanski in the year 2014 stressed that the definition of RIF given by most researchers in the last two decades has been based on one or two criteria or a combination of them, the first being the number of unsuccessful cycles alone, and the second being the number of embryos transferred, Some definitions included the type of embryo transferred (fresh or frozen), and the quality (usually highlighting that the transferred embryo must

be of good quality). There were also a few studies which mentioned the stage of embryo transfer. Polanski and his group concluded that “RIF should be defined as the absence of implantation itself defined by a negative serum hCG, 14 days after oocyte collection, following two consecutive cycles of IVF, ICSI or frozen embryo transfer, in which the cumulative number of embryos transferred was at least four cleavage-stage embryos, or at least two blastocysts, with all embryos being of good morphological quality and in the appropriate stage of development. He also pointed out that there are many differing definitions of RIF used in these studies and no justification was given for the use of any particular one (Polanski et al., 2014).

One of the last definitions of RIF was based on publications by PGD Consortium (a specialized group of ESHRE – European Society of Human Reproduction and Embryology) and publication by Coughlan et al. 2014. This definition includes all the important factors: RIF is a failure to achieve pregnancy after  $\geq 3$  embryo transfers (ET) of high-quality embryos in women  $< 40$  years, or transfers of  $\geq 10$  embryos in total in multiple transfers. Presence or absence of pregnancy is diagnosed by an ultrasound examination after the 5th week (Bender Atik et al. 2018; Coughlan et al. 2014; Thornhill et al. 2005).

According to ESHRE data, as of 2019, the rate of clinical pregnancies showing implantation success has increased to 34.6% and the rate of live births is up to 30.7% (Gliozheni et al. 2023), which clearly indicates that this problem is very relevant and there is still no exact answer about the reasons and tactics for patients with RIF.

As can be seen from the above literature review, however, there are two main reasons or their combination, such as embryo incompetence and endometrial incompetence, the success of implantation depends on these factors.

### **The aim, objectives, and hypotheses of the work**

The aim of this work is to assess how additional examination of the embryo before embryo transfer (with methods such as TLM, morphological examination of the embryo and the development of embryo division), as well as the genetic stability of healthy embryos can affect embryo implantation, including specifying the quality and competence of the endometrium affects the embryo implantations and the use of both methods affects IVF results. In order to achieve the set goal, the following tasks were set:

1. To evaluate the ability of time-lapse microscopy/imaging to improve the outcome of the IVF procedures.
2. To evaluate if endometrial receptivity array and invasive embryo preimplantation genetic testing for aneuploidies used alone or in combination can improve the outcome of IVF treatment in a group of patients with recurrent implantation failure.
3. To assess the utility of preimplantation genetic testing for aneuploidies in IVF procedures in specific group of patients – the carriers of balanced structural chromosomal rearrangements.

4. To clarify, standardize and operationally define non-invasive and invasive methods of embryo selection to decrease recurrent implantation failure rate and to promote the recommendations to other researchers and clinical professionals.

The following hypotheses were put forward:

1. Embryo selection is a vital step to increase the success of ART treatment. Selection of high implantation potency embryos using both methods- non-invasive Time-lapse microscopy/ imaging and invasive preimplantation genetic testing for aneuploidies, can improve the outcome of the treatment.
2. Embryonal and endometrial factors in combination are the likely cause of failed implantation. Applying the combination of methods (Time-lapse microscopy and invasive preimplantation genetic testing together with adjusting implantation window) can reduce the number of recurrent implantation failure and early pregnancy loss.
3. Applying the preimplantation genetic testing for aneuploidy to the patients with structural imbalances in their karyotypes and determining the percentage of normal embryos have a great effect on implantation.

### **The novelty of the work**

This study is both diverse and comprehensive. It pursues a multivariate approach, where the author in the first uses the combinations of these methods to investigate the main factors involved in recurrent implantation failure and early pregnancy loss.

The study determine which methods and factors are significant and which ones can be recommended in clinical practice for implementation in standard IVF work-up. It also identifies those methods that remain questionable. Based on the results of this study, a proposal is made to develop national guidelines for IVF treatment that will help the reproductive medical society in Latvia and abroad to improve results, of importance both for medical research and for demographical work. At the present time the standardized guidelines from ESHRE are not available.

### **Place of work performance**

The doctoral thesis was carried out at the reproduction clinic iVF Riga from January 15, 2014, to December 9, 2021.

### **Structure and scope of work**

The doctoral thesis is written in English, it consists of the thesis itself, written on 98 pages, 2 summaries (48 pages in Latvian and 49 pages in English) and 4 attached publications. The work contains 10 tables, 33 images and 7 appendices. The bibliography contains 245 literary sources, the oldest of which was published in 1979, and the newest in 2023.

**Work ethic**

All patients were informed about the purpose and methods of the study before inclusion and signed the informed consent. The study met the standards set by the Declaration of Helsinki. The Latvian Central Medical Ethics Committee has approved the study (Approval No. 01-29.1.2/1735).

# 1. LITERATURE REVIEW

## 1.1. Etiology of RIF

Successful embryo implantation is an interactive process between the blastocyst and the uterus. Synchronized development of embryos with uterine differentiation to a receptive state is necessary to facilitate pregnancy.

Implantation failure may occur even at early stages during the embryo attachment or migration.

As a result, there will be no objective evidence of a pregnancy, i.e., negative urine or blood pregnancy tests (human chorionic gonadotrophin, hCG). Alternatively, the embryo can migrate through the luminal surface of the endometrium and start to produce hCG, which may be detected in the blood or urine. But even at this stage, the process could be disrupted before the formation of an intrauterine gestational sac. Clinically this condition is called biochemical pregnancy.

The International Committee Monitoring Assisted Reproductive Technologies and the World Health Organization defined biochemical pregnancy as the detection of hCG in blood or urine without subsequent clinical signs of pregnancy (Maesawa et al. 2015).

Coughlan et al. (2014) noted that the term “implantation failure” can be understood in two ways. The first relates to women in whom there have never been quantifiable signs of implantation, such as increased levels of hCG. The second refers to women who have evidence of implantation (detectable hCG production) which did not proceed beyond the formation of a gestational sac, visible on the ultrasonography 2 weeks later (C. Coughlan et al. 2014). From that information, it can be concluded, that biochemical pregnancy falls into the category of implantation failure (Coulam and Roussev 2002).

From the clinical point of view as defined by the ASRM, implantation is considered successful when there is ultrasonographic evidence of an intrauterine gestational sac or by histopathological examination (Bashiri, Halper, and Orvieto 2018). Despite the acceptance of the general implantation definition, many authors still unsure about the optimal hCG level and in many publications, numbers vary between  $> 5$  to  $> 25$  mIU/ml (Zeadna et al. 2015; R. Yang et al. 2015; Bates and Ginsburg 2002).

With hundreds of potential causes to consider, etiology of implantation failure is still a complex challenge for reproductologists. To reduce the complexity, some researchers attempted to summarise all the putative variables and factors involved in RIF which are helpful, because they facilitate the proposal of new, beneficial solutions for the treatment and management of patients. For example, T. Timeva et al. (2014) has divided RIF causes into three main groups:

- 1) The first group is “Multifactorial RIF” having the subgroups of maternal (endocrine diseases such as thyroid, diabetes mellitus, thrombophilia), male, hormonal or metabolic disorders, infections and thrombophilias.
- 2) The second group is “Endometrial RIF”, which results from thin ( $\leq 6$  mm) endometrium, with or without variations in vascularity.
- 3) The third group is “Idiopathic RIF”, which is unexplained failure to achieve pregnancy after ET of good quality embryos, without any anatomical and histological changes in uterine cavity and endometrium, and without any other disturbances in the patient, the patient-partner and the embryos (Timeva, Shterev, and Kyurkchiev 2014).

Other authors have divided etiologic groups into decreased endometrial receptivity, defective embryonic development, and also multifactorial causes – which include endometriosis, hydrosalpinxes and suboptimal ovarian stimulation (Margalioth et al. 2006).

Setting aside the many inconsistencies and free creativity of the authors in attempts to classify causes, two main causes of implantation failure are salient in all of those publications.

They are Uterine and Embryonic factors.

Embryo factors usually include embryo quality and embryo aneuploidy.

Regarding the uterus, a lot of biological factors can alter the process of successful implantation, for example, uterine cavity abnormalities – Mullerian anomalies, fibroids, polyps and also tubal disorders. At the micro level, there are altered expressions of adhesive molecules, and specific immunological factors (Bashiri, Halper, and Orvieto 2018).

Other studies also analyze conception success in RIF patients. Statistical data from these are important as they provide reproductology professionals with better understanding of the problems they face, and help them to find better solutions for these problems. Bord et al. (2016) conducted a retrospective study of 184 patients in which they analyzed a group of young patients. They came to the conclusion that women with RIF that have a normal ovarian reserve and satisfactory ovarian response to superovulation should be encouraged to pursue IVF, even though the probability to conceive is relatively low, compared to the general IVF population (Bord et al. 2016).

## **1.2. Uterine factors**

### **1.2.1. Endometrial receptivity**

The endometrial environment plays a crucial role in embryo implantation and early placental development. There is a specific period of endometrial maturation during which the trophoblast of the blastocyst can attach to the endometrial epithelial cells and subsequently proceed to invade the endometrial stroma and the vasculature. This is called

endometrial receptivity (Lessey and Young 2019a). This complex process facilitates the embryo to attach normally, implant and continue to develop.

In the menstrual cycle, there is a short period of time during which endometrial receptivity is optimal and embryo implantation is possible. This period is called the “window of implantation” (WOI). Studies with donor embryos in humans have shown that this receptive period starts at day 6 post-ovulation and continues 4–5 days (or 3–6 days) within the secretory phase being days 20–24 of the cycle) in most healthy women. In certain pathologic conditions, this window is narrowed or shifted, which disrupts normal implantation, leading to infertility or pregnancy loss (Lessey and Young 2019b; Bergh and Navot 1992).

Endometrium is unique in its ability to block embryos from implanting, except during this narrow window of receptivity, where the endometrium undergoes morphological, cytoskeletal, biochemical, and genetic changes (Mahajan 2015). As shown in the mouse models (and as is common to other species), the “implantation window”, is regulated by ovarian steroid hormones. In the receptive endometrium, crucial hormones are progesterone (P4) and 17 $\beta$ -estradiol (E2) (Jian Tan et al. 1999; Paria, Huet-Hudson, and Dey 1993).

In the mice models, researchers observed, that on the first day of pregnancy E2, which is preovulatory secreted, a proliferation of uterine epithelial cells was induced. From the formed corpus luteum, P4 levels start to increase, and on day 3, stromal cell proliferation is initiated. Due to rising levels of P4 and E2 secretion, the pre-receptive uterus becomes receptive on the fourth day (Huet-Hudson YM, Andrews GK 1989; Matsumoto 2017; Talbi et al. 2006). Devroey et al., and Papanikolaou EG et al., also discussed the importance of these two hormones in humans and stated that there is increasing evidence that endometrial function in the stimulated cycle is adversely affected by levels of estrogen and premature secretion of progesterone (Devroey et al. 2004; Papanikolaou et al. 2010).

In one of the studies of 189 pregnancies, the risk of early pregnancy loss increased with later implantation ( $P < 0.001$ ). Among the 102 blastocysts that implanted by the ninth day, 13% ended with early loss. This proportion rose to 26% with implantation on day 10, and to 52% on day 11, then to 82% following day 11. The conclusion of this is that most successful human pregnancies and blastocyst implantations occur 8 to 10 days after ovulation. The risk of early pregnancy loss increases with later implantation (Wilcox, Baird, and Weinberg 1999).

### **1.2.2. Human Endometrium transcriptomics**

The transcriptome reflects the genes that are actively expressed at any given time within a specific cell population or tissue (Garrido-Gómez et al. 2013).

Human endometrial receptivity transcriptomes pose a rather complex issue and the quantity of crucial genes that play a main role in receptivity is still being debated. Despite the many publications on this, that revealed hundreds of simultaneously up- and down-regulated genes, the number of selected genes in all these publications, has varied widely.

Early research on mouse models began with the identification of a few genes of receptivity, such as leukemia inhibitory factor-LIF, Homeobox protein X3, genes of embryo response – Cyclooxygenase 2-COX 2; and decidualization – Interleukin-11 Receptor-IL-11R. Dominguez compared the gene expression patterns of 375 human cytokines, chemokines, and related factors in receptive and prereceptive human endometrium. They identified IGF-1 (an insulin-like growth factor-binding protein) as a new endometrial receptivity gene (Dominguez et al. 2003).

Then comparative studies started to appear. For example, Zhang et al., analyzed 3 past microarray studies (Talbi et al. 2006; Burney et al. 2007; Hever et al. 2007) and proposed 148 receptivity biomarkers (D. Zhang et al. 2012). Tapia et al. investigated 7 microarray studies that led to a proposed list of 61 receptivity biomarkers (Tapia et al. 2011).

A publication done by Riesewijk et al. in 2003, compared pre-receptive endometrium 2 days after the LH peak with receptive endometrium 7 days after the LH peak, by sequencing whole-genome expression profiles. Bhagwat et al. studied 179 genes that can be called Receptivity Associated Genes (RAGs). These Genes were compiled from a Human Gene Expression Endometrial Receptivity database (HGExERdb) (Bhagwat et al. 2013).

The HGExERdb database, created by Bhagwat et al. should be reported separately, as it contains information about 19,285 genes, in 312 datasets retrieved from 51 publications of human studies. It also provides information on the relative expression level of individual genes, when compared under different physiological conditions.

One of the most valuable meta-analyses of transcriptomic biomarkers for receptivity was done in 2016 and published by Altmäe in “Nature”. In this study 14 publications were selected for qualitative analysis from 57 eligible publications. In this meta-analysis, a robust rank aggregation (RRA) method was used (Kolde et al. 2012), using enrichment analysis to identify the meta-signature of highly presumed biomarkers of endometrial receptivity (Altmäe et al. 2017).

A statistically significant meta-signature of 52 up-regulated and five down-regulated genes was identified. The highest scores in receptive-phase endometrium reserved 5 up-regulated transcripts – GADD45A, SPP1, PAEP, GPX3 and MAOA. The five down-regulated transcripts, receptivity-associated genes were SFRP4, EDN3, OLFM1, CRABP2 and MMP7. SPP1 was detected in all transcriptome studies that were included in meta-analysis, together with ANXA4, CLDN4, DPP4, GPX3, MAOA, and PAEP (D. Zhang et al. 2012; Horcajadas, Pellicer, and Simón 2007; Tseng et al. 2010; Tapia et al. 2011; Bhagwat et al. 2013).

Interestingly, commercial ERA by Igenomix (Maria Ruiz-Alonso et al. 2013; Díaz-Gimeno et al. 2011) shares 47 genes in common with the identified 57 putative receptivity biomarkers.

Several other molecules are currently being studied as potential biomarkers for endometrial receptivity. They include mucin (MUC-1), trophinin, L-selectin, Wingless (Wnt) family members, etc. (Lessey 2011).

### 1.2.3. Endometrial Receptivity Array

Traditional histologic endometrial dating criteria defined by Noyes (Noyes, Hertig, and Rock 1975), have been a standard procedure over the last 60 years. Despite the historical importance, because of the questioned accuracy and functional relevance of this procedure, a scientific group from the Igenomix company has attempted to make an analyses to clinically improve detection of embryonic–endometrial desynchrony due to accelerated or delayed endometrial luteal-phase differentiation. In 2009, after more than 10 years of research for transcriptomic signatures of endometrial receptivity, an “Endometrial receptivity array” was designed, developed and patented (Díaz-Gimeno et al. 2011). Garrido Gomez from Igenomix developed a clinical algorithm with a computational predictor which tests results, based on the expression analysis of 248 genes (see Figure 1.2.3.). Expression profiling was accomplished by assaying mRNA levels with microarrays or next-generation sequencing technologies (RNA-seq), that allowed identification of the transcriptomic signature of the window of implantation (WOI) (Hung and Weng 2017).

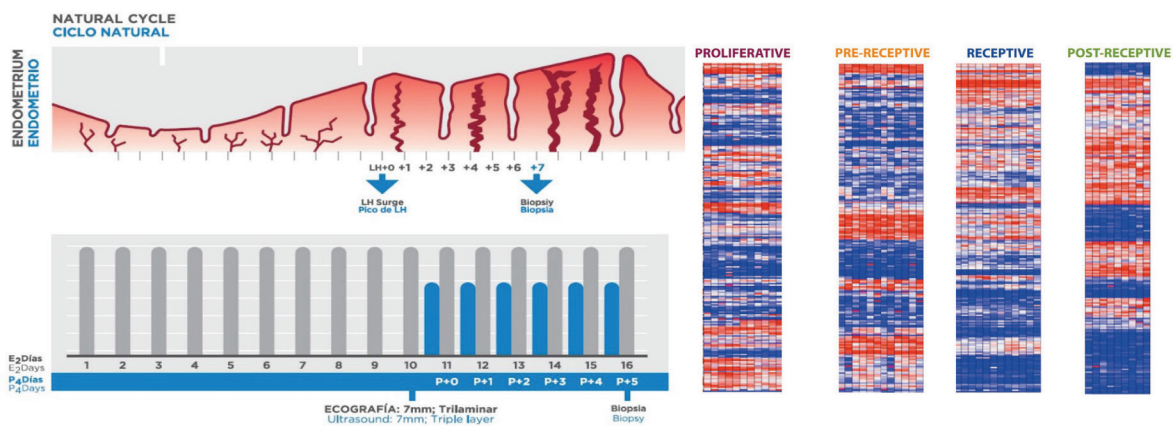


Figure 1.2.3. Igenomix endometrial receptivity analysis (Maria Ruiz-Alonso et al. 2013; Díaz-Gimeno et al. 2011)

The reproducibility of ERA results depends on two points. First, the test must be performed repeating the exact same conditions that match embryo transfer cycle of a particular patient. It should always be hormone replacement therapy cycles (HRT) or natural cycles. Controlled ovarian stimulation cycles are not allowed.

The second point, is the exact time in which endometrial biopsy must be done. In HRT cycle first endometrial biopsy should be performed after 5 full days of progesterone administration (or 120 hours of progesterone administration). In the natural cycle biopsy should be done at 7<sup>th</sup> day of hCG triggering (or after 168 hours of hCG administration). Since the ERA checks the endometrium at the moment of implantation, in the case of day-3 embryo transfers, instructions for biopsy are the same (P+5 or hCG+7).

After sequencing the mRNA from an endometrial biopsy, the patient will receive her answer about the endometrium receptivity – the endometrium can be receptive or non-receptive (pre-receptive, post-receptive, or proliferative).

If the endometrium is receptive, the blastocyst should be transferred at P+5, or at P+3 in the case of day-3 embryo. In 10% of cases the second biopsy is needed in the specific day designated by the first ERA test.

Detecting specific point in time of endometrial cycle in which the window of implantation starts, allows doctors to perform personalized embryo transfer (pET), after which each patient receives personal recommendations that are based on her specific endometrial profile. Moreover, the term personalized embryo transfer and the procedure itself started to exist because of ERA (Garrido-Gómez et al. 2013).

The accuracy and consistency of the ERA test had been demonstrated in multiple trials, that showed that the ERA test is a reliable and reproducible method for determination of the exact time of the WOI that can be used with better results in comparison to histological dating of endometrial receptivity (Díaz-Gimeno et al. 2013).

A pilot study was conducted by Igenomix of 17 RIF patients, who underwent oocyte donation and routine embryo transfer (ET) but were then treated with pET after the personalized diagnosis of their WOI. This study demonstrated that embryo-endometrial synchronization within an optimal time-frame increases the chances of success in an assisted reproductive treatment (M Ruiz-Alonso et al. n.d.) . The same group showed that patients with at least three previous failed ovum donation cycles, and IVF patients aged <40 years, with at least three failed IVF cycles with a receptive ERA diagnosis resulted in a 62.8% pregnancy rate (Garrido-Gómez et al. 2013).

Other groups also showed increased probability of having successful implantations and pregnancies following pET in accordance with the ERA result. Results in the Indian population revealed an endometrial factor in 27.5% RIF patients, which was significantly greater than the non-RIF group 15% ( $P = 0.04$ ) (Mahajan 2015). In another study 93% of patients achieved pregnancy after ERA in their next ET. All 8 patients whose ET cycles were adjusted due to ERA results achieved pregnancy in their next cycle, as did 6/7 with receptive ERA and unadjusted cycles. 71% of pregnancies resulted in live births or are currently ongoing (Pasternak, Schattman, and Rosenwaks 2018). Taguchi et al. after recruiting 506 patients of the 550 planned, showed, that the implantation in the DET (deferred embryo transfer) group rate was 8.7% and 19.0% in the pET group (Taguchi et al. 2018). It is interesting to note that one study showed that the transcriptomic profile of the mid-secretory phase endometrium does not change substantially between cycles, even during relatively long periods of the women's reproductive life (Díaz-Gimeno et al. 2013).

Another Japanese group – Hashimoto et al. also showed that there is no statistical significance in pregnancy rate between receptive and non-receptive groups of patients after performed pET (Hashimoto et al. 2017) but there is certainly increased percentage of

non-receptive ERA test in women with RIF with comparison group patients undergoing IVF (Maria Ruiz-Alonso et al. 2013; J. Tan et al. 2018; Mahajan 2015).

#### **1.2.4. Endometrial tests for specific conditions**

Regarding tests for women with specific conditions, a new non-invasive ReceptivaDx test for detecting endometriosis had been developed by team from the CiceroDx company from Greenville, South Carolina.

Endometriosis is an inflammatory condition that is associated with progesterone (P4) resistance and cell proliferation. Unlike ERA, the recently developed ReceptivaDx identifies endometrial receptivity defects associated with P resistance, usually due to endometriosis. This test is based on the finding of overexpression of endometrial human proto-oncogene BCL6 that is responsible for increase in cell proliferation through the inhibited expression of p53 and p300 (Evans-Hoeker et al. 2016; Phan and Dalla-Favera 2004) As mice experiments have shown, the BCL6 protein together with the histone deacetylase protein SIRT1, bind to and suppress the promoter of GLI1, a critical mediator of progesterone action. Data suggest, that SIRT1 and BCL6 are coordinately over-expressed in eutopic endometrium of women with endometriosis and likely participate in the pathogenesis of certain condition (Yoo et al. 2017).

A clinical study was conducted in 2017, which suggested that BCL6 high expression is a strong predictor of poor reproductive outcomes in IVF cycles in women with UI. The live birth rate was 58.8% vs 11.5% in women with low BCL6 expression vs high BCL6 expression (Almquist et al. 2017). The successful treatment for these infertile women could be GnRH agonist suppression or surgery for endometriosis before IVF (Liles et al. 2019).

#### **1.2.5. Uterine peristalsis**

Rhythmic peristaltic contractions of the uterine muscular wall guide the movement of spermatozoa during follicular phase. During that phase uterine peristalsis, and thus directed sperm transport, are controlled systemically by the estradiol, which is secreted from the dominant follicle, enhanced by oxytocin and goes into the utero-ovarian countercurrent system (Wildt et al. 1998).

However, in the luteal phase, uterine peristaltic activity decreases in frequency and intensity, mainly because of rising progesterone secretion, which inhibits the uterine contractility, but contractions did not completely disappear (Lyons et al. 1991; Leyendecker et al. 1996; Fanchin et al. 2001). The human blastocyst, after getting into the uterine cavity around 4 days following ovulation, remains in the high fundal part of the uterus without having close contact to the endometrium for about 2 days (Croxatto 2002). This orientation of the blastocyst is not random, and is also present in many mammals and remains highly specific in each species (Wiebold and Becker 1987; Louton, v. Domarus, and Hartmann 1988).

Uterine peristalsis during the luteal phase facilitates the fundal implantation of the blastocyst predominantly ipsilateral to the site of the dominant ovarian structure. There is a theory, that suggests this specific implantation area serves as the defense against the infiltration and inflammation of the upper genital tract, and thus prevents the implanted embryo from degradation (Kunz et al. 2006).

If there is a timing failure in contraction patterns in the late follicular phase, or through mid-luteal hypercontractility, it might interfere with fertility (Kuijsters et al. 2017). To measure uterine contractions in research trials, mainly intrauterine pressure is measured by a catheter placed in the uterine cavity with visual assessment by transvaginal ultrasound (TVUS). Also, complex techniques such as hysterosalpingo-radionuclide scintigraphy or magnetic resonance imaging-based techniques have been tested, but these methods are not that popular because of their expense, the time-consumption involved, and inability to measure the amplitude of contractions.

In various studies based on TVUS it is shown that in controlled ovarian stimulation (COS) cycles uterine contractions are higher and more prolonged in the early luteal phase compared to natural cycles with an established relaxation 2 days later (Ayoubi et al. 2003; Lin Zhu, Li, and Xu 2012). Evidence indicates that reasons for increased contractions may lay in the supraphysiological levels of steroids in COS (Lin Zhu, Li, and Xu 2012; Kolde et al. 2012) and mechanical stimulation, such as touching the fundus, inducing bleeding, or usage of rigid catheters or tenaculum (Altmäe et al. 2017), during a difficult embryo transfer.

The higher the frequency ( $\geq 3$  UC/min) of UP around the time of embryo transfer, the poorer reproductive outcome will be with fresh or frozen embryos (Fanchin, Righini, et al. 1998; L. Zhu et al. 2014; Chung et al. 2017).

#### **1.2.6. Uterine Microbiome**

Joshua Lederberg in 2001 defined the human microbiome as the totality of microorganisms and their collective genetic material present in or on the human body (Mor, Driggers, and Segars 2015).

Due to the fact that a lot of new information had emerged from the studies of the gut microbiome, and how it can affect other systems organs, especially the impact of gut bacteria on such conditions as obesity (R. Liu et al. 2017) and interaction between gut and the brain. Due to the increased interest in bacterial topics in recent years the interest in the effect of uterine microbiome on women reproductive health and implantation also started to grow.

Until recently, the medical community assumed that the uterus is a perfectly sterile place. The explanation for that may be that most of the past data on the reproductive tract in the past were based on vaginal samples.

Normal microbiome in healthy women primarily consists of hydrogen peroxide-producing species – *Lactobacilli* species (Ravel et al. 2011). From an ecological point of view, communities that are less stable can be more susceptible to invasion by pathogenic organisms

(Dunstan and Johnson 2006). This instability and many variations of shifts in bacterial communities may depend upon factors such as age, sexual activity and changes in hormonal milieu (Gajer et al. 2012).

For example, during infancy the vaginal flora consists of aerobic and anaerobic bacterial populations, including *Streptococcus* and *Staphylococcus*, *Prevotella* and *Enterobacteria* species (Huang et al. 2014). When puberty comes, the estrogen production causes glycogen to rise and pH to decrease with subsequent domination by *Lactobacilli* species.

Identifying bacterial communities in the reproductive tract was always done by the culturing method. But this method gave a much less accurate insight on the microbiome and was replaced by the microbiome pyrosequencing of 16S rDNA that is capable of identifying taxa that rarely grow in cultured media. Research using this method, has also shown, that the microbiomes of all reproductive organs (vagina, cervix, Fallopian tubes, and ovaries) are significantly correlated (Walther-António et al. 2016).

Ravel et al. in his study implied, that instead of a single most frequent microbiome, there are multiple core microbiomes, and they can be distinguished on the basis of two criteria: (i) whether the constituent communities are dominated by *Lactobacillus*, and (ii) by the presence of particular *Lactobacillus* species.

They classified the vaginal microbiome into five groups or community state types (CST). 73% of women demonstrated vaginal microbiota dominated by variety of *Lactobacillus* species, corresponding to group I, II, III and V. A smaller proportion of women in group IV with the highest median pH ( $5.3 \pm 0.6$ ) had a lower percentage of *Lactobacilli* and dominance of anaerobic bacteria including *Aerococcus*, *Atopobium*, *Dialister*, *Gardnerella*, *Megasphaera*, *Prevotella* and *Sneathia*. Communities in group I were dominated by *L. crispatus* and had the lowest median pH ( $4.0 \pm 0.3$ ). Whereas groups II, III, and V were dominated by *L. gasseri*, *L. iners*, and *L. jensenii*, respectively and had slightly higher pH, ranging from 4.4 (group III) to 5.0 (group II). This might indicate, that communities dominated by species of *Lactobacillus* other than *L. crispatus* may not produce as much lactic acid as those of group I or might have different buffering capabilities (Ravel et al. 2011).

Studies also indicate that lower diversity in the microbiome show better outcomes. It is logical, given the fact that so called 'normal state' has prevalence of *Lactobacilli* taxa and has low diversity (Hyman et al. 2012). Between gravid and nongravid vaginal microbiome, several studies found differences in stability and diversity (Prince et al. n.d.; 2014; Aagaard et al. 2012). It seems that gravid vaginal microbiome tends to be more stable and less diverse through all periods of pregnancy (Romero, Hassan, Gajer, Tarca, Fadrosh, Nikita et al. 2014) with major changes such as increases in the dominance of four *Lactobacillus spp.* (*L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. vaginalis*) and a decrease in the amount of anaerobic species (Romero, Hassan, Gajer, Tarca, Fadrosh, Bieda, et al. 2014).

Regarding endometrial microbiome of women with RIF, RM or both, in 19 patients investigated, *Bacteroides* and *Proteobacteria phyla* were the most frequent. But one patient

had microbiota dominated by *L. crispatus* and another had polymicrobial community including *Prevotella*, *Mobiluncus curtisii*, *Dialister*, *Porphyromonas*, *Atopobium vaginae* and *Peptostreptococcus phylotypes* (Verstraelen et al. 2016). Meta-analysis done in 2013 also proves the point of previous results by pointing out that dysbiotic shifts are more frequent in subfertile population. (van Oostrum et al. 2013). Many other publications have shown that *Enterobacteriaceae*, *Streptococcus*, *Staphylococcus*, *Escherichia coli* and Gram-negative bacteria, from the transfer catheter tip have negative effect on implantation and pregnancy rates (Egbase et al. 1996; Fanchin, Harmas, et al. 1998; Egbase et al. 1999; Moore et al. 2000; Selman et al. 2007).

Despite that, one study demonstrated that there is no significant bacterial difference between groups of women with ongoing pregnancies and those without. Also, as expected, *Flavobacterium* and *Lactobacillus* represented the majority of the bacterium seen in both groups (Franasiak et al. 2016).

Shifts in the species bacteria were also found between the cancer and hyperplasia cases. Several taxa were found to be significantly enriched in samples belonging to the endometrial cancer cohort: *Firmicutes*, *Spirochaetes*, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* (Walther-António et al. 2016).

### **1.3. Immunologic factors**

An imbalance in uterine immune cells and their secreted cytokines has been suggested as a potential reason for RIF in healthy women who have transferred high-quality euploid embryos. This suggests that the uterine environment may not be conducive to successful implantation, despite the embryo being of good quality.

It is believed that the presence of certain immune cells in the uterus is necessary for successful implantation. However, an imbalance in these cells and their secreted cytokines can result in an environment that is hostile to the embryo. This can lead to the failure of implantation, even when the embryo is of good quality.

#### **1.3.1. Lymphocytes Th1/Th2 Profile and Th17**

T-lymphocytes are types of immune cells that originate from bone marrow and develop into specific classes in the thymus gland. One of the populations of lymphocytes are T-helpers, which secrete CD4 and divide it into subpopulations. Subpopulations like Th1 and Th2 are characterized by cytokines that secrete and also have a cellular immunity function. Th1 subpopulation in general tend to be proinflammatory and secrete such cytokines as interferon- $\gamma$  to, TNF- $\alpha$  and interleukins (ILs) 1, 2, 12, 15 and 18. Th2 subpopulations on the other hand, cancel out Th1 subpopulations and serve as anti-inflammatory agents that

secrete ILs 4, 5, 6, 10, 13 and also the granulocyte-macrophage colony-stimulating factor (Wegmann et al. 1993; Kalu et al. 2008).

During pregnancy, the milieu of Th cells in normal circumstances shifts towards the Th2 subpopulation. This happens due to the rise in Progesterone, which inhibits the secretion of Th1 cytokines. In immunologic profiles the levels of IL-4 and IL-6 go up and levels of IL-2, IL-12 and interferon- $\gamma$  go down. When the child is born it also has a Th2-dominant cytokine milieu, which quickly changes because of microbial colonization (Raghupathy 1997; Del Prete, Maggi, and Romagnani 1994). If the milieu reverses towards Th1 cell dominance it could impact on the pregnancy by causing a Cytokine-triggered abortion due to thrombotic/inflammatory processes in the maternal uteroplacental blood vessels.

In 1999, an experiment on pregnant mice demonstrated that the injection of Th1 cytokines, specifically TNF- $\alpha$  or IFN- $\gamma$ , led to abortion, while the administration of TNF- $\alpha$  antagonists reduced fetal loss (Clark et al. 1998). However, detecting this specific problem in women is not easy due to several factors.

During the peri-implantation period, the prevalence of Th1 cells in the peripheral blood is normal, and no laboratory differences can be detected between healthy women and those who experience cytokine shift during pregnancy. Additionally, there is no correlation between cytokine expression and serum hormone levels. Therefore, screening tests cannot predict imbalances in future pregnancies (N. Macklon 2017; Lim et al. 2000).

Many studies that compare the pro- and-anti-inflammatory cytokine balance were performed by analyzing women having normal pregnancies and women who have pregnancy failures. In 2015 a study by Liang PY et al. compared the ratios of pro and anti-inflammatory cytokines IFN- $\gamma$ /IL-4, IFN- $\gamma$ /IL-10, IFN- $\gamma$ /TGF- $\beta$ 1, TNF- $\alpha$ /TGF- $\beta$ 1 in the RIF patients and women having successful pregnancies. They discovered a shift towards pro-inflammation in the RIF group (Liang et al. 2015). Another group also indicated, that without any difference in gestational age, the pro-inflammatory cytokine levels, such as TNF- $\alpha$ , IFN- $\gamma$ , IL-6 and IL-10 are significantly higher in euploid miscarriages, than in healthy pregnant women at 10–14 weeks of pregnancy (Calleja-Agius et al. 2012).

As the role of relevance of Th1/Th2 population in fertility is supported by more and more evidence, the role of third population of cells like Th17 also received attention.

Th17 cells are types of T-helper lymphocytes that are characterized by pro-inflammatory cytokine IL-17 production. Signals such as the Transforming growth factor beta (TGF- $\beta$ ), interleukin 6 (IL-6), interleukin 21 (IL-21) and interleukin 23 (IL-23) cause Th17 formation in mice and humans while at the same time these signals inhibit regulatory T cell differentiation (Ivanov et al. 2006; Hartigan-O'Connor et al. 2011). IL-17 is a cytokine which promotes the expression of various mediators of inflammation while playing an important role in maintaining pathogen free mucosal surfaces. The loss of Th17 cell populations on mucosal surfaces is related to chronic inflammation and microbial translocation.

In a study done by Nakashima et al. it was demonstrated that the levels of Th17 cells in peripheral blood lymphocytes do not change in women with normal pregnancy (Nakashima et al. 2010) but another study showed that the proportion of Th17 cells in the peripheral blood and decidua significantly increases in unexplained recurrent spontaneous abortion (RSA) patients (Wang et al. 2010; Lee et al. 2011).

### **1.3.2. TNF-a**

Overproduction of TNF-a could have toxic effect on pregnancy despite its necessity to assure endometrial receptivity.

By analyzing cytokine profiles of 210 women undergoing IVF with the endometrial secretion analysis technique, higher TNF-a and IL-1 $\beta$  levels have been detected in patients having a previous history of implantation failure who later achieved clinical pregnancy (Boomsma et al. 2009). This could emphasize and prioritize that proinflammatory cytokines are also needed for successful transfer, for pregnancy to occur and for sustaining immunologic balance in the uterus.

### **1.3.3. IL-11**

In 1998 a study on mice that lacked IL-11 receptors had fertility problems that occurred in the post-implantation response to the implanting blastocyst (Robb et al. 1998). IL-11 have anti-inflammatory properties and could be critical for the regulation of trophoblast invasion, suggesting its involvement in placentation (Evdokia Dimitriadis et al. 2005).

### **1.3.4. Regulatory T Cells**

Regulatory T cells, formally known as suppressor T cells or Tregs express the CD4, FOXP3, and CD25 biomarkers and develop from the same lineage as T-helper cells (Curiel 2007). These cells are best known for their function to generally suppress Th1- and Th17-mediated immunity. In other words, they suppress autoreactive and alloreactive immune responses, thereby preventing autoimmune diseases and allograft rejection (Sakaguchi et al. 1995). These cells also modulate the immune system and downregulate induction and proliferation of effector T cells (CD8+).

Tregs are currently one of the most studied immune cell populations due to their involvement in the regulation of local maternal tolerance towards the fetus. Schumacher and Zenclussen (2014) say, that their concentration expands within 2 days of conception in normal human pregnancy.

From phenotypical and functional observations of lymphoid cell populations in pregnant uterus mucosa and decidua in early normal pregnancies, one of the mechanisms by which a maternal immunologic reaction against the semi-allogenic fetus is prevented, comes from local selective inhibition of surface expression of the T cell receptor /CD3 complex (Mincheva-Nilsson, Hammarström, and Hammarström 1992). Also expression of IL-10 and

TGF- $\beta$  by Tregs and its suppression potential is significantly reduced in patients with unexplained recurrent spontaneous miscarriages when compared with fertile patients (Bao et al. 2011). Tregs also regulate the CD8+ T cell differentiation by significantly reducing the expression of perforin and granzyme B in the decidua compared to peripheral blood EM CD8+ T cells, which may also play a crucial role in establishing the maternal immune tolerance cells (Tamara Tilburgs et al. 2010).

Studies observed Treg cell increase during the first and second trimester in peripheral blood compared to controls (Heikkinen et al. 2004) and even higher concentrations of these cells in the decidua (T. Tilburgs et al. 2006).

An unusually high proportion of CD4+, CD25+ regulatory T cells were found in the uterus of pregnant mice. This finding might indicate that suppressor T cells are actively recruited or their population is expanded by the organism having the purpose of sustaining normal pregnancy and depletion of CD4+, CD25+ Tregs leads to gestation failure (Aluvihare, Kallikourdis, and Betz 2004). A study in 2011 showed the decrease in CD4+, FOXP3+ lymphocytes in non-pregnant women with idiopathic recurrent pregnancy loss in comparison to age-matched controls (Lee et al. 2011).

Kim DJ et al. (2014) showed that patients with recurrent miscarriages and cellular immune imbalance could be treated with intravenous Immunoglobulin G, which increases the quantity of Th17 and FOXP3+ Treg cells.

### **1.3.5. Natural Killers (NK) and Uterine NK (uNK)**

In most cases parents who want to have a child are not tissue-matched and, if they want their pregnancy to be successful, the mother's immune system has to be suppressed. NK cells seem to play an important role in this process (Lash, Robson, and Bulmer 2010).

Natural killer cells, or NK cells, are a type of cytotoxic lymphocytes. In the uterus there are a special type of Natural Killers that consists of two main subsets – CD56+, CD16+ cells (10% of uNK) with dim phenotype and CD56+, CD16+ cells (90% of uNK) with bright phenotype (Lachapelle et al. 1996). The bright type, the norm, should be the dominant cell subset. They have low cytotoxic ability, are potent at cytokine secretion and have a slightly different receptor profile than the peripheral CD56 bright NK cells (Lash, Robson, and Bulmer 2010).

IL-15 and IL-18 are involved in the maturation of uNK. As the potential markers of uNK activity, it has been shown that levels of IL-15 and IL-18 in patients having implantation failure are positively correlated with uNK levels (Lédée et al. 2011).

uNK cells increase in number and account for about 70% to 83.2% of the uterine leukocytes during the mid- to late luteal phase and first trimester of pregnancy (Nishikawa et al. 1991; Flynn et al. 2000). In some studies, it has been established that uterine NK cells play a role in decidual vascularization. The elevation of uNK CD56 cells density could contribute to increased angiogenesis in the pre-implantation period, leading to reduced uterine

artery resistance to blood flow, endometrial oedema and as a result, to implantation failure (Tuckerman et al. 2010; Quenby et al. 2008). Abnormal decidual vascularization and the increase in angiogenic factors contributes to the development of miscarriages and implantation failures.

Another study by Fukui et al. (2017) revealed the significant lowering of the uterine NKp46+ cell population in both women with RPL and in pregnant women with a history of RPL. The percentages of tumor necrosis factor- $\alpha$ - and/or interferon- $\gamma$ -producing uterine endometrial NK cells were significantly lower in women with RPL compared with controls (Fukui et al. 2017).

The systematic review and meta-analyses of 22 studies conducted in 2014 was more focused on RM but included studies that analyzed both peripheral NK and uNK cells versus fertile women. These showed no significant difference between the two groups and no significant difference in studies that analyzed only uNK cells in women with RM compared with controls (SMD 0.40; 95% CI -1.24, 2.04;  $P = 0.63$  (Seshadri and Sunkara 2014).

### **1.3.6. Autoantibodies**

Antiphospholipid syndrome (APS) is an autoimmune condition that is associated with thrombosis and morbidity in pregnancy (T. da S. Santos et al. 2017). The pathogenic pathway by which whose conditions occur are not yet fully understood. It is known, however, that mechanisms may be heterogeneous. The main Antiphospholipid Antibodies (aPLs) found in APS are lupus anticoagulant (LA), anticardiolipin antibodies (aCLs) and anti- $\beta$ 2glycoprotein I antibodies (a $\beta$ 2GPI) (Kutteh and Hinote 2014).

The international Sapporo criteria that were created in 1999 and revised in 2006 emphasized main diagnostic criteria for APS diagnosis. APS is diagnosed if at least one of three antiphospholipid antibodies is detected on two or more occasions during a 12-week interval and associated with a clinical condition, such as thrombosis or morbidity in pregnancy. Morbidity in pregnancy includes preterm delivery due to eclampsia, preeclampsia, unexplained stillbirths at  $\geq 10$  weeks of gestation, or placental insufficiency, and three or more consecutive miscarriages (Miyakis et al. 2006).

Recurrent early pregnancy loss (EPL) is one of the antiphospholipid syndrome (APS) obstetrical features having an incidence of aPLs in patients with recurrent EPL between 15% and 20% (Kutteh and Hinote 2014).

The most decisive meta-analysis regarding recurrent miscarriages was done by T. da S. Santos et al., in 2017. In seven of nine studies they analyzed a relationship between aPLs and recurrent EPL was found. Two other studies found no significant correlation between these two factors in their analyses.

The authors, however, noted that one of them did not investigate the relationship between aPLs and recurrent EPL while the other investigated the relationship between antiphosphatidylethanolamine and EPL in aPLs. They concluded that the meta-analysis demonstrated a

significant association between aPLs and/or APS with recurrent EPL, with an overall combined OR of 0.279 (95% CI: 0.212–0.366) (T. da S. Santos et al. 2017).

As regards recurrent implantation failure, there is little scientific data and it has to be studied further, as these specific antibodies could be present in patients with recurrent implantation failure.

## **1.4. Altered expression of associated molecules**

Several other molecules have been identified that could play a role in endometrial receptivity and implantation. These include prostaglandins (PG) and cellular adhesion molecules (CAMs), as well as LIF and apolipoprotein A-I.

Prostaglandins and CAMs are involved in the inflammatory response and are believed to play a role in the recruitment and adhesion of immune cells to the endometrium. LIF, a cytokine-like protein, is known to play a key role in implantation by promoting embryo adhesion and invasion. Apolipoprotein A-I has also been shown to be involved in embryo implantation, although the mechanisms involved are not yet fully understood.

### **1.4.1. Prostaglandins**

Prostaglandins are group of physiologically active lipid compounds called eicosanoids that derive from arachidonic acid. Their function is to sustain homeostasis, and to mediate pathogenic mechanisms by stimulating a reaction in one tissue (inflammatory response) while inhibiting the same reaction in another tissue. These reactions are determined by the type of receptor the prostaglandin binds to. Prostaglandins are synthesized from Arachidonic acid by the action of cyclooxygenase (COX) isoenzymes (Ricciotti and Fitzgerald 2011).

Decidual cells also secrete prostaglandins that later interacts in complex reactions with other cytokines, growth factors and hormones like progesterone, prolactin, relaxin.

Achache et al. (2010) suggested that decreased prostaglandin synthesis might be a key factor in altered endometrial receptivity, and therefore play a role in implantation failure. Levels of cytosolic phospholipase A2 (cPLA2a) and COX-2, measured by PCR and Western blot tests, were found to be decreased in patients having RIF compared with the fertile group. He suggested, that implantation may be detrimental when both enzymes are missing. Interestingly, secretory phospholipase A2 (sPLA2-IIA) was overexpressed in response to the decreased function and presence of these enzymes. This overexpression is most likely a form of compensation to maintain the release of arachidonic acid (Achache et al. 2010).

Other data on RIF patients show, that they express defective endometrial PG on days 21–24 of the cycle. It has been estimated at both mRNA and protein level, that previously

mentioned enzymes, such as cyclooxygenase-2 (COX-2), secretory phospholipase A2 group: IIA, V, and IB (sPLA2-IIA, sPLA2-V, sPLA2-IB) and other molecules like glypican-1, PG-E synthase, PG-E receptors, and lysophosphatidic acid receptor 3 (LPA3), play an important role in PG synthesis and are found in very low concentrations in 85% of RIF patients (Achache et al. 2010).

#### **1.4.2. HOX-a and E-cadherin**

Certain types of transcription factors, such as HOXA-10 and E-Cadherin might also play important roles in the implantation process. HOXA-10 and E-Cadherin are localized in the glandular epithelium cells of endometrium. Specifically, HOXA-10 is in the nuclei of stroma cells and E-Cadherin is in the cytoplasm of glandular epithelium cells.

Regarding HOXA-10 and E-cadherin expression, a positive correlation was established between significantly reduced levels of HOXA-10 and E-cadherin expression in women having a history of recurrent implantation failure and RM when compared with the control group (Y. Yang et al. 2017).

#### **1.4.3. Leukemia inhibitory factor**

LIF or leukemia inhibitory factor was primarily identified as a macrophage differentiation inducer of the myeloid leukemia M1 cell line (Hilton, Nicola, and Metcalf 1988). Later LIF started to be considered as the first cytokine shown to play a critical role in mice blastocyst development and implantation (Stewart et al. 1992). The other functions of LIF included proliferation, differentiation and cell survival (Metcalf 1992). LIFs mRNA is expressed during days 18–28 of the menstrual cycle in the endometrium of fertile women which suggests it has its role in human implantation (E. Dimitriadis, Salamonsen, and Robb 2000). LIF expression is regulated by Progesterone. This is also proven by the fact, that treating women with the progesterone receptor antagonist, immediately after ovulation, reduces immunoreactivity of LIF at the expected time of implantation (Gemzell Danielsson, Swahn, and Bygdeman 1997).

Evidence for the role of LIF in human fertility was described by Laird and Ledee-Bataille. They showed that low concentrations of LIF in the uterine flushing fluid at day 26 may be a good predictor of successful implantation, while at the same time endometrial explants from infertile women and women with recurrent miscarriages secrete less LIF than those from fertile women (Laird et al. 1997; Lédée-Bataille et al. 2002; Evdokia Dimitriadis et al. 2005; Guzeloglu-Kayisli, Kayisli, and Taylor 2009).

#### **1.4.4. Apolipoprotein A-I**

From the proteomic analysis of endometrial biopsies collected between LH+5 to LH+10 a new molecule was found, that could be a potential predictor of endometriosis and RIF patients.

Apolipoprotein A-I (Apo A-I) was identified as an anti-implantation protein, that is secreted by differentiating endometrium. It seems that higher expression of Apo A-I can be found in ectopic secretory endometrium in patients having endometriosis. This statement implies that dysregulation of certain molecule secretions might be a significant factor in pathogenesis of endometriosis and a crucial point for RIF (Brosens et al. 2010). More data on this new “fingerprint” is needed to be able to apply it to the future diagnoses.

## 1.5. Embryonal factors

In cases of RIF, the investigation of embryo quality becomes crucial for patients undergoing IVF treatment. According to the Istanbul Consensus recommendations, evaluating the morphokinetic properties of embryos using the Embryo grade system is essential for selecting high-quality embryos. Additionally, testing for embryo ploidy status is necessary.

By evaluating morphokinetic properties, embryologists can observe the development of embryos in real-time and assess key parameters such as cell division rates, fragmentation, and blastocyst formation. This information can be used to determine which embryos are of the highest quality and most likely to result in successful implantation.

Testing for embryo ploidy status involves assessing the number of chromosomes present in the embryo. Embryos with an abnormal number of chromosomes (aneuploid) are less likely to result in successful implantation and may lead to recurrent pregnancy loss (Balaban et al. 2011).

### 1.5.1. Embryo quality

Blastocysts (day-5 embryos) are graded according to expansion and quality of the inner cell mass and trophectoderm. Other criteria include blastomeres being of equal size and regular in distribution, distribution of cytoplasm without granularity and less than 10% fragmentation (Cutting et al. 2008). A good-quality embryo needs to have the correct number of cells corresponding to the day of its development. The present stage of embryo evaluation, including the description of all its related pathological elements and the development of time-lapse imaging took over 4 decades of research.

The Gardner grading system, which is one of the most commonly used methods for embryo grading, was first described by Gardner and Schoolcraft in 1999 and is still the most widely used, but there are still several different grading systems (Gardner, David K.; Schoolcraft 1999; Gardner and Sakkas 2003; Lai et al. 2020).

The grading system involves the assessment of three main quality parameters: the degree of blastomere (embryonic cell) expansion, Inner Cell Mass (ICM) and Trophectoderm (TE).

**Blastomere expansion** is assessed on a scale of 1–6, where:

- Grade 1: All blastomeres are tightly packed and the embryo has a smooth appearance.

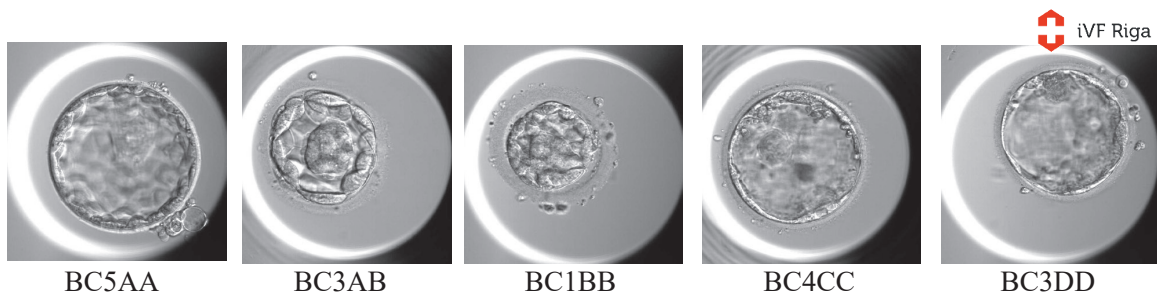
- Grade 2: All blastomeres are still tightly packed, but the embryo has a slightly irregular appearance.
- Grade 3: The embryo has started to compact, but some blastomeres are still visible as individual cells.
- Grade 4: The embryo is starting to expand and blastomeres are no longer tightly packed.
- Grade 5: Hatching blastocyst.
- Grade 6: Hatched blastocyst.

**ICM** – the cells inside the embryo that will form into a fetus is assessed by a letter grade designating the quantity and quality:

- A: many cells tightly compacted;
- B: several cells loosely adhered;
- C: very few cells;
- D: no cells or degenerate or necrotic cells.

**TE** – the cells lining the embryo that will become the placenta assessed by second a second letter grade designating the quantity and quality:

- A: continuous layer of small identical cells;
- B: noncontinuous layer with fewer cells;
- C: noncontinuous layer with few small cells and large cells;
- D: sparse distribution of large or flat or degenerate cells.



*Figure 1.5.1. An example of blastocysts (BC) of different grades.*  
Images from iVF Riga archive

An example (Figure 1.5.1.) of an embryo with a grade of BC3AB would indicate that it has started to compact, with some blastomeres still visible as individual cells, and has moderate fragmentation.

Embryo grading is an important tool used to select the best embryos for transfer in IVF cycles. However, it is important to note that while embryo grading can provide valuable information about the developmental potential of an embryo, it is not a guarantee of success, and other factors such as embryo ploidy status and endometrial receptivity should also be considered.

As a start morphological evaluation of the embryo was the only criterion for estimating developmental potential of the embryo and predicting the probability of achieving pregnancy (Baczkowski, Kurzawa, and Głabowski 2004). The history behind the science of current time-lapse embryo imaging goes back to 1997 when Payne et al. documented the use of time-lapse video cinematography to observe the initial events in 38 normally fertilized human oocytes and compare these events with day 3 embryo development.

Based on Paynes work, ten years later, Mio and Maeda (Mio and Maeda 2008) extended the analysis period to blastocyst stage and obtained 286 images of human oocytes and embryos. In the same year, Lemmen and colleagues (Lemmen, Agerholm, and Ziebe 2008) analyzed the events that occur during the first day of the development after fertilization of 102 oocytes using a microscope with an enclosed camera system. They were the first group that studied embryo kinetic properties, by finding a link between the early disappearance of pronuclei after fertilization, early first cleavage, and many blastomeres on day 2 of the development.

Publications began to appear after some time in which authors suggested that kinetic properties needed to be included as additional morphological evaluations, like the timing of embryonic cell divisions (Sakkas et al. 1998; Lundin, Bergh, and Hardarson 2001).

At first the main focus was on the timing of the first embryonic division or early cell division at which point an embryo became a 2-cell organism. The correlation between pregnancy rate and time of early cell division was first studied by Edwards et al. in 1984. They had concluded that the transfer of embryos that cleaved 24–26 h after insemination resulted in higher implantation and pregnancy rates than embryos transferred which had delayed division. Later many other publications showed that too fast or too slow cleavage has a negative impact on the embryo (M. Meseguer et al. 2011; Cruz et al. 2012; Natalia Basile and Meseguer 2012; Chamayou et al. 2013; Tsai et al. 2002; Edwards et al. 1984).

The design of those studies varied, based on the structure of the study and factors studied. One group compared IVF cycles that didn't have early cleaving embryos with cycles that had at least one early cleaving embryo (Tsai et al. 2002). Others examined correlations between early cleavage embryos and pregnancy rates (Salumets et al. 2001). Many argued that this kind of study approach is not fully valid as one should not compare relationships of embryo kinetics with successful cycles, because early cleavage is not an independent predictor of pregnancy and should be evaluated together with embryo morphology, cell number and genetics. It is important to keep in mind that conclusions in some studies about higher implantation rates in early cleaving embryos may be false-positive. A more accurate approach is to compare the times of early cleavage with embryo quality. Several studies have shown that early cleavage embryos have significantly higher numbers of cells than late cleaving embryos (Sakkas et al. 1998; Lundin, Bergh, and Hardarson 2001; Fenwick et al. 2002).

The reason for time variability of cell division in different embryos is still being debated. It is likely that divisions that occurred on time reflect the good quality of the cytoplasmic

component of the embryo, an optimal level of energy provided by mitochondria, They also reflect properly reacted components of activation, such as  $Ca^{2+}$  oscillations (Milewski and Ajduk 2017). It had been proven in several mouse studies that  $Ca^{2+}$  oscillations impact on embryo growth many days after fertilization. More specifically, they increase ATP levels in mitochondria. They could be related to culture conditions, as well as to intrinsic factors of sperm, age, genetic competence and metabolic conditions (Lundin, Bergh, and Hardarson 2001).

### **1.5.2. Time-lapse microscopy**

Time-lapse microscopy (TLM) is a noninvasive embryo selection method for evaluation of embryonic development. This method provides 24-h monitoring of the embryo morphology and timing of cleavages without disrupting the constant culturing environment. The TLM method is based on embryo selection according to cell division timing, excluding morphologically normal embryos with discordant cleavage timing.

Modern time-lapse observation systems are developed for more optimized and accurate selection of viable embryos that include morphological grading with the possibility of registering kinetic parameters (M. Meseguer et al. 2011). Time-lapse imaging has its own benefits, like low light exposure in relation to traditional morphology observation methods (Ottosen, Hindkjær, and Ingerslev 2007) and the possibility to observe the embryo inside the incubator without moving it. This provides stable and uninterrupted conditions, beneficial to the final result (Marcos Meseguer et al. 2012).

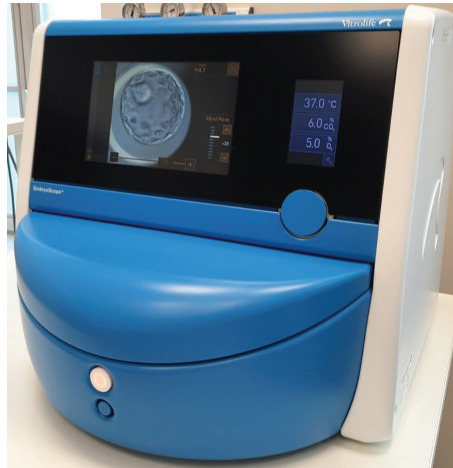
An Embryoscope™ (Figure 1.5.2.) is a new type of incubator that enables embryologists to monitor embryo cell divisions while the embryos remain in the incubator. The incubator is equipped with a built-in camera that captures pictures of the embryos at set intervals. These images are then compiled to create a time-lapse video that displays the embryo's development at each crucial stage. By observing when and how the embryos divide, our embryology team can evaluate whether normal development is occurring.

An incubator simulates the physiological conditions in a woman's body as closely as possible to create an optimal environment for the embryos while they are in the IVF laboratory.

Step by step, evidence started to accumulate that implantation rates are better in TLM (Marcos Meseguer et al. 2012; Aparicio, Cruz, and Meseguer 2013; I. Rubio et al. 2014; Fodina et al. 2015), however, the conclusions regarding significant impact on pregnancy and implantation rates still vary (Cruz et al. 2011; Kirkegaard et al. 2013).

Many predictive models that are based on different statistical methods of analysis were created which can predict parameters like blastocyst stage development and quality. Algorithms can be very simple, with only one parameter that needs to be calculated, or they could have a complex hierarchical structure, to give more predictive value than simple algorithms and be more popular in reproductive medicine (Milewski and Ajduk 2017). Such morphokinetic algorithms were described by Cruz, Motato Yang et al. (Cruz et al.

2012; Motato et al. 2016; S. T. Yang et al. 2015). Morphokinetic algorithms that could predict successful implantation and biochemical or clinical pregnancy were described by Meseguer, Liu and Motato et al. (M. Meseguer et al. 2011; Y. Liu et al. 2016; Motato et al. 2016). ESHRE also described the positive effect of TLM, but acknowledged that it is difficult to compare the outcomes of different studies since the methodologies used are not consistent (Apter et al. 2020).



*Figure 1.5.2. Embryoscope+™, Vitrolife, ES-P1. 3063. Images from iVF Riga archive*

### **1.5.3. Embryo aneuploidy**

Through life people are affected by many negative factors in the external environment.

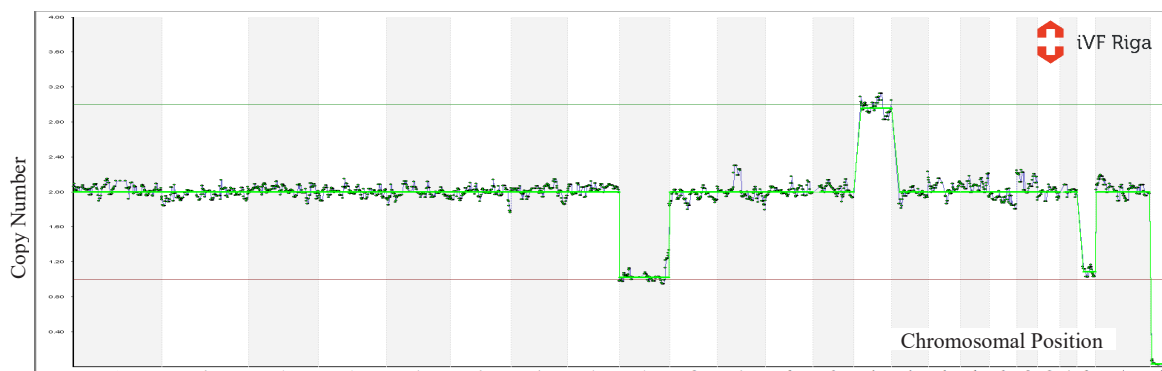
As people age, body defense systems are not always able to protect all the cells properly. This results in errors in chromosome segregation during meiosis I and II. Some reports claim that segregation errors in meiosis II occurs more frequently than in meiosis I (Alan H. Handyside et al. 2012). Because each woman from birth has limited reserve of oocytes, all errors lead to increase of embryos having abnormal chromosome numbers. The reasons for these chromosome segregation errors involve many factors, like incorrect formation of bivalents, deterioration of cohesins, sister kinetochores separation by large distances and incorrect attachment of spindle microtubules to kinetochores during meiosis (Webster and Schuh 2017). Despite the decreasing quality of oocytes that correlates with age, many women in current times, especially in high income countries, more frequently delay having their first child until later. (Webster and Schuh 2017).

Detection and management of embryo anomalies that occur due to age, poor quality oocytes or sperm abnormalities, may be accomplished by performing preimplantation genetic testing (PGT) that allows selection and transfer of blastocysts having normal genetic constitution (Figure 1.5.3.).

The history of PGT goes back to 1989 when A. Handyside performed the first pre-implantation genetic diagnostic (PGD) study detecting a Y chromosome-specific region

with PCR in a case having X-linked adrenoleukodystrophy and X-linked mental retardation (A. H. Handyside et al. 1990). Now, defining embryo gender is known as sexing and can complement genetic testing of monogenic disorders linked to the sex chromosomes (Coonen et al. 2020).

With time, PGT underwent significant methodological and approach changes, starting from polar body testing and blastomere analysis to adapting trophectoderm biopsies with subsequent blastocyst freezing (Renwick et al. 2006). In early days, the blastomeres were analyzed using FISH method for chromosomes X, Y, 18, 13, and 21. At that time it was shown that women of advanced age, after PGT, had significantly lower live birth rates as compared with those undergoing IVF without PGT (Maxwell and Grifo 2018). The analysis of more than just single cells led to comprehensive downstream molecular investigations, which resulted in development of the blastocyst stage biopsy strategy (Cimadomo et al. 2016). Molecular genetic testing started as analysis of single loci by the PCR method and grew to become sophisticated single cell whole genome amplifications (Fiorentino 2012). Instead of using PGT, many groups have tried to develop algorithms to detect ploidy based on morphokinetic properties. There have been several attempts to create such algorithms using time-lapse monitoring. The idea was based on the assumption that embryos display different cleavage dynamics depending on their genetic material, but research has not yet fully documented this (Chavez et al. 2012; Vera-Rodriguez et al. 2015).



**Figure 1.5.3. Preimplantation genetic testing result profile of aneuploid embryo.**  
Images from iVF Riga archive

PGT consists of two main tests: PGT-A and PGT-M. PGT-A (Preimplantation Genetic Testing for Aneuploidy) is a procedure that allows one to determine the chromosomal status of IVF embryos by screening all 23 pairs of human chromosomes including sex chromosomes. Many different methods are used, which include the array of comparative genomic hybridization (aCGH), quantitative PCR (qPCR), single nucleotide polymorphism array (SNP array) and next-generation sequencing (high and low resolution) (NGS). The difference between those methods is in the quantity of genomic amplification, the ability to detect balanced or unbalanced translocations, partial aneuploidies, polyploidy,

and mosaicism. For example, Array CGH, SNP array, and high resolution NGS use whole genome amplification (WGA) of genomic DNA but at the same time, can introduce an artefact. Quantitative PCR and low resolution NGS are not able to amplify the whole genome and due to their low genomic coverage, small deletions or duplications could not be detected (Maxwell and Grifo 2018).

After the PGT-A embryos can be diagnosed three ways – as euploids with the normal number of chromosomes; as aneuploid with abnormal numbers of chromosomes, and as mosaics – where 2 different cell lines are present in the same embryo (often one euploid cell line and one aneuploid cell line). Regarding mosaic embryos, it has been shown that mosaicism rates decrease with extended embryo cultures. This could happen due to the embryos ability to self-correct, or because euploid cell lines predominate at later developmental stages (M. A. Santos et al. 2010).

PGT-M is a Pre-implantation genetic testing of embryos for monogenic (or single gene) diseases. PGT-M is part of the IVF process in couples having hereditary genetic disorders.

Balanced rearrangements represent one of the most common forms of genetic abnormality affecting approximately 1 in every 500 (0.2%) individuals. Difficulties processing the abnormal chromosomes during meiosis lead to an elevated risk of chromosomally abnormal gametes, resulting in high rates of miscarriage and/or children with congenital abnormalities (Alfarawati et al. 2022). This rearrangement in embryos may occur as a *de novo* event or can be the product of an abnormal karyotype in one of the parents. Carriers of balanced translocations are most often identified by karyotyping or by genetic analysis of products of conception after an embryonic demise which necessitated the subsequent karyotyping of parents (Sundheimer et al. 2018). Two types of balanced structural chromosomal rearrangements are known – Robertsonian translocations and reciprocal translocations. Robertsonian translocations (ROBs) are chromosomal rearrangements that result from the fusion of the entire long arms of two acrocentric chromosomes. Robertsonian translocations are among the most frequent reorganizations in human genes. Most of the cases analyzed correspond to rearrangements with chromosomes from the D-group (chromosomes 13, 14 and 15), whereas some rare Robertsonian translocations are seldom found in the literature, except those with both chromosomes from the G-group (chromosomes 21 and 22) and those involving chromosomes from both groups (D; G translocations) (Anton, Blanco, and Vidal 2010; Zhao et al. 2015; Alfarawati et al. 2022). Reciprocal translocation is a type of chromosome rearrangement that is involved in the exchange of chromosome segments between two chromosomes that do not belong to the same pair of chromosomes (H. G. Zhang et al. 2016).

ESHRE guidelines indicates the necessity of the genetic screening of the embryos before embryo transfer with different methods (PGT-A, PGT-SR, PGT-M), the scientific review still continues for different group of patients (recurrent implantation failure, recurrent pregnancy loss, for patients with the structural aberrations in their karyotype or rare inherited gene diseases) (Coonen et al. 2020).

## 1.6. Other factors

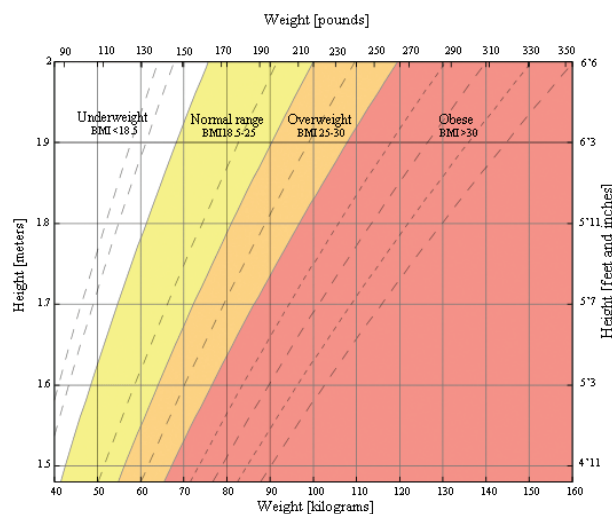
### 1.6.1. Female factor

**Age.** The notion that age correlates with meiotic chromosome segregation errors, and that quality of genetic material in oocytes decreases over time is a well-researched fact found in many of scientific publications, over many years.

One of the world largest studies on aneuploidy was performed in 2011. More than 20 000 oocytes were obtained from 2830 patients having an average age of 38.8 years. Their polar bodies were examined using the FISH method. 20% of aneuploidies in women 35 years of age were detected that increased to nearly 60% of oocytes in women over 43 years of age. Of all the tested oocytes, almost half (46.8%) were aneuploid (Kuliev et al. 2011).

Oocyte donation is an effective way of dealing with age related problems. Sauer et al. in 1992 proved its effectiveness by creating two groups of women 40 years old and over. One group of women underwent a standard IVF procedure and embryo transfer using their own oocytes while the other group who had premature ovarian failure used donor oocytes. In the second group, cycles resulted in better implantation and ongoing pregnancy than in the group having their own oocytes (Sauer, Paulson, and Lobo 1992).

**BMI.** Body mass index – by definition, an overweight person has a BMI of 25 or above. Obesity is defined as having a body mass index (BMI) equal to or greater than 30. These medical terms were established by the World Health Organization (WHO). According to the WHO, over 1.9 billion adults worldwide are affected by excess body weight, with more than 650 million classified as obese. What was once a problem primarily seen in high-income countries become a global issue affecting most countries around the world (“World Health Organization. Facts on Obesity.” 2021).



**Figure 1.6.1. BMI categories chart for adults, based on the WHO data. The dashed lines represent subdivisions within a major categorization (Maple Tech International, n.d.)**

Obesity poses significant health risks, including an increased likelihood of developing chronic diseases such as diabetes, cardiovascular disorders, also presents risks to fertility and reproductive health. It is associated with an increased risk of infertility in both men and women, as well as a higher likelihood of experiencing pregnancy complications and suboptimal outcomes, such as miscarriage and gestational diabetes. Managing obesity and maintaining a healthy weight can play a crucial role in improving fertility outcomes and reducing the associated risks.

To investigate the impact of obesity and morbid obesity on IVF outcome, Doody et al. conducted a study of 822 women in 2003, and grouped them into four WHO BMI categories all having mean age ranges of 33.1 to 33.7 years. The results showed significantly lower ongoing pregnancy rates and implantation rates in the > 35 BMI group compared to the other groups (Doody et al. 2003). Similar results of significant reduction in both implantation and pregnancy rates during the same year was confirmed by Ryley et al. in a study of 6,827 cycles (Ryley et al. 2004).

Bellver evaluated 419 cycles in women having BMI > 30 (mean 33.6). The implantation rate, pregnancy and live birth rate were clearly critically lower in obese women with the tendency to worsen progressively with each unit of BMI (Bellver et al. 2010).

In 2007 a comprehensive study on this topic was conducted by the Society for Assisted Reproductive Technology (SART). They analyzed cycles of one or more transferred embryos, taking height, and weight from the 345 ART centers (about 90% of U.S. ART centers). From a total of 45,163 cycles, the investigators found that higher BMI was associated with lower clinical pregnancy rates, especially in women under age 35 using their own oocytes (Luke et al. 2011).

**Ovarian response to stimulation.** The goal of ovarian stimulation is the collection of multiple dominant follicles in an effort to compensate for inefficiencies of embryology culture, and embryo selection, thus improving chances for successful conception in IVF (N. S. Macklon et al. 2006). The definition of poor ovarian response (POR) should be understood as the essential inability of a woman's ovaries to react properly to the selected stimulation.

The patients who were first described as poor responders were noted 28 years ago to have low E2 which is an indicator of poor response and resulting in few oocytes being retrieved (Garcia et al. 1983).

Since then many publications have appeared having descriptions of POR causes, symptoms and recommended treatments (Surrey and Schoolcraft 2000; Loutradis, Vomvolaki, and Drakakis 2008; Kyrrou et al. 2009). Those publications however, had small heterogeneous samples. This precluded the treatments prescribed to improve POR outcomes to be counted as evidence based.

As ESHRE together with the American Society for Reproductive Medicine already defined diagnostic criteria for the polycystic ovarian syndrome. The same approach was used for the definition of poor ovarian response (POR). The ESHRE Campus Workshop

was organized March 19–20, 2010 in Bologna. Its main purpose is to establish agreement on a standardized definition and diagnosis for POR. Special Interest Groups (SIGs) and the majority of researchers who contributed to the field the most were involved in the decision making. This large scientific community developed the first standardized definition of poor ovarian response (POR).

In this POR definition, at least two of the following three features must be present:

- Advanced maternal age ( $\geq 40$  years) or any other risk factor for POR;
- A previous POR ( $\leq 3$  oocytes with a conventional stimulation protocol);
- An abnormal ovarian reserve test (i.e. AFC 5–7 follicles or AMH ,0.5–1.1 ng/ml) (Ferraretti et al. 2011).

AMH, (Anti-Mullerian Hormone) is a dimeric glycoprotein, that is, a member of the transforming growth factor-beta superfamily that is produced in granulosa cells, whose main roles are tissue growth, differentiation and folliculogenesis (Cate et al. 1986).

Before AMH treatment, many methods were used to measure ovarian reserve. They included physical testing such as Ovarian volume measurements by TVS, antral follicle count measurements; endocrine biomarkers – Day 3 FSH, Estradiol, FSH:LH ratio as indirect measures and Inhibin B, Clomiphene citrate challenge test, The Gonadotropin-releasing Hormone Agonist Test (GAST) (Roudebush, Kivens, and Mattke 2008). Following this, a lot of data began to appear that showed strong correlations between the number of retrieved oocytes and basal AMH serum levels in women undergoing ovarian stimulation. In conclusion, both AMH and AFC (antral follicle count) were selected as the ideal markers for measuring ovarian reserve (Broer et al. 2009; La Marca et al. 2009).

As with the RIF definition, many approaches to treat and define POR without standardized criteria are still being used, as a 2015 worldwide survey from 196 IVF centers clearly demonstrated (Patrizio et al. 2015). The lack of standardization is a problem as treatment options are subjective and not evidence based.

Many risk factors may cause poor ovarian response: short menstrual cycle, single ovary, ovarian cystectomy, chronic smoking, unexplained infertility, previous chemotherapy and/or radiotherapy treatment, family history of premature menopause (genes); X chromosome derangements, fragile X mental retardation 1 pre-mutation (Younis, Ben-Ami, and Ben-Shlomo 2015) BRCA1, pelvic infection.

Pelvic inflammatory disease with severe pelvic adhesions is associated with poor response to gonadotropin stimulation by confirmed tubal damage and positivity to Chlamydia IgG antibody testing (Molloy et al. 1987; Keay et al. 1998). Ovarian responsiveness is also reduced in women with ovarian endometriomas and patients who have undergone unilateral ovarian cystectomy (Nargund, Cheng, and Parsons 1996; Garcia-Velasco and Somigliana 2008).

Chemotherapy with the use of an alkylating agent, has been reported to significantly reduce the ovarian pool reserve and is associated with some degree of risk for primary ovarian insufficiency (Oktem and Oktay 2007; De Vos, Devroey, and Fauser 2010).

The response of each individual's ovaries to this stimulation was quite variable. If a woman's first stimulation was unsuccessful that does not necessarily mean that her results will be the same on the second attempt. As shown by Klinkert (Klinkert et al. 2004), the incidence of poor response in the second stimulation cycle corresponds to 62.4%, which means that one third of women succeeded after the second try, and that the percentage of ongoing pregnancy is higher after three cycles than after the first cycle (Ferraretti et al. 2011).

The POSEIDON (Patient-Oriented Strategies Encompassing Individualized Oocyte Number) criteria were developed in 2016 to identify women who are at a higher risk of having poor ovarian response during IVF treatment. The criteria aim to individualize the treatment approach based on the patient's characteristics and ovarian reserve (Esteves et al. 2016; Alviggi et al. 2016).

For example, patients with low ovarian reserve may benefit from higher doses of FSH during ovarian stimulation, while patients with a history of poor response may benefit from alternative stimulation protocols such as mini IVF or natural cycle IVF (Roque et al. 2021; Esteves et al. 2018).

*Table 1.6.1. The POSEIDON criteria (Alviggi et al. 2016)*

Group	Age	AFC	AMH	No. of oocytes retrieved in previous OS cycles
Group 1	< 35	≥ 5	≥ 1.2 ng/ ml	
Subgroup 1a				< 4
Subgroup 1b				4–9
Group 2	≥ 35	≥ 5	≥ 1.2 ng/ ml	
Subgroup 2a				< 4
Subgroup 2b				4–9
Group 3	< 35	< 5	< 1.2 ng/ml	
Group 4	≥ 35	< 5	< 1.2 ng/ml	

### 1.6.2. Male factor

Male factor infertility can affect pregnancy outcomes through a variety of mechanisms, including DNA damage in sperm, abnormal sperm chromatin structure, altered gene expression, oxidative stress and the immune system's response. Several studies have reported an association between male factor infertility and RIF and EPL. A recent meta-analysis of 19 studies found that male factor infertility was associated with a decreased live birth rate in IVF cycles (Agarwal et al. 2015).

Poor sperm motility, morphology, and concentration, which decreases the likelihood of successful fertilization and implantation, can also lead to male factor infertility. Abnormalities in the epididymis, testes, and vas deferens can also contribute to male

infertility and may lead to poor sperm quality. In addition, certain lifestyle factors, such as smoking, alcohol consumption, and obesity, have been linked to male infertility and may also impact pregnancy outcomes.

The male factor infertility does not always preclude successful pregnancy outcomes, as assisted reproductive technologies such as ICSI, can often overcome some of these obstacles. However, even with these interventions, male factor infertility can still have an impact on pregnancy rates and outcomes, highlighting the importance of addressing and treating male infertility as part of the overall fertility evaluation and management plan.

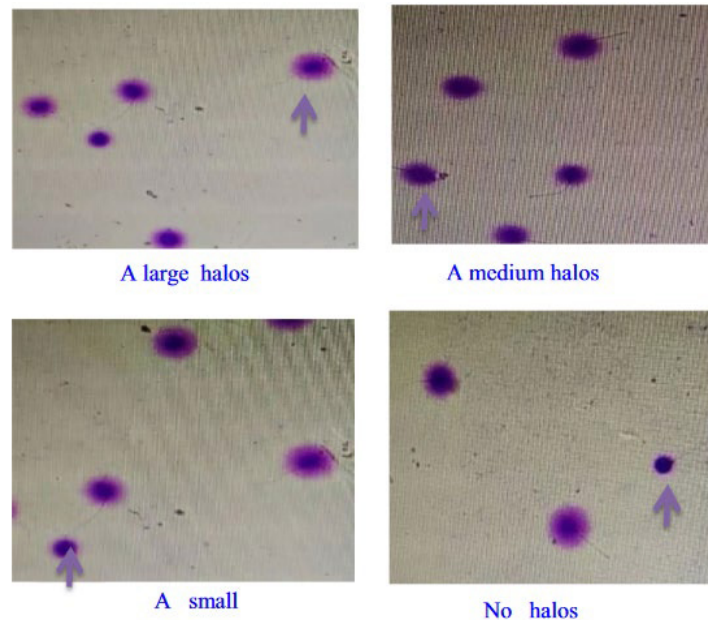
In the last two decades, more emphasis has been placed on assessing sperm functional parameters and their predictive value for male fertility potential (Figure 1.6.2.). These parameters are mainly related to the challenges that sperm must overcome inside the female reproductive system, particularly in the cervix and oviduct, to achieve successful fertilization of the oocyte. The gold standard for human semen analysis is the World Health Organization Manual. The most recent edition is referred to as WHO 6 (World Health 2021).

	N	Centiles									
		2.5th	5th	(95% CI)	10th	25th	50th	75th	90th	95th	97.5th
Semen volume (ml)	3586	1.0	1.4	(1.3-1.5)	1.8	2.3	3.0	4.2	5.5	6.2	6.9
Sperm concentration (10 <sup>6</sup> per ml)	3587	11	16	(15-18)	22	36	66	110	166	208	254
Total sperm number (10 <sup>6</sup> per ejaculate)	3584	29	39	(35-40)	58	108	210	363	561	701	865
Total motility (PR + NP, %)	3488	35	42	(40-43)	47	55	64	73	83	90	92
Progressive motility (PR, %)	3389	24	30	(29-31)	36	45	55	63	71	77	81
Non-progressive motility (NP, %)	3387	1	1	(1-1)	2	4	8	15	26	32	38
Immotile spermatozoa (IM, %)	2800	15	20	(19-20)	23	30	37	45	53	58	65
Vitality (%)	1337	45	54	(50-56)	60	69	78	88	95	97	98
Normal forms (%)	3335	3	4	(3.9-4.0)	5	8	14	23	32	39	45

**Figure 1.6.2. Distribution of semen examination results from men in couples starting a pregnancy within one year of unprotected sexual intercourse leading to a natural conception. From Campbell et al.; fifth percentile given with variability (95% confidence interval) and as presented in the WHO 6 (2021) manual**

The exact mechanisms by which male factor infertility affects RIF and EPL are not fully understood. However, several hypotheses have been proposed. One possible mechanism is through the DFI, the DNA fragmentation index of sperm. DFI is a measure of the amount of damaged DNA in sperm, which can negatively affect fertility outcomes. Several studies have found that high DFI is associated with lower pregnancy rates and increased miscarriage rates (Simon et al. 2017; Robinson et al. 2012).

The Sperm chromatin dispersion (SCD) test does not rely on the determination of color or fluorescence intensity. Instead, it measures the endpoint by determining the percentage of spermatozoa with nondispersed or dispersed nuclei, which can be easily and reliably observed by the naked eye (K. S. Liu et al. 2023). Spermatozoa with nondispersed nuclei exhibit very small halos or no halos at all (Figure 1.6.3.).



*Figure 1.6.3. SCD test of sperm (K. S. Liu et al. 2023)*

Some studies, however, have failed to support a hypothesis that sperm DNA integrity is an important factor in RIF (Carol Coughlan et al. 2015). Additionally, male factor infertility has been linked to abnormalities in the paternal epigenome, which can lead to embryo development issues and pregnancy loss (Jenkins et al. 2014).

Research has also suggested that male factor infertility can affect the immune system's response during pregnancy, potentially leading to RIF and EPL. Abnormalities in seminal plasma cytokines, such as interleukin-6 (IL-6), have been linked to immune dysfunction in the uterus and increased rates of pregnancy loss (Tremellen et al. 2000; Robertson and Sharkey 2016).

Another possible mechanism is through sperm chromatin structure assay (SCSA), which measures the degree of DNA compaction in sperm. Studies have found that abnormal SCSA results are associated with decreased pregnancy rates and increased miscarriage rates (Zini et al. 2008; Evenson and Wixon 2006).

Further research is needed to fully understand the relationship between male factor infertility and RIF and EPL, and to develop effective interventions to improve outcomes for couples affected by these conditions.

## 2. OBJECTIVES OF THE STUDY

This study focused on patients having recurrent early pregnancy loss and recurrent implantation failure. The **aim** of the work is to assess the ability of different methods and approaches to improve the outcome of the IVF treatment, and to decrease recurrent implantation failure and early pregnancy loss.

The following objectives have been set for achieving this aim:

1. To evaluate the ability of time-lapse microscopy/imaging to improve the outcome of the IVF procedures.
2. To evaluate if endometrial receptivity array and invasive embryo preimplantation genetic testing for aneuploidies used alone or in combination can improve the outcome of IVF treatment in a group of patients with recurrent implantation failure.
3. To assess the utility of preimplantation genetic testing for aneuploidies in IVF procedures in specific group of patients – the carriers of balanced structural chromosomal rearrangements.
4. To clarify, standardize and operationally define non-invasive and invasive methods of embryo selection to decrease recurrent implantation failure rate and to promote the recommendations to other researchers and clinical professionals.

### **3. RESEARCH HYPOTHESES**

1. Embryo selection is a vital step to increase the success of ART treatment. Selection of high implantation potency embryos using both methods- non-invasive Time-lapse microscopy/imaging and invasive preimplantation genetic testing for aneuploidies, can improve the outcome of the treatment.
2. Embryonal and endometrial factors in combination are the likely cause of failed implantation. Applying the combination of methods (Time-lapse microscopy and invasive pre-implantation genetic testing together with adjusting implantation window) can reduce the number of recurrent implantation failure and early pregnancy loss.
3. Applying the preimplantation genetic testing for aneuploidy to the patients with structural imbalances in their karyotypes and determining the percentage of normal embryos have a great effect on implantation.

## 4. SCIENTIFIC VALUE

Novelty of this study: existing studies and publications regarding the different factors that affect outcomes of IVF procedures are controversial and present conflicting evidence. Some studies suggest different methods assessing embryonal or uterine factors to improve the outcome of ART and to decrease implantation failure and early pregnancy loss. Some of the studies do not recommend these methods and question the credibility of this procedure. The purpose of this study is to investigate and evaluate this important issue. Large samples of patients were studied, and different embryonal (morphological, genetical) and uterine methods were used, which are widely known and commonly used in research and clinical practice.

This study is both diverse and comprehensive. It pursues a multivariate approach, where the author in the first uses the combinations of these methods to investigate the main factors involved in recurrent implantation failure and early pregnancy loss.

The study determine which methods and factors are significant and which ones can be recommended in clinical practice for implementation in standard IVF work-up. It also identifies those methods that remain questionable. Based on the results of this study, a proposal is made to develop national guidelines for IVF treatment that will help the reproductive medical society in Latvia and abroad to improve results, of importance both for medical research and for demographical work. At the present time the standardized guidelines from ESHRE are not available.

## 5. MATERIALS AND METHODS

This study was conducted in the reproductive clinic iVF Riga from January 15, 2014 until December 9, 2021.

Different approaches were used to support the hypotheses of the effect of embryonic and endometrial factors on implantation failure. In the first study (Paper I) evaluated the effect of time-lapse imaging on embryo morphokinetics with further influence on implantation outcome. The second study (Paper II) evaluated the effect of selecting embryos with the PGT-A method on implantation results, as well as the impact of endometrial receptivity as a single factor, and in combination with embryonic factors on implantation outcomes. The third study (Paper III) compared the PGT-A results of patients with structural imbalances in their karyotypes to determine the percentage of normal embryos and their effect on implantation.

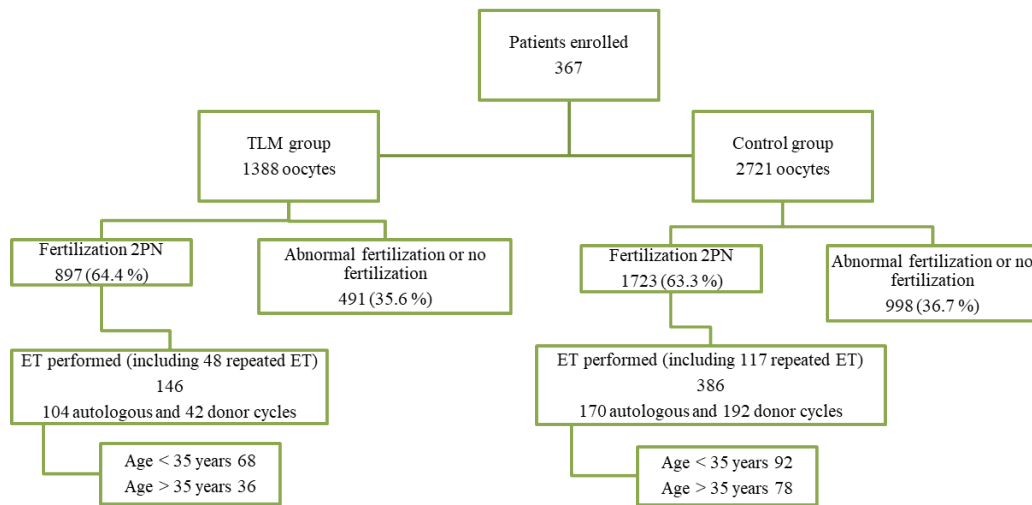
All patients were informed of the goal and methods of the study before enrolment and signed the informed Consent form before being included in the study. The study conformed to the standards set by the Declaration of Helsinki. The Latvian Central Medical Ethical Committee has approved the study (approval No. 01-29.1.2/1735, available in the Appendix I).

### 5.1. Patients (Sample Inclusion / Exclusion Criteria)

For the study (The study No. 1) that evaluated the effect of time-lapse imaging of 367 patients. In this part of the study recruited couples received autologous or oocyte donation ICSI cycles. Study group included 897 embryos analyzed using TLM for 146 embryo transfers. Control group included 1723 embryos cultivated/ selected conventionally using traditional static observations method prior to 386 embryo transfers (ETs) (Figure 5.1.1.).

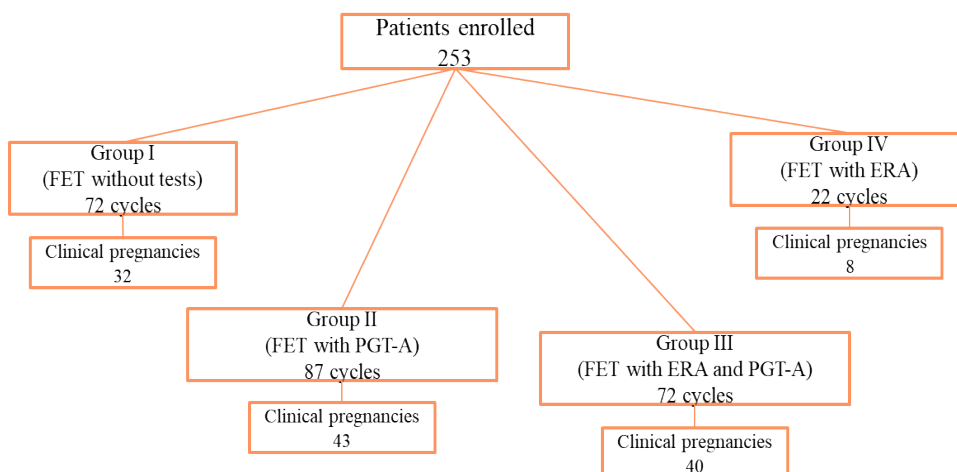
- Inclusion criteria:
  - ▷ Patient aged 35–39 years;
  - ▷ Patients who had failed to achieve a clinical pregnancy after transfers of at least three good-quality embryos in different single fresh or frozen embryo transfers;
  - ▷ IVF cycles only with ICSI procedure;
  - ▷ Only single embryo transfers and double embryo transfer;
  - ▷ Only good quality embryos were transferred, *i.e.* those that had the correct number of cells corresponding to the day of development – the day-5 embryos (blastocysts).
- Exclusion criteria:
  - ▷ Man with obstructive azoospermia and oligospermia;

- ▷ Patients with abnormal karyotypes (translocations, inversions), or severe endocrine disorders, or atrophic endometrium;
- ▷ Embryo transfers with three embryos.



**Figure 5.1.1. Patients enrolled for the Time-lapse microscopy or imaging study (The study No. 1)**

For the study (The study No. 2) that evaluated the effect of selecting embryos with the PGT-A and endometrial receptivity in total 253 cycles of the assisted reproduction by means of ICSI were included, and further randomly divided into 4 groups: Group I – frozen embryo transfers without any additional tests or procedures (FET),  $n = 72$  cycles; Group II – FET with PGT-A,  $n = 87$ ; Group III – FET with PGT-A and ERA,  $n = 72$ ; Group IV – FET with ERA,  $n = 22$  (Figure 5.1.2.).

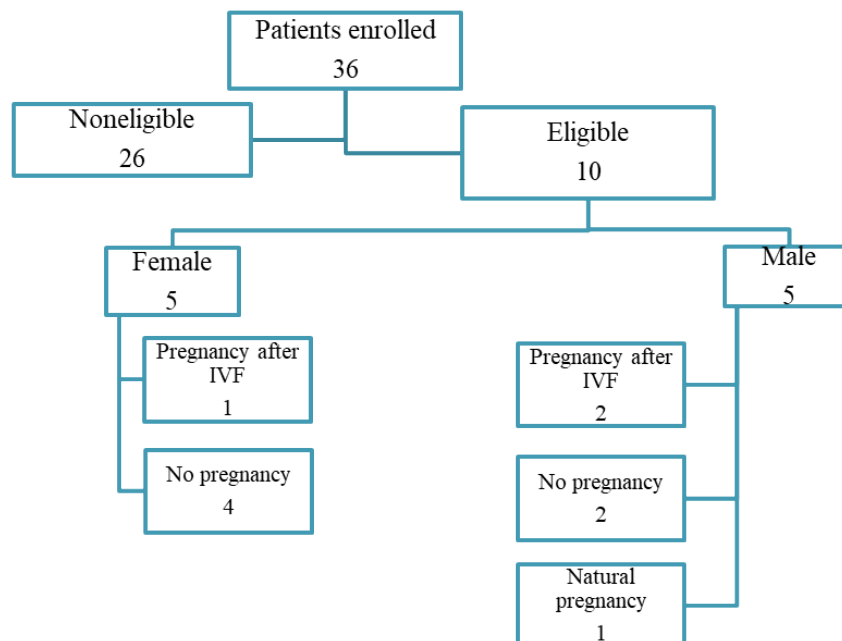


**Figure 5.1.2. Patients enrolled for the preimplantation genetic testing for aneuploidies and endometrial receptivity array in a group of recurrent implantation failure in the intra-cytoplasmic sperm injection cycles (The Study No. 2)**

- Inclusion criteria: Patients aged 34–36 with at least one previous implantation failure in their medical history.
- Exclusion criteria:
  - ▷ Patients with poor ovarian reserve (less than 5 oocytes);
  - ▷ abnormalities in the endometrium such as inflammation, Asherman's syndrome, organic changes, polyps, and submucosal myomas;
  - ▷ Patients with aberrations in the karyotype.

For the study (The study No. 3) about the patients with structural imbalances, from the 36 patients with changes in karyotypes that were found in iVF Riga clinic from period 2014–2018, 10 patients undergoing IVF with PGT-A were eligible for the study. Three patients were with the most frequent type of Robertsonian translocation from the D group (between 13 and 14 chromosomes), and 7 patients with various reciprocal translocations. As noted above: 180 oocytes were collected. 140 fertilized normally. 90 of these 140 embryos were frozen. Of these 90, 69 were biopsied, and of these 69, 48 were analyzed with PGT-A (Figure 5.1.3.).

- Inclusion criteria: patients with structural imbalances.



**Figure 5.1.3. Enrolled patients for the application of preimplantation genetic testing for aneuploidies for carriers of balanced structural chromosomal rearrangements (The study No. 3)**

## 5.2. Time-lapse microscopy or imaging

In this part of the study, 367 couples participated, receiving treatment involving autologous or oocyte donation ICSI cycles. Study group included 897 embryos analyzed using TLM (EmbryoScope, Vitrolife) for 146 embryo transfers.

Control group included 1723 embryos cultivated/ selected conventionally using traditional static observations method before 386 embryo transfers (ETs).

Embryos were classified in four groups: complete trophectoderm and high cell number compact inner cell mass – group A; incomplete trophectoderm and several grouped cells – group B; few cells in trophectoderm or inner cell mass – group C, sparse distribution of large or flat or degenerate cells in trophectoderm and no cells or degenerate or necrotic cells in ICM – group D. Top embryos were selected for day 3 ET – control group only. Human blastocysts with trophectoderm cells and inner cell mass in A or B groups were selected for the embryo transfer or vitrification on day 5 (Figure 5.2.1.). Embryo evaluation and selection with morphokinetic method was performed by analysis of time-lapse images of each embryo on an external computer with software developed for time-lapse image analysis (EmbryoViewer workstation; Unisense Fertilitich A/S, Aarhus, Denmark). The Meseguer classification was used (Marcos Meseguer et al. 2012) to describe morphokinetic classification depending on embryo cell division timing: time of cleavage to two-blastomere embryo ( $t_2$ ), time of cleavage to three-blastomere embryo ( $t_3$ ), time of cleavage to four-blastomere embryo ( $t_4$ ), and time of cleavage to five-blastomere embryo ( $t_5$ ). Additionally, the duration of the second cell cycle ( $cc_2$ ) – that is, the duration of the two-blastomere embryo phase ( $t_3-t_2$ ) – and synchrony ( $s_2$ ) in divisions from a two-blastomere embryo to a four-blastomere embryo ( $t_4-t_3$ ) were calculated.

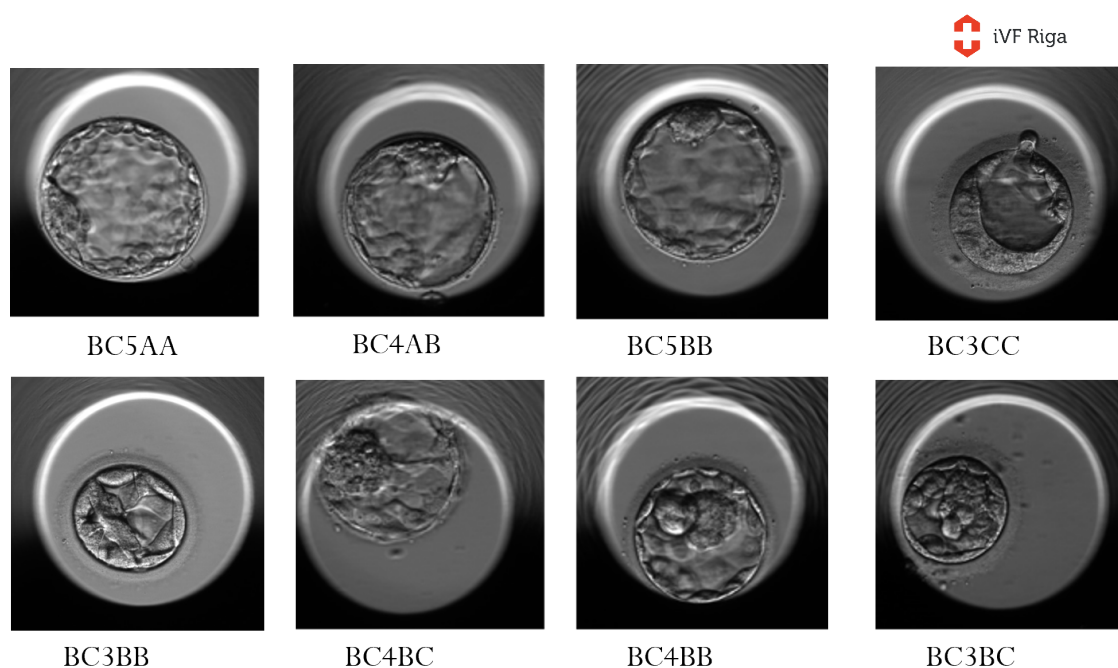


Figure 5.2.1. Different types of blastocysts (BC). Images from iVF Riga archive

### 5.3. Preimplantation genetic testing for aneuploidies (PGT-A) and endometrial receptivity array (ERA) in a group of RIF in intra-cytoplasmic sperm injection (ICSI) cycles

In this study, the ICSI results were evaluated from couples with RIF. The patients who failed to achieve a clinical pregnancy after transfers of at least three good-quality embryos in different single fresh or frozen embryo transfers were considered RIF.

#### 5.3.1. Whole Genome Amplification (WGA) and chromosomal analysis – preimplantation genetic testing for aneuploidies

PGT-A was performed as described elsewhere (C. Rubio et al. 2013). Sureplex reagent kit (Illumina) was used for WGA of trophectodermal cells. Electrophoresis and DNA quantification with Qubit was followed to evaluate the success of amplification. PGT-A was done either by aCGH (array Comparative Genomic Hybridization) with Illumina 24Sure arrays from 2015 to 2017 (Figure 5.3.1.) or Illumina VeriSeq PGS library preparation kit from 2018 to 2019 due to methodology change (Figure 5.3.2.). Both methods allow to detect imbalanced aberrations of autosomes 1–22 and X, Y sex chromosomes in preimplantation embryos, but due to technical differences patients were divided in groups after methods used – aCGH or NGS.

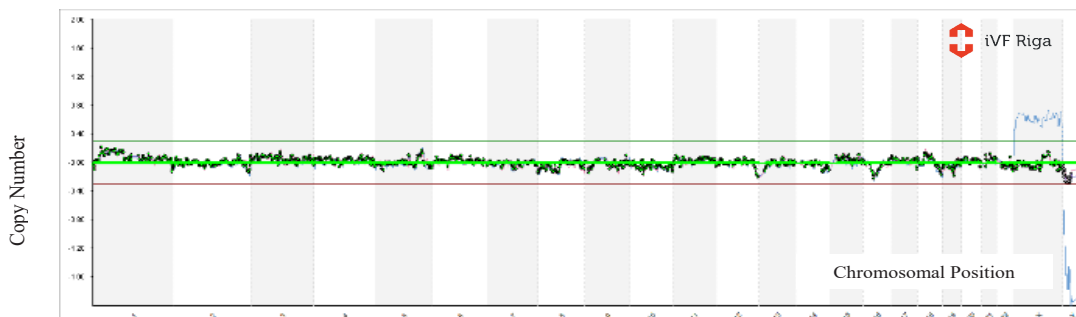


Figure 5.3.1. Preimplantation genetic testing for aneuploidies with aCGH. Images from iVF Riga archive

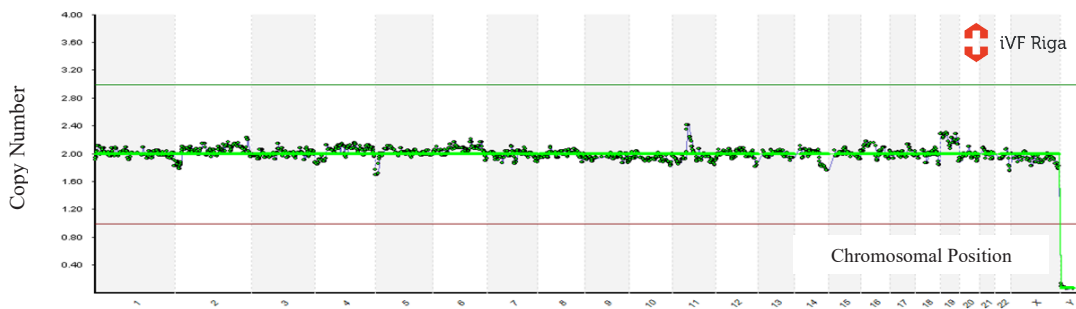


Figure 5.3.2. Preimplantation genetic testing for aneuploidies with NGS. Images from iVF Riga archive

Low level mosaicism, uniparental disomy, 69XXX variant and aberrations under 20 Mb cannot be determined. Copy number variations (CNV) analysis in both cases was performed with Bluefuse software. At least two molecular geneticists and at least one clinical geneticist interpreted the results. Chromosomal aberrations were classified according to guidelines: euploid, low level mosaic (20–50%), high level mosaic (50–80%), aneuploid. Decision regarding transfer was made according to chromosomes involved and type of aberration (whole chromosome versus partial). Embryos with aneuploidy were not transferred.

### 5.3.2. Endometrial Receptivity Array (ERA)

Endometrial biopsies were collected from the uterine fundus following the manufacturer's protocol (Díaz-Gimeno et al. 2011), and samples were sent for the analysis by iGenomix (Figure 5.3.4.). Endometrium were classified by expression profile as receptive, pre- or post-receptive based on transcriptome signature (Díaz-Gimeno et al. 2011).

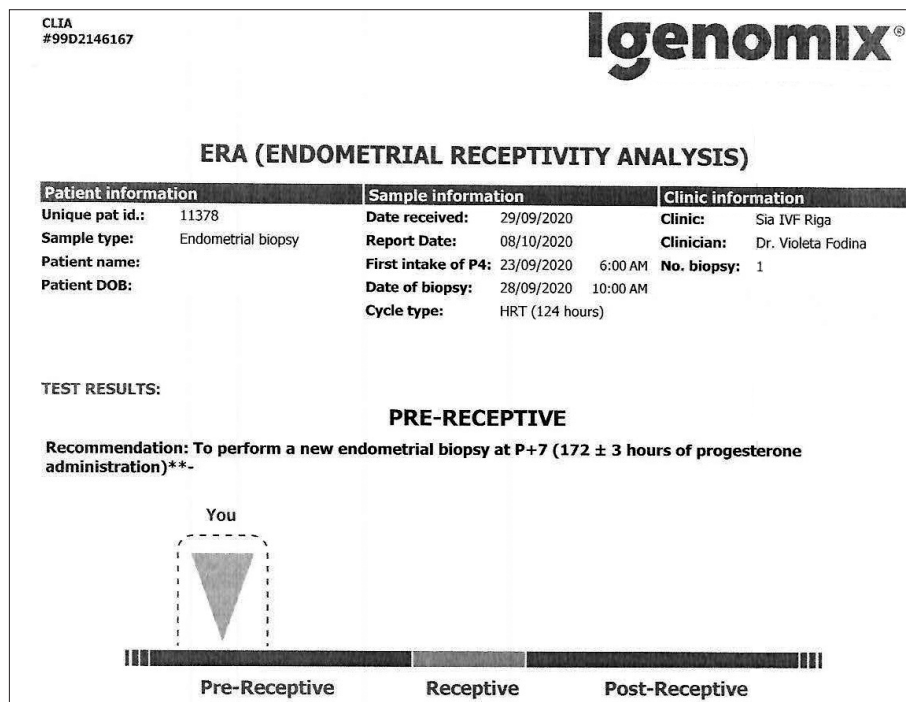
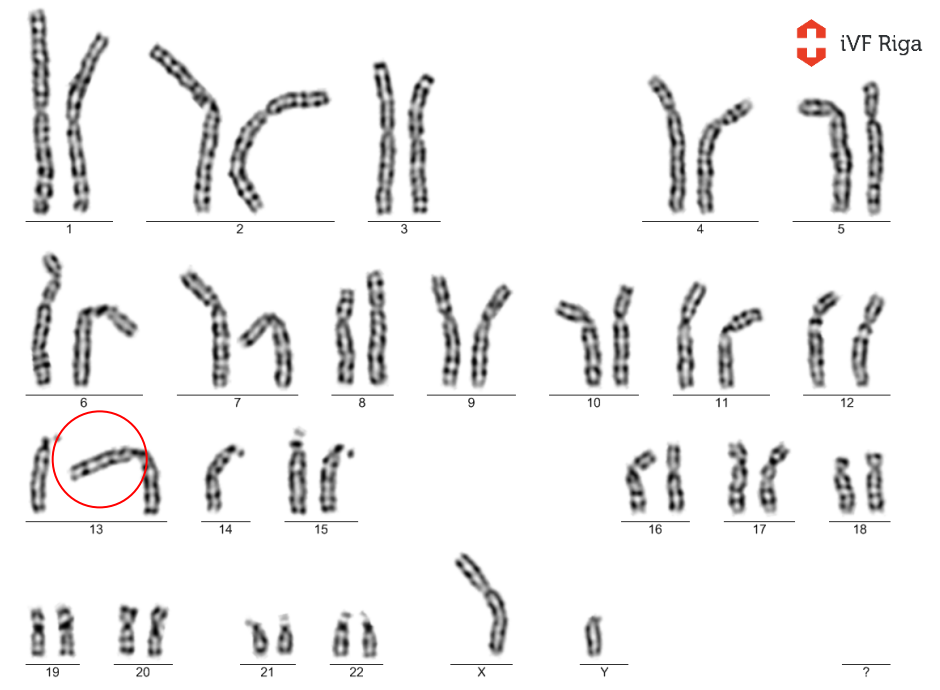


Figure 5.3.4. Igenomix endometrial receptivity analysis report (full version of this report is available in the Appendix II)

## 5.4. The application of PGT-A for carriers of balanced structural chromosomal rearrangements

The most frequent type of Robertsonian translocation is presented in figure 5.4.1.

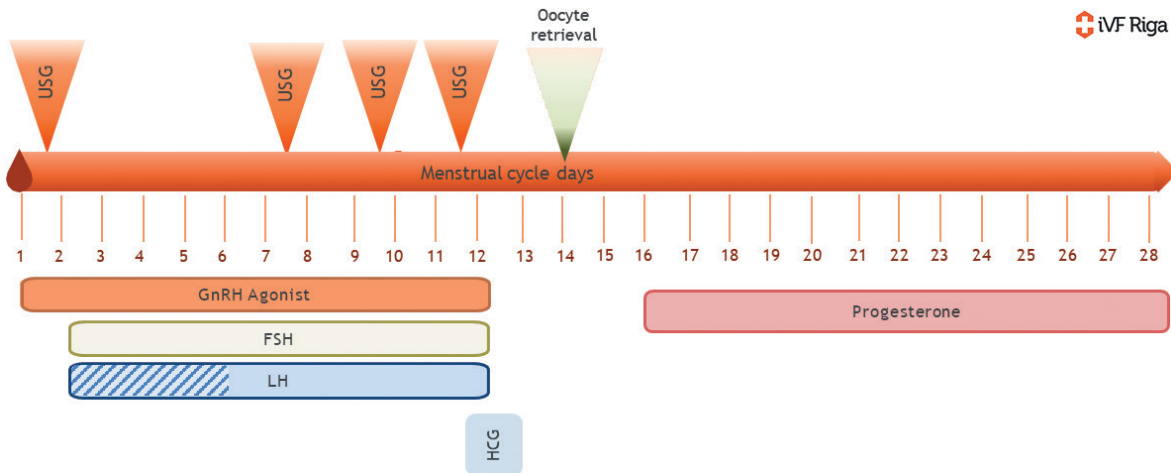


**Figure 5.4.1. Karyotype with Robertsonian translocation.**  
Images from iVF Riga archive

Whole Genome Amplification (WGA) and chromosomal analysis – preimplantation genetic testing for aneuploidies was performed as described above. Parental karyotyping was performed using classical G band cytogenetic approach. Peripheral blood cells (lymphocytes) were cultivated for 72 h in the PB-MAX™ Karyotyping Medium. The colcemide solution was added and put to a thermostat to stop the cell division. Fixative, consisting of methanol and acetic acid, was added to each sample of cells. Processed cells in their metaphase state were fixated on the glass slide with chromosome staining using Giemsa stain. At least 15 metaphases analyzed for each patient with a microscope using Lucia Kario software program. Karyotype was analyzed based on the International System for Human Cytogenomic Nomenclature (ISCN 2016) criteria.

## 5.5. Stimulation protocols

Human chorionic gonadotropin (hCG) trigger was administered when leading follicle reached mean diameter of 18 mm un transvaginal ultrasound. Oocyte retrieval was scheduled 36 h after the hCG administration.

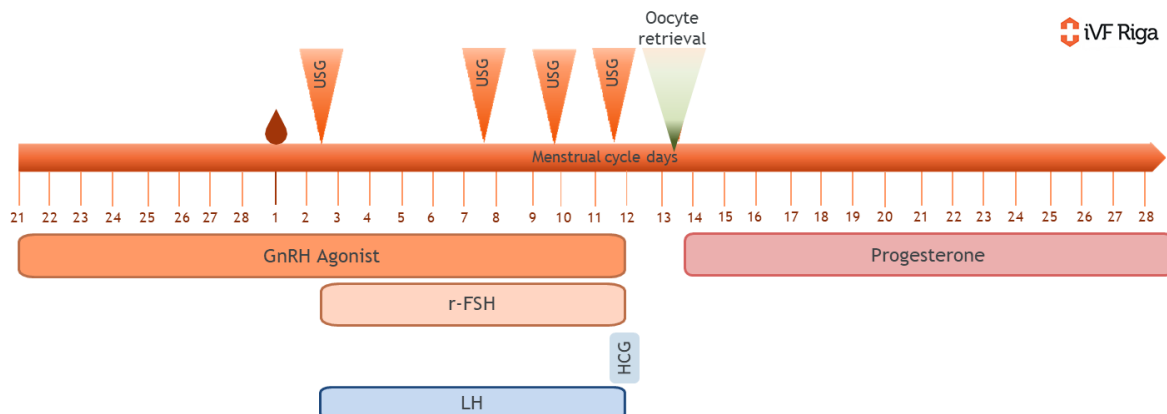


*Figure 5.5.1. Short ovarian stimulation protocol.*  
Images from iVF Riga archive

## 5.6. Common protocols for all the parts of the study

### 5.6.1. Ovarian stimulation

The long (Figure 5.5.1.) or short (Figure 5.5.2.) controlled ovarian stimulation protocols were used for both autologous and donor cycles with recombinant follicle stimulating hormone (rFSH).

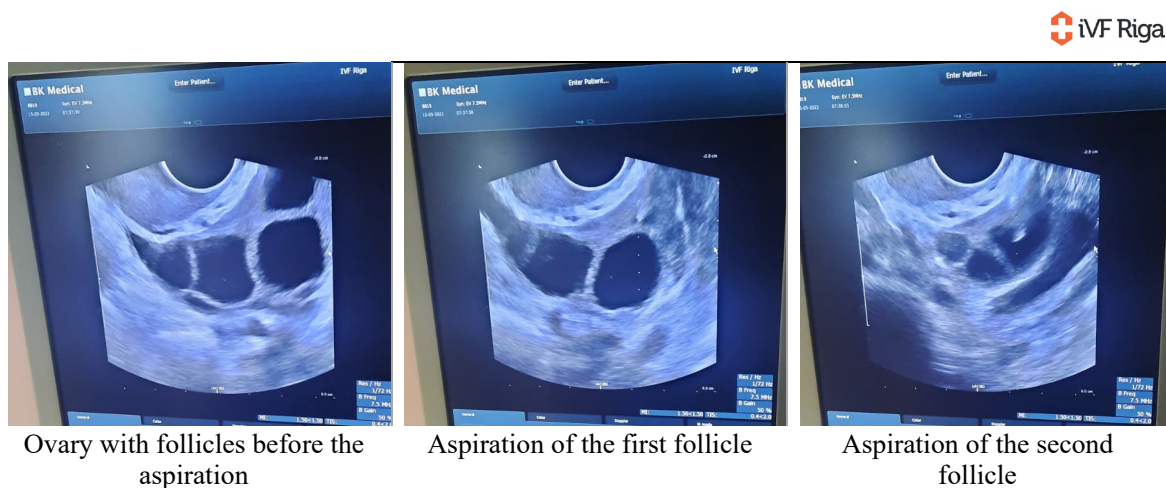


*Figure 5.5.2. Long ovarian stimulation protocol.*  
Images from iVF Riga archive

Controlled ovarian stimulation was performed using recombinant follicle-stimulating hormone (Follitropin Alfa) and gonadotropin-releasing hormone antagonist (Ganirelix acetate injection or Cetrorelix acetate). All the dosages were used considering ovarian reserve and anti-mullerian hormone values in patients' medical histories. When the lead follicle reached its mean diameter 18–20 mm, 6500 IU human chorionic gonadotropin (hCG) agonist were injected subcutaneously for ovulation induction.

### 5.6.2. Oocyte retrieval

Oocyte retrieval was performed 35–36 h after hCG injection. Follicles were aspirated, and oocytes were washed in mHTF washing medium (Life Global, USA) with human serum albumin supplement (Figure 5.6.1.). After washing, oocytes were cultured in fertilization medium with human serum albumin supplement (Life Global, USA) at 5.5% CO<sub>2</sub> and 37 °C (Planer benchtop BT-37 incubator) for 2 h before oocyte denudation. Oocyte denudation was performed by mechanical pipetting 40 IU/ml hyaluronidase in the same medium and incubated at 5.5% CO<sub>2</sub> and 37 °C (Planer benchtop BT-37 incubator) for 2 h before intracytoplasmic sperm injection.



*Figure 5.6.1. Oocyte retrieval manipulation. Images from iVF Riga archive*

### 5.6.3. Intracytoplasmic sperm injection (ICSI)

ICSI was performed in a medium with human serum albumin supplement (Life Global, USA) at 200 x magnification using Nikon Ts-i microscope (Figure 5.6.2.). Immediately after ICSI, the injected oocytes for time-lapse microscopy cycles were placed individually in pre-equilibrated culture dishes (EmbryoSlide; Unisense Fertilitech A/S) with 25 µl medium supplemented with human serum albumin (Life Global, USA) under oil (Lite Oil, Life Global) at 37 °C and 5% O<sub>2</sub>, 6% CO<sub>2</sub> in a time-lapse incubator (EmbryoScope). Zygotes for conventional incubator (Planer benchtop BT-37) cycles were placed in normal Petri dishes

(Nunc, Roskilde, Denmark) containing culture media (Life Global, USA) with human serum albumin supplement under oil (Lite Oil, Life Global) at 37 °C and 5% O<sub>2</sub>, 6% CO<sub>2</sub>.



Figure 5.6.2. Intracytoplasmic sperm injection (ICSI). Images from iVF Riga archive

**5.6.4. Embryo cultivation**

All embryos were incubated at 37 °C and 5% O<sub>2</sub>, 6% CO<sub>2</sub> and were cultured individually (Figure 5.6.3.) until embryo transfer or embryo biopsy (Figure 5.6.4.) and freezing.

Embryo #	Day 1	Day 2	Day 3	Day 4	Day 5	Result
1						B/FR
2						FR
5						B/FR
6						B/FR
8						Discard
10						B/FR

Figure 5.6.3. Time-lapse view of embryos development by days. Images from iVF Riga archive

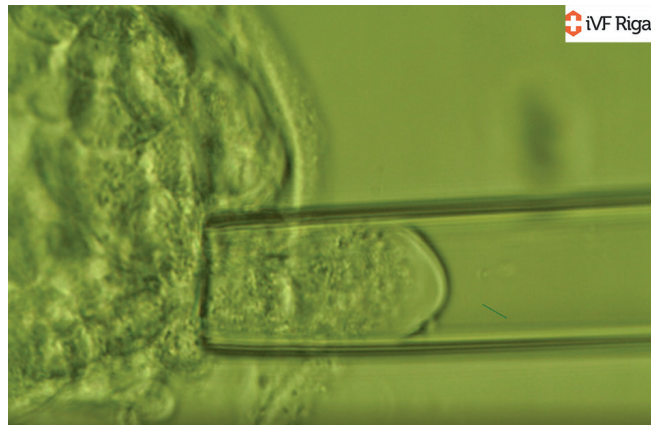


Figure 5.6.4. **Biopsy of the blastocyst.** Images from iVF Riga archive

## 5.7. Outcome assessments

The main end-points were divided into:

1. No pregnancy – no symptoms or signs of pregnancy (“How To Be Reasonably Certain That a Woman Is Not Pregnant” 2016);
2. Biochemical pregnancy (defined as the occurrence of a low peak in  $\beta$ -hCG (< 100 mIU/mL), a rapid decline in urinary or serum  $\beta$ -hCG concentration, and the absence of a significant delay in the onset of the next menstrual period (Annan et al. 2013) (not proceeding further to the clinical pregnancy);
3. Clinical pregnancy (diagnosed by an increasing level of hCG and by ultrasonography of at least one fetus with the presence of heart beat (Zegers-Hochschild et al. 2009));
4. Pregnancy loss (Spontaneous pregnancy demise before 10 weeks of gestational age or before 8<sup>th</sup> developmental week (Kirk et al. 2020).

## 5.8. Statistical analysis

Descriptive statistics are presented using medians, interquartile ranges and percentages. Differences between groups were assessed using Chi-square tests and Fisher’s exact test. Multivariate regression analysis was used to assess the results for the covariates. Potential covariates tested included the age of the female, the number of acquired oocytes after the pick-up procedure, and the number of acquired blastocysts.

In the multivariate regression analysis, the results of Groups II–IV were compared with the “reference” Group I (FET without any additional genetic tests). To compare the results of the ICSI cycles, the end-points b), c) and d) were compared with an end-point a) – no pregnancy: in order to assess the ability of each approach to achieve better clinical outcomes as

compared to the reference group FET approach, and as compared with the negative outcome (no pregnancy).

Two-tailed,  $\chi^2$  tests were used,  $p < 0.05$  was the significance level. Statistical analyses were performed using SPSS 20.0 [SPSS Inc., Chicago, IL, USA].

## 5.9. Equipment used in the study

- Micro manipulator RI Integra 3, laser system Saturn, Cooper Surgical, SN 0195/14;
- EmbryoScope, Vitrolife, SN ES-D2.491;
- Biosafety cabinet Mars 1200 LAF, Cooper Surgical, SN O.02131333;
- Biosafety cabinet Fortuna 1800E IVF Dual, Cooper Surgical, SN O.03131357;
- Biosafety cabinet Fortuna 1200E IVF Clean Air, Cooper Surgical, SN O.03131356;
- Biosafety cabinet BSC Class II LA2-4L1-E, ESCO, SN 2015-94905;
- Biosafety cabinet BSC Class II AC2-3SB, ESCO, SN 2015-99755;
- Microscope Eclipse Ci-L, Nikon, SN 500395;
- Microscope Eclipse Ti, SMZ-1500, Nikon, SN 1633793;
- Incubator Isotherm IFA-32-8-SS, ESCO, SN 2015-T01911;
- Incubator Planer benchtop BT-37, Planer, SN 27970-2012;
- Incubator Planer benchtop BT-37, Planer, SN 27946-2012;
- Next generation sequencing system MiSeq, Illumina, SN 1527131090;
- Genetic analyser 3500, Applied biosystems, SN 27131090;
- Operation theater, iVF Riga;
- Ultrasound systems Voluson P8 BT16, General electrics, SN VP8004017;
- Ultrasound scanner 1202, flexFocus 400, BK medical, SN 1903274;
- Ultrasound scanner 1202, flexFocus 400, BK medical, SN 1902018;
- Hysteroscope Visera OTV-S7V, Digital processor, Olympus, SN 7231193.

## 6. RESULTS

### 6.1. Time lapse microscopy (TLM) or imaging study

In the study of time-lapse based morphokinetic (TLM) evaluation of the embryonic development, selection of high implantation potency embryos was found to improve treatment outcomes, and to decrease EPL and multiple pregnancy rate when implementing selective single embryo transfers (SET).

Three hundred and sixty-seven female patients participated in the study. The TLM group comprised 98 patients. The control group included 269 patients (Figure 6.1.1.).

The median age of control group patients was 39.2. It was 35.9 years for the TML group, which is significantly lower ( $p < 0.0001$ ).

Data on sperm, oocyte and embryo characteristics per ET are summarized in Table 6.1.1.

Table 6.1.1. Sperm, oocyte, and embryo characteristics per ET

Parameters	TLM		Control		p value
	%	n	%	n	
ET from autologous oocytes	71.2	104	44.3	170	< 0.0001
Autologous oocytes		10.7		7.4	< 0.0001
Donor oocytes		13.6		11.8	0.07
Fertilization rate (2PN)	64.4	897/1388	63.3	1723/2721	0.41
2 PN		7.4		6.2	0.004
ET day		4.7±0.7		4.4±0.9	0.002
ET day 3	13		22.3		0.054
ET day 5	81.5		71.5		
Embryo per ET		1.7±0.5		1.7±0.5	0.8
SET	33.6		34.7		0.8
DET	66.4		65.3		
Sperm quality					
Concentration > 18	84.9	124	86	332	0.75
Concentration < 18					
18–10	68.2	15	33.3	18	0.005
10–5	13.6	3	35.2	19	0.06
> 5	13.6	3	29.6	16	0.15
DNS fragmentation		33		13	
35–50	33.3	11	53.8	7	0.21
> 50	15.2	5	0	0	0.14
ET difficult	8.3	12	10.9	42	0.38

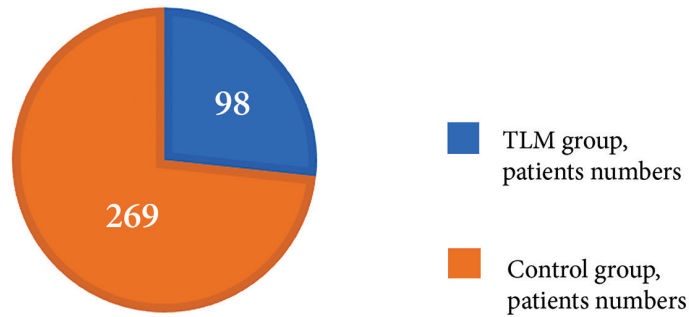


Figure 6.1.1. Patient distribution in the Time lapse microscopy (TLM) study

ETs resulting from autologous oocyte were more common in the TLM group than in the control group (71% versus 44.3%,  $p < 0.0001$ ). In the TLM group more oocytes were retrieved per transfer (10.7 versus 7.4,  $p < 0.0001$ ). There was no significant difference in retrieved donor oocytes (13.6 versus 11.8,  $p = 0.07$ ) (Figure 6.1.2.).

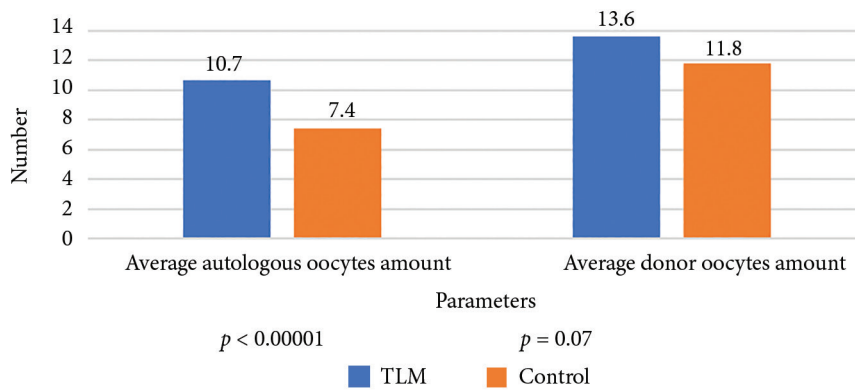


Figure 6.1.2. TLM group and control group oocytes characteristics

Sperm used for fertilization was good quality. There were no statistically significant differences in the sperm concentration (Figure 6.1.3.).

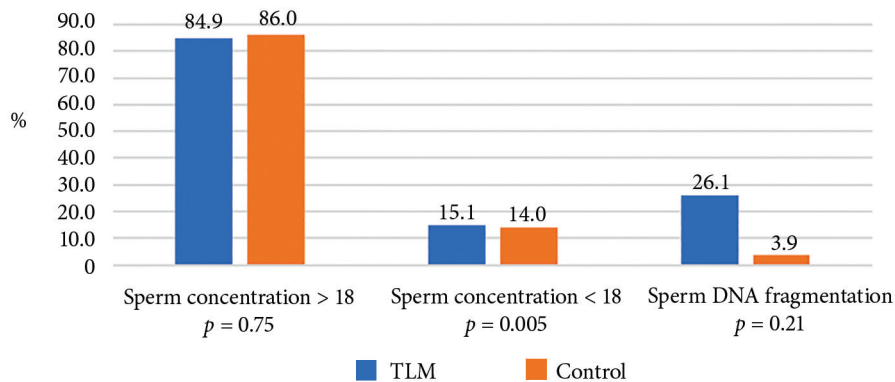


Figure 6.1.3. Characteristics of the sperm quality in TLM group and control group

There was no significant difference in the fertilization rates (64.4% versus 63.3%,  $p = 0.41$ ). In the TLM group however, there were 7.4 fertilized oocytes (with 2 pronucleus: 2 PN) per transfer compared to 6.2 in control group ( $p = 0.004$ ) (Figure 6.1.4.).

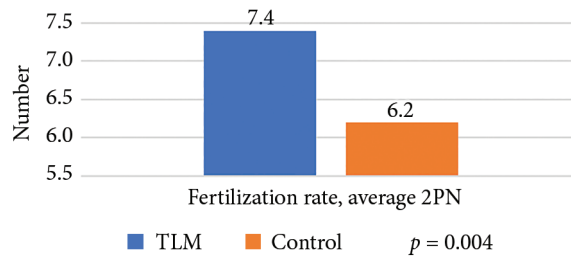


Figure 6.1.4. TLM group and control group oocytes fertilization rates

In both groups blastocysts were transferred more often than day-3 embryos. DET (double ET) were performed on 66.4% of the TLM group and 65.3% of the control group, but SET (single ET), found in 33.6% and 34.7%, is not statistically significant (Figure 6.1.5. and in the Table 6.1.1.). The biochemical pregnancy rate per ET in the TLM group was 50.7% versus 50.3% for the control group ( $p = 0.93$ , OR = 1.02, 95% CI = 0.7–1.49).

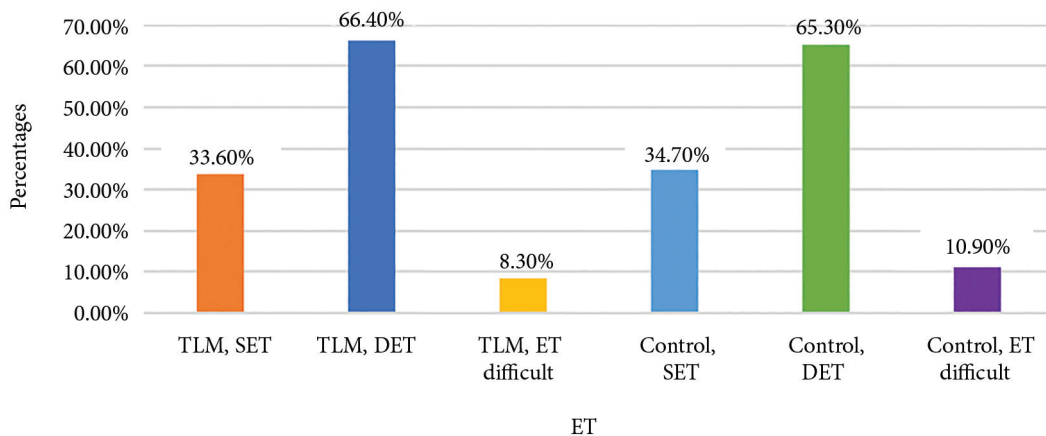


Figure 6.1.5. Transfer characteristics in TLM group and control group

The clinical pregnancy rate per ET were both increased in the TLM group (Table 6.1.2).

Table 6.1.2. Outcome measures

Parameters	TLM		Control		p value	Odds ratio	95% CI
	%	n	%	N			
ET		146		386			
Biochemical pregnancy per ET	50.7	74	50.3	194	0.93	1.02	0.70–1.49
Clinical pregnancy per ET	41.1	60	36.6	141	0.33	1.21	0.82–1.79
Ongoing pregnancy per ET	36.3	53	29.8	114	0.13	1.36	0.91–2.03
Early clinical pregnancy loss (of all clinical pregnancies)	12.1	7	20.7	27	0.23	0.58	0.24–1.42
Biochemical loss (of all biochemical pregnancy)	18.9	14	27.5	54	0.14	0.61	0.31–1.17
Early pregnancy loss (biochemical and clinical loss)	28.4	21	41.8	81	0.045	0.55	0.31–0.99
All transferred embryos		243		638			
Implantation rate (of all transferred embryos)	31.7	77	28.1	179	0.31	1.19	0.86–1.64
Early pregnancy loss (of all implanted embryos)	9.1	7	19.1	35	0.043	0.41	0.17–0.97
<b>Autologous cycles younger than 35 years</b>							
ET		68		92			
Biochemical pregnancy per ET	54.4	37	47.8	44	0.41	1.3	0.69–2.44
Clinical pregnancy per ET	39.7	27	38	35	0.83	1.07	0.56–2.04
Ongoing pregnancy per ET	39.7	27	31.5	29	0.28	1.43	0.74–2.76
Early clinical pregnancy loss (of all clinical pregnancies)	0	0	17.1	6	0.03	0.53	0.41–0.68
Biochemical loss (of all biochemical pregnancy)	27	10	20.5	9	0.49	1.44	0.51–4.04
Early pregnancy loss (biochemical and clinical loss)	27	10	34.1	15	0.5	0.72	0.28–1.86
All transferred embryos		110		161			
Implantation rate (of all transferred embryos)	35.5	39	29.8	48	0.33	1.29	0.77–2.17
Early pregnancy loss (of all implanted embryos)	0	0	25	12	0.001	0.48	0.38–0.61

Table 6.1.2. Outcome measures. Continued

Parameters	TLM		Control		p value	Odds ratio	95% CI
	%	n	%	N			
<b>Over 35 years, only with autologous oocytes</b>							
ET		36		78			
Biochemical pregnancy per ET	33.3	12	30.8	24	0.79	1.13	0.48–2.62
Clinical pregnancy per ET	27.8	10	21.8	17	0.49	1.38	0.56–3.42
Ongoing pregnancy per ET	25	9	15.4	12	0.22	1.83	0.69–4.85
Early clinical pregnancy loss (of all clinical pregnancies)	10	1	29.4	5	0.24	0.27	0.03–2.70
Biochemical loss (of all biochemical pregnancy)	16.7	2	29.2	7	0.41	0.49	0.08–2.81
Early pregnancy loss (biochemical and clinical loss)	25	3	50	12	0.15	0.33	0.07–1.54
All transferred embryos		63		121			
Implantation rate (of all transferred embryos)	15.8	10	14	17	0.75	1.15	0.49–2.70
Early pregnancy loss (of all implanted embryos)	10	1	29.4	5	0.24	0.27	0.03–2.70

The clinical pregnancy rate per ET was 41.1% for the TLM group versus control group 36.6% (OR = 1.21, 95% CI = 0.82–1.79,  $p = 0.33$ ). The ongoing pregnancy rate per ET in TLM group was 36.3% versus the control group 29.8% (OR = 1.36, 95% CI = 0.91–2.03,  $p = 0.13$ ). The biochemical (TLM group 18.9% versus control group 27.5% OR = 0.61, 95% CI = 0.31–1.17,  $p = 0.14$ ) and the clinical pregnancy loss rates (TLM group 12.1% versus control group 20.7% OR = 0.58, 95% CI = 0.24–1.42,  $p = 0.045$ ) rates were significantly reduced in the TLM group.

The total EPL rate in the TLM group was significantly reduced (TLM 28.4% versus control 41.8% OR = 0.55, 95% CI = 0.31–0.99,  $p = 0.043$ ) (Figure 6.1.6.).

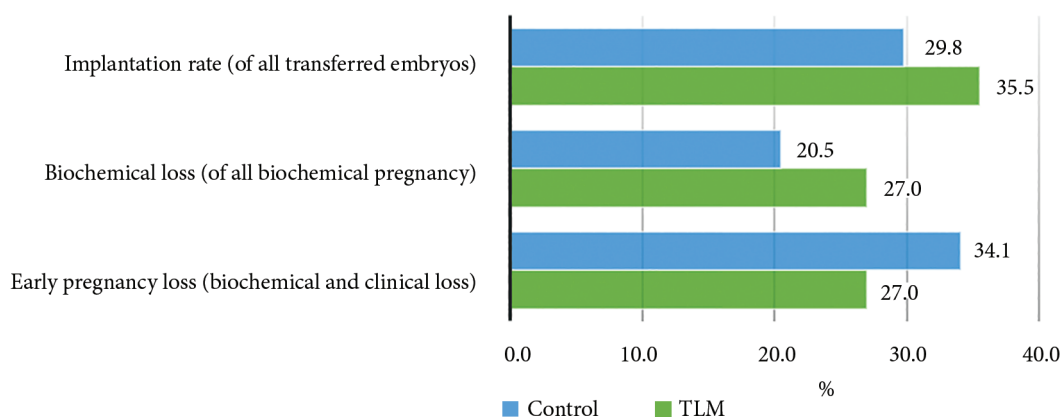
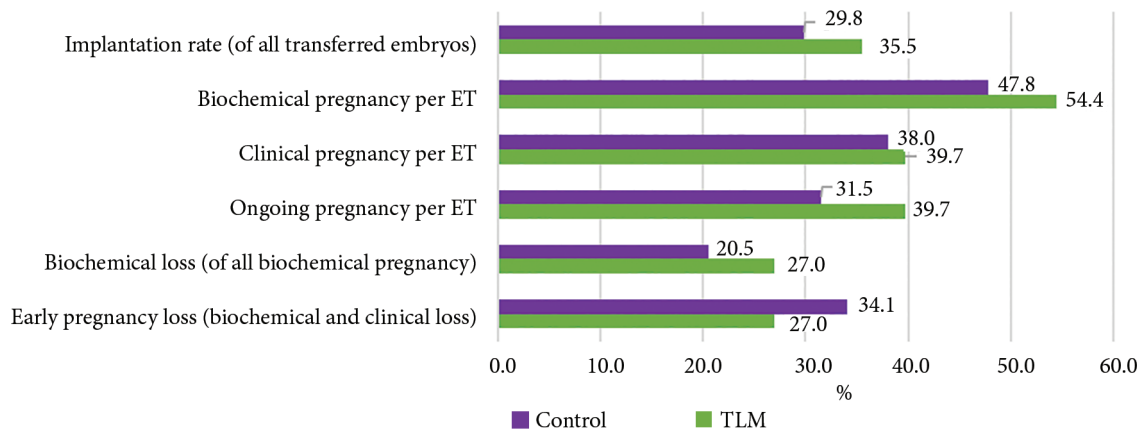


Figure 6.1.6. Implantation and EPL rates in TLM and control group

The implantation rates per ET in both groups were not significantly different (TLM group 31.7% versus control group 28.1% OR = 1.19, 95% CI = 0.86–1.64,  $p = 0.31$ ), but EPL rate per ET was significantly reduced in the TLM group (TLM 9.1% versus control 19.1% OR = 0.41, 95% CI = 0.17–0.97,  $p = 0.043$ ).

To reduce the maternal age influence on EPL, patients in both the groups were divided into two sub-groups: younger than 35 years and 35 years and older (autologous cycles only).



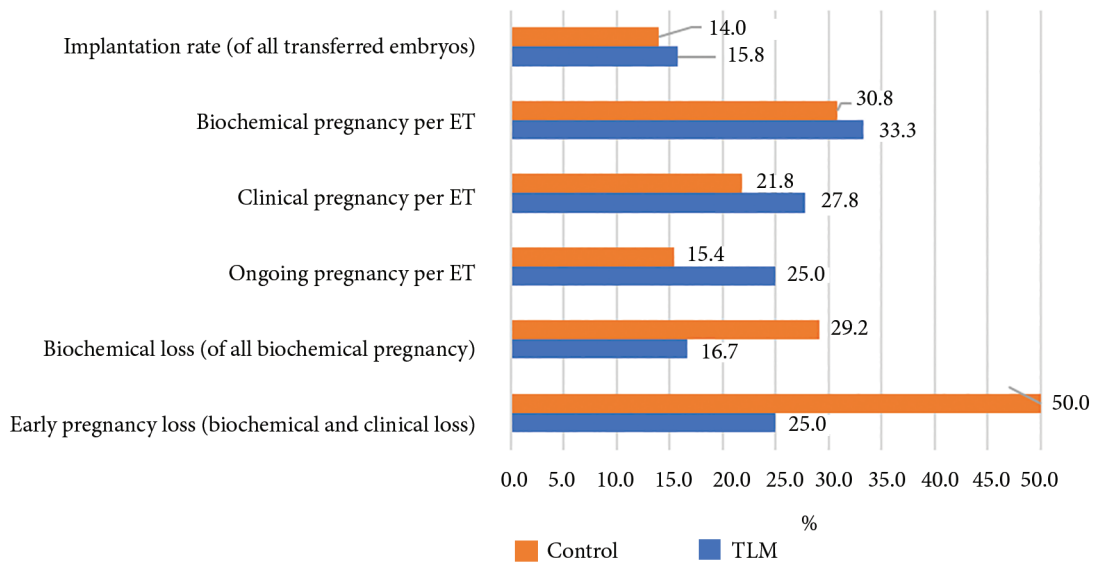
**Figure 6.1.7. Autologous cycles patients younger than 35 years implantation, biochemical and clinical pregnancy rates and EPL rate in the TLM group and control group**

In autologous cycles the patients younger than 35 years became pregnant biochemically and clinically with no statistically significant difference between them, but the ongoing pregnancy rate was increased in the TLM group (TLM 39.7% versus control 31.5% OR = 1.43, 95% CI = 0.74–2.76,  $p = 0.28$ ) (Figure 6.1.7.). It should be noted that only biochemical pregnancy loss was documented in the TLM group (TLM 27% versus control 20.5% OR = 1.44, 95% CI = 0.51–4.04,  $p = 0.49$ ), but clinical pregnancy loss was not observed in the TLM group (TLM 0% versus control 17.1% OR = 0.53, 95% CI = 0.41–0.68,  $p = 0.03$ ).

The implantation rate per ET was 35.5% for the TLM group and 29.8% for the control group (OR = 1.29, 95% CI = 0.77–2.17,  $p = 0.33$ ), which is not statistically significant, but EPL per ET was greatly decreased in the TLM group (TLM 27.0% versus control 34.1% OR = 0.48, 95% CI = 0.38–0.61,  $p = 0.001$ ).

Autologous cycle patients older than 35 years had decreased outcome measures:

- biochemical pregnancy rate (TLM group 33.3% versus control group 30.8% OR = 1.13, 95% CI = 0.48–2.62,  $p = 0.79$ );
- clinical pregnancy rate (TLM group 27.8% versus control group 21.8% OR = 1.38, 95% CI = 0.56–3.42,  $p = 0.49$ );
- ongoing pregnancy rate (TLM group 25% versus control group 15.4% OR = 1.83, 95% CI = 0.69–4.85,  $p = 0.22$ ). Biochemical, clinical pregnancy, implantation and EPL rates per ET were not significantly different. Main outcome measures are summarized in Figure 6.1.8.



**Figure 6.1.8. Autologous cycles patients older than 35 years implantation, biochemical and clinical pregnancy rates and EPL rate in the TLM group and control group**

Logistic regression analysis was done for the TLM group and control group, and, adjusted for age and autologous oocyte rates. There were no significant differences in biochemical, clinical, and ongoing pregnancy rates per ET. However, the EPL rate per ET was significantly lower: OR = 0.41, 95% CI = 0.17–0.98,  $p = 0.044$  (Table 6.1.3).

**Table 6.1.3. Logistic regression analysis adjusted by age and autologous oocytes.**

Parameters	<i>p</i> value	Odds ratio	95% CI
<b>Per ET</b>			
Biochemical pregnancy	0.6	1.12	0.74–1.68
Clinical pregnancy	0.18	1.33	0.88–2.01
Ongoing pregnancy	0.11	1.42	0.93–2.17
<b>Per cycle</b>			
Early clinical pregnancy loss	0.39	0.67	0.27–1.68
Biochemical pregnancy loss	0.11	0.57	0.29–1.14
Early pregnancy loss (biochemical and clinical pregnancy)	0.07	0.57	0.31–1.04
<b>Per ET</b>			
Implantation rate	0.31	1.19	0.85–1.66
Early pregnancy loss	0.044	0.41	0.17–0.98

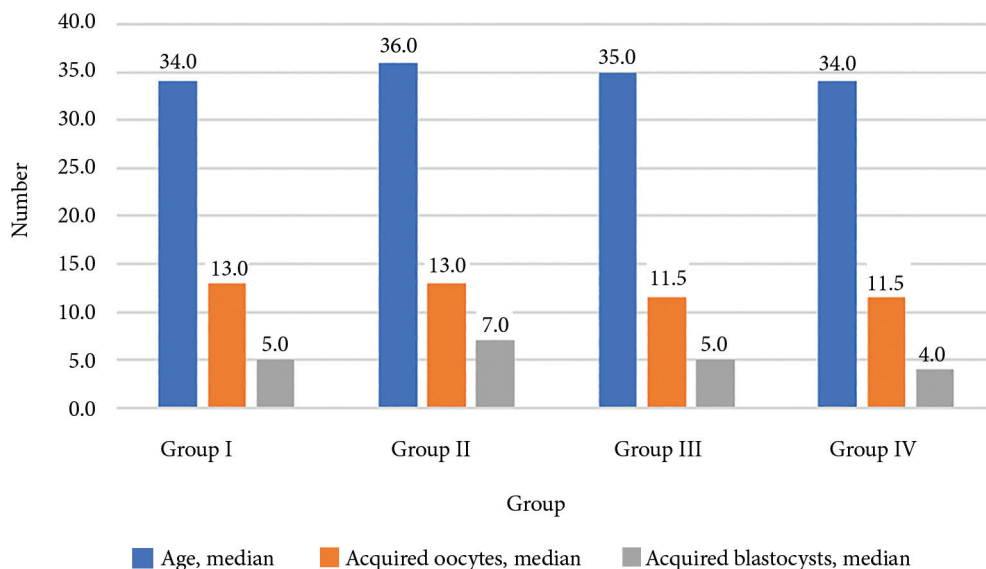
## 6.2. Preimplantation genetic testing for aneuploidies (PGT-A) and endometrial receptivity array (ERA) in a group of RIF in ICSI cycles

The present research suggests that RIF depends on several factors. Successful embryo implantation is an interactive process between the blastocyst and the uterus. Synchronized development of an embryo with uterine differentiation to a receptive state is a prerequisite for attaining pregnancy. The main reasons for implantation failure are summarized in this study.

**Table 6.2.1. Descriptive statistics for the study groups. (Group I – cycles with FET; Group II – cycles with FET+PGT-A; Group III – cycles with FET+PGT-A+ERA test; Group IV – cycles with FET+ERA test)**

Variable	Group I	Group II	Group III	Group IV	<i>p</i>
Age (median, IQR)	34.0 (37.0–32.0)	36.0 (38.0–34.0)	35.0 (37.0–33.0)	34.0 (38.0–32.5)	0.21
Acquired oocytes (median, IQR)	13.0 (16.0–10.0)	13.0 (17.0–9.0)	11.5 (18.0–8.0)	11.5 (17.5–6.8)	0.83
Acquired blastocysts (median, IQR)	5.0 (7.0–3.0)	7.0 (9.0–3.0)	5.0 (7.0–3.0)	4.0 (5.5–3.0)	0.02

The median age of the entire study group for the females was 35 years (Figure 6.2.1.). Female age and acquired oocytes counts showed no differences between the all Groups I–IV. Table 6.2.1. shows the all the statistics for this study cohort.



**Figure 6.2.1. Statistics for the study groups (Group I – cycles with FET; Group II – cycles with FET+PGT-A; Group III – cycles with FET+PGT-A+ERA test; Group IV – cycles with FET+ERA test)**

The counts of acquired blastocysts were higher for Group II, compared both to the Group I ( $p = 0.03$ ) and Group IV ( $p = 0.004$ ) (Table 6.2.2.). All these factors were used as covariates in multivariate regression analysis.

**Table 6.2.2. Comparative analysis (p values) for counts of acquired blastocysts between the Groups I-IV**

	Group I	Group II	Group III	Group IV
Group I		<b>0.03<sup>1</sup></b>	0.52	0.13
Group II			0.11	<b>0.004<sup>1</sup></b>
Group III				0.07
Group IV				

Table 6.2.3. shows the clinical outcomes (no pregnancy, biochemical pregnancy, clinical pregnancy, and pregnancy loss) in Groups I-IV. Group I – cycles with FET; Group II – cycles with FET+PGT-A; Group III – cycles with FET+PGT-A+ERA test; Group IV – cycles with FET+ERA test.

**Table 6.2.3. Clinical outcomes in Groups I-IV**

Parameters		Group				Total
		Group I	Group II	Group III	Group IV	
No pregnancy	Number	35	19	30	11	95
	%	48.6	28.4	41.7	50.0	40.8
Biochemical pregnancy	Number	4	12	1	1	18
	%	5.6	17.9	1.4	4.5	7.7
Clinical pregnancy	Number	32	33	40	8	113
	%	44.4	49.3	55.6	36.4	48.5
Pregnancy loss	Number	1	3	1	2	7
	%	1.4	4.5	1.4	9.1	3.0
Total	Number	72	67	72	22	253

Analysis with chi-square test of the clinical outcomes of biochemical pregnancy, clinical pregnancy and pregnancy loss to compare with the end-point of “no pregnancy”, Group II (FET+PGT-A) showed a statistically significant higher chance of achieving both biochemical ( $p = 0.01$ , OR = 5.5) and clinical pregnancy ( $p = 0.049$ , OR = 2.3), as compared with the Group I (FET with no additional tests).

The more detailed analysis of the results by the Fisher’s exact test was done to compare the Group II (FET+PGT-A) and the Group III (FET+PGT-A+ERA) biochemical pregnancy statistically significant

results, which showed that ERA test statistically significantly decreases biochemical pregnancy loss (Table 6.2.4.).

*Table 6.2.4. Statistical analysis of the clinical outcomes in Groups I-IV*

	Group I	Group II	Group III	Group IV
Group I		<b>0.005<sup>1</sup></b>	0.37	0.99
Group II			<b>0.001<sup>1</sup></b>	0.07
Group III				0.49
Group IV				

The clinical pregnancy was statistically significant in the Group III (FET+PGT-A+ERA) comparing to the Group II (FET+PGT-A).

*Table 6.2.5. Statistical analysis of the clinical outcomes in Groups I-IV*

	Group I	Group II	Group III	Group IV
Group I		<b>0.08<sup>1</sup></b>	0.18	0.99
Group II			<b>0.002<sup>1</sup></b>	0.43
Group III				0.33
Group IV				

### **6.3. The application of PGT-A for carriers of balanced structural chromosomal rearrangements**

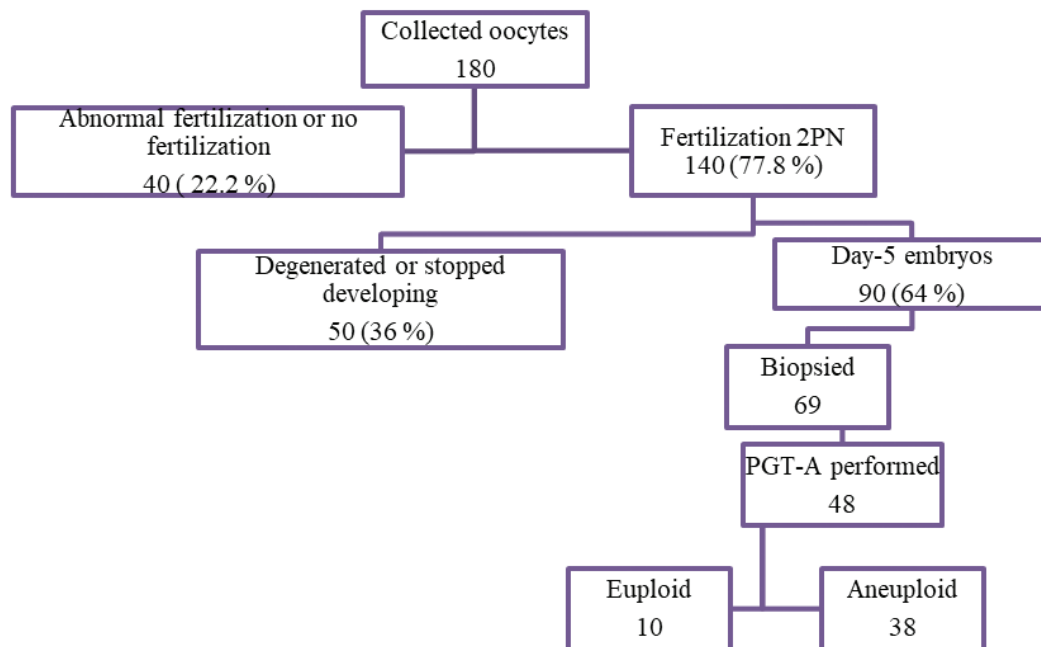
Balanced rearrangements represent one of the most common forms of genetic abnormality. This study examined 10 couples who had abnormalities in their karyotypes and previous histories of reproductive results. One of the main factors of recurrent pregnancy loss and implantation failure is aneuploid embryos. The study shows that expectancy of total embryo aneuploidy rates is higher in those carriers, than in people with normal karyotypes (Table 6.3.1., full information is available in the Appendix III).

A total of 180 oocytes were collected. 140 (77.8%) fertilized normally, resulting in 90 (64%) of the embryos reaching the maturation stage on day 5. A biopsy was performed on 69 blastocysts and PGT-A was done for biopsies from 48 embryos. The total abnormality rate from the 48 embryos was 79.2% (including 10 – euploid, 38 – having chromosomal structural aberrations or aneuploidies) (Figure 6.3.2.).

*Table 6.3.1. All the patient’s pregnancy history with data of ovarian stimulation, insemination, embryo biopsy with current results*

<sup>1</sup> Statistically significant

Pair No.	Karyotype	Number of euploid embryos	Number of aneuploid embryos	Results
1	46,XX,t(1;6) (p22;p25)	1	3	Pregnancy did not occur
	46,XY			
2	46,XX	2	2	Natural pregnancy
	46,XY,t(4;12) (q31;q13)			
3	46,XX	4	2	Pregnant after IVF
	45,XY,der(13;14) (q10;q10)			
4	46,XX,t(9;17) (p10;p10)	1	2	1 child after IVF
	46,XY			
5	46,XX,t(6;10) (p21.3;q22.3)	0	1	Needs new stimulation
	46,XY			
6	46,XX	0	2	Needs new stimulation
	45,XY,der(13;14) (q10;q10)			
7	46,XX	2	2	Pregnant after IVF
	46,XY,t(1;3) (q12;q29) [4]/46,XY[15]			
8	46,XX,t(13;20) (q34q13)	0	6	Needs new stimulation
	46,XY			
9	46,XX,t(8;11) (p23;p15)	0	4	Needs new stimulation
	46,XY			
10	46,XX	0	4	Needs new stimulation
	45,XY,der(13;14) (q10;q10)			



**Figure 6.3.1. Diagram of study flow, data of collected oocyte and embryo biopsy with current results**

Twenty two embryos, were analyzed from the carriers of Robertsonian translocations.

Of these 22, the rate of unbalanced translocations was 81.8%, whereas from the reciprocal translocation carrier group, the unbalanced translocation rate of embryos was 76.9%.

Unbalanced translocation rates in embryos resulting from woman were higher than those from men, 88.9% versus 73.3%. This study involved 18 embryos from 5 couples where the female was a carrier of balanced translocation and 30 embryos from 5 couples where the male was carrier.

The women ranged from 31–36 years of age with a median age of 34 and the men ranged 28–46 years with a median age of 39.

The highest euploidy rate was in the male carrier group – 26.7%, and the lowest was in the Robertsonian translocation carrier group – 18.2%. Sporadic aneuploidy of 68.2% was also highest in the Robertsonian translocation carrier group, and lowest in the female carrier group – 11.1%.

The unbalanced translocation rate in embryos was highest in the female carrier group – 77.8%, and lowest in Robertsonian group – 13.6%.

One female patient with a reciprocal translocation between 13 and 20 chromosomes had unbalanced chromosomal translocations inherited from her mother in all six embryos tested. One male patient with type D Robertsonian translocation between 13 and 14 chromosomes had aneuploidy in 12 embryos from 16 that were tested.

After PGT-A results were obtained, 5 of 10 female patients needed new stimulation as PGT-A showed 0 euploid embryos. In 1 female patient with reciprocal translocation, pregnancy did not occur after FET. One patient, whose husband was a reciprocal translocation carrier, conceived naturally. Two patients having husbands being Robertsonian and reciprocal translocation carriers, got pregnant after iVF treatment. One child was born from a woman carrier of -reciprocal translocation (Detailed information is available in the Table 6.2.3. in the Appendix III).

## 7. DISCUSSION

Infertility is a widespread problem that affects many couples worldwide. It has been estimated that up to 15% of couples experience infertility. It can be caused by many different factors, including problems with ovulation, sperm quality, or the reproductive system itself. One of the most common causes of infertility is a recurrent implantation failure, which occurs when embryos fail to implant in the uterus after transfer. Recurrent implantation failure is an important challenge in assisted reproduction, as it significantly reduces the chances of a successful pregnancy. In this thesis, strategies and techniques are discussed that can be used to decrease the incidence of early pregnancy loss and increase the chances of successful pregnancy in patients with infertility. The research focused on three studies that investigated the use of different methods and combination of them to reach the result in the fastest way. The idea of investigation of different levels (embryonic and endometrial) of the IVF processes may provide the answers to the questions which factors affect the implantation most and what we need to avoid or identify in the group of patients with recurrent implantation failure. The studies that are included into the present thesis investigated the following embryonic factors such as morphology, kinetics and genetics, which represent the life perspective and potential of the embryo. One of the uterine factors, such as endometrium receptivity also reflects the women's health condition and have been studied by the author of the thesis.

The main goal of assisted reproductive technologies is to improve quality and effectiveness of infertility treatment in IVF cycles, and to reduce RIF and complications during procedures. For this purpose several methods of embryo selection were tested – one of them is time-lapse system, another – PGT-A and clarification of the endometrium functionality. As complies with the research of the both authors (Cozzolino et al. 2020; Lessey and Young 2019b). Altogether they elucidate the individual capabilities of patients to produce high-quality gametes, the ability of these gametes to fertilize and further divide, as well as the individual characteristics of a woman regarding implantation of the fertilized gametes or – an embryo. It is established that the quality of the gametes and the quality of the embryos strongly correlates (Campbell et al. 2013). The “gold standard” for preparing and conducting an IVF procedure is the evaluation of three fundamental factors – the embryonic, uterine and woman's health factors (immune factor, coagulation factors), upon which the outcome of the IVF directly depends.

Study I – during this study the main focus was to understand whether the TLM (time-lapse system) can affect the fertilization of the oocytes and developing of the embryos in comparison with the control group, in which the embryos were cultivated in the standard incubator, and also how the incubation in the closed system (TLM) can affect pregnancy outcomes and pregnancy loss until seventh week of pregnancy. Also, utilizing a TLM system can offer additional advantages, such as improved and flexible management of laboratory

workload. The selection of embryos based on visual indicators of presumed quality has mostly relied on subjective application of a decision tree. The incorporation of multiple visual parameters has resulted in enhanced outcomes, and the widespread adoption of the widely known 'Gardner criteria' serves as a good illustrative example (Apter et al. 2020). Kirkegaard group and later on Milewski group also showed that TLM system positively affected fertilization and cultivation of embryo till blastocyst stage in comparison with the standard incubation (Kirkegaard et al. 2013; Milewski and Ajduk 2017). Considering that the embryonic factor is one of the main factors influencing implantation (Balaban et al. 2011), one of the main tasks is to ensure the quality of gametes (oocytes and spermatozoa).

Thus, the specific selection of embryos is fundamental to the process of obtaining positive results in the IVF cycle. To accomplish successful outcome, precise standardized methods need to be employed, including the selection of embryos according to three parameters. The first being morphology, the second – kinetic properties of the embryo, and the third – genetic evaluation of the blastocyst.

The study found that the time-lapse imaging and monitoring system is a safe non-invasive method to improve reproductive outcomes. Stable and controlled incubation conditions and embryo monitoring *in-situ* provide a consistent milieu for the developing embryo. Changes in temperature, air and pH which the embryo is exposed to in conventional incubators can be harmful (De Mouzon et al. 2010). The study demonstrated increased biochemical, clinical, and ongoing pregnancy rates, and a statistically significant decrease in early pregnancy loss rates.

The median age of patients involved in IVF treatment ( $37\pm 2$  years) (Eurostat, "Fertility Statistics – Statistics Explained," n.d.) has increased to  $38\pm 6$  years in recent years, which is a risk factor for obtaining an insufficient number of good quality oocytes. To improve this aspect various modified, personalized protocols are used to acquire a larger quantity and usually better quality of cells. In the Study I the median age of the patients which were involved in to the study was 35.9 years for the TLM group and 39.2 years for the control group, that is statistically different ( $P < 0.0001$ ). That why in this study patients were divided in two subgroups – younger than 35 and over 35 years. The quality and the age of the oocytes are one of the most important factors for the future embryo quality, its ability to cleavage and evolve, and the possibility of implantation later on. As indicated by many authors, maternal age have been associated with a strong decline in the production of healthy and high-quality oocytes which results in reduced fertility in women that are older than 35 years (Mikwar, MacFarlane, and Marchetti 2020).

Also, the quality of the sperm can affect the prospects of the embryo development and quality. In the Study I only two characteristics of sperm from male partners was tested – the sperm concentration and DNA integrity, and in the both groups (TLM and control group), the parameters of the sperm were the same ( $p = 0.21$  and  $p = 0.14$ , respectively). Since the preparation of the sperm includes swim-up method that selects the most motile sperm in

the upper fraction and leaves low or no motility sperm in the lower fraction, the concentration of the sperm does not play a crucial role in the fertilization. It is known that sperm DNA integrity plays more important role in fertilization and proper division of embryos than the sperm concentration (Bungum et al. 2007), as it affects the fragmentation of the blastocysts and their quantity and directly their ability for implantation. Therefore, the obtained results from the TLM and control groups are comparable.

The fertilization of the oocytes till 2 PN stage in both subgroups (younger 35 and over 35 years of age) in the TLM group shows statistically significant better results ( $p = 0.004$ ) comparing to the controls. This confirms that the comfortable environment, which is provided by TLM system influences the fertilization rate and embryo developing. Interestingly, higher cleavage rate till the 3<sup>rd</sup> day and the 5<sup>th</sup> day was statistically higher in the TLM group in comparison with control group. It is in line with the study of the Milewski group (Milewski and Ajduk 2017). The results of the study support the use of the TLM system as successful tool for improving the fertilization of oocytes to the 2PN stage, as well as for the cultivation of embryos up to 3–5 days in all study groups (donor cells, patients younger and older than 35 years) compared with the controls.

The TLM selection model has been validated in several other studies (I. Rubio et al. 2014; Pribenszky et al. 2010; Wong et al. 2010). It also specifies the conditions for excluding impaired embryos from the transfer, in order to prevent early pregnancy loss. TLM system is not only a high-quality incubator, which provides correct media for better embryo development, but also the specific artificial intelligence, which contributes the algorithm impeding correct choice of the embryo for the transfer. As described in the Basile research the current methods of embryo evaluation relies mostly on static observations of the morphology. These observations are critically restricted to specific times and, as the development of the embryo is a dynamic process, many critical stages may go unnoticed. This imaging system in the embryology and the possible use of it is a novel selection tool (Natalia Basile and Meseguer 2012). This approach is also supported by ESHRE guidelines (Apter et al. 2020).

ESHRE guidelines provide the long list of recommended condition for embryologist regarding education of the staff, finding the appropriate system settings. During Study I the effectiveness, utility and accessibility of the technology was validated and proved, as well as counsel patients alongside offering described method.

The use of TLM and the selection of high-quality of embryos directly affect the further onset of pregnancy and its outcome. In the Study I it has been shown that in spite of similar implantation rate in all transfer groups, the early pregnancy loss significantly decreases in the TLM group in comparison with the controls ( $p = 0.043$ ). The same outcome have been demonstrated by Meseguer, as clinical pregnancy rate for treatments using TLM, comparing to standard incubator, significantly increased (Marcos Meseguer et al. 2012).

Therefore, TLM can be recommended for the implementation in the clinical routine of IVF clinics. Especially important is TLM ability to decrease early pregnancy loss rates, which

is a devastating clinical problem for both the couple and the physician. Implementing this non-invasive method would be the first step to improve the outcomes in IVF treatment, and to decrease EPL rates.

Genetic testing of embryos for chromosome composition is one of the fundamental criteria for the quality of embryos, as well as factors affecting implantation and reducing the number of unsuccessful cycles. It is worth noting that both methods – non-invasive TLM and invasive PGT-A – are interconnected and give the best result only in combination. Further, an invasive method for examining embryos at the blastocyst stage requires TLM due to the impossibility to coordinate the time of the trophectoderm biopsy.

Chromosomal aberrations in embryos occur for several reasons, the most common of which are an insufficient maturity of the oocytes or age-related changes in the oocytes associated with a weakening of the spindle apparatus, as well as various hormonal and immune factors. The male factor plays an important role in the formation and further development of the embryo. Abnormal DNA integrity can cause aneuploidy in embryos. It is well known that the percentage of euploid and aneuploid embryos depends on the woman's age: in the age group up to 35 years, every 5<sup>th</sup> oocyte leads to the formation of an euploid embryo; in the age group 35–39 years – every 9<sup>th</sup> oocyte, and after 39 years – every 16<sup>th</sup> oocyte (Esteves et al. 2018). The POSEIDON criteria were developed to provide a more detailed picture of patients with poor ovarian response and to help physicians in the management of these patients (Roque et al. 2021). The new standards are challenging the current criteria of patients with poor ovarian response in favor of the newly defined concept of “low prognosis” (Esteves et al. 2018). Some of the authors implies that the selection of the embryos until the blastocysts stage is completely enough to achieve the clinical pregnancy (Cruz et al. 2011). Thus, the use of the PGT-A method makes it possible to reduce the number of obviously negative embryo transfers (or transfers with aneuploid embryos) in genetically healthy couples. It also makes it possible to find embryos free from chromosomal defects in patients having an altered karyotype, thereby reducing the percentage of implantation failure.

For the purpose to improve the results of IVF treatment and to decrease early pregnancy loss, in the Study II two invasive methods were tested: preimplantation genetic testing for aneuploidies and endometrial receptivity array – which both focus on patients having recurrent implantation failure. It is well established that maternal age is associated with a rapid decline in the production of healthy and high-quality oocytes and euploid embryos which results in reduced fertility in women older than 35 years (Mikwar, MacFarlane, and Marchetti 2020). In the last ESHRE guidelines edition (ESHRE PGT Consortium good practice recommendations for the detection of structural and numerical chromosomal aberrations) it is recommended to use PGT-A method as one of the treatment factors for patients with recurrent implantation failure and recurrent pregnancy loss (Coonen et al. 2020).

In this study the women of all groups were the similar age ( $p = 0.21$ ) with recurrent implantation failure in the medical history were analyzed. The embryonic outcomes in four

groups showed statistical differences ( $p = 0.02$ ), due to variations in the quantity of acquired blastocysts. However, as the single embryo transfer (SET) for patient in each group was performed, the results are comparable. Furthermore, in the study groups, additional examination was provided for both the embryo and the endometrium. In the control group – Group I, SET was performed without any additional examination of the embryo (PGT-A) or the endometrium (ERA test).

In the Group II PGT-A method was provided to determine the chromosomal status of an embryo prior to transfer. An euploid embryo refers to an embryo that possesses 23 haploid chromosome pairs and 2 sex chromosomes. On the other hand, aneuploid embryos are those that exhibit alterations in the number or structure of chromosomes.

In this study for Groups II and III the euploid embryos have been used, but for Groups I and IV PGT-A test was not provided. Therefore, the chromosomal status of embryo in the latest groups was not clear. From another studies (Cozzolino et al. 2020) it is known that the euploid embryos have highest possibility of implantation, less percent of early pregnancy loss, higher percent of live birth and the similar results have been shown in this thesis. The possibility of the implantation considerably reduces in a case of embryos aneuploidy. For example, the changes in the 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> chromosomes stops the development of the embryo in the first 8 days. The changes in the 16<sup>th</sup>, 19<sup>th</sup>, and 22<sup>nd</sup> chromosomes predict the obturation of the pregnancy around week 7–8<sup>th</sup> of pregnancy (C. Rubio et al. 2013). The missing one of the pair of the chromosomes blocks the development of the embryo in the early stages (Thornhill et al. 2005). The results of the studies presented in this thesis confirmed these data as well. Therefore PGT-A is considered essential for women over 35 to improve IVF outcomes and to decrease RIF rates.

In Group III the PGT-A and ERA test were provided in combination. ERA tests determination of the implantation windows in the endometrium is one of the possible factors to reduce implantation failure rates. The implantation windows are forming in the endometrial structure in day 7 after ovulation. The different factors such as inflammation process in the uterus, Asherman syndrome, as well as organic changes as polyps or submucosal myomas, can affect implantation process negatively (Bender Atik et al. 2022). Therefore, patients with these confounding factors were excluded from the present study. Based on that, the ERA test, which provides genetic profiling of the endometrium and is not directly linked to the factors mentioned above. The ERA test is more precise and more important method to determine the day and hour for the implantation, as the result patients with recurrent pregnancy loss, have higher rate for clinical pregnancy and significantly decrease biochemical pregnancy loss, if this method combined with PGT-A. Ruiz-Alonso with the group also shows decrease in the early pregnancy loss with the ERA tests (Maria Ruiz-Alonso et al. 2013). In the current ESHRE guidelines the effect of the ERA test for the recurrent pregnancy loss patient group was not described. General focus in ESHRE Recurrent pregnancy loss guidelines was made on the morphological and immunological condition of the endometrium, but the

genetic status of the endometrium was not reviewed. (Bender Atik et al. 2022). However, data from Study II confirm that combination of several factors play a crucial role in recurrent implantation failure, including embryo quality (both morphological and genetic) and also endometrial receptivity (window of implantation). Two groups that showed the best results of the achieving clinical pregnancy were the Groups when combined treatment methods were used (FET+PGT-A 49.3% and FET+PGT-A+ERA 55.6%, respectively). The clinical pregnancy rate is the main criterion for determining treatment effectiveness. These results demonstrate that the combination of genetic testing of the embryo (PGT-A) prior embryo transfer and genetic assessment of the endometrium (ERA test), which establish the exact time of the embryo transfer are the best treatment model, as they decrease biochemical loss and increase the clinical pregnancy rate. Combined implementation of the PGT-A and ERA test improves biochemical and clinical pregnancies rates either in patients with recurrent implantation failure, compared to cycles where embryo transfers were carried out without additional tests or if the methods were provided one at a time.

The results in the Group IV possibly can be explained by the small number of patients for whom only the ERA test was applied. Therefore, the use of the ERA test alone is still debatable. However its use in combination with PGT-A enhances the results of implantation, as PGT-A gives complete enlightenment about the chromosomal structure of the transferable embryo, its potential for implantation and development. Clarification of the day and time of transfer is determined by the ERA test.

As mentioned before, other putative factors can have an impact on fertility treatment, which were not investigated in this study, including uterine microbiome, different immunological factors such as increase of pro-inflammation interleukins, overproduction of the TNF- $\alpha$ , abnormal function of natural killers (NK) and specifically uterine NK cells along with non-immunological factors. Also, as mentioned earlier, the male factor or sperm DNA integrity is thought to play a role in RIF (Zini et al. 2008). Other studies, however, failed to confirm this (Carol Coughlan et al. 2015). Assessing of their relative importance is crucial. Such studies would help clinicians reduce recurrent implantation failure and assist couples to achieve successful pregnancies. Perspective studies should be continued.

Study III also demonstrated that PGT-A becomes important not only for a group of patients with recurrent implantation failure having normal karyotypes, but more importantly, for a group of patients having balanced translocations. The results showed that rates of aneuploid embryos in these patients are higher when compared to patients with normal karyotypes. Therefore, they are at significantly higher risk of developing RIF – especially if a female is the carrier of a translocation. This is considered as good practice and has been strongly recommended by the ESHRE PGT Consortium (Coonen et al. 2020).

Comparing male and female carriers, the total aneuploidy rates higher in the maternal group, and the percentage of embryos related to translocation is 4.7 times higher in the maternal group than in the paternal one, even taking maternal age into account. This

explains the phenomenon, that female translocations carriers have higher percentage of miscarriages and fetal abnormalities than partners of male carriers. This difference in the rate of embryos with unbalanced translocations between male and female translocation carriers presented in this study is statistically significant (12% vs. 24%,  $p < 0.05$ ), which is in concordance to other authors (Idowu et al. 2015).

There are only a few studies investigating patients with balanced translocations and comparing them for all possible aspects of IVF outcomes (Idowu et al. 2015). Usually, the number of the patients is small as this is a rare disease. The present study provides additional data to this rare condition, as well support the evidence of mandatory implementation of PGT-A in the infertile treatment patients with Robinson translocation. More effective consultations and patient evaluations are needed regarding prognostic accuracy of aneuploid embryos. More data on balanced chromosomal changes are required.

In summary, the reasons for implantation failure are multifactorial. The most common reasons are chromosomal aberrations of an embryo, endometrium abnormality, different kinds of coagulopathies, endocrine disorders, and combinations and interplay between these factors. The present work conducted during the last years analyzed and observed the patients facing these problems. In these 3 studies the most important factors of implantation – as embryonic and endometrial – were investigated and analyzed. Taking into consideration review of the literature and the results of described studies, the algorithm of fertility treatment is outlined in chapter 9. This unique algorithm provides detailed practical clinical steps for IVF procedures regarding fertility treatment of patients having an recurrent implantation failure in the medical history. To date, the ESHRE Recurrent Implantation Failure Guidelines, have not delineated specific recommendations for patients having implantation failure, however, the ESHRE group is currently updating these guidelines.

For patients having 2 failed pregnancies during the IVF cycles, the fundamental recommendation is a detailed examination of both partners including karyotyping them to exclude different kinds of aberrations in their own chromosomes. It is also important to make detailed examination of the embryos, including embryo cultivation until the day 5 (blastocyst stage) through daily or on-line monitoring using time-lapse microscopy. This step is expected to improve the process of excluding embryos with increased fragmentation and of morphologically poor quality. The use of this standardized algorithm and performing the appropriate selection (kinetic and morphological) will increase ET implantation potential. This method provides information about kinetics of embryo development (or embryo meiosis rate) using different validated algorithms (M. Meseguer et al. 2011; I. Rubio et al. 2014; N. Basile et al. 2015). It also provides a good image of embryo development following its transfer to the uterus cavity.

PGT-A is the most promising method in the evaluation of embryo quality and prognosis for implantation. It is worth noting that more precise prognostic results are obtained analyzing material from the embryo trophectoderm and not from the blastomere (3<sup>rd</sup> day embryo).

Examination of specifically those cells gives reliable testing results. This method improves pregnancy outcomes in patients having implantation failure. It also reduces most of the serious diseases that might occur in their future offspring. Accordingly, the complete examination of the embryo is the first step for patients having 2 failed pregnancies during IVF cycles.

One of the aspects for patients with recurrent implantation failure is the detailed examination of the endometrium, which includes evaluation of endometrial thickness in the first and second phase of the menstrual cycle, to exclude the presence of organic damage like polyps, hyperplasia, adhesions as well as myomas, adenomyosis and congenital anomalies. Observation of patients with implantation failure confirms that better prognostic potential requires examination of the endometrium regarding the implantation window. Different factors, like abortion, inflammatory processes of endometrium, severe or chronic hormonal imbalance, can lead to a significant shift of the window of implantation. The examination of window of implantation can be recommended in cases of implantation failure as an additional examination.

It is important to stress that the recommendations mentioned do not guarantee absolute or ultimate success following embryo transfer. More studies are necessary including endometrial T regulatory lymphocytes, plasma cell (CD138+) infiltration, and the state of immunity of the endometrium.

## 8. CONCLUSIONS

Based on the results of this thesis, the following conclusions are reached:

1. The TLM selection model helps to exclude impaired embryos for transfer and prevents early pregnancy loss due to embryo morphological and kinetic developmental changes. Implementation of TLM increases biochemical, clinical pregnancy and decreases early pregnancy loss rates. It might serve as the step one in the treatment algorithm for the patients with recurrent implantation failure.
2. Use of the TLM system is essential as it provides the next step of embryo examination - pre-implantation selection of the embryos of aneuploidy or PGT-A.
3. Excluding transfer of aneuploid embryo, the PGT-A procedure positively affects infertility care and reduces recurrent implantation failure, even if implemented as a single treatment method.
4. The combination of morphological, kinetical and genetical embryo assessment and clarification of the implantation window by ERA test greatly decreases the implantation failure rate and increases the rate of clinical pregnancy.
5. For patients with the balanced translocations in their karyotype, pre-implantation selection of the embryos of aneuploidy is a “Gold standard” in the case of recurrent implantation failure, as it increases the pregnancy rate and decreases biochemical and clinical pregnancy loss.
6. Based on results obtained, recommendations for clinical practice have been developed and are described in Chapter 9.

## 9. CLINICAL APPLICATIONS FOR FUTURE PRACTICE

The reasons for implantation failure are multifactorial. The most common causes are chromosomal aberrations of embryos, endometrium failure, different kinds of coagulopathies, endocrine disorders, and the combination or interaction between mentioned determinants. This research conducted in the iVF Riga clinic during the last 10 years, including clinical analyses and observation of patients has resulted in the development of a new algorithm for the examination and management of IVF patients (Figure 9.1.).

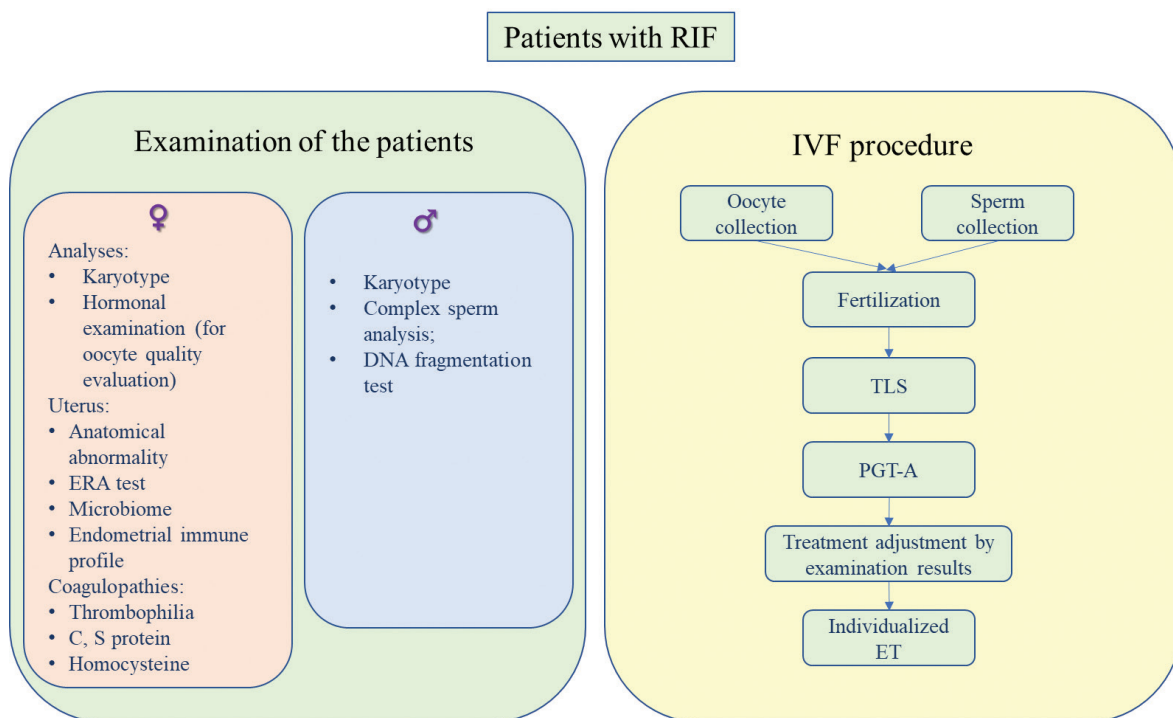


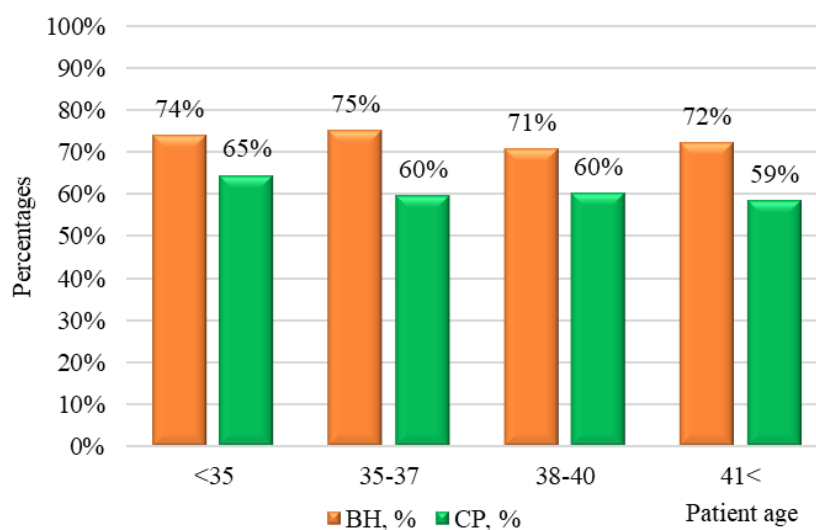
Figure 9.1. Algorithm for examination of patients having an implantation failure

Patients with 2 or more implantation failures in IVF cycles must be examined before undergoing new IVF cycles.

1. At the first stage of examination, karyotyping of both partners is required to assess the risk of developing abnormal embryos.
2. Detailed examination of the man includes sperm analysis using the DNA fragmentation test. Both factors work to avoid defects during fertilization and embryo development, and increase good morphological embryos.

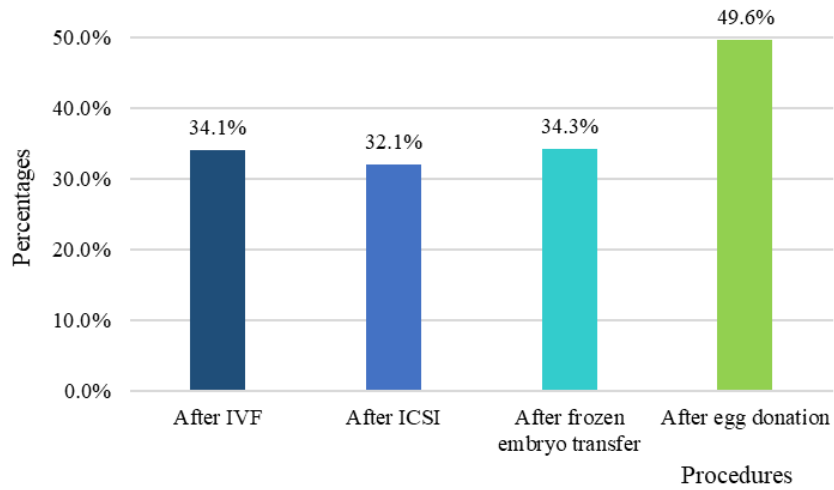
3. Other factors in examination of the woman includes hormonal analyses and coagulation criteria, which affect the maturation quality of the oocyte and also help professional to choose the best strategy for the stimulation.
4. In IVF cycles it is recommended to use TLM for the embryo selection by morphological, kinetic factors and also to specify the day and time of the embryo biopsy.
5. The PGT-A method is recommended for patients who have normal karyotypes but have a complicated medical history. This method is essential for patients with balanced translocations in their own karyotype.
6. A combination of TLM and PGT-A with the ERA test is an effective approach for improving the outcome of IVF treatment, decreasing implantation failure and early pregnancy loss in patients with normal karyotypes and appliance of the PGT-A in patients with balanced translocations provides positive results in IVF treatment.

This algorithm design has been used for infertility treatments of patients who have a medical history of recurrent implantation failure. The clinical and biochemical pregnancy rate, achieved at iVF Riga in 2022 is shown in Figure 9.2. For comparison, the data provided by the ESHRE registry are presented in Figure 9.3. (ESHRE 2022).



**Figure 9.2. Statistical data of iVF Riga biochemical and clinical pregnancy outcomes, 2022**

This data involved FET results present higher clinical pregnancy success rates than European mean pregnancy rates in the Figure 9.3. below.



*Figure 9.3. Statistical data of ESHRE clinical pregnancy outcomes, 2018*

Further studies are recommended to identify other reasons for recurrent implantation failure, including uterine microbiome, immunological and male factors.

## 10. RELATED PUBLICATIONS, LECTURES AND PRESENTATIONS

### **This thesis is based on the following publications:**

- Fodina, V.,** Andzane, D., Pimane, E., Miskova, A. (2015). How to decrease EPL rate treating infertility? *Gynecol Endocrinol*, 31(S1): pp. 51–54.
- Fodina, V.,** Dudorova, A., Alksere, B., Dzalbs, A., Vedmedovska, N., Andersone, S., Conka, U., Erenpreiss, J., Berzina, D. (2019). The application of PGT-A for carriers of balanced structural chromosomal rearrangements, *Gynecol Endocrinol*, 35:sup1, pp. 18–23.
- Fodina, V.,** Dudorova, A., Erenpreiss, J. (2021). Evaluation of embryo aneuploidy (PGT-A) and endometrial receptivity (ERA) testing in patients with recurrent implantation failure in ICSI cycles, *Gynecol Endocrinol*, 37:sup1, pp. 17–20.
- Fodina, V.,** Dudorova, A., Erenpreiss, J. (2021). Reasons and Mechanisms of Recurrent Failed Implantation in IVF. In: Wei-Hua Wand, ed. “Infertility and Assisted Reproduction,” ISBN 978-1-83962-825-2. IntechOpen, doi: 10.5772/intechopen.98301. <http://mts.intechopen.com/articles/show/title/reasons-and-mechanisms-of-recurrent-failed-implantation-in-ivf>

### **Results of this thesis were presented in the following conferences and lectures:**

- Fodina, V.,** Miskova, A., Voložonoka, L. Embryo selection impact on early pregnancy loss. ESHRE annual meeting, July 15–19, 2015, Lisbon, Portugal.
- Fodina, V.** Reduction of reproductive loss in the treatment of infertility, The health of reproduction is the national security guarantee, IV International Congress of obstetricians and gynecologists, October 16–17, 2015, Yekaterinburg, Russia.
- Fodina, V.** Understanding IVF failures in patients with complicated reproductive history, 3<sup>rd</sup> Annual Baltic Fertility Society Meeting, May 20–21, 2016, Riga, Latvia.
- Fodina, V.** Endometrium receptivity as one of the improving factors of ART, StemcellBio-2016, November 10, 2016, Saint Petersburg, Russia.
- Fodina, V.** How to deal with patients who have had a miscarriage, iVF Riga clinic organized lecture cycle on “Innovations in Latvian medicine”, February 28, March 28, April 4, 18, May 2, 16, June 6, 2017, Riga, Latvia.
- Fodina, V.** Genetics and infertility: how it can help in practice, iVF Riga clinic offsite seminar, May 30, 2017, Ventspils, Latvia and June 13, 2017, Daugavpils, Latvia.
- Fodina, V.** How to help couples who are unable to conceive on their own, or families with sick children. How to avoid? MFD conference, March 29, 2018, Riga, Latvia.

- Fodina, V.** “Neveiksmīgas implantācijas iemesli IVF ciklos. Diagnostikas taktika. Problēmu risināšana”. 8. Latvijas Dzemdību speciālistu un Ginekologu kongress, 2018. 20.–21. aprīlī, Rīgā. Mutiskā prezentācija un tēzes.
- Fodina, V.** Diagnostic options and treatment tactics in complicated IVF cases, January 26, 2019, Stockholm, Sweden.
- Fodina, V. et al.** Prior miscarriage and preterm delivery in assisted reproductive technology pregnancies, RĪGA STRADIŅŠ UNIVERSITY INTERNATIONAL CONFERENCE on Medical and Health Care Sciences “Knowledge for Use in Practice”, April 1–3, 2019, Riga, Latvia.
- Fodina, V.** Female infertility diagnosis and new treatment options for various pathologies. Action plan in cases when the development of the embryo stops within the IVF cycle. Novel Avenues in the treatment of female and male infertility, Scientific Practical Conference, June 6–7, 2019, Vilnius, Lithuania.
- Fodina, V.** Diagnostic options and treatment tactics in complex IVF cases, E-visit lecture, February 2, 2020, Riga, Latvia.

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# APPENDIXES

## Appendix I

### Centrālā medicīnas ētikas komiteja

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*Par atļaujas saņemšanu pētījumam  
"GENĒTISKO VARLĀCĻU IDENTIFIKĀCIJA  
PACIENTIEM AR REPRODUKTĪVIEM  
TRAUCĒJUMIEM MĒRĶTIECĪGAS  
ĀRSTĒŠANAS STRATĒGIJAS IZVĒLĒ"*

Centrālā medicīnas ētikas komiteja izskatīja Rīgas Stradiņa Universitātes Molekulārās ģenētikas zinātniskās laboratorijas vadošā pētnieka, Dr. Med. Jura Ērenpreiņa pieteikums par pētījumu "GENĒTISKO VARLĀCĻU IDENTIFIKĀCIJA PACIENTIEM AR REPRODUKTĪVIEM TRAUCĒJUMIEM MĒRĶTIECĪGAS ĀRSTĒŠANAS STRATĒGIJAS IZVĒLĒ" (reģistrēts Veselības ministrijā 2021. gada 3. martā Nr.3605).

Atbilstoši Centrālā medicīnas ētikas komitejas 2021. gada 18. marta sēdes protokola Nr. 2021-10 II. daļas "PRECIZĒTIE IESNIEGUMI" 4. punktam tiek sniegts atzinums, ka pētījums nav pretrunā bioētikas normām.

Centrālās medicīnas ētikas  
komitejas priekšsēdētājs

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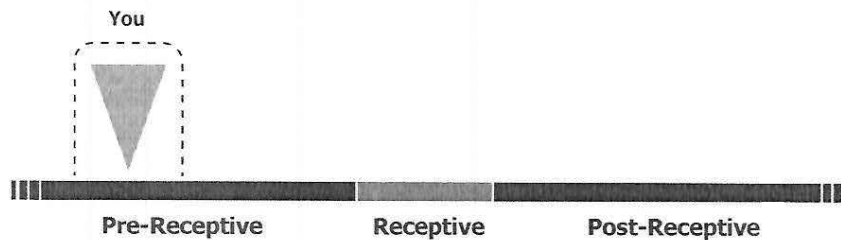
## ERA (ENDOMETRIAL RECEPTIVITY ANALYSIS)

Patient information	Sample information	Clinic information
<b>Unique pat id.:</b> 11378	<b>Date received:</b> 29/09/2020	<b>Clinic:</b> Sia IVF Riga
<b>Sample type:</b> Endometrial biopsy	<b>Report Date:</b> 08/10/2020	<b>Clinician:</b> Dr. Violeta Fodina
<b>Patient name:</b>	<b>First intake of P4:</b> 23/09/2020 6:00 AM	<b>No. biopsy:</b> 1
<b>Patient DOB:</b>	<b>Date of biopsy:</b> 28/09/2020 10:00 AM	
	<b>Cycle type:</b> HRT (124 hours)	

### TEST RESULTS:

#### PRE-RECEPTIVE

**Recommendation: To perform a new endometrial biopsy at P+7 (172 ± 3 hours of progesterone administration)\*\*-**



### INTERPRETATION OF YOUR RESULT:

The gene expression profile is concordant with an endometrium that has not yet reached its receptive stage, meaning this patient may have a displaced window of implantation. A new endometrial biopsy is recommended to validate this displacement and to guide the personalized embryo transfer. The new biopsy should be performed 2 days later than the time that this endometrial biopsy was taken (P+7).

\*\* This recommendation is only applicable to the same type of cycle treatment as the one used for this endometrial biopsy and if the endogenous progesterone measured prior to the first progesterone intake is <1ng/ml.

### TEST DESCRIPTION:

ERA (Endometrial Receptivity Analysis) is a molecular tool used to determine if the endometrium (the mucous membrane lining the womb) exhibits a receptive profile after 5 days of progesterone exposure, the time at which the endometrium is typically ready for embryo implantation. This molecular diagnosis method is based on measuring the gene expression profile of endometrial tissue. Therefore, ERA helps to determine when the endometrium presents the ideal condition for embryo implantation, increasing the possibility of a successful in vitro fertilization treatment.

### COMMENTS

None

### INTERPRETATION OF ERA ANALYSIS RESULTS:

#### Receptive:

This gene expression profile is compatible with a normal receptive endometrium. In this case, it is recommended to perform a blastocyst(s) transfer following the same protocol utilized during this Endometrial Receptivity Analysis (ERA).

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MUE-00422124 1 | 3  
SPA\_L\_F\_RP\_ERA\_002\_EN v2.0

Igenomix is present in Los Angeles | Miami | Sao Paulo | Valencia | Dubai | New Delhi | Mexico | New York | Montreal | London | Tokyo | Buenos Aires | Marostica | Istanbul

CLIA  
#99D2146167

Patient name:  
Patient DOB:  
Report Date: 09/10/2020 14:15



**Early Receptive:**

This gene expression profile is concordant with an endometrium is at the beginning of the receptive stage. In this case, it is recommended to administer progesterone (HRT cycle) or rest (natural cycle) for 12 hours more relative to when the biopsy was taken before performing a blastocyst(s) transfer.

**Late receptive:**

This gene expression profile is concordant with an endometrium is at the end of the receptive stage. In this case, it is recommended to administer progesterone (HRT cycle) or rest (natural cycle) for 12 hours less relative to when the biopsy was taken before performing a blastocyst(s) transfer.

**Pre-receptive:**

This gene expression profile is concordant with an endometrium at a pre-receptive stage due to the potential displacement of the window of implantation. To confirm this result, the analysis of a second biopsy on the recommended day could be required.

**Post-receptive:**

This gene expression profile is concordant with an endometrium at a post-receptive stage due to the potential displacement of the window of implantation. To confirm this result, the analysis of a second biopsy on the recommended day is needed.

**Proliferative:**

This gene expression profile is concordant with an endometrium at a proliferative stage. It is recommended to contact the ERA laboratory to evaluate the protocol in which this endometrial biopsy was performed.

**Non-informative:**

The profile analyzed does not match the control gene expression profiles present in the ERA predictor. It is recommended to contact the ERA laboratory to evaluate the protocol in which this endometrial biopsy was performed.

**Insufficient RNA:**

It was not possible to determine the gene expression profile of the sample because there was not enough genetic material. This occurs in approximately 2.5% of samples received. It is necessary to evaluate a new endometrial biopsy.

**Invalid RNA:**

It was not possible to determine the gene expression profile of the sample due to the poor quality of genetic material obtained. This occurs in approximately 3% of samples received. It is necessary to evaluate a new endometrial biopsy.

**TEST LIMITATIONS:**

The aim of this test is to provide physicians with an objective molecular diagnosis of the patient's endometrial reproductive health. Depending on the result of this analysis, the physician may use it to guide personalized embryo transfer.

Following the recommendations indicated in the ERA report does not guarantee successful implantation. Failed implantation may be caused by other factors such as poor embryo quality, genetic abnormalities, or previous pathologies.

**TESTING METHODOLOGY:**

The ERA test was developed and patented by Igenomix (PCT/ES2009/000386).

The ERA test simultaneously analyzes the expression of 248 genes selected for their endometrial receptivity profile. Analysis of the expression of these genes utilizes Next Generation Sequencing in conjunction with a bioinformatics tool (computational predictor) that gives an endometrial receptivity diagnosis with a specific diagnostic probability.

After receiving the endometrial biopsy and extracting the genetic material (RNA), for each sample minimum quality requirements are evaluated before use of the ERA diagnosis tool.

**LEGAL/QUALITY**

IGENOMIX SPAIN LAB, SLU will only release the report once a completed Test Requisition Form is received. The clinic/clinician/certified health professional requesting the test is responsible for obtaining and taking custody of "Informed Consent" from the patient as depicted by national guidelines and/or legislation.

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**EXEMPTION CLAUSE OF DIAGNOSTIC LIABILITY**

The genetic diagnosis services carried out by IGENOMIX SPAIN LAB, SLU are exclusively intended to be interpreted by qualified/certified health professionals. The result obtained by this test and the information that could be derived from it, cannot be considered in any case as substitute of genetic counselling or medical treatment by a trained professional neither represent itself a medical enquiry. Any result should be interpreted in the context of all available clinical findings, within the general context of a medical enquiry, which must be conducted by genetic diagnosis and / or clinical trained professionals. IGENOMIX SPAIN LAB, SLU is not responsible for the use of their services made by the contracting party, nor the results obtained by means of their study analysis, nor the harmful temporary consequences diverted by its use, making specific discretion of taking appropriate legal measures assuming an improper use of those mentioned studies and analysis.

**INFORMATION:**

Development of the ERA test was published by Díaz-Gimeno et al., 2011 (Fertil Steril. 2011 Jan;95(1):50-60, 60.e1-15. doi:10.1016/j.fertnstert.2010.04.063). Accuracy and reproducibility of the ERA test was demonstrated in Díaz-Gimeno et al., 2013 (Fertil Steril. 2013 Feb;99(2):508-17. doi: 10.1016/j.fertnstert.2012.09.046). Clinical applicability in patients with repeated implantation failures was demonstrated in Ruiz-Alonso et al., 2013 (Fertil Steril. 2013 Sep;100(3):818-24. doi: 10.1016/j.fertnstert.2013.05.004) and Ruiz-Alonso et al., 2014 (Hum Reprod. 2014 Jun;29(6):1244-7. doi: 10.1093/humrep/deu070).

**SIGNED & COUNTERSIGNED**

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Table 6.3.1. All the patient's pregnancy history with data of ovarian stimulation, insemination, embryo biopsy with current results

Pair No.	Karyotype	Pregnancy	Childbirth	Children	Pregnancy with fetal abnormality	Life-born child with pathology	Obtained oocytes	Fertilized oocytes	Obtained blastocysts	Biopsied blastocysts	Blastocysts analyzed	Euploidy	Aneuploidy	Results
1	46,XX,t(1;6) (p22;p25)	0	0	0	0	0	18	9	8	5	4	1	3	Pregnancy did not occur
	46,XY													
2	46,XX	1	0	0	0	8	8	5	4	4	4	2	2	Natural pregnancy
	46,XY,t(4;12) (q31;q13)													
3	46,XX	0	0	0	0	1 cycle - 13	1 cycle - 11	1 cycle - 4	1 cycle - 4	1 cycle - 4	1 cycle - 4	4	2	Pregnant after IVF
	45,XY,der(13;14) (q10;q10)													
4	46,XX,t(9;17) (p10;p10)	0	0	0	0	18	14	8	4	4	3	1	2	1 child after IVF
	46,XY													
5	46,XX,t(6;10) (p21.3;q22.3)	1	1	1	0	13	5	5	4	4	1	0	1	Needs new stimulation
	46,XY													
6	46,XX	1	1	1	0	1 cycle - 11	1 cycle - 9	1 cycle - 9	1 cycle - 2	1 cycle - 2	1 cycle - 2	0	2	Needs new stimulation
	45,XY,der(13;14) (q10;q10)													
7	46,XX	0	0	0	0	24	20	16	15	4	4	2	2	Pregnant after IVF
	46,XY,t(1;3) (q12;q29)[4]/46,XY[15]													
8	46,XX,t(13;20) (q34q13)	2	0	0	1	8	8	8	6	6	6	0	6	Needs new stimulation
	46,XY													
9	46,XX,t(8;11) (p23;p15)	3-5	1	1	0	25	18	5	4	4	4	0	4	Needs new stimulation
	46,XY													
10	46,XX	2	0	0	0	17	17	9	9	4	4	0	4	Needs new stimulation
	45,XY,der(13;14) (q10;q10)													

Table 6.3.2. Patients' data of karyotype, number and grade of obtained blastocysts with PGT-A results

No.	Gender	Age	Karyotype	Blasto- cysts analysed	Blastocysts grade	PGT-A results
1	Female	35	46,XX,t(1;6) (p22;p25)	4	1) BC3BB 2) BC3BB 3) BC4AA 4) BC3AB	1) arr1p36.331p22.1(102664-92589017)x1, 6p25.3p24.3(103351-887441)x3; 2) arr1p36.331p22.1(102664-92589017)x3,6p25.3p24.3(103351-887441)x1; 3) arr(1-22)x2,(XY)x1; 4) arr1p36.331p22.1(102664-92589017)x1,6p25.3p24.3(103351-887441)x3
2	Male	46	46,XY,t(4;12) (q31;q13)	4	1) BC3BB 2) BC3BA 3) BC2BB 4) BC3AB	1) arr4q32.3q35.2(169102980-190815481)x1,12q21.1-q24.33(72408753-133435933)x3 2) arr16p13.3p12.2(137326-23760229)x3 3) arr(1-22,X)x2 4) arr(1-22,X)x2
3	Female	34	46,XX,t(9;17) (p10;p10)	3	1) BC2BB/3/D 2) BC2BB/2/A 3) BC2BB/5/A	1) arr9p24.3q13(104476-66534426)x1, 17p13.3p11.2(556474-20230594)x1 2) arr(9)x1 3) arr(1-22)x2, (XY)x1
4	Female	32	46,XX,t(6;10) (p21.3;q22.3)	1	1) BC3BB	1) arr(2)x1~2,6p25.3p21.32(672168-3233433100)x1,10p23.1q26.3(82574455-135336967)x3,(12-22,X)cx
5	Male	28	46,XY,t(1;3) (q12;q29)[4]/ 46,XY[15]	4	1) BC2BB/4/A 2) BC3BB/4/A	1) seq[GRCh37]dup(4)(p16.3q31.2)chr4:g.602,531_144,926,382 del(4)(q31.21q35.2) chr4:g.146,137,523_190,874,077 2) seq[GRCh37](1-22)x2, (XY)x1
6	Female	31	46,XX,t(13;20) (q34q13)	6	1) BC2BC/5/A 2) BC2BB/5/A 3) BC2AB/5/A 4) BC2AB/4/A 5) BC3BB/5/BG 6) BC2BB/5/A	1) seq[GRCh37](1-22)cx dup(13)(q12.11q33.3) chr13:g.19,812,720_108,833,167 del(13) (q34q34)chr13:g. 110,994,782_114,494,829 del(20) (p13q13.2) chr20:g.621,362_52,103,980 dup(20) (q13.2q13.33) chr20:g. 53,221,087_62,715,666 2) seq[GRCh37] (1-22,X)cx dup(13) (q12.11q33.3) chr13:g.19,812,720_108,833,167 del(13) (q34q34) chr13:g. 110,994,782_114,494,829 del(20) (q13.2q13.33) chr20:g.53,221,087_62,715,666 3) seq[GRCh37]dup(13) (q12.11q33.3) chr13:g.19,812,720_108,833,167 del(13) (q34q34) chr13:g.110,994, 782_114,494,829 del(20)(p13q13.2) chr20:g.621,362_52,103,980 dup(20)(q13.2q13.33) chr20:g.53,221,087_62,715,666 4) seq[GRCh37](1)x1del(13) (q34q34) chr13:g.110,994,782_114,494,829(17)x1 dup(20) (q13.2q13.33) chr20:g. 53,221,087_62,715,666 del(20) (q13.2q13.33) chr20:g.53,221,087_62,715,666 5) seq[GRCh37]dup(13)(q12.11q33.3) chr13:g.19,812,720_108,833,167 del(13) (q34q34) chr13:g.110,994,782_114,494,829 6) seq[GRCh37]del(13)(q34q34) chr13:g.110,994,782_114,494,829 dup(20) (q13.2q13.33) chr20:g.53,221,087_62,715,666

Table 6.3.2. Patients' data of karyotype, number and grade of obtained blastocysts with PGT-A results. Continued

No.	Gender	Age	Karyotype	Blastocysts analysed	Blastocysts grade	PGT-A results
7	Female	36	46,XX,t(8;11)(p23;p15)	4	1) BC2AB/5/A 2) BC2BC/ CC/5/A 3) BC5BC/ CC/1/D 4) BC3AB/2/A	1) seq[GRCh37]del(8)(p23.3p23.1) chr8:g.663,894_9,478,357, dup(11)(p15.5p15.4) chr11:g.721,142_6,682,406,(13)x1, (14)x1 2) seq[GRCh37]dup(X)(p22.33p22.12)chrX:g.3,354,528_21,343,697 3) seq[GRCh37] dup(8)(p23.3p23.1) chr8:g.663,894_9,478,357, del(11) (p15.5p15.4) chr11:g.663,894_6, 682,406 4) seq[GRCh37]del(6) (q26q27)chr(6):g.161, 462,989_170,976,006,cxmos
8	Male	46	45,XY,der(13;14)(q10;q10)	1 <sup>st</sup> cycle - 4 2 <sup>nd</sup> cycle - 12	1) BC2BB/5/D 2) BC2BB/5/A 3) BC2BB/5/A 4) BC2BB/4/A 1) BC2BB/5/A 2) BC2BB/2/A 3) BC3BB/4/A 4) BC3AB/5/A 5) BC3AA/5/A 6) BC3BC/3/D 7) BC3AB/1/C 8) BC3AA/1/C 9) BC3BB/5/A 10) BC3BB/5/A 11) BC2BB+cells/2/A 12) BC3BB/2/A	1) arrmos(2)x1, 11q23.2q25(114304322-134564974)x1 2) arr(15)x1,(16)x3 3) arr(16)x1 4) arr(13)x1,(16)x1 1) arr(16)x1 2) arr18q21.2q23(49776191-77856022)x1_2 3) arr2q12.2q37.3(10656227-242058943)x1_2,6q23.3q27(138010907-170931603)x1_2; 4) arr(1-22),(XY)x1 5) arr(1-22),(XX)x1 6) arr(22)x1 7) arr(1-22,X)x2 8) arr(16)x3 9) arr(4)x3,(16)x3 10) arr(1-22)x2,(XY)x1 11) arr(4)x1, (19)x3 12) arr(16)x3
9	Male	40	45,XY,der(13;14)(q10;q10)	1 <sup>st</sup> cycle - 2 2 <sup>nd</sup> cycle - 0	1) BC2AB 2) BC3BB	1) seq[GRCh37]6q13q27 (75,871,171-170,976,006)x1 2) seq[GRCh37](1-22)cx
10	Male	35	45,XY,der(13;14)(q10;q10)	4	1) BC2BB 2) BC3AB 3) BC3BB 4) BC3BB	1) seq[GRCh37](1-22,X)cx(13)x1,(21)x3 2) seq[GRCh37](1-22)cx (21)x2~3 3) seq[GRCh37]del(12)(q12q24.33) chr12:g. 44, 629,644_133,380,179 4) seq[GRCh37](1-22)cx(13)x1~2,(16)x1