Optimizing IVF by controlling for both embryonic aneuploidy and endometrial receptivity using genetic testing

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning.

Tiffany Stankewicz

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outcomes following day 5 embryo biopsy with fresh day 6 transfer. I thought your research was brilliant and discussed your work on endometrial receptivity with my Medical Director at the IVF clinic when I got home. Very soon after we started implementing changes to our PGT-A transfer program, which resulted in great success. Little did I know that several years later I would be joining you at Igenomix and have the opportunity to work closely with you. Thank you for your commitment to this field and more specifically your research regarding the endometrium, you are truly a pioneer. Finally, thank you for making me a better scientist and teaching me how to conduct research in the best way possible.

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Incorporation of published work

This thesis largely incorporates published work from several papers and uses text from them. Alterations are made where appropriate and to suit a thesis style, format. The following is a comprehensive list of my published, "in press" and "in preparation" works and those indicated in bold type represent research that has been performed during my registration period (2014-2021).

Taylor TH, Stankewicz T, Katz SL, Patrick JL, Johnson L, Griffin DK. Preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm. Zygote. 2020 Apr;28(2):93-96. Chapter 5.0/specific aim a

Leondires M, Akopians AL, Nakhuda G, Stankewicz T. Improved pregnancy rates following endometrial receptivity analysis and personalized embryo transfer in patients with previous failed implantation after euploid embryo transfer. (In preparation for submission). Chapter 6.0/specific aim b

Stankewicz T, Ruiz-Alonso M, Soler-Ibañez M, Simón C, Valbuena D. Are the clinical outcomes different in day 5 versus day 6 single embryo transfer when endometrial factor is controlled? – Published: November 17, 2021DOI:https://doi.org/10.1016/j.rbmo.2021.11.010. Chapter 7.0/specific aim c

Stankewicz T, Ruiz-Alonso M, Valbuena D. Evaluating clinical outcomes associated with pET guided by transition phase results: do small shifts lead to big outcomes? (In preparation for submission). Chapter 8.0/specific aim d

What is the narrowest window of implantation? A case report – submitted to Fertility Research and Practice (submitted to Fertility Research and Practice, October 4, 2021). Chapter 9.0/specific aim e

Stankewicz T, Valbuena D, Ruiz-Alonso M. Inter-cycle consistency versus test compliance in endometrial receptivity analysis test. J Assist Reprod Genet. 2018 Jul;35(7):1307-1308. Chapter 10.0/specific aim f

Stankewicz T, Ruiz-Alonso M, Valbuena D. Evaluating the clinical utility of ERA for Gestational Carriers. (In preparation for submission). Chapter 11.0/specific aim g

In addition, the following is a list of abstracts published in learned journals that I have accrued during my career. Again, the ones relevant to this thesis are **highlighted in bold.**

Stankewicz-McKinney TL, Taylor TH, Glassner MJ, Orris JJ, Brasile DR, Griffin DK. 2015. Preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm. PGDIS. Oral. Chapter 5.0/specific aim a

Leondires M, Akopians AL, Stankewicz T, Gomez E, Snider A, Harton G, Valbuena D, Simón C. 2018. Improved pregnancy rates following endometrial receptivity analysis and personalized embryo transfer in patients with previous failed implantation after euploid embryo transfer. ESHRE, Barcelona, Spain. P-480. Chapter 6.0/specific aim b

Stankewicz T, Adaniya G, Cinnioglu C, Ruiz M, Valbuena D, Bopp B, Colver R, Reuter L, Will M, Will E, Simón C. 2019. Intramuscular versus vaginal progesterone: can we expect differences in endometrial receptivity? PCRS, Palm Springs, CA. P-30.

Taylor TH, Stankewicz TL, Hanshew KK, Orris JJ, Glassner MJ, Anderson SH. 2012. Significantly higher implantation and pregnancy rates with day 5 biopsy and day 6 fresh transfer following array comparative genomic hybridization when compared to day 5 transfer alone. ASRM, San Diego, CA. P-88.

Taylor TH, Gilchrist JW, Hanshew KK, Stankewicz TL, Orris JJ, Anderson SH. 2011. Aneuploidy rates of women under 35 years old, undergoing array comparative genomic hybridization (aCGH) for the sole purpose of family balancing. Fertil Steril. 96;6:S224.

List of abbreviations

aCGH	Array Comparative Genomic Hybridization
AH	Assisted Hatching
AMA	Advanced Maternal Age
ART	Assisted Reproduction Technology
ASRM	American Society for Reproductive Medicine
BPR	Biochemical Pregnancy Rate
CAMs	Cell Adhesion Molecules
CGH	Comparative Genomic Hybridization
cET	Customized Embryo Transfer
COS	Controlled Ovarian Stimulation
CPM	Confined Placental Mosaicism
CSC	Continuous Culture Medium
EFT	Endometrial Function Test
EMMA	Endometrial Microbiome Metagenomic Analysis
ER	Estrogen Receptor
ERA	Endometrial Receptivity Analysis
ERT	Endometrial Receptivity Test
ES	Endometrial Scratch
eSET	Elective Single Embryo Transfer
ESHRE	European Society of Human Reproduction and Embryology
ET	Embryo Transfer
FET	Frozen Embryo Transfer
FISH	Fluorescent *in situ* Hybridization
FSH	Follicle Stimulating Hormone
GC	Gestational Carrier
GIFT	Gamete Intrafallopian Transfer
GnRH	Gonadotropin Releasing Hormone
hCG/βhCG	Human Chorionic Gonadotropin/β Human Chorionic Gonadotropin
hr-NGS	High-Resolution Next Generation Sequencing
HRT	Hormone Replacement Therapy
ICSI	Intracytoplasmic Sperm Injection
IP	Intended Parent
IR	Implantation Rate
IVF	*In vitro* Fertilization
ICM	Inner Cell Mass
LBR	Live Birth Rate
LH	Luteinizing Hormone
MII	Metaphase II
mCGH	Metaphase Comparative Genomic Hybridization
mtDNA	Mitochondrial DNA
mRNA	Messenger RNA
NGS	Next Generation Sequencing

niPGT-A	Non-Invasive Preimplantation Genetic Testing for Aneuploidies
NIPT	Non-Invasive Prenatal Testing
OPR	Ongoing Pregnancy Rate
P4	Progesterone
pb	Polar Body
PCOS	Polycystic Ovarian Syndrome
pET	Personalized Embryo Transfer
pFET	Personalized Frozen Embryo Transfer
PIO	Progesterone in Oil
PGCs	Primordial Germ Cells
PGDIS	Preimplantation Genetic Diagnosis International Society
PGT	Preimplantation Genetic Testing
PGT-A	Preimplantation Genetic Testing for Aneuploidies
PGT-M	Preimplantation Genetic Testing for Monogenic Disorders
PGT-SR	Preimplantation Genetic Testing for Structural Rearrangements
pn	Pronuclei
PR	Progesterone Receptor
PR	Pregnancy Rate
pWOI	Personalized Window of Implantation
RCT	Randomized Controlled Trial
RIF	Repeated Implantation Failure
RPL	Recurrent Pregnancy Loss
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SBM	Spent Blastocyst Media
SET	Single Embryo Transfer
SNP	Single Nucleotide Polymorphism
SSS	Serum Substitute Supplement
SVM	Support Vector Machine
TE	Trophectoderm
unK	Uterine Natural Killer
UPD	Uniparental Disomy
WOI	Window of Implantation
ZIFT	Zygote Intrafallopian Transfer
ZP	Zona Pellucida

Abstract

Successful implantation following *in vitro* fertilization (IVF) requires the transfer of a good quality and competent blastocyst to a receptive endometrium. Since the introduction of IVF, emphasis has been placed mainly on embryo development and quality, and this has guided decision-making regarding what day to perform an embryo transfer and how many embryos to transfer. IVF lab techniques to improve culture conditions contributed to an improvement in embryo quality and implantation rates. In recent years, an increase in Preimplantation Genetic Testing for Aneuploidy (PGT-A) involving transfer of euploid embryos has further enhanced these rates. Despite the transfer of good quality euploid blastocysts however, implantation failure still occurs in approximately one third of PGT-A cycles. In order to optimize implantation rates, it is necessary to focus both on the embryonic contribution, and on the role of the endometrium. In this regard, the most likely cause of implantation failure is alteration or desynchrony of endometrial receptivity. Until recently, there has been no objective and reliable diagnostic test to assess endometrial receptivity accurately and provide reproducible results with a clear clinical directive. This, combined with the widely accepted assumption that all women possessed a consistent window of implantation (WOI), meant that meaningful assessment of endometrial receptivity was often omitted from the standard IVF work-up. However, the implementation of personalized medicine to IVF has expanded our understanding of endometrial receptivity and introduced the concept of a personalized WOI (pWOI). A prime example is Endometrial Receptivity Analysis (ERA), a robust molecular test that deciphers the gene expression pattern of an endometrial biopsy sample and employs a bioinformatic predictor that accurately diagnoses the phase of receptivity, providing a recommendation for a personalized embryo transfer (pET).

The overall aim of this thesis is to answer the question: can implantation following IVF be optimized when controlling for both embryonic factor with PGT-A and endometrial factor with ERA? To address this question, the work described here investigates the impact of controlling for embryo aneuploidy and endometrial receptivity, both independently and together, and their effects on clinical outcomes following IVF. This thesis comprises of seven specific aims: a) to provide a preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm; b) to establish whether there are improved pregnancy rates following endometrial receptivity analysis and personalized embryo transfer in patients with previous failed implantation after euploid embryo transfer; c) to ask the question of whether the clinical outcomes are different in day-five versus day-six single embryo transfer when endometrial factor is controlled; d) to evaluate clinical outcomes associated with personalized embryo transfers guided by transition phase results: to ask whether small shifts can lead to big outcomes; e) to ask, through a single case report, what is the narrowest window of implantation by examining the evolution of the endometrial phases via transcriptomic profiling of biopsies taken at various hours of progesterone exposure; f) to provide a novel reanalysis of published data presented pertaining to inter-cycle consistency versus test compliance in endometrial receptivity analysis testing; and g) to assess the prevalence of a displaced WOI in gestational carriers and the clinical utility of applying ERA.

This thesis was largely successful in the fulfillment of these aims in that, the research presented herein provided novel insight into the basic understanding of chromosomal aneuploidy in embryos and endometrial receptivity, along with the clinical impact of controlling for each of these factors, both independently and collectively. My work determined a) a difference in

aneuploidy rates between biopsies taken from the mural versus the polar trophectoderm, possibly suggesting an underlying mechanism to embryo implantation and further considerations when studying mosaicism within preimplantation embryos. b) When controlling for both embryonic aneuploidy and endometrial receptivity, significantly improved implantation rates were achieved in patients with a previous failed euploid transfer, demonstrating the necessity of proper embryo-endometrial synchrony to successful implantation. c) when properly controlling for endometrial receptivity with ERA, similar clinical outcomes between day-five versus day-six blastocysts were observed, suggesting equal viability of blastocysts regardless of blastulation timing. This contributes to our basic understanding of embryo development and potential, and it allows other factors of embryo viability to be prioritized when selecting the best embryo for transfer. d) novel understanding into the WOI was achieved, demonstrating that small shifts of only 12 hours lead to improved outcomes, along with e) showing the existence of an extremely narrow WOI (lasting less than 12 hours). Both these observations clearly epitomize how unique the WOI is in each woman, along with the advantages that precision medicine has to offer in terms of improving clinical IVF outcomes by providing a personalized recommendation for transfer timing based on a woman's unique genetic profiling. f) It is also demonstrated the consistency of receptivity timing from cycle to cycle, increasing our confidence in transcriptomic analysis for endometrial dating and embryo transfer planning. g) Endometrial transcriptomic studies in gestational carriers (GC) (i.e., women with proven fertility) at time of expected receptivity revealed an unexpectedly high rate of non-receptivity at the standard transfer time in hormone replacement therapy (HRT) cycles, suggesting that receptivity may be commonly altered in this population outside of natural conception cycles. Following the clinical outcomes in

GCs when applying a pET, especially when also controlling for embryo aneuploidy, exceptionally high rates of implantation were achieved, suggesting the significance of applying both these technologies, even in a “fertile” population of patients, and again undoubtedly demonstrating the clinical relevance of controlling for both embryo aneuploidy and endometrial receptivity to optimize IVF outcomes.

1.0 Introduction

1.1 Infertility

Most couples achieve pregnancy within six months to a year of unprotected intercourse. However, for approximately 1 in 6 couples, normal pregnancy is not achieved within this timeframe (Agarwal et al., 2015; de Krester 1997; O'Flynn-O'Brien et al., 2010). Numerous causes of infertility have been established, with approximately one-third of infertility cases caused by female reproductive issues and another one-third caused by male reproductive issues (Centers for Disease Control and Prevention 2009). Known causes of infertility include tubal factor, ovulatory dysfunction, diminished ovarian reserve, endometrial anomalies, hormonal imbalances, advanced maternal age, and male factor affecting semen parameters. With that said, up to 30% of couples are diagnosed with unexplained infertility (Practice Committee of the American Society for Reproductive Medicine 2006), in which a direct cause cannot be determined. In any case, it is imperative to understand normal reproductive function as to allow potential sources of infertility to be adequately investigated.

1.2 Normal Female Reproductive Function

1.2.1 Oogenesis

Females are born with all the eggs that they will ever have. Oogenesis, the process by which mature ova are produced, begins around the 8th week of gestation with the migration of primordial germ cells (PGCs) in the germinal epithelium (De Felici 2010). Between 8 to 11 weeks of gestation, PGCs multiply by mitosis and are now referred to as oogonia (Garcia *et al.*, 1987). By gestational week 20, clusters of oogonia have formed within the fetal ovary. In order to

produce haploid gametes necessary for reproduction, oogonia must undergo DNA replication followed by two reduction divisions referred to as meiosis I and meiosis II (Bulcun-Filas and Handel, 2018).

1.2.1.1 Meiosis I

As mentioned above, oogonia first undergo DNA replication, or interphase, in which the oogonium becomes a primary oocyte consisting of 46 pairs of chromosomes (Gondos et al., 1986). At this stage the oocyte contains four chromatids of each chromosome: 1. a chromatid from the mother, 2. a duplication of mother's chromatid (sister chromatid), 3. a chromatid from the father, 4. a duplication of the father's chromatid (sister chromatid; Figure 1). From this point the primary oocyte can enter meiosis I.

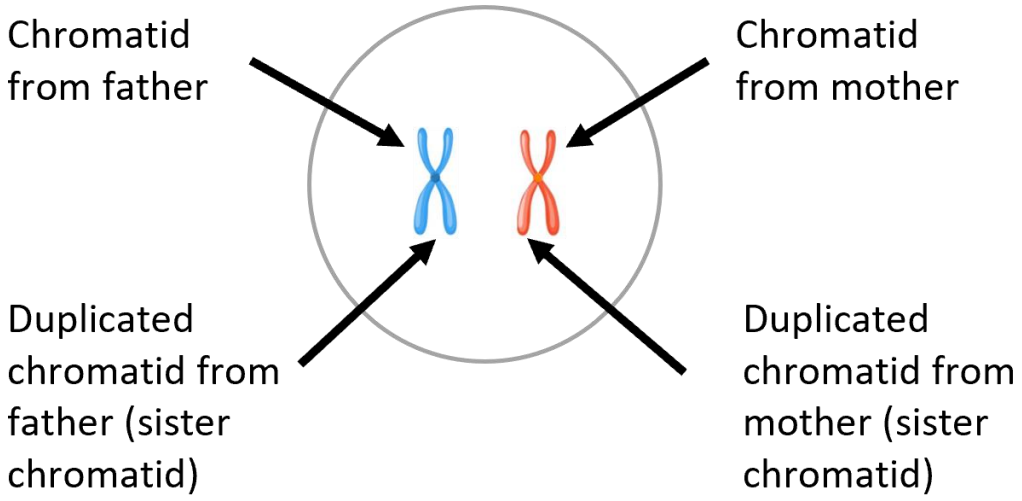


Figure 1. Primary oocyte chromosomal content. At this stage primary oocytes consists of 46 pairs of chromosomes. Each chromosome contains four chromatids: 1. A chromatid from the father; 2. A duplicated chromatid from the father (sister chromatid); 3. A chromatid from the mother; 4. A duplicated chromatid from the mother (sister chromatid).

Meiosis I consists of four main phases: prophase I, metaphase I, anaphase I, and telophase I (Figure 2). In prophase I (consisting of six stages itself), homologous chromosomes undergo synapsis where they pair and fuse together. It is in the diplotene phase of prophase I where primary oocytes will remain with all duplicated chromosomes until the onset of puberty (Bulcun-Filas and Handel 2018).

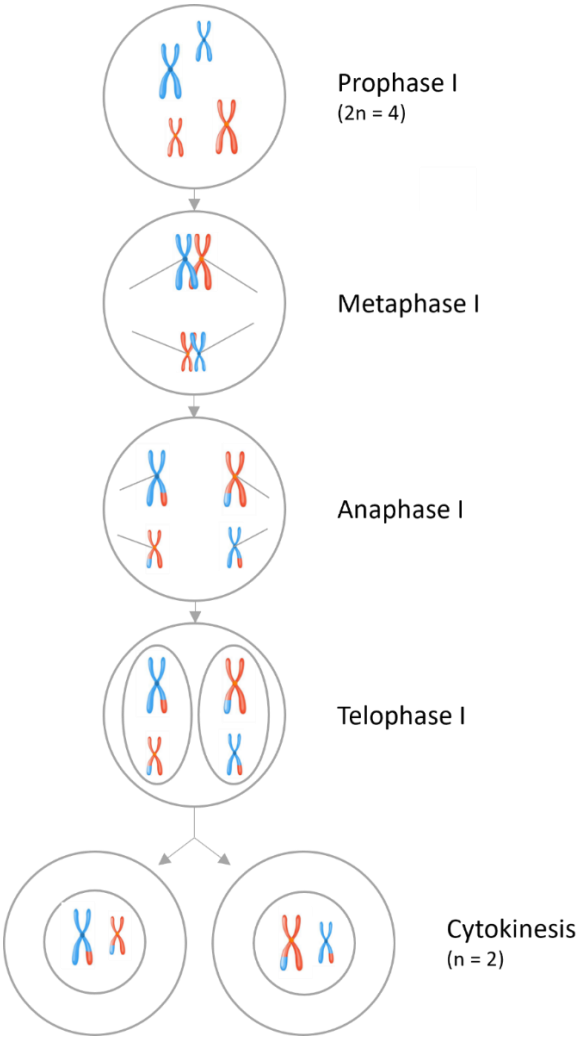


Figure 2. Phases of meiosis I. Meiosis I begins with a diploid (2n = 4) cell and ends with two haploid (n = 2) cells. In humans (2n = 46), who have 23 pairs of chromosomes, the chromosome number is reduced by half at the end of meiosis I (n = 2). Meiosis I stars with prophase I: homologous chromosomes pair and are said to be in synapsis. Metaphase I: the bivalents will align on the metaphase plate. Anaphase I: the chiasmata separate allowing for each chromosome to move to separate poles. Telophase I: the nuclear envelop reforms. Cytokinesis: two complete daughter cells are generated.

Upon puberty, monthly hormonal signals in the form of luteinizing hormone (LH) and follicular stimulating hormone (FSH) are produced by the pituitary gland and act on the follicles where the oocytes still remain in meiotic arrest. Together, these hormones stimulate the primordial follicle to undergo changes leading to the simultaneous maturation of the follicle and oocyte and the resumption of meiosis I (Sánchez and Smitz 2012). In metaphase I, the bivalents align on the metaphase plate leading to the initiation of anaphase I, where the chiasmata (the link between two chromatids where crossing over and exchange of genetic material between parental chromosomes occur) separate allowing for each chromosome to move to separate poles. Telophase I involves the reformation of the nuclear envelop permitting cytokinesis to take place resulting in two complete daughter cells, which in the case of female gametogenesis, corresponds to the secondary oocyte and first polar body (Gilbert 2000).

1.2.1.2 Meiosis II

Immediately following meiosis I, the secondary oocyte undergoes meiosis II. Meiosis II comprises of prophase II, prometaphase II, metaphase II, anaphase II, and telophase II (Bulcun-Filas and Handel, 2018) (Figure 3). During prophase II, the chromosomes condense, and the centromeres move toward opposite poles forming new spindles. In prometaphase II, the spindle is now fully established, and each sister chromatid forms an individual kinetochore that attach to microtubules from the opposite poles. Just as in meiosis I, the oocyte also arrests during meiosis II, however in this case the process is halted at metaphase II and is only resumed upon fertilization. Once resumed, the oocyte undergoes metaphase II, where the sister chromatids become maximally condensed and align at the equator of the cell. Anaphase II then commences, and the sister chromatids are pulled apart by the kinetochore microtubules and move toward

opposite poles. Lastly, telophase II coupled with cytokinesis occurs in which the chromosomes begin to decondense and become surrounded by a nuclear envelope, at this point meiosis II is complete, resulting in an ovum and the second polar body (Gilbert 2000).

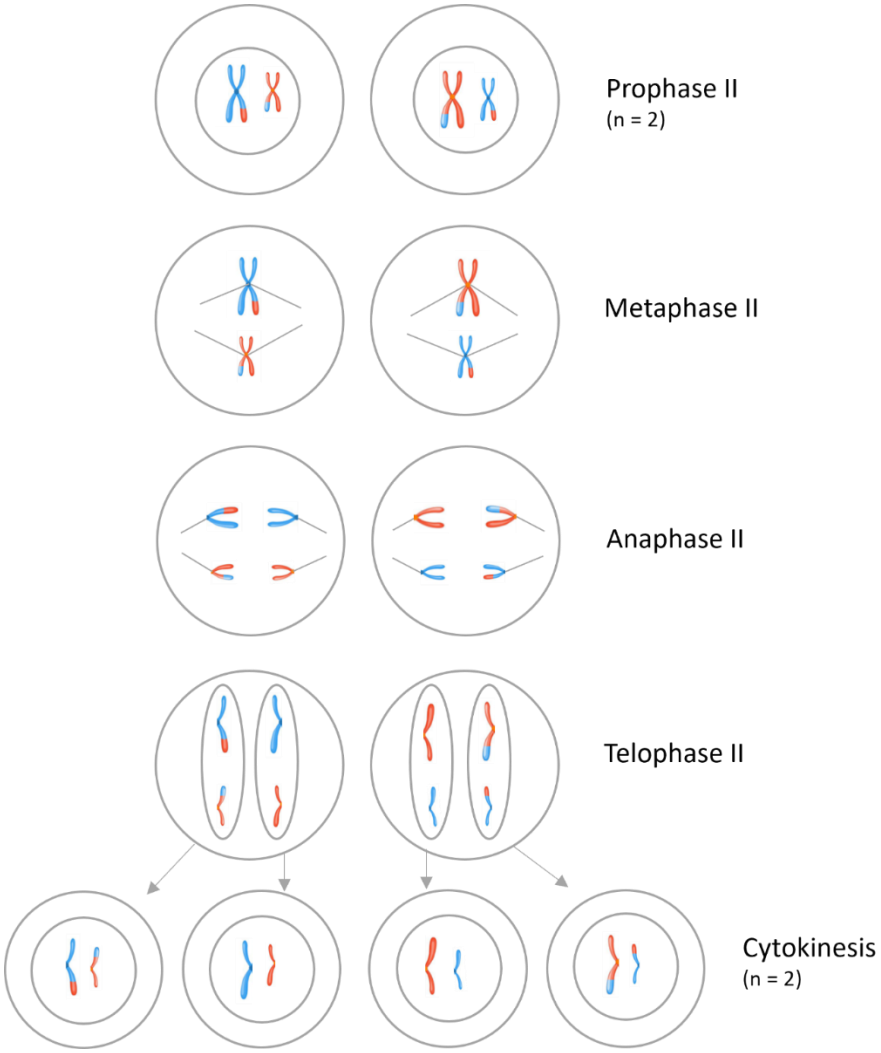


Figure 3. Phases of meiosis II. Meiosis II begins with two haploid cells (n = 2) and end with four haploid cells (n = 2). In humans (2n = 46), who have 23 pairs of chromosomes, the chromosome number remains unchanged throughout meiosis II (n = 2). Meiosis II begins with prophase II: the chromosomes condense, and the centromeres move toward opposite poles forming new spindles. Metaphase II: the sister chromatids are maximally condensed and align on the metaphase plate. Anaphase II: the sister chromatids are pulled apart by the kinetochore microtubules and move toward opposite poles. Telophase II: chromosomes begin to decondense and become surrounded by a nuclear envelope. Cytokinesis: separates the two cells into four unique haploid cells.

1.2.1.3 Meiosis in Females versus Males

The process of meiosis in female and male gametes is similar at the fundamental level, however the timing of meiotic events differs immensely. In females, as previously stated, oogenesis begins early in fetal development where a finite number of oocytes will be formed prior to birth. On the other hand, spermatogenesis initiates at the onset of puberty, where millions of sperm will be continuously produced throughout a male's lifetime (Feng et al., 2014). Not only is the onset of meiosis different between the two sexes, but also the duration of the process itself. Although meiosis in females is initiated in the womb, the actual process does not conclude until fertilization occurs; this can translate to decades of meiotic arrest before completion, whereas spermatogenesis is completed in approximately 72 days (Muciaccia et al., 2013). A primary concern in the oocyte pertains to an increased risk of meiotic errors due to the extended period of meiotic arrest. During which, oocytes are susceptible to prolonged exposure to environmental insults such as the accumulation of reactive oxygen species (Tarin et al., 1996) and carbonyl stress (Tatone et al., 2011). These insults can potentially lead to defective spindle formation and chromosome segregation during meiosis (Tarin et al., 1996; Tatone et al., 2011), affecting the final chromosome numerical content in oocytes and consequently leading to aneuploidy in resulting embryos, an area of concern which will be discussed in later sections. Lastly, the number of gametes produced per completed meiotic event differs, in females a single round of meiosis results in a single oocyte whereas in males four spermatocytes are generated (Bulcun-Filas and Handel 2018). Figure 4 summarizes the differences discussed above.

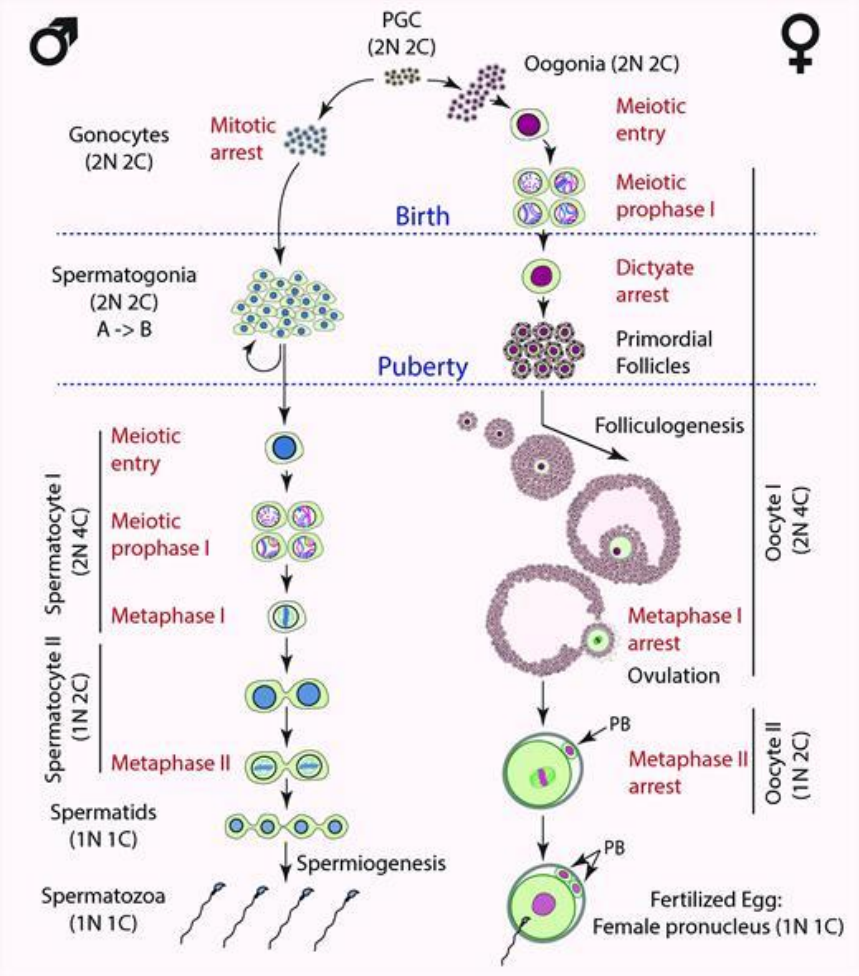


Figure 4. Comparison of Gametogenesis and Meiosis in Males Versus Females. Primordial germ cells (PGCs) are initially sexually undifferentiated, and the sex-specific differentiation initiates once PGCs colonize the fetal gonads. In males (left panel), germ cells undergo mitotic proliferation and then arrest, forming quiescent gonocytes. Gonocytes remain arrested until after birth when they resume mitotic divisions and establish spermatogonial stem cells. Type A spermatogonia either self-renew or differentiate to type B spermatogonia, which are committed to enter meiosis, thus initiating the spermatocyte stage. The diploid spermatocytes progress through prophase I, completing meiotic recombination, and, without interruption, undergo the first and second meiotic divisions. The haploid spermatids thus formed undergo spermiogenic differentiation to form mature spermatids (termed spermatozoa after passage to the epididymis). In females (right panel), after a limited period of mitotic proliferation, oogonia enter meiotic prophase I during fetal development. The diploid oocytes complete meiotic recombination around birth and begin a protracted arrest stage known as dictyate. Shortly after birth, somatic pre-granulosa cells surround arrested oocytes to form primordial follicles. During or after puberty, when a primordial follicle is activated, it grows in size through both granulosa cell proliferation and increase in size of the oocyte, which remains arrested at dictyate. Prior to ovulation, the oocyte resumes meiosis and arrests at metaphase I. Upon ovulation, first meiotic division is completed, and the first polar body (PB) is extruded into the space under the zona pellucida. The ovulated oocyte arrests again, at metaphase II, until fertilization, which triggers the second meiotic division and extrusion of the second PB. Taken from Bolcun-Filas and Handel, 2018.

1.2.2 Menstrual cycle

Menarche is the first menstrual period in a female adolescent, occurring between 12 to 13 years of age (Chumlea et al., 2003). Menstruation itself marks the first day of a female's menstrual cycle in which the functional layer of the uterine lining is shed in the absence of a pregnancy. The average menstrual cycle in humans is around 28 days and is divided into two phases, the follicular and luteal phases (in reference to the ovary) or the proliferative and secretory phases (in reference to the uterus), with ovulation occurring in between (Vollman 1977) (Figure 5).

During the follicular/proliferative phase, low serum levels of estradiol and progesterone allow for an increase in pulsatile gonadotropin-releasing hormone (GnRH) levels, resulting in a rise in FSH and LH (Filicori et al., 1986; Adams et al., 1994; Taylor et al., 1995). FSH stimulates ovarian follicular maturation, where in turn the growth of ovarian follicles leads to increasing estradiol levels. Also, as mentioned above, this period of the menstrual cycle coincides with the resumption of meiosis I in the primary oocyte. The role of estradiol during the follicular/proliferative phase includes thickening of the inner layer of the uterine cavity, or endometrium, and, after reaching a critical level, producing a positive feedback effect on LH, generating a significant surge in it is level (Filicori et al., 1984; Filicori et al., 1986; Taylor et al., 1995).

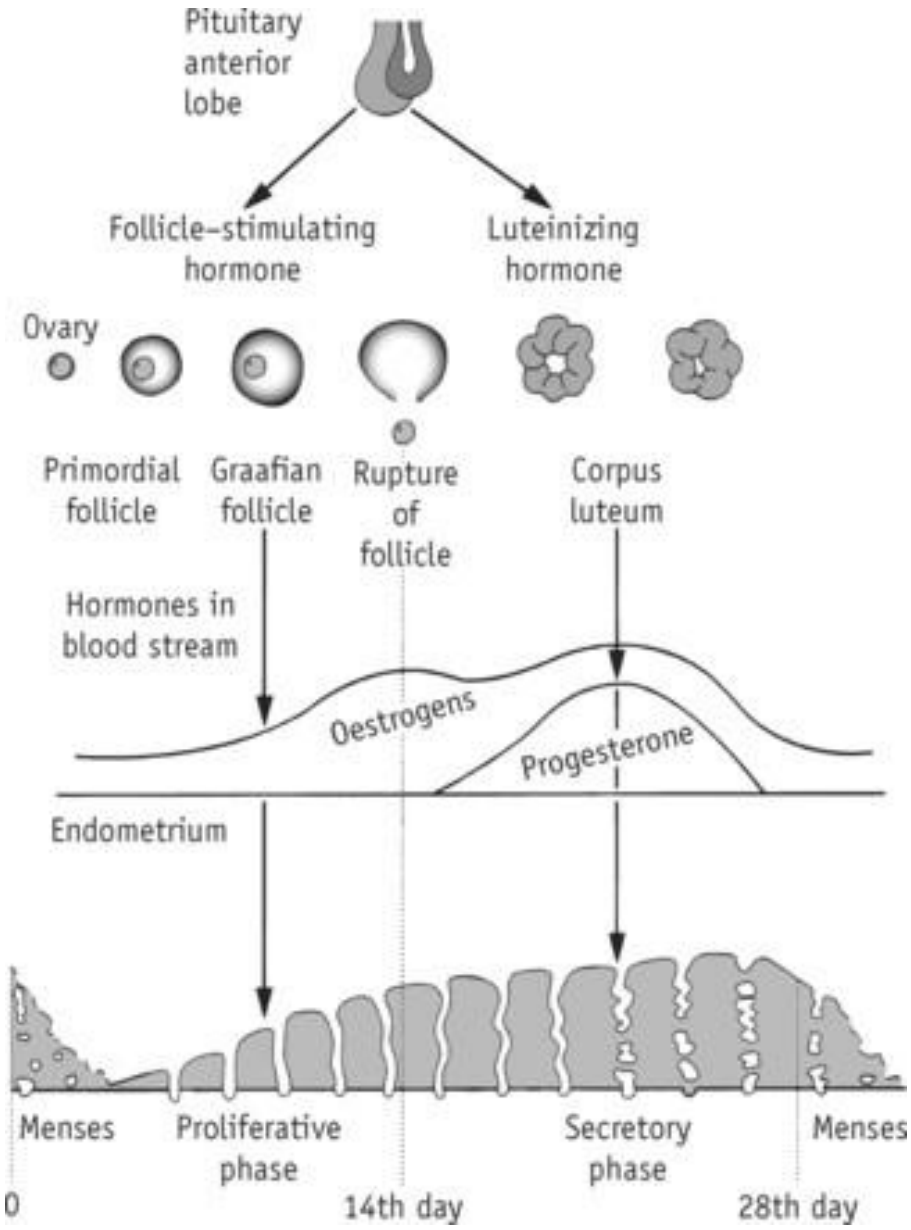


Figure 5. The uterine cycle. The endometrial cycle is influenced by changing patterns of gonadotropins (follicle-stimulating hormone and luteinizing hormone) released by the pituitary anterior lobe and ovarian hormones (oestrogens and progesterone). There are three phases of the uterine cycle: menses, the proliferative phase, and the secretory phase. Menses is marked by a thinning of the uterine lining due to shedding of the endometrial tissue. In the proliferative phase, the endometrium is rebuilt in the presence of oestrogens. Following ovulation around day 14 of the cycle, the secretory phase is initiated, where progesterone is the main hormone regulating endometrial receptivity for blastocyst implantation. If implantation and pregnancy is achieved, progesterone levels remain elevated to maintain the endometrial thickness. If implantation and pregnancy is not achieved in the secretory phase, both oestrogens and progesterone levels fall, and menses begins again with shedding of the endometrial layer. Adapted from Henderson, C & Macdonald, S. 2004; Mayes' Midwifery: A Textbook for Midwives (13th ed.), Ballière Tindall, Edinburgh, Fig. 6.1, p. 89.

Ovulation occurs approximately 36 hours after the aforementioned LH surge, in which follicular rupture leads to the release of the oocyte into the infundibular region of the fallopian tube (Adams et al., 1994). In a 28-day menstrual cycle, ovulation occurs around day 14. The remnants of the ruptured follicle, referred to as the corpus luteum, releases progesterone, which has a negative feedback effect on the release of FSH and LH. At the endometrial level, progesterone induces plasma membrane transformation, initiating the onset of the secretory state (Adams et al., 1994; Stocco et al., 2007). It is during the secretory phase, where an embryo can implant into the uterine lining and produce a pregnancy. In this case, following successful implantation, the endometrium will secrete human chorionic gonadotropin (hCG), referred to as the 'pregnancy hormone,' which maintains the corpus luteum and hence sustains progesterone production (Filicori et al., 1986; Taylor et al., 1995). On the other hand, in the absence of pregnancy, declining levels of LH contribute to decreasing levels of progesterone and estradiol, inducing menstruation and thus the start of a new menstrual cycle (Filicori et al., 1984; Taylor et al., 1995).

1.2.3 Conception

1.2.3.1 Fertilization

Life begins with the fusion of two gametes, in a process referred to as fertilization. In natural conception the site of fertilization is located within the oviduct (fallopian tube(s)). Ejaculated sperm make the long journey through the female reproductive tract, in which only a very small fraction will actually reach the egg. Once the sperm encounter the egg, the environment becomes quite competitive, with only one sperm actually penetrating the egg, in the case of a

normal fertilization. However, before a sperm can accomplish this great feat, it must first undergo a process called capacitation (Allouche-Fitoussi and Breitbart 2020). A capacitated sperm has the capability to make its way through the cumulus cells that surround the egg and bind to the zona pellucida (ZP), the glycoprotein layer that encapsulates the oocyte. Once bound to the ZP, a sperm then undergoes the acrosome reaction, in which the release of enzymes breakdown the thick ZP, allowing the sperm to reach the plasma membrane of the egg (Brucker and Lipford 1995). At this point fusion of the sperm to the plasma membrane can occur, allowing the transmission of the sperm nucleus into the ooplasm of egg. The fusion of the sperm to the egg membrane causes the egg to undergo the cortical reaction, imperative to normal fertilization as it prevents the fusion of other sperm to the membrane that may lead to a defect known as polyspermy (Wong and Wessel 2006).

1.2.3.2 Embryonic Development

Approximately 16 to 18 hours following the fusion of a sperm to the cell membrane of an oocyte, the signs of fertilization become apparent, and the resulting conceptus is now referred to as a zygote. Normal fertilization is marked by the presence of two pronuclei (Figure 6), one from each parent, with each possessing 22 autosomes and one sex chromosome. Another marking of normal fertilization is the extrusion of the second polar body (pb) (as stated previously, the completion of meiosis within the oocyte only occurs upon fertilization) (Gilbert, 2013).

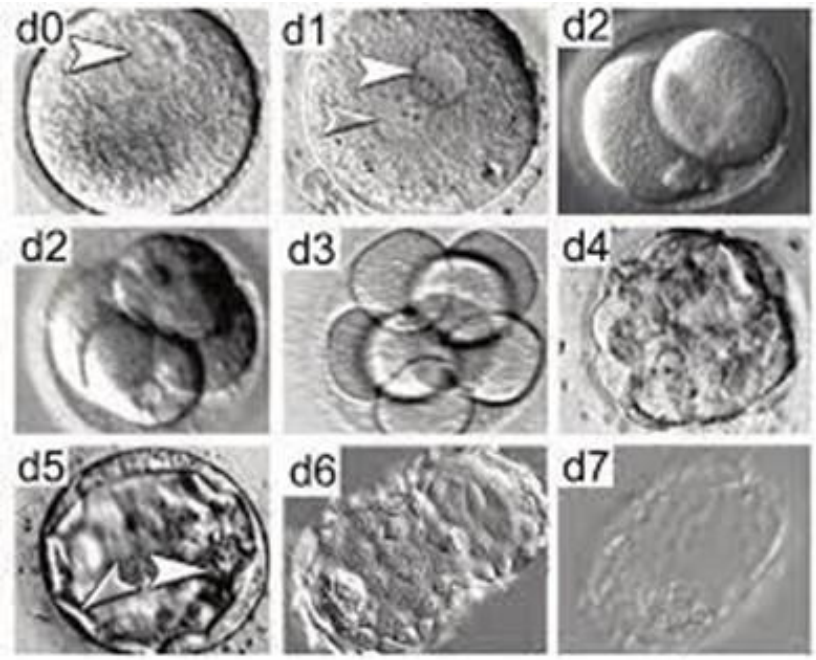


Figure 6. The stages of preimplantation embryonic development day 1 through day 7. Beginning with the upper left picture, d0, shows an immature oocyte, with the arrow pointing the germinal vesical. D1 is a zygote embryo, where each arrow is pointing to a single pronuclei, in which the presence of two pronuclei serve as a marker of normal fertilization. Both the upper right picture and center-row left picture display a day 2 embryo that has undergone the first mitotic division to form a two-cell embryo and the second mitotic division to form a four-cell embryo, respectively. D3 is a day 3 embryo consisting of eight cells. D4 is a morula containing approximately sixteen cells. D5 is a day 5 embryo, referred to as a blastocyst. The left arrow in d5 is pointing to the trophectoderm, whereas the right arrow is pointing to the inner cell mass. D6 shows an expanded blastocyst that is hatching out of the zona pellucida. D7 shows a hatched blastocyst that has completely escaped the zona pellucida. Taken from Niakan et al., 2012.

Occasionally the egg may be abnormally fertilized, such as in the case with polyspermy (the fusion of more than one sperm), in which three or more pronuclei will be observed (Figure 7), signifying extra sets of chromosomes (called polyploidy), a lethal scenario for the resulting conceptus (Papale et al., 2012).

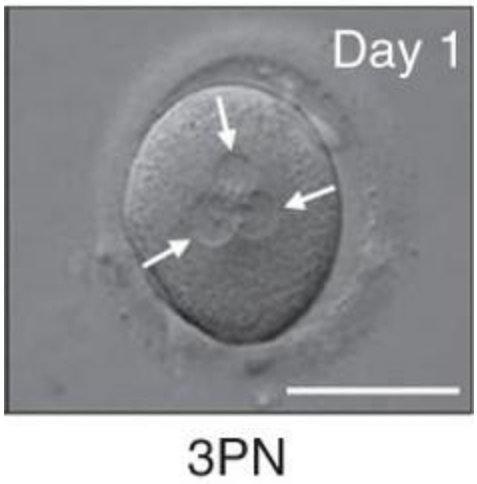


Figure 7. Abnormally fertilized embryo (3pn). Each arrow points to an evenly sized pronuclei (pn). Normal fertilization is marked by the presence of two evenly sized pns, with the assumption that one pn was contributed by the oocyte and the other pn by the sperm. The presence of three evenly sized pn represents and embryo with three complete sets of DNA, also referred to as triploidy, where three copies of every chromosome is present instead of the normal two. In this case the likely scenarios that resulted in this abnormal fertilization are: two sets of chromosomes contributed by the oocyte (diploid oocyte) and one set from the sperm, or two sets of chromosomes contributed by the sperm (either a diploid sperm or simultaneous fertilization by two different sperm (polyspermy)) and one set from the oocyte. Adapted from O'Leary et al., 2013.

The zygote continues its way down the fallopian tube, while at the same time undergoing multiple cell divisions, where it is now referred to as an embryo. The divisions and subsequent cellular proliferation are result of mitosis, in which a single cell (mother cell) divides into two identical daughter cells. Mitosis itself consists of four phases: prophase, metaphase, anaphase, and telophase, though to note prior to the initiation of this process, the chromosomes are first replicated while in interphase (Figure 8). Signaling the start of mitosis, prophase is when the chromatin in the nucleus begins to condense. Additionally, the nuclear membrane breaks down and spindle fibers begin to form. In metaphase, the spindle fibers align the chromosomes at the metaphase plate at center of the cell nucleus. Next, during anaphase, the paired chromosomes separate at the kinetochores and move to opposite sides of the cell. Telophase then occurs when

chromatids arrive at opposite poles of the cell and a new membrane form around each of the daughter nuclei. At the conclusion of telophase, cytokinesis occurs generating two daughter cells (Gersen and Keagle 2013).

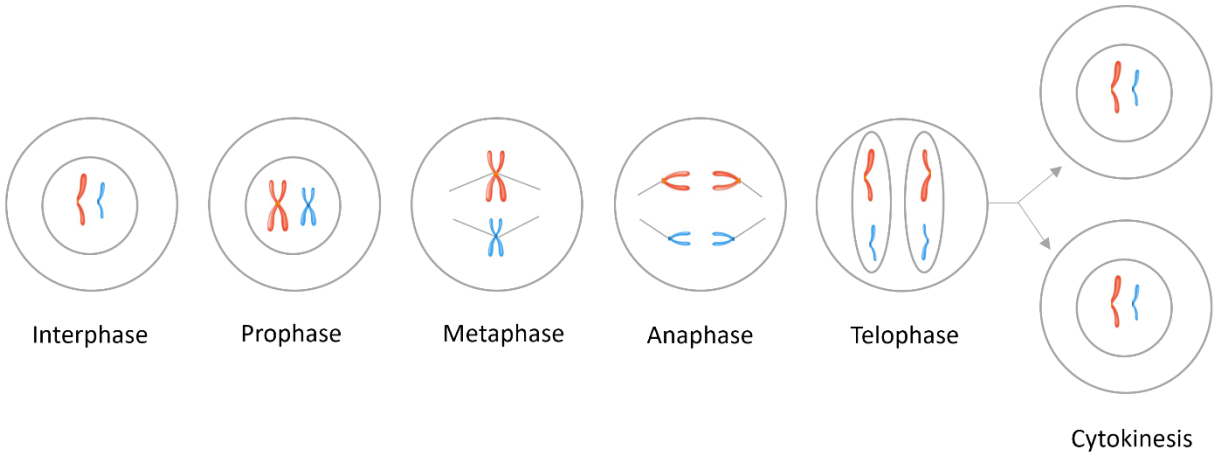


Figure 5. Phases of mitosis. In preparation for mitosis, chromosomes are first replicated during interphase. Prophase the chromatin in the nucleus begins to condense and the nuclear membrane breaks down and spindle fibers begin to form. Metaphase: the spindle fibers align the chromosomes at the metaphase plate at center of the cell nucleus. Anaphase: the paired chromosomes separate at the kinetochores and move to opposite sides of the cell. Telophase: the chromatids arrive at opposite poles of the cell and a new membrane form around each of the daughter nuclei. Cytokinesis: two daughter cells are generated.

The first mitotic division, occurs within the first 24 hours post-fertilization, usually resulting in a 2-cell embryo. The embryo continues to cleave, reaching the 6-8 cell stage the following day. By day 4 of embryonic development, the embryo is comprised of approximately 16 cells, or blastomeres, when it transforms into a sold mass, referred to as a morula. From this point mitotic divisions rapidly increase, expanding the size of the embryo, and coincides with the formation a fluid filled cavity called the blastocoel (Zhai et al., 2022).

By approximately day 5 of embryonic development, the embryo reaches the uterus, where it is has proliferated to over 100 cells and is now termed a blastocyst. At this point the cells within in the blastocyst have differentiated into two distinct lines, the trophectoderm (TE) which will eventually become the placenta and the inner cell mass (ICM) which will become the fetus. The TE forms cells on the outside of the embryo, while the ICM lies within the fluid filled blastocoel cavity. Within the uterus, the cells of the blastocyst will continue to divide, leading to blastocyst expansion and consequent thinning of the ZP. Eventually the ZP will thin out enough to where the blastocyst can hatch out and completely escape. Once the blastocyst is fully hatched, it can now attach and burrow into to the uterine wall to begin the implantation process (Zhai et al., 2022). Figure 9 summarizes the journey from fertilization to implantation (Gasser 1975).

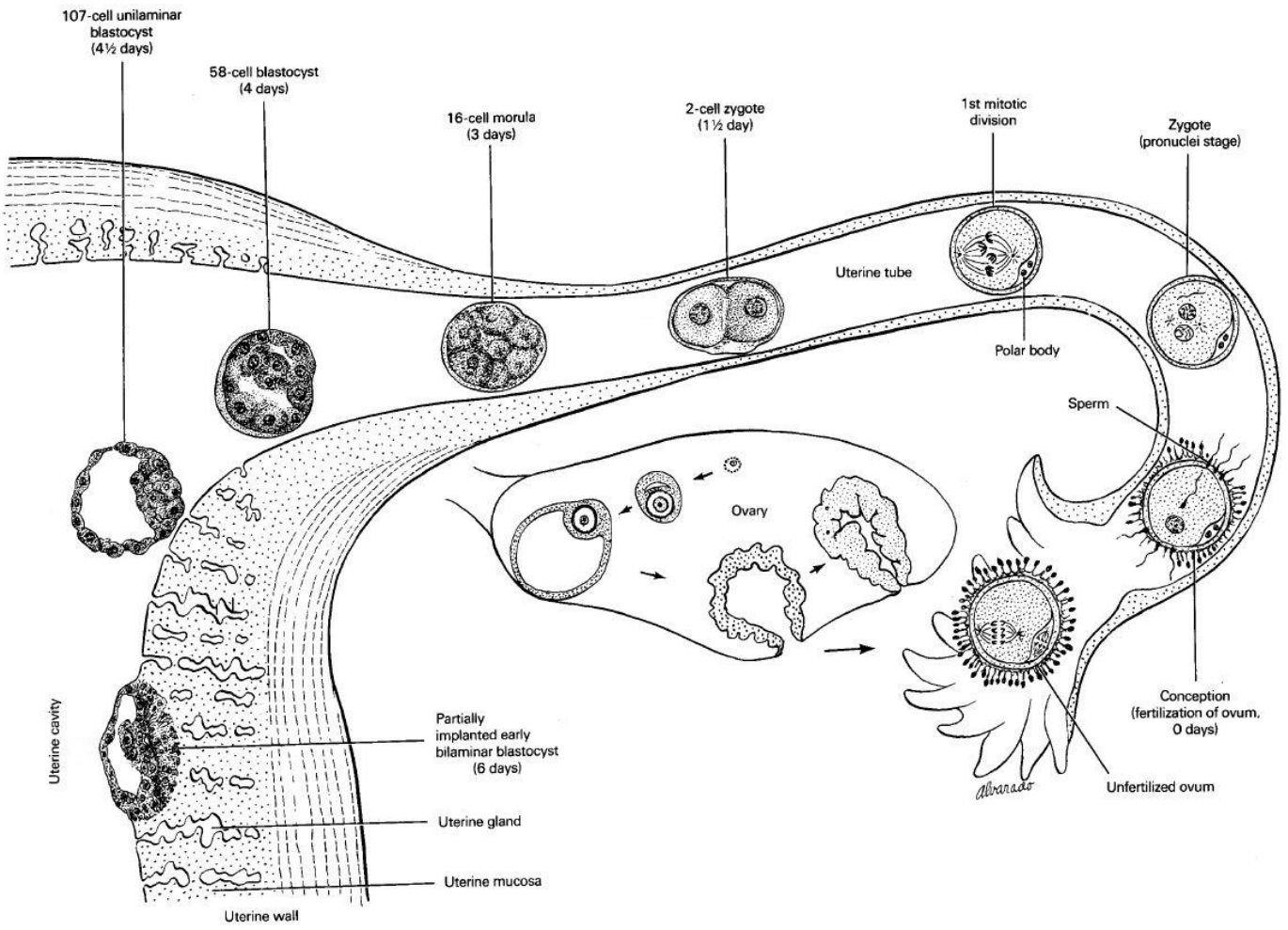


Figure 6. The developmental events and migration of the embryo during the first week of life. Oocytes reside in the ovary, where progression is marked by the arrows representing maturation from a primary oocyte to a secondary oocyte and finally the follicular rupture of a mature ootid. Each menstrual cycle, several follicles will grow, however, typically only one will grow to adequate proportion and release (ovulate) an egg. The egg is released into the infundibular region of the fallopian tube, where conception (fertilization by the sperm) takes place. Successful fertilization is marked by the presence of two pronuclei, one derived from the egg and the other by the sperm, and two polar bodies. At this point the resulting conceptus is called a zygote. The zygote will undergo its first mitotic division, while moving down the fallopian tube with the assistance of hair-like projections called cilia, resulting in a 2-cell embryo. Further cell division takes place as the embryo continues to move within the fallopian tube, where it transforms to a 16-cell morula around day 3. Eventually cavitation occurs, with fluid collecting between the cells forming a blastocoel. Additionally, the cells start to differentiate giving rise to an inner cell mass and a trophectoderm, at this point the embryo is referred to as a blastocyst and consists of over 100 cells. Coinciding with blastocyst formation is the migration of the embryo to the uterine cavity where implantation takes place. The embryo will position itself along the endometrium and attach around day 6, with invasion of the luminal epithelium following shortly after. Taken from Gasser RF, 1975, 2. Atlas of Human Embryos. Copyright © 1975 RF Gasser, PhD.

1.2.3.3 Implantation

Embryo implantation is imperative for pregnancy to be achieved and is a significant rate limiting step in human reproduction (Edwards 2006). This process largely hinges on the quality and status of both the embryo and endometrium and the interplay and dialogue between them (Cha et al., 2012).

1.2.3.3.1 Endometrial Anatomy and Physiology

The endometrium is the tissue that lines the inside of the uterine cavity. This is the location where the embryo implants and the resulting pregnancy resides for 9 months. In response to steroid hormones (estrogen and progesterone), this highly dynamic tissue undergoes physiological changes each cycle in order to prepare to receive an implanting blastocyst (Fitzgerald et al., 2021).

The endometrium is divided into two layers: the functional layer and the basal layer. The functional layer develops throughout the menstrual cycle to prepare for the arrival of an embryo, undergoing proliferation and secretion, and if a pregnancy is not attained, tissue degradation (Fitzgerald et al., 2021). Composed of two main cellular compartments, an epithelial cell lining and stroma, the functional layer accounts for two-thirds of the endometrial thickness (Beier and Beier-Hellwig 1998, Diedrich et al., 2007, Bulun and Adashi 2009). The basal layer possesses a regenerative capability and is responsible for regenerating the functional layer following menses (Diedrich et al., 2007, Bulun and Adashi 2009, Hawkins and Matzuk 2008).

As reviewed above, the uterine cycle can be divided into two phases: the proliferative phase and secretory phase (Figure 5). During the proliferative phase the endometrium rebuilds in response

to gradually increasing estradiol levels, leading to proliferation of stromal cells and glands and the elongation of spiral arteries. Following ovulation, the secretory phase begins, where an increase in progesterone further prepares the endometrium for an implanting embryo by inducing a receptive state. If an embryo successfully implants, then a pregnancy will be achieved, however if a pregnancy does not occur, then steroid hormone levels decrease, and menstruation will occur, where the endometrial lining will be shed (Hawkins and Matzuk 2008).

1.2.3.3.2 Embryo Implantation

Implantation occurs when a blastocyst attaches and infiltrates the endometrium. This process is integral to achieving pregnancy. Implantation itself is comprised of three phases: apposition, attachment, and invasion (Figure 10). During apposition, the blastocyst properly positions itself in front of the endometrial epithelium. Attachment follows with the blastocyst adhering to the endometrial basal lamina and the stromal extracellular matrix. Finally, the blastocyst penetrates and invades the luminal epithelium (Enders 1967).

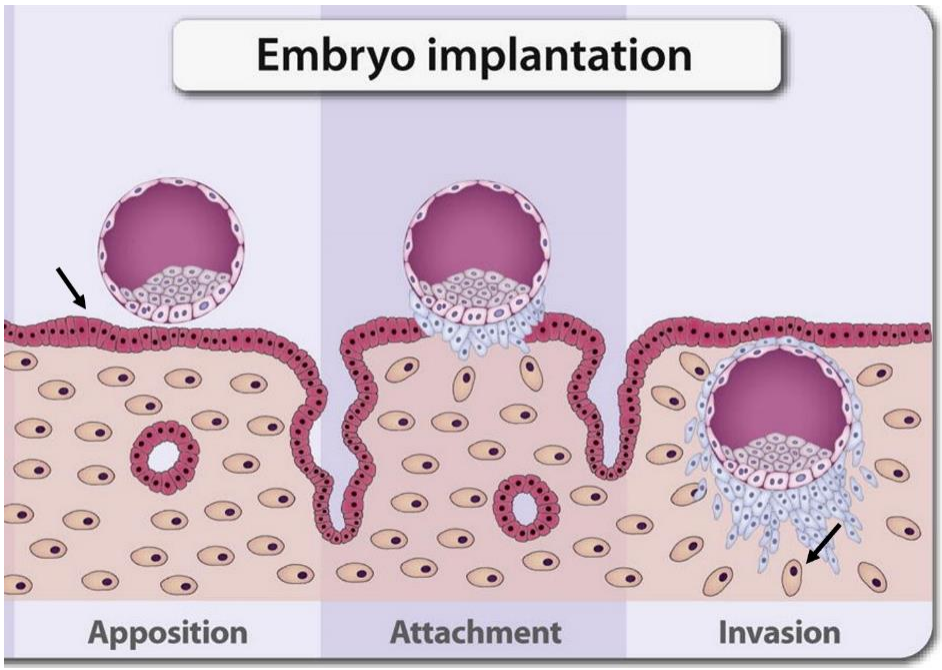


Figure 7. Blastocyst implantation process. During apposition the blastocysts aligns itself with the inner cell mass facing the endometrial epithelium (arrow pointing to endometrial epithelial cells). Next, the blastocyst attaches to the endometrial basal lamina and stromal extracellular matrix via the polar trophectoderm. Lastly, the blastocyst invades stromal cell compartment (arrow pointing to a stromal cell). Adapted from Weimar et al., 2013.

Both functionality and synchronization are necessary for successful implantation. Appropriate embryonic development must coincide with a receptive endometrium (Simón and Pellicer 2000, Ruiz et al., 2012). The uterus typically becomes receptive during the mid-luteal phase of the menstrual cycle (between cycle days 19 to 23), a time commonly referred to as the window of implantation (WOI) (Paria et al., 2001). The WOI is only open for a finite period, placing stringent time constraints on this process each month. Comparatively, in non-menstruating species, such as rodents and rabbits, implantation is controlled by the embryo, allowing more flexibility in the process, and hence greater overall efficiency in achieving pregnancy (Simón and Giudice 2017).

1.2.3.3.3 The Window of Implantation (WOI)

The existence of the WOI was first proposed in the mid-1950s (Hertig et al., 1956). Some studies have suggested that the window may last up to 10 days post-ovulation (Lenton et al., 1982), however, these earlier studies poorly defined the day of ovulation, resulting in a systemic bias to their conclusions (Wilcox et al., 1999, Direito et al., 2012). Later studies redefined the length of the WOI based on what was optimal versus what was possible, demonstrating superior clinical rates during a 2-day window (Prapas et al., 1998). More recently, in a study based on transcriptomic assessment of the WOI, a slightly shorter window has been defined lasting on average 29-36 hours, and in some women less than 24 hours (Rincon et al., 2018). In any case, the WOI concludes upon initiation of stromal cell decidualization, subsequently permitting trophoblast invasion and placental formation. It is not completely known whether there is an individual or inter-cycle variation in the duration of the WOI within each cycle, though molecular studies have found that the transcriptomic profiles pertaining to endometrial receptivity remain constant for at least 40 months when replicating cycle protocols and conditions (Díaz-Gimeno et al., 2013).

1.2.3.3.4 Attaining Endometrial Receptivity

Endometrial receptivity and the WOI is the product of a well-orchestrated and integrated interaction of paracrine signaling with ovarian hormones, growth factors, lipid mediators, transcription factors, and cytokines (Cha et al. 2013). Progesterone is the single most important hormone pertaining to receptivity and the WOI. Once progesterone levels reach a critical threshold, a well-timed and orderly secretory transformation begins. Even very low levels of serum progesterone (as low as 2.5ng/ml) have the capacity to trigger differential expression of

key genes associated with the inception of secretory transformation and ultimately the control of the WOI (Usadi et al., 2008; Mesen and Young 2015).

1.2.3.3.4.1 Ovarian Steroid Hormones

The endometrium is a hormonally regulated tissue wherein the acquisition of endometrial receptivity is chiefly regulated by estradiol and progesterone. The actions of these hormones are mediated by the presence of both progesterone receptors (PR-A and PR-B) and estrogen receptors (ERα and ERβ) expressed within the epithelium and stromal compartments of the endometrium. During implantation, signaling of these receptors are executed by juxtacrine, paracrine, and autocrine factors that are coordinated by various growth factors, cytokines, lipid mediators, homeobox transcription factors and morphogens (Cha et al 2013).

Estrogen is first to exert its effects on the endometrium. During the first half of the cycle, increasing estrogen levels promote endometrial cell proliferation. Ovulation occurs mid-cycle leading to the release of progesterone from the resultant corpus luteum, subsequently bringing about endometrial epithelial differentiation and maturation. As previously stated, progesterone is by far the single most important hormone pertaining to the establishment and maintenance of pregnancy. The initiation of the WOI by progesterone has been demonstrated clinically (Navot et al., 1991), epidemiologically (Wilcox et al., 1999), and morphologically (Murphy 2004).

1.2.3.3.4.2 Plasma Membrane Transformation

In order for implantation to take place, intercellular interaction between the apical membranes of the endometrial epithelium and the trophoblastic ectoderm must occur. In the endometrium it is imperative for the luminal epithelium to undergo a plasma membrane transformation from a

non-adhesive surface to an adhesive one. The endometrial remodeling bringing about this transformation requires the meticulous coordination between multiple systems and encompasses the integration of numerous molecules. Some of the members belonging to the molecular repertoire include prostaglandins, selectins, integrins, mucins, cadherins, cytokines, and immune response, all of whose contributions are detailed below. Together these molecules, among others, define a complex network, the balance of which promotes endometrial receptivity and successful implantation.

1.2.3.3.4.2.1 Prostaglandins

Prostaglandins are molecules commonly found in tissues throughout the human body and are active participants in numerous biological processes, such as in the endometrium where they appear to induce epithelial cell proliferation (Milne 2003; Battersby et al., 2004). Although the presence of prostaglandins persists throughout the entire menstrual cycle, their influence on endometrial receptivity have been documented. One study investigating endometrial fluid found a significant increase in the concentration of specific prostaglandins (PGE_2 and PGF_{2a}) during the WOI in fertile women versus infertile women. Additionally, when carrying out the same measurements 24 hours before embryo transfer in IVF cycles, higher concentrations of these prostaglandins were exhibited in those women who became pregnant versus those who did not (Vilella et al., 2013). These findings, along with others, such as a correlation between defective prostaglandin synthesis and repeated implantation failure (RIF) (Achache et al., 2010), suggest the effect of these specific molecules during the WOI.

1.2.3.3.4.2.2 Selectins

Belonging to the family of cell adhesion molecules (CAMs), selectins are gylcoproteins that mediate leukocyte-endothelium interactions. On the endometrium, L-selectin interacts with oligosaccharide ligands to mediate tethering and rolling of leukocytes on the endothelium (Fazleabas and Kim 2003; Genbacev et al., 2003). Additionally, L-selectin also has been found to be highly expressed on the surface of hatched blastocysts (Genbacev et al., 2003). The dual presence of this molecule suggests the importance of L-selectin, and its ligands, in early implantation to facilitate the adhesion of a floating blastocyst to the endometrium. Further supporting this claim, abnormal expression of selectins and their ligands have been associated with infertility. For instance, in the case of RIF, an absence of L-selectin ligand MECA-79 expression during the mid-luteal phase has been linked to nil or extremely low chance of pregnancy (Foulk et al., 2007). Abnormal expression has also been implicated in cases of endometriosis (Margarit et al., 2009) and ectopic pregnancies (Li et al., 2014).

1.2.3.3.4.2.3 Integrins

Integrins, also a member of the CAM family, are transmembrane cell adhesion molecules, found in the endometrium within the luminal and glandular epithelium (Lessey et al., 1992; Lessey et al., 1994a,b; Klentzeris et al., 1993). Regulated by steroids and cytokines, integrins seem to play a critical role in embryo-endometrial interaction at time of implantation. Receiving much attention, αvβ3 is an integrin expressed during the midsecretory phase (along with other integrins) and has been employed as a purported marker for the WOI. The expression of αvβ3 is rate limited by the production of the β3 subunit, which is regulated by HOXA10, a transcription factor whose expression in the uterus is imperative for fertility and decidualization (Benson et

al., 1996). An increase β3 mRNA subunit is observed after cycle day 19, though not before (Apparao et al., 2001). The absence of the β3 subunit has been implicated in endometriosis (Lessey et al., 1994a), whereas its aberrant expression is associated with unexplained infertility (Lessey et al., 1995), both of these observations serve as the basis for the diagnostics applied in the E-tegrity test that analyzes endometrial samples for endometriosis and luteal phase defects.

1.2.3.3.4.2.4 Mucins

Another member of the CAM family, mucins are transmembrane glycoproteins that line the apical surface of the endometrial epithelium. There are three mucins found within the endometrium, MUC1, MUC4, and MUC16, and are the major constituents of mucus wherein their primary functions include lubrication and protection. MUC1 possesses anti-adhesion properties in which it has been proposed that the physical mucus barrier created by the high density of MUC1 may prevent blastocyst attachment by masking the adhesion molecules present on the endometrial epithelium (Aplin et al., 1996). Intuitively, a downregulation of MUC1 during implantation would be expected, as observed in the mouse model, though the opposite has been reported in humans. In fact, fertile women experience a progesterone-mediated increase in MUC1 levels during the WOI (Meseguer et al., 1998; Horne et al., 2006) whereas women with RIF typically do not (Bastu et al., 2015). A potential explanation for this contradiction includes recognition of MUC1-associated glycans by the embryo, potentially resulting in a blastocyst-induced localized clearance of MUC1 during adhesion to facilitate implantation at that specific site (Meseguer et al., 1998). Based on this proposed theory, MUC1 could essentially act as a gatekeeper, preventing the implantation of low-quality embryos that possess poor reproductive potential.

1.2.3.3.4.2.5 Cadherins

Cadherins are CAM glycoproteins responsible for calcium-dependent cell-to-cell adhesion and consist of 3 subclasses: E, P, and N. In mice, E-cadherin appears to play a role in proper trophoblast invasion as a significant enrichment in E-cadherin expression is observed at the apical membranes of uterine epithelial cells just prior to implantation (Jha et al., 2006). Additionally, the presence of a E-cadherin gene mutation has been reported to result in defective embryo implantation (Riethmacher et al., 1995). In humans, E-cadherin mRNA expression have also been shown to significantly increase post-ovulation in response to progesterone (Fujimoto et al., 1996) and is the dominant molecule found in the adherens junction, which play a critical role in in the mediation of cell-to-cell adhesion (Berx et al., 1998). However, surprisingly, immunohistochemical analyses have failed to detect cyclic variations in E-cadherin protein levels (van der Linden et al., 1995; Béliard et al., 1997; Dawood et al., 1998; Poncelet et al., 2002). Complete understanding of this molecule's role in human receptivity and implantation still requires further investigation.

1.2.3.3.4.2.6 Cytokines

Cytokines are multifunctional glycoproteins that act as signaling molecules via their interaction with specific cell surface receptors. Found throughout the endometrium, including the luminal and glandular epithelium and the stroma, cytokines enable communication between endometrial cells as well as between the endometrium and embryo. A number of different cytokines have been implicated in uterine receptivity and implantation (including a specific role in decidualization) as marked by increasing levels during the mid to late secretory phases. It has been suggested that appropriate cytokine equilibrium must be maintained for these processes to

be carried out successfully. In mouse models, cytokines IL-12, IL-15, and IL-18 are essential for proper activation of uterine natural killer (uNK) cells (the loss of which is associated with unexplained infertility, recurrent pregnancy loss (RPL), and endometriosis (Giuliani et al., 2014)), and control of angiogenesis. However, to note, when out of balance these cytokines can exert deleterious effects (Croy et al., 2003a, b). In humans, mutations, abnormal expression patterns, and deficiencies of certain cytokines and their receptors have been associated with unexplained infertility, RPL, and implantation failure (Dimitriadis et al., 2000; von Wolff et al., 2000; Krüssel et al., 2003; Steck et al., 2004).

1.2.3.3.4.2.7 Immune Response

Multiple immune regulators have been recognized as playing an essential role in endometrial receptivity and implantation. Directly regulated by estrogen and progesterone, there is an influx of immune cells to the endometrium during the secretory phase that converts local immunity from adaptive to innate (Gellersen et al., 2007). In other words, this conversion leads to a shift from protecting the host from invaders to permitting the invasion of a blastocyst and subsequent maintenance of pregnancy to occur (Robertson 2000). As such, during the mid to late secretory phase a majority of immune cells present within the endometrium belong to the innate immunity compartment and include uNK cells (making up 65-70% of the immune cells present at this time), macrophages and dendritic cells, though, adaptive immune T cells are also detected (Loke et al., 1995; Bulmer and Lash 2005; Dosiou and Giudice 2005; Hanna et al., 2006; Vacca et al., 2011). Uterine NK cells provide numerous growth factors and cytokines that are integral to inducing local secretion of angiogenic factors by endometrial cells and building a healthy placenta (Ashkar et al., 2003; Hanna et al., 2006). Not surprisingly, abnormal uterine levels of

uNK cells have been associated with unexplained infertility, RPL, and endometriosis (Giuliani et al., 2014). Although uNK cells appear to be necessary for successful implantation and the resulting pregnancy, poor prognostic value pertaining to uNK cell count has inhibited any sort of clinically relevant diagnostic test involving this immune cell.

1.2.3.3.5 Implantation Failure

In general, conception is most likely to occur in the first month of trying, based on an associated 30% conception rate (Taylor 2003). However, as discussed previously, implantation is a major rate limiting step to achieve a successful pregnancy (Edwards 2006), and compared to other mammals, this process in humans is quite inefficient. At the very basic level, the major contributors to failed implantation pertain to abnormalities in the embryo and/or endometrium.

1.2.3.3.5.1 Chromosome Abnormalities in Human Embryos

1.2.3.3.5.1.1 Aneuploidy

Aneuploidy refers to a numerical chromosome abnormality that deviates from the normal karyotype (46 chromosomes in humans). This includes whole chromosome gains (trisomies) and losses (monosomies) along with partial gains and losses, referred to as segmental aneuploidies. A single aneuploidy within an embryo may exist, or combination of aneuploidies, yielding a complex aneuploid result. Aneuploidy in embryos is strongly correlated with maternal age (Kahrahman et al., 2000; Hassold and Hunt 2009; Rubio et al., 2017) and can result in poor embryo development, failed implantation, miscarriage, or the birth of a baby with congenital anomaly/ies (Hassold and Hunt 2001; Hall et al., 2006; Hassold et al., 2007).

Aneuploidy can arise from both meiotic (in gametes) and mitotic (in embryos) errors and has been reported to occur in all 23 chromosomes (Fragouli and Wells 2011). As there is a well-established correlation between advancing maternal age and increasing incidence of embryo aneuploidy, we expect a high percentage of embryo aneuploidy to originate from meiotic abnormalities in oocytes. In a study by Franasiak et al., (2014), the authors demonstrated that the percentage of aneuploid blastocysts increased from 20.7% in women in their 20s to upwards of 90% in women nearing their mid-40s (Figure 11). Biologically, this makes sense, as women are born with all of the eggs they will ever have, which remain in meiotic arrest until just before ovulation. This extended meiotic arrest translates to prolonged exposure to environmental insults (Tarín et al., 1996; Tatone et al., 2011), leaving oocytes vulnerable to errors that result in abnormal spindle formation and chromosome malsegregation. Although the true incidence of aneuploidy remains unknown in natural conceptions, the relatively high rates documented in *in vitro* fertilization (IVF) implies that aneuploidy is a key contributor to failed cycles, especially for women with advanced maternal age (AMA) (Bronsens et al., 2014), and is a prominent cause of RIF (Rubio et al., 2013).

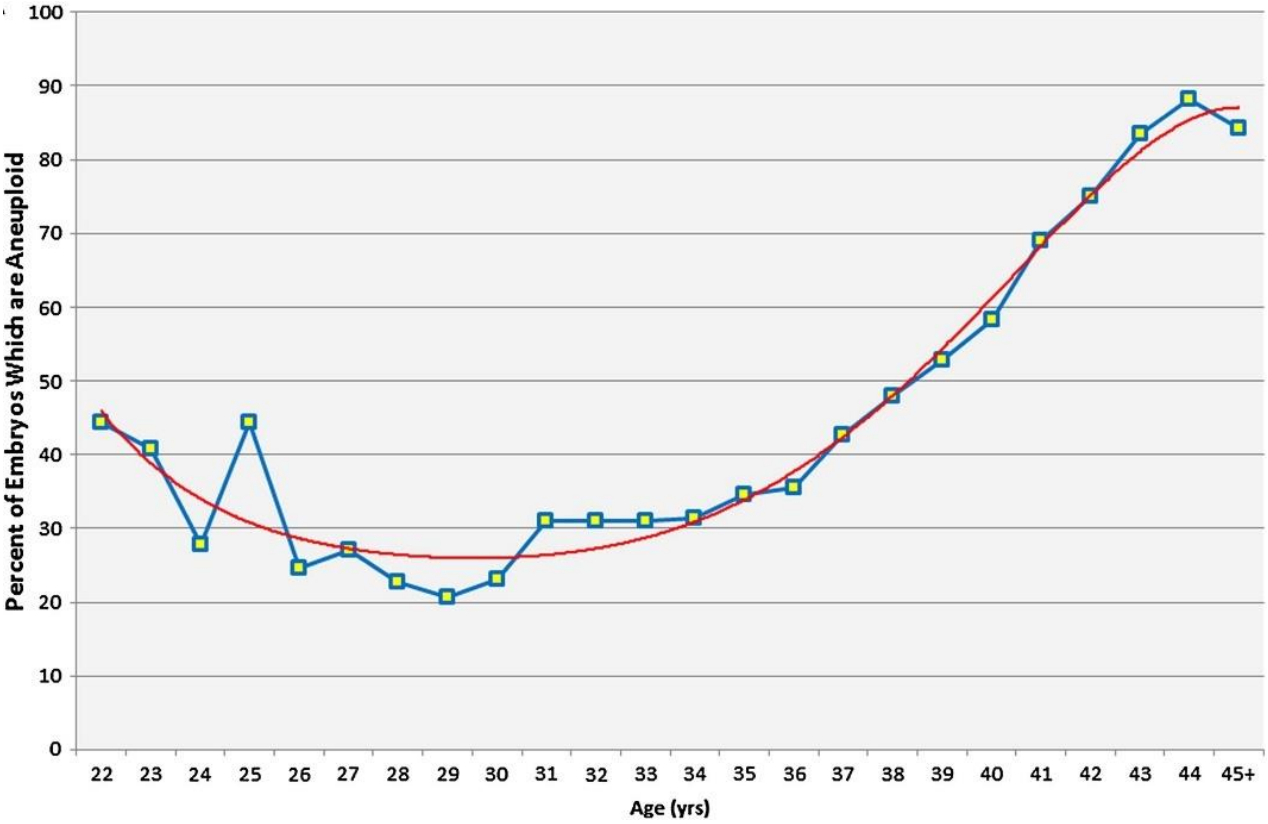


Figure 8. Percent of embryos which are aneuploid versus maternal age. The prevalence of aneuploidy relative to female age is lowest for women in their mid to late twenties. Significantly higher rates of aneuploidy were observed in women that were in their earlier twenties and also those who were beyond their late twenties. The authors determined that the relationship between aneuploidy and maternal age was best predicted by a fifth order polynomial. This polynomial regression line is shown in red. Adapted from Franasiak et al., 2014.

1.2.3.3.5.1.2 Mosaicism

Aneuploidy is often described as a chromosome abnormality (or abnormalities) that is uniformly persistent throughout all cells of a conceptus. However, it is more accurate to expand this description to include mosaicism, defined as the presence of two or more chromosomally distinct cell lines. The common prevalence of mosaic aneuploidy in human preimplantation embryos was first documented in the early 1990s in studies applying interphase fluorescent *in situ* hybridization (FISH) for sexing (Griffin et al., 1992; Delhanty et al., 1993). Mosaicism itself is

usually the product of a mitotic error generally thought to be derived from one of three main mechanisms: mitotic non-disjunction, anaphase lagging, or endoreplication (Figure 12). Mitotic non-disjunction is the failed separation of sister chromatids, resulting in one cell with a monosomy and another cell with a trisomy. Anaphase lagging, believed to be the main mechanism leading to mosaicism (Coonen et al., 2004; Ioannou et al., 2012; Capalbo et al., 2013), is the failure of a single chromatid to be incorporated into the nucleus, resulting in one cell possessing a monosomy of that chromosome and another cell possessing a disomy. Endoreplication is the replication of a chromosome without division, resulting in one cell with a trisomic chromosome and the other with a disomic chromosome (Taylor et al., 2014b).

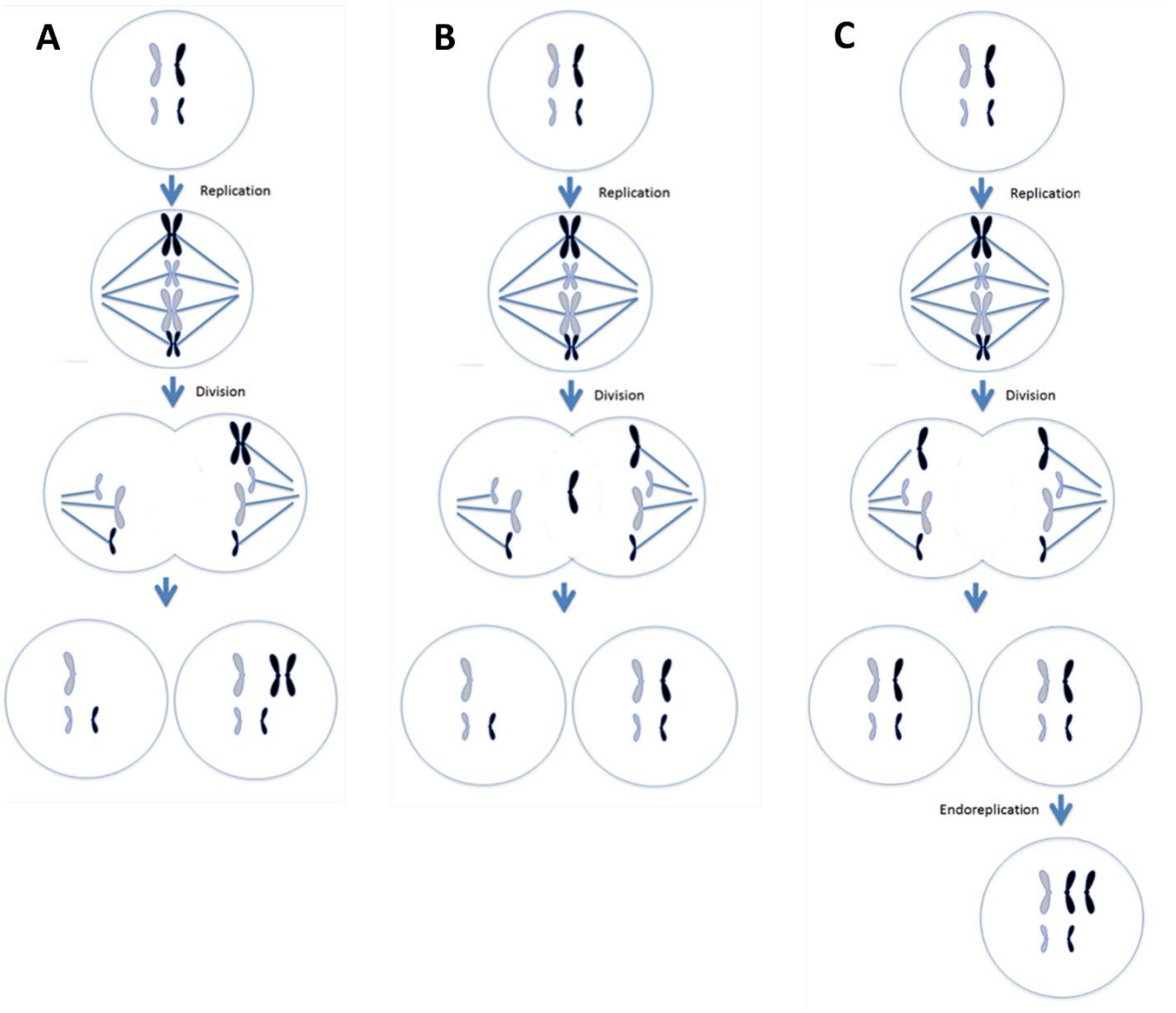


Figure 9. Three main mechanisms leading to mosaicism in embryos. (A) Mitotic non-disjunction event, where the failure of sister chromatids to separate during mitosis, leading to a monosomy in one cell and a trisomy in another. (B) Anaphase lagging event, where there is the failure of a single chromatid to be incorporated into the daughter cell nucleus, leading to a disomy in one cell and a monosomy in another. (C) Endoreplication event, where there is a replication of a chromosome without division, resulting in a trisomy in one cell and a disomy in the other. Adapted from Taylor et al., 2014b.

Although most mosaic events purportedly arise from purely post-zygotic, mitotic errors, some may originate from an initial meiotic abnormality. In the case of trisomy rescue, a cell that possesses a meiotically-derived trisomy is corrected during an anaphase lagging event, resulting in a diploid chromosome complement (Figure 13). The frequency of trisomy rescue is not well

established; however, this mechanism may account for the incidence of some uniparental disomies (UPDs) leading to presence of two copies of maternal chromosomes or two copies of paternal chromosomes, instead of one copy of each (Taylor et al., 2014b).

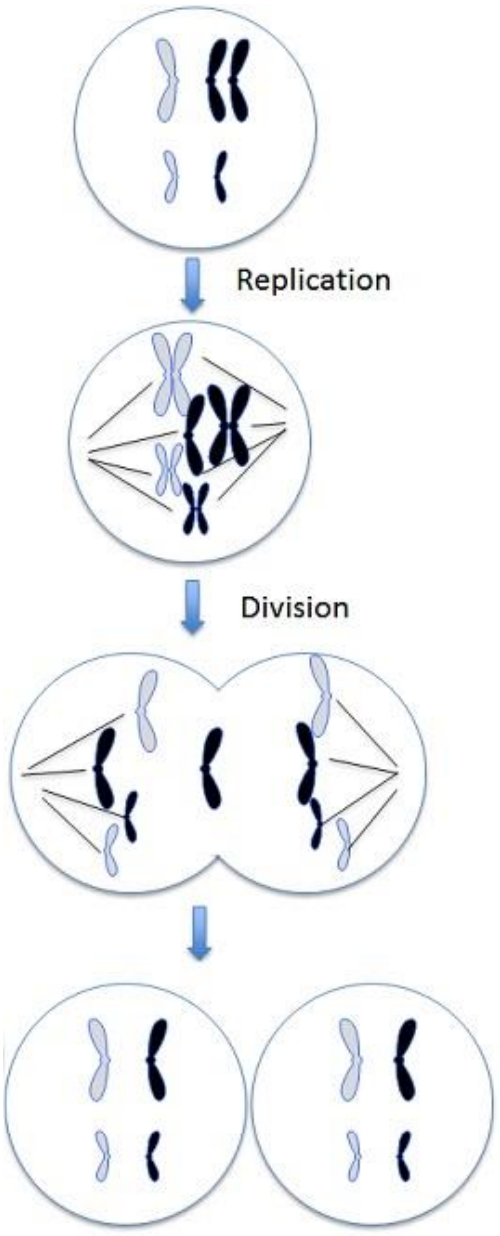


Figure 10. Trisomy rescue of a meiotically-derived trisomy. A trisomic rescue event where a trisomic chromosome undergoes an anaphase lagging event, where a chromatid fails to be incorporated during the final stages of mitosis, subsequently resulting in two disomic cells. Adapted from Taylor et al., 2014b.

When assessing the severity of a mosaic event, it is not only necessary to consider the causative mechanism, but also the timing of the error itself. A mitotic error occurring in early embryo development, prior to cellular differentiation, will result in general mosaicism where the presence of different cell lines persists throughout the entire organism. The same holds true for "meiotic" mosaicism (i.e., trisomy rescue), in which more severe clinical outcomes are expected due to a higher number affected cells. Conversely, a mosaic event occurring later in development will result in confined mosaicism, in which the affected cells are localized to a specific area of an organism. A common example of confined mosaicism in reproduction is confined placental mosaicism (CPM) resulting in a different chromosomal status between the fetus and placenta (Ledbetter et al., 1992).

1.2.3.3.5.2 Endometrial Abnormalities

Pathological modification to the uterine cavity (hyperplasia, submucosal myomas, endometrial polyps, endometritis, among others), accounts for 18-27% of repeated implantation cases (Demirol and Gurgan 2004). In the case of pathological uterine anomalies such as endometrial polyps, surgical interventions can be applied and in the case of chronic endometritis, antibiotics can be administered to rid the endometrium of pathogenic bacteria (Cinelli et al., 2015).

Nevertheless, patients can still endure implantation failure without ever identifying a cause. It was previously implied that the WOI was constant in timing for all women, even those with RIF, however, we now know that both woman with (Li et al., 1993, Ruiz-Alonso et al., 2013) and without a history of implantation failure (Simón et al., 2020), can have displaced WOI at the expected time of endometrial receptivity. Classic morphometric analysis applied in earlier studies, found that RIF patients undergoing insemination had slowly evolving endometrium in

relation to their cycle timing (Li et al., 1993). Later genomic studies further validated this trend observing altered gene expression patterns at the anticipated time of receptivity in women with RIF versus fertile controls (Tapia et al., 2008, Koler et al., 2009), discovering aberrations in hormonal regulation of endometrial genes (Bersinger et al., 2008) and endometrial prostaglandin synthesis (Achache et al., 2010).

1.2.3.3.5.3 Repeated Implantation Failure

Patients suffering with RIF experience reduced reproductive outcomes (Das and Holzer 2012; Mitri et al., 2016), making RIF a major cause of infertility in otherwise healthy women. Although RIF ultimately affects success, even following the application of assisted reproductive technologies (ART) such as IVF (Margalioth et al., 2006, Simón and Laufer 2012), it remains largely unaddressed and poorly characterized. This in turn leads many patients to drop out of the infertility treatment process and consider second opinions. Various definitions of RIF exist (Stephenson and Fluker 2000), for instance in the IVF setting, RIF is commonly defined as the failure in more than 3 IVF cycles involving the transfer of morphologically high-quality embryos (Simon and Laufer 2012). It is generally agreed that after the failure of 3 or more IVF cycles, with the transfer of one or two morphologically good embryos, special protocols should be employed. However, since RIF has not yet been fully investigated and there lacks hard evidence from RCTs demonstrating the clinical value of current approaches (Stephenson and Fluker 2000; Margalioth et al., 2006, Simón and Laufer 2012), patients are often subjected to empirical treatments that are unproven and potentially harmful.

1.3 *In vitro* Fertilization

IVF was first carried out in animals and dates back to the late 1800's with the report of the first known case of embryo transplantation in rabbits (Biggers and Heape 1991). The first human IVF pregnancy did not occur until about 80 years later in 1973 in Melbourne Australia. Unfortunately, this pregnancy ended less than one week later (De Kretzer et al., 1973). However, soon after, the world welcomed the first ever IVF baby, Louise Brown, on July 25, 1978 (Steptoe and Edwards 1978). Since then, millions of babies have been born worldwide following IVF treatment and as a field we are continually exploring ways to optimize outcomes of this treatment, by introducing new technologies and gaining a deeper understanding of basic science underlying human reproduction.

1.3.1 IVF Overview

The first step of IVF involves stimulating ovarian follicular development and oocyte maturation with the administration of exogenous gonadotropins (Pacchiarotti et al., 2016). After approximately 10 days of stimulation, the oocyte retrieval takes place, and the collected oocytes are then fertilized in the IVF laboratory. Fertilization can be performed using two different techniques. One technique is intracytoplasmic sperm injection (ICSI) where one sperm is injected into the cytoplasm of one egg. The second method is through conventional insemination where sperm are introduced to a petri dish containing the eggs. Resulting embryos are cultured in the laboratory for typically a week, when at this time they can either be transferred directly back to the uterus or cryopreserved for future transfer in a frozen embryo transfer (FET) cycle. As the

embryo transfer represents the initiation of the implantation in the IVF setting, this section will review various aspects pertaining to this specific procedure and the advances realized.

1.3.2 The Embryo Transfer

1.3.2.1 Day of Embryonic Development

In the earlier days of IVF, modified versions of the "transfers" we know today, were carried out in order to improve the chance of a successful pregnancy in patients undergoing ART. These consisted of gamete intrafallopian transfers (GIFT) and zygote intrafallopian transfers (ZIFT). With GIFT, multiple oocytes are retrieved from the ovary and mixed *in vitro* with sperm. The oocyte-sperm mixture is then placed into a catheter where it is then injected directly into the fallopian tubes via a laparoscopic procedure (Pace-Owens 1989). ZIFT on the other hand, also involves the retrieval of several oocytes, however fertilization is observed in the laboratory and only the resulting zygotes will be introduced laparoscopically into the fallopian tubes, one day post-retrieval (Devroey et al., 1986). These methods are no longer commonly practiced, as limitations, such as increased pelvic infection following laparoscopic transfer and a high risk of multiple pregnancy, outweigh the benefits, with reported live birth rates as low as 15.9% with GIFT (Rombauts et al., 1997) and miscarriage rates as high as 22.5% with ZIFT (according to the Society for Assisted Reproductive Technology registry results from 1994).

Still carried out today, albeit at a much lower frequency, the transfer of cleavage stage embryos directly to the uterus on either day 2 or more commonly day 3 of embryonic development, was the primary stage of transfer in IVF until recent years. Transferring a cleavage stage embryo over a zygote, provided the advantage of transferring the 'best' embryos, as not only does a visualized

cell division validate embryo viability in early development, but morphological assessment in terms of appropriate cell number, cell symmetry, and percent fragmentation present is performed to choose which embryos would likely have a higher chance of success (De Placido et al., 2020). In a direct comparison to ZIFT, the ability to select the best embryos at the cleavage stage for transfer not only provides improved clinical rates, in terms of pregnancy and implantation, (Jaroudi et al., 2004), but has been shown to be overall more efficient and affordable (Shahrokh Tehraninejad et al., 2015), with a lower risk for post-procedural complications, as anesthesia and laparoscopic transfer are not required for a cleavage stage transfer.

Currently, blastocyst transfer is the preferred method of transfer in modern IVF, with associated implantation rates estimated to be 25%-35% higher than cleavage stage transfer (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology). Although the first report of a pregnancy resulting from a blastocyst transfer was in 1995 (Edwards and Brody 1995), its widespread practice has only taken place in recent years. The transition to blastocyst transfer was fundamentally made possible by the introduction of improved culture media and incubation systems that enhanced the progression of embryo development to this phase *in vitro*. Transferring at the blastocyst stage furthers the concept of choosing the 'best' embryo for uterine replacement, as not all embryos will reach this developmental phase, lending to the notion of selecting only those embryos demonstrating ongoing viability for transfer and hence higher implantation potential (Jones et al., 1998; Racowsky et al., 2000). Additionally, the prevalence of chromosomal aneuploidy in day 5 blastocysts has been shown to be significantly less when comparing day 3 cleavage stage embryos (Staessen et al., 2004). From an *in vivo*

perspective, another benefit of transferring a blastocyst over a cleavage stage embryo pertains to the uterine environment. In the case of natural conception, the embryo does not reach the uterus before the morula phase (Croxatto et al., 1972). With differing metabolic requirements between a cleavage-stage embryo and a blastocyst, which are adequately and uniquely addressed by the fallopian tube and uterus (Gardner et al, 1996), respectively, exposing a cleavage-stage embryo prematurely to the uterus may cause embryonic stress, potentially reducing its implantation potential. Considering all of the above, it is clear to understand the significantly higher clinical rates reported with blastocyst transfer over cleavage-stage transfer (Papanikolaou et al., 2005; Papanikolaou et al., 2006; Zech et al., 2006).

1.3.2.2 Number of Embryos Transferred

The evolution to blastocyst transfer has not only resulted in increased success following IVF treatment but has altered the number of embryos typically transferred per cycle, which has ultimately led to the current movement and objective of an elective single embryo transfer (eSET). Prior to 2002, only 1% of fresh IVF transfers in the United States were eSET (Harbottle et al., 2015). In the earlier days of IVF, multiple embryos were commonly transferred in order to maximize the likelihood of success, however this often gave rise to high rates of multiple pregnancy, the most common adverse ART outcome (ESHRE Campus Course Report, 2001). The risks of a multiple pregnancy not only exist for the mother (Ombelet et al., 2005), but also extends to the resulting infants, with an increase occurrence of premature birth, low birthweight, disability, and death (Boulet et al., 2008). Fortunately, due to the multiple advancements realized in the IVF field over the last few decades, including blastocyst culture and PGT-A, eSETs can be performed without reducing live birth rates.

1.3.2.3 Embryo Transfer Cycle Types

A fresh embryo transfer occurs in the same cycle as ovarian stimulation and oocyte retrieval, typically performed on day 3 or day 5 post ovum collection, in which a day 3 cleavage stage embryo or a day 5 blastocyst, respectively, is replaced. However, embryo transfers have been performed anytime between 1 to 6 days post-retrieval, depending on factors such as number of embryos in culture, quality of embryos, and if complementary procedures such as PGT-A have been applied. It was previously thought that "fresh is best" when it came to transferring embryos, however this mindset has now shifted, with most clinics stepping away from fresh embryo transfers and increasingly offering FETs. This shift was in large part made possible by the advancement in embryo freezing protocols, specifically the introduction of vitrification (rapid cooling that prevents deleterious ice crystal formation within embryos) (Rienzi et al., 2017). Limitations of fresh embryo transfers include desynchrony with the uterine environment, as ovarian stimulation is associated with prematurely advancing endometrial receptivity (Hoozemans et al., 2004; Martínez-Conejero et al., 2007; Horcajadas et al., 2008), confining the number of blastocysts available for transfer (since fresh blastocyst transfers typically occur on day 5, meaning only those embryos reaching the blastocyst stage by this day are considered, hence omitting the selection of day 6 blastocysts), and the inability to have PGT-A performed (as fresh day 6 transfers post-PGT-A are not commonly performed due to embryo-endometrial desynchrony, added expense (reference labs charge more for fresh PGT-A results), and simply because many PGT-A reference labs no longer offer the option for next-day PGT-A results).

A FET occurs in a different cycle than ovarian stimulation and oocyte retrieval. To carry out a FET, patients will first undergo endometrial preparation in either a natural or hormone replacement

therapy (HRT) cycle, where monitoring by bloodwork and ultrasound is performed to help ensure proper endometrial conditions, such as pattern (trilaminar appearance) and thickness. Once endometrial conditions are suitable for implantation, a frozen embryo will be selected, warmed (thawed), evaluated for viability and quality, and then transferred into the uterus. Previously, FETs were mainly conducted when patients underwent a subsequent transfer with surplus embryos following a fresh embryo transfer (either in the case of an initial failed fresh cycle or for another baby following a successful fresh transfer), if they could not transfer in a fresh cycle due to ovarian hyperstimulation syndrome that required a "freeze all" in the fresh cycle, or in cases where preimplantation genetic testing for monogenic disorders (PGT-M) were performed that required a "freeze all" post-biopsy due to a prolonged turnaround time for results. The rise in the proportion of FETs versus fresh transfers over the last decade coincided with the widespread implementation of vitrification into the IVF lab, which has improved post-thawing (warming) survival rates in embryos and has been found to be superior to slow freezing/thawing in terms of clinical outcomes (Rienzi et al., 2017). Some of the advantages of FETs over fresh transfers include improved endometrial-embryo synchrony, increased cohort of blastocysts to select from (now can choose from day 5 or day 6 blastocysts), ability to perform PGT-A, and reportedly higher outcome rates (Acharya et al., 2018), though a recent Cochrane meta-analysis was unable to demonstrate the superiority of FETs over fresh transfers in regard to cumulative live birth rate and ongoing pregnancy rate (Zaat et al., 2021).

1.3.2.4 Embryo Transfer Selection

1.3.2.4.1 Embryonic Morphology

Morphology is the primary criteria employed in the IVF lab when attempting to select the most optimal embryos for transfer. Numerous grading systems exist, including globally recognized systems commonly applied throughout the IVF field and custom systems developed "in-house" that are uniquely applied within a particular IVF clinic. Different criterial factors are assessed depending on the developmental stage of the embryo (i.e., day 3 versus day 5). Once all of a patient's embryos are evaluated, a clear ranking based on quality can be established, and the best embryo(s) are selected for transfer back into the uterus. Transferring of excellent quality embryos have been associated with significantly higher live birth rates when compared to poor quality embryos, and even average and good quality embryos. It has also been demonstrated that poorer quality embryos have a statistically higher rate of miscarriage, even when controlling for embryonic aneuploidy (Irani et al., 2018). Lastly, it is important to keep in mind the subjective nature of this method, not only between different clinics (using different grading systems), but even between different embryologists working within the same IVF lab.

The most common factors assessed on day 3 include: cell number, cell symmetry, multinucleation, and degree of fragmentation. A high-quality cleavage stage embryo possesses a suitable number of cells for day of development (2-4 cells on day 2 or 6-8 cells on day 3), the cells themselves are symmetrical, with all being the same size and shape, lacking multinucleation, and ideally there is little to no cell fragmentation observed within the confines of ZP. Other factors that may be considered include the presence of vacuoles, granularity of the cytoplasm, and ZP thickness (Nasiri and Eftekhari-Yazdi 2015).

In terms of the blastocyst, perhaps the most widely used system for grading is the Gardner Score (Gardner and Schoolcraft 1999). Considered the gold standard for blastocyst assessment, this system incorporates three different quality scores pertaining to degree of expansion, quality of ICM, and quality of TE. Blastocyst expansion is graded on a scale of 1 to 6, with a 1 referring to a blastocyst with the least degree of observed expansion and 6 indicating a blastocyst that has completely hatched out of its ZP (Table 1). For blastocysts with expansion scores between 3-6, the ICM (Table 2) and TE (Table 3) are assigned a grade of A, B, or C, with A being the highest grade and C being the lowest. The final score is a compilation of all three grades, where the expansion score is listed first, followed by the ICM score, and finally the TE score. An example of a score corresponding to a superior quality hatched blastocyst would be "6AA." In addition to using morphological grading to select the best blastocysts for transfer, this system can also be used to select which embryos would be suitable to undergo PGT-A testing and/or cryopreservation.

Table 1. Gardner scoring for blastocyst expansion.

Expansion Score	Degree of Expansion
1	Early blastocyst - with blastocoel less than half of the volume of the embryo
2	Blastocyst - with blastocoel that is half or greater than half of the volume of the embryo
3	Full blastocyst - blastocoel completely filling the embryo
4	Expanded blastocyst – blastocoel volume larger than that of the early embryo, with thinning ZP
5	Hatching blastocyst – TE starting to herniate through the ZP
6	Hatched blastocyst – blastocyst has completed escaped from the ZP

Table 2. Gardner scoring for ICM quality.

ICM Grade	ICM Description
A	Tightly packed, many cells
B	Loosely packed, several cells
C	Very few cells

Table 3. Gardner scoring for TE quality.

TE Grade	TE Description
A	Many cells, forming a cohesive epithelium
B	Few cells, forming a loose epithelium
C	Very few large cells

1.3.2.4.2 Morphokinetics

A more recent method for embryo assessment is time-lapse microscopy. This involves the continuous imaging of cultured embryos in the IVF lab. Time-lapse systems may be introduced into existing laboratory incubators or marketed as stand-alone benchtop incubator units with imaging components already integrated. In either case, time-lapse microscopy provides the ability to monitor embryonic development without disturbance (unlike traditional microscopic assessment where embryos must be removed from the incubator and placed on a microscope for observation), hence maintaining optimal culture conditions such as a stable pH, oxygen concentration, and temperature. In theory, this undisturbed culture should contribute to better embryo development and quality, as fluctuations to the embryo's environment can lead to

metabolic stress (Fuiwara et al., 2007; Swain et al., 2016; Wale and Gardner 2016). Another advantage that time-lapse has over traditional microscopic assessment, is the continual monitoring of embryonic development and quality. With traditional microscopic assessment, embryos are usually evaluated once a day, if at all, leading to significant events being missed, such as abnormal or late fertilization, early pronuclear breakdown, and atypical cell divisions, a characteristic linked to lower rates of implantation (Rubio et al., 2012). However, in terms of embryo selection for transfer, perhaps the greatest advantage of time-lapse microscopy is the associated algorithm that provides an objective assessment of the reproductive potential of embryos, based not only on embryo quality, but also timing and rates of developmental events, referred to as morphokinetics (Meseguer et al., 2011; Conaghan et al., 2013; Basile et al., 2015; Peterson et al., 2016). Several studies, including RCTs, have demonstrated statistically significant increases in ongoing pregnancy rates when embryos are selected for transfer based on their morphokinetic score versus classic morphological assessment made by an embryologist (Rubio et al., 2013; Yang et al., 2014). However, a recent Cochrane meta-analysis concluded that there is still lacking evidence to prove that time-lapse scoring algorithms are superior to conventional microscopic grading by an embryologist to select those embryos with the highest reproductive potential (Armstrong et al., 2019). With that said, a growing number of studies linking specific morphokinetic parameters to embryonic ploidy (Chavez et al., 2012; Campbell et al., 2013a; Basile et al., 2014) paired with the other apparent benefits of time-lapse culture (such as an undisturbed embryonic environment), the role and utility of this method within the IVF lab continues to be actively investigated.

1.3.2.4.3 Preimplantation Genetic Testing for Aneuploidies (PGT-A)

Selecting by morphology alone however does not take into account that chromosomally abnormal embryos are commonplace in humans, most of which would not develop, and correlations between morphology and chromosomal status are patchy at best (Dahdouh et al., 2015). In other words, it is important to keep in mind that chromosomally abnormal embryos are often indistinguishable from chromosomally normal embryos when studied using a microscope (Munné 2006; Sandalinas et al., 2001; Fesahat et al., 2017).

Not only does aneuploidy affect women with AMA age (Kahrahman et al., 2000; Hassold and Hunt 2009; Rubio et al., 2017), but elevated rates have also been observed in younger women and even ovum donors (Franasiak et al., 2014). Additionally, the effects of aneuploidy can be deleterious to the success of IVF treatment and the resulting fetus (Hassold and Hunt 2001; Hall et al., 2006; Hassold et al., 2007). To overcome this, PGT-A was developed as an adjunct to an IVF cycle comprehensively to screen for aneuploidy in all 24 chromosomes (22 autosomes and 2 sex chromosomes), allowing for the subsequent transfer of an embryo diagnosed as euploid. Briefly, during the PGT-A cycle, embryos are generally cultured to the blastocyst stage. At this point, approximately 5 TE cells are biopsied and sent to a reference PGT-A lab for analysis. Based on the PGT-A lab results, the IVF clinic can then select those embryos that are euploid for transfer, while typically discarding the aneuploid embryos. Transfer of confirmed chromosomally normal (euploid) embryos are usually performed in FET cycles, though some are still carried out in fresh embryo cycles, in the case of day 5 biopsy with fresh day 6 transfer, or less commonly after either a day 3 blastomere biopsy or polar body (PB) biopsy.

The main indications for PGT-A are AMA, recurrent pregnancy loss (RPL), RIF (Findikli et al., 2006) and severe male factor infertility (Kahraman et al., 2006; Coates et al., 2015). However, considering the reported benefits of PGT-A, including improved implantation rate, reduction in miscarriage rate, and increased chance of delivering a healthy baby (Gianaroli et al., 1999; Munné et al., 1999; Yang et al., 2012; Forman et al., 2013; Scott et al., 2013a; Rubio et al., 2017), more patients than ever are opting to screen their embryos with PGT-A prior to transfer. In theory, selecting embryos for transfer based on confirmed euploidy should also result in increased live birth rates, as aneuploid (chromosomally abnormal) embryos are associated with lower implantation potential and increased rate of miscarriage (Gianaroli et al., 1999; Munné et al., 1999; Scott et al., 2013a; Rubio et al., 2017). Additionally, some authors have reported that embryo transfer selection based on PGT-A shortens treatment time and reduce overall treatment costs compared to transferring without aneuploidy screening (Neal et al., 2018; Somigliana et al., 2019). Although PGT-A is extensively used throughout the IVF field to select the best embryos for transfer, it is still widely debated whether or not the benefit of an improved live birth rate is actually achieved with PGT-A for euploid embryo selection.

1.3.2.4.3.1 Biopsy

Although seldom performed today, in the past PGT-A was mostly achieved by analyzing embryo biopsy samples obtained from either pbs or blastomeres. Pb biopsy was first introduced in 1990 (Verlinsky et al., 1990) and involves the consecutive or simultaneous removal of pbs from unfertilized oocytes and/or zygotes. Although this method represents the least invasive biopsy technique, the limitation to detect only maternally derived meiotic errors, and lack to detect both paternally derived meiotic errors and mitotic errors, contributes to pb biopsy having the

lowest predictive value of embryo euploidy and implantation potential compared to other biopsy types (Salvaggio et al., 2014).

Blastomere biopsy is performed on day 3 of preimplantation development, in which 1 to 2 cells are removed from the embryo. Though blastomere biopsy is the oldest and historically the most widely applied biopsy technique (Harper et al., 2012), considerable limitations pertaining to a high presence of mosaicism at the cleavage stage (Munné and Wells 2017), associated detrimental effects of the procedure on the developmental potential of embryos (Scott et al., 2013b), and reports of adverse effects of day 3 biopsy on IVF outcome (Mastenbroek et al., 2007), has resulted in blastomere biopsy being a largely omitted practice in clinical human IVF.

Blastocyst biopsy involves the removal of several TE cells from embryos and is most frequently performed on day 5 or day 6 of preimplantation development. Although this technique has existed for quite some time, the widespread use of blastocyst biopsy is relatively recent, coinciding with the advancement of embryo culture conditions in the IVF lab and successful implementation of vitrification (Rienzi et al., 2017). This method overcomes all of the aforementioned limitations of previous biopsy techniques, with one of the most obvious being the capability of TE biopsy to detect maternally and paternally derived meiotic errors in addition to mitotic errors. Regarding the advantages of blastocyst biopsy over cleavage stage, these include a significantly declined incidence of mosaicism at the blastocyst stage (Johnson et al., 2010a; Santos et al., 2010; Munné and Wells 2017), intrinsically lower detrimental effects of the procedure on embryonic development and viability (Scott et al., 2013b) (as only several cells are removed from the 100+ cell blastocyst versus 1-2 cells from a typical 6-8 cell day 3 embryo), and a marked improvement in implantation and delivery rates (Scott et al., 2013a). Other primary

benefits of blastocyst biopsy include the capability to report mosaic results and the potential cost savings for patients, due to a reduced cohort of embryos eligible for biopsy, as not all embryos that developed to a day 3 cleavage embryo will develop to a blastocyst.

1.3.2.4.3.2 Diagnostic Platforms

1.3.2.4.3.2.1 Fluorescence in situ Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) involves the use of target-specific DNA probes labeled with distinct fluorochromes that bind to their complementary sequence that allows for subsequent visualization and chromosome counting using a microscope (Figure 14). FISH was first applied clinically in PGT-M cases for sex-linked diseases (Delhanty et al., 1993; Griffin et al., 1993). The first utilization of FISH for PGT-A was performed soon thereafter, involving the assessment of five chromosomes, 13, 18, 21, X, Y, which are those chromosomes that can result in the live birth of an affected infant if an aneuploidy is present (Munné et al., 1993). Although widely utilized for over two decades, this method of testing is now rarely performed due to limitations such as inconsistencies in spreading and fixation methods and the inherent subjectivity pertaining to the visual analysis. However, conceivably the most significant limitation pertains to the number of chromosomes able to be screened using FISH. Due to the limited number of probes that can be used per round of FISH and limited number of rounds that can be performed before decreasing the efficiency of the technique (Ruangvutilert et al., 2000), the routine number of chromosomes analyzed by FISH ranges from only 5 to 12. Being that aneuploidy can occur in any of the 24 chromosomes (Fragouli and Wells 2011), even with 12-probe FISH, a reported 20% of aneuploid embryos will go undetected (Munné et al., 2010). These particular drawbacks certainly contributed to the criticism of PGT-A and demise of FISH

itself, as RCTs incorporating this method into their study design, have demonstrated either no improvement, or worse, an adverse effect, on live birth rates following PGT-A (Mastenbroek et al., 2007; Twisk et al., 2007).

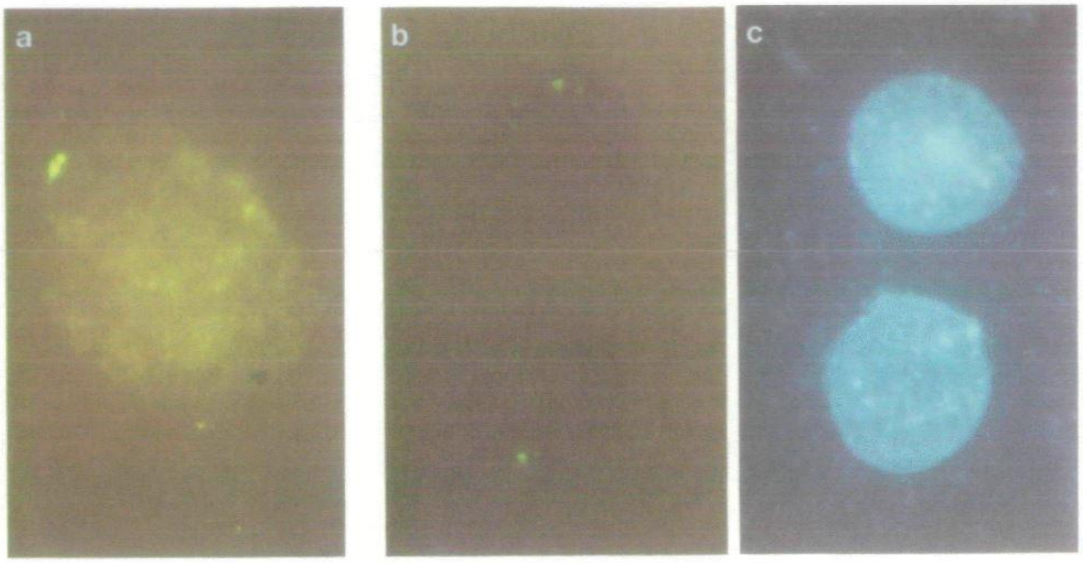


Figure 11. First report of FISH on interphase nuclei of blastomeres from human preimplantation embryos for sexing. (a) A single hybridization signal in a single nucleus, using the Y chromosome-specific probe, pHY.21 (specific for the long arm of the Y chromosome), indicating a male embryo. (b) A single hybridization signal seen in two separate nuclei, using the X chromosome-specific probe, pBamX7 (specific for the centromeric region of the X chromosome), indicating the presence of only one X chromosome and hence a male embryo. (c) UV illumination allowing for the visualization of DAPI (4',6-diamidino-2-phenylindole) stained nuclei and revealing their exact position. Taken from Griffin et al., 1991.

1.3.2.4.3.2.2 Comparative Genomic Hybridization (CGH)

In order to address the limitations of FISH, Comparative Genomic Hybridization (CGH), also known as metaphase CGH (mCGH), was introduced to the field of IVF in the late 1990s, allowing for the analysis of all 24 chromosomes in a single test. This technique relies on the amplification of DNA with whole genome amplification (WGA), followed by the simultaneous hybridization of

both sample DNA and reference DNA from a normal control, to normal metaphase chromosome spreads. The sample DNA and reference DNA are each differentially labeled with two fluorochromes. Changes in the ratio of the intensities of these two fluorochromes allows for the detection of both whole chromosome gains and losses, in addition to segmental deletions and duplications. Although the advantages of CGH appeared to be clear, there were obvious shortcomings such as a difficult and technically challenging protocol, described as arduous and time consuming (Wells et al., 2002). The extended protocol timing of CGH (up to 5 days), required either freezing post blastomere biopsy, which at the time consisted of slow-freezing embryos at the cleavage stage, yielding much poorer survival rates than current day vitrification methods (Rienzi et al., 2017), or polar body biopsy with the potential of a fresh transfer, however this option omitted the detection of both paternal meiotic errors and mitotic errors in the resulting embryos. Nevertheless, these limitations combined prevented routine application of this technique.

1.3.2.4.3.2.3 Array-CGH (aCGH)

A further adaptation of CGH was array-CGH (aCGH), which, although quite similar in protocol, has significant differences, the most notable being that, with aCGH, the DNA is hybridized to microarrays (a glass slide consisting of small fragments of DNA arranged in specific pattern) instead of metaphase chromosomes. The advantages that aCGH offers over CGH include higher resolution, increased automation within the protocol, and a significantly shorten testing time, in turn allowing for day 5 TE biopsy with fresh day 6 transfer. Following the first live birth after the application of aCGH (Fishel et al., 2010), this became the predominant PGT-A method in the early 2010s being applied to polar body, blastomere, and TE biopsies. However, as with each

approach, there were limitations such as the inability to detect haploidy and polyploidies, mosaicism, balanced chromosome arrangements and UPDs.

1.3.2.4.3.2.4 Single Nucleotide Polymorphism Microarrays (SNP arrays)

Single nucleotide polymorphisms (SNPs) are areas within the genome where a single nucleotide in the DNA sequence differs within the population. A majority of SNPs are biallelic, meaning they exist in one of two forms, this along with the frequency of millions of SNPs occurring throughout the entire genome, allows for the detection of genetic variation. SNP microarrays assess hundreds of thousands of SNPs and then using a computer software determines the number of copies of each chromosome present in a sample (Treff et al., 2010). Some of the advantages of SNP arrays over aCGH is the capability to detect parental origin of chromosome abnormalities, UPD, ploidy status (haploidy and triploidy), distinguishes between meiotic and mitotic errors, and permits for the simultaneous testing of single gene mutations (for PGT-M) and aneuploidy (Johnson et al., 2010b; Northrop et al., 2010). However, drawbacks such as the need for parental DNA prior to testing, which adds time and cost to testing and can be exceptionally difficult to obtain when using donor gametes, combined with a lack of reported improvement in clinical rates versus other methods, contribute to the reasons why SNP has never evolved as the primary approach for PGT-A testing.

1.3.2.4.3.2.5 Real-Time Quantitative PCR (RT-qPCR)

Real-time quantitative PCR (RT-qPCR) was introduced to clinical IVF in the early 2010s following its initial validation on TE biopsies for comprehensive chromosome screening (CCS) (Treff et al., 2012). This method offers a rapid turnaround time of approximately 4 hours (versus other

methods that exceed 12 hours), allowing for same day biopsy and aneuploidy screening with fresh blastocyst transfer, supporting the likelihood of suitable embryo-endometrial synchrony (van Voorhis and Dokras 2008). The primary advantage of this approach is that PCR is performed directly on the biopsy sample without the need for WGA. Not only does this save time within the procedure but omits the shortcomings of WGA that is associated with variable accuracy in both aneuploidy screening and genotyping applications (Treff et al., 2011). Like SNPs, RT-qPCR also allows for simultaneous testing of aneuploidy and single gene mutations. Limitations of this technique involve the lack of segmental aneuploidy and mosaicism detection and an increased cost pertaining to the processing of multiple embryos in parallel. As is also the case with SNPs, this method is not as widely used as others today, especially considering that the distinctive advantage of fast testing time is no longer broadly relevant as a high percentage of transfers are preferably carried out in frozen cycles.

1.3.2.4.3.2.6 Next Generation Sequencing (NGS)

Next Generation Sequencing (NGS) is currently one of the most (if not the most) commonly applied method for PGT-A. Following the birth of the first NGS baby in 2013, this approach was made clinically available shortly after and has since replaced earlier technologies such as aCGH (Florentino et al., 2014; Wells et al., 2014; Zheng et al., 2015). Briefly, the NGS protocol first begins with WGA and barcoding, followed by library preparation, purification, and templating, then loading and sequencing. From this point, the sequenced reads are then aligned to a human reference genome in which the data can be analyzed, and reporting can be carried out (Garcia et al., 2020).

The prime advantages of NGS are its high-throughput which results in lower cost and reduced workload, expanding the accessibility of PGT-A to a greater number of patients undergoing IVF and its capability to detect mosaicism, an important consideration as mosaicism has been reported in both cleavage stage (Mertzanidou et al., 2013) and blastocyst stage (Johnson et al., 2010a) embryos. As there is no clear association to maternal age (van Echten-Arends et al., 2011; Mertzanidou et al., 2013; Turner et al., 2016), mosaicism is a concern for all patients undergoing IVF, especially as it has been implicated in preimplantation embryo wastage, miscarriage, and genetic diseases (Hassold and Hunt, 2001). With that in mind there is still much debate about the fate of known IVF mosaic embryos, considering similar clinical rates are observed between the transfers of low-level mosaics and euploid embryos (Spinella et al., 2018; Figure 15) and an overall lack of underlying basic science associated with these findings.

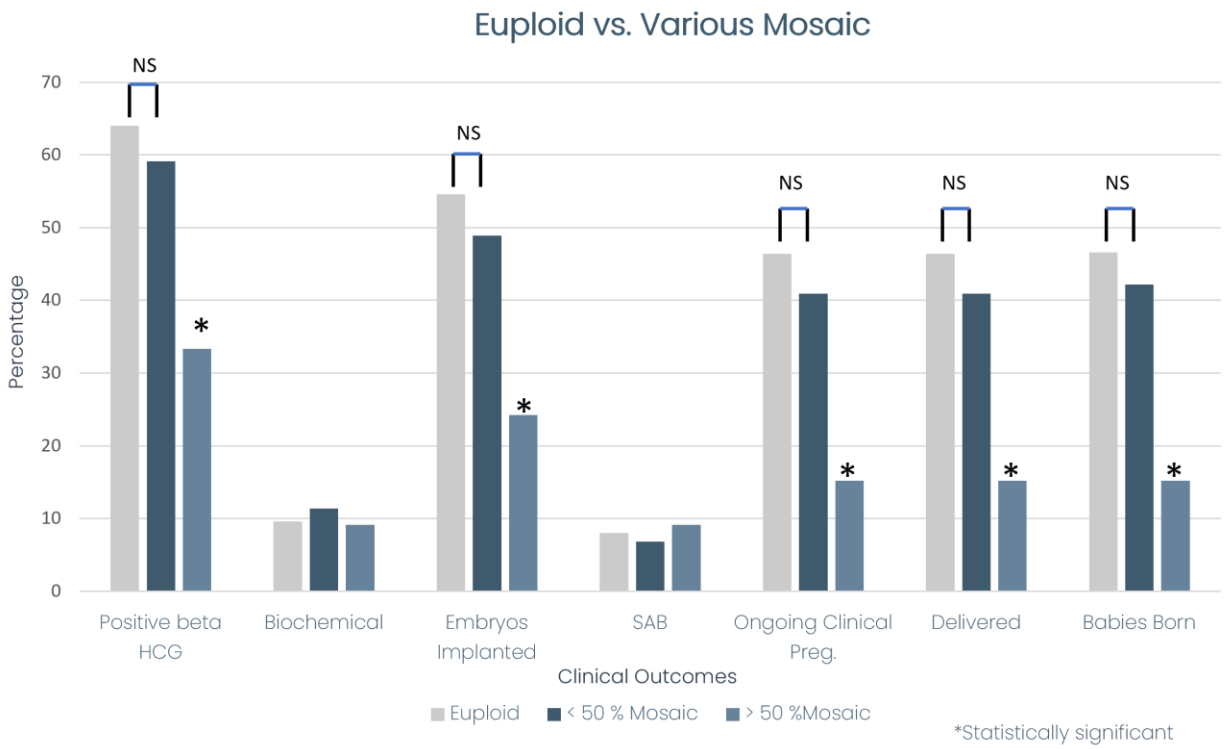


Figure 12. Comparison of clinical outcomes following the transfer of euploid, low mosaic (<50%), and high mosaic (>50%) embryos. Clinical rates associated with embryo transfers of either euploid or low mosaic PGT-A testing embryos were shown to be similar across all clinical outcomes assessed. In contrast, embryo transfer involving high mosaic embryos were associated with significantly poorer rates of positive beta hCG, implantation, ongoing clinical pregnancy, delivery, and live birth. Similar outcome rates were observed for all three groups for biochemical pregnancies and spontaneous abortions (SAB). Adapted from Spinella et al., 2018.

Other highlights of NGS include the ability to determine haploid/polyploid status, and simultaneously assessment of mitochondrial DNA (mtDNA) copy numbers and single gene mutations for PGT-M. The combined benefits of NGS may prove to positively influence clinical IVF rates by providing patients and clinicians with the information to make informed decisions regarding the most optimal embryos to transfer, thereby decreasing miscarriage rates (Maxwell et al., 2016) and increasing the likelihood of a successful pregnancy. However, like any technique, downfalls exist, for example, differences in mosaic reporting among PGT-A reference laboratories due to customized calling algorithms and independently established mosaic cutoffs

(Figure 16), can result in over diagnosing of mosaic embryos, leading to the reduction in embryos available for transfer (Ioannou et al., 2018; Monahan et al., 2019). In any case, it is widely accepted that NGS is now the gold standard when validating the clinical utility of PGT-A and future advances in aneuploidy screening.

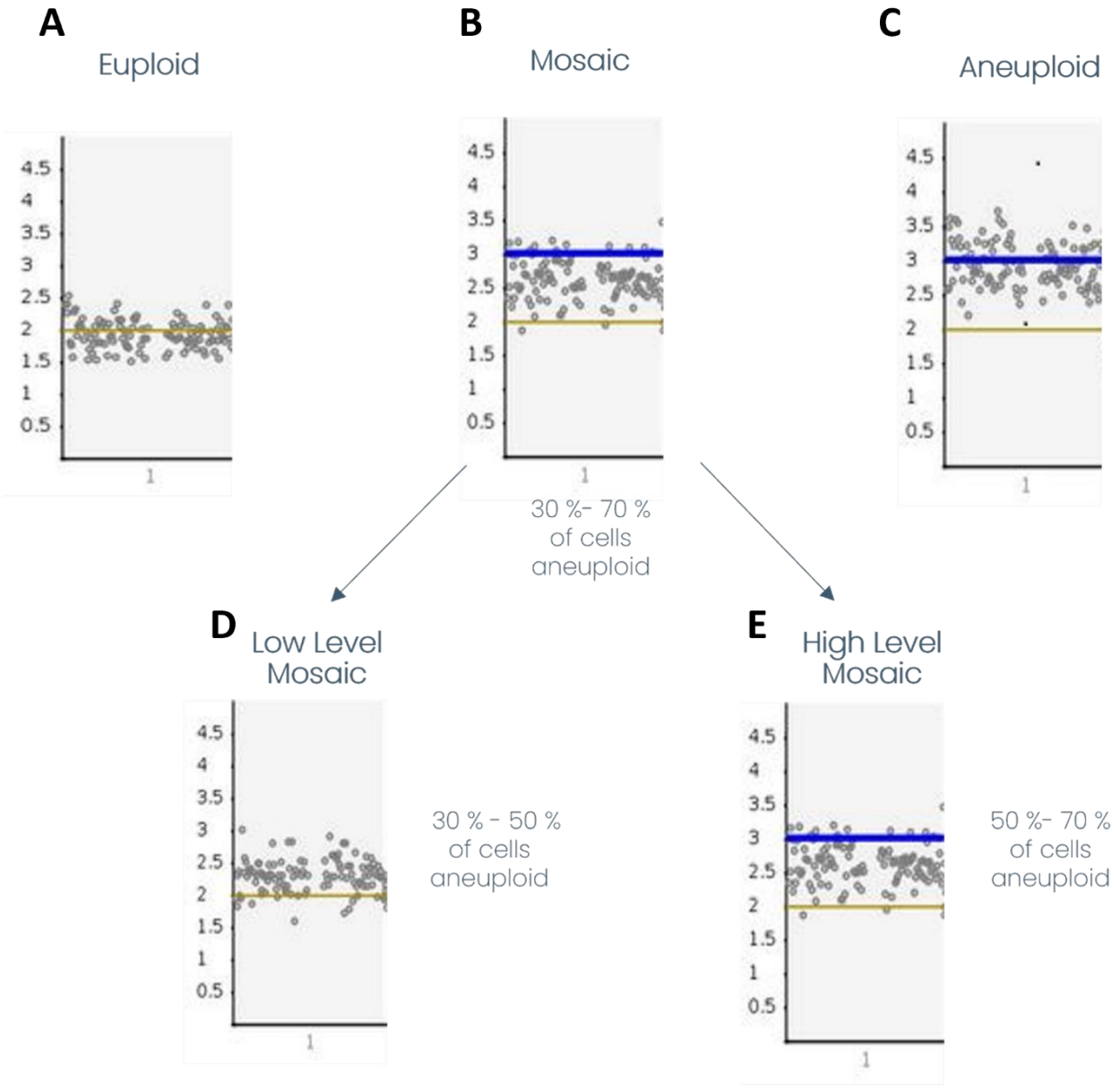


Figure 13. NGS profiles associated with euploid, mosaic, and aneuploid PGT-A results. (A) NGS profile, with reads concentrated around the line associated with the presence two copy numbers, indicating a euploid PGT-A result for chromosome 1, as defined by Igenomix (<30% cells aneuploid). (B) NGS profile, with reads concentrated in between the lines associated with the presence of two and three copy numbers, indicating a mosaic PGT-A result for chromosomes 1, as defined by Igenomix (30-70% cells aneuploid). (C) NGS profile, with reads concentrated around the line associated with the presence of three copy numbers, indicating an aneuploid PGT-A result for chromosome 1, as defined by Igenomix (>70% cells aneuploid). (D) NGS profile indicating a low-level mosaic PGT-A result for chromosome 1 as defined by Igenomix (30-49% cells aneuploid). (E) NGS profile indicating a high-level mosaic PGT-A result for chromosome 1 as defined by Igenomix (50-70% cells aneuploid). Courtesy of Igenomix.

1.3.2.4.3.3 Validating PGT-A for Widespread Clinical Practice

There have been multiple reports over the years of improved reproductive outcomes following the utilization of PGT-A in IVF cycles (Gianaroli et al., 1999; Munné et al., 1999; Scott et al., 2013a; Rubio et al., 2017). Selecting only those embryos that are euploid for transfer should provide an advantage in terms of increased implantation and decreased miscarriage rates. However, the lack of an RCT that clearly demonstrates a significant increase in live birth rate, hinders PGT-A from being universally accepted and practiced. To date the largest PGT-A RCT carried out has been the STAR trial (Munné et al., 2019), which incorporated 661 IVF cycles, and randomized patients to either the treatment group where they underwent a SET with a known euploid embryo by PGT-A or the control group where SET selection was carried out according to standard morphological grading. No statistical difference in ongoing pregnancy rate was detected between the two groups, in addition there was no significant difference in pregnancy and miscarriage rates. However, when stratifying the data to look specifically at women with AMA, a significantly higher ongoing pregnancy rate per embryo transfer was achieved in the PGT-A group (51%) versus the control group (37%), further supporting the use of PGT-A for women over 35 years old undergoing IVF. Limitations of this study largely pertained to the high variability regarding the quality of embryos transferred, number of samples arising from each enrolling clinic along with the degree of proficiency of the embryologists performing the lab procedures, and differences in PGT-A calling parameters and expertise among the different reference labs involved (Munné et al., 2019). With that said, although to date we are still pending an RCT that clearly demonstrates the benefit of PGT-A in terms of live birth rate, this does not mean that a benefit does not exist. In a 'straw man' exercise presented by Griffin and

Sheldon (2017), the authors propose that the benefits of PGT-A may already be evident without an RCT. They continue to state that although RCTs are the gold standard when validating a practice, a randomized study does not automatically mean it is a good study, especially if performed badly, just as a non-randomized study does not automatically mean it is bad one.

1.3.2.4.3.4 The Future of PGT-A – Non-Invasive PGT-A (niPGT-A)

A common criticism and stated weakness of the current PGT-A protocol is the requirement for an invasive embryo biopsy, as this removal of cells have been shown to adversely affect IVF outcomes (Cimadomo et al., 2016). To add to this, long-term studies investigating the effects of embryo biopsy in humans have not been carried out, though animal studies have shown a negative influence on adrenal and neural development (Zeng et al., 2013; Zhao et al., 2013; Wu et al., 2014). Over the last decade, various efforts have been made to develop a non-invasive approach to PGT (Palini et al., 2013; Assou et al., 2014; Gianaroli et al., 2014; Galluzi et al., 2015), however the implementation of a non-invasive method for PGT-A in clinical IVF has been fairly recent. Current commercial niPGT-A procedures involve the culture of embryos in individual microdrops (~10µl) from day 3 to day 5 or day 6 (depending on the specific reference lab protocol) of embryonic development. After the blastocysts are removed from their culture drops for vitrification, the spent media is collected, placed in a PCR tube, and sent to the PGT-A reference lab for analysis with NGS. Several studies have been carried out to determine the concordance rate between the niPGT-A method and traditional PGT-A with TE biopsy (Xu et al., 2016; Ho et al., 2018; Vera-Rodriguez et al., 2018; Rubio et al., 2020). In a large, multicenter, prospective study involving 1301 blastocysts, concordance rates as high as 86% were achieved when ensuring proper laboratory practice to reduce contamination from both maternal

(cumulus cells) and embryologist sources (Rubio et al., 2020). Although the lack of 100% concordance between the current gold standard (TE biopsy) and the non-invasive method may be viewed as a shortcoming of niPGT-A, we should instead question if the non-invasive method actually better defines the true chromosomal status of an embryo, as the DNA analyzed may be derived from the entire embryo, including the ICM, versus only the TE. Further supporting this idea, Rubio et al., (2018) reported a higher rate of miscarriage when the embryo was determined to be euploid by TE biopsy, but aneuploid with niPGT-A versus when both the TE and non-invasive technique yielded a euploid result (Table 4). Currently further studies are being carried out better determine the concordance rate between the two methods, along with an RCT comparing the clinical rates following PGT-A with TE biopsy versus niPGT-A. In any case, niPGT-A is being applied in IVF clinics today to help select the embryos with the highest implantation potential and has already resulted in the birth of babies worldwide.

Table 4. Clinical outcome results for euploid TE/euploid niPGT-A results versus euploid TE/aneuploid niPGT-A results. This table shows higher clinical rates for the transfer of embryos where the aneuploidy screening results for both the TE biopsy and spent blastocyst media (SBM) indicate a euploid result versus the transfer of embryos where the aneuploidy screening results for the TE biopsy indicates a euploid result, but the SBM indicates an aneuploid result. A statistical difference in ongoing implantation rate was achieved. Courtesy of Igenomix.

Clinical Outcome	Euploid TE/ Euploid SBM	Euploid TE/ Aneuploid SBM	TOTAL
Number of transfers	17	12	29
Mean maternal age (SD)	37.5 (2.5)	37.4 (2.3)	37.5 (2.4)
Positive hCG	11 (64.7)	4 (33.3)	15 (51.7)
Clinical pregnancy rate (%)	9 (52.9)	4 (33.3)	13 (44.8)
Biochemical miscarriage	2 (18.2)	0	2 (13.3)
Clinical miscarriage	0	2 (50.0)	2 (15.4)
Ongoing implantation rate (%)*	9 (52.9)	2 (16.7)	11 (37.9)

1.3.2.4.4 Other Molecular Markers for Embryo Selection – Mitochondrial DNA

PGT-A studies have shown that over one third of transfers involving morphologically high quality, euploid embryos fail to implant (Yang et al., 2012; Forman et al., 2013b), highlighting the impact of other factors, beyond embryo morphology and chromosome content, have on the success of an IVF cycle. One of these potential factors is the effect of an embryo's energy supply on its viability. Most cellular energy originates from the mitochondria, commonly referred to as the powerhouse of the cell. In human embryos, the mitochondrial content is derived from the maternal mitochondria present in the oocyte. It has been postulated that following mitochondrial DNA (mtDNA) replication in the metaphase II (MII) oocyte (Jansen and Boer 1998) there are no additional replication events that occur between fertilization and early preimplantation development (Pikó and Taylor 1987). Based on this, the total amount of mtDNA

present in the MII oocyte must be distributed among all cells of a developing embryo, with a proportional decrease in cell copy number occurring with each division. Retrospective studies comparing mtDNA copy numbers in euploid embryos to corresponding clinical outcomes found that a high mtDNA copy number is associated with lower implantation potential (Diez-Juan et al., 2015; Fragouli et al., 2015). The authors suggest that elevated mtDNA levels are linked to reduced embryo viability, resulting from reduced metabolic fuel during oocyte development and/or elevated metabolism. From this work, several PGT-A labs have offered mitochondrial scoring for euploid embryos to further aid in the selection of the most viable embryos for transfer. With that said larger prospective studies are still necessary to evaluate the true predictive value of this biomarker, as the previous findings discussed above have not been reproduced when carried out by other groups (Victor et al., 2017; Treff et al., 2017).

1.3.2.5 Endometrial Preparation for Transfer

Traditionally, embryo development had been the primary factor guiding the timing of a transfer in an IVF cycle. This practice assumes that the WOI is constant in all women, initiated on cycle day 19 or 20 and remaining open for 4 to 5 days (Lessey 2011). In the case of natural conception, embryonic development and the WOI are controlled by the follicle, where following ovulation, progesterone begins to rise and if both the resulting embryo and endometrium develop normally, then they will be synchronous, and implantation may occur. However, in an IVF cycle, this natural coordination may be lost, even when controlling for appropriate embryo development on day of transfer. Supraphysiological levels of steroid hormones present during controlled ovarian hyperstimulation have been shown to deteriorate uterine receptivity (Hoozemans et al., 2004; Martínez-Conejero et al., 2007; Horcajadas et al., 2008). Progesterone

has been observed to rise prematurely, and at a much faster and more vigorous rate following an ovulation trigger in an IVF cycle versus an LH surge that occurs in a natural cycle, leading to an early secretory transformation (Silverberg et al., 1991). Consequently, these factors can shift the WOI by 16-24 hours, resulting in a dyssynchrony. Delayed blastulation, especially in older and low responders, may be another source of dyssynchrony. Interestingly, when late blastulating embryos are cryopreserved and transferred in a FET cycle, improved clinical rates are observed, further highlighting the need for proper synchronization (Shapiro et al., 2008; Forman et al., 2013a; Franasiak et al., 2013). Unfortunately, it is difficult to ensure physiological synchronization prior to an IVF cycle start and even replicate it from cycle to cycle.

1.3.2.5.1 Monitoring of Hormone Levels

Serum hormone levels and ultrasonography are the two classic parameters used to monitor the WOI (Shapiro et al., 1993; Hofmann et al., 1996; Remohi et al., 1997; Paulson 2011). In regard to serum hormone levels, estradiol and progesterone concentrations are the primary hormonal determinants of embryo transfer timing, with specific serum thresholds correlated with IVF success (Silverberg et al., 1991, Bosch et al. 2010). Often exogenous estradiol and progesterone are administered to offer further control during treatment, though optimal administration type, dose, and duration have not been established (Glujuovsky et al., 2010; Sharm and Majumdar 2016).

1.3.2.5.2 Assessment of Endometrial Thickness

Endometrial thickness, often measured by transvaginal ultrasound, is a standard parameter assessed in ART cycles to indicate when the endometrium is adequate for embryo transfer. An

atrophic, or exceedingly thin endometrium (<6mm), has been associated with poorer clinical outcomes following IVF (Kovacs et al., 2003). On the other hand, it has been demonstrated that patients with a hypertrophic endometrium (≥14 mm), also suffer from lower clinical rates (Weissman et al., 1999). To the contrary, others have found that neither an atrophic nor hypertrophic lining is associated with decreased ART success (Oliveira et al., 1997; Dietterich et al., 2002). In the end although ultrasonography is used to monitor the endometrial WOI, it should be agreed upon that endometrial thickness is not a reliable diagnostic tool for endometrial receptivity. Furthermore, due to its limited capacity to identify patients with a decreased probability of conceiving following an embryo transfer (Kasius et al., 2014), its utility in guiding the fate of an IVF cycle in general should be reassessed.

1.3.2.5.3 Endometrial Scratching

Endometrial scratching is the deliberate injury made to the endometrial lining that is performed to improve implantation rates (Barash et al., 2003; Raziel et al., 2006). The "scratch" itself can be accomplished through endometrial manipulation during a hysteroscopy (Pundir et al., 2014), an endometrial biopsy (Karimzadeh et al., 2009) or curettage (Shohayeb and El-Khayat 2012). It is postulated that the biological response to the endometrial scratch is the basis for improved implantation rates, whether due to inducing stromal cell decidualization (Li and Hao 2009), an associated inflammatory response (Gnainsky et al., 2010), or as a mechanism to delay endometrial maturation in controlled ovarian hyperstimulation cycles to encourage appropriate embryo-endometrial synchrony. However, endometrial scratching in ART remains controversial. The lack of a clear consensus on when within the cycle to perform the scratch, how long before embryo transfer to perform the scratch, and the best method for performing the scratch,

coupled with results showing no clinical value (Simón and Bellver 2014), in particular an RCT involving 1,364 women undergoing IVF (Lensen et al., 2019), translates to endometrial scratching not being a part of standard ART practice. Lastly, it is critical to consider the cost to the patient when performing this procedure, including pain (Nastri et al., 2013), monetary expense, and potential iatrogenic damage, ultimately classifying this treatment as empirical, unproven, and possibly harmful.

1.3.2.5.4 Methods for Assessing Endometrial Receptivity in IVF

1.3.2.5.4.1 Histology

Until recently, histology was considered the gold standard for endometrial dating. First applied in the 1950's, this method was developed according to the criteria established from the laboratory observations of approximately 8,000 endometrial biopsy samples (Noyes et al., 1950). The criteria are based upon variations occurring throughout the endometrial phases and include gland mitosis, pseudo stratification of nuclei, basal cell vacuolation, secretion, stromal edema, pseudodecidual reactions, stromal mitosis, and leukocytic infiltration (Noyes et al., 1950; Noyes et al., 1975). As an example (Figure 17), the evolution of stromal gland variation can be seen beginning in the proliferative phase where they appear straight and tubular, then become more convoluted in the pre-receptive phase, and ultimately more irregular with a papillary or sawtooth appearance in the receptive phase (Noyes et al., 1950; Noyes et al., 1975). Although the association of certain histological features to specific phases of receptivity have been demonstrated the clinical utility and accuracy of this method has been vehemently questioned. A number of randomized (Coutifaris et al., 2004; Murray et al., 2004) and prospective studies (Balasch et al., 1985; Scott et al., 1988; Gibson et al., 1991; Balasch et al., 1992; Scott et al.,

1993) have shown extensive intersubject, intrasubject, and interobserver variability, this paired with innate biases to protocol itself, such as tissue-fixation artefacts, suggests that histology lacks the reliability to adequately evaluate the luteal phase and subsequently guide the treatment of women undergoing ART.

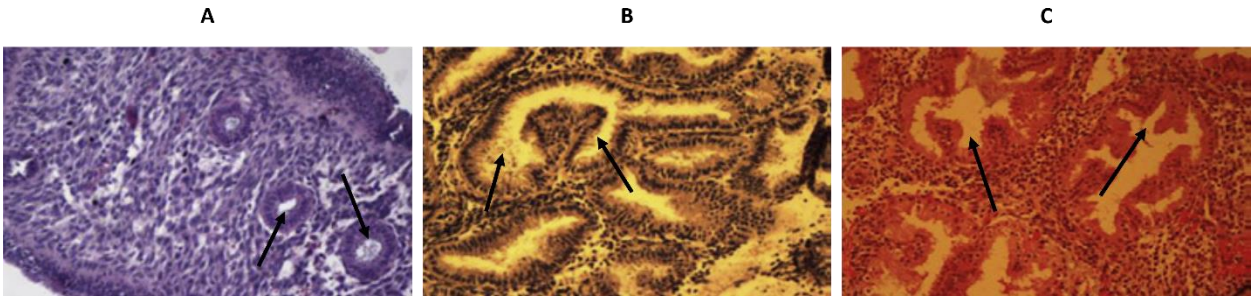


Figure 14. Evolution of stromal gland appearance from proliferative phase to pre-receptive phase to receptive phase according to Noyes criteria. (A) Proliferative phase: 10x, arrows pointing to stromal glands which appear short, straight, and narrow. (B) Pre-receptive phase: 20x, arrows pointing to stromal glands that have become more convoluted and possess a frayed boarder. (C) Receptive phase: arrows pointing to glands that are now more irregular with a papillary or sawtooth appearance. Adapted from Diaz-Gimeno et al., 2013.

1.3.2.5.4.2 Biochemical Markers

The 1990s and early 2000s gave rise to the exploitation of biochemical markers for the prediction of endometrial receptivity. Proposed informative markers included integrins (Lessey et al., 1992), MUC1 (Meseguer et al., 1998), calcitonin (Kumar et al., 1998), leukemia inhibitory factor (Stewart et al., 1998), HOXA11 (Taylor et al., 1999), and prostaglandin E2 and interleukin-1beta (Davis et al., 1999), just to name a few. This trend gave rise to several commercially available tests, such as E-tegrity and the endometrial function test (EFT). E-tegrity is based on the expression of β_3-integrin protein in endometrial biopsies collected during the presumed WOI, between cycle day 20-24, paired with histological assessment. An absence of the β3 integrin

subunit has been linked to endometriosis (Lessey et al., 1994a,b) whereas abnormal expression of this subunit is seen in women with unexplained infertility (Lessey et al. 1995), both indicating defective uterine receptivity as a potential underlying cause. With E-tegrity, three different patterns can be detected: (1) normal pattern (β_3 subunit detected with an "in-phase" histological result), (2) luteal phase defect pattern (β_3 subunit not detected with an "out-of-phase" histological result), (3) pattern associated with unexplained infertility, minimal or mild endometriosis, or hydrosalpinx (β_3 subunit not detected with an "in-phase" histological result). EFT, on the other hand, is an immunohistochemistry test based on the expression of cyclin E and p27 proteins as signifiers for endometrial development and hence receptivity status, in which an abnormal result can guide subsequent treatment often in the form of an intervention or change in stimulation protocol. (Dubowy et al., 2003). With that said, none of these biochemical markers have been successfully translated into clinical practice (Aghajanova et al., 2008), due to a lack of a clear understanding about their interactions, not to mention endometrial receptivity itself is a complex and multifactorial process in which it is impossible to summarize with the analysis of just one or a few molecules. Furthermore, using single biochemical markers to draw associations with RPL, implantation failure, or unexplained fertility is not practical due the multifactorial origins of these infertility factors.

1.3.2.5.4.3 Transcriptomics

Transcriptomics is the study of the transcriptome, which is the full range of messenger RNA (mRNA) molecules expressed from the genes of an organism (Assis et al., 2014). The analysis of gene expression patterns in particular tissues, under distinct conditions, allows not only for the

diagnosis of pathological conditions (Nevins and Potti 2007), but also physiological processes like endometrial receptivity (Díaz-Gimeno et al., 2011).

The study of endometrial transcriptomics has led to hundreds of publications, and consequently an improved understanding and assessment of pathologies relating to endometrial cancer (Doll et al., 2007), endometriosis (Matsuzaki 2011), and RIF (Koler et al., 2009). Transcriptomic profiling of the endometrium throughout its various phases (Borthwick et al., 2003; Ponnampalam et al., 2004; Talbi et al., 2005) has provided further insight to the general physiology of this tissue and the opportunity to innovate new clinical tools that leverage this science. In the field of IVF, by far one of the most advantageous uses of endometrial transcriptomics has been its utilization in studying endometrial receptivity in order to increase the probability of implantation following ART treatment. In an early study by Reisewilk et al., (2003), the investigators set out to elucidate the transcriptomic signature relating to receptivity by comparing gene expression profiles from endometrial biopsies taken from fertile women at LH+2 (presumed pre-receptive endometrium) and LH+7 (presumed receptive endometrium) within the same cycle, finding that 211 genes were differentially expressed between these two phases. A subsequent study proclaimed the receptive or mid-secretory phase to be a "transcriptional awakening process" as most genes were found to be upregulated at this time compared to the pre-receptive phase (Díaz-Gimeno et al., 2011). Based on the molecular classification of endometrial phases greater objective and reliable diagnostics are now possible.

1.3.2.5.4.3.1 Endometrial Receptivity Analysis (ERA)

The implementation of personalized medicine to IVF has expanded our understanding of endometrial receptivity and introduced the concept of a personalized WOI (pWOI). A prime

example of this is Endometrial Receptivity Analysis (ERA), a robust molecular test that deciphers the gene expression pattern of an endometrial biopsy sample and employs a bioinformatic predictor that accurately diagnoses the phase of receptivity and provides a recommendation for a personalized embryo transfer (pET) (Diaz-Gimeno et al., 2011). Currently the ERA test applies NGS to examine the transcriptomic profile of the endometrium that is based on a panel of 248 genes associated with receptivity in humans. Established differences in gene expression profiles between receptive and non-receptive endometrium is the basis for diagnosis, resulting in an actionable directive for a more individualized approach to embryo transfer in order to enhance synchrony between an implanting embryo and receptive endometrium, with the objective to improve implantation.

1.3.2.5.4.3.1.1 Development of ERA

Previous studies indicated the possibility of accurately classifying and diagnosing the human endometrial cycle, including the receptive phase, based upon various gene expression profiles obtained (Ponnampalam et al., 2004; Talbi et al., 2005; Carson et al., 2002; Kao et al., 2003; Borthwick et al., 2003; Riesewijk et al., 2003; Mirkin et al., 2005; Haouzi et al., 2009; Mirkin et al., 2004; Simón et al., 2005; Horcajadas et al., 2008; Horcajadas et al., 2006). Built on this concept, the ERA test was developed, which initially employed a customized microarray to assess the expression of 238 genes associated with endometrial receptivity in humans. When developing the ERA, it was first necessary to select a specific panel of genes that would define the test. To accomplish this, different transcriptomic profiles were analyzed from 20 biopsies sampled from receptive (LH + 7 (n =5)) and prereceptive (LH+1 (n=5), LH+3 (n=5), LH+5 (n=5)) endometrium. After performing a t-test, only those genes showing an absolute fold-change >3 and a false

discovery rate <0.05 were selected, resulting in 238 genes shown to be differentially expressed during the receptive phase (Diaz-Gimeno et al., 2011). Figure 18 shows a heatmap of the ERA gene expression profiles within each endometrial phase.

It was then necessary to develop a bioinformatic tool in order to date the endometrium and define the transcriptomic signature. The gene expression profiles from a total of 68 endometrial biopsy samples, collected from fertile women during various times within the menstrual cycle (signifying a proliferative, prereceptive, or receptive endometrium), were used to effectively train the predictor to classify samples into the different endometrial phases. The established specificity and sensitivity of the ERA predictor to classify an LH+7 endometrium (indicative of endometrial receptivity) at the transcriptomic level, is 0.8857 and 0.99758, respectively (Diaz-Gimeno et al., 2011).

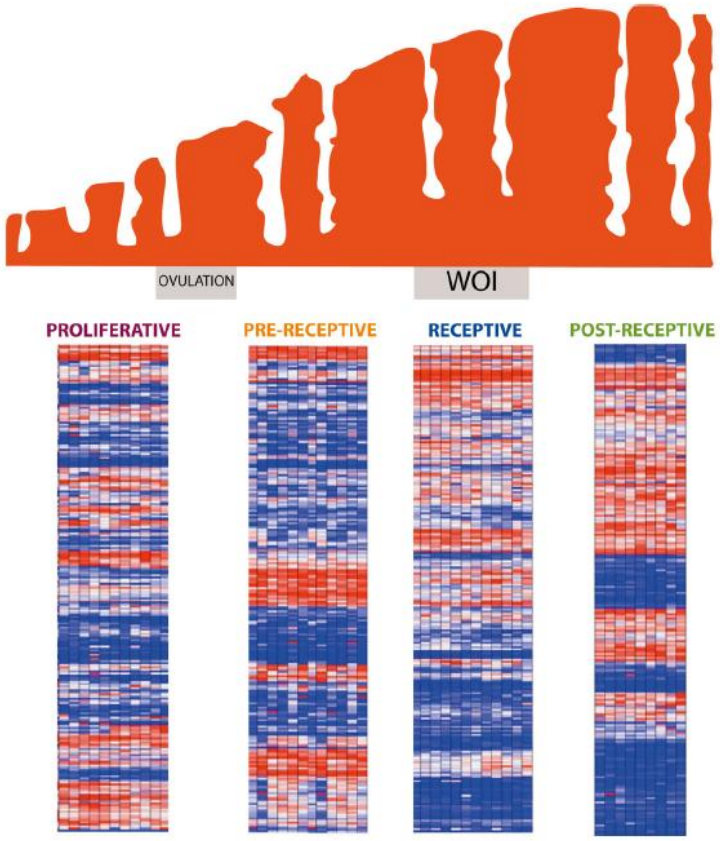


Figure 15. Evolution of endometrial tissue, showing increasing thickness from the time of ovulation to the WOI, and the associated gene expression value at each phase. The heatmap shows ERA gene expression profiles in the proliferative, pre-receptive, receptive, and post-receptive phases. Color indicates gene expression value intensities, in which blue = low gene expression value intensity and red = high gene expression value intensity. The WOI has been referred to as a 'transcriptional awakening,' marked in the above heatmap by an increased frequency of red and decreased frequency of blue during the receptive phase. Taken from Diaz-Gimeno et al., 2014.

1.3.2.5.4.3.1.2 ERA versus Histology

One of the initial validations of the ERA test was the comparison of results to those obtained from histological examination of endometrial biopsy samples, the gold standard for endometrial dating at that time. In a study by Diaz-Gimeno and colleagues (2013), 49 endometrial biopsy samples were taken from Caucasian ovum donors at various times during a natural cycle and dated by both ERA and histology. For histological assessment, two separate pathologists, both

blinded, evaluated 20 distinct histological features according to the Noyes criteria. Concordance rates were analyzed by Kappa index values, where a value between 0.61-0.80 was considered good and a value between 0.81-1.00 was considered very good. When comparing dating results to timing of LH surge (the indicator for endometrial progression in a cycle), pathologist 1 and 2 obtained values of 0.618 and 0.685, respectively, whereas the ERA test obtained a value of 0.922. Concordance between pathologist 1 and 2 was also considered, in which a value of 0.622 was obtained, indicating a poorer interobserver variability than previous reports (Coutifaris et al., 2004; Murray et al., 2004). Only one case was not properly dated by the ERA, but was properly dated by both pathologists, which represented an endometrium in the proliferative phase. Ultimately, it was concluded that the ERA was superior to traditional histology in detecting the receptive, pre-receptive, and post receptive endometrial phases, concluding that the molecular platform offered objective results with greater precision (Figure 19).

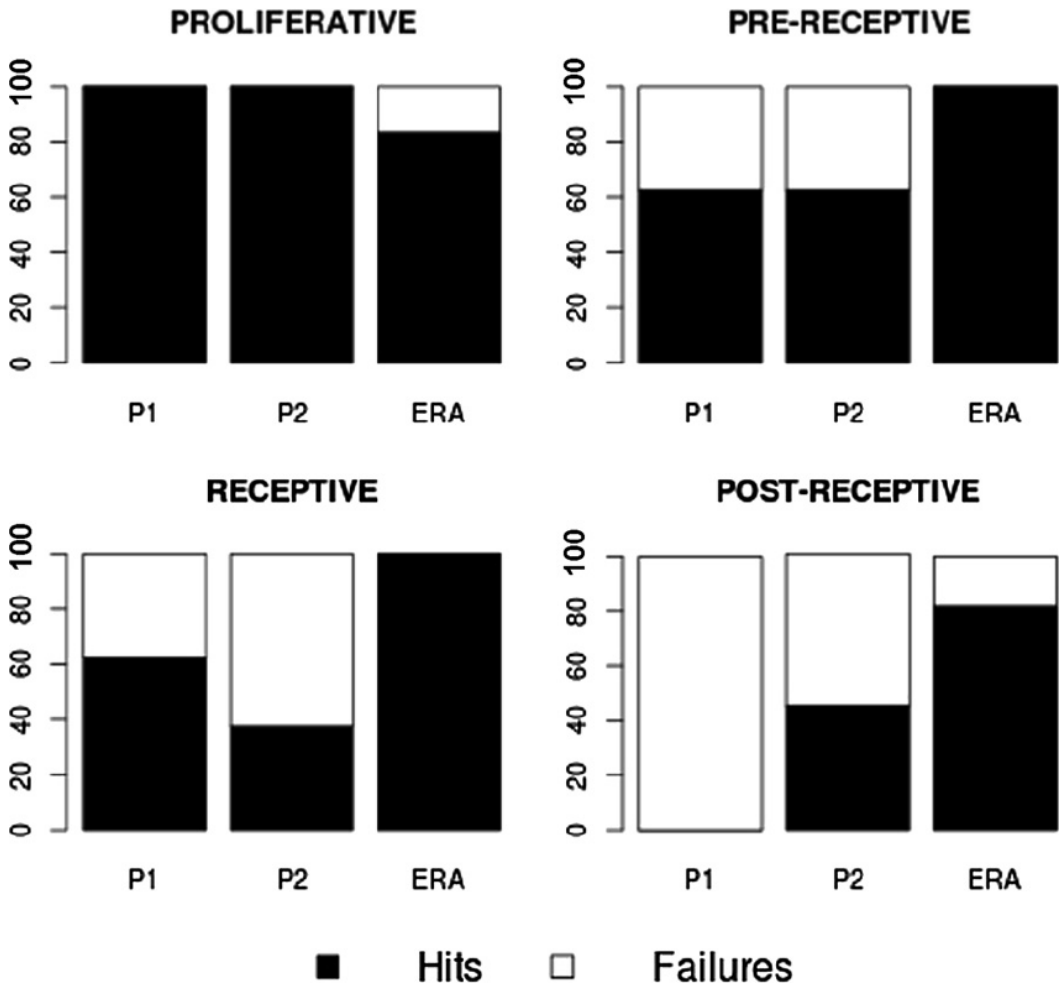


Figure 16. Endometrial dating calls during proliferative, pre-receptive, receptive, and post-receptive phases made by pathologist 1 (P1) versus pathologist 2 (P2) versus ERA. Hits and Failures are shown as percentages for each pathologist and ERA. Taken from Diaz-Gimeno et al., 2013.

1.3.2.5.4.3.1.3 ERA Reproducibility

Results from the ERA test has been found to be reproducible up to 40 months (Díaz-Gimeno et al. 2013), warranted that the cycle protocol is replicated and there is no significant alteration to the woman's uterine cavity or body mass index (BMI). In the study by Díaz-Gimeno and colleagues (2013), an endometrial biopsy for ERA in a natural cycle was performed on 7 fertile women. Twenty-nine to 40 months later, the same women underwent a second biopsy on the same day of menstrual cycle as the initial biopsy. Although there were some gene-to-gene

differences, the transcriptomic profiles overall remained consistent, even after 3 plus years (Figure 20). Interestingly, the transcriptomic profiles from the same woman, taken 29-40 months apart, were found to be much closer, compared to the profiles of the control samples within the same menstrual phase. The small sample size of the aforementioned study does preclude a definitive conclusion; however, it appears that unlike histology, in which variations up to 60% have been observed in secretory features between cycles (Murray et al., 2004), transcriptomic variation from cycle to cycle is relatively uncommon (Stankewicz et al., 2018).

A

Consistency pair	Endometrial phase	1st ERA test (diagnostic probability)	Months between sampling	2nd ERA test (diagnostic probability)
AA	LH+7(R)	R (0.908)	29	R (0.908)
BB	LH+7(R)	R (0.908)	30	R (0.908)
CC	LH+7(R)	R (0.908)	35	R (0.908)
DD	Day 8(PF)	NR (0.864)	36	NR (0.864)
EE	Day 9(PF)	NR (0.864)	40	NR (0.864)
FF	LH+7(R)	R (0.908)	35	R (0.908)
GG	LH+11(PS)	NR (0.109)	32	NR (0.109)

B

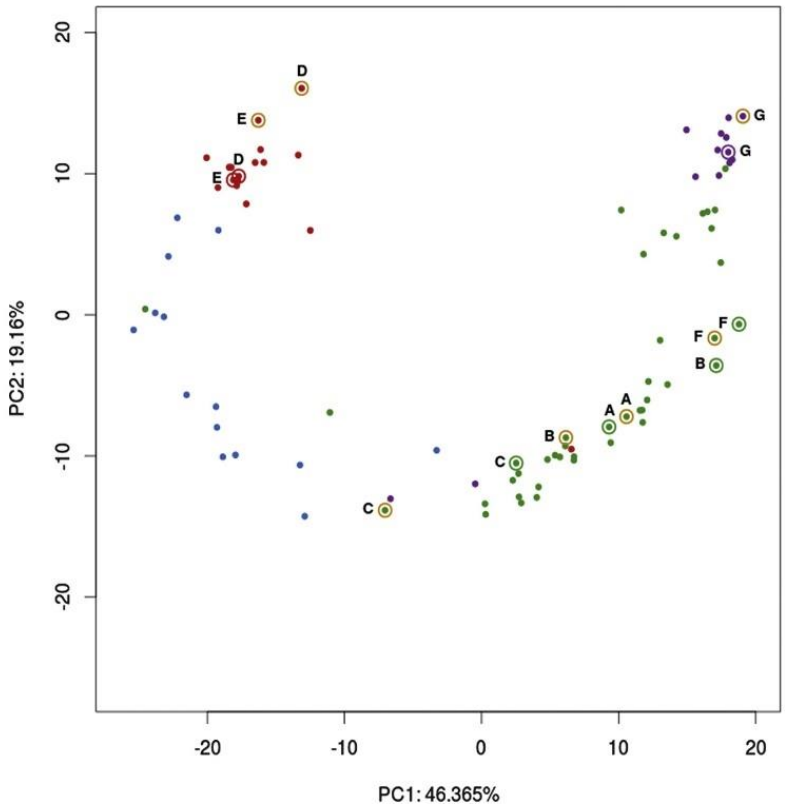


Figure 17. Consistency of ERA results over two different cycles, ranging 29-40 months apart. (A) Results of the first and second ERA biopsy over the indicated timeframes for 7 women undergoing a biopsy at a specified time during the menstrual cycle. The findings indicate that the same result (receptive = R versus non-receptive = NR) was obtained across all of the study participant's biopsies. (B) Principal component analysis showing the placement of genomic profiles obtained for each biopsy and from each of the study subjects (A-G). Taken from Diaz-Gimeno et al., 2013.

1.3.2.5.4.3.1.4 ERA in RIF Population

Earlier studies have demonstrated that women with RIF present with altered endometrial gene expression profiles (Koler et al., 2009). Ruiz and colleagues (2013) were the first to apply the ERA test to this population women, finding that approximately 1 in 4 (25.9%) women had a displaced WOI at the expected time of receptivity. Subsequent studies all showed similar rates of displacement in the RIF group, with the overall trend of patients being pre-receptive, requiring additional progesterone exposure time prior to obtaining endometrial receptivity at the transcriptomic level (Mahajan 2015; Hashimoto et al., 2017; Tan et al., 2018). In the aforementioned study, the ERA was also applied to a control group, with no history of RIF, showing a displacement rate of only 12% (Ruiz et al., 2013). When adjusting accordingly to the time of optimal receptivity, as determined by ERA, similar pregnancy and implantation rates were achieved in both the RIF and control groups, posing the question of whether RIF of endometrial origin should be considered a pathology or simply a lack of properly investigating and detecting the unique WOI in each woman (Ruiz-Alonso et al., 2013).

It is imperative to point out that although many studies have corroborated the same findings of improved implantation in the RIF population when ERA is applied, others have failed to determine any benefit of performing ERA in this group of patients. A retrospective, multicenter cohort study set to determine the clinical usefulness of ERA in patients with moderate (failed implantation after the transfer of 3 or more embryos in at least 3 SETs) and severe RIF (failed implantation after the transfer of 5 of more embryos in at least 3 SETs) showed no benefit of ERA in either group in terms of implantation or ongoing pregnancy (Cozzolino et al., 2020).

1.3.2.5.4.3.1.5 ERA in Good Prognosis Patients

Although the application of ERA in the RIF population appears evident, its utility in good prognosis patients has yet to be fully established. A retrospective study comparing outcomes in good prognosis patients, without a history of RIF, who underwent ERA with subsequent pET versus those who transferred at the standard FET timing, showed no difference in ongoing pregnancy rates (35.2% vs. 39%, respectively). The authors did acknowledge the limitation of their findings, mostly related to the retrospective design of their study, which unavoidably comes with its own inherent biases. Based on this, they necessitated the need for larger prospective studies to further elucidate the role of ERA in good prognosis patients (Bassil et al., 2018).

In an attempt to answer the question of clinical utility of ERA in the good prognosis population, a large RCT was carried out on patients at the 1st IVF appointment (Simón et al., 2020). All participants were overall good prognosis patients, ≤37 years old, with BMI between 18.5 – 30, normal ovarian reserve, and free of any pathologies affecting the endometrial cavity. Four hundred and fifty-eight patients were randomized to 1 of 3 groups: fresh embryo transfer arm, FET arm, or pET guided by ERA arm. The primary objective measured was live birth rate, with secondary objectives including cumulative live birth rate, pregnancy rate, implantation rate, cumulative pregnancy rate, and cumulative implantation rate. Patients randomized to the ERA group had superior clinical rates across the board versus those randomized to the fresh or frozen embryo arms. With that said, a statistically significant live birth rate was not attained in the ERA arm, versus either the fresh and frozen arms, though a 13.8 percentage point and 10.5 percentage point increase in this rate was achieved in the pET group versus the other two groups, respectively. The authors pointed out that an unexpected patient dropout rate of 50%

rendered the study underpowered, contributing to the lack of statistical significance achieved. It is clear that further studies need to be conducted in this population of patients, as the potential exists for ERA to diagnose the endometrial factor in the work-up of the infertile couple.

1.3.2.5.4.3.1.6 ERA and Effects of Endometriosis

Endometriosis can be an extremely painful disorder in which endometrial tissues grows outside of the uterus. This abnormal tissue growth occurs most frequently on the ovaries, fallopian tubes, and the tissue lining the pelvis, however, albeit less common, can spread beyond the pelvic organs to other parts of the body. Meta-analyses have reported that women diagnosed with endometriosis have significantly lower ART success rates, exhibiting an inverse relationship with increasing severity of the disease itself (staged I-IV) (Barnhart et al., 2002). Biologically it would be assumed that endometrial factor would be the source of these poorer rates, however, increasing evidence suggests that the inferior implantation rates found within this population of patients may instead be due to poorer embryo quality. In a study carried out in an ovum donation sharing program, patients with advanced stages of endometriosis (III and IV) who received eggs from healthy donors demonstrated similar clinical outcomes to recipients without endometriosis (Diaz et al., 2000). In another study involving donor oocytes, poorer pregnancy rates were reported in infertile recipients receiving eggs from donors with endometriosis versus donors without endometriosis (Simón et al., 1994). When specifically studying endometrial receptivity by applying ERA, no difference in gene expression profiles were found between women with the disease (regardless of stage) versus the control group of women without the disease, further supporting that endometriosis does not impair endometrial receptivity (Garcia-Velasco et al., 2015).

1.3.2.5.4.3.1.7 ERA and Effects of Body Mass Index (BMI)

In the United States, approximately 2 out of 3 women of reproductive age are overweight (BMI ≥25kg/m^2) or obese (BMI ≥30kg/m^2) (Flegal et al., 2012). Obesity is associated with inferior fecundity and poorer reproductive outcomes in ART (Maheshwari et al., 2007). Endometrial receptivity has been shown to be impaired in obese patients undergoing IVF. A study assessing the ART outcomes in obese recipients of donor oocytes from non-obese donors, reported a statistically significant inverse relationship between declining clinical rates and increasing BMI (Bellver et al., 2013). Transcriptomically, obese women have been reported to experience greater endometrial gene dysregulation versus women with a normal BMI, a trend further exemplified in obese women with PCOS (Bellver et al., 2011). Comstock and colleagues (2017) leveraged the ERA test to study the effect of increasing BMI on endometrial gene expression patterns in infertile patients. They reported a higher incidence of a non-receptivity as BMI increased, though not statistically significant. Based on differences in gene expression profiles between the receptive endometrium of obese versus non-obese patients, 9 obesity biomarkers were identified. The altered genes related to protein binding and other functions necessary for endometrial development. The dysregulation of these 9 genes, were further exaggerated as BMI increased and an additional 4 biomarkers were identified in obese patients presenting with a metabolic syndrome. Currently, the effect of weight loss on the normalization of endometrial receptivity and gene expression is unknown.

1.3.2.5.4.3.1.8 ERA and Biochemical Pregnancy

The ERA test has undergone several refinements since it was first introduced. One of these refinements, with the aim to improve the training set population, was performed by further

stratifying the transcriptomic signature of the WOI (Diaz-Gimeno et al., 2017). To accomplish this, the authors investigated the specific reproductive outcomes of patients whose genomic profiles were initially classified as receptive. This resulted in the stratification of four different subsignatures pertaining to the WOI, where optimal receptivity was associated with an ongoing pregnancy rate (defined as the number of ongoing gestations confirmed by visualization of a gestational sac, divided by the total number of positive pregnancy tests) of 80% and a biochemical pregnancy loss rate (defined as the gestational loss after a positive pregnancy test without the visualization of a gestational sac) of 6.6% (Figure 21). Conversely, the newly branded late receptive profile was associated with an ongoing pregnancy rate of 33.3% and a biochemical pregnancy loss rate of 50% and distinguished by the dysregulation of 22 genes (Diaz-Gimeno et al., 2017). The late receptive profile itself is indicative of endometrium that is close to the end of the WOI. These findings corroborate with earlier clinical results where the risk of pregnancy loss increased with later implantation (Wilcox et al., 1999). The late receptive phase now provides a recommendation for transfer 12 hours earlier in order to ensure that the embryo encounters the endometrium at the time of optimal receptivity.

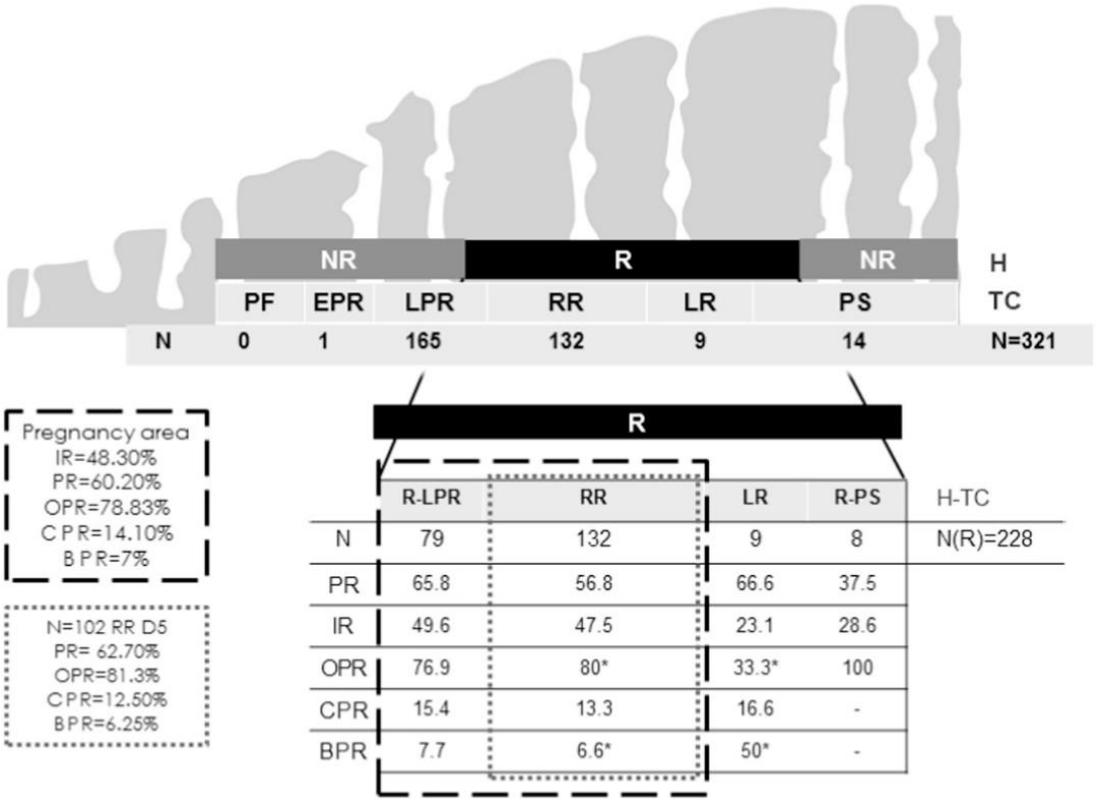


Figure 18. Clinical outcomes obtained from patients diagnosed as receptive (R) according to the initial ERA predictor based on hormone levels (H), prior to the reclassification of the receptive profile and the introduction of the transition phase subsignatures (early and late receptive). The new model was supervised by transcriptomic clusters (TC) based on the clinical follow up set, with the number of samples classified in each profile (N). ERA profiles: NR = non-receptive; PF = proliferative; EPR = early pre-receptive; LPR = late pre-receptive; RR = receptive; LR = late receptive; PS = post-receptive. Mixed ERA profiles: R-LPR = receptive-late pre-receptive; R-PS = receptive-post-receptive. Clinical outcomes: PR = pregnancy rate; IR = implantation rate; OPR = ongoing pregnancy rate; CPR = clinical pregnancy rate; BPR = biochemical pregnancy rate. Asterisks signify statistical significance with Fisher's exact test (P<.05). Under the newly classified late receptive (LR) phase, a significantly higher risk for biochemical pregnancy was detected versus both the receptive-late prereceptive phase (R-LPR) and optimal receptive (RR) phase. Taken from Diaz-Gimeno et al., 2017.

1.3.2.5.4.3.1.9 Transcriptomics and Genomics Combined – ERA + PGT-A

As implantation requires both a competent embryo and receptive endometrium, in theory the optimization of both of these factors would only contribute further to the enhancement of implantation success in IVF. Currently, several RCTs are being conducted to understand if a benefit does indeed exist when the combination therapy of PGT-A for aneuploidy in embryos

plus ERA to determine the ideal timing for transfer of said euploid blastocysts is executed. Until then, this question remains largely unanswered, as mixed results from retrospective studies have been reported.

When studying patients with at least one previously failed euploid FET, Tan et al., (2018) found higher implantation and ongoing pregnancy rates, albeit not statistically significant, in patients who underwent a pET guided by ERA compared to those patients who did not have ERA performed and underwent transfer at the standard FET timing (73.7%; 63.2% vs. 54.2%; 41.7%, respectively). In this study, an obvious limitation is the retrospective design, however an absence of a control arm in order to understand if the transfer had occurred outside the WOI as determined by the ERA, also contributed to the lack of clear clinical translation of their findings. With that said, the authors bring up a valid point, that when implantation failure occurs in the case where both aneuploidy and endometrial receptivity had been controlled for, it is essential to investigate other etiologies, with a prime example being the endometrial microbiota (Moreno et al., 2016).

A retrospective, multicenter cohort study set to determine the clinical usefulness of PGT-A plus ERA testing in patients with moderate (failed implantation after the transfer of 3 or more embryos in at least 3 SETs) and severe RIF (failed implantation after the transfer of 5 of more embryos in at least 3 SETs) showed no benefit of this combined treatment in terms of implantation and ongoing pregnancy. However, due to an extremely small sample size of patients that underwent both ERA and PGT-A (n=15 in the moderate RIF group and n = 4 in the severe RIF group), the preclusion of any definitive clinical indication of their findings is apparent (Cozzolino et al., 2020).

1.3.2.5.4.3.2 Other Transcriptomic Tests for Endometrial Receptivity

Although the ERA is the most widely used commercial test that investigates endometrial receptivity at the transcriptomic level, similar tests have recently come into existence, as it is now clear that women possess unique WOIs, which is best assessed with the use of gene expression profiling.

1.3.2.5.4.3.2.1 Win-Test

The Win-Test (Window Implantation Test), also marketed as ADHESIO^{RT}, was the first commercialized transcriptomic assay for endometrial dating. Applying RT-qPCR to 62 endometrial biopsy samples from 31 normoresponder patients, taken on both LH+2 and LH+7, the developers validated the expression profiles of 5 new marker genes that were specifically modified during the WOI, all of which were shown to be overexpressed during the mid-secretory phase (LH+7), or the presumed time of receptivity, when compared to the early-secretory phase (LH+2) (Haouzi et al., 2009). Ultimately, the commercially available test incorporated the 11 most up-regulated genes to predict endometrial receptivity status. Based upon the mean expression of these 11 genes, an algorithm classifies the samples as receptive when mean expression is ≥70%, partially receptive when between 50-70%, and non-receptive when <50% (Haouzi et al., 2021). Following this classification, a recommendation for a personalized FET (pFET) or customized embryo transfer (cET) can be provided in the case of a receptive diagnosis, whereas a recommendation for subsequent biopsy and retesting is made when a partially receptive or non-receptive result is reported.

Recently, a multicenter, prospective, interventional study was carried out to explore the clinical benefit of the Win-Test. The study involved 217 women with a history of RIF, all who underwent

endometrial biopsy and evaluation with the Win-Test. One hundred fifty-seven of these patients underwent a cET according to the Win-Test results, whereas the other 60 patients served as controls and underwent transfer according to the standard timing. A significantly higher implantation rate per cycle was achieved by the cET group than by the control group (22.7% vs 7.2%), as well as live birth rates per patient (31.8% vs. 8.3%). The authors concluded that timing of the WOI is patient dependent, yielding the need for proper embryo-endometrial synchronization, which can be achieved by assessing endometrial status by applying the Win-Test, as shown evident by a significant improvement in implantation and live birth (Haouzi et al., 2021).

1.3.2.5.4.3.2.2 ERPeak/ERMap

The ERPeak, as known in the United States, or ERMap, as referred to elsewhere, analyzes the expression of 40 genes using RT-qPCR to predict endometrial receptivity status. The genes involved in this test relate to processes taking place on the endometrium during the WOI, specifically relating to endometrial proliferation and the maternal immune response associated with embryonic implantation, as opposed to genes relating more so to endometrial receptivity status, as with ERA. With that said, ERPeak/ERMap only share 7 common genes with the ERA test. Another difference between ERPeak/ERMap and ERA is the molecular platform utilized by each test. According to its developers, RT-qPCR was chosen, as it is considered the gold standard for gene expression analysis, as it possesses the widest dynamic range, lowest quantification limits, and least biased results (Incisor et al., 2018).

The validation of ERPeak/ERMap involved a total of 312 endometrial samples taken from both fertile (n=96) and infertile women (n=120) at LH+2 (pre-receptive) and LH+7 (receptive) in a

natural cycle and after 5 days of progesterone exposure in an HRT cycle, respectively. The samples derived from the fertile subjects were used to define the endometrial transcriptomic signature of the test, whereas the samples from the infertile subjects underwent testing with both the resulting ERPeak/ERMap test and ERA in order to evaluate concordance. Initially, the expression of 184 genes, which were selected after an extensive literature search pertaining to genes involved in endometrial receptivity and immune response, were analyzed. Eight five of these genes were found to be significantly, differentially expressed between the presumed pre-receptive and receptive phases. After applying principal component analysis and discriminant function analysis, it was shown that 40 of the 85 genes accounted for more than 99.5% of total sample variance and allowed for accurate classification of samples according to receptivity status (proliferative, pre-receptive, receptive, or post-receptive), leading to these 40 genes defining the receptive transcriptomic signature of the ERPeak/ERMap. The biopsy samples from the infertile group then underwent simultaneous testing with ERPeak/ERMap and ERA, in which a concordance rate of 97.59% was obtained for the training set and a 91.67% for the testing set (Incisor et al., 2018). Currently, there are no published papers, clinically validating the ERPeak/ERMap test.

1.3.2.5.4.3.2.3 beREADY

With the application of NGS, the beREADY commercial test evaluates the expression of 67 genes associated with endometrial growth, maturation, and receptivity in order to properly phase the status of the endometrium. During its initial development, the transcriptomic profiles of 57 genes associated with endometrial receptivity were used to properly define the various phases of the menstrual cycle at the molecular level. Endometrial tissue samples from 78 women were

taken at various times during the menstrual cycle (including menses, proliferative, early secretory, mid-secretory, and late-secretory phases). In order to establish a machine learning support vector machine (SVM) model to differentiate between the early and mid-secretory phases, 54 paired endometrial biopsy samples, from 27 women with proven fertility were collected at both the early and mid-secretory phase and sequenced. Sequencing data from the initial 78 biopsies resulted in 4 distinct clusters corresponding to menses, proliferative, early + mid-secretory, or a late secretory endometrial phase. When the SVM model was applied, further differentiation was made possible between the early and mid-secretory phase samples. The authors emphasized the importance of transcriptomic tools, like the beREADY test, to not only properly characterize the endometrial status and phase, but to also facilitate the discovery of true disease related markers (Saare et al., 2019). To date, there are no publications clinically validating the beReady test.

1.3.2.5.4.3.2.4 YK-ERT

The endometrial receptivity test (ERT) analyzes the expression of 100 genes related to endometrial receptivity with NGS to predict the receptive state. Like most other tests of its kind, the biopsy is taken in either a natural cycle on LH+7 or in an HRT cycle on P+5. At this time, there are no publications pertaining to the development or clinical validation of this method.

1.3.2.5.4.3.2.5 BioER

Also employing NGS, Bio-ER evaluates the gene expression of 72 genes associated with endometrial receptivity and maternal immune response associated with embryonic implantation

to provide a recommendation for embryo transfer on the most optimal day. Such as YK-ERT, no publications currently exist that describe the development or validation of this commercial tool.

1.3.2.5.5 Beyond Receptivity, Molecular Testing to Optimize the Endometrial Microbiome

Recently the human microbiome, that is the combined genetic material of microbes (bacteria, fungi, viruses) inhabiting our body, has gained attention, showing links to overall health status and diseased states, such as diabetes, obesity, allergies, autism, and depression. From a reproductive stance, the presence of pathogenic bacteria within the reproductive tract can lead to infertility and obstetric complications. For instance, chronic endometritis, which is the inflammation of the endometrium commonly caused by the presence of bacterial pathogens in the uterine cavity, has been demonstrated to be prevalent in women suffering from RPL and RIF, with rates up to 60% (Cicinelli et al., 2014) and 66% (Cicinelli et al., 2015), respectively. Classically, it was assumed that a healthy uterine cavity was sterile, devoid of any bacterial species. However, we now know that like other parts of the human body, such as the vagina, the presence of good bacteria, specifically a high density of Lactobacillus, in the endometrium is associated with superior reproductive outcomes (Moreno et al., 2016).

In the study by Moreno and colleagues (2016), they compared the clinical outcomes of patients undergoing a pET guided by ERA to the endometrial bacterial profiles previously obtained at time of biopsy. The authors reported that those patients with a Lactobacillus dominated microbiome (90% or more of the microbiome consisting of Lactobacillus) experienced significantly higher pregnancy rates, implantation rates, and ongoing pregnancy rates than those patients with a non-Lactobacillus dominated microbiome (<90% of the microbiome composed of Lactobacillus). Not only does it appear that Lactobacillus plays an important role in establishing a pregnancy,

but also maintaining a pregnancy, as miscarriage rates differed between the groups, with a rate of 16.7% in the Lactobacillus dominated group versus 60% in the non-Lactobacillus dominated group. Furthermore, an adverse effect on reproductive outcomes, in terms of no pregnancy or miscarriage, was more evident in those patients presenting with a higher percentage of the bacterial pathogens, Gardnerella and Streptococcus. Based on these findings, a relatively new molecular test is now available, the Endometrial Microbiome Metagenomic Analysis (EMMA), that evaluates the endometrium at the microbiological level, including specific screening for chronic endometritis. The EMMA test employs RT-PCR (in the United States) or NGS (elsewhere worldwide) to examine the bacterial profile of the endometrium, assessing not only the presence of bacterial DNA from pathogens in an endometrial biopsy sample, but also the level of Lactobacillus. The results provide insight to the bacterial ecosystem of a woman's endometrium, allowing targeted therapy to be applied when pathogenic bacteria are detected and proper restoration of the Lactobacillus population, creating an optimal environment for embryo implantation and the resulting pregnancy. Further optimization may be achieved by combining the EMMA with ERA, in which a single biopsy can provide a recommendation for transfer based on both optimal receptivity and an optimal microbial status.

1.4 The Purpose of this Thesis

1.4.1 Perspectives

Since the introduction of IVF, the field has focused on optimizing the embryo in order to improve outcomes. Although it seems likely that we are approaching the pinnacle of our understanding of how to optimize embryonic factors and development fully, we have made extraordinary leaps allowing us to achieve significantly higher clinical rates today versus those forty plus years ago at the start of clinical human IVF. By contrast, a lesser emphasis has been placed on optimizing the endometrium during an IVF cycle. We have largely relied on archaic, and quite accurately, non-diagnostic markers such as endometrial thickness and hormone levels to decipher the status and health of the endometrium. However, with the recent wide introduction of personalized medicine into ART, the innovation of new technologies has provided more robust, reliable, and objective endometrial tests that provide a clinical directive. Specifically, the ERA which leverages transcriptomics to study the endometrium, has not only been associated with improved outcomes, but has also given us greater insight into endometrial receptivity and how unique this process can be in each woman. The ERA, along with other commercially offered transcriptomic endometrial receptivity tests, has brought to the forefront the importance of endometrium in implantation.

Implantation remains the limiting factor to the success of an IVF cycle, requiring both a competent embryo and receptive endometrium. With that said, in order to increase implantation success further, we must redirect our focus on the interplay between the embryo and endometrium, not treating them as separate entities and hence independent routes of treatment. Recognizing that a relatively high rate of euploid embryos do not implant, in addition

to that the fact that the endometrium will only allow for implantation to occur during a finite period of time each cycle, we should theoretically increase implantation potential of IVF transfer cycles by confirming the transfer of a euploid embryo into a receptive endometrium. Although quite intuitive, current research and the available data documenting this theory is quite sparse. It is my belief that when we simultaneously control for both of these factors that we will achieve consistently greater overall IVF outcomes for all patients. It is imperative therefore that we understand the basic science behind embryo aneuploidy and the endometrial receptivity, asking questions if mosaicism is present in all embryos and if so, does this represent an underlying mechanism necessary for implantation or what is the length of the WOI and how specific is the timing of receptivity in some women? By answering these questions, we can exploit these two players to their maximum potential, subsequently allowing us to assess the resulting outcomes in order to implement a new standard of care for patients undergoing IVF treatment.

Although it seems reasonable to suggest that generally higher clinical rates will be achieved across all patients when controlling for both aneuploidy and endometrial receptivity, we are unlikely reach the apex of implantation optimization with this combined treatment alone. Once we routinely control for both embryo aneuploidy and endometrial receptivity during transfers, there is even more to gain, and hence fine-tune and optimize the IVF process. For instance, when a failed euploid transfer occurs in a confirmed receptive endometrium, we can then search out more elusive etiologies. One possibility currently being investigated is the endometrial microbiome, which ensures an optimal microbial status at time of transfer that further supports successful implantation and ongoing pregnancy. Though personalized medicine is still in its infancy in the field of IVF, it will guide us to a deeper understanding of human reproduction,

allowing us to enhance our practices as much as possible by offering customized treatment plans based on the unique genetic constitute of each patient.

1.4.2 Specific Aims of the Thesis

As previously stated, for the most part, the field as a whole has classically focused on the impact of the embryonic factor on success rates while typically forgoing meaningful assessment of endometrial receptivity. This practice was largely in part to the widely accepted dogma that the WOI was consistent between all women as well as a lack of an objective and reliable diagnostic test to accurately assess endometrial receptivity and provide reproducible results with a clear clinical directive. The purpose of the work described in this thesis was to investigate if implantation during IVF treatment can be optimized by controlling for both embryonic factor with aneuploidy screening and endometrial factor though transcriptomic assessment of receptivity. With the above in mind, the specific aims of this thesis were as follows:

a) To provide a preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm
b) To establish whether there are improved pregnancy rates following endometrial receptivity analysis and personalized embryo transfer in patients with previous failed implantation after euploid embryo transfer
c) To ask the question of whether the clinical outcomes are different in day 5 versus day 6 single embryo transfer when endometrial factor is controlled
d) To evaluate clinical outcomes associated with personalized embryo transfers guided by transition phase results: to ask whether small shifts can lead to big outcomes

e) To ask, through a single case report, what is the narrowest window of implantation by examining the evolution of the endometrial phases via transcriptomic profiling of biopsies taken at various hours of progesterone exposure

f) To provide a novel reanalysis of published data presented pertaining to inter-cycle consistency versus test compliance in endometrial receptivity analysis testing

g) To assess the prevalence of a displaced WOI in gestational carriers and the clinical utility of applying ERA

2.0 Materials and Methods

2.1 Specific aim a) To provide a preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm

2.1.1 Study Design

This study was deemed exempt by Sterling IRB because it only incorporated routine IVF procedures. Only patients who were undergoing IVF with PGT between January 2012 and April 2013 at the Reproductive Endocrinology Associates of Charlotte (Charlotte, North Carolina, USA) were included in this study. All biopsy specimens were sent to Genesis Genetics (Detroit, Michigan, USA) where samples underwent NGS. Briefly, all fertilized oocytes were cultured to day 3 and assisted hatching (AH) was performed. Embryos were placed back into incubator and cultured to the blastocyst stage. Embryos whose trophectoderm was hatching out of the ZP underwent the biopsy procedure. Biopsied blastocysts were divided into three groups depending on which area (polar, mid, or mural) of the trophectoderm was protruding from the ZP and biopsied.

2.1.2 Egg retrieval and embryo culture

All retrieved oocytes were designated for intracytoplasmic sperm injection (ICSI). Oocytes were retrieved, trimmed of blood, and stripped of cumulus cells as described by Taylor and colleagues (Taylor et al., 2008). Oocytes were separated based on maturity and placed into a 60 mm dish (Thermo Scientific, Rochester, New York, USA) with approximately 100 µl drops of continuous culture medium (CSC; Irvine Scientific, Santa Ana, California, USA) supplemented with 10% serum

substitute supplement (SSS; Irvine Scientific, Santa Ana, California, USA) and overlaid with oil (Irvine Scientific, Santa Ana, California, USA). After grading, the dish containing the oocytes was placed into an incubator at 37°C, 6% CO2 and 5% O2 in air for 2–3 h. After 2 h, all oocytes presenting with a polar body underwent ICSI as described by Nagy et al. (1995), placed back into the same dish and put back into the incubator.

The day following oocyte retrieval, 16–18 h post ICSI, oocytes were evaluated for true fertilization. Embryos that exhibited two pronuclei were group cultured in a fresh dish of CSCþ10% SSS overlaid with oil and placed back into the incubator. Embryos were not viewed on day 2.

On day 3, the embryos were removed from the incubator, graded and AH was performed on all cleaving embryos with the aid of a laser (Zilos-tk, Hamilton Thorne, Beverly, Massachusetts, USA). Using a pulse of 610 µs, the ZP was breached with two or three shots of the laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA). The ZP was breached where there were no blastomeres that could be directly affected by the laser pulse. After breaching the ZP with the laser, the embryos were left in the same drop and placed back into the incubator.

On the morning of day 5 (112–115 h post insemination) and day 6 (136–139 h post insemination), embryos were removed from the incubator, and blastocysts were graded based on Schoolcraft and colleagues (1999) and those blastocysts that had a good or fair trophectoderm protruding from the ZP, along with good or fair quality ICM, were biopsied. Blastocysts were only viewed once in the morning and at no other times. If the blastocysts were not suitable for biopsy in the morning of day 5, they were re-evaluated on the morning of day 6.

Blastocysts were biopsied on day 5 or day 6, depending on the day they met the biopsy criteria. If embryos did not meet the criteria for biopsy on day 6, they were discarded. There was no morphological difference between blastocysts that were biopsied on day 5 or day 6 other than the embryos needed an extra day to reach the proper stage for biopsy.

2.1.3 Trophectoderm biopsy

Blastocysts that presented with a good or fair quality ICM and trophectoderm were placed in a drop of modified human tubal fluid (Irvine Scientific, Santa Ana, California, USA) þ 10% SSS (Irvine Scientific, Santa Ana, California, USA). Suction was applied to the blastocysts via a holding pipette (Humagen, Charlottesville, Virginia, USA). A biopsy pipette (Humagen, Charlottesville, Virginia, USA) gently aspirated the trophectoderm into the biopsy needle. A laser (Zilos-tk, Hamilton Thorne, Beverly, Maine, USA), with a pulse length of 610 µm, was used to 'cut' the trophectoderm from the blastocyst, without exposing the trophectoderm to unnecessary laser pulses. The piece of trophectoderm was prepped for NGS.

2.2 Specific aim b) To establish whether there are improved pregnancy rates following endometrial receptivity analysis and personalized embryo transfer in patients with previous failed implantation after euploid embryo transfer

2.2.1 Study Design

A prospective pilot study in three independent reproductive centers (Table 5) was performed from December 2016 to May 2018. Endometrial receptivity and clinical outcome following a pET was assessed in 26 women of reproductive age, without history of successful pregnancy, and with at least one failed, frozen euploid embryo transfer in an HRT cycle. Clinical follow up data collected included pregnancy rate, implantation rate, clinical pregnancy rate, biochemical and clinical miscarriage rate, and live birth rate. Approval for this study was obtained from Advarra Institutional Review Board (Igenomix Laboratories – IGS001: Igenomix Research Study on PGS with ERA testing). Verbal and written informed consent were obtained from all patients prior to enrollment.

Table 5. Number of patients enrolled per clinic site.

Clinic	Number of Patients Enrolled (n=26)
A	12
B	11
C	3

2.2.2 Patient population

There was a single test group, with all patients receiving the same treatment. Careful adherence to inclusion and exclusion criteria was necessary for this study design. Patients with no successful previous pregnancies and having at least one failed euploid frozen blastocyst transfer were invited to participate. Patients needed a minimum of one euploid blastocyst, diagnosed by either high resolution NGS (hr-NGS), aCGH, or SNP, available for transfer in a subsequent cycle. No specific age range was defined for this study, though all participants were of reproductive age. An earlier study found significantly increased non-receptivity rates in women with a BMI >30 (Comstock *et al.*, 2017; Bellver *et al.*, 2018), hence only patients with a BMI of 18.5-30 were included. Other inclusion factors comprised of a normal ovarian reserve, as defined by an antral follicle count of ≥8 or an FSH <8, and no pathology affecting the endometrial cavity (such as polyps/submucosal myomas, intramural myomas > 4 cm, or hydrosalpinx) or with previous surgical correction.

Patients were excluded from this study if they had undergone prior aneuploidy screening and achieved pregnancy, even if that pregnancy subsequently miscarried; had a history of multiple miscarriages or whom had never had previous aneuploidy screening. Other exclusion factors included a male partner with severe male factor (spermatozoa <2million/ml), having underwent preimplantation genetic testing for monogenetic defects (PGT-M) or structural rearrangements (PGT-SR), such as translocations or inversions, and if a different sperm and/or oocyte source was used in the previous failed euploid transfer cycle.

2.2.3 ERA mock cycle and endometrial biopsy

An endometrial biopsy for ERA was performed by a physician within the IVF clinic, in an HRT cycle identical to one carried out during the failed euploid embryo transfer cycle, with respect to the type and dosage of progesterone. Although all HRT cycles incorporated both estradiol and progesterone, the type and dosage of progesterone varied slightly depending on the clinic's standard of care, however these factors were controlled for during all three HRT cycles (failed transfer cycle prior to enrollment, mock-transfer ERA cycle, pET transfer cycle as part of the study). The standard HRT cycle recommended by Igenomix includes start of estradiol (6mg/day orally or two patches every two days) on the 1st or 2nd day of the menstrual cycle. An office visit is then conducted 7-10 days later that includes ultrasound assessment to validate a trilaminar endometrium measuring >6mm and bloodwork to ensure endogenous progesterone is <1ng/ml. Within the next 24 hours luteal support begins using 400mg of micronized vaginal progesterone every 12 hours (800mg/day), with first intake of progesterone day defined as "P+0". The endometrial biopsy is obtained on "P+5", after a full 5 days of progesterone intake, equating to 120 hours of progesterone exposure.

The endometrial biopsy procedure involved the extraction of a small piece of endometrial tissue (70mg) from the uterine fundus with the use of a Pipelle catheter or similar. The endometrial biopsy sample was immediately transferred to a cryotube containing 1.5ml of RNAlater (Qiagen), vigorously shaken for 10 seconds, and kept at 4-8°C for a minimum of 4 hours. Specimens were then shipped at room temperature to Igenomix (Valencia, Spain) for analysis.

2.2.4 ERA analysis and interpretation

The ERA molecular diagnostic tool currently utilizes NGS to analyze the expression levels of 248 genes related to the status of endometrial receptivity. RNA extraction from the tissue is performed using the RNeasy mini kit (Qiagen). All samples are DNAse treated, and cDNA is obtained by retrotranscription and analyzed by targeted RNA-Seq assay on IonTorrent NGS for 248 ERA genes in an Ion S5 system. Sequencing files are used as the input of the ERA predictor (Clemente-Ciscar et al., 2018) to quantify the expression of the ERA genes and to assess the endometrial receptivity status of each sample. Briefly, the reads are mapped to the hg19 human genome transcriptome using the STAR read aligner (Dobin et al., 2013). To count the number of reads that could be assigned to each gene, the HTSeq tool (Anders et al., 2015) with the union option is used. The ERA gene counts are used by the prediction model to classify each sample in an endometrial receptivity class as proliferative, pre-receptive, receptive, or post-receptive.

The various result profiles yielding an informative result include receptive, early receptive, late receptive, pre-receptive 1 day, pre-receptive 2 days, and post-receptive. A receptive result indicates that the gene expression profile is concordant with a normal receptive endometrium. Embryo transfer will be recommended to be performed at the same timing at which the biopsy was taken. Early and late receptive results are both referred to as transition phases, where the endometrial transcriptomic profile is in a transition period between a non-receptive phase (either pre-receptive or post-receptive) and the receptive phase. In these instances, the WOI is either delayed by 12 hours (as in the case of an early receptive result) or it is advanced by 12 hours (as in the case of a late receptive result), the recommendation will be to transfer 12 hours later or 12 hours earlier, respectively. A pre-receptive result corresponds to a gene expression

profile that is concordant with an endometrium at that has not yet reached the WOI. This profile can be further classified as pre-receptive 1 day, where the WOI is delayed by 24 hours or pre-receptive 2 days, where the WOI is delayed by 48 hours. Lastly, a post-receptive result corresponds to a gene expression profiles that is concordant with an endometrium that has already passed the WOI, usually by 24 hours. At this time, a direct recommendation for transfer can be made based upon all the informative profiles, except for pre-receptive 2 day and post-receptive profiles, where instead a second biopsy will be requested to validate the corresponding prediction of receptivity.

Less commonly, non-informative results are obtained with the ERA. These include proliferative, invalid RNA, insufficient RNA, and non-informative profiles. In the case of a proliferative result, the gene expression profile is concordant with an endometrium that is still within the proliferative phase, that has not yet been exposed to progesterone. An invalid RNA result specifies that it was not possible to determine the gene expression profile of the sample due to poor quality of genetic material obtained, whereas an insufficient RNA result specifies that it was not possible to determine the gene expression profile of the sample because there was not enough biopsy material present. A non-informative result is associated with a profile that does not match the reference gene expression profiles in the ERA predictor. In all these cases, a new biopsy will be requested in order to obtain a valid and informative result.

2.2.5 pET cycle and clinical outcome

Once the patient's WOI was identified and a recommendation for transfer was made, the patient then proceeded with a pET guided by the ERA results (Figure 22). The report itself provided a distinct hour for blastocyst transfer, with a +/-3-hour window. The same protocol that was used in the failed euploid transfer cycle and the ERA mock cycle(s) was replicated in the pET cycle, with the exception of progesterone exposure time, if indicated by the ERA pET recommendation. Special consideration was taken to ensure proper control of endogenous progesterone levels and similar uterine conditions among all cycles. One or two vitrified, euploid blastocysts were warmed and transferred.

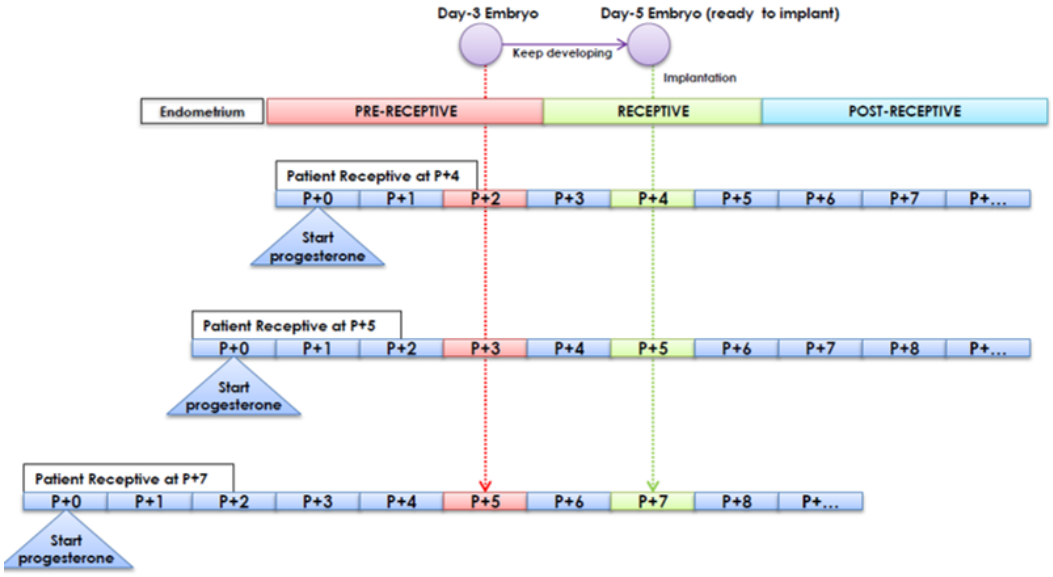


Figure 19. A pET recommendation chart. A pET is provided to allow proper synchronization between an implanting embryo and a receptive endometrium. The figure shows the recommended timing for both day 3 cleavage embryo transfer and blastocyst transfer for patients found to be post-receptive, receptive, and pre-receptive, respectively, at the standard time of expected receptivity. Taken from Gardner, D.K., & Simón, C. (Eds.). (2017). Handbook of *In vitro* Fertilization (4th ed.). CRC Press. https://doi.org/10.1201/9781315157269.

Data were reported as pregnancy rate (percentage of patients with βhCG positive from the total of patients with pET), implantation rate (percentage of embryos that successfully implanted as number of gestational sacs observed by vaginal ultrasound at the 5-6th week of pregnancy divided by the number of transferred embryos), ongoing pregnancy rate (percentage of pregnancies that continued beyond the 20th week from the total of patients with pET) and live birth rate (percentage of pregnancies resulting in live birth from the total of patients with pET). The percentages of biochemical pregnancies and clinical miscarriage were also documented. An additional analysis was performed comparing the outcome between the participants who received an initial "receptive" result and participants who had a pET following a "non-receptive" result.

2.2.6 Statistical analysis

Statistical analysis was performed using SPSS 26.0 software (IBM, MD, USA). Descriptive analysis was done in all clinical variables, as well as in variables involved with the controlled ovarian stimulation (COS) cycle. A comparison of quantitative variables was done using independent samples Student´s *t*-test. For comparing categorical data, the non-parametric Fisher´s Exact proportion test was applied because of the low number data. Those comparisons were made between the first HRT cycle and the ERA cycle and between these two and the pET cycle. Also, ET characteristics and clinical outcome comparisons were made between the first failed ET cycle and the second ET after ERA (pET). Clinical outcome was compared between receptive patients at day P+5 and non-receptive in this day. $P < .05$ was considered statistically significant.

2.3 Specific aim c) To ask the question of whether the clinical outcomes are different in day 5 versus day 6 single embryo transfer when endometrial factor is controlled

2.3.1 Study Design

This is a retrospective data study, analyzing the clinical outcomes of 260 patients, from 14 different fertility centers, who underwent a SET with either a day 5 or day 6 blastocyst in a pET cycle guided by ERA between January 2017 and December 2019 (Table 6). All patients previously underwent ERA testing in either an HRT or natural cycle in which a receptive result was obtained at time of biopsy. The following parameters were assessed: patient BMI, embryo origin, developmental day of transferred embryos, morphological embryo quality, utilization of PGT-A, and resulting clinical outcome (number of implanted sacs, bHCG, pregnancy results). Details pertaining to the ERA and pET cycle timing and protocol were also included to ensure reproducibility and proper execution of the ERA recommendation provided by the results. The study with code 2021/18 was approved 25th February by the Research Committee of the Biomedical Research Institute INCLIVA and the Ethic Committee of the Hospital Clínico Universitario de Valencia. All participants provided written informed consent for research.

Table 6: Number of day 5 versus day 6 embryos transferred per fertility clinic.

Clinic	Day of transferred embryos d5 n=183	Day of transferred embryos d6 n=77	Total n=260
A	68	9	77
B	31	22	53
C	26	11	37
D	12	9	21
E	13	6	19
F	9	2	11
G	4	5	9
H	7	0	7
I	2	5	7
J	2	4	6
K	4	1	5
L	3	0	3
M	1	2	3
N	1	1	2

2.3.2 Endometrial preparation and biopsy

Endometrial preparation for ERA was performed by a physician within the IVF clinic, in either an HRT or natural FET mock cycle. Although all HRT cycles incorporated both estradiol and progesterone, administration types and doses are clinic specific. The standard HRT cycle recommended by Igenomix includes start of estradiol (6mg/day orally or two patches every two days) on the 1st or 2nd day of the menstrual cycle. An office visit is then conducted 7-10 days later that includes ultrasound assessment to validate a trilaminar endometrium measuring >6mm and

bloodwork to ensure endogenous progesterone is <1ng/ml. Within the next 24 hours luteal support begins using 400mg of micronized vaginal progesterone every 12 hours (800mg/day), with first intake of progesterone day defined as “P+0”. The endometrial biopsy is obtained on “P+5”, after a full 5 days of progesterone intake, equating to 120 hours of progesterone exposure.

In a natural ERA cycle the time of biopsy was based on either an LH surge or an hCG trigger. In the case of an LH surge, once the dominant follicle reaches 16mm, serum or urine LH levels should be measured daily until a negative-positive-negative pattern is observed. Using this pattern, the LH surge date can accurately be identified and denoted as “LH+0,” biopsy is then performed 7 days later on “LH+7”. If a human chorionic gonadotropic (hCG) trigger shot is used, day of trigger is considered “hCG+0” and biopsy occurs 7 days after on “hCG+7”, or 168 hours from administration. During a natural cycle, exogenous supplementation with progesterone may or may not have been used.

The endometrial biopsy procedure involves the extraction of a small piece of endometrial tissue (70mg) from the uterine fundus with the use of a Pipelle catheter or similar. The endometrial biopsy sample is immediately transferred to a cryotube containing 1.5ml of RNAlater (Qiagen), vigorously shaken for 10 seconds, and kept at 4-8°C for a minimum of 4 hours. Specimens are then shipped at room temperature to Igenomix (Valencia, Spain) for analysis.

2.3.3 ERA laboratory protocol and results

The ERA molecular diagnostic tool currently utilizes NGS to analyze the expression levels of 248 genes related to the status of endometrial receptivity. RNA extraction from the tissue is

performed using the Rneasy mini kit (Qiagen). All samples are DNAse treated, and cDNA is obtained by retrotranscription and analyzed by targeted RNA-Seq assay on IonTorrent NGS for 248 ERA genes in an Ion S5 system. Sequencing files are used as the input of the ERA predictor (Clemente-Ciscar et al., 2018) to quantify the expression of the ERA genes and to assess the endometrial receptivity status of each sample. Briefly, the reads are mapped to the hg19 human genome transcriptome using the STAR read aligner (Dobin et al., 2013). To count the number of reads that could be assigned to each gene, the HTSeq tool (Anders et al., 2015) with the union option is used. The ERA gene counts are used by the prediction model to classify each sample in an endometrial receptivity class as proliferative, pre-receptive, receptive, or post-receptive.

The various result profiles yielding an informative result include receptive, early receptive, late receptive, pre-receptive 1 day, pre-receptive 2 days, and post-receptive. A receptive result indicates that the gene expression profile is concordant with a normal receptive endometrium. Embryo transfer will be recommended to be performed at the same timing at which the biopsy was taken. Early and late receptive results are both referred to as transition phases, where the endometrial transcriptomic profile is in a transition period between a non-receptive phase (either pre-receptive or post-receptive) and the receptive phase. In these instances, the WOI is either delayed by 12 hours (as in the case of an early receptive result) or it is advanced by 12 hours (as in the case of a late receptive result), the recommendation will be to transfer 12 hours later or 12 hours earlier, respectively. A pre-receptive result corresponds to a gene expression profile that is concordant with an endometrium at that has not yet reached the WOI. This profile can be further classified as pre-receptive 1 day, where the WOI is delayed by 24 hours or pre-receptive 2 days, where the WOI is delayed by 48 hours. Lastly, a post-receptive result

corresponds to a gene expression profiles that is concordant with an endometrium that has already passed the WOI, usually by 24 hours. At this time, a direct recommendation for transfer can be made based upon all the informative profiles, except for pre-receptive 2 day and post-receptive profiles, where instead a second biopsy will be requested to validate the corresponding prediction of receptivity.

Less commonly, non-informative results are obtained with the ERA. These include proliferative, invalid RNA, insufficient RNA, and non-informative profiles. In the case of a proliferative result, the gene expression profile is concordant with an endometrium that is still within the proliferative phase, that has not yet been exposed to progesterone. An invalid RNA result specifies that it was not possible to determine the gene expression profile of the sample due to poor quality of genetic material obtained, whereas an insufficient RNA result specifies that it was not possible to determine the gene expression profile of the sample because there was not enough biopsy material present. A non-informative result is associated with a profile that does not match the reference gene expression profiles in the ERA predictor. In all these cases, a new biopsy will be requested in order to obtain a valid and informative result.

2.3.4 pET and outcomes

A pET was carried out in a subsequent FET cycle. The endometrial preparation protocol used in the ERA mock cycle in which a receptive result was achieved was replicated and timing of transfer was guided by the ERA recommendation. In this study we only considered those patients without a displacement, where recommended time of transfer was the same as biopsy time, as this reflected a transcriptomic result indicating optimal receptivity with highest

confidence. A single day 5 or day 6 blastocyst (previously frozen according to clinic-specific criteria, including appropriate degree of expansion, whether that be on day 5 or day 6 of development), selected based on morphological quality and in some cases PGT-A results, was warmed, and transferred.

Clinical outcomes pertaining to the first pET in terms of pregnancy rate, implantation rate, and ongoing pregnancy rate following transfer of day 5 blastocysts versus day 6 blastocysts were compared. Pregnancy rates were based on βhCG results following transfer. Ongoing pregnancy rates were defined as those pregnancies that persisted with positive identification of gestational sac(s) after 12 weeks of gestation. Implantation rates were calculated dividing the total number of gestational sacs present by the total number of embryos transferred, which in this case was 1 as all patients underwent a SET.

2.3.5 Statistical analysis

Student's t-test was used to compare quantitative variables and Chi-Square and Fisher tests were used to compare categorical variables between groups. A multivariant binomial regression was made considering the control variables that could interfere on the principal studied variable (ongoing pregnancy rate). Significance was set at $P < 0.05$.

2.4 Specific aim d) To evaluate clinical outcomes associated with personalized embryo transfers guided by transition phase results: to ask whether small shifts can lead to big outcomes

2.4.1 Study design

This is a retrospective study data review, analyzing the clinical outcomes of 567 patients, from 17 different IVF clinics (Table 7), who underwent a pET guided by ERA between February 2017 and September 2019. All patients previously underwent ERA testing in an HRT cycle in which a result indicating a direct recommendation for transfer (either with or without a displacement) was obtained. Clinical rates were compared between pETs associated with a transition phase result (12 hours displacement) versus pETs associated with a receptive phase result (no displacement). The data was further stratified to compare clinical rates between the two different types of transition phases, with pETs guided by either an early receptive result (+12 hours) or by a late receptive result (-12 hours). The study with code 2021/18 was approved 25th February by the Research Committee of the Biomedical Research Institute INCLIVA and the Ethic Committee of the Hospital Clínico Universitario de Valencia. All participants provided written informed consent for research.

Table 7. Number of patient cases analyzed per clinic site.

IVF Clinics	Number of patients (n=567)
A	136
B	115
C	75
D	61
E	45
F	26
G	25
H	18
I	17
J	12
K	11
L	10
M	9
N	3
O	2
P	1
Q	1

2.4.2 Endometrial preparation and biopsy

Endometrial preparation for ERA was performed by a physician within the IVF clinic, in either an HRT or natural FET mock cycle. Although all HRT cycles incorporate both estradiol and progesterone, administration types and doses are clinic specific. The standard HRT cycle

recommended by Igenomix includes start of estradiol (6mg/day orally or two patches every two days) on the 1st or 2nd day of the menstrual cycle. An office visit is then conducted 7-10 days later that includes ultrasound assessment to validate a trilaminar endometrium measuring >6mm and bloodwork to ensure endogenous progesterone is <1ng/ml. Within the next 24 hours luteal support begins using 400mg of micronized vaginal progesterone every 12 hours (800mg/day), with first intake of progesterone day defined as "P+0". The endometrial biopsy is obtained on "P+5", after a full 5 days of progesterone intake, equating to 120 hours of progesterone exposure.

In a natural ERA cycle the time of biopsy was based on either an LH surge or an hCG trigger. In the case of an LH surge, once the dominant follicle reaches 16mm, serum or urine LH levels should be measured daily until a negative-positive-negative pattern is observed. Using this pattern, the LH surge date can accurately be identified and denoted as "LH+0," biopsy is then performed 7 days later on "LH+7". If a human chorionic gonadotropic (hCG) trigger shot is used, day of trigger is considered "hCG+0" and biopsy occurs 7 days after on "hCG+7", or 168 hours from administration. During a natural cycle, exogenous supplementation with progesterone may or may not be used.

The endometrial biopsy procedure involves the extraction of a small piece of endometrial tissue (70mg) from the uterine fundus with the use of a Pipelle catheter or similar. The endometrial biopsy sample is immediately transferred to a cryotube containing 1.5ml of RNAlater (Qiagen), vigorously shaken for 10 seconds, and kept at 4-8°C for a minimum of 4 hours. Specimens are then shipped at room temperature to Igenomix (Valencia, Spain) for analysis.

2.4.3 ERA laboratory protocol and results

The ERA molecular diagnostic tool currently utilizes NGS to analyze the expression levels of 248 genes related to the status of endometrial receptivity. RNA extraction from the tissue is performed using the RNeasy mini kit (Qiagen). All samples are DNAse treated, and cDNA is obtained by retrotranscription and analyzed by targeted RNA-Seq assay on IonTorrent NGS for 248 ERA genes in an Ion S5 system. Sequencing files are used as the input of the ERA predictor (Clemente-Ciscar et al., 2018) to quantify the expression of the ERA genes and to assess the endometrial receptivity status of each sample. Briefly, the reads are mapped to the hg19 human genome transcriptome using the STAR read aligner (Dobin et al., 2013). To count the number of reads that could be assigned to each gene, the HTSeq tool (Anders et al., 2015) with the union option is used. The ERA gene counts are used by the prediction model to classify each sample in an endometrial receptivity class as proliferative, pre-receptive, receptive, or post-receptive.

The various result profiles yielding an informative result include receptive, early receptive, late receptive, pre-receptive 1 day, pre-receptive 2 days, and post-receptive. A receptive result indicates that the gene expression profile is concordant with a normal receptive endometrium. Embryo transfer will be recommended to be performed at the same timing at which the biopsy was taken. Early and late receptive results are both referred to as transition phases, where the endometrial transcriptomic profile is in a transition period between a non-receptive phase (either pre-receptive or post-receptive) and the receptive phase. In these instances, the WOI is either delayed by 12 hours (as in the case of an early receptive result) or it is advanced by 12 hours (as in the case of a late receptive result), the recommendation will be to transfer 12 hours later or 12 hours earlier, respectively. A pre-receptive result corresponds to a gene expression

profile that is concordant with an endometrium at that has not yet reached the WOI. This profile can be further classified as pre-receptive 1 day, where the WOI is delayed by 24 hours or pre-receptive 2 days, where the WOI is delayed by 48 hours. Lastly, a post-receptive result corresponds to a gene expression profiles that is concordant with an endometrium that has already passed the WOI, usually by 24 hours. At this time, a direct recommendation for transfer can be made based upon all the informative profiles, except for pre-receptive 2 day and post-receptive profiles, where instead a second biopsy will be requested to validate the corresponding prediction of receptivity.

Less commonly, non-informative results are obtained with the ERA. These include proliferative, invalid RNA, insufficient RNA, and non-informative profiles. In the case of a proliferative result, the gene expression profile is concordant with an endometrium that is still within the proliferative phase, that has not yet been exposed to progesterone. An invalid RNA result specifies that it was not possible to determine the gene expression profile of the sample due to poor quality of genetic material obtained, whereas an insufficient RNA result specifies that it was not possible to determine the gene expression profile of the sample because there was not enough biopsy material present. A non-informative result is associated with a profile that does not match the reference gene expression profiles in the ERA predictor. In all these cases, a new biopsy will be requested in order to obtain a valid and informative result.

2.4.4 pET and outcomes

A pET was carried out in a subsequent FET cycle. The endometrial preparation protocol used in the ERA mock cycle was replicated and timing of transfer was guided by the ERA recommendation. In this study we specifically examined the outcomes of only those patients without a displacement (receptive) and those with a displacement of only 12 hours (early or late receptive).

Clinical outcomes pertaining to the first pET in terms of pregnancy rate, implantation rate, and ongoing pregnancy rate were compared. Pregnancy rate was based on βhCG results following transfer. Ongoing pregnancy rates was defined as those pregnancies that persisted with positive identification of gestational sac(s) after 12 weeks of gestation. Implantation rate was calculated dividing the total number of gestational sacs present by the total number of embryos transferred.

2.4.5 Statistical analysis

Student's t-test and Wilcoxon-Mann-Whitney test were used to compare quantity variables and Fisher test was used to compare categorical variables between groups. Significance was set at $P < 0.05$.

2.5 Specific aim g) To assess the prevalence of a displaced WOI in gestational carriers and the clinical utility of applying ERA

2.5.1 Study design

This is a retrospective data review assessing the receptivity rates of 39 GCs who underwent the ERA test at a single IVF clinic between July 2019 and November 2020. Furthermore, clinical outcomes following the first pET cycle involving the transfer of a single blastocyst were evaluated. The study with code 2021/18 was approved 25th February by the Research Committee of the Biomedical Research Institute INCLIVA and the Ethic Committee of the Hospital Clínico Universitario de Valencia. All participants provided written informed consent for research.

2.5.2 Endometrial preparation and biopsy

Endometrial preparation for ERA was performed by a physician within the IVF clinic, in an HRT cycle. Although all HRT cycles incorporate both estradiol and progesterone, administration types and doses are clinic specific. The standard HRT cycle recommended by Igenomix includes start of estradiol (6mg/day orally or two patches every two days) on the 1st or 2nd day of the menstrual cycle. An office visit is then conducted 7-10 days later that includes ultrasound assessment to validate a trilaminar endometrium measuring >6mm and bloodwork to ensure endogenous progesterone is <1ng/ml. Within the next 24 hours luteal support begins using 400mg of micronized vaginal progesterone every 12 hours (800mg/day), with first intake of progesterone

day defined as “P+0”. The endometrial biopsy is obtained on “P+5”, after a full 5 days of progesterone intake, equating to 120 hours of progesterone exposure.

The endometrial biopsy procedure involves the extraction of a small piece of endometrial tissue (70mg) from the uterine fundus with the use of a Pipelle catheter or similar. The endometrial biopsy sample is immediately transferred to a cryotube containing 1.5ml of RNAlater (Qiagen), vigorously shaken for 10 seconds, and kept at 4-8°C for a minimum of 4 hours. Specimens are then shipped at room temperature to Igenomix (Valencia, Spain) for analysis.

2.5.3 ERA laboratory protocol and results

The ERA molecular diagnostic tool currently utilizes NGS to analyze the expression levels of 248 genes related to the status of endometrial receptivity. RNA extraction from the tissue is performed using the RNeasy mini kit (Qiagen). All samples are DNAse treated, and cDNA is obtained by retrotranscription and analyzed by targeted RNA-Seq assay on IonTorrent NGS for 248 ERA genes in an Ion S5 system. Sequencing files are used as the input of the ERA predictor (Clemente-Ciscar et al., 2018) to quantify the expression of the ERA genes and to assess the endometrial receptivity status of each sample. Briefly, the reads are mapped to the hg19 human genome transcriptome using the STAR read aligner (Dobin et al., 2013). To count the number of reads that could be assigned to each gene, the HTSeq tool (Anders et al., 2015) with the union option is used. The ERA gene counts are used by the prediction model to classify each sample in an endometrial receptivity class as proliferative, pre-receptive, receptive, or post-receptive.

The various result profiles yielding an informative result include receptive, early receptive, late receptive, pre-receptive 1 day, pre-receptive 2 days, and post-receptive. A receptive result

indicates that the gene expression profile is concordant with a normal receptive endometrium. Embryo transfer will be recommended to be performed at the same timing at which the biopsy was taken. Early and late receptive results are both referred to as transition phases, where the endometrial transcriptomic profile is in a transition period between a non-receptive phase (either pre-receptive or post-receptive) and the receptive phase. In these instances, the WOI is either delayed by 12 hours (as in the case of an early receptive result) or it is advanced by 12 hours (as in the case of a late receptive result), the recommendation will be to transfer 12 hours later or 12 hours earlier, respectively. A pre-receptive result corresponds to a gene expression profile that is concordant with an endometrium at that has not yet reached the WOI. This profile can be further classified as pre-receptive 1 day, where the WOI is delayed by 24 hours or pre-receptive 2 days, where the WOI is delayed by 48 hours. Lastly, a post-receptive result corresponds to a gene expression profiles that is concordant with an endometrium that has already passed the WOI, usually by 24 hours. At this time, a direct recommendation for transfer can be made based upon all the informative profiles, except for pre-receptive 2 day and post-receptive profiles, where instead a second biopsy will be requested to validate the corresponding prediction of receptivity.

Less commonly, non-informative results are obtained with the ERA. These include proliferative, invalid RNA, insufficient RNA, and non-informative profiles. In the case of a proliferative result, the gene expression profile is concordant with an endometrium that is still within the proliferative phase, that has not yet been exposed to progesterone. An invalid RNA result specifies that it was not possible to determine the gene expression profile of the sample due to poor quality of genetic material obtained, whereas an insufficient RNA result specifies that it was

not possible to determine the gene expression profile of the sample because there was not enough biopsy material present. A non-informative result is associated with a profile that does not match the reference gene expression profiles in the ERA predictor. In all these cases, a new biopsy will be requested in order to obtain a valid and informative result.

2.5.4 pET and outcomes

A pET was carried out in a subsequent FET cycle. The endometrial preparation protocol used in the ERA mock cycle was replicated and timing of transfer was guided by the ERA recommendation. All GC-pETs consisted of a single frozen blastocyst. Clinical outcomes pertaining to the first pET in terms of pregnancy rate, implantation rate, and ongoing pregnancy were assessed. Pregnancy rates were based on βhCG results following transfer. Implantation rates were calculated dividing the total number of gestational sacs present by the total number of embryos transferred, which in this case was 1 as all patients underwent a SET. Ongoing pregnancy rates were defined as those pregnancies that persisted with positive identification of gestational sac(s) after 12 weeks of gestation.

3.0 Preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm (specific aim a).

For this specific aim, the following published works are presented:

Taylor TH, Stankewicz T, Katz SL, Patrick JL, Johnson L, Griffin DK. Preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm. Zygote. 2020 Apr;28(2):93-96.

My personal contributions to this work are collecting oocytes, performing ICSI, culturing resulting embryos, performing assisted hatching on embryos, performing trophectoderm biopsies, collecting PGT-A result data, reviewing drafts of the manuscript, and giving an oral presentation of the data at Preimplantation Genetic Diagnosis International Society (PGDIS) Conference 2015.

Stankewicz-McKinney TL, Taylor TH, Glassner MJ, Orris JJ, Brasile DR, Griffin DK. 2015. Preliminary assessment of aneuploidy rates between the polar, mid and mural trophectoderm. PGDIS Chicago 2015. Oral presentation.

3.1 Summary of this Chapter

The objective of this study was to compare aneuploidy rates between three distinct areas of the human trophectoderm: mural, polar and a region in between these two locations termed the 'mid' trophectoderm. This is a cohort study on IVF patients undergoing comprehensive chromosome screening at the blastocyst stage at a private IVF clinic. All embryos underwent assisted hatching on day 3 with blastocyst biopsy and comprehensive chromosome screening. Biopsied blastocysts were divided into three groups depending on which area (polar, mid, or mural) of the trophectoderm was protruding from the zona pellucida and biopsied. Aneuploidy rates were significantly higher with cells from the polar region of the trophectoderm (56.2%) compared with cells removed from the mural region of the trophectoderm (30.0%; P = 0.0243). A comparison of all three areas combined also showed a decreasing trend, but this did not reach clinical significance, polar (56.2%), mid (47.4%) and mural trophectoderm (30.0%; P = 0.1859). The non-concordance demonstrated between polar and mural trophectoderm can be attributed to biological occurrences including chromosomal mosaicism or procedural differences between embryologists.

3.2 Chapter Introduction

As pointed out in section 1.2.3.3.5.1.1, aneuploidy refers to the presence or absence of whole chromosomal abnormalities. For a euploid live birth to occur, chromosomes must divide equally in the developing fetus. Any abnormal division during development can have disastrous

downstream effects (reviewed further in section 1.2.3.3.5.1.1). Therefore, PGT-A has been created to test for aneuploidy prior to implantation, thereby allowing the transfer of euploid embryos. The benefits of transferring euploid embryos have demonstrated (see section 1.3.2.4.3), though previous studies are limited to good prognosis patients or are not based on 'intent to treat'. More recent research has demonstrated that embryos diagnosed as mosaic and even embryos diagnosed as aneuploid can produce live births (Munné et al., 2017; Patrizio et al., 2019).

As cited in section 1.2.3.2, the blastocyst represents the first stage of differentiation in preimplantation development. The blastocyst differentiates into the inner cell mass (ICM), which will become the fetus and the trophectoderm, which will become the placenta. The trophectoderm itself is subdivided into two areas based on the location of the ICM: the mural trophectoderm, the area furthest away from the ICM and the polar trophectoderm, the area adjacent to the ICM. Typically, during PGT, cells are removed from the mural trophectoderm to not expose the ICM to the damage caused by the laser (Taylor et al., 2014a). However, blastocyst biopsy is not standardized, and this lack of standardization can lead to inter- and intra-differences with embryologists in terms of the area biopsied. It has been suggested that ploidy is consistent throughout the trophectoderm (i.e., that all cells have the same karyotype) (Northrop et al., 2010; Capalbo et al., 2013). Therefore, cells removed from the mural trophectoderm should mirror the chromosome content of the remaining cells.

To test this hypothesis, the following sub-aims were as follows:

ai. To biopsy 166 blastocysts, taking samples one of the following TE regions: mural TE (n = 40), polar TE (n=48), or a region in between these two locations termed the mid TE (n=78).

aii. To analyze all 166 TE samples for PGT-A with NGS.

aiii. To perform statistical analysis with chi-squared test to compare aneuploidy rates between all three regions, thereby testing the hypothesis that they differ.

3.3 Materials and Methods

Refer to section 2.1 for more detail regarding materials and methods used for this study.

3.4 Results of this Chapter

In total, 166 blastocysts were biopsied, 48 from the polar trophectoderm, 78 from the mid trophectoderm and 40 from the mural trophectoderm. There was no significant difference in maternal age between the three groups, i.e., 35.8 ± 4.9 years, 34.9 ± 4.4 years, and 35.2 ± 5.1 years, for the polar, mid and mural trophectoderm, biopsied groups respectively (Table 8; P = 0.8024). Aneuploidy rates were 27/48 in the polar trophectoderm group (56.2%), 37/78 in the mid trophectoderm group (47.4%) and 12/40 in the mural trophectoderm group (30.0%; Table 8; P = 0.1859). In a direct comparison between mural and polar trophectoderm, aneuploidy rates were significantly higher (Table 9; P = 0.0243).

Table 8. A comparison of aneuploidy rates between the polar, mid and mural trophectoderm.

	Polar	Mid	Mural	P-value
Average age (years)	35.8 ± 4.9	34.9 ± 4.4	35.2 ± 5.1	0.8024[a]
No. blastocysts	48	78	40	0.1859[b]
No. aneuploid	27 (56.2%)	37 (47.4%)	12 (30.0%)	

[a]Kruskal-Wallis test; [b]chi-squared test.
Prepared in collaboration with Tyl Taylor.

Table 9. A comparison of aneuploidy rates between polar and mural trophectoderm.

	Polar	Mural	P-value
Average age (years)	35.8 ± 4.9	35.2 ± 5.1	0.8417[a]
No. blastocysts	48	40	0.0243[b]
No. aneuploid	27 (56.2%)	12 (30.0%)	

[a]Kruskal-Wallis test; [b]chi-squared test.
Prepared in collaboration with Tyl Taylor.

3.5 Chapter Discussion

The hypothesis that aneuploidy is evenly distributed throughout the trophectoderm cannot be supported by this study. Aneuploidy rates were significantly higher when cells were taken from the polar region of the trophectoderm (56.2%) compared with cells removed from the mural region of the trophectoderm (30.0%; Table 9). These data also demonstrated a strong trend in decreasing aneuploidy from the polar (56.2%), mid (47.4%) and mural trophectoderm (30.0%; Figure 23). The non-concordance demonstrated between polar and mural trophectoderm can be attributed to biological occurrences or procedural differences.

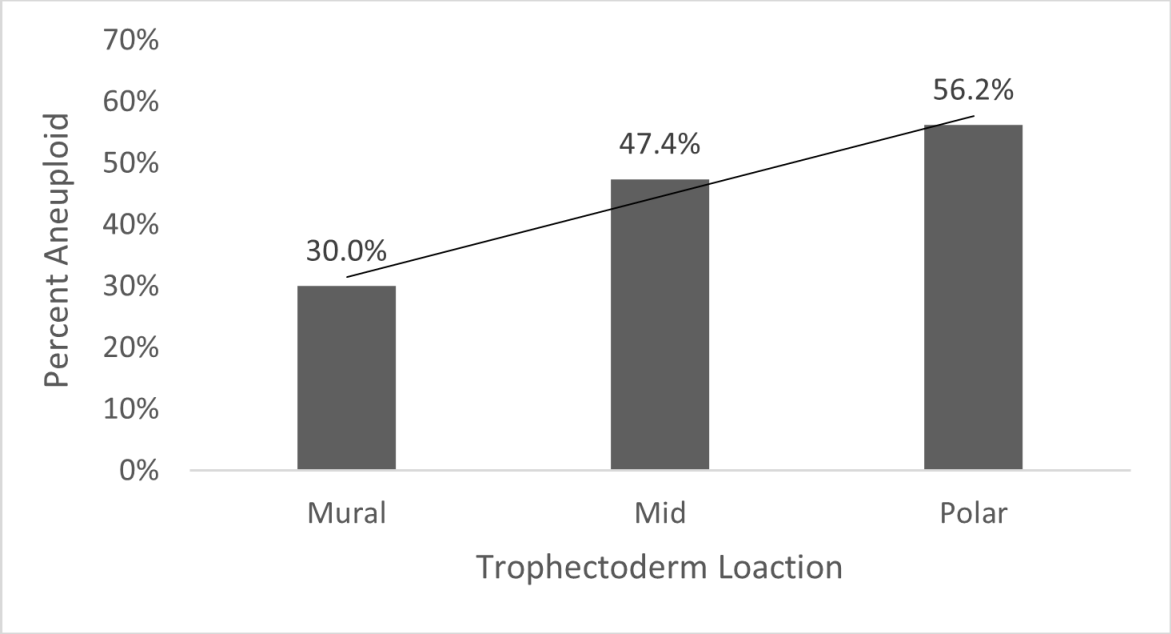


Figure 20. Aneuploidy rates between polar, mid, and mural trophectoderm. An increasing trend in percent aneuploidy observed from the mural trophectoderm to the mid trophectoderm to the polar trophectoderm.

Biologically, Hogan and Tilly (1978) dissected mouse ICM from the trophectoderm and left the ICM in culture. Within 5 days, some of the individual ICMs had the appearance of a blastocyst. Moreover, the individual ICMs derived trophoblast giant cells. These studies suggested that cells from the ICM feed the trophectoderm. It is unknown if this mechanism is present in human embryos, however if it were this could explain these data. If the ICM were mosaic and contained equal proportions of aneuploid and euploid cells, then aneuploid cells would feed into the trophectoderm at the same rate as euploid cells. Once in the trophectoderm, the euploid cells would proliferate at a faster rate than aneuploid cells (Ruangvutilert et al., 2000). Therefore, the blastocyst could have a higher proportion of aneuploid cells in the polar region compared with the mural trophectoderm, which these data support (Figure 24). Conversely, this theory would suggest that the blastocyst may be able to allocate aneuploid cells to the trophectoderm,

thereby correcting its chromosome state by the elimination of aneuploid cells from the ICM. Research using FISH and array-based techniques have found no evidence of this correction mechanism in place for human blastocysts (Evsikov and Verlinsky, 1998; Johnson et al., 2000; Derhaag et al., 2003; Fragouli et al., 2008; Northrop et al., 2010).

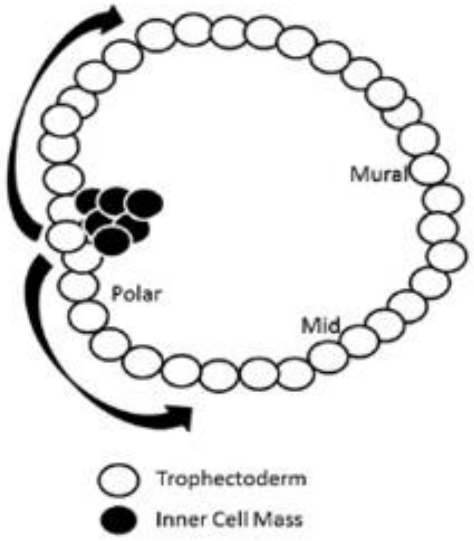


Figure 21. Direction of cellular migration from the inner cell mass out into the trophectoderm. Courtesy of Tyl Taylor.

Another biological reason for the discrepancy between regions of the trophectoderm could be the blastocyst preparing for implantation. During implantation, the blastocyst embeds itself with the ICM (polar trophectoderm) against the uterine wall. To invade the uterine wall, the cytotrophoblasts, which are located in the polar region, have been shown to induce aneuploidy (Weier et al., 2005). These data suggest that aneuploidy is higher in the polar region, possibly because the embryo is undergoing chromosomal changes to prepare for implantation. Unfortunately, this study did not examine implantation rates between the three different

categories, so it is unknown if aneuploidy in the polar region is detrimental. However, transfers of 'aneuploid' or mosaic blastocysts have resulted in euploid live births suggesting that some aneuploidy and mosaicism may not be clinically significant (Scott et al., 2012; Taylor et al., 2014b; Greco et al., 2015; Munné et al., 2017; Patrizio et al., 2019). Both of these biological occurrences suggest that mosaicism is a common phenomenon within the human blastocyst (Taylor et al., 2014b).

The published literature is currently lacking in terms of the effects of the biopsy procedure on the outcomes of PGT cycles. For example, in this study, the embryologist has to biopsy from the mural trophectoderm. Because of its proximity to the ICM, it is possible that some ICM cells were removed with the trophectoderm during the biopsy. Unfortunately, the level of contamination between the ICM and trophectoderm during the biopsy is unknown. However, this may not affect the PGT result, as research has indicated a high concordance between the two regions (Johnson et al., 2000; Capalbo et al., 2013). Interestingly, with the advent of NGS and its increase in the detection of mosaicism, the biopsy procedure has become a variable. If embryologist 'A' biopsies two cells from the blastocyst and both are normal or abnormal, mosaicism will not be detected. However, if embryologist 'B' biopsies 10 cells from the blastocyst and six cells are aneuploid and four cells are euploid, mosaicism will be detected simply due to the increased number of cells biopsied. Research has also suggested that the majority of abnormalities at the blastocyst stage are mitotic in origin, suggesting that with enough cells present, PGT results could be altered (McCoy et al., 2015).

Ideally, one should biopsy from the polar, mid and mural trophectoderm from a single blastocyst; however, this was not possible because these were patients undergoing IVF and not

blastocysts donated to research. Northrop et al. (2010) examined three separate trophectoderm sections from the same blastocyst and demonstrated a concordance rate of 80% (40/50 blastocysts), but this study did not record the location of the trophectoderm samples in relation to the ICM. Another limitation was performing AH on day 3. AH allows for premature hatching which may disrupt the true chromosomal makeup within the embryo or influence cell distribution. It is possible that the heat generated from the laser could disrupt cell junctions and affect further embryological development, possibly allowing for the premature expulsion of cells (White et al., 2018). However, research in the mouse demonstrates that embryos hatch equally from the polar, mid and mural trophectoderm, suggesting a limited effect on the AH procedure (Schimmel et al., 2014). Our data are similar, for the 166 blastocysts there was no difference between which area (polar, mid, or mural) hatched out of the blastocyst, 37.8%, 30.7% and 31.5%, respectively (P = NS). Further research is needed whereby AH is not performed and blastocysts are not exposed to the laser until biopsy, at day 5 or 6.

Most of the research with mosaicism at the blastocyst stage deals with the reanalysis of aCGH samples or the mixing of known cell lines to determine the per cent mosaicism present in the entire blastocyst (Ruttanajit et al., 2016). The only way we can understand aneuploidy and blastocyst morphology is to isolate individual cells within the blastocyst and effectively 'map' the cells, creating a virtual image of the blastocyst (Taylor et al., 2016). This study has already been performed and, although the cost was prohibitive, larger studies are certainly warranted.

In conclusion, these data do not support the hypothesis that aneuploidy is evenly distributed throughout the trophectoderm. This study adds to the pool of data that may help patients and

clinicians understand why some embryos diagnosed as ‘euploid’ fail to implant. Further research is needed to better understand aneuploidy at the blastocyst stage and its clinical consequences.

4.0 Improved pregnancy rates following endometrial receptivity analysis and personalized embryo transfer in patients with previous failed implantation after euploid embryo transfer (specific aim b)

For this specific aim, the following published works are presented:

Leondires M, Akopians AL, Stankewicz T, Gomez E, Snider A, Harton G, Valbuena D, Simón C. 2018. Improved pregnancy rates following endometrial receptivity analysis and personalized embryo transfer in patients with previous failed implantation after euploid embryo transfer. ESHRE, Barcelona, Spain. P-480.

My personal contributions to this work are writing and maintaining the IRB study protocol, training clinicians in the study protocol, reviewing eligibility for recruited patient subjects, collecting data, analyzing data, writing, and submitting the above abstract to the European Society for Human Reproduction and Embryology Conference (ESHRE), preparing the accepted poster presentation for ESHRE 2018 conference, writing the manuscript.

Manuscript in progress:

Stankewicz T, Leondires M, Akopians AL, Nakhuda G. Improved pregnancy rates following endometrial receptivity analysis and personalized embryo transfer in patients with previous failed implantation after euploid embryo transfer. In preparation to submit to RBMO. Submission expected January 2022.

4.1 Summary of this Chapter

The objective of this study was to determine the additional value of using a combination of ERA testing and aneuploidy screening in infertile patients with at least one failed implantation after the FET of an euploid embryo analyzed by PGT-A. We performed a prospective pilot study in three independent reproductive centers from December 2016 to May 2018. Twenty-six patients underwent ERA testing in a mock cycle replicating the previous failed euploid FET cycle(s), with endometrial biopsy occurring at approximately the same progesterone exposure time as the previous ET(s). The reproductive outcomes following transfer of PGT-A-tested embryos in a pET cycle, versus euploid FET controls were assessed as well as the percentages of displacements of the WOI diagnosed by ERA in this patient population. The ERA test revealed a displaced WOI in 17 out of 26 patients (65.4%) and a standard WOI in 9 patients (34.6%). Clinical follow up was possible for 25 patients: 88% (22/25) had a positive pregnancy test (βhCG); overall implantation rate was 62.96% (17/27); and overall live birth rate was 48% (12/25). Being that all previous clinical rates were 0% in the previous euploid transfer cycles, the associated pET outcomes achieved were statistically higher. Our findings demonstrates that a large proportion of patients with previous failed transfer of euploid embryo(s) have a displaced WOI and may benefit from ERA testing to guide a pET. When utilizing this two-tiered approach of PGT-A plus ERA, we observed improved implantation and overall reproductive success, offering both physicians and patients subsequent treatment options when aneuploidy screening alone does not suffice to achieve implantation.

4.2 Introduction to Chapter

As stated in section 1.2.3.3, successful implantation following IVF requires the transfer of a good quality blastocyst to a receptive endometrium. For patients who experience implantation failure, reproductive endocrinologists may consider genetic testing to guide further treatment in order to achieve pregnancy. In many cases, this is accomplished through PGT-A and subsequent selection and transfer of euploid embryos with a normal number of chromosomes. The negative impacts of aneuploidy on reproductive outcomes along with the benefits of performing PGT-A to permit for the transfer of known euploid embryos have been reviewed in previous section 1.3.2.4.3. To note, chromosomally abnormal embryos are often indistinguishable from chromosomally normal embryos when studied using a microscope (Sandalinas *et al.*, 2001; Munné, 2006; Fesahat *et al.*, 2017), therefore, embryo morphology alone cannot be used to evaluate chromosome abnormalities.

Despite the transfer of a good quality euploid blastocyst, implantation failure still occurs in approximately one third of these cycles (Fragouli *et al.*, 2010; Yang *et al.*, 2012; Forman *et al.*, 2013). Following an unsuccessful euploid embryo transfer, it is imperative to explore other sources of implantation failure. Besides embryo aneuploidy the other most likely candidate implicated is alteration/desynchrony of endometrial receptivity. For some women experiencing implantation failure, infertility is caused by a uterus that is not receptive to embryo implantation at the typical time of transfer in an IVF cycle. Endometrial gene expression signatures permit reproductive endocrinologists to diagnose a woman's endometrial receptivity status as another reproductive health parameter. This diagnosis is now offered through commercial tests such as the ERA (see section 1.3.2.5.4.3.1).

The efficacy of ERA in patients with RIF (section 1.3.2.5.4.3.1.4) along with the utility of the test in the good prognosis patient population have been established (section 1.3.2.5.4.3.1.5). Additionally, a retrospective study (Tan et al., 2018) showed benefit of ERA for patients with previous failed euploid transfers, however, results from a prospective study, clearly demonstrating the advantage of PGT-A combined with ERA, have not yet been published.

To determine the value of applying a combination of ERA testing and aneuploidy screening in patients undergoing IVF, the sub-aims of this chapter were as follows:

bi. To select patients for study having no previous successful pregnancy and with at least one failed implantation after the FET of an euploid embryo analyzed by PGT-A.

bii. To conduct an ERA mock cycle, replicating the same HRT protocol employed in the previous failed euploid FET, and perform endometrial biopsy at same progesterone exposure timing that the previous failed transfer took place.

biii. To analyze the transcriptomic profiles of the endometrial biopsy samples with ERA to diagnose endometrial receptivity timing.

biv. To transfer one or two chromosomally normal blastocysts in a pET cycle guided by the ERA results.

bv. To perform statistical analysis to compare reproductive outcomes following transfer of PGT-A-tested embryos in a pET cycle, versus previous euploid FET(s), thereby testing the hypothesis that controlling for both embryo aneuploidy and endometrial receptivity in an IVF transfer will lead to improved outcomes.

4.3 Materials and Methods

Refer to section 2.2 for more detail regarding materials and methods used for this study.

4.4 Results of this Chapter

Patients' baseline clinical and COS cycle characteristics from where the embryos came from are summarized in Table 10. Age average was 34.69 ± 3.07 and BMI 22.67 ± 3.43, 23 patients had regular menstrual cycles, and all presented normal ovarian reserve, 12.83 ± 6.86 MII oocytes were extracted and 5.54 ± 2.32 PGT-A analyzed embryos were vitrified.

Table 10. Demographic, clinical and COS patient characteristics at baseline.*

Clinical and COS characteristics		mean + SD or nº (%)
Age (y)		34.69 ± 3.07
BMI		22.67 ± 3.43
Ethnicity (%)	Asian	5 (19.2)
	Caucasian	15 (57.7)
	Hispanic/Latino	1 (3.8)
	Middle Eastern	3 (11.5)
	South Asian	2 (7.7)
Regular menstrual cycles (%)		23 (88.5)
FSH (mIU/mL)		6.79 ± 2.51
AMH (ng/mL)		3.14 ± 2.98
Nº of previous pregnancies		0.58 ±1.03
Nº of previous IVF failed		2.31 ± 2.36
Nº of previous failures with PGS		1.35 ± 0.69
Sperm concentration (million/mL)		55.10 ± 63.28
FSH doses (UI)		2479.17 ± 1059.45
Nº of Antral Follicles		16.33 ± 8.54
Ovulation induction (%)	Analog	3 (11.5)
	Analog + hCG	3 (11.5)
	hCG	18 (69.2)
Nº of Oocytes MII		12.83 ± 6.86
Nº of fertilized oocytes		9.62 ± 5.33
Nº of embryos PGT-A analyzed		5.68 ± 2.32
Nº of embryos vitrified		5.54 ± 2.32

*Data are expressed as mean ± SD or n (%). Descriptive analysis of quantitative and qualitative variables was done.
§The body-mass index is the weight in kilograms divided by the square of the height in meters.
Prepared in collaboration with Diana Valbuena.

4.4.1 ERA Results

Twenty-six patients with at least one failed euploid embryo transfer, underwent endometrial biopsy and ERA testing in an HRT cycle replicating the protocol employed in the failed euploid transfer cycle. The ERA test yielded a displaced WOI in 17 out of 26 patients (65.4%) and a standard WOI in 9 patients (34.6%). The group of patients with displaced WOI were further classified by the ERA predictor, indicating the specific endometrial stage. Eight patients (30.8%) were found to be pre-receptive by 24 hours, and 5 patients more than 24 hours pre-receptive (19.2%), both indicating the need for additional progesterone exposure prior to transfer. Three patients (11.5%) were considered to have a transitional profile between pre-receptive and receptive, referred to early receptive, requiring only an additional 12 hours of progesterone exposure prior to transfer. Only one patient (3.8%) was reported to have a post-receptive result, with a recommendation to perform another biopsy with 24 hours less progesterone administration than the previous transfer/biopsy (Figure 25).

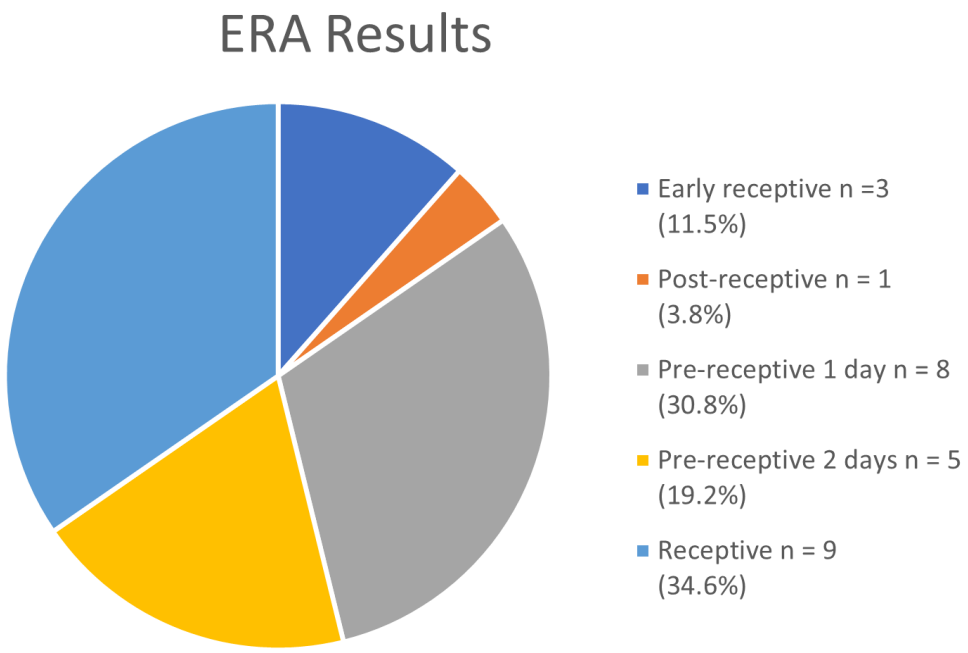


Figure 22. Summary of ERA results. Pre-receptive 2 days: 5 cases (19.2%) 145-168 hours of progesterone recommended by ERA, Pre-receptive: 8 cases (30.8%) 133-144 hours of progesterone recommended by ERA, early receptive: 3 cases (11.5%) 127-132 hours of progesterone recommended by ERA, receptive: 9 cases (34.6%) 115-126 hours of progesterone recommended by ERA, late receptive: No cases (0%) 109-114 hours of progesterone recommended by ERA, post-receptive: 1 case (3.8%) 96-108 hours of progesterone recommended by ERA.

4.4.2 HRT cycle characteristics

HRT cycle details for failed ET, ERA and pET are explained in Table 11. Total days of estradiol treatment, nor endogenous progesterone value before its supplementation showed differences among the cycles. The route of progesterone used was similar in all cycles. Total hours of progesterone did not match among the failed ET (118.83 ± 5.21), the ERA cycle (121.62 ± 6.79), the ERA recommendation (133.31 ± 12.78) and the pET cycle (135.12 ± 10.46). Although it was thought the ERA cycle would be repeated exactly the same as the failed ET cycle, the total hours of progesterone intake were significantly lower than the ERA cycle (p value 0.04). Also, the

timing of progesterone supplementation was shorter in the ERA cycle than the recommendation of the results (p value <0.0001). As was expected, the progesterone intake was lower in the ET cycle versus the pET cycle (p value <0.0001). Finally, similar amount of progesterone hours recommended by ERA were used in the pET cycle (p value 0.55), this last comparison shows the fact that pET accurately replicated the ERA recommendations (Table 11).

Table 11. HRT cycle characteristics in failed embryo transfer (ET), ERA biopsy and personalized embryo transfer (pET).*

	Transfer failed cycle ET (n=26)	ERA cycle (n=26)	ERA Recommendation (n=26)	pET cycle (n=25)	ET vs ERA		ERA vs Recommendation		ET vs Recommendation		ET vs pET		Recommendation vs pET		ERA vs pET	
					p-value	Mean difference /Relative risk (95% CI)	p-value	Mean difference /Relative risk (95% CI)	p-value	Mean difference /Relative risk (95% CI)	p-value	Mean difference /Relative risk (95% CI)	p-value	Mean difference /Relative risk (95% CI)	p-value	Mean difference /Relative risk (95% CI)
Days of Estradiol	17.39 ± 3.79	19.15 ± 4.07		20.16 ± 5.76	0.084	-1.81 (-2.14 to 0.14)					0.02	-2.54 (-3.80 to -0.38)			0.21	-1.29 (-2.60 to 0.60)
Endo P4 Value before P4 administration (ng/mL)	0.44 ± 0.31	0.39 ± 0.27		0.40 ± 0.29	0.19	1.38 (-0.05 to 0.22)					0.49	0.72 (-0.12 to 0.23)			0.97	-0.04 (-0.12 to 0.11)
Total hours P4	118.83 ± 5.21	121.62 ± 6.79	133.31 ± 12.78	135.12 ± 10.46	0.043	-2.15 (-5.90 to -0.10)	<0.0001	-4.40 (-17.17 to -6.22)	<0.0001	-4.28 (-19.36 to -6.72)	<0.0001	-5.54 (-20.82 to -9.45)	0.55	-0.61 (-1.22 to 0.66)	<0.0001	-5.78 (-18.24 to -8.64)
P4 route of administration																
Vaginal	5 (19.2)	2 (7.7)		2 (8)	0.42	1.53 (0.87 to 2.69)					0.42	1.50 (0.8 to 2.62)			1	0.98 (0.35 to 2.71)
IM	0	3 (11.5)		2 (8)	0.23	0 (infinity)					0.23	0 (Infinity)			1	1.2 (0.55 to 2.60)
IM/vaginal	19 (73.1)	20 (76.9)		21 (84)	1	0.90 (0.50 to 1.64)					0.50	0.74 (0.42 to 1.30)			0.73	0.81 (0.45 to 1.47)
Unknown	2 (7.7)	1 (3.8)		0												

*Values are expressed as mean ± SD or n (%). Fisher's Exact test (two-sided) was used to compare these results. Mean difference /Relative risk (95% CI) was calculated using the approximation of Katz. Paired-Samples Student's t-test was applied when quantitative variables were compared; relative risk and 95% CI were calculated using Bonferroni and Dunnett T3 Post Hoc test. Prepared in collaboration with Diana Valbuena.

4.4.3 Embryo transfers data and clinical outcome

Every contemplated variable that reflects the ET cycles in both, the previous failed ET and the pET cycle were compared and showed in Table 12. There were no differences between the embryo day of the transfer, number of embryos transferred, embryo stage or quality. The information from the COS cycle is the same for both transfers because the pET embryos originated from the same initial oocyte retrieval cycle.

Table 12: Embryo transfer data in failed embryo transfer (ET) versus personalized embryo transfer (pET).*

		Failed embryo transfer ET (n=26)	Personalized embryo transfer pET (n=25)	p-value	Mean difference /Relative risk (95% CI)
Embryo transfer day	3	1 (3.8)			
	5	6 (23.1)	9 (36)	0.37	0.72 (0.36 to 1.43)
	6	18 (69.2)	11 (44)	0.09	1.71 (0.92 to 3.18)
	7				
	Unknown		0		
Number of embryos transferred		1.15 ± 0.46	1.08 ± 0.28	0.33	1 (-0.085 to 0.24)
Embryo stage	CB	1 (3.8)	5 (20)	0.09	0.3 (0.05 to 1.83)
	EB	6 (23.1)	4 (16)	0.73	1.23 (0.68 to 2.23)
	HingB	15 (57.6)	13 (52)	0.78	1.12 (0.65 to 1.94)
	HedB	2 (7.7)	3 (12)	0.67	0.77 (0.25 to 2.32)
	Unknown	2 (7.7)	0		
ICM quality	A	9 (34.6)	11 (44)	0.57	0.82 (0.46 to 1.47)
	B	11 (42.3)	9 (36)	0.78	1.14 (0.66 to 1.95)
	C	4 (15.4)	5 (20)	0.73	0.85 (0.39 to 1.86)
	Unknown	2 (7.7)	0		
TE quality	A	4 (15.4)	6 (24)	0.50	0.75 (0.33 to 1.68)
	B	14 (53.8)	12 (48)	0.78	1.12 (0.65 to 1.93)
	C	5 (19.2)	7 (28)	0.52	0.77 (0.37 to 1.61)
	Unknown	2 (7.7)	0		
Pregnancy rate		0	22/25 (88)		
Number of implanted embryos		0	17/27 (62.96)		
Pregnancy outcome					
	OGP	0	12/22 (54.55)		
	Biochemical pregnancy	0	5/22 (22.73)		
	Spontaneous abortion	0	5 /22 (22.73)		
	Live Birth rate/transfer	0	12/25 (48)		
	Gestational Age (Weeks)		39.25 +1.17		

*Values are expressed as mean ± SD or n (%). Fisher's Exact test (two-sided) was used to compare these results. Mean difference /Relative risk (95% CI) was calculated using the approximation of Katz. Paired-Samples Student's t-test was applied when quantitative variables were compared; relative risk and 95% CI were calculated using Bonferroni and Dunnett T3 Post Hoc tests. Prepared in collaboration with Diana Valbuena.

Clinical follow up pertaining to the first pET cycle after ERA testing, was possible in 25 out of the 26 patients. The patient who received a post-receptive result, left the participating clinic, and did not repeat biopsy to validate her pWOI to undergo a pET. For the patients who did undergo transfer, one or two previously vitrified, euploid blastocysts were warmed and transferred in an HRT cycle at the time designated as receptive by the ERA test. Twenty-two of the 25 patients (88%) had a positive pregnancy test (βhCG); pregnancy rate was significantly different between 7/9 (77.78%) patients who initially resulted as receptive and 15/16 (93.75%) patients who needed a progesterone timing correction according to their WOI displacement (p value 0.006). The overall implantation rate was 62.96% (17/27); 44.4% (4/9) for patients with standard WOI and 72.22% (13/18) for patients with a displaced WOI. An overall 48% (12/25) live birth rate was achieved; 44.4% (4/9) for patients with standard WOI and 50% (8/16) for patients with displaced WOI. Biochemical pregnancy rate and miscarriage rate were also recorded (Table 13). It is important to note that displaced patients presented a high rate of spontaneous abortions (33.33%), so the live birth rate was similar between the two groups.

Table 13: Embryo transfer data in pre-receptive versus receptive patients.*

		Pre-receptive patients (n=16)	Receptive patients (n=9)	p-value	Mean difference /Relative risk (95% CI)
Days of Estradiol		21.81 ± 5.91	17.22 ± 4.35	0.05	2.03 (-0.08 to 9.26)
Endo P4 Value before P4 administration (ng/mL)		0.34 ± 0.19	0.49 ± 0.42	0.39	0.89 (-0.50 to 0.21)
Total hours P4 for pET		141.81 ± 6.12	123.22 ± 3.07	0.0001	8.47 (14.05 to 23.13)
Total hours P4 for failed ET		117.08 ± 6.33	121.22 ±1.72		
Total hours P4 for ERA		121.23 ± 9.56	122.89 ± 1.96		
Embryo transfer day	5	6 /16 (37.5)	3/9 (33.3)	1	1.07 (0.59 to 1.94)
	6	5/16 (31.3)	6/9 (66.7)	0.12	0.58 (0.29 to 1.17)
	7	5/16 (31.3)	0		
Number of embryos transferred		1.13 ± 0.34	1 ± 0	0.29	1.09 (-0.11 to 0.36)
Embryo stage	CB	4/16 (25)	1/9 (11.1)	0.62	1.33 (0.76 to 2.35)
	EB	3/16 (18.8)	1/9 (11.1)	1	1.21 (0.63 to 2.34)
	HingB	7/16 (43.8)	6/9 (66.7)	0.41	0.72 (0.39 to 1.31)
	HedB	2/16 (12.5)	1/9 (11.1)	1	1.05 (0.44 to 2.48)
ICM quality	A	7/16 (43.8)	4/9 (44.4)	1	0.99 (0.55 to 1.79)
	B	5/16 (31.3)	4/9 (44.4)	0.67	0.81 (0.41 to 1.58)
	C	4/16 (25)	1/9 (11.1)	0.62	1.33 (0.76 to 2.35)
TE quality	A	3/16 (18.8)	3/9 (33.3)	0.63	0.73 (0.31 to 1.72)
	B	8/16 (50)	4/9 (44.4)	1	1.08 (0.60 to 1.95)
	C	5/16 (31.3)	2/9 (22.2)	1	1.17 (0.64 to 2.12)
Pregnancy rate		15 (93.75)	7 (77.78)	0.006	6.82 (1.04 to 44.76)
Implantation rate		13/18 (72.22)	4/9 (44.4)	0.22	1.53 (0.78 to 3)
Pregnancy outcome	OGP	8/15 (53.33)	4/7 (57.14)	1	0.95 (0.54 to 1.68)
	Biochemical pregnancy	2/15 (13.33)	3/7 (42.86)	0.27	0.52 (0.17 to 1.58)
	Spontaneous abortion	5/15 (33.33)	0		
Live Birth rate/transfer		8/16 (50)	4/9 (44.4)	1	1.08 (0.60 to 1.95)
Gestational Age (Weeks)		39.37 ± 0.91	39 ± 1.70	0.62	0.51 (-1.27 to 2.02)

*Values are expressed as mean ± SD or n (%). Fisher's Exact test (two-sided) was used to compare these results. Mean difference /Relative risk (95% CI) was calculated using the approximation of Katz. Independent-Samples Student's t-test was applied when quantitative variables were compared; relative risk and 95% CI were calculated using Bonferroni and Dunnett T3 Post Hoc tests. Prepared in collaboration with Diana Valbuena.

The question arises why results between displaced and non-displaced patients were not different, except by the pregnancy rate. Receptive patients had better results than their own previous failed cycle (PR = 0%), looking for the possible variables involved, we found that endometrial biopsy to diagnose the WOI was taken a little later than when the ET was performed (122.89 ± 1.96 and 121.22 ±1.72, respectively; p value 0.09), and pET was made even later than ERA (123.22 ± 3.07), in this last comparison a non-significant result was seen, possibly due to the increased hours of progesterone in pET cycle, and/or the small number of cases involved.

4.5 Chapter Discussion

In this study it was demonstrated that the utilization of ERA in patients with at least one previous failed euploid embryo transfer, showing that a high percentage (65.4%) of these patients had a displaced WOI at time of transfer. Traditionally, embryonic numerical chromosome abnormalities are the first potential source of implantation failure examined following IVF failure. However, even when an euploid embryo is transferred, successful implantation may not be obtained, or a miscarriage can occur, a very frustrating scenario for patients and physicians. For this subset of patients, other reasons for failed implantation or miscarriage must be explored. One example would be the uterine microbiome, since it has been recently reported that receptive patients could have a significant decrease in implantation, pregnancy rate, and live birth rate when a non-*Lactobacillus*-dominated microbiota is detected (Moreno *et al.*, 2016). On the other hand, a study reported that 66% of RIF patients were diagnosed with chronic endometritis and realized increased pregnancy and live birth rates following appropriate

treatment (Cicinelli *et al.*, 2015). These reasons could provide a clue to as why we faced a 33.33% miscarriage rate when displaced patients were corrected in the present study.

In this study we observed a high rate of displaced WOI in patients with at least one failed euploid embryo transfer, indicating a significant endometrial contribution to past implantation failure(s). To establish a WOI displacement as a potential cause of previous implantation failure, the ERA test was performed during a mock cycle that replicated the failed transfer HRT cycle protocol, with endometrial biopsy taken on the same progesterone exposure day (i.e., P+5 day) as the previous transfer. It is probable that the high displaced percentage could be the source of prior implantation failure. WOI displacement in other patient subgroups have been previously studied, Ruiz-Alonso and colleagues examined non-receptivity in patients with RIF, reporting that approximately 1 in 4 of these patients were non-receptive at the standard time of embryo transfer (Ruiz-Alonso *et al.*, 2013). It is important to make note that in this earlier study, embryo aneuploidy was not controlled for, meaning that ploidy status of the embryos transferred was unknown, owning to another potential cause for implantation failure. This fact likely explains the reason for increased non-receptivity observed in our study, since embryonic aneuploidy, and not necessarily endometrial factor, may have been a significant cause of implantation failure in the earlier study. To further support this idea, it is imperative to emphasize the considerable contribution that embryo aneuploidy has on poor reproductive clinical outcome and alternatively, the improvement of clinical outcome with euploid embryo transfer (Kahraman *et al.*, 2003; Munné *et al.*, 2003; Rubio *et al.*, 2003; Rubio *et al.*, 2017). In another study, endometrial receptivity was examined in a population of patients at the first IVF appointment, without a history of RIF. Results of this RCT demonstrated that approximately one in three

patients have a displaced WOI at the typical time of blastocyst transfer (Simón *et al.*, 2020). By comparison, these results further highlight the role that endometrial factor plays in patients with implantation failure following euploid embryo transfer. The subset of patients enrolled in our study have the highest reported displaced WOI rate to date.

In this study, we demonstrated improved reproductive outcome with pET of euploid blastocysts, as guided by ERA. All patients enrolled in this study had never achieved a successful pregnancy, (PR = 0%) even following euploid transfer. Total pregnancy rate (both with standard and displaced WOI) after the first pET cycle was 88% which was shown to be significant comparing to their previous results (p= <0.0001).

When comparing reproductive outcomes between patients with and without WOI displacement, there was a significant difference in pregnancy rate (93.75%% vs 77.78%), however because of a high rate of spontaneous abortions there were no differences in other clinical outcomes. It is interesting to note a significant improvement in reproductive outcome in those patients found to be receptive, being that embryo transfer theoretically occurred at approximately the same time in the pET cycle as it did in the initial failed transfer cycle. A possible explanation is the improved preciseness in the number of hours of progesterone exposure, as indicated by the ERA results in the pET versus the previous failed transfer cycle. The ERA results are reported in number of hours and not days, so that in the pET cycle the transfer should occur within a 6-hour timeframe based on where the receptive phase is found. With this said, although transfer in the pET cycle occurred on the same "P+" day as in the failed cycle, the actual number of progesterone exposure hours differed between both cycles depending on actual progesterone start time and transfer time in each of the cycles. Differences in hours of progesterone exposure, may be more

consequential for those patients with a narrow WOI who possess a receptive phase lasting less than the expected 24-48 hours in human reproduction (Wilcox *et al.*, 1999). Hence, transferring according to progesterone exposure hours instead of progesterone exposure days, help ensure that the embryo is transferred into a receptive endometrium, leading to improved reproductive outcome as observed in not only the displaced WOI group but also the standard WOI group in our study.

Although it has been suggested that local injury to the endometrium during the ERA biopsy may help to increase implantation success in the subsequent month or the following two or three months, in our study the average time between biopsy and pET was 1.6 ± 1.03 months (range from one to five months). Moreover, in an earlier study from our group, we examined clinical results of the first pET in receptive patients up to 6 months following the ERA test. The pregnancy and implantation rates in the first month after biopsy were 36.4% and 23.8% and in the sixth month, 50.0% and 33.3%, clearly demonstrating no improvement in reproductive outcome the month following biopsy and therefore showing no benefit of the endometrial scratch itself (Ruiz-Alonso *et al.*, 2013). Likewise, this has been repeated in the most recent RCT, in which patients had their pET up to 9 months after their ERA biopsy and pregnancy rate and live birth rate were not modified (Simón *et al.*, 2020). There is not enough evidence for using endometrial scratching before IVF to improve reproductive outcome and its beneficial effect was concluded to be doubtful by at least 6 meta-analysis (Vitagliano *et al.*, 2018; Nastri *et al.*, 2012; Potdar *et al.*, 2012; El-Toukhy *et al.*, 2012; Nastri *et al.*, 2015; Panagiotopoulou *et al.*, 2015), many opinion reviews (Simón and Bellver 2014; Ko *et al.*, 2016; Zygula *et al.*, 2016; Santamaría *et al.*, 2016; Bellver and Simón, 2018) and hundreds of publications. Moreover, the same group

who initially reported the beneficial effect of endometrial scratching on ART (Barash *et al.*, 2003), recently published a retrospective matched controlled study, concluding that the scratch did not improve implantation and pregnancy rates (Levin *et al.*, 2017), reflecting that endometrial scratching is an artifact and not a true mechanism to improve embryo implantation.

Given our results, we have demonstrated that a significant proportion of patients with previous failed transfer of euploid embryo(s) have a displaced WOI and may benefit from ERA testing to guide a pET. When utilizing this two-tiered approach of PGT-A plus ERA, we observed improved implantation and overall reproductive success. These data can offer physicians and patients subsequent treatment options, in terms of combined ERA testing and pET, when aneuploidy screening alone does not suffice to achieve implantation. Currently our group is conducting a larger randomized study to validate the initial findings of this pilot study.

5.0 Are the clinical outcomes different in day 5 versus day 6 single embryo transfer when endometrial factor is controlled? (specific aim c)

For this specific aim, the following published works are presented:

Stankewicz T, Ruiz-Alonso M, Soler-Ibañez M, Simón C, Valbuena D. Are the clinical outcomes different in day 5 versus day 6 single embryo transfer when endometrial factor is controlled? – Published: November 17, 2021DOI:https://doi.org/10.1016/j.rbmo.2021.11.010

My personal contributions to this work are assisting with establishing and maintaining IRB approval for this study, collecting the clinical follow up data, analyzing data, writing the manuscript, submitting the manuscript to Reproductive BioMed Online for publication, and addressing all reviewers edits and resubmitting for final publication.

5.1 Summary of this Chapter

The objective of this study is to evaluate if there is a difference in clinical outcomes between day 5 versus day 6 blastocysts when controlling for endometrial receptivity by transferring in a pET cycle guided ERA. This is a multicenter, retrospective study involving 260 patients who underwent a SET with either a day 5 or day 6 blastocyst in a pET cycle between January 2017 and

December 2019. Of those embryos transferred, 183 (70.4%) were day 5 blastocysts and 77 (29.6%) were day 6 blastocysts. Clinical outcomes were comparable when transferring day 5 blastocysts versus day 6 blastocysts: pregnancy rate was 75.4% (138/183) and 70.1% (54/77) (P = 0.465), implantation rate was 67.8% (124/183) and 63.6% (49/77) (P = 0.476), and ongoing pregnancy rate was 57.9% (106/183) and 58.4% (45/77) (P = 0.728), respectively. Our data suggests that the clinical potential of day 5 and day 6 blastocysts are similar, as no difference in clinical outcomes are observed when transferring at the time of optimal endometrial receptivity as determined by transcriptomic analysis with ERA.

5.2 Introduction to Chapter

The field of ART has evolved considerably with realized advancements in stimulation protocols, enhanced embryo culture techniques, emergence of highly developed technologies such as PGT-A, and the introduction of personalized medicine, all of which contributing to improved patient outcomes. By far, one stage of the IVF process that has undergone the greatest transformation is the ET procedure. Progression from the transfer of cleavage stage embryos to blastocysts has resulted in significantly higher implantation rates (Cruz et al., 1999; Gardner et al., 1998a; Gardner et al., 1998b; Gorrill et al., 1999). A decrease in the number of embryos routinely replaced has reduced the incidence of multiples (Thurin et al., 2004) and the associated obstetrical and neonatal complications (Anvon et al., 2019; Conde-Agudelu et al., 2000; Meyer et al., 2018; Wu et al., 2019), while at the same time maintaining similar clinical rates (Kresowik et al., 2011; Ryan et al., 2007). Lastly, a shift in mindset from “fresh is best” to frozen is better

(Roque et al., 2019; Zhang et al., 2018), made possible in large part to improvements in embryo freezing protocols, specifically the transition from slow freezing to vitrification (Rienzi et al., 2017), translates to more patients than ever undergoing FETs. In summary, a typical IVF ET now consists of a single blastocyst, warmed, and transferred during an FET cycle.

As previously discussed in section 1.3.2.3, there are numerous advantages of FETs over fresh ETs including the opportunity to select an embryo for transfer from the entire cohort of blastocysts, not just what is available on day 5 of culture, the ability to add-on PGT-A testing, and improved synchrony between the transferred embryo and the endometrium, especially considering that in fresh cycles, ovarian hyperstimulation coupled with elevated estrogen levels can adversely affect the endometrium, leading to impaired receptivity (Ozgur et al., 2013; Shapiro et al., 2011; Shapiro et al., 2013; Shapiro et al., 2014).

With regard to endometrial receptivity, until recently, there has been no objective and reliable diagnostic test to accurately assess endometrial receptivity and provide reproducible results with a clear clinical directive. However, the implementation of personalized medicine to IVF has expanded our understanding of endometrial receptivity and introduced the concept of a pWOI. A prime example is ERA (see section 1.3.2.5.4.3.1).

To understand the clinical potential of day 5 versus day 6 blastocysts better when properly controlling for endometrial receptivity at time of transfer, the sub-aims were as follows

ci. To collect clinical outcome data from patients who transferred either a single day 5 or day 6 blastocyst in a pET cycle guided by ERA.

cii. To perform statistical analysis to compare reproductive outcomes following the transfer of day 5 versus day 6 blastocysts in a pET cycle, to test the hypothesis that the

clinical potential of day 5 and day 6 are similar when properly controlling for endometrial receptivity using transcriptomics.

5.3 Materials and Methods

Refer to section 2.3 for more detail regarding materials and methods used for this study.

5.4 Results of this Chapter

Two hundred and sixty patients underwent a single blastocyst transfer in a pET cycle guided by ERA between January 2017 and December 2019. The pET cycles were divided into two groups according to the developmental day of the blastocyst transferred: day 5 (n = 183) and day 6 (n = 77). HRT was the endometrial preparation protocol used for both the ERA mock cycles and subsequent pETs, with the exception of one patient in the day 5 blastocyst group who underwent a natural cycle determined by LH surge.

Table 14 summarizes all patient characteristics considered. The average patient age was slightly higher in the day 5 blastocyst group (37.6 ± 5.55) versus the day 6 blastocyst group (36.1 ± 4.7) (P = 0.035); however, BMI (23.9 ± 4.9 vs 24.3 ± 6; P = 0.716) and timing of endometrial receptivity and transfer were similar (125.74 ± 11.83 hours vs 129.29 ± 15.01 hours, P = 0.057). Number of previous failed cycles were also comparable between both groups: 27.7% (41/148) and 34.6% (18/52) with no previous failures, 25% (37/148) and 21.2% (11/52) with 1 previous

failure, 12.2 % (18/148) and 15.4% (8/52) with 2 previous failures, and 35.1% (52/148) and 28.8% (15/52) with 3 or more failures (P = 0.660).

In regard to the blastocysts transferred, embryo origin differed between each group, with a higher percentage of donor oocyte derived embryos in the day 5 blastocyst group (62/182; 34.1%) compared to the day 6 blastocysts group (11/77; 14.3%), and thus a higher percentage of autologously-derived embryos in the day 6 group (66/77; 85.7%) than in the day 5 group (120/182; 65.9%) (P = 0.001). The utilization of PGT-A testing was similar between the day 5 and day 6 groups, in which 60.1% (110/183) and 67.5% (52/77) of transfers involved an euploid embryo. A single mosaic transfer occurred in the day 6 group (1/77; 1.3%). The remaining transfers consisted of embryos of unknown ploidy status (73/183; 39.9% vs. 24/77; 31.2%; P = 0.124). Lastly, there was no significant difference in morphological quality of the embryos transferred as there were similar percentages of optimal (76/115; 66.1% vs. 39/68; 57.4%), medium (30/115; 26.1% vs 20/68; 29.4%) and suboptimal (9/115; 7.8% vs. 9/68; 13.2%) quality blastocysts in the day 5 and day 6 groups (P = 0.361= 0.361).

Table 14: Patient characteristics.

	Day of transferred embryos d5 n=183	Day of transferred embryos d6 n=77	p-value
	SET Day 5	SET Day 6	
Age	37.6 ± 5.55	36.08 ± 4.68	.035*
Body-Mass Index[a]	23.94 ± 4.96	24.32 ± 6.04	.716
No. previous failed cycles			
0	41 (27.7)	18 (34.6)	
1	37 (25)	11 (21.2)	
2	18 (12.2)	8 (15.4)	
≥3	52 (35.1)	15 (28.9)	.660
Timing with P4 HCG	125.74 ± 11.83	129.29 ± 15.01	.057
Embryo origin			
Ovum donation	62 (34.1)	11 (14.3)	
Own oocytes	120 (65.9)	66 (85.7)	.001*
PGT-A			
Euploid	110 (60.1)	52 (67.5)	
Mosaic	0 (0)	1 (1.3)	
Not analyzed	73 (39.9)	24 (31.2)	.124
Morphological embryo quality			
Optimal	76 (66.1)	39 (57.4)	
Medium	30 (26.1)	20 (29.4)	
Suboptimal	9 (7.8)	9 (13.2)	.361

Data are expressed as mean ± SD unless indicated otherwise.

[a] Body-mass index is the weight in kilograms divided by the square of the height in meters.

Student's t-test, Chi-Square and Fisher tests were used to compare quantity and categorical variables between groups. Significance was set at P <0.05.

P4: progesterone, HCG: human chorionic gonadotropin, PGT-A: preimplantation genetic testing for aneuploidies

Prepared in collaboration with Diana Valbuena.

As shown in Table 15, there were no significant differences in clinical outcome rates between those patients who transferred a day 5 blastocyst versus those who transferred a day 6 blastocyst. Pregnancy rates were 75.4% (138/183) per transfer in the day 5 blastocyst group and 70.1% (54/77) per transfer in the day 6 blastocyst group (P = 0.465). The implantation rates per transfer were 67.8% (124/183) in the day 5 blastocyst group and 63.6% (49/77) in the day 6 blastocyst group (P = 0.476). Finally, ongoing pregnancy rate per transfer were 57.9% (106/183) in the day 5 blastocyst group and 58.4% (45/77) in the day 6 blastocyst group (P = 0.728). In order to clarify if either chromosomal embryo selection or source of oocyte is relevant in this comparison, we compared the results from those PGT-A studied embryos and also those derived from donor eggs. Results are summarized in Table 16 and Table 17, respectively, which show that no statistically significant differences were found between the two groups.

Table 15: Clinical outcome of patients undergoing a pET with either a day 5 or day 6 blastocyst.

	Day of transferred embryos d5 n=183	Day of transferred embryos d6 n=77	p-value
Implantation rate	124/183 (67.8)	49/77 (63.6)	.476
Pregnancy rate	138/183 (75.4)	54/77 (70.1)	.465
Ongoing pregnancy (Ongoing + LB)	106/183 (57.9)	45/77 (58.4)	.728

Chi-Square and Fisher tests were used to compare the variables between groups. Significance was set at P <0.05.
Prepared in collaboration with of Diana Valbuena.

Table 16: Clinical outcome of PGT-A patient undergoing a pET with either a day 5 or day 6 known euploid blastocyst.

	Day of transferred embryos d5 n=110	Day of transferred embryos d6 n=52	p-value
No. Implanted sacs	0.73 ± 0.45	0.6 ± 0.5	.108
Implantation rate	80/110 (72.7)	31/52 (59.6)	.134
Pregnancy rate	88/110 (80.0)	35/52 (67.3)	.117
Ongoing pregnancy rate (Ongoing + LB)	67/110 (60.9)	29/52 (55.8)	.652

Data are expressed as mean ± SD unless otherwise specified.
Student's t-test, Chi-Square and Fisher tests were used to compare quantity and categorical variables between groups.
Significance was set at P <0.05.
Prepared in collaboration with Diana Valbuena.

Table 17: Clinical outcome of patients undergoing a pET with either a day 5 or day 6 donor oocyte derived blastocyst.

	Day of transferred embryos d5 n=62	Day of transferred embryos d6 n=11	p-value
No. Implanted sacs	0.76 ± 0.43	0.73 ± 0.47	0.841
Implantation rate	47/62 (75.8)	8/11 (72.7)	1
Pregnancy rate	50/62 (80.6)	9/11 (81.8)	1
Ongoing pregnancy rate (Ongoing + LB)	41/62 (66.1)	7/11 (63.6)	1

Data are expressed as mean ± SD unless otherwise specified.
Student's t-test, Chi-Square and Fisher tests were used to compare quantity and categorical variables between groups.
Significance was set at P <0.05.
Prepared in collaboration with Diana Valbuena.

A multivariant binomial logistic regression was made considering the control variables that could interfere with the principal variable studied (ongoing pregnancy rate). This included age, embryo origin, number of previous failed cycles, PGT-A, morphological embryo quality, and day of development of the transferred embryos. None of these factors significantly affected the ongoing pregnancy. Table 18.

Table 18: Multivariant binomial regression to assess the impact of control variables on the ongoing pregnancy rate.

Variables	p-value	Std. Error	OR 95% CI
Day of transferred embryo			
D6	0.467	0.416	1.353 (0.603 3.098)
Age	0.082	0.041	1.074 (0.992 1.167)
Embryo origin			
Own oocytes	0.956	0.58	0.968 (0.305 3.04)
Timing with P4/HCG	0.632	0.022	1.011 (0.969 1.058)
PGT-A			
Not analyzed	0.602	0.415	0.805 (0.356 1.829)
Morphological embryo quality			
Optimal	0.153	0.446	1.89 (0.793 4.595)
Suboptimal	0.975	0.797	0.975 (0.196 4.85)
Number of previous failed cycles	0.248	0.104	1.128 (0.924 1.398)

A multivariant binomial regression was made considering the control variables that could interfere on the principal studied variable (ongoing pregnancy rate). Significance was set at $P < 0.05$.
P4: progesterone, HCG: human chorionic gonadotropin, PGT-A: preimplantation genetic testing for aneuploidies.
Prepared in collaboration with Diana Valbuena.

5.5 Chapter Discussion

The aim of this study was to understand the clinical potential of day 5 versus day 6 blastocysts. As successful implantation requires appropriate synchronization between a competent embryo and a receptive endometrium, controlling for the endometrial WOI allows for a more definitive assessment of embryonic viability. Therefore, in this study the clinical outcomes following a SET of a day 5 or day 6 blastocyst during the time of optimal receptivity as confirmed by ERA were considered.

We detected no significant difference in pregnancy rates, ongoing pregnancy rates, and implantation rates, suggesting that the clinical potential of day 5 and day 6 blastocysts is similar when properly controlling for endometrial receptivity. These results are striking given that more patients transferring a day 5 embryo were recipients of ovum donation. In contrast is the fact that the transfers of day 6 blastocysts had more euploid embryos (although not significant).

Our findings challenge current clinical practices of embryo selection for FETs in which day 5 blastocysts are often prioritized for transfer over day 6. This concept is largely based upon findings of earlier studies which reported superior clinical rates of day 5 embryos, implying delayed blastocyst development may be indicative of reduced viability (Barrenetxea et al., 2005; Khorram et al., 2000; Shapiro et al., 2001). However, these studies were carried out in fresh IVF transfer cycles, creating a strong bias as ovarian stimulation advances the endometrium, leading to an asynchronous embryo-endometrial environment (Kolb and Paulson, 1997; Kolibianakis et al., 2002), especially in those cases where transfer was deferred to 6 days post retrieval. Another bias innate to these earlier studies was the transfer of multiple embryos at various stages of expansion and quality (Shapiro et al., 2001), an issue that is now easily overcome by vitrification of blastocysts with appropriate expansion and quality with subsequent transfer in an FET cycle.

More recent studies attempted to overcome bias relating to asynchrony by assessing outcomes in FET cycles. Frozen transfers omit the effects of ovarian stimulation while simultaneously allowing for those embryos that reached the blastocyst stage on various days of culture to be transferred on the same day of endometrial preparation. Under this study design, similar findings to the earlier studies referred to above were reported in which embryos reaching the blastocyst stage by day 5 resulted in significantly higher implantation rates, clinical pregnancy rates, and live birth rates than those that formed by day 6 (Desai et al., 2016; Ferreux et al., 2018; Haas et al., 2016). Increased aneuploidy in slower developing embryos (Alfarawati et al., 2011; Campbell et al., 2013b; Rubio et al., 2007; Taylor et al., 2014) may be an explanation for inferior clinical rates of day 6 blastocysts. Irani *et al* controlled for aneuploidy in their study by analyzing the outcomes in single euploid frozen transfer of either a day 5 or day 6 blastocyst,

reporting significantly higher implantation and live birth rates in the day 5 group, though no differences were detected in miscarriage rates between the two groups. They proposed that factors pertaining to metabolic or epigenetic health may play a role in the reduced clinical potential of slower blastulating embryos (Irani et al., 2018).

It is important to consider that transfers of day 6 blastocysts can occur due to reasons other than slowed development, such as waiting for PGT-A results in fresh cycles, thawing in day 5 and transferring one day later for administrative reasons, or surely more prevalent in the future because of noninvasive PGT-A (that requires culture media from day 4 to day 6 to have adequate DNA from the embryo). In all these scenarios, day 6 does not imply poor quality, and hence the conclusions on implantation potential must be deeply studied, as conditions for day 5 and day 6 embryos really could be of comparable quality.

Although a number of studies, including those designed in order to attempt greater control over endometrial receptivity, have demonstrated a disparity in clinical rates between day 5 and day 6 blastocysts, other groups have reported conflicting results, leading to a lack of clear clinical translation and consistency. Shapiro *et al* analyzed outcomes following 377 fresh ETs and 106 FETs, finding higher clinical pregnancy rates in the fresh transfer of day 5 embryos versus day 6, but similar outcomes between the two groups following frozen transfers, further supporting those findings in fresh studies were likely a result of suboptimal embryo-endometrial synchrony as opposed to rate of blastocyst development (Shapiro et al., 2008). A meta-analysis of 15 controlled studies of 2504 transfers revealed no difference in ongoing pregnancy and live birth rates between day 5 and day 6 blastocysts possessing the same morphological quality at the time of freezing (Sunkara et al., 2010). Due to inconsistent findings and perhaps to a larger

extent the lack of veritable control over endometrial receptivity in past designs, it is not possible to make any definitive conclusion regarding embryonic potential of day 5 versus day 6 blastocysts. PGT-A studies have shown that euploid embryos fail to implant more than a third of the time (Forman et al., 2013), suggesting source of failure beyond the embryo. Displacement of the WOI is strongly correlated with reduced implantation, an important association to consider as non-receptivity at conventional timing of transfer has been demonstrated not only in patients suffering with RIF (Hashimoto et al., 2017; Mahajan, 2015; Ruiz et al., 2013; Tan et al., 2018) but also patients at the first IVF appointment (Simón et al., 2020). When properly adjusting for displacements, significantly improved implantation, pregnancy, and live birth rates are observed (Ruiz-Alonso et al., 2013; Simón et al., 2020).

This study has limitations. The first being the retrospective design which unavoidably comes with its own inherent biases, including a lack of control of the distribution of patient demographics within each group. Secondly, the data analyzed was collected from patient cycles carried out at multiple IVF clinics, all implementing different protocols and culture conditions, among other factors, which may affect overall clinical rates. Third, the study size of 260 patients precludes any definitive conclusion. With that said, a larger, prospective study with greater control over patient population to ensure proper distribution of characteristics is needed. Controlling for additional influencers of implantation, such as embryo aneuploidy and endometrial microbiota, would further assist with objectively assessing the effects of blastulation day on clinical outcomes.

To the best of our knowledge this is the first report comparing clinical outcomes of day 5 versus day 6 blastocysts when transferring into a known receptive endometrium. Implementing our design, no difference in clinical rates is observed between transfer of a day 5 versus a day 6

blastocyst when transferring at the time of optimal endometrial receptivity. The clinical translations of our findings are clear. First, since the viability of day 5 and day 6 embryos appear to be similar, other embryonic characteristics that demonstrate a strong association with improved clinical rates can take priority in the embryo selection process. For instance, higher blastocyst morphology grades, shown to be correlated with superior pregnancy outcomes (Irani et al., 2017; Irani et al., 2018), should be prioritized, and evaluated prior to day of blastocyst formation. Other markers that have potential to refine the embryo selection process include morphokinetic events during early embryo culture (Campbell et al., 2013b), mitochondrial load (Diez-Juan et al., 2015), and the utilization of noninvasive PGT-A (Rubio et al., 2019). Another clinical translation of our findings is the omission of unnecessary additional fresh cycles in attempt to obtain day 5 blastocysts. This not only reduces the cost and time of the IVF process for patients, but also relieves stress for those patients with only day 6 embryos available. Lastly, we have highlighted the importance of properly controlling for endometrial receptivity, further emphasizing that successful implantation not only requires a competent embryo but also a properly synchronized endometrium. By default, our method and findings also underline the contribution and importance of the application of personalized medicine in the field of IVF.

6.0 Evaluating clinical outcomes associated with pET guided by transition phase results: do small shifts lead to big outcomes? (specific aim d)

Manuscript in progress:

Stankewicz T, Ruiz-Alonso M, Valbuena D. Evaluating clinical outcomes associated with pET guided by transition phase results: do small shifts lead to big outcomes? In preparation to submit to RBMO. Submission expected February 2022.

My personal contributions to this work are assisting with establishing and maintaining IRB approval for this study, collecting the clinical follow up data, analyzing data, and writing the manuscript.

6.1 Summary of this Chapter

The aim of this study was to evaluate the clinical outcomes associated with pETs guided by transition phase results (12-hour shift) by comparing these rates to those associated with pETs based on a receptive phase result, answering the question: can small shifts lead to big outcomes? This is a retrospective study data review, involving 567 patients who underwent a pET, in an HRT cycle, guided by either a receptive ERA result or transition phase ERA result (early

or late receptive) between February 2017 and September 2019. There were no significant differences in the clinical outcome rates between patients who underwent a pET guided by a receptive phase versus a transition phase result (pregnancy rates 75.52% (290/384) versus 70.49% (129/183) (p = 0.221), implantation rates 59.49% (307/516) versus 57.51% (134/233) (p = 0.630), and ongoing pregnancy rate 58.07% (223/384) versus 57.38% (105/183) (p =0.928)). Furthermore, no significant difference in clinical rates were observed between pETs guided by an early receptive result and those guided by a late receptive result (pregnancy rates 70.04% (107/152) versus 70.97% (22/31) (p = 1), implantation rates 58.64% (112/191) versus 52.38% (22/42) (p = 0.493), and ongoing pregnancy rates 57.89% (88/152) versus 58.84% (17/31) (p = 0.843)). Given our results, we have demonstrated that transition phase profiles provided by the ERA test are effective in predicting the optimal receptive phase and provide accurate guidance in regard to time of transfer, signifying that small shifts can lead to big outcomes.

6.2 Introduction to Chapter

As previously stated in section 1.2.3.3, successful implantation remains one of the foremost limiting factors in effective treatment in an IVF cycle and although an embryo has the ability to readily implant in other human tissues, uterine implantation requires a precise synchronization between a competent blastocyst and a receptive endometrium. It was previously thought that endometrial receptivity was consistent among all women (see section 1.2.3.3.3), this, combined with the lack of an objective and reliable diagnostic test to accurately date the endometrium

omitted receptivity assessment from the IVF workup. However, with the introduction of the ERA test, we now understand that women possess unique pWOIs.

The utility and efficacy of the ERA test relies on the advances realized in the field of genetics, and more specifically the study of transcriptomics. To take full advantage of the latest developments, the ERA has been continuously refined since it was first introduced clinically in 2011. Initially, the test was performed using a customized microarray, however in 2017 NGS replaced this technology, leading to further improvements in clinical rates (Clemente-Ciscari et al., 2018). Another notable refinement was the redefining of the receptive phase, giving rise to transition phase profiles (Diaz-Gimeno et al., 2017). These new subsignatures, early receptive and late receptive, indicate a displacement of only 12 hours pre or post receptive, respectively. Furthermore, a correlation between the late receptive phase and increased risk of biochemical pregnancy has been established (Diaz-Gimeno et al., 2017).

To evaluate the efficacy of transition phase transcriptomic profiles and their corresponding pET recommendations, hence answering the question can small shifts lead to big outcomes, the following sub-aims are presented:

di. To collect clinical outcome data from patients who transferred based on a transition phase (early or late receptive) ERA profile and patients who transferred based on a receptive phase (indicating optimal receptivity) ERA profile.

dii. To perform statistical analysis to compare reproductive outcomes following a pET based on a transition phase result and recommendation versus a receptive phase result and recommendation, testing the hypothesis that small shifts in the WOI can occur and

when adjusted accordingly can result in similar outcomes to those patient's transferring at time of optimal receptivity.

dii. To perform statistical analysis to compare reproductive outcomes following a pET based on an early receptive phase result and recommendation versus a late receptive phase result and recommendation, testing the hypothesis that small shifts in the WOI can occur earlier or later than expected and when adjusted accordingly result in similar outcomes.

6.3 Materials and Methods

Refer to section 2.4 for more detail regarding materials and methods used for this study.

6.4 Results of this Chapter

Five hundred and sixty-seven patients underwent a pET guided by ERA between February 2017 and September 2019. Three hundred and eighty-four (67.7%) patients transferred according to a receptive phase result and 183 (32.3%) patients transferred according to a transition phase result. Of the transition phase group, there was a higher number of patients with an early receptive profile (152; 83.1%) than a late receptive profile (31; 16.9%). HRT was the endometrial preparation protocol used for both the ERA mock cycle and the subsequent pET in all patient cases.

Patient demographics were similar between the receptive and transition phase groups. The average patient age was 36.68 ± 5.38 years and 36.12 ± 5.05 years (p = 0.238), average BMI was

25.23 ± 5.64 versus 24.11 ± 5.31 (p = 0.080), and number of previous failed cycles per each group were also similar (27.42% (85/384) and 30.58% (37/183) with no previous failures, 24.52% (76/384) and 25.62% (31/183) with 1 previous failure, 15.16% (47/384) and 14.88% (18/183) with 2 previous failures, and 32.9% (102/384) and 28.93% (35/183) with 3 or more failures (p = 0.849)) (Table 19).

In regard to the embryos transferred in the pET cycles, these parameters too were largely all similar (with the exception of day of development of the transferred embryos) between the receptive group and the transition phase group. The proportion of autologous and donor ovum derived embryos was 65.54% (251/384) and 34.46% (132/384) in the receptive group and 70.49% (129/183) and 29.51% (54/184) in the transition phase group (p = 0.252). Utilization of PGT-A was also similar between both groups, with 49.74% (191/384) and 50.82% (93/183) of transfers involving known euploid embryos,

48.7% (187/384) and 48.63% (89/183) of transfers with embryos of unknown euploidy status, and 1.56%

(6/384) and 0.55% (1/183) involving the transfer of a mosaic embryo (p = 0.683). We classified embryonic morphology as “optimal,” “medium,” and “suboptimal” and observed a similar distribution of transfers with these various grades of embryos in the receptive group versus the transition phase group (62.63% (181/384) vs. 67.3% (107/183), 28.03% (81/384) vs. 24.53% (39/183), 9.34% (27/384) vs. 8.18% (13/183) (p = 0.647)). The number of transferred embryos were also similar with 1.34 ± 0.52 embryos transferred in the receptive group and 1.27 ± 0.47 embryos transferred in the transition phase group (p = 0.108). As mentioned previously, the

developmental day of the embryos transferred differed between the receptive and transition phase groups, although the trends were similar with a majority of transfers involving a day 5 blastocyst (73.18% (281/384) and 62.64% (114/183)), followed by day 6 blastocysts (26.82% (103/384) and 35.16% (64/183)), and lastly day 3 cleavage embryos (0% (0/384) and 2.2% (4/183)) ($p = 0.001^*$) (Table 19).

Table 19: Demographic, clinical, and outcome data in transition phase versus receptive patients.

	Transition Phase n=183	Receptive n=384	p-value
Age	36.12 ± 5.05	36.68 ± 5.38	.238
Body-Mass Index	25.23 ± 5.64	24.11 ± 5.31	.080
No. previous failed cycles			
0	37 (30.58)	85 (27.42)	
1	31 (25.62)	76 (24.52)	
2	18 (14.88)	47 (15.16)	
≥3	35 (28.93)	102 (32.9)	.849
Timing with P4 HCG	124.03 ± 12.92	126.1 ± 11.99	.073
Embryo origin			
Ovum donation	54 (29.51)	132 (34.46)	
Own oocytes	129 (70.49)	251 (65.54)	.252
PGS			
Mosaic	1 (0.55)	6 (1.56)	
Euploid	93 (50.82)	191 (49.74)	
Not analyzed	89 (48.63)	187 (48.7)	.683
Morphological embryo quality			
Medium	39 (24.53)	81 (28.03)	
Optimal	107 (67.3)	181 (62.63)	
Suboptimal	13 (8.18)	27 (9.34)	.647
Day of transferred embryos			
Day 3	4 (2.2)	0 (0)	
Day 5	114 (62.64)	281 (73.18)	
Day 6	64 (35.16)	103 (26.82)	.001*
No. of transferred embryos	1.27 ± 0.47	1.34 ± 0.52	.108
No. of implanted sacs	0.73 ± 0.62	0.8 ± 0.64	.232
Implantation rate	134/233 (57.51)	307/516 (59.49)	.630
Pregnancy rate per transfer	129/183 (70.49)	290/384 (75.52)	.221
Ongoing pregnancy rate per transfer	82/183 (44.81)	193/384 (50.26)	.243
Ongoing pregnancy rate (ongoing + LB)	105/183 (57.38%)	223/384 (58.07%)	.928

*Data are expressed as mean ± SD or n (%). Level of statistically significance between groups is <0.05. There were significant differences among groups in Day of transferred embryos. No significant differences were found in the other variables. Student's t-test was used to compare quantity variables between groups. Fisher Exact tests was used to compare categorical variables. Normality is assumed due to sample size. Prepared in collaboration with Diana Valbuena.

In the comparison of only early receptive versus late receptive phase groups, average age was 36.01 ± 5.21 years and 36.68 ± 4.25 years (p = 0.446), average BMI was 25.13 ± 5.92 and 25.64 ± 4.41 (p = 0.287), and number of previous failed cycles per each of the transition phase groups were similar (33.01% (34/152) and 16.67% (3/31) with no previous failures, 26.21% (27/152) and 22.22% (4/31) with 1 previous failure, 13.59% (14/152) and 22.22% (4/31) with 2 previous failures, and 27.18% (28/152) and 38.89% (7/31) with 3 or more failures (p = 0.366)) (Table 20). All embryo parameters were similar except for developmental day of the embryos transferred. In the early receptive phase and late receptive there was no significant difference in the proportion of autologous (71.05% (108/152) and 67.74 (21/31)) versus donor-oocyte derived embryos (28.95% (44/152) and 32.26% (10/31)) (p = 0.829), euploid (53.29% (81/152) and 38.71% (12/31)) versus mosaic (0% (0/152) and 3.23% (1/31) versus unknown euploidy status (46.71% (71/152) and 58.06% (18/31)) (p = 0.063), morphological quality (optimal: 67.65% (92/152) and 65.22% (51/31); medium: 23.53% (32/152) and 30.43% (7/31); suboptimal: 8.82% (12/152) and 4.35% (1/31)) (p = 0.761), and number of embryos transferred (1.26 ± 0.45 and 1.35 ± 0.55) (p = 0.398). Again, a significant difference in the proportion of day 5 (62.91% (95/152) and 61.29% (19/31)) versus day 6 (36.42% (55/152) and 29.03% (9/31)) versus day 3 (0.66% (1/152) and 9.68% (3/31)) embryos transferred among the early and late receptive phase groups, but the same trend that was observed in the comparison of receptive versus transition phase transfers was also observed here (p = 0.023*) (Tables 19 and 20).

There were no significant differences in the clinical outcome rates between patients who underwent a pET guided by a receptive phase result and those who underwent a pET guided by a transition phase result. Pregnancy rates were 75.52% (290/384) versus 70.49% (129/183) (p =

0.221), implantation rates 59.49% (307/516) versus 57.51% (134/233) (p = 0.630), and ongoing pregnancy rate 58.07% (223/384) versus 57.38% (105/183) (p =0.928) (Table 19). Furthermore, no significant difference in clinical rates were observed between pETs guided by an early receptive result and those guided by a late receptive result. Pregnancy rates were 70.04% (107/152) versus 70.97% (22/31) (p = 1), implantation rates 58.64% (112/191) versus 52.38% (22/42) (p = 0.493), and ongoing pregnancy rate 57.89% (88/152) versus 58.84% (17/31) (p = 0.843) (Table 20).

Table 20: Demographic, clinical, and outcome data in early receptive versus late receptive patients.

	Early Receptive N=152	Late Receptive N=31	p-value
Age	36.01 ± 5.21[a]	36.68 ± 4.25[a]	.446
Body-Mass Index	25.13 ± 5.92[b]	25.64 ± 4.41[b]	.287 (t= .771)
No. previous failed cycles			
0	34 (33.01)	3 (16.67)	
1	27 (26.21)	4 (22.22)	
2	14 (13.59)	4 (22.22)	
≥3	28 (27.18)	7 (38.89)	.366
Timing with P4 HCG	120.7 ± 5.49[b]	139.8 ± 22.93[b]	.000* (t= .000)
Embryo origin			
Ovum donation	44 (28.95)	10 (32.26)	
Own oocytes	108 (71.05)	21 (67.74)	.829
PGS			
Mosaic	0 (0)	1 (3.23)	
Euploid	81 (53.29)	12 (38.71)	
Not analyzed	71 (46.71)	18 (58.06)	.063
Morphological embryo quality			
Medium	32 (23.53)	7 (30.43)	
Optimal	92 (67.65)	15 (65.22)	
Suboptimal	12 (8.82)	1 (4.35)	.761
Day of transferred embryos			
Day 3	1 (0.66)	3 (9.68)	
Day 5	95 (62.91)	19 (61.29)	
Day 6	55 (36.42)	9 (29.03)	.0229*
No. of transferred embryos	1.26 ± 0.45[b]	1.35 ± 0.55[b]	0.398 (t= .357)
No. of implanted sacs	0.74 ± 0.62[b]	0.71 ± 0.64[b]	0.813 (t= .830)
Implantation rate per transfer	112/191 (58.64)	22/42 (52.38)	.493
Pregnancy rate per transfer	107/152 (70.04)	22/31 (70.97)	1
Ongoing pregnancy rate per transfer	67/152 (44.08)	15/31 (48.39)	.695
Ongoing pregnancy rate (ongoing + LB)	88/152 (57.89)	17/31 (58.84)	.843

*Data are expressed as mean ± SD or n (%). Level of statistically significance between groups is <0.05. There were significant differences among groups in Day of transferred embryos and timing with P4 HCG. No significant differences were found in the other variables. Student's t-test (superscript a) and Exact Wilcoxon-Mann-Whitney test (superscript b) were used to compare quantity variables between groups. Normality was assessed with Shapiro-Wilk test and homogeneity of variance with Levene's test, then nonparametric Wilcox exact test was applied on quantitative data that does not accomplish normality and/or variance homogeneity. Due to the sample size and small count in some categories we use fisher exact test on categorical data. Prepared in collaboration with Diana Valbuena.

6.5 Chapter Discussion

The aim of this study was to evaluate the clinical outcomes associated with pETs guided by transition phase results (12-hour shift), in attempt to answer the question: can small shifts lead to big outcomes? As a receptive phase result indicates optimal timing of endometrial receptivity and has been linked to significantly better live birth rates (Diaz-Gimeno et al., 2017), we used the pET outcomes of these cycles as a comparison group in order to assess the efficacy of transition phase ERA recommendations. We further stratified the data to compare the pET outcomes associated with early and late receptive results.

We detected no significant difference in pregnancy rates (75.52% vs. 70.49%; p= 0.221), implantation rates (59.49% vs. 57.51%; p = 0.630), and ongoing pregnancy rates (58.07% vs. 57.38%; p= 0.928) between the receptive and transition phase groups. These findings demonstrate that capability of the ERA test to accurately predict the optimal timing of endometrial receptivity with superior preciseness and the efficacy of the resulting pET recommendation, even when there is only a very minor shift (i.e., 12 hours). Additionally, no significant difference in pregnancy rates (70.04% vs. 70.97%; p= 1), implantation rates (58.64% vs. 52.38%; p = 0.493), and ongoing pregnancy rates (57.89% vs. 58.84%; p= 0.843) were observed between the early versus late receptive groups, revealing equivalent accuracy of the two result types and their corresponding predictions pertaining to timing of optimal receptivity.

The clinical rates shown here were similar to those by Clemente-Ciscar et al (2018), who reported pET outcomes in 261 patients, realizing a 70.9% pregnancy rate and 55.7% implantation rate. All patients in their study had a diagnosis of RIF and while our study did include patients without previously failed cycles, a majority of patients did have at least one prior

IVF failure. Considering the substantial proportion of patients with implantation failure, emphasizes the importance of precise analysis of endometrial receptivity at the transcriptomic level, for when the transfer time was adjusted by just 12 hours, high clinical rates were attained. With that said, the utilization of ERA in patients without a history of failed cycles should not be discarded, as a recent RCT has shown that 1 in 3 women at the 1st IVF appointment have a displaced WOI. When transfers were adjusted according to the time of optimal receptivity, superior clinical rates, beyond those resulting from a standard FET timing, were achieved (Simón et al., 2020). Since our study included patients without prior IVF failure, we can only solidify the indication of ERA to all patients undergoing ART, in order to define the optimal time of endometrial receptivity and prevent the loss of valuable embryos.

The importance of identifying transition phase profiles is further underscored when comparing our clinical findings to those reported by Ohara et al (2020), who in their study, employed an alternative endometrial receptivity test based on the analysis of 48 genes by RT-qPCR. They observed a pregnancy rate of 46.0% and implantation rate of 23.8% in patients who possessed a displaced WOI and transferred according to the results. The endometrial test applied in the aforementioned study differs from the ERA test, in that it lacks transition phase profiles. Instead, detected displacements and transfer recommendations are based on 24-hour increments. The absence of greater preciseness and the inability to identify minor shifts most certainly contributed in part to the poorer clinical rates obtained in their study.

The integration of the transition phases into the ERA test was a result of the refinement to the endometrial WOI transcriptomic signature. New subsignatures were defined which included an optimal receptive profile correlated with an ongoing pregnancy rate of 80%, an early receptive

phase, and a late receptive phase that consisted of 22 dysregulated genes and corresponded to a 50% risk for biochemical pregnancy (Diaz-Gimeno et al., 2017). The increased incidence of biochemical pregnancies in late receptive cases corroborated with earlier studies showing that the risk for pregnancy loss increased with later implantation (Wilcox et al., 1999). Our findings not only highlight the efficacy of transferring according to a transition phase recommendation, but also the value of identifying these displacements, particularly in the case of the late receptive phase, to avoid adverse clinical outcomes like biochemical losses.

Leveraging the capability to identify transition phase profiles has provided a better understanding of implantation. The WOI was previously described as opening around day 19 or 20 of the menstrual cycle and lasting for 4 to 5 days (Harper 1992; Lessey 2011), however, conflicting findings (Mcculloh et al., 2015) necessitated the need for a more concrete understanding. A recent study based on ERA results of paired samples, effectively measured the length of the WOI, by computing the duration from when the window of implantation opens (early receptive phase) to when it closes (late receptive phase). They established that the WOI typically lasts 29-36 hours and in some patients can be <24 hours (Rincon et al., 2018). Since the WOI is shorter than initially thought, transferring according to the time of optimal receptivity is imperative (especially in those patients possessing exceptionally narrow WOIs), clearly verifying the role and significance of the transition phase signatures.

Given our results, we have demonstrated that transition phase profiles provided by the ERA test are effective in predicting the optimal receptive phase and provide accurate guidance in regard to time of transfer. This in turn has resulted in improved clinical rates, in addition to further mitigating the risk of biochemical loss. It is safe to say that small shifts can lead to big outcomes.

7.0 What is the narrowest window of implantation? A case report (specific aim e)

This work is based on a manuscript submitted for publication:

Glassner M, Stankewicz T, Ruiz-Alonso M, Valbuena D. What is the narrowest window of implantation? A case report. – submitted to Fertility Research and Practice (October 4, 2021).

My personal contributions to this work are obtaining patient consent to publish this case report, collecting clinical follow up data pertaining to each biopsy cycle, reviewing the transcriptomic profiles associated with the biopsy samples, writing the manuscript, and submitting the manuscript to Fertility Research and Practice for publication.

7.1 Summary of this Chapter

This article reports a case of a very narrow WOI as defined by endometrial transcriptomic profiling, and the proceeding live birth following a pET accounting for this unusually short period of time. A 40-year-old patient suffering from primary infertility and diminished ovarian reserve, with one previous failed euploid embryo transfer cycle, underwent a total of 9 endometrial biopsies to assess her personalized window of receptivity at the transcriptomic level. She was found to have a very narrow WOI, lasting less than 12 hours. Using her only remaining euploid embryo, she underwent a pET according to her unique window of receptivity, which subsequently resulted in a healthy live birth. Although it was previously accepted that the WOI

was constant in most women, remaining open for several days, here we report a case of endometrial receptivity lasting less than 12 hours. We not only underscore the value of effective evaluation of endometrial receptivity at the transcriptomic level, but also the contribution of the endometrium in the implantation process and the advantages that personalized medicine offers to the field of IVF.

7.2 Background

It has been well established that embryo implantation requires precise synchronization between a competent blastocyst and a receptive endometrium (section 1.2.3.3). However, the widely accepted assumption that the WOI is constant in all women, opening around day 19 or 20 of the menstrual cycle and lasting for 4 to 5 days (Harper 1992, Lessey 2011), paired with a lack of a reliable diagnostic test, lead to the noted absence of endometrial receptivity assessment in patients undergoing IVF. With that said, a new era of precision medicine based on objective molecular fingerprinting of different functions has led to the development of the ERA test that relies largely on precise timing to identify optimal endometrial receptivity. This in turn has provided us with a deeper understanding of endometrial receptivity, not only in the terms of its personalized placement but also its variable duration.

Pursuit of the following sub-aims attempted to identify the shortest WOI identified by transcriptomic as follows:

ei. To review the ERA results from a patient who underwent nine endometrial biopsies.

eii. To provide a pET recommendation that accounts for a WOI lasting less than 12 hours.

eiii. To demonstrate functional evidence of an extremely narrow WOI by documenting the recommended pET timing and resulting live birth.

7.3 Case presentation

In April 2018, a 40-year-old patient attended our reproductive clinic presenting with primary infertility and diminished ovarian reserve. She had undergone treatment at another clinic that included an FET of a known euploid blastocyst of high morphological quality, at standard transfer timing in an HRT cycle, which subsequently failed to implant. The patient presented with normal BMI (22.5kg/m2), AMH of 0.9 ng/ml, FSH levels 6.4 IU/mL, and normal IVF workup (hysterosalpingography, hysteroscopy, semen analysis, normal patient and partner karyotypes).

In March 2017, after counseling, the patient underwent molecular endometrial receptivity work-up using the ERA test. Her first biopsy was performed in an HRT cycle at 108 hours of progesterone exposure. Estradiol priming protocol consisted of Estrace 2mg tablets every 12 hours and two Vivelle transdermal patches every 3 days. Prior to progesterone start, endogenous progesterone was routinely measured ensuring the level was <1ng/ml. Exogenous progesterone was started with 1cc of progesterone in oil (PIO) followed by alternating doses of 400mg of vaginal progesterone. The first analysis yielded a pre-receptive profile with recommendation to perform a second biopsy 24 hours later (at 132 hours). In April 2017, a second biopsy was obtained in a medicated protocol replicating the previous mock cycle at 131 hours of progesterone exposure. It was observed then that the endometrial transcriptomic profile still was pre-receptive, so it was reported with a recommendation to rebiopsy 24 hours

later, at 155 hours. In May 2017, a third biopsy was taken at 156 hours of progesterone exposure, again replicating the previous two HRT cycle protocols. This time an early receptive profile with direct recommendation to transfer 12 hours later (at 168 hours) was obtained. Due to these special circumstances, to validate that the patient's WOI was accurately predicted by the previous result, the physician and patient opted to take a fourth biopsy the following month, forgoing the recommendation for a pET as indicated by that most recent report. This biopsy was taken 169 hours after progesterone start in a replicated ERA mock cycle protocol. A receptive profile was obtained with recommendation to transfer at 169 hours of progesterone administration. Following the receptive diagnosis, the patient intended to undergo a pET at 169 hours of progesterone administration, using her only remaining euploid, frozen blastocyst. Unfortunately, the blastocyst did not survive the vitrification/warming process and the transfer was canceled. Subsequently, in March 2018, the clinician elected to repeat the ERA test, but this time in a natural cycle. The biopsy was performed 156 hours after hCG trigger, and an early receptive result was reported with recommendation to transfer 12 hours later, or 168 hours after hCG trigger.

The patient changed care at this time to our fertility clinic, and first underwent a new fresh IVF cycle to acquire more euploid embryos. During the fresh cycle, two blastocysts were biopsied for PGT-A, in which one was deemed euploid. Then, in order to validate the initial IVF clinic's findings in regard to endometrial receptivity, a sixth ERA mock cycle was carried out in May 2018, ensuring the HRT protocol utilized in ERA tests one through four was replicated exactly, and with biopsy taken at 170 hours of progesterone exposure. A late receptive profile was reported, and the ERA bioinformatic predictor delivered a recommendation to transfer 12 hours

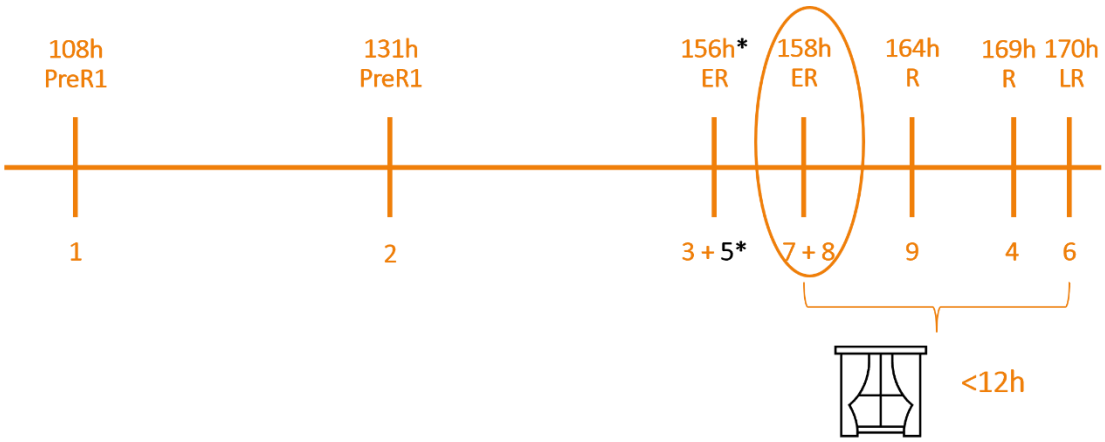
earlier at 158 hours. This was a noteworthy finding as we could clearly see the transition between the receptive phase at 169 hours and the late receptive phase initiating just one hour later at 170 hours, distinctly signifying the end of receptivity.

Since the patient had a previous early receptive result at 156 hours of progesterone exposure, at request of the patient, a seventh biopsy was sampled in June 2018 at 158 hours of progesterone exposure to validate this previous finding. An early receptive result was reported. Based on the three transition phase results (two early receptive profiles at 156 and 158 hours, and a late receptive profile at 170 hours) obtained, it was now inferred that this patient possessed a narrow WOI of less than 12 hours. In order to account for this observation, a more personalized recommendation (beyond the standard recommendation offered by the ERA predictor) was given, advising transfer to occur during the early receptive phase, at 158 hours, to allow the blastocyst adequate time to implant before the window closed.

Even with a more personalized recommendation proposed, the patient was adamant to better understand her WOI and in July 2018 chose to repeat the biopsy once again at 158 hours. Not surprisingly, an early receptive result was reported. Based on previous findings, a recommendation to transfer during the early receptive phase at 158 hours, again allowing for adequate time for the blastocyst to implant, was provided.

Still determined to achieve another receptive result, the patient opted to undergo a ninth ERA biopsy at 164 hours. This time, a receptive result was reported, however based on the multiple data points previously collected for this case and an observed narrow WOI of less than 12 hours, the recommendation to transfer during the early receptive phase, instead of the time of

receptivity determined by the algorithm, was still indicated in order to allot adequate time for the implantation process. Figure 26 summarizes the endometrial profiles of the nine biopsies.



**Bx #5 taken in a Natural (hCG) cycle at 156 hours and reported as Early Receptive. Only biopsy results taken under exact replicated protocols can be correlated.*

Figure 23. Summary of transcriptomic profile results obtained from the patient's nine endometrial biopsy samples. PreR1 = Pre-Receptive 1 day (+24 hours progesterone exposure recommended); ER = Early Receptive (+12 hours progesterone exposure recommended); R = Receptive (no shift in hours of progesterone exposure recommended); LR = Late Receptive (-12 hours progesterone exposure recommended). The collation of all ERA results taken together indicate a WOI that lasts less than 12 hours. Based on this finding, it was recommended to transfer a blastocyst at 158 hours, during the Early Receptive phase to allow adequate time for blastocyst implantation.

Now satisfied with the compilation of results, the patient underwent a pET in August 2018, replicating the HRT cycle protocol used in eight of the nine ERA mock cycles. Exogenous progesterone was initiated on August 23, 2018, at 11:00pm and a single euploid blastocyst was transferred on August 30, 2018, at 1:25pm, yielding a total progesterone exposure time of 158 hours and 25 min. A positive serum pregnancy test was detected 10 days later and following an unremarkable pregnancy, a healthy baby girl was born in May 2019.

Written consent to publish this case report was obtained from the patient.

7.4 Chapter Discussion

Here we report a case of a very narrow WOI, less than 12 hours wide, as determined by molecular profiling of endometrial receptivity using ERA. ERA provides a recommendation for a pET based on specific timing; however, this case further highlights the exemplary precision of molecular testing for receptivity, following the enhanced degree of personalization and the resulting live birth.

The ERA test determines optimal receptivity by examining the transcriptomic profile of an endometrial biopsy sample taken at a specific time in either an HRT or natural cycle. The sample is then classified as receptive or non-receptive, and a recommendation for a pET is given. Although the ERA test does not specifically define the duration of the WOI, this information can sometimes be derived if multiple biopsies are performed at various hours of progesterone exposure. Rincon et al (2018) presented their data based on ERA results of paired samples, where they effectively measured the length of the WOI by computing the time from when the window of implantation opens (the early receptive phase) to when it closes (the late receptive phase), finding that the WOI typically lasts 29-36 hours, and in some patients can be <24 hours long. This differs from previous understanding that the implantation window remained open for 4 to 5 days (Harper 1992; Lessey 2011), again, only further demonstrating the preciseness of molecular endometrial assessment along with the true complexity and uniqueness of the WOI possessed by each woman.

The case reported here not only underscores the value of effective evaluation of endometrial receptivity at the transcriptomic level, but also the contribution of the endometrium in the implantation process and the advantages that personalized medicine offers to the field of IVF. In

an earlier case report by Ruiz-Alonso et al (2014), where they presented the successful pET outcome in a patient possessing a WOI displaced by 48 hours, they stated “what a difference two days make.” Here we take this one step further, proclaiming “what a difference a few hours make.”

8.0 To reanalyze a set of previously published data pertaining to inter-cycle consistency compared to test compliance in an endometrial receptivity analysis test (specific aim f)

For this specific aim, the following published work is presented:

Stankewicz T, Valbuena D, Ruiz-Alonso M. Inter-cycle consistency versus test compliance in endometrial receptivity analysis test. J Assist Reprod Genet. 2018 Jul;35(7):1307-1308.

My personal contributions to this work are reviewing the results and recommendation with the referring clinic, reviewing the transcriptomic profiles associated with each biopsy, and writing the manuscript.

8.1 Review

This chapter involved the reviewing, a letter by Cho et al. (2018). In this letter, the authors questioned the consistency of the diagnosis of the window of implantation (WOI) using the endometrial receptivity analysis (ERA) test in one patient through four different biopsies over four different months. After careful examination of the data provided by the authors in their letter, my co-authors and I felt the need to thank them for actually showing the consistency of ERA testing outcomes and prediction. In this case, the authors performed an ERA test in one patient and, after receiving the report with explicit instructions on how to proceed, instead

performed the subsequent endometrial biopsy at a moment completely discordant from the original recommendation. Cho and co-authors conclude that the ERA test shows variability from month to month following various biopsies in one patient. Our conclusion, which we aim to set forth in this letter, is quite the contrary. According to our review of this case and the ERA concept, it illustrates how consistent ERA results are between different cycles within this patient and between different cycles as originally described (Diaz-Gimeno et al., 2013).

It is recommended to all clinicians that the first endometrial biopsy should be obtained after 120 hours of progesterone administration, because this is the optimal time frame where any displaced WOI will be identified. As any other sampling test requires special conditions (i.e., glycemia analysis requires a fasting period), ERA requires analysis to be performed in this time frame in order to interpret the results appropriately. In this case, the authors performed the first biopsy after 106 hours of progesterone exposure, the ERA predictor recognized a pre-receptive endometrium, and the resultant report indicated that the personalized WOI for this patient was estimated to lie at 154±3 hours of progesterone administration (Figure 27(A)). For this cycle, the report clearly suggested a confirmatory endometrial biopsy at the stated progesterone exposure timing to confirm receptivity as measured by the ERA test. Instead of obtaining the second biopsy following the recommendations, the second endometrial biopsy was performed after 194 hours of progesterone exposure, a full 40 hours later than suggested and outside the normal WOI in humans (Figure 27(B)). The ERA predictor identified this second biopsy as post-receptive; however, as it was taken after so many hours of progesterone exposure, the report recommended another biopsy 24 hours earlier because it was out of the normal range established to have an endometrial biopsy for ERA (P+3 to P+7). It is not a surprise that a pre-

receptive endometrium became post-receptive during this time frame, as the duration of time between the first and second biopsies was 88 h (3 days and 16 hours).

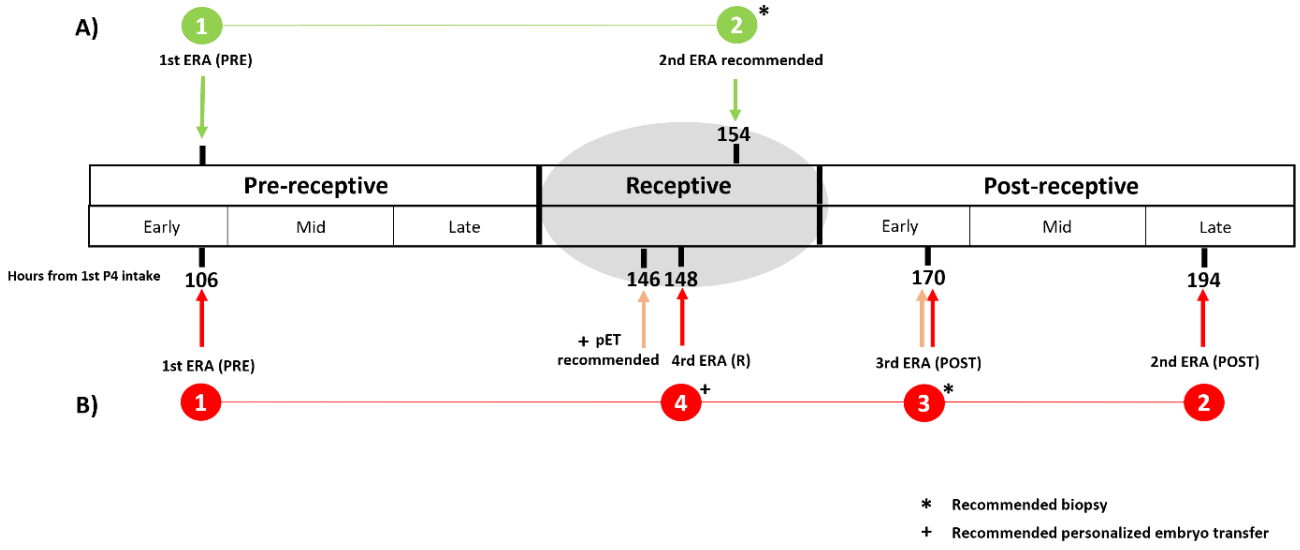


Figure 24. Endometrial profiling based on ERA results. A) Igenomix initial prediction. B) Biopsies performed by Cho et al.

A third biopsy was then performed on this same patient after 170 hours of progesterone exposure. Here, the ERA results again showed a post-receptive uterine lining; however, for this biopsy, the predictor recommended a personalized ET at 146±3 hours (Figure 27(B)). Instead of performing the personalized ET based on this recommendation, the authors performed a fourth endometrial biopsy following 148 hours of progesterone exposure. Here, the results of the third and fourth biopsies were consistent and, interestingly, match up quite nicely with the initial recommendation (Figure 27(A)). During this particular ERA diagnostic case, we established a direct line of communication with the doctor leading the case.

It should be noted that the WOI duration varies from 12 to 48 hours, depending on each patient. An ERA result indicates that the endometrium is receptive at a specific moment of the cycle (with a time frame of ±3 hours), but it does not define the total length of the WOI. Therefore, it is highly probable that if the recommendation given for the first ERA were followed, receptivity would have been confirmed following the second biopsy. Furthermore, it has been documented that the implantation window does not vary within an individual once it has been identified, lasting this way for up to at least 40 months (Diaz-Gimeno et al., 2013). In addition, there are several cases in which patients have achieved a second pregnancy up to 3 years apart by following the same initial ERA recommendation used for the first pregnancy, again showing consistency of the detected WOI.

Independent publications by Hashimoto et al. (2017), Mahajan (2015), and Tan et al. (2018) have demonstrated the clinical value of the ERA test in patients with implantation failure of endometrial origin.

Here, my co-authors and I presented a case to suggest that Cho et al. have misinterpreted their own data and suggested that the ERA test is not accurate nor is it reproducible. We have pointed out a number of questionable clinical decisions made by the Cho team which led them to perform four different endometrial biopsies in one patient after receiving a recommendation for the best way forward in this case. Biopsies two, three, and four all corroborate our initial finding that the patient was receptive with approximately 150 hours of progesterone exposure. The most important question that remains to be answered is whether this 44-year-old patient, presented in the Cho et al. letter, went on to achieve pregnancy following transfer of embryos in the predicted WOI.

9.0 To assess the prevalence of a displaced WOI in GCs and the clinical utility of applying ERA (specific aim g)

Manuscript in progress:

Stankewicz T, Leondires M, Ruiz-Alonso M, Valbuena D. A preliminary evaluation of the clinical utility of ERA for Gestational Carriers. In preparation for RBMO. Submission expected February 2022.

My personal contributions to this work are assisting with establishing and maintaining IRB approval for this study, collecting the clinical follow up data, analyzing data, writing the manuscript.

9.1 Summary of this Chapter

The objective of this study was to evaluate the potential benefit of performing transcriptomic analysis of the WOI to guide the timing of transfer in gestational surrogacy cycles. This is a retrospective data review assessing the receptivity rates of gestational carriers (GCs) who underwent the ERA test at a single IVF clinic between July 2019 and November 2020. Furthermore, clinical outcomes were assessed in GCs following a single blastocyst transfer in the first pET guided by ERA. Surprisingly, a high rate of non-receptivity (73.0%) at the expected time of the WOI was observed in the GCs. A pregnancy rate of 81.1% (30/37), implantation rate of

70.3% (26/37), and ongoing pregnancy rate of 56.8% (21/37) were achieved. This preliminary data demonstrates that a significant proportion of GCs may have a displaced WOI while undergoing the IVF transfer and may benefit from ERA testing to guide a pET as high clinical rates were attained. Evaluating endometrial receptivity timing in GC cycles may contribute to increased efficiency in these cycles, a particularly important concept when considering the unique psychological and financial circumstances associated with gestational surrogacy.

9.2 Introduction to Chapter

A GC is a woman who is contracted by an intended parent (IP) or parents, to carry a pregnancy, usually initiated through IVF (Ethics Committee of the American Society for Reproductive Medicine, 2013). The first successful pregnancy using a GC was reported in 1985 (Utian et al., 1985) and since then the number of GC cycles have increased year after year (Perkins et al., 2016; Murugappan et al., 2017). In general, carriers tend to be younger than 35 years old (Perkins et al., 2016) and are often only considered when presenting with a normal weight, are free of any serious medical condition, and without a history of infertility or recurrent miscarriage (McGovern, 2017). It is also recommended by the American Society for Reproductive Medicine (ASRM) that GCs have had at least one prior, full-term, uncomplicated pregnancy (Ethics Committee of the American Society for Reproductive Medicine, 2018).

The proportion of IVF cycles in the U.S. utilizing GCs continue to rise at a significant pace (Perkins et al., 2016). Additionally, the percentage of non-U.S. residents undergoing GC cycles in the U.S. has also increased, nearly tripling from 2006 to 2013 (Perkins et al., 2016), a likely result of the exclusion of GCs in many countries (Bromfield et al., 2014) along with the U.S. being one of the

few industrialized countries that does not federally prohibit compensated gestational surrogacies (though state regulations do differ) (Armour 2012; Burrell and Edozien 2014). Overall, a growth in the number of IVF clinics performing carrier cycles (Perkins et al., 2016), the expanding number of states implementing a legal framework to accommodate these types of cycles (Creative Family Connections Surrogacy Law by State, 2015), and the general acceptance of the practice, have further contributed to the overall increased number of gestational surrogates.

The most common indications for a GC include absence of a uterus, RPL, RIF, poor obstetric history, medical conditions that make pregnancy not safe or possible for the IP, and same sex couples (Murugappan et al., 2018; Brinsden, 1999). The two most common infertility factors associated with carrier cycles are diminished ovarian reserve and uterine factor (Perkins et al., 2016).

When examining clinical outcomes, statistically higher implantation rates, clinical pregnancy rates, and live birth rates in GC cycles vs. non-GC cycles have been reported (Perkins et al., 2016; Murugappan et al., 2018). When using a GC for uterine-factor infertility, the impact on clinical rates is even greater, compared to using a GC for non-uterine factor diagnoses (Murugappan et al., 2018), a finding that only further emphasizes the integral contribution of the uterus to successful implantation and overall fertility.

The prime component pertaining to the uterus and successful implantation is receptivity. Although finding a displaced WOI is not unexpected in the RIF population, in theory we would expect a displaced WOI to be absent or rare in the fertile population, particularly in GCs with proven fertility. However, the results of a recent RCT involving patients at their first IVF

appointment have shown comparable rates of non-receptivity to the RIF population (Simón et al., 2020), suggesting that a displacement can be present in women without previous implantation failure.

In a review by McGovern, 2018, discussing GC use and uterine role in infertility, the author stated, "we need to develop better methods of assessing the functional reproductive competence of the uterus in ART if we are to truly achieve maximum success." With this goal in mind, we sought to examine the potential benefit of transcriptomic analysis of the WOI by ERA in gestational surrogacy cycles, by the following sub-aims:

gi. To assess the ERA results and percent displacement in the GC population.

gii. To calculate clinical pET outcomes in the GCs to demonstrate the clinical efficacy in this population.

9.3 Materials and Methods

Refer to section 2.5 for more detail regarding materials and methods used for this study.

9.4 Results of this Chapter

A total of 39 GCs underwent an endometrial biopsy for ERA between July 2019 and November 2020. The average timing of the ERA biopsy was at 119.7 hours of progesterone exposure (range 112-121 hours). When calculating receptivity rates, only those cases with a valid ERA result were considered (n=37). A 73.0% (27/37) non-receptive rate and a 27.0% (10/37) receptive rate were

obtained. A majority of non-receptive GCs were found to be pre-receptive (96.3% (26/27)), requiring an additional 24 hours of progesterone prior to ET. Figure 28.

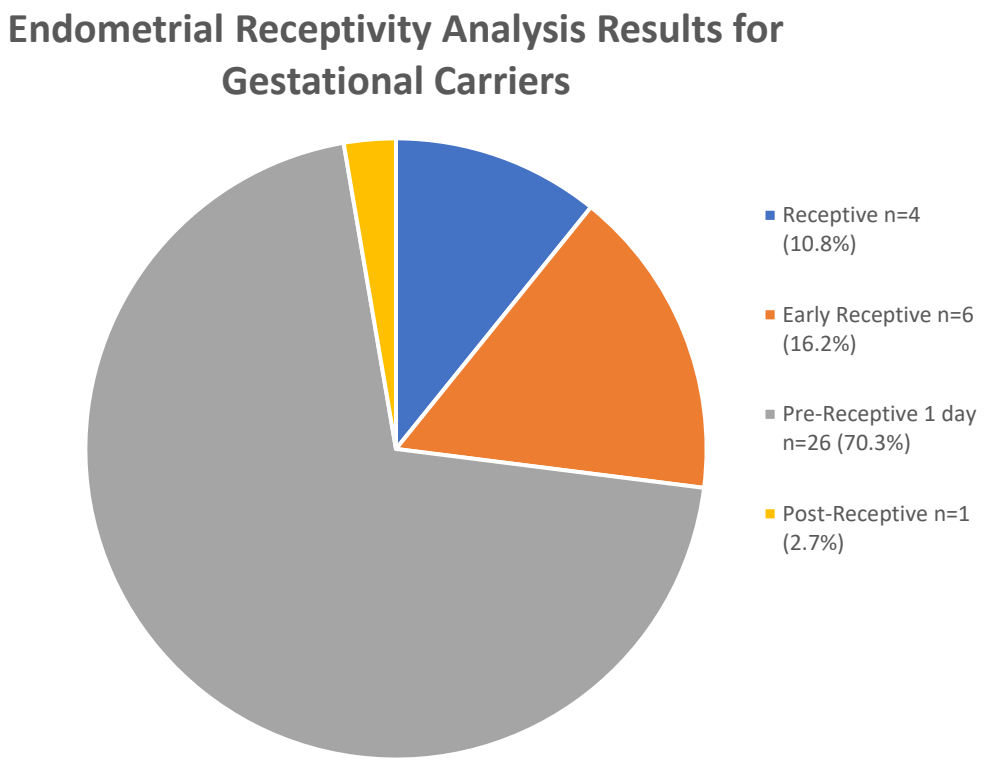


Figure 25. Endometrial profiling for GCs based on ERA results. Classified Receptive: Receptive: 4 cases (10.8%), Early receptive: 6 cases (16.2%); Classified Non-receptive: Pre-receptive 1 day: 26 cases (70.3%), Post-receptive: 1 case (2.7%).

Clinical follow up was possible for all 37 GCs who underwent a pET cycle guided by the ERA recommendation. Only the first pET immediately following the ERA test was considered for each case. The pregnancy rate was 81.1% (30/37), implantation rate was 70.3% (26/37), and ongoing pregnancy rate was 56.8% (21/37). Considering that 33/37 (89.2%) of GC-pET cycles were with a known euploid PGT-A embryo, the outcome data was further stratified. In these specific cases,

the pregnancy rate was 81.8% (27/33), implantation rate 72.7% (24/33), and ongoing pregnancy rate was 60.6% (20/33). Table 21.

Table 21: Clinical outcomes of GC's undergoing a pET.

	Overall pET outcome rates n=37	pET + PGT-A outcome rates n=33
Pregnancy rate	30/37 (81.1)	27/33 (81.8)
Implantation rate	26/37 (70.3)	24/33 (72.7)
Ongoing pregnancy rate	21/37 (56.8)	20/33 (60.6)

9.5 Chapter Discussion

The aim of this study was to explore the potential benefit of ERA in gestational surrogacy cycles. Given that a vast majority of GCs are considered fertile with at least one prior, full term, uncomplicated, pregnancy, we would assume they possess a low rate of displacement at the expected time of endometrial receptivity. Clinical outcome rates in the first subsequent GC-pET cycle were analyzed to further assess the utility and efficacy of the ERA and personalization of transfer in this group.

To our surprise, we detected a high rate of endometrial non-receptivity in the GCs tested. This well exceeded the 1 in 4 non-receptivity rate previously reported for the RIF population (Ruiz-Alonso at al., 2013). Intuitively, we would expect a low rate of WOI displacement in the GC population, as these women are assumed fertile. A possible explanation for this observation may relate to the HRT protocol itself. As the ERA test is only reproducible for the type of cycle in which the biopsy is taken, the results cannot be translated from an HRT cycle to a natural cycle,

or vice versa. With that said, it is possible that carriers are more likely to be found receptive in a natural cycle at the standard WOI timing than in an HRT cycle, wherein hormones are altered from normal physiological levels, potentially affecting endometrial receptivity timing. This is important to consider, as it is often assumed that previous reproductive success (typically defined through natural conception) in GCs is a positive predictor of success following IVF. Additionally, as in earlier studies (Ruiz-Alonso et al., 2013; Ruiz et al., 2014; Hashimoto et al., 2017; Tan et al., 2018), there was an overall trend of prereceptivity, with a majority of GCs requiring one more day of progesterone exposure prior to reaching the WOI.

A pregnancy rate of 81.1%, implantation rate of 70.3%, and ongoing pregnancy rate of 56.8% were achieved in the first GC-pET cycles following ERA testing. These rates are superior to previous outcomes reported in gestational surrogacy cycles (Anchan et al., 2013; Murugappan et al., 2018; Fuchs et al., 2020). For instance, a review of SART (Society for Assisted Reproductive Technology) registry cycle data revealed a clinical pregnancy rate of 48.4% for GC-FET cycles (Murugappan et al., 2018), however, this data pertains to GC cycles taking place between January 2004 and December 2013, whereas our study was carried out between July 2019 through November 2020, leading to obvious bias due to more recent technological advancements within the IVF process (embryo vitrification, PGT-A, culture systems) paired with other factors such as the progression to eSETs with blastocyst embryos, hence omitting the possibility of a more direct and meaningful comparison.

The data was further stratified to examine outcomes associated with known euploid pETs, in which a slight improvement in clinical rates were observed. This trend was not unexpected, as numerous studies have demonstrated the benefits of PGT-A and subsequent transfer of euploid

embryos (Gianaroli et al., 1999; Munné et al., 1999; Scott et al., 2013; Rubio et al., 2017). However, despite the transfer of good quality euploid blastocysts in previous studies, implantation failure still occurred in approximately one third of cycles (Fragouli et al., 2010; Yang et al., 2012; Forman et al., 2013). Considering our results, we further demonstrate the importance of both a competent embryo (as defined by PGT-A) and receptive endometrium (as defined by ERA) in achieving successful implantation. Similar to our findings, Tan and colleagues (2018) reported improved clinical rates when controlling for both aneuploidy with PGT-A and endometrial receptivity with ERA. Currently, our group is conducting a large, multicenter, RCT evaluating the value of PGT-A in combination with a pET guided by ERA.

Optimizing gestational surrogacy cycles is imperative as the number of these cycles have quadrupled over the last decade, accounting for tens of thousands of infants born (Perkins et al., 2013). Integration of technological advances, such as ERA, to not only the IVF cycles involving infertile patients but also GCs may be crucial to accomplishing this. Although the clinical rates in our study exceed those previously reported for GCs, and are further enhanced when applying PGT-A, there is still room for improvement. Statistically higher reproductive rates in infertile women undergoing IVF are achieved when ensuring a healthy endometrial microbiome composed of a high percentage of lactobacillus and void of bacterial pathogens (Moreno et al., 2016). The presence of bacterial pathogens in the uterine cavity should not only be considered for the infertile population but also the fertile population, as the prevalence of bacterial induced diseases such as chronic endometritis has been reported to be as high as 19% in this specific group (Cicinelli et al., 2005; Farooki 1967). Hence, applying techniques to assess the reproductive potential of a women should not be limited to just those with infertility but should

also be applied to the fertile population. Based on our data, relying on a history of pregnancy through natural conception in GCs as a positive prognosticator for IVF success, may not translate as clearly as we once thought. Unfortunately, this assumption may have hindered us in the past to optimize carrier cycles to a greater degree.

Not only do we need to consider clinical outcomes in GC cycles, but overall management of these cases, including factors such as time, finances, and the physical and psychological health of all parties involved. By ensuring transfer of embryos to a GC with a receptive endometrium, we are potentially decreasing the number of failed cycles. The cost-effectiveness estimation per baby at home at the first transfer attempt in IVF patients has previously been performed, showing a savings of approximately $8,000 when undergoing a pET guided by ERA versus a standard FET at conventional timing (Simón et al., 2020). Based on the findings presented here, we can assume that the same general cost benefit of pET over FET would apply to GC cycles, but perhaps to an even greater extent, as there are additional costs associated with GC cycles (travel costs, agency costs, etc), beyond those of non-GC cycles.

The cost benefit is not the only advantage of a reduced number of transfer cycles in order to achieve a baby at home. The psychological and physical effects experienced by both IPs and GCs should also be considered. For instance, if a healthy pregnancy can be achieved sooner, the likelihood of transferring multiple embryos at a time, and hence the chance of multiple pregnancy, will be reduced. Based on our own experience, IPs and GCs will consider transferring more than one embryo at a time, as the number of failed cycles increase, an observation also reflected by previous reports that show 80% of GC cycles in the early 2000s involved the transfer of 2 or more embryos, leading to high multiple birth rates, and consequently increased obstetric

and neonatal risk (Conde et al., 2000; Francois et al., 2005; Spiliopoulos et al., 2011; Perkins et al., 2016). Fortunately, the field as a whole has shifted to eSET, though the transfer of multiple embryos in both GC and non-GC cycles still occur today.

This study has limitations. The first being the retrospective design which unavoidably comes with its own inherent biases. Secondly, the small sample size along with the lack of a control group of GCs without a pET, precludes any definitive conclusion. With that said, a larger, prospective study that includes a comparable control group is needed. Controlling for additional influencers of implantation, such as endometrial microbiota, would further assist with objectively evaluating the benefits offered by advanced techniques in endometrial assessment and personalized medicine in IVF to this group of patients

To the best of our knowledge this is the first report evaluating the utility of ERA in GCs in order to detect the timing of endometrial receptivity. We demonstrated that a significant proportion of GCs have a displaced WOI and may benefit from ERA testing to guide a pET. Furthermore, when controlling for both embryonic aneuploidy and endometrial receptivity, clinical rates improved further. The implication of our findings not only translates to better clinical rates, but also enhances efficiency of treatment as a whole by possibly reducing the psychological and financial burdens that are inherent to gestational surrogacy cycles. As the number of carrier IVF cycles increase year after year, optimization is key. Continuing to exploit technological advancements in the field of IVF and applying them in GCs will most certainly lend to further improvements in these cycles.

10.0 General Discussion

The field of IVF has evolved considerably since the birth of the first IVF baby nearly 44 years ago (Steptoe and Edwards, 1978). The practice itself has become more efficient, resulting in improved clinical rates (Eskew and Jungheim 2017). With that said, live birth rates following IVF are still only 25-30% per started cycle worldwide (Adamson et al. 2018). Over the last four decades, a great deal of emphasis, and hence advancements in the field, has related to optimizing the embryo; and although vast improvements have been achieved, we can still do better. Recently, the introduction of precision medicine has refocused our efforts to providing targeted individualized treatment, wherein we should not only consider and apply this fundamental principle to the embryo, but to the entire IVF process itself. As implantation is the major rate limiting step to the success of an IVF transfer cycle (Edwards 2006), it is imperative that we start here, by applying technologies that leverage personalized medicine to the two crucial components of this process: the embryo and the endometrium.

10.1 Progress with Respect to Specific Aims

This thesis consists of seven specific aims that cover the impact of controlling for embryo aneuploidy and endometrial receptivity, both independently and together, and their effects on clinical outcomes following IVF. This thesis was successful in the fulfillment of these aims in that, the research presented herein provided novel insight into the basic understanding of chromosomal aneuploidy in embryos and endometrial receptivity, along with demonstrating the positive clinical impact when controlling for both of these factors. In summary:

- I provided a preliminary assessment of aneuploidy rates between the polar, mid and mural TE in which higher aneuploidy rates were observed in the polar TE, possibly suggesting a mechanism necessary for human implantation (Taylor et al., 2019).
- I established an improved pregnancy rate following a euploid pET guided by ERA in patients with previous failed implantation after euploid ET, showing an optimization in outcome when controlling for both embryo aneuploidy and endometrial receptivity together (Leondires et al., 2018).
- I demonstrated similar clinical outcomes in day 5 versus day 6 SETs when endometrial factor is controlled, suggesting an equal clinical potential between blastocysts formed on day 5 and day 6 (Stankewicz et al., 2021).
- I reported similar clinical outcomes in pETs guided by transition phase results versus those guided by a transcriptomic result representing optimal receptivity, further verifying the efficacy of transition phase profiles and the significance in small shifts to endometrial receptivity timing.
- I reported the narrowest window of implantation documented by transcriptomic profiling, lasting less than 12 hours, indicating the importance of identifying the pWOI and representing the uniqueness of the WOI among women.
- I showed the reliability and reproducibility of transcriptomic profiling for endometrial receptivity, further supporting the clinical utility of this method for endometrial dating and subsequent transfer planning (Stankewicz et al., 2018).
- I showed a high displacement of the WOI in fertile patient population, by means of examining GCs, signifying that all women can suffer a displacement when undergoing IVF

treatment and suggesting clinical utility of applying ERA to all ART patients in order to properly account for WOI timing to help ensure optimal outcomes.

In my work looking at aneuploidy rates in various areas of the trophectoderm (chapter 3), there was a clear trend indicating a higher rate of chromosome abnormalities seen the further the biopsy was taken from the ICM. Given that mosaicism is somewhat common in preimplantation blastocysts (McCoy et al., 2015; Starostik et al., 2020), it is necessary to comprehend if and where its occurrence is normal and hence necessary for proper embryonic development and implantation. Addressing this concept will help support the introduction of a standard biopsy procedure in the IVF laboratory, one that is deemed both effective and safe, and that reduces the potential of 'sampling error,' thus providing more consistent reporting and clinical outcomes among clinics. This standardization may increase the utilization of PGT-A by remedying some of the concerns held by those who currently oppose the practice, such as a presumed high false positive rate (Gleicher et al., 2021) and adverse effect of the biopsy procedure on reproductive potential (Gleicher et al., 2017), which in turn may lead to greater widespread, optimization of the IVF process by ensuring the transfer of a predetermined 'euploid' embryo. Additionally, not only is standardization necessary within the IVF laboratory, but also across PGT-A reference laboratories. For instance, inconsistencies in rates of aneuploidy and clinical outcome in the STAR Trial (Munné et al., 2019) were not solely due to differences among the IVF clinics participating in this study, but also the various PGT-A reference labs used. Unfortunately, these inconsistencies ultimately contributed in some part to the failure of this RCT to show an obvious benefit of PGT-A for all patients undergoing IVF. With that stated, different reference labs use different testing platforms, algorithms, and cut-offs for aneuploidy and mosaicism calling. The

implementation of the most optimal standard practice throughout the aneuploidy screening process is necessary to promote the initiative of PGT-A on a universal scale.

On the other hand, the findings in chapter 3 may also explain why not all euploid embryos implant (Yang et al., 2012; Forman et al., 2013b), as this so called 'sampling error' can occur in which the euploid TE biopsy sample does not accurately represent the chromosomal status of the ICM (Gleicher et al., 2017). With that said, research has indicated a high concordance between these two regions (Johnson et al., 2000; Capalbo et al., 2013), so this may be a minute concern to begin with. However, we still need to better address concordance within the TE itself, especially considering that the aforementioned studies did not distinguish where within the TE biopsy occurred, potentially skewing the observed concordance rates. Additionally, with the advent of NGS and its increase in the detection of mosaicism (Garcia et al., 2020), the biopsy procedure has become an even more important variable to consider. For instance, not only do we need to contemplate the location of biopsy but also the number of TE cells sampled. The following scenario describes an example of a 'sampling error' in regard to the number of cells biopsied and the resulting discrepant results. If embryologist 'A' biopsies two cells from the blastocyst and both are normal or abnormal, mosaicism will not be detected. Yet, if embryologist 'B' biopsies 10 cells from the blastocyst and six cells are aneuploid and four cells are euploid, mosaicism will be detected simply due to the increased number of cells biopsied. Emerging research suggests that the majority of abnormalities at the blastocyst stage are mitotic in origin, suggesting that with enough cells present, PGT-A results could be altered (McCoy et al., 2015). Defining the location within the TE that would give the most accurate ploidy status associated with the resulting fetus and defining the optimal number of cells to biopsy, while at the same

time determining the significance of aneuploid cells in the implantation process, would certainly contribute to confirming the best way to standardize embryo biopsy to improve implantation following IVF. Once defined, this best practice could be applied in IVF laboratories and incorporated into guidelines set forth by societies such as PGDIS, to further ensure the safety and accuracy of PGT and its application into clinical practice. Nevertheless, other sources of implantation failure beyond embryonic aneuploidy exist, such as endometrial factor (Zeyneloglu et al. 1998; Simón and Pellicer 2000; Demirol and Gurgan 2004; Penzias 2012; Ruiz et al. 2012) or embryonic mitochondrial stress (Diez-Juan et al., 2015; Fragouli et al., 2015) that still need to be taken into consideration.

In my work pertaining to evaluating IVF cycle outcomes while controlling for both embryo aneuploidy and endometrial receptivity (chapter 4), a high rate of displacement of the WOI was observed in patients with at least one previous failed euploid ET. However, when adjusting for this displacement and transferring a PGT-A tested euploid embryo at the time of established receptivity, an 88% pregnancy rate was achieved (Leondires et al., 2018). These findings demonstrate the importance of controlling for endometrial receptivity during ET, in addition to suggesting that a fair rate of failed PGT-A transfers may not be due solely to an innate drawback relating to PGT-A, such as a 'sampling error,' but instead due to our lack of personalizing transfers based on the patient's unique timing of her WOI (Ruiz et al., 2013; Ruiz et al., 2014). When considering the results of chapters 3 and 4 together, patients may benefit from a combined treatment that involves a more standardized practice of PGT-A for effective euploid embryo selection and analysis of endometrial receptivity to decipher the best timing for ET. This dual treatment plan would increase the likelihood of implantation success, while decreasing the

occurrence of euploid embryo wastage, due to transfer into a non-receptive endometrium. The model of controlling for both embryo aneuploidy and endometrial receptivity was previously explored in a retrospective study by Tan et al., (2018) who also showed that a significant proportion of patients with a history of implantation failure of a known euploid embryo had a displaced WOI, and when subsequently transferred according to their pWOI realized improved clinical outcomes. With that said, another group reported no significant improvement in outcome when applying ERA to euploid FET (Cozzolino et al., 2020), though as shown in chapter 8, test compliance is necessary when employing ERA (Stankewicz et al., 2018), meaning it is unclear from retrospective studies if proper protocol replication, control of endogenous progesterone, and execution of the pET recommendation was carried out in each cycle analyzed. In any case, this combined treatment plan could be put into practice quite easily, as many IVF cycles, particularly those involving cases of RIF, currently incorporate either PGT-A or ERA, albeit not simultaneously. At the very least, this treatment option should be made available to patients who have already suffered a failed euploid transfer, as the data suggests that there is a high likelihood that these women possess a displaced WOI.

Furthermore, not only did my research reveal a high rate of endometrial displacement in infertile patients with previous failed euploid transfers, but I also demonstrated a high non-receptivity rate in the fertile population when studying GCs. This finding was surprising as many GCs have proven fertility (Ethics Committee of the American Society for Reproductive Medicine, 2018), though generally through natural conception, hence, suggesting a possible induced state of altered receptivity when undergoing HRT in preparation for the ART transfer. Taken together, the results of chapter 4 and 9 demonstrate the existence of a pWOI among all women, again,

emphasizing the importance of controlling for endometrial receptivity during the implantation process. These findings are especially important to consider as PGT-A and GCs represent "last resort" treatment options, often only implemented following the failure of more rudimentary forms of therapy (Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology, 2018; Ethics Committee of the American Society for Reproductive Medicine, 2018). Additional research regarding GCs and endometrial receptivity is imperative, as the number of gestational surrogacy IVF cycles continue to increase year after year (Perkins et al., 2016; Murugappan et al., 2017), along with the innate intricacies that are exclusively associated with GC cycles, making optimization key on many levels (costs, time, psychological considerations). Moreover, the data supports, implementing endometrial dating via transcriptomic profiling for all women undergoing IVF, as even a previous successful pregnancy does not appear to be a reliable marker for future reproductive ART success.

The idea of a unique WOI was further exhibited in my work studying patients presenting with a transition phase transcriptomic profile (chapter 6) and in the case study I reported on a patient who possessed a narrow WOI that lasted less than 12 hours (chapter 7). Both studies support earlier work that demonstrated the existence of small alterations to a patient's WOI (Diaz-Gimeno et al., 2017), in addition to debunking the classical assumption that the WOI remains open for 4 to 5 days (Lessey 2011). Additionally, my work further validates transcriptomic studies that have proclaimed the existence of a much narrower WOI, that lasts on average 29-36 hours (Rincon et al., 2018). The data presented here provides clinical proof on the basis of successful implantation when properly accounting for these small shifts at time of embryo transfer. These findings are important, as patients can benefit from understanding the exceptionally unique

factors pertaining to their WOI, like a narrow WOI, ensuring a personalized and precise ET at the optimal time to facilitate successful implantation. This concept could be exceptionally useful in cases where other advanced methods of treatments (such as PGT-A and GCs, as in chapter 4 and 9) have been applied without success (Yang et al., 2012; Forman et al., 2013b; McGovern et al., 2017), while further preventing unnecessary wastage of good quality embryos.

Although displacements have been detected with transcriptomic analysis and efficacy of personalized transfers based on the results have been shown (Ruiz et al., 2013, Ruiz et al., 2014, Simón et al, 2020), it is necessary to assess the reproducibility and confidence of these findings in the clinical setting to further accept the practice and derive greater understanding that will assist in optimizing IVF outcomes. Through the novel review of a patient's results (Cho et al., 2018) (chapter 8), I was able to solidify further the clinical utilization of transcriptomic analysis for endometrial receptivity by showing consistency of results from cycle to cycle and at various timings of endometrial biopsy. In an earlier study by Diaz-Gimeno et al., (2012) endometrial biopsies from the same patient, under the same cycle conditions, were performed up to 40 months apart and tested for receptivity with ERA. The authors concluded that ERA for endometrial dating and receptivity provided completely reproducible results over time, with 100% consistency rates achieved. This confirmation of reproducibility in turn, allows for other factors of the IVF transfer to be more reliably studied, as in the case with my research assessing the clinical potential of day 5 versus day 6 blastocysts (chapter 5). When ensuring a transfer into a receptive endometrium, we can better examine the embryonic contribution to implantation. Here I established similar clinical rates between the pET of day 5 versus day 6 blastocysts, indicating similar clinical potential regardless of day of blastulation (Stankewicz et al., 2021).

Based on these results we can expand our research, focusing on alternative or perhaps even novel parameters of embryo quality and potential when selecting the best embryo(s) for transfer. Examples of this include morphology (Irani et al., 2017; Irani et al., 2018), morphokinetics (Campbell et al., 2013b), mitochondrial load (Diez-Juan et al., 2015; Fragouli et al., 2015), and niPGT-A (Rubio et al., 2020), all of which, for instance, have been shown to distinguish euploid embryos with the greatest implantation potential from those with lower potentials. However, in many IVF laboratories the current scenario involving the prioritization of blastulation day over the above-mentioned methods, may be providing a disservice to patients, in terms of reduced clinical rates per transfer and unnecessary oocyte retrieval cycles to obtain more day 5 blastocysts. Fortunately, as supported by the data presented, this practice can be easily adjusted, as any one or a combination of the selection methods mentioned above can be readily incorporated into any IVF laboratory setting.

All in all, the findings making up my thesis demonstrate how we can further optimize the IVF process through the implementation of personalized medicine and controlling for both embryo aneuploidy and endometrial receptivity. Here I have added to the work that supports the objective of exploring ways to overcome the major rate limiting step of IVF, that is, by increasing the chance of implantation for all women undergoing ART (Figure 29).

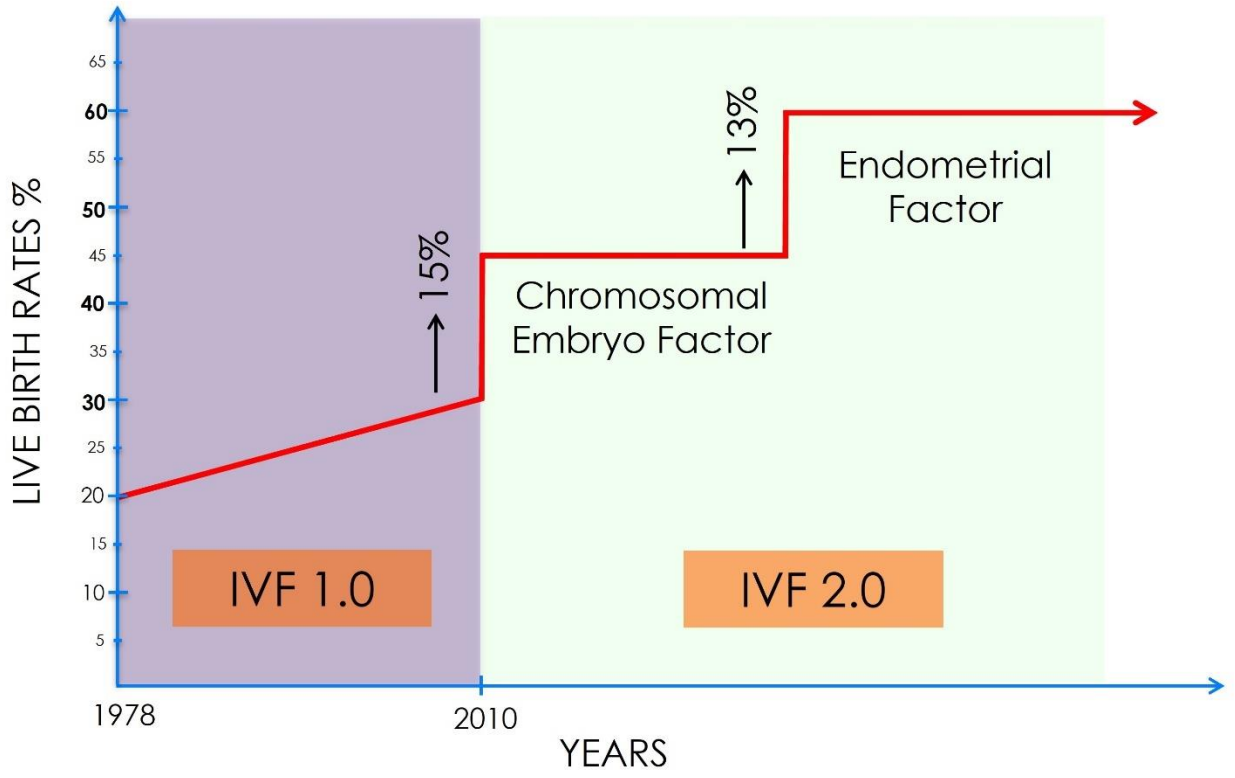


Figure 26. Optimization of human IVF since the birth of the world's first IVF baby in 1978. Prior to 2010, aneuploidy screening with PGT-A and endometrial receptivity analysis by transcriptomic profiling was not commonplace in IVF treatment. In a period referred to a "IVF 1.0" live birth rates reached about 30%. In 2010, when controlling for chromosomal factor was more commonly employed in IVF, a 15% increase in live birth rate is observed. A few short years later, controlling for endometrial factor in terms of receptivity was introduced, leading to a 13% increase in live birth rates. Considering that at a basic level, implantation requires a chromosomally competent embryo and receptive endometrium, when controlling for both factors simultaneously ("IVF 2.0"), IVF live birth rates can be enhanced to a greater degree.

10.2 Future work

There are still questions that need to be answered in order to optimize IVF treatment outcomes to the highest possible degree. Much of this research starts with the basic science underlying embryo development, endometrial receptivity, and human implantation. Due to the nature of these subjects, it is somewhat difficult to carryout basic research within this arena (Legro 2011). Many times, we rely on clinical based research to guide new technologies and treatment plans in

our field, with recent examples including observational studies examining the endometrial microbiome (Moreno et al., 2016; Moreno et al., 2022) and niPGT-A concordance trials (Rubio et al., 2020). However, addressing the basic science, such as determining the occurrence and role of embryonic mosaicism, will allow us to leverage this knowledge appropriately in the clinical setting.

As previous research has suggested mosaicism is a common feature in early human development (McCoy et al., 2015; Starostik et al., 2020), one question we must answer: is mosaicism present in all preimplantation embryo? If so, what is the role of mosaicism? Perhaps, as previously mentioned, we will confirm that a line of abnormal cells in an otherwise normal embryo is necessary to facilitate proper invasion during uterine implantation, a theory based on a report of induced aneuploidy of the cytotrophoblast (Weier et al., 2005)? This theory makes sense as aneuploidy helps to evade a host's immune response (Davoli et al., 2017) and, more specifically, in the case of human embryonic implantation upregulates immune response genes (Starostik et al., 2020). This particular question may be answered, in part, by the widespread implementation of niPGT-A. Recent studies suggest that the cell free DNA found in the spent blastocyst culture media arise from both the ICM and TE (Rubio et al., 2018; Rubio et al., 2020), as clinical outcomes were significantly better when patients transferred an embryo diagnosed euploid by both TE biopsy and niPGT-A versus an embryo diagnosed euploid by TE biopsy, but aneuploid by niPGT-A (Rubio et al., 2018). Currently, research pertaining to cell mapping via electron microscopy and live imaging is being carried out by the Plachta lab at University of Pennsylvania to better understand the origin of cell free DNA. If confirmed that both the ICM and TE

contribute cell free DNA to spent culture medium, we can reassess the presence and patterns of mosaicism in human blastocysts.

Perhaps in the future, possessing a better understanding of mosaicism patterns in conjunction with the widespread employment of niPGT-A and cell mapping at the clinical level, cell free DNA originating from the ICM and TE can be sorted through an application based on a methodology similar to noninvasive prenatal testing (NIPT). In the case of NIPT, a maternal blood sample is taken as early as 10 weeks gestation. The maternal and fetal DNA is sorted, and the fetal DNA is then assessed for the presence of chromosomal aneuploidies (Bianchi et al., 2012). Employing a similar method to cell free DNA arising from ICM and TE, could permit the selection of a blastocyst for transfer based upon a confirmed euploid ICM result, as again it is the ICM that eventually gives rise to the fetus (Goldstein and Kiehart 2015). Cell mapping plus niPGT-A can also be applied to understand mosaic patterns within the TE itself, confirming if induced aneuploidy within the polar TE is a normal event necessary to facilitate endometrial invasion during implantation.

Deciphering patterns in mosaicism can also assist with establishing the best practice for DNA sampling for PGT-A. If the niPGT-A method allows for the analysis of DNA from both the ICM and TE, then we can gather a better understanding of the reproductive potential of that embryo versus analyzing DNA from the TE alone. Additionally, there is evolving evidence indicating that the TE biopsy procedure may be a significant contributor to artefactual PGT-A results (Popovic et al., 2020). Recently, data was presented showing that 40.5% of embryos that were initially diagnosed as chaotic, defined as having 6 more chromosomal aneuploidies, were subsequently

diagnosed as euploid when re-biopsied (Rodrigo et al., 2021). Technical limitations such as low number of cells in the TE biopsy or presence of damaged cells in the biopsy were proposed sources of the discrepancies detected. To test this theory, both niPGT-A and TE biopsies can be performed for the rebiopsy of the initially diagnosed chaotic blastocyst, allowing for a comparison of the results and omission of an artefactual finding associated with the TE biopsy. These data would support the replacement of TE biopsy with niPGT-A, or at the minimum the dual supplementation of both methods, in an attempt to increase the accuracy of the test and lead to improved clinical outcomes. Still, more research needs to be conducted, as niPGT-A comes with its own inherent drawbacks such as the requirement for culture to day 6 and the significant effect that maternal cell contamination from the presence of cumulus cells within the spent medium has on the accuracy of this method (Rubio et al., 2020). However, addressing these shortcomings could lead to an even greater understanding of mosaicism, wherein if we could test the cell free DNA earlier in preimplantation development, then based on the extent of mosaicism detected on day 3 of day 4 of development, we could potentially determine which embryos are likely to have confined mosaicism versus general mosaicism, again allowing for the selection of the most optimal embryo(s) for transfer.

As previously stated, the research of mtDNA levels in preimplantation development as a prospective marker for reproductive potential has stalled. Future work could involve establishing the relationship between mtDNA and mosaic levels. Earlier mtDNA studies applied aCGH, which did not detect the presence of mosaicism (Diez-Juan et al., 2015; Fragouli et al., 2015). Using NGS to effectively detect mosaicism, it is possible that we would observe increasing mtDNA levels (signifying greater mitochondrial stress, and poorer reproductive outcome) with increasing

percent of mosaicism. This would make sense, as low-level mosaic embryos (<50%) have comparable clinical rates to embryos diagnosed as euploid and in comparison, high level mosaic embryos (>50%) have significantly poorer clinical rates (Spinella et al., 2018; Capablo et al., 2021). Determining if a relationship exists could not only reveal a feasible and biological cause and effect explanation between mitochondrial stress and mosaicism but would also lend the opportunity for future research with the objective to reduce mtDNA levels, such as reducing environmental stressors in preimplantation culture, and potentially rate of mosaicism. In any case, all of the abovementioned reasons would support the reintroduction of mtDNA levels as a marker embryonic implantation potential.

Reducing the rate of mosaicism and possibly aneuploidy in the first place would be a monumental achievement for human reproduction in the IVF setting. Studies show that a high percentage of preimplantation aneuploidy is due to mitotic errors (McCoy et al., 2015). This most likely indicates an origin other than advanced maternal age. A probable external source for mitotic error pertains to culture conditions. For instance, supplementing culture media with antioxidants is associated with higher clinical outcome rates (Ueno et al., 2021). Perhaps the biological explanation for these findings relates to the reduction of mitotic errors during embryo culture. As environmental insults such as the accumulation of reactive oxygen species can potentially lead to defective spindle formation and chromosome segregation during meiosis (Tarin et al., 1996), conceivably the presence of reactive oxygen species in culture media can have the same effect on mitosis. This would be a relatively straightforward study performed in the clinical IVF laboratory in conjunction with PGT-A, and if shown a benefit, can be easily

implemented on a global scale, as culture media containing antioxidants are already commercially available.

Furthering our knowledge on embryo-endometrium crosstalk and implantation will also allow us to optimize treatment options further. However, we are limited to *in vivo* models due to the obvious ethical concerns surrounding the study of human implantation at the *in vitro* level and the inability to incorporate subjects outside of clinical treatment cycles who are undergoing IVF with the purpose of achieving conception (Legro 2011). Nevertheless, transcriptomic studies have permitted us to broaden our understanding of endometrial receptivity and implantation (Borthwick et al., 2003; Reisewilk et al., 2003; Ponnampalam et al., 2004, Talbi et al., 2005). The use of gene expression profiling has revealed the transcriptomic signature of the WOI (Reisewilk et al., 2003), defining this specific period of time as a "transcriptional awakening process," in which most genes are found to be upregulated (Díaz-Gimeno et al., 2011). One area I touched upon in my thesis, though requires additional study, is personalization according to not only the placement of the WOI but its duration. Not only is defining a narrow WOI important, for obvious clinical reasons, but understanding which patients have an extended WOI can also be helpful. For instance, my work showed a significant WOI displacement in GCs, who represent a seemingly fertile population of women. However, this does not make sense, as previous studies have reported higher clinical rates when using GCs (Anchan et al., 2013; Murugappan et al., 2018; Fuchs et al., 2020), implying that embryonic-endometrial dysynchrony should not be an issue for this particular group. I proposed that perhaps the HRT protocol induces a state of non-receptivity in this group (Silverberg et al., 1991), as these women often have proven fertility through natural conception (Ethics Committee of the American Society for Reproductive Medicine, 2018).

Though, it is also possible that fertile women possess a longer WOI, allowing for implantation to occur over many hours or even days versus infertile women who have a more restrictive timeframe for implantation, thus requiring enhanced precision of the timing of transfer. Currently, the only way to derive the length of the WOI is through the acquisition of multiple data points from endometrial biopsies performed at various times. This is not an ideal practice as the patient must undergo multiple biopsies, enduring discomfort, in addition to expending time and finances. Yet, if we can derive this data by an alternative means, then we can better understand the WOI length in different populations of women, while at the same time providing enhanced guidance regarding pETs. One method that may assist with answering this question is the use of endometrial fluid for ERA, thus omitting the need for endometrial tissue extraction. In this scenario, multiple endometrial fluid samples can be taken per cycle and without additional discomfort, allow us to not only define where the WOI exists, but also its duration.

Beyond determining the WOI length via the examination of multiple endometrial fluid samples, this method of sample collection needs to be studied further. A prime advantage of this method would be the potential time and cost savings for the patient. A previous study demonstrated the benign nature of this collection method, when the authors sampled endometrial fluid from a patient that unbeknownst to them was 4 weeks pregnant (Moreno et al., 2020). This pregnancy continued uneventfully and resulted in a healthy, full-term live birth. Based on this, endometrial fluid could be sampled and examined in the same cycle as the intended transfer, without inflicting a detrimental effect on a resulting pregnancy, to determine the optimal implantation timing. Furthermore, endometrial fluid analysis has been shown to effectively assess the endometrial microbiome (Moreno et al., 2021), wherein its analysis at the molecular level is

already proving to be helpful in optimizing IVF outcomes (Cicinelli et al., 2015; Moreno et al., 2016; Moreno et al., 2022). With that said, if it was possible to verify that the endometrial microbiome was optimal for transfer in the same month of sampling, this could lead to further improved outcomes, as the stability of the microbiome can shift over time (Gejar et al., 2012) due to factors such as, hormone fluctuations, sexual intercourse, hygiene, and antibiotic use; meaning what we find in one cycle may not represent the microbial status in another.

Further regarding the endometrial microbiome, previous work demonstrates that patients with a Lactobacillus dominated microbiome (90% or more of the microbiome consisting of Lactobacillus) experienced significantly higher pregnancy rates, implantation rates, and ongoing pregnancy rates than those patients with a non-Lactobacillus dominated microbiome (<90% of the microbiome composed of Lactobacillus) (Moreno et al., 2016). Not only does it appear that Lactobacillus plays an important role in establishing a pregnancy, but also maintaining a pregnancy, as miscarriage rates differed between the groups, with a rate of 16.7% in the Lactobacillus dominated group versus 60% in the non-Lactobacillus dominated group (Moreno et al., 2016). One area of study that our group is currently exploring is the endometrial microbiome at time of miscarriage when the fetus is determined euploid by products of conception testing. In any case, an optimal microbiome in other areas of the human body is associated with overall improved health status and even a decrease in symptoms when the 'good' bacteria are restored, as is the case with autism (Marijnissen et al., 2020). To that extent it appears obvious that the same effects could be expected in the endometrium during pregnancy (Moreno et al., 2016; Moreno et al., 2022).

Future work to normalize the WOI should be conducted. In the United States, approximately 2 out of 3 women of reproductive age are overweight (BMI ≥25kg/m2) or obese (BMI ≥30kg/m2) (Flegal et al., 2012). Obesity is associated with inferior fecundity and poorer reproductive outcomes in ART (Maheshwari et al., 2007). Previous work has shown that the rate of endometrial receptivity by ERA decreases with increasing BMI (Bellver et al., 2013). As the endometrium is a hormonally regulated tissue (Cha et al., 2013) and BMI can affect the metabolism of hormones (Singla et al., 2010), in theory the transcriptomic signature may be affected by a shift in a woman's BMI. To date the effects of weight loss on endometrial receptivity at the transcriptomic level have not been studied. The hypothesis to test here would be does the WOI normalize in women after weight loss and who procure a normal BMI? The study design would require an endometrial biopsy to be taken prior to losing weight and second biopsy following significant weight loss and/or reaching a normal BMI.

Lastly optimizing IVF goes beyond just achieving better clinical outcomes. It is imperative that we make this process as efficient as possible from all perspectives, including patient time and money. Although independent cost-effectiveness studies have been carried out in the past regarding PGT-A (Neal et al., 2018; Somigliana et al., 2019) and ERA (Simón et al., 2020), there still lacks an analysis when utilizing both of these methods together. This would be quite straightforward to carry out and would provide additional guidance for patients and physicians when making clinical decisions regarding treatment plans. Moreover, other technologies to optimize IVF outcomes could also be considered in these analyses, such as the addition of endometrial microbiome testing or the application of niPGT-A methods with endometrial control.

10.3 Personal Perspectives

I sincerely believe that the primary means to optimize IVF outcomes for patients is through precision medicine. This approach is currently being carried out in other medical fields including cancer screening and treatment, therapeutic options for chronic diseases such as Cystic Fibrosis, and as mentioned above, individualized intervention for autism. Already we are realizing the benefits of personalized treatment plans with IVF, including the existing standard practice of appropriate hormone protocols for ovarian stimulation based on an individual's baseline levels, antral follicle count, and overall physiological response. Taking this a step further and leveraging genetics can give us advanced insight regarding the best treatment plans for each patient undergoing ART; a concept demonstrated here when considering the combined results of chapter 4 and 7 in which patients benefitted from controlling for both embryo aneuploidy and their pWOI during the IVF transfer to optimize implantation. With this in mind, if we want to achieve significant improvements in IVF outcomes, we first must accept the uniqueness that pertains to all patients and treat accordingly.

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