ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
ΙΑΤΡΙΚΗ ΣΧΟΛΗ
&
ΠΑΝΕΠΙΣΤΗΜΙΟ ΔΥΤΙΚΗΣ ΑΤΤΙΚΗΣ
ΤΜΗΜΑ ΜΑΙΕΥΤΙΚΗΣ

ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ
«ΕΡΕΥΝΑ ΣΤΗ ΓΥΝΑΙΚΕΙΑ ΑΝΑΠΑΡΑΓΩΓΗ»

ΤΙΤΛΟΣ ΜΕΤΑΠΤΥΧΙΑΚΗΣ ΔΙΠΛΩΜΑΤΙΚΗΣ ΕΡΓΑΣΙΑΣ

(MASTER THESIS TITLE)

Investigating the Value of Age 35 as a Cut-off Point regarding Decision Making in In Vitro Fertilization (IVF) Treatment A Retrospective Data Analysis

Διερεύνηση της Αξίας της Ηλικίας των 35 ετών ως Κατώφλιο Όριο στην Λήψη Κλινικών Αποφάσεων κατά τη διάρκεια της Θεραπείας Υποβοηθούμενης Αναπαραγωγής (IVF): Αναδρομική Μελέτη Παρατήρησης

Όνομα φοιτητή: Γρηγοριάδης Σωκράτης B.Sc. (First-Class Honours)

Ιδιότητα: Βιολόγος-Ειδικευόμενος Κλινικός Εμβρυολόγος

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Αθήνα, 2019

Η παρούσα Μεταπτυχιακή Διπλωματική Εργασία είναι
Αφιερωμένη ως ένδειξη απεριορίστου Σεβασμού και
Ευγνωμοσύνης στην Οικογένεια μου

Στην Μητέρα μου Κωνσταντίνα
Στον Πατέρα μου Βασίλη
Στον Αδελφό μου Θεοφάνη-Δημήτρη

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Πρόλογος

Η παρούσα Μεταπτυχιακή Διπλωματική Εργασία εκπονήθηκε στο Εργαστήριο Πειραματικής Φυσιολογίας της Ιατρικής Σχολής του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών σε συνεργασία με την Ιδιωτική Κλινική ΄΄Γένεσις Αθηνών΄΄, στο πλαίσιο του Προγράμματος Μεταπτυχιακών Σπουδών ΄΄Έρευνα στην Γυναικεία Αναπαραγωγή΄΄, της Ιατρικής Σχολής του Ε.Κ.Π.Α, υπό την επίβλεψη της Επίκουρης Καθηγήτριας της Ιατρικής Σχολής Δρ. Μάρας Σιμοπούλου κατά το Ακαδημαϊκό Έτος 2018-2019. Με την ολοκλήρωσή της θα ήθελα να ευχαριστήσω θερμά την επιβλέπουσα καθηγήτριά μου που με δέχτηκε στην ερευνητική της ομάδα με σκοπό να πραγματοποιήσω τα πρώτα μου βήματα στον χώρο της Υποβοηθούμενης Αναπαραγωγής, για την εμπιστοσύνη που μου έδειξε καθ' όλη την διάρκεια της συνεργασίας μας, και για την ουσιαστική καθοδήγηση και τις πολύτιμες συμβουλές που μου παρείχε, οι οποίες αποτελούν πολύτιμα εφόδια για την μελλοντική μου πορεία όχι μόνο ως επιστήμονα αλλά και ως άνθρωπο.

Θα ήθελα να εκφράσω τις θερμές μου ευχαριστίες στα μέλη της τριμελούς εξεταστικής επιτροπής, στον Καθηγητή Ενδοκρινολογίας και Διευθυντή του Π.Μ.Σ ΄΄Έρευνα στη Γυναικεία Αναπαραγωγή΄΄ Δρ. Γεώργιο Μαστοράκο και στον Καθηγητή Πειραματικής Φυσιολογίας και Διευθυντή του Εργαστηρίου Πειραματικής Φυσιολογίας Δρ. Μιχαήλ Κουτσιλιέρη για την ύψιστη τιμή προς το πρόσωπό μου να συμμετέχουν στην επιτροπή. Μετά απεριορίστου σεβασμού θα ήθελα να εκφράσω την εκτίμηση και τις ευχαριστίες μου προς τα πρόσωπά τους για τις γνώσεις και την καθοδήγηση που μου παρείχαν. Εξαιρέτως, θα ήθελα να ευχαριστήσω τον Καθηγητή Δρ. Γεώργιο Μαστοράκο για την στενή παρακολούθηση και τις πολύτιμες συμβουλές που μου παρείχε ακούραστα καθ' όλη τη διάρκεια της φοίτησής μου.

Ιδιαίτερη αναφορά θα ήθελα να πραγματοποιήσω στους υποψήφιους Διδάκτορες της Ιατρικής Σχολής Ευάγγελο Μαζιώτη, Άννα Ραπάνη και Πολίνα Γιαννέλου και στην Μεταπτυχιακή Φοιτήτρια Πετρούλα Τσιούλου που αποτέλεσαν τους οδηγούς μου σε αυτό το όμορφο ''ταξίδι'' που πραγματοποίησα. Τους ευχαριστώ ιδιαίτερα για την εμπιστοσύνη, την επιείκεια και την υπομονή που έδειξαν προς το πρόσωπό μου, και για τις πολύτιμες συμβουλές και γνώσεις που μου παρείχαν. Όλοι θα μείνουν ανεξίτηλα γραμμένοι στην μνήμη μου, όχι μόνο για τα επιστημονικά εφόδια που απλόχερα μου παρείχαν, αλλά κυρίως για την ευφυΐα και το ΗΘΟΣ που τους διακρίνει. Το σημαντικότερο πράγμα που μου δίδαξαν είναι ότι η αμοιβαία εμπιστοσύνη και κατανόηση είναι το κλειδί της επιτυχίας.

Ένα μεγάλο ευχαριστώ θα ήθελα να εκφράσω σε όλα τα μέλη του Εργαστηρίου Πειραματικής Φυσιολογίας και στον Δρ. Αθανάσιο Παππά της Κλινικής ΄΄Γένεσις Αθηνών΄΄ για την καθοδήγηση και την υποστήριξη προς το πρόσωπό μου.

Ολοκληρώνοντας θα ήθελα να ευχαριστήσω θερμά την οικογένειά μου, τη μητέρα μου Κωνσταντίνα, τον πατέρα μου Βασίλη, και τον μικρό μου αδερφό Θεοφάνη-Δημήτρη για την αμέριστη στήριξη, υπομονή και ηθική συμπαράσταση που έδειξαν όλα τα χρόνια της ζωής μου και στους φίλους μου και συνεργάτες μου που ήταν πάντα δίπλα μου και με στήριζαν ακούραστα.

Abstract

Introduction: Clinicians are called to overcome age-related challenges in decision making during In Vitro Fertilization (IVF) treatment. The aim of this study was to assess the value of age 35 as a cut–off point regarding decision making in IVF in women of good prognosis.

Methods: Medical records between 2010 and 2017 of patients fulfilling the strict inclusion criteria were identified and analyzed. The study group consisted of women diagnosed with tubal factor infertility only. The sample size was divided in three categories at 34, 35 and 36 years of age. The single cycle concluded with one Embryo Transfer including two embryos performed on Day 5. Comparisons were performed regarding hormonal profile, response to stimulation, quality of transferred embryos, implantation and clinical pregnancy rates.

Results: A total of 353 women were eligible to participate. One-hundred and thirty four women were 34 years old, 108 were 35 years old and the remaining 111 were 36 years old. No statistically significant difference was observed between the three age groups regarding the hormonal profile, the number of oocytes collected and the quality of embryos transferred. Regarding the implantation rate, women aged 36 years old presented with a statistically significant decreased implantation rate in comparison to women aged 34 and 35 years old (RR: 0.62, 95% CI: 0.46-0.84, RR: 0.62, 95% CI: 0.46-0.82). Evaluating the clinical pregnancy rate, women aged 36 years old presented with a statistically significant decreased clinical pregnancy rate, in comparison to women aged 35 years old (RR: 0.74, 95% CI: 0.56-0.96).

Conclusions: Results indicate that implantation and clinical pregnancy rates are decreased for the patients aged 36 in comparison to the patients aged 35 when two embryos are transferred, indicating that the age 35 may serve as a valid cut-off point in the respective embryo transfer decision.

Περίληψη

ΕΙΣΑΓΩΓΗ: Την τελευταία δεκαετία παρατηρείται μία συνεχής αύξηση του μέσου όρου ηλικίας απόκτησης πρώτου τέκνου σε πληθυσμούς γυναικών σε όλον τον κόσμο. Ως αποτέλεσμα της παρατηρούμενης αυτής τάσης, ο μέσος όρος ηλικίας όπου οι γυναίκες καταφεύγουν για πρώτη φορά στην Εξωσωματική Γονιμοποίηση (IVF) ανέρχεται σήμερα στα 35 έτη. Παρά το γεγονός πως η επίδραση της ηλικία της υποψήφιας μητέρας στην έκβαση της IVF έχει διερευνηθεί εκτενώς, τα συμπεράσματα που έχουν εξαχθεί σχετικά με τα ηλικιακά όρια που θεωρούνται ως κατώφλι για την έκβαση της IVF είναι αντικρουόμενα.

ΣΚΟΠΟΣ: Η διερεύνηση της υπόθεσης πως η ηλικία των 35 ετών λειτουργεί αποτελεσματικά ως κατώφλιο όριο αναφορικά με τις αποφάσεις στη διαχείριση ενός κύκλου IVF.

ΥΛΙΚΟ-ΜΕΘΟΔΟΣ: Στην παρούσα αναδρομική μελέτη συμπεριλήφθηκαν 353 γυναίκες ηλικίας 34 (134), 35 (108) και 36 (111) ετών, με διάγνωση πρωτοπαθούς υπογονιμότητας σαλπιγγικής αιτιολογίας, που υποβλήθηκαν σε Εξωσωματική Γονιμοποίηση κατά τα έτη 2010-2017. Πραγματοποιήθηκε διαχωρισμός του δείγματος σε τρεις βασικές ομάδες ανάλογα με την ηλικία των ασθενών. Ακολούθησε στατιστική ανάλυση και σύγκριση με γνώμονα το ορμονικό προφίλ των ασθενών, την απάντηση στην διέγερση των ωοθηκών, την ποιότητα των μεταφερόμενων εμβρύων, το ποσοστό επίτευξης βιοχημικής και κλινικής εγκυμοσύνης.

ΑΠΟΤΕΛΕΣΜΑΤΑ: Δεν παρατηρήθηκε καμία στατιστικά σημαντική διαφορά μεταξύ των ηλικιακών ομάδων όσον αφορά το ορμονικό προφίλ, την απάντηση στην διέγερση και την ποιότητα των μεταφερόμενων εμβρύων. Οι γυναίκες ηλικίας 36 ετών παρουσίασαν στατιστικά μικρότερο ποσοστό επίτευξης βιοχημικής εγκυμοσύνης σε σύγκριση με τις γυναίκες ηλικίας 35 και 34 ετών (RR: 0.62, 95% CI: 0.46-0.84, RR: 0.62, 95% CI: 0.46-0.82). Ανάλογα, μικρότερο ήταν και το ποσοστό επίτευξης κλινικής εγκυμοσύνης στις γυναίκες ηλικίας 36 ετών σε σύγκριση με αυτές που ήταν 35 ετών (RR: 0.74, 95% CI: 0.56-0.96).

ΣΥΜΠΕΡΑΣΜΑΤΑ: Η ηλικία των 35 ετών της υποψήφιας μητέρας μπορεί να θεωρηθεί ως κατώφλιο όριο όσον αφορά την έκβαση της IVF και την λήψη κλινικών αποφάσεων που σχετίζονται με τον ιδανικό αριθμό των μεταφερόμενων εμβρύων σε ένα κύκλο IVF.

Chapter 1: Introduction

Chapter 1: Introduction

1.1 Infertility Definition and Causes

Based on the latest international glossary, infertility is a disease identified by the failure to establish a clinical pregnancy following 12 months of regular, unprotected sexual intercourse or due to an impairment of a person's capability to reproduce, either as an individual or along with his/her partner (Zegers-Hochschild et al., 2014). Regular sexual intercourse is a determinant factor for the occurrence of pregnancy.

Infertility can be used as a synonym of sterility in some cases since the outcome is sporadically occurring spontaneous pregnancies. The major factor affecting individual spontaneous pregnancy is the timing of unwanted non-conception which determines the grading of subfertility. Most of the pregnancies occur in the first six cycles with intercourse in the fertile phase (80%). Following that, serious subfertility must be assumed to the extent of 10%. Nonetheless, following 12 unsuccessful cycles- live birth rates among them will reach nearly 55% in the next 36 months. Thereafter, (48 months), approximately 5% of the couples are definitive infertile with a nearly zero chance of becoming spontaneously pregnant in the future. With age, cumulative probabilities of conception decline because heterogeneity in fecundity increases due to a higher proportion of infertile couples. In truly fertile couples cumulative probabilities of conception are probably age independent (Gnoth et al., 2005).

1.1.1 Primary and Secondary Infertility

Infertility is further categorized into primary and secondary. The primary infertile female is a woman who never achieved a clinical pregnancy (Figure 1). Secondary female infertility describes women unable to establish a clinical pregnancy at present but that have achieved a clinical pregnancy in the past (Figure 2) (Zegers-Hochschild et al., 2017). The same categorization is applicable to the male partner as well, due to his participation in the initiation of a pregnancy.

Infertility is common, with recent publications quoting a 9 to 18% prevalence in the general population (Aghajanova et al., 2017). According to the National Summary Report from the Society for Assisted Reproductive Technology, there were 190.394 cycles initiated in 2014 for oocyte retrieval, frozen embryo transfer and frozen oocyte thawing which indicates the impressive amount of women undergoing fertility

treatment (SART, 2014). Since the late 1970s when ART became available, the clinical focus is committed on meeting the immediate reproductive needs of patients with infertility.

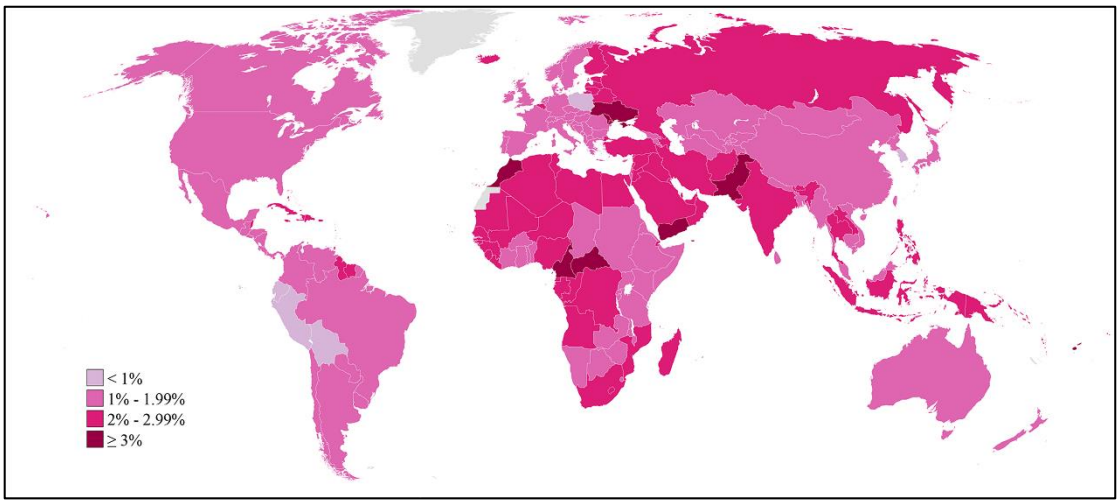

Figure 1. Prevalence of primary infertility among women who seek a child, in 2010 (Mascarenhas et al., 2012).

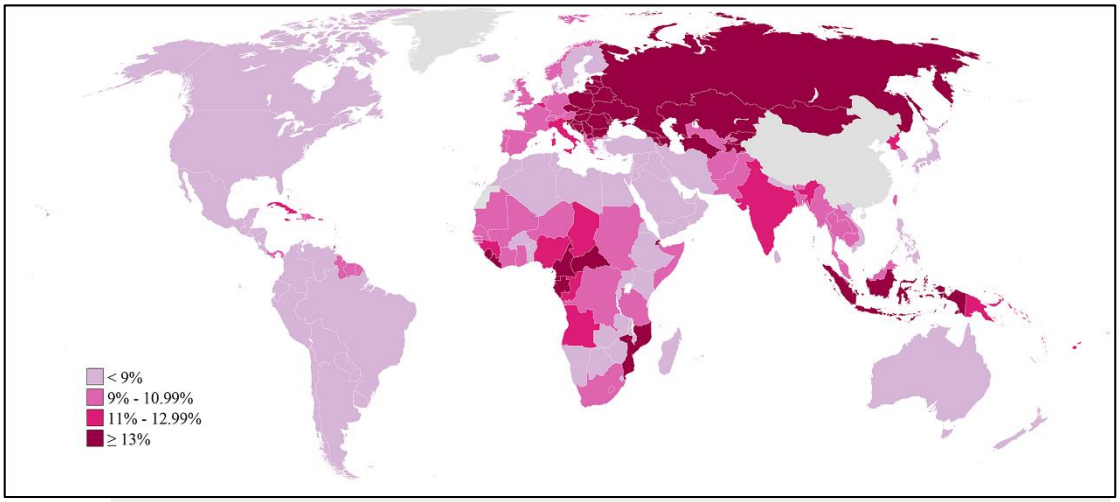

Figure 2. Prevalence of secondary infertility among women who have had a live birth and seek another, in 2010 (Mascarenhas et al., 2012).

1.1.2 Categories of Female Factor Infertility

The prevalence of the pathologies related to the reproductive system failure has increased over the years. Regarding female infertility, it is well documented that several factors could negatively affect the female reproductive dynamic. From anatomical abnormalities, and pathological conditions related to the endocrine system functionality, to systemic autoimmune disorders, and lifestyle/environmental related factors, the pallet of the usual suspects in affecting fertility covers a wide range. In addition, a high prevalence of Premature Ovarian Failure (POF), Polycystic Ovary

Syndrome (PCOS), and endometriosis is also observed in several female populations studied all over the word (Pantou et al., 2019) (Figure 3).

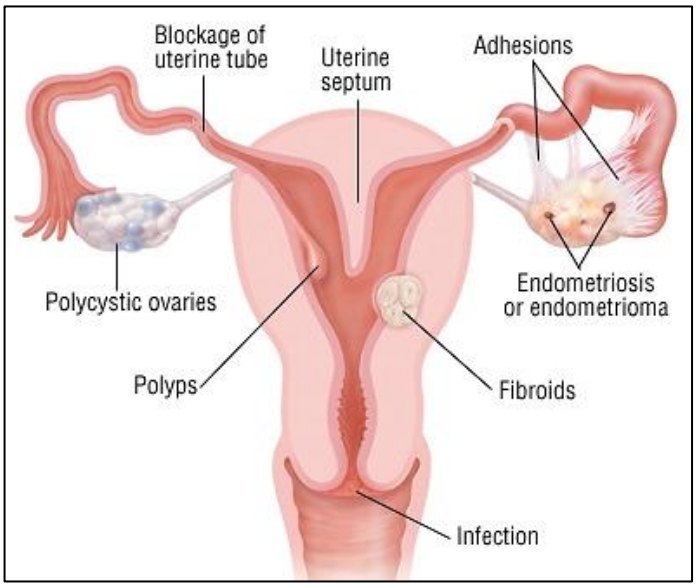

Figure 3. Possible causes of female infertility (modified by https://www.health.harvard.edu)

Anatomical abnormalities including tuboperitoneal abnormalities, endometriosis, myomas distorting the uterine cavity and congenital uterine anomalies are usually responsible for female infertility. Of these, the most common is tuboperitoneal abnormalities, as it occurs in 25-35% of women with infertility problems, and the main cause of tubal damage is Pelvic Inflammatory Disease (PID), mainly due to *Chlamydia trachomatis* infection (Ahmad et al., 2006; Rodgers et al., 2011). The infection is usually asymptomatic, so women do not know that they suffer from tubal disease which is responsible for their infertility (Rodgers et al., 2011). Equally important infertility factors in women are those that deform the uterine cavity, and may be either congenital or acquired. Irrespective of their origin, these factors cause infertility, embryo implantation failure and repeated failure of pregnancy (Steinkeler et al., 2009). The most common congenital dysplasia of the uterus is the septate uterus, associated with pregnancy failure rates of about 60% in women (Grimbizis et al., 2001; Homer et al., 2000). Respectively, uterine myomas are the most frequent acquired factor causing deformity of the uterine cavity. The intramuscular and submucosal myomas adversely affect the success of pregnancy either by physical or in vitro fertilization (IVF) (Olive and Pritts, 2010). Endometrial adhesions are responsible for the partial or total destruction of the uterine cavity leading to amenorrhea and hence infertility (Thomson et al., 2009).

Another cause of compromising tubal patency and function is endometriosis. Endometriosis is an asymptomatic condition, although it causes pain and infertility in women. Is a benign gynecological disorder affecting women of reproductive age, which may be asymptomatic or related to dysmenorrhea, dyspareunia, non-cyclical pelvic pain, and infertility. It has been classified as a polymorphic and multifocal disease with no known cure, or preventive mechanisms that affects approximately 10% of reproductively healthy women. It is a chronic, estrogen-dependent disease, characterized by the intrusion and development of endometrial-like tissues outside the uterine cavity, especially in pelvic floor. Three possible pathophysiological mechanisms have been proposed as the possible mechanisms leading to endometriosis namely, retrograde menstruation theory, theory of coelomic metaplasia, and the embryonic rests theory. Additionally, several mutations in genes encoding proteins related to the immune system regulation, and in genes encoding extracellular elements, have been described in patients suffering from endometriosis. The mechanism and the effects of endometriosis on fecundity are unclear. Data provided indicate that endometriosis negatively affects fertility and IVF outcome. The detrimental effects of this association are clearly evident regarding the advanced stages of the disease. There are numerous reasons endometriosis mainly contributes to fertility compromise as changes within the immunologic milieu of the peritoneal cavity create an unfriendly environment for gamete interaction, early embryo development, and implantation. Furthermore, studies indicate that the ectopic endometriotic lesions into the peritoneal cavity are trigger inflammation, which compromise oocyte quality leading to a reduction of fertilization and implantation rates (Pantou et al., 2019).

Apart from the anatomic factors, there are studies that correlate the female infertility to hormonal factors, which are mainly responsible for the failure of the embryo implantation into the endometrium (Fox et al., 2016). For a successful implantation, it is necessary to ensure a receptive endometrium, as well as a functional embryo at the blastocyst stage and a synchronized interaction between embryonic and maternal tissues (Cakmak and Taylor, 2011; Makrigiannakis and Minas, 2007; Paulson, 2011). For this procedure it is imperative to regulate hormonal secretion of the endometrium during each menstrual cycle. In each cycle, there is a period also known as “implantation window” during which the endometrium is capable of receiving the embryo, depending on progesterone. The endometrial changes occurring in each cycle are energy-demanding, so glucose metabolism is pivotal for the preparation of the

endometrium during embryo implantation (Frolova et al., 2011; Frolova and Moley, 2011; Kim and Moley, 2009). Changes in the endometrial stromal cells depend on the increase of the expression of both glucose and its transporter (GLUT) increasing the probability of embryonic implantation and a successful pregnancy (Frolova et al., 2009). In addition, insulin resistance and hyperinsulinemia have proved to suspend endometrial stromal cell decidualization, thereby reducing the implantation rate (Giudice et al., 1992).

Two metabolic disorders related to infertility are obesity and polycystic ovary syndrome (PCOS). More specifically, several studies have shown that obesity in women is related to infertility, increases rates of miscarriage and, despite assisted reproductive technology, reduces implantation, pregnancy and live birth rates (Boots and Stephenson, 2011; Luke et al., 2011; Wise et al., 2010). According to Bellver J. et al. (2010), as the woman's BMI increases, there is a decrease in the rate of implantation, pregnancy and live births. In addition, in recent studies, it has been reported that obese women present with significant changes in uterine receptivity, in implantation markers and in stromal cell differentiation markers, suggesting the dysfunction of the molecular mechanisms of the endometrium (Bellver et al., 2011, 2007).

Health implications of PCOS have been thoroughly documented, including an increased risk of endometrial cancer, metabolic disorders, cardiovascular disease, obesity, and a predisposition to insulin resistance or outright diabetes (Ghaffarzad et al., 2016; Kvaskoff et al., 2015; Mastorakos et al., 2002; Polat et al., 2015). Since PCOS has ten times more of a chance of making women to develop infertility, it is crucial to understand the connections between a diagnosis of PCOS and overall health. The nature of PCOS makes the study of this disease process challenging. There is a significant overlap between PCOS, infertility, and the risk factors of poor overall health that often accompany this diagnosis. Appropriate patient counseling and attempts to encourage healthy lifestyle in women with PCOS may help mitigate some of the health risks associated with PCOS. Nonetheless, significant confounders remain in studies which evaluate the role of PCOS, infertility, and health outcomes.

1.1.3 Categories of Male Factor Infertility

In addition to female infertility, high prevalence of male infertility is also reported in several studies (Figure 4). However, the causes of male infertility still remained largely unknown (Winters and Walsh, 2014). The cause of male infertility is known in some

cases (e.g. cryptorchidism, specific genetic causes and medical conditions). Further to that, male factor infertility may be classified according to semen analysis (e.g. oligozoospermia, asthenozoospermia or teratozoospermia–singular abnormalities or in combination. Poor quality of sperm is principally due to genetic factors (O'Flynn O'Brien et al., 2010), chromosomal abnormalities (Ferlin et al., 2007), and mainly aneuploidy, chromosomal translocations (Carrell, 2008; Gianaroli et al., 2002), and chromosome deletions of genes associated with spermatogenesis and the development of male gonads (Reynolds and Cooke, 2005).

Specifically, cryptorchidism is the absence of one or both testes from the scrotum and constitutes one of the most common congenital defects of the male reproductive system. It afflicts approximately 2-4% of new-born boys and may have an impact at the health of the male adult. The cause of the abnormal descent may be genetic, including mutations on the Insulin-like factor 3 (INSL3) gene, chromosomal alterations or polymorphisms (Foresta et al., 2008). Environmental reasons may be another important cause of cryptorchidism including maternal lifestyle habits like increased alcohol consumption (Damgaard et al., 2007), and smoking (Jensen et al., 2007). Urogenital track infections comprise the cause of almost 15% of male infertility cases. The infections may impact on different locations of the urogenital track, as the testes, the epididymis and the accessory male glands (Pellati et al., 2008). The spermatozoa may be affected during the different stages of their development resulting in reduced vitality and reduced sperm count. Moreover, a smaller percentage of male infertility is due to endocrine disorders. The causes of the endocrine disturbances may be genetic or acquired. Acquired causes include tumors, inflammation or injury of the hypothalamus or the pituitary.

In addition, genetic syndromes that include an abnormal chromosomal number are a cause of infertility. Reciprocal translocations, ring chromosomal abnormalities, Robertsonian translocations, inversions and reciprocal translocations are associated with male infertility. Mutations affecting the Y chromosome are possible causes of male infertility such as the AZF deletions (Shamsi et al., 2011). Another type of male infertility is Immune Infertility which is an autoimmune disease caused by anti-sperm antibodies. Abnormalities of the blood-testis barrier may cause the production of anti-sperm antibodies. The anti-sperm antibodies may inhibit sperm functions that are important for fertilization mostly affecting zona and oolemma binding (Bohring and Krause, 2003). Ultimately, lifestyle habits or work conditions may also have an impact

in male fertility. Gasoline, lead and zinc fumes have been found to jeopardize male fertility. Smoking, obesity and drugs may also be a cause of infertility (Sharma et al., 2013).

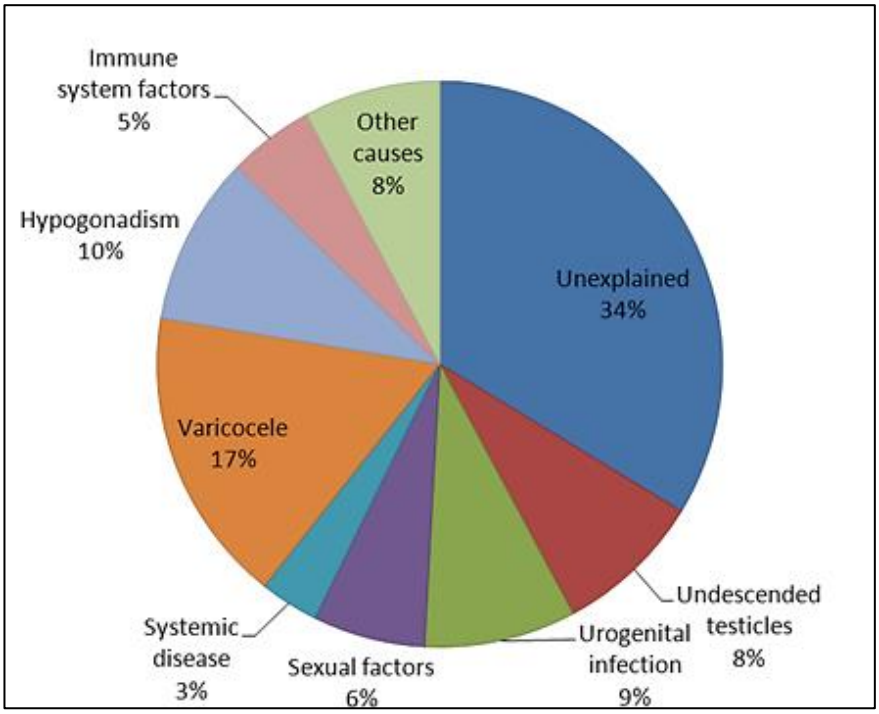

Figure 4. Possible causes of male infertility (modified by http://www.drsonubalhara.in)

1.2 Assisted Reproductive Technology (ART) Treatment Methods

Since the birth of the first infant in the United Kingdom which was conceived through assisted reproductive technology (ART) in 1978, the use of advanced technologies to overcome infertility has increased, as has the number of fertility clinics providing ART services and procedures in the most western societies. ART includes a wide spectrum of technologies such as infertility treatment, In Vitro Fertilization (IVF) and surrogacy arrangement (Figure 5) (Gardner et al., 2009; Nagy et al., 2012; Strauss and Barbieri, 2009). Less invasive methods include Ovulation Induction (OI), Intrauterine Insemination (IUI) or Artificial Insemination (AI) and donor treatment. Advanced invasive techniques involve IVF, Intracytoplasmic Sperm Injection (ICSI) and Preimplantation Genetic Screening (PGS).

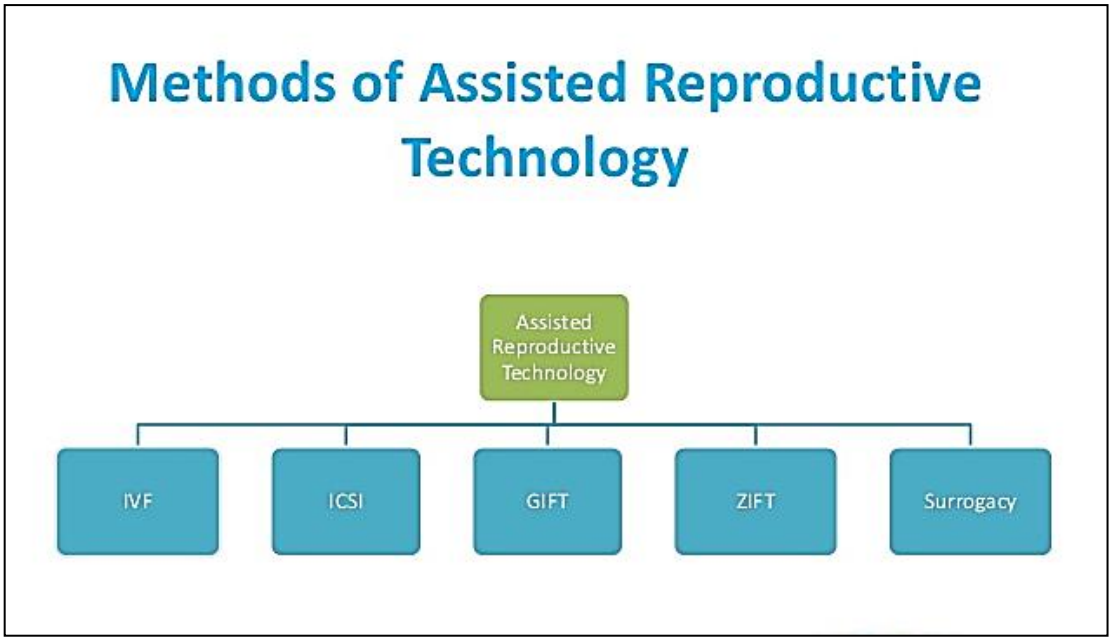

Figure 5. Major types of ART methods employed (modified) (Neo-Est Scandinavian Fertility Centre, 2014).

1.2.1 Intrauterine Insemination (IUI)

Intrauterine Insemination (IUI) is one of the most common assisted reproduction methods and has been broadly used for treating couples with subfertility. This technique requires a fine catheter which is used to place highly concentrated motile sperm into the uterus through the cervix (Figure 6). The semen extracted from the male patient is being processed prior to the insemination occurs in order to provide a sample of improved motility and concertation (Figure 4) (Vichinsartvichai et al., 2015). The prerequisites for IUI being suggested to an infertile couple are young female age, higher total motile sperm per ejaculation, higher number of dominant follicles, better ovarian reserve and the female patient having at least one potent fallopian tube. IUI is appealing to infertile couples since it is more affordable and accessible than other assisted reproductive technologies (ART) and can be repeated in short intervals. This method's success rate per cycle ranges from 5% to 20% (Speyer et al., 2013). Before resulting to a more costly method such as IVF and intracytoplasmic sperm injection ICSI) a patient should be informed appropriately by a reproduction specialist about the benefits of repeating IUI cycles for assisted reproduction since it can result to emotional distress for the couple after it has been repeated several times with no results. The cost-effectiveness is definitely a big plus since it makes it more affordable and approachable to infertile couples but it should be mentioned that the cumulative cost is what matters,

not the cost per cycle since it can be proven to be costly if ineffective. Also the time consumed is of great importance. If the method is proven unsuccessful all the above should be considered in order to move onto a higher success rate protocol for achieving pregnancy in the best possible moment. For that reason many assisted reproduction centers have a limited number of IUI attempts before adopting another protocol. The number varies from three, four, six to even nine IUI cycles (Vichinsartvichai et al., 2015).

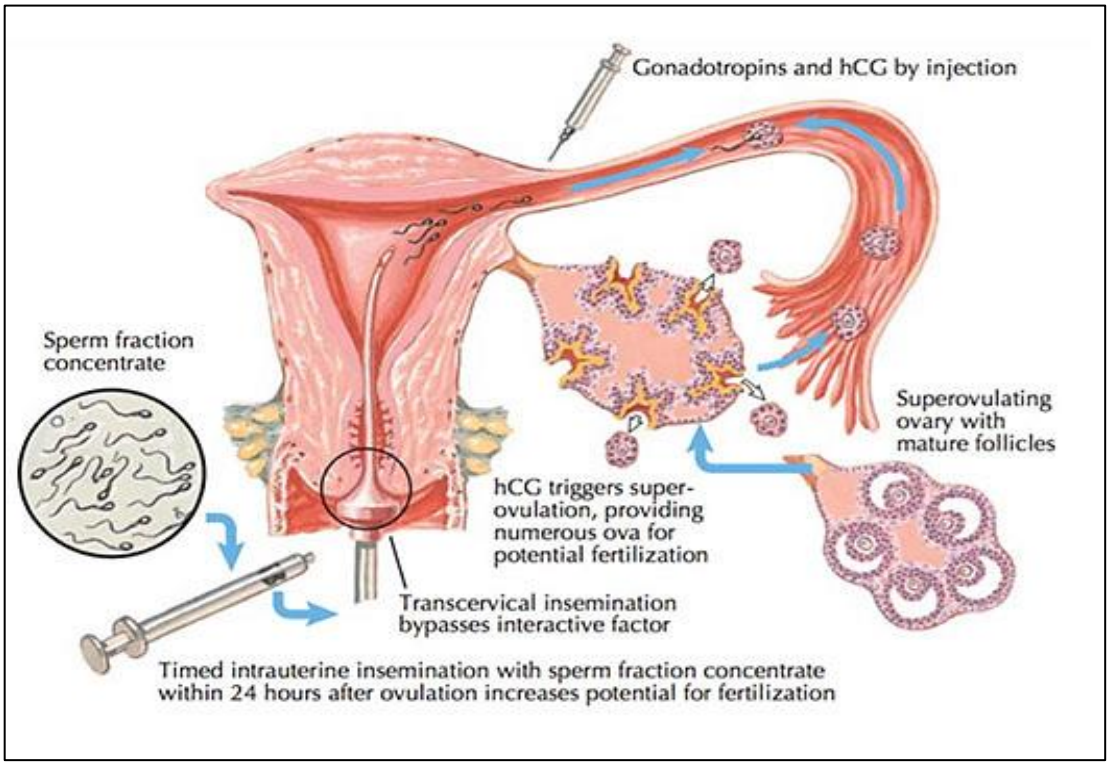

Figure 6. IUI procedure methodology (modified by https://medigence.com)

1.2.2 In Vitro Fertilization (IVF)

The first successful IVF took place in 1978 creating many new opportunities for couples suffering from infertility (Steptoe and Edwards, 1978). IVF procedure includes four major stages namely, Controlled Ovarian Hyperstimulation (COH), oocyte retrieval and sperm collection, in vitro fertilization, and Embryo Transfer (ET) (Figure 7). COH is the method to induce the development of multiple follicles in the same menstrual cycle. Women are injected with shots of Follicle-stimulating hormone (FSH) and are checked by monitoring their estradiol levels with diagnostic tests and their follicular growth with the use of ultrasound examination. During stimulation it is critical to suppress early ovulation by administrating Gonadotropin-Releasing Hormone (GnRH) agonist or GnRH antagonist in order to prevent the endogenous luteinizing hormone (LH)

production. When follicles reach a suitable size ranging from 18-20 mm, an injection of Human Chorionic Gonadotropin (hCG) is performed in order to induce ovulation. hCG acts like the endogenous LH promoting ovulation that would occur approximately two days following hCG administration. Follicular fluid is transvaginally aspirated from the follicles using an ultrasound-guided needle. The retrieved follicular fluid is examined in order to identify the aspirated oocytes. The process is usually taking place under mild or general anesthesia. The oocytes and sperm, that have been previous collected, are prepared for fertilization in a procedure called gamete washing. Sperm and oocyte are incubated together in culture media and fertilization takes place "in vitro". Fertilization success is noted when two pronuclei are microscopically visible in the zygote. Fertilized oocytes are cultured in a special growth medium and left for about 2-6 days until ET. Embryos' culture can be performed in an artificial culture medium, which contains numerous components in order to assist and improve embryonic growth or in an endometrial cell culture which consisting of the top cell layer from the woman's own endometrium. The duration of culture is until the cleavage (3 days following fertilization) or the blastocyst stage (5-6 days following fertilization). The selection of the best embryos is mostly based on certain attributes regarding morphology, cell number and the overall "architecture" of the embryo. Embryos are evaluated through multiple assessments at each developmental stage. In particular the morphological criteria have been shown to have some predictive value regarding pregnancy rate. A scoring system studies certain traits in regards to oocyte morphology, appearance of cytoplasm and zona pellucida, polar body, zygote quality (pronuclear scoring system), and blastomeres.

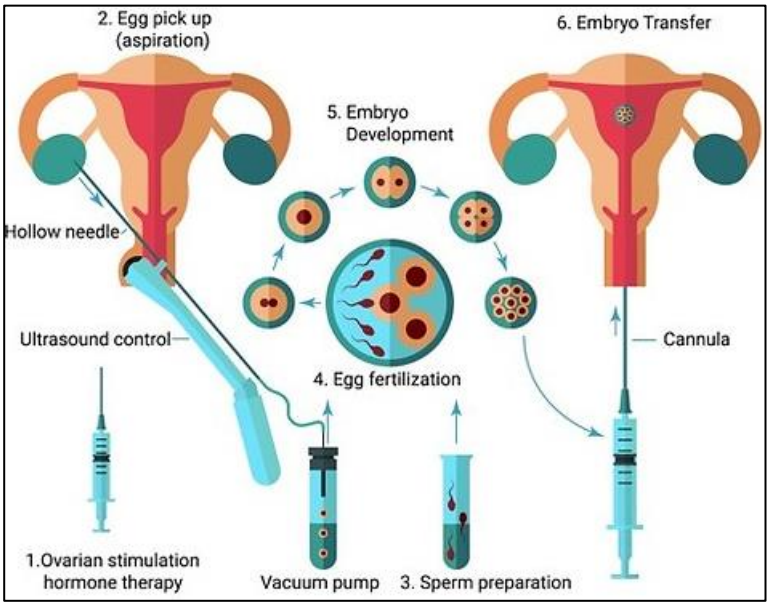

Figure 7. IVF procedure methodology (modified by https://www.practo.com)

1.2.3 Intracytoplasmic Sperm Injection (ICSI)

During the 1980s, there was an advancement in procedures used to address dysfunctions of the male gamete which caused infertility. Some of the most important are zona softening (Gordon et al., 1988; Kiessling et al., 1988), partial zona dissection (Cohen et al., 1988), zona drilling (Gordon and Talansky, 1986) and subzonal injection which was considered the most efficient since the procedure was allowing for an injection of a single spermatozoon into the perivitelline space (Laws-King et al., 1987; Palermo et al., 1992; Palermo and Van Steirteghem, 1991). During the performance of a subzonal injection of an oocyte the oolemma was accidentally breached which lead to the spermatozoon being delivered into the ooplasm instead. That led to the establishment of the ICSI as an assisted reproductive technology treatment method and it is still performed in humans to this day (Palermo et al., 1996a). ICSI entails the injection of a single sperm cell directly into the ooplasm (Figure 8). The capabilities of the ICSI treatment include the utilization of spermatozoa with low progressive motility and microsurgical collection of gametes from the epididymis and testis in azoospermic patients among others (Palermo et al., 1995, 1996a, 1999). ICSI can also be applied in cases where there are low oocyte yields. ICSI is broadly used. In European countries such as Italy and Germany where the law restricts the number of oocytes that can be inseminated, ICSI is the preferred method of choice (Benagiano and Gianaroli, 2004; Trappe, 2017). Since cryostress can lead to a premature exocytosis of cortical granules and zona hardening disallowing spermatozoa to penetrate naturally, ICSI is being used in order to fertilize oocytes that were previously cryopreserved (Johnson, 1989; Porcu et al., 1997; Schalkoff et al., 1989; Van Blerkom and Davis, 1994; Vincent et al., 1990). ICSI is preferred in the cases when only a restricted number of oocytes is available for insemination, as well as a means to eliminate the possibility of polyspermy. Furthermore, by employing and handling a single spermatozoon, the exposure of the oocyte at the time of insemination employing ICSI is limited. This contributes considerably, so as the chance of HBV, HIV, HCV and other viruses and diseases transmission to be significantly reduced. Thus ICSI is the prevalent method for insemination in patients with risk of HIV (Mencaglia et al., 2005; Peña et al., 2002; Sauer and Chang, 2002).

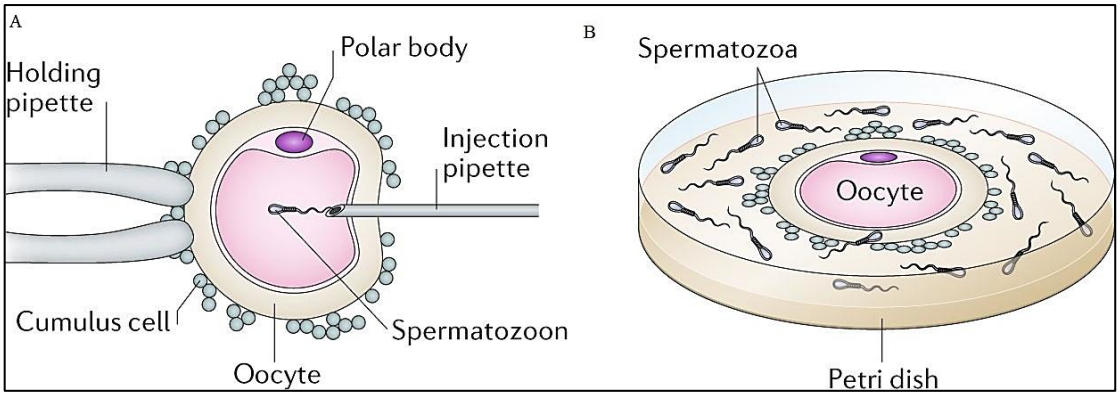

Figure 8. Assisted Reproduction methods: ICSI (A) vs IVF (B) (modified by: (Esteves et al., 2018))

As mentioned above, ICSI is unaffected by the immaturity of the male gamete since the spermatozoa are being directly retrieved from the epididymis or testis. These spermatozoa have some distinct characteristics, they have an underdeveloped cell membrane and an incomplete flagellum (Palermo et al., 1999, 1996b). In all situations where surgical retrieval is required such as in men with complete azoospermia, cryptozoospermia or virtual azoospermia, ICSI prevailed where other methods failed to achieve successful pregnancies by utilizing these spermatozoa (Ron-El et al., 1997).

In the last decade ICSI presented to be the most popular ART technique employed globally. From a cross-sectional survey by the International Committee for Monitoring Assisted Reproductive Technologies (ICMART), on Assisted Reproductive Technology (ART) procedures performed in 60 countries in 2010 the results showed that 63.0% of all cycles utilized ICSI ranging from a 58.4% prevalence in Asia to a virtual totality of 98.4% in the Middle East (Dyer et al., 2016). There was another recent publication with purpose to analyze ART trends in the USA from 1996 to 2012 which identified an increase in the use of ICSI from 36.4% in 1996 to 76.2% in 2012 (Boulet et al., 2016). It was also similarly observed in other studies that there has been a progressive increase in ICSI utilization from 1993 onwards. More analytically, 32.2% in 1993 rising to 48.8% in 1995, 73.6% by 2002 and 79.29% in 2016 (Dyer et al., 2016; Palermo et al., 2015).

ICSI managed to achieve fertilization with such characteristics on spermatozoa making the process puzzling for scientists initially. Despite that though, ICSI has helped in the advancement of ART procedures and has lead research into the processes involved in successful fertilization with more focus on cases where dysfunctional spermatozoa have been delivered into the ooplasm. The disparity between the success of ICSI and classic semen parameter thresholds has induced the development of new bioassays aiming to

qualify the male gamete from a genetic and epigenetic point of view (Palermo et al., 2017).

1.2.4 ART in Natural Cycle

Although it is known that the first successful IVF treatment was performed in a natural cycle, the practice was abandoned because of the subsequent high cancellation rates and premature LH surges it had. Despite this fact, there has been an increased interest in the protocol over the past few years. This protocol has been used for patients with poor ovarian response (POR) following two or more attempts with gonadotropin stimulation since it has been suggested that IVF following a natural cycle may be an alternative for these patients where other protocols are not successful (Figure 9).

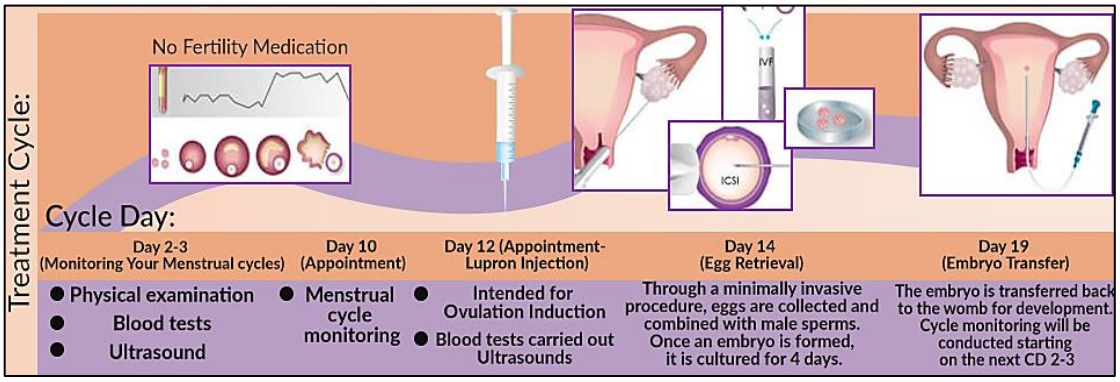

Figure 9. IVF/ICSI following natural cycle (modified by https://sofatinfertility.com)

The biological advantages of the natural cycle may only provide a single oocyte but the oocyte is of better quality and so, it allows the transfer of a healthier embryo into a more receptive endometrial environment. It is also more time-efficient and less expensive than other ovarian stimulation protocols and it avoids most of the risks and complications of ovarian stimulation while it spares the endometrium from the adverse effects of ovarian stimulation. It is also more psychologically friendly to the patient (Barri et al., 2000; Loutradis et al., 2007).

Although pregnancy rates with natural IVF cycles are lower, for some patients with POR to classical and new stimulation strategies, the return to the natural cycle is possibly the last hope for these patients (Bassil et al., 1999).

Improving ovarian stimulation response has always been challenging and a variety of protocols have been employed in order to achieve just that. Until now there is no optimal protocol yet to be defined but reproductive specialists have multiple protocols at their disposal. These protocols have been used as treatment for poor responders.

There is a variety on the efficacy of various controlled hyperstimulation (COH) regimens with focus in the most promising.

1.3 The Impact of Maternal Age on Women Reproductive Dynamic

It is important to differentiate between human fecundity and fertility. Understanding the difference will help explain why some women who conceive are unable to carry a pregnancy up to delivery (pregnancy loss) and some couples are unable to conceive within 6 months (conception delay) or after 12 months (infertility) following unprotected intercourse. From a population research perspective, we can define fecundity as the biologic capacity to reproduce in complete independence by any pregnancy intentions, while fertility is demonstrated fecundity as measured by live or even still births. Couples' fecundity is dynamic and variable because either partner could experience difficulties at any trying attempt, which can be resolved spontaneously by following treatment, after changing partner, or may remain unresolved. This observation signifies the couple-dependent nature of fecundity. Unlike fertility, which is easily measured by births, fecundity cannot be directly measured at the population level and requires reliance on proxy measures. Some commonly utilized measures to assess fecundity in women include menstruation and ovulation, hormonal profiles and biomarkers of follicular reserve such as the Anti-Müllerian hormone (AMH) (Steiner, 2013). In men, fecundity can be assessed based on clinical measures of testicular volumes, semen quality and hormonal profiles (Olsen and Ramlau-Hansen, 2014). A couple's fecundity is measured by the number of calendar months or menstrual cycles required to achieve a pregnancy; with the underlying premise being that a shorter time-to-pregnancy (TTP) indicates higher fecundity.

The question of whether human fecundity is changing divides scientists based on the interpretation of existing data. Because it is nearly impossible to distinguish whether fewer births reflect reduced fecundity (i.e. the biological capacity to reproduce) or changing reproductive behavior (e.g. contraception, delayed childbearing), some authors believe that the question is not answerable (Sallmén et al., 2005). Still, there are salient reasons to address this issue given that reduced fecundity has a profound psychological and financial costs for individual couples. Furthermore, a growing volume of evidence suggests that both fecundity and fertility are markers of somatic health and longevity, including cardiovascular disease (Eisenberg et al., 2016), higher risks of reproductive and non-reproductive cancers (Brinton et al., 2005; Eisenberg et

al., 2016; Hanson et al., 2016; Jacobsen et al., 2000; Winters and Walsh, 2014), and mortality for men and women (Eisenberg et al., 2014; Jensen et al., 2009), indicating that they might have potential usage in health screening (Omu, 2013; Ventimiglia et al., 2015). Moreover, such findings have implications for previously described research paradigms that assess the role of fecundity in the pathway to later onset diseases as conceptualized in the testicular dysgenesis syndrome, and ovarian dysgenesis syndrome (Louis et al., 2011; Skakkebaek et al., 2001).

1.3.1 Advanced Maternal Age is related with infertility and adverse pregnancy outcomes

By categorizing maternal age, an observation can be made; infertility and pregnancy risks are minimal until the age of 35 and then can abruptly increase after the age of 35, 40 or 45 (Figures 10). This approach conceals other trends within age categories and it can underestimate age-related risks for women in younger age groups. Furthermore, it can overestimate risks for mothers in older age groups. Age 35 achieved a threshold status employed in 1970 since it was the age at which the risk of pregnancy loss due to amniocentesis was equal to the risk of Down syndrome (Littlefield, 1970) (Figure 11). However, this does not imply that this approach has been horizontally applied regarding other important pregnancy and birth outcomes. Nonetheless, age 35 may be particularly meaningful. Moreover and although analyses in perinatal epidemiology are often conducted in cohort studies, in which absolute measures of effect are estimable, studies of maternal age at pregnancy have generally presented odds ratios even for common outcomes. Odds ratios are less useful for clinical decision-making and public health policies than absolute risks and may be particularly unclear in this area due to varying exposure and referent group definitions. Because of the insufficiency of available literature, childbearing families and clinicians are unable to understand year-by-year differences in pregnancy and birth risks on the absolute scale.

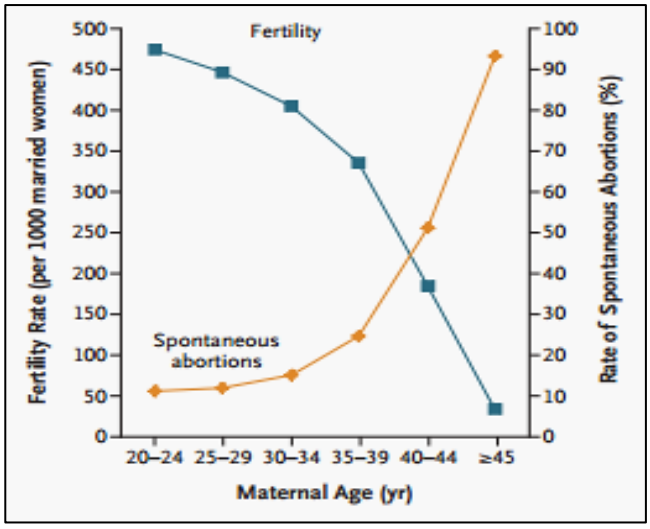

Figure 11. Fertility and Miscarriage Rates as a Function of maternal age (modified by http://www.denverholisticmedicine.com

Maternal Age at Delivery (yr)	Risk of Down's Syndrome	Risk of Any Chromosomal Abnormality
20	1/1667	1/526
25	1/1200	1/476
30	1/952	1/385
35	1/378	1/192
40	1/106	1/66
45	1/30	1/21

Figure 10. Risk of Down's syndrome and Chromosomal Abnormalities at Live Birth, According to maternal age (modified by http://www.denverholisticmedicine.com)

Advanced maternal age continues to be associated with a range of adverse pregnancy outcomes including pre-term birth (Delbaere et al., 2007a; Jolly et al., 2000; Joseph et al., 2005), low birth weight (Aliyu et al., 2008; Jolly et al., 2000; Joseph et al., 2005), stillbirth unexplained fetal death (Flenady et al., 2011; Hoffman et al., 2007; O'Leary et al., 2007) and increased rates of Caesarean section (Janssens et al., 2008) (Figure 12). However, whilst the volume of literature in this area is impressive and while the majority of studies suggest an increased risk of adverse pregnancy outcome in advanced age women, some studies indicate inconsistency when it comes to conclusions about specific outcomes adversely affected by maternal age and the strength of the association (Wang et al., 2011a). In addition, there isn't a common consensus as to the precise maternal age when the increase in the risk of adverse pregnancy outcome becomes clinically important. Some studies have reported that ≥35 years is the cut-off for increased risk (S. Cnattingius et al., 1992; Delbaere et al., 2007a) while others reckon that the risk of adverse pregnancy outcome becomes important at age greater than 40 years (Nybo Andersen et al., 2000). These conflicting findings might be representing the fact that many of the datasets used in the literature contain data on births that took place 25–30 years ago (Aliyu et al., 2008; Hoffman et al., 2007; Jolly et al., 2000). Such data do not reflect recent demographic changes in the antenatal population which may also influence the outcome. For example, contemporary older mothers tend to be well educated (Carolan, 2003), of higher socio-economic status (Ales et al., 1990) and of lower parity than the same category of mothers from the recent past. In addition,

assisted reproductive technology most possibly also contributed to the rise in the number of pregnancies achieved by women in their forties. It has been suggested that social advantage may ameliorate some of the adverse effect of advanced maternal age on perinatal outcome (Carolan, 2003; O'Leary et al., 2007). In recent years, older women who become pregnant are of a better socio-economic status while in the past they were of more low socio-economic status (Chan and Lao, 2008). Moreover, few contemporary studies also demonstrate that pregnancies might have risen because of the difference in socioeconomic status between then and now but there are some other variables to take into consideration, such as body mass index (BMI) and parity that may also influence pregnancy outcome.

	Maternal age 30–34 years	Maternal age 35–39 years	Maternal age 40+ years
Outcome	**Adjusted [a]RR (95% CI)**	**Adjusted [a]RR (95% CI)**	**Adjusted [a]RR (95% CI)**
Emergency Caesarean section			
Most deprived	1.26 (1.20–1.31)	1.39 (1.32–1.47)	1.61 (1.46–1.78)
Least deprived	1.28 (1.23–1.33)	1.42 (1.35–1.48)	1.62 (1.50–1.76)
Elective Caesarean section			
Most deprived	1.44 (1.38–1.50)	**1.69 (1.61–1.78)**	**1.80 (1.64–1.97)**
Least deprived	1.43 (1.37–1.48)	**1.82 (1.74–1.89)**	**2.16 (2.02–2.30)**
Stillbirth			
Most deprived	1.18 (0.97–1.43)	1.45 (1.14–1.83)	1.67 (1.08–2.60)
Least deprived	1.33 (1.05–1.69)	1.41 (1.08–1.86)	2.17 (1.43–3.27)
VSGA			
Most deprived	**0.96 (0.89–1.03)**	1.01 (0.91–1.11)	**1.25 (1.04–1.50)**
Least deprived	**0.80 (0.74–0.87)**	0.89 (0.81–0.98)	**0.90 (0.74–1.09)**
VLGA			
Most deprived	**1.34 (1.24–1.44)**	**1.48 (1.35–1.61)**	1.52 (1.28–1.81)
Least deprived	**1.22 (1.14–1.30)**	**1.29 (1.20–1.38)**	1.38 (1.22–1.57)
Preterm delivery			
Most deprived	**1.11 (1.04–1.18)**	**1.36 (1.26–1.47)**	1.29 (1.10–1.51)
Least deprived	**0.99 (0.93–1.06)**	**1.11 (1.03–1.20)**	1.14 (1.00–1.31)

Figure 12. Crude and adjusted relative risks of the association between maternal age and adverse pregnancy outcome (modified by (Kenny et al., 2013a)).

1.3.2 Advanced Maternal Age is related with Chromosomally Unbalanced Embryos

It is crucial to establish the relationship between embryo aneuploidy rate and maternal age (Figure 13). Franasiak and colleagues examined the relationship between age and the probability that no euploid embryos would be available for transfer. The oocyte retrieval was getting lower while the maternal age was growing higher and they specifically performed more biopsies between the 30-40 age group where it was

determined that the percentage of euploid embryos minimizes while the maternal age grows with age 39 being the point where the euploid embryos percentage was less than the aneuploidy embryos one. At age 47 there was still a small percentage of euploid embryos and at ages 48 and beyond the rate of aneuploid embryos was very high. Women in their mid-30s and above had an excessive increase in embryo aneuploidy rate that begun at 30% and reached 90% towards their late 40s, prior to menopause (Capalbo et al., 2017; Franasiak et al., 2014).

Careful consideration is required when performing IVF treatment in advanced maternal age (AMA) patients because of the high risk of miscarriage, chromosomally abnormal pregnancy and implantation failure. The probability of conceiving a chromosomally-normal baby is minimal, more specifically the production of a chromosomally-normal blastocyst in women older than 43 can be as low as 5% (Ubaldi et al., 2017; Vaiarelli et al., 2018). The decline of oocyte and embryo competence along with the minimization of the ovarian reserve in AMA women, both defining the ability to produce a baby, are the two major factors where this low percentage can be attributed to (Cimadomo et al., 2018; Keefe et al., 2015; Miao et al., 2009).

In order to help mitigate the risks, there are a few processes that have been suggested over the course of the last couple of decades. The spindle-assembly checkpoint (SAC) for instance, where during mitosis and meiosis it maintains the genome stability by delaying cell division until an accurate chromosome segregation can be guaranteed. Accuracy requires that chromosomes become correctly attached to the microtubule spindle apparatus via their kinetochores. When not correctly attached to the spindle, kinetochores activate the spindle assembly checkpoint network, which in turn blocks cell cycle progression. Once all kinetochores become stably attached to the spindle, the checkpoint is inactivated, which alleviates the cell cycle block and thus allows chromosome segregation and cell division to proceed (Kolano et al., 2012; Lara-Gonzalez et al., 2012; Nagaoka et al., 2011; Steuerwald et al., 2001).

Telomeres' shortening is another process leading to genome instability. Telomerase is capable of extending cell lifespan and nowadays it is well known that it is capable of immortalizing human somatic cells. By reversing the shortening of telomeres through temporary activation of telomerase a potent means to slow aging is indicated (de Lange, 2009; Keefe et al., 2015). Similarly through impaired mitochondrial metabolic activity since various studies on the aging process point out that mitochondria are one of the key regulators of longevity. The increasing age in all mammals relates to increased

levels of mtDNA mutations along with a deteriorating respiratory chain function (Eichenlaub-Ritter, 2012; Van Blerkom, 2011).

Lastly, dysfunctional cohesins is another parameter affecting chromosomal stability. Cohesin is a protein structure that encircles DNA. Cohesin affects many processes that occur on chromosomes such as segregation, DNA replication, double-strand break repair, condensation, chromosome organization, and gene expression. Mutations in the genes that encode cohesin and its regulators cause human developmental disorders and cancer. It is shown that cohesin is essential for cell division, but partial loss of function can alter gene expression, DNA replication and repair, gametogenesis, and nuclear organization (Cheng and Liu, 2017; Singh and Gerton, 2015). All the processes mentioned above are attempting to modulate embryo competence since they are directly or indirectly involved in chromosome segregation (Cimadomo et al., 2018).

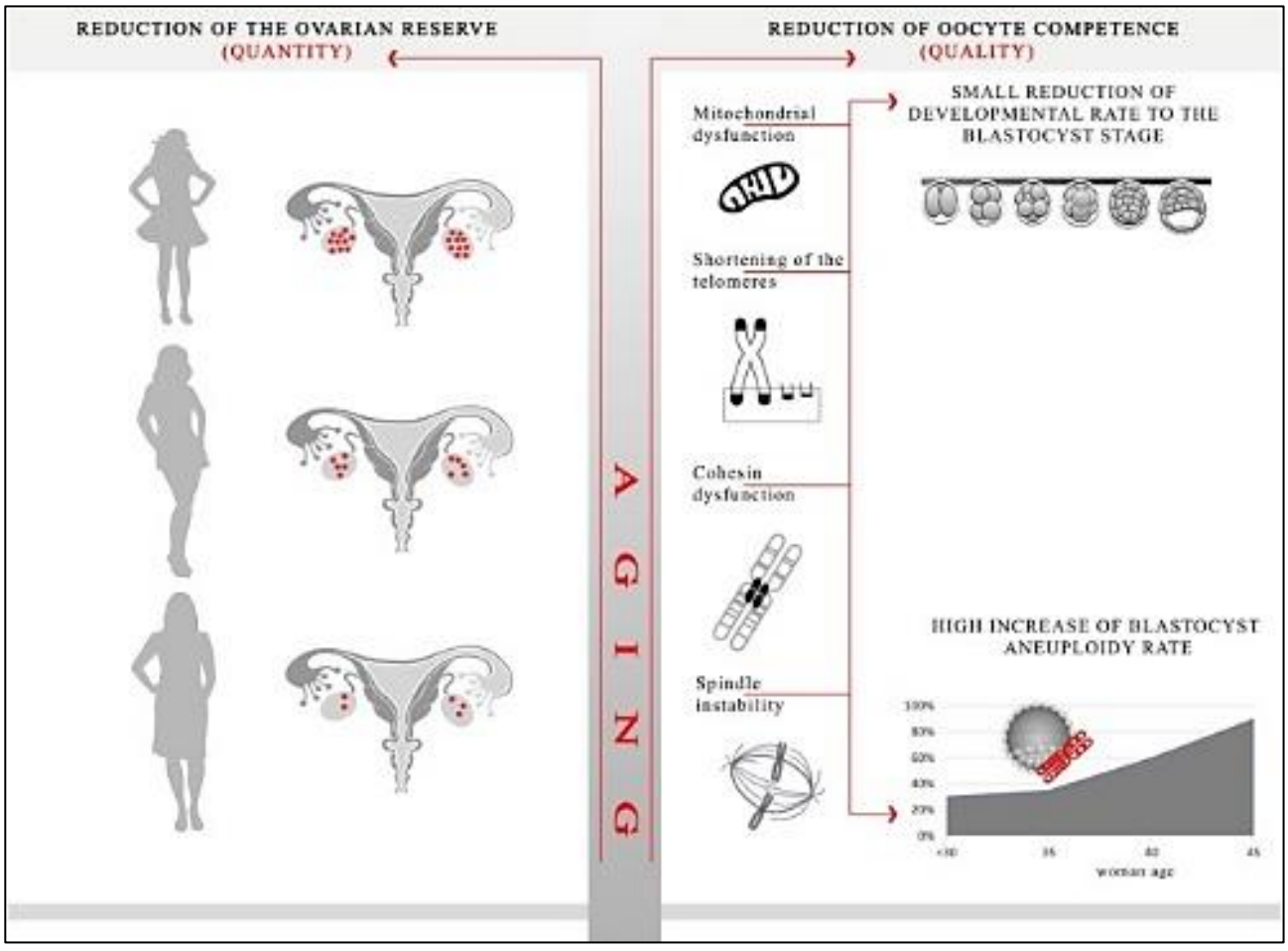

Figure 13. Effect of advanced maternal age on oocyte/embryo competence and putative mechanisms impaired by aging. Aging in women causes both a reduction of the ovarian reserve and of the oocyte competence. All the processes impaired may result into a lower energy production/balance involving a small reduction of embryo developmental rate to the blastocyst stage, as well as a higher frequency of chromosome missegregation during maternal meiosis leading to a high increase in blastocyst aneuploidy rate (modified by (Cimadomo et al., 2018).

1.3.3 IVF/ICSI Success Rates are strongly correlated with Maternal Age

Many studies and plenty of data up to this stage indicate that increasing age is associated with a decrease in pregnancy rate either via assisted reproductive techniques (ART) or natural conception (Figure 14). With advancing age comes a decrease in ovarian reserve which leads to a decrease in the number of oocytes and poor oocyte quality. Therefore, age is one of the most important limiting factors in the success rate of assisted reproduction in older women. Despite that, there is a subgroup of patients with adequate follicular response to stimulation which can yield higher success rates with the application of assisted reproduction (Dal Prato et al., 2005; Osmanagaoglu et al., 2002). In other words, oocyte utilization rate is strongly affected by female age but also dependent on ovarian response. Therefore, oocyte utilization in older women with minimal or moderate response leads to oocytes with increased reproductive potential. Based on studies, there is a suggestion that mild IVF approaches might be more effective in women <35 years who yielded a single mature oocyte. Therefore, IVF presents the best opportunity for achieving a successful pregnancy in this group of patients with older maternal age. Also, women >35 years require a longer period to achieve conception than younger women, and a higher percentage of older than younger women will never achieve pregnancy. In addition, the rate of early pregnancy wastage increases substantially for women in their thirties, and is >50% after the age 40 years. More studies need to be conducted to support this conclusion because of the extremely low pregnancy rates of this age group (Frederick et al., 1994; Telli et al., 2013).

In vitro fertilization (IVF) is recognized as the last resort for infertile couples who would like to have biological children, and is accepted as the most efficient treatment for infertility. Intracytoplasmic sperm injection (ICSI) is the most efficient technique for the treatment of male factor infertility and while the science advances, the scope of ICSI has been widened to include other causes of infertility (Ahmed et al., 2015).

When it comes to ICSI, outcomes such as oocytes retrieved, fertilized oocytes, embryos transferred and the rate of successful induction of pregnancy depends primarily on the age of the woman, where the optimal outcomes are observed in women <30 years of age. Also, findings indicate that optimal IVF outcomes like the number of oocytes retrieved was highest among women aged <30 years, with a reduced number of oocytes retrieved per cycle, lower pregnancy and live birth rates seen among older women. The poor oocyte quality, higher embryo implantation failure, uterine problems, decreasing ovarian reserve and ovulatory dysfunction due to poor hormonal environment were

all effects of the aging process that can have a detrimental effect on the efficacy of IVF/ICSI (Ahmed et al., 2015).

Putting both techniques into perspective and by comparing data for these two, an observation can be made. More pregnancies have been achieved with IVF in comparison to ICSI in both women of advanced age and poor responders groups. In other words, if there is a normal range of sperm counts in an infertile couple IVF should be preferable over ICSI (Koifman et al., 2008).

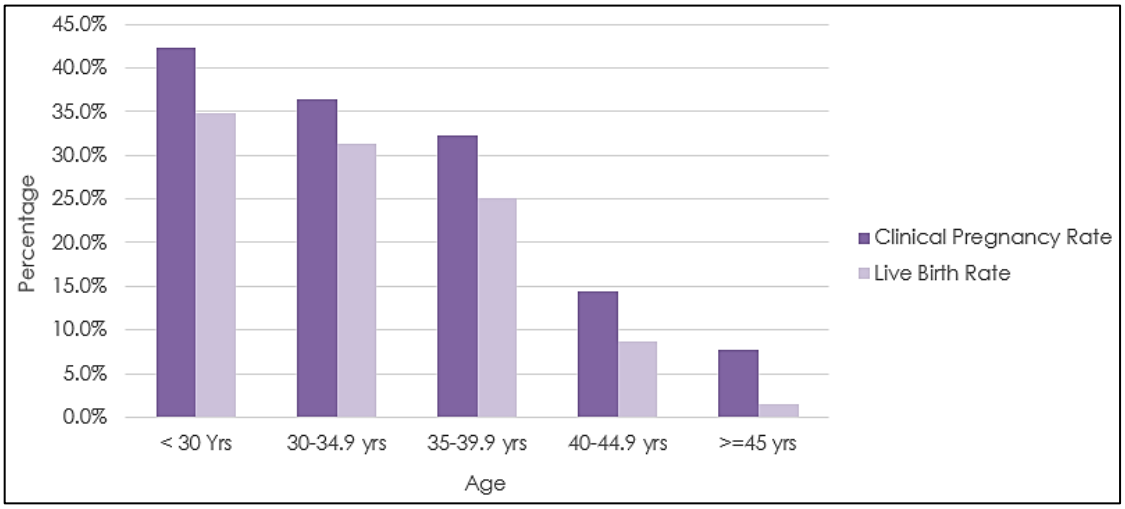

Figure 14. Clinical Pregnancy Rate and Live Birth Rate following IVF/ICSI treatment according to maternal age (modified by https://www.ivf.com.au).

1.3.4 Patient management according to Maternal Age

Although infertility is classified as a disease the means for treating patients varies. It is based on patient-specific indications and scientific evidence and in order to achieve the best outcome the couple's will and potential is crucial, especially when referring to AMA patients (Figure 15) (Zegers-Hochschild et al., 2014). Woman's age, ovarian reserve and some additional factors are to be considered in order to achieve the best outcome.

A higher number of oocytes is required in patients with AMA in order to pick at least one euploid embryo since there is increased risk of aneuploidy, a chromosomal anomaly where there is one more or one less chromosome than normal. The risk increases with the decreasing ovarian reserve AMA patients present (Vaiarelli et al., 2018).

In order to maximize the ovarian response in AMA patients, optimization of the ovarian response, accurate estimation of the ovarian reserve and collecting a consistent number of oocytes is required. In order to prevent a putative reduction in embryo and oocyte quality in women older than 35 some mild ovarian stimulation protocols were

recommended despite the fact that there is an increased cycle cancellation rate due to limited/no ovarian response, potentially higher cost since it requires more hormonal stimulation cycles and since oocyte retrievals and the number of those oocytes that can be fertilized is limited (Fauser et al., 1999; Mansour et al., 2003; Revelli et al., 2011). Because of these factors there was a necessity to maximize the number of oocytes retrieved and hence, tailored ovarian stimulation protocols emerged.

A direct correlation exists between the sequential number of oocytes collected and the cumulative live birth rate (CLBR) on each IVF cycle (Briggs et al., 2015; Drakopoulos et al., 2016; Magnusson et al., 2018; Polyzos et al., 2018). Although it is harder to achieve the desired outcome, some groups reported that there was a higher quality in embryos retrieved and better outcomes achieved from a fresh ET perspective (Baker et al., 2015; Borges et al., 2017; Yilmaz et al., 2013). One great concern is that Controlled Ovarian Stimulation (COS) increases hormonal levels which potentially can impair endometrial receptivity.

In order to minimize infertility, oncological patients are being informed of means to preserve their fertility in a younger age. One of those methods to preserve fertility in a younger age is embryo freezing. Cryopreservation has been improved over the past few years because of the introduction of the vitrification approach in clinical practice. Vitrification is a fast, and efficient protocol regarding oocyte cryopreservation and currently referred to as the optimal choice regarding fertility preservation (Practice Committees of American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2013). Obviously, the age of the female patient when the oocyte cryopreservation occurs and the number of those oocytes stored are crucial for the cost-effectiveness of oocyte cryopreservation, with the efficacy being higher on patients < 35 years of age and the upper limit set to 37 (Doyle et al., 2016). In situations where there is a clear lack of ovarian reserve or recurring IVF failures the oocyte donation protocol can be utilized. To achieve the optimal results in AMA patients and minimize all the risks associated with greater age when attempting pregnancy through IVF, PGT-A is also recommended.

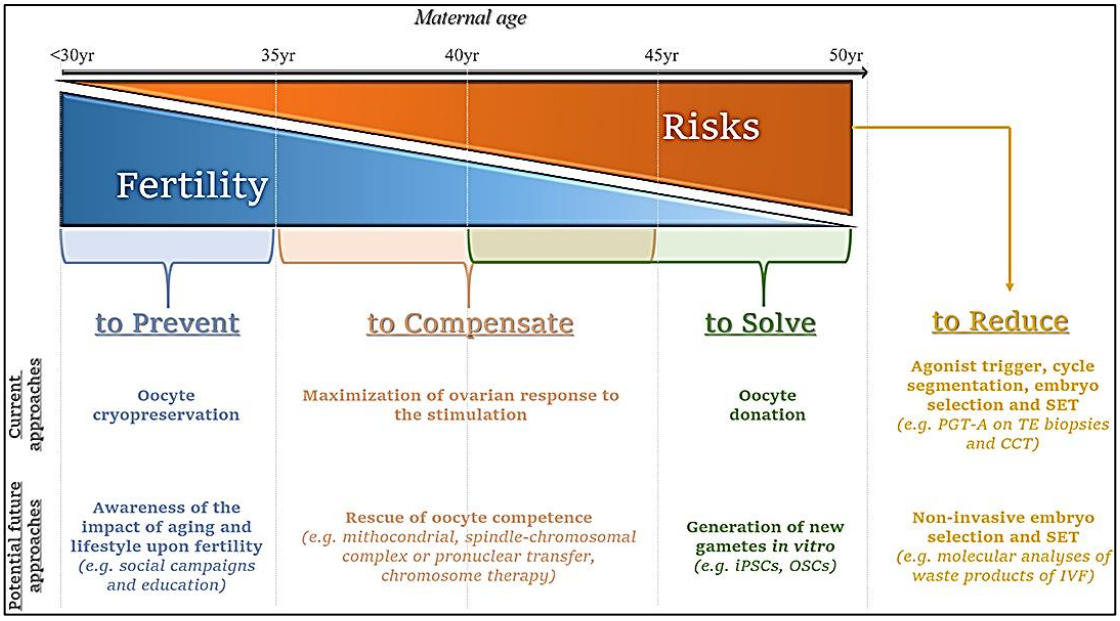

Figure 15. Summary of the current and potential future approaches to prevent, compensate or solve the issues related with advanced maternal age on infertility, while limiting the putative concurrent risks (modified by (Ubaldi et al., 2019).

1.4 How Old is Too Old? Challenges Faced regarding Age Cut-Off Points in ART

Achieving a pregnancy over the age of 35 has surfaced as a trend especially in high-income countries over the past decades. Advanced maternal age (AMA) is the apposite term describing the phenomenon of delayed parenthood (Huang et al., 2008). In the USA, motherhood in women aged over 35 years was recorded to be almost eight times higher comparing data from 1970 to 2006. Similar trends are also observed in Europe. It is well documented in literature that AMA in women pursuing fertility treatment is a challenging trend that practitioners are called to overcome in providing successful treatment. Delaying pregnancy appears as a new life style, as couples postpone marriage and opt to pursue advanced education, financial stability and higher career goals (Mills et al., 2011). In addition, the improvement of assisted reproduction technologies (ART) such as oocyte cryopreservation and oocyte donation further contribute towards establishing this new revolutionary era of reproduction (Mills et al., 2011).

Despite the improvement in social–economic circumstances and advances in medically assisted reproduction, several epidemiological studies indicate that AMA is correlated with a plethora of pregnancy complications including: gestational diabetes, preeclampsia, placental dysfunction, placental abruption, fetal growth restriction, pre–term birth and stillbirth (Carolan et al., 2012; J Cleary-Goldman et al., 2005; Giri et al., 2013; Kenny et al., 2013b; Khalil et al., 2013; Samantha C. Lean et al., 2017; Martinelli

et al., 2018; Salihu et al., 2008). AMA is also associated with an increased risk of operative delivery through cesarean sections (Bayrampour and Heaman, 2010), higher miscarriage rates and increased incidence of congenital abnormalities (George and Kamath, 2010; Rubio et al., 2017; Wang et al., 2008). Advanced age women treated with In Vitro Fertilization (IVF) tend to present with poor response in ovarian stimulation and lower implantation rate (Spandorfer et al., 1998; Younis et al., 2015). All the aforementioned risks pose as major concerns.

AMA patients are increasingly resorting to IVF in order to achieve a pregnancy. Thus, health providers face several dilemmas, ethical conflicts and challenges concerning management and assessment of possible risks and benefits. Clinicians make decisions regarding the appropriate age cut–off points to employ regarding several aspects of ART including the decisions in employing donor eggs or embryos and gestational surrogacy. Despite guidelines (ASRM, 2016; Dondorp et al., 2012), health providers address these challenges employing an array of various approaches highlighting the need for implementation of a common strategy worldwide (Klitzman, 2016). An issue of particular interest is the maximum number of embryos transferred. The guidelines suggest that age 35 is a cut–off point regarding the number of embryos transferred in a single cycle and recommend the practice of elective single embryo transfer (eSET) for patients under the age of 35 years ("Guidelines on number of embryos transferred," 2009). In addition, most countries in Europe abide by a legal framework regarding the number of embryos transferred in a single cycle. In some of these countries such as Belgium for patients under 36 years this number may be as few as one, or in Germany for patients under 37 years as many as three (mostly two). In Greece, the legal framework sets the age cut off point to be at 35 years. For women below this age threshold the transfer of two embryos is allowed strictly under specific circumstances. Limitations regarding embryo transfer number in the UK or the Netherlands are age related, while eSET is preferred. No more than two embryos can be transferred in France or Sweden ("Regulation and legislation in assisted reproduction," 2017).

Despite the large number of publications investigating fertility in women of AMA, there is a lack of consensus among practitioners concerning the age cut–off points implemented in several aspects of ART (Figure 16) (Berkowitz et al., 1990; Klitzman, 2016; Wang et al., 2011b). Some studies denoted that the age of 40 should be the cut–off point in which the correlation between AMA and adverse pregnancy outcome

becomes significant (Andersen, 2000) and some others reported that age 35 is the cut–off point for increased risk (S Cnattingius et al., 1992; Delbaere et al., 2007b).

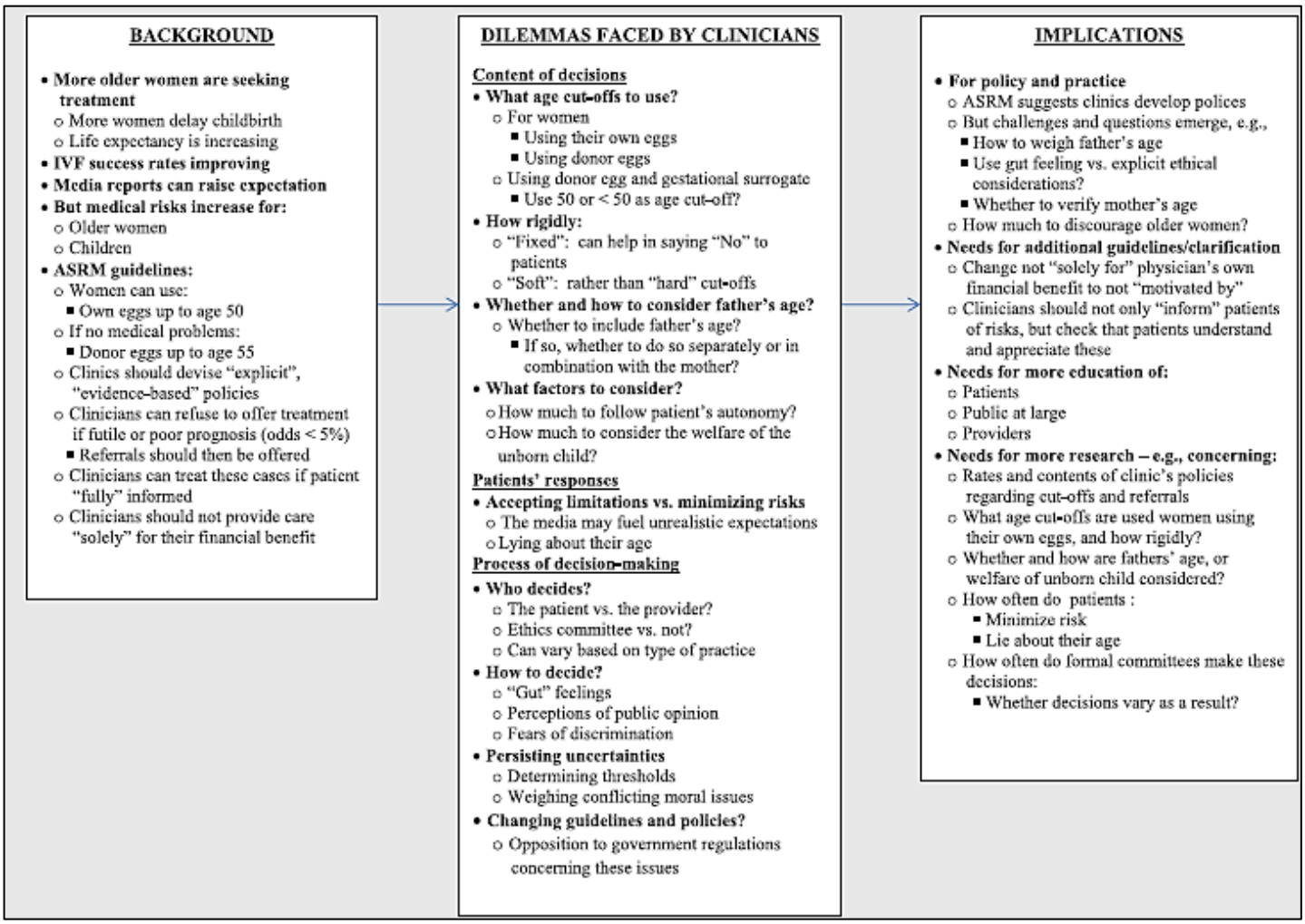

Figure 16. Dilemmas faced by clinicians regarding the appropriate management of AMA patients (modified by Klitzman et al., 2016).

Chapter 2: Aim & Scope

Chapter 2: Aim & Scope

Based on the data and the varying practices globally it becomes clear that the practitioners would majorly benefit from studies indicating with further certainty the clinical value associated with all ages surrounding the well-known cut-off point. Could the age 34 and 36 be significantly differentiated to the cut-off point of age 35 when it comes to deciding the number of embryos included in the embryo transfer? When aiming to minimize complications such as multiple pregnancies and equally increase the chances of a successful treatment how does exact age weigh in the equation? Such concerns render this manuscript timely and essential. The scope of this study is to investigate the value of age 35 as a cut-off point regarding the IVF outcome in women presenting with good response to stimulation. To our knowledge this is the first report on investigating the effect of age in good prognosis IVF patients referring to and investigating the distinct calendar years 34, 35, 36 versus age cohorts of a wider age range as we are accustomed to identify in the literature.

Chapter 3: Material & Methods

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The patients of this retrospective data analysis were recruited from medical records between 2010 and 2017. The Hospital Ethics Board approved the study protocol in accordance to the Helsinki declaration. These women were diagnosed with tubal factor infertility and were subsequently submitted to a single cycle of IVF treatment. The inclusion criteria for recruitment described: women diagnosed with tubal factor primary infertility confirmed by hysterosalpigography characterized as fallopian tube(s) blockage, or removed salpinx following hydrosalpinx diagnosis. The etiology was selected when designing the study due to its favorable prognosis. These women reported regular length of menstrual cycles 24-35 days. The pharmaceutical Controlled Ovarian Hyperstimulation (COH) protocol of choice was the standard Gonadotropin-Releasing Hormone (GnRH) long agonist protocol: The protocol included administration of 0.1 mg GnRH agonist on cycle–day 21. Administration of gonadotropin at 300 international units (IU) was employed in a daily dose. The adjustment of gonadotropin dose was based on sonographically assessed follicular development. Thirty six hours following administration of Human Chorionic Gonadotropin (HCG) injection oocyte retrieval was performed. Luteal support through progesterone administration was opted for. Good ovarian response was defined as a collection of ≥10 oocytes. The sample size of this study was divided in three major categories according to patients' age, namely 34, 35 and 36 years of age. Cycles were concluded with embryo transfers including two blastocyst stage embryos, performed on day 5. Patients were further subcategorized regarding embryo quality at the time of Embryo Transfer (ET) on day 5 in three categories. Category D5A included two top quality blastocysts both 4AA or 5AA or 6AA. Any other grading of the blastocyst embryos was classified as non-top. Category D5B included a top quality blastocyst and another non-top quality blastocyst and category D5C included two blastocysts evaluated as non-top quality. Grading criteria for blastocyst stage embryos were according to Gardner's blastocyst grading system (Figure 17) (Gardner et al., 2000). As stated above the insemination technique was standard IVF. Male factor or any additional etiology regarding the infertility status of the couple was excluded in an effort to dismiss any parameters that could knowingly hinder the positive outcome of a good prognosis patient, and hence act as confounders for the study. The above analyzed inclusion criteria ascertained a patient's profile of good prognosis and favorable

chances towards achieving a pregnancy following a single IVF treatment. All patients who met the inclusion criteria were divided in the three age groups as described above. The three age groups were statistically compared to each other with respect to the basic hormonal profiling of the patients namely Follicle Stimulating Hormone's (FSH), Luteinizing Hormone's *(*LH) levels, Estradiol's levels, and Anti-Müllerian Hormone's (AMH) levels. FSH and LH Analysis was performed on the day 3 of the menstrual cycle by using chemiluminescent microparticle immunoassay on a Roche Immunoanalyser (Roche Cobas e 411). Estradiol's levels analysis was performed on the day of the HCG trigger by using chemiluminescent microparticle immunoassay on a Roche Immunoanalyser (Roche Cobas e 411). AMH levels analysis was performed on the day 3 of the menstrual cycle by using AMH Gen II chemiluminescent microparticle immunoassay on a Roche Immunoanalyser (Roche Cobas e 411). Further to that the three age groups were statistically compared to each other with respect to the following: The number of oocytes collected, the number of normally fertilized oocytes, the quality of embryos transferred, the implantation rate and the clinical pregnancy rate. Implantation rate represents the percentage of the cases in which a positive beta human chorionic gonadotropin test (beta hCG) in maternal serum was observed seven days following blastocyst transfer. Clinical pregnancy rate represents the percentage of the cases in which pregnancy was confirmed by an ultrasound detection of fetal heart beat 6 to 7 weeks following the last menstrual period.

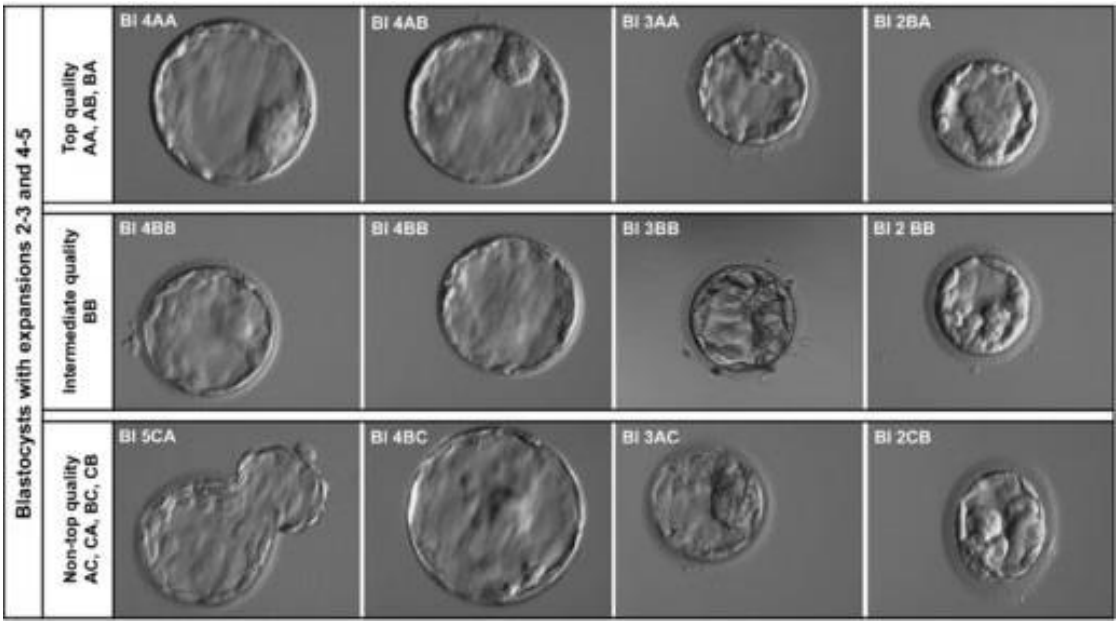


Figure 17. Morphological grading classification of blastocyst morphology according to the Gardner score (Gardner et al. 2000).

All data analyses were performed using the R Programming Language for Statistical Purposes. The hormonal profiling, the number of oocytes collected and the number of normally fertilized oocytes of the patients among the three age groups were compared with One Way ANOVA test and Bonferroni Correction Post-hoc analysis. The comparison regarding the transferred embryos' quality was performed applying Fisher's exact test. Concerning the implantation and clinical pregnancy rate, the three age groups were statistically compared to each other by evaluating the Risk Ratio (RR) with respect to not achieving implantation or a clinical pregnancy status, following a single cycle of IVF treatment. RR indicates the multiple of risk of the outcome measured (not achieving implantation or clinical pregnancy status) in one group (Age 36 group) compared to another group (Age 35 group and Age 34 group, respectively). Therefore, classification of results as "decreased risk to achieve implantation or clinical pregnancy status" corresponds to increased chances of achieving the aforementioned status. Confidence Intervals (CI) of 95% were calculated for each variable and *P*-value < 0.05 was considered statistically significant.

Chapter 4: Results

Chapter 4: Results

A total of 353 women were eligible to participate in the current study. One-hundred and thirty four women were 34 years old during their first attempt, 108 were 35 years old and the remaining 111 were 36 years old. Tubal factor was diagnosed as the sole infertility etiology in all our patients in order to ensure the fact that the sample included good prognosis patients not harboring idiopathic or unexplained infertility which could serve as a confounder for the study. The mean levels of estradiol, on the day of the HCG trigger for our patients was 1800.85 ± 1167.84 pg/ml, ranging from 500 to 6890 pg/ml. AMH levels were ranging from 1.7 to 7.93 ng/ml, with an average of 5.86 ± 2.85 ng/ml. The mean levels for FSH and for LH were 5.39 ± 0.98 mIU/ml and 3.58 ± 0.97 mIU/ml respectively. FSH levels ranged from 3.8 to 7.5 mIU/ml and LH levels ranged from 2.1 to 6.5 mIU/ml. No statistically significant difference was established between the three age groups regarding the hormonal profile of our patients.

An average of 14.71 ± 9.21 oocytes was collected per retrieval, ranging from 10 to 28. No statistically significant difference was observed among the three age groups regarding the number of retrieved oocytes (15.76 ± 9.14 vs 13.88 ± 9.73 vs 14.24 ± 8.24). Oocyte fertilization rates also presented with no statistically significant difference, with an average of 8.26 ± 5.94 oocytes being fertilized normally. Hormonal levels for each group of patients as well as number of retrieved oocytes and two-pronuclear (2PN) zygotes are presented in Table 1.

Table 1. Average ± Standard Deviation of hormonal levels, number of oocytes retrieved and normally fertilized oocytes for each age group.

	Age 34	**Age 35**	**Age 36**
E2 (pg/ml)	1708.19 ± 989.18	2002.88 ± 1378.11	1715.39 ± 1101.88
AMH (ng/ml)	5.98 ± 3.17	5.84 ± 2.14	5.76 ± 3.04
FSH (mIU/ml)	5.24 ± 0.90	5.52 ± 1.05	5.39 ± 1.06
LH (mIU/ml)	3.33 ± 0.87	3.72 ± 1.05	3.74 ± 0.97
Oocytes Retrieved	15.76 ± 9.74	13.89 ± 9.73	14.24 ± 8.64
2PN Zygotes	8.22 ± 5.76	8.39 ± 6.67	8.18 ± 5.38

No statistically significant difference was observed among the three age groups regarding the frequency of A, B, or C quality of transferred blastocysts, as presented in Table 2.

Table 2. Frequency of embryo transfer at day 5 regarding the blastocysts' quality for each age group.

	Age 34	**Age 35**	**Age 36**
D5A†	50 (78) (58.2%)	37 (52) (48.15%)	32 (52) (46.85%)
D5B‡	28 (34) (25.4%)	17 (25) (23.15%)	24 (29) (26.13%)
D5C§	12 (22) (16.4%)	17 (31) (28.7%)	23 (30) (27.02%)
†: Category D5A included two top quality blastocysts 4AA, 5AA or 6AA			
‡: Category D5B included a top quality blastocyst and another non-top quality blastocyst			
§: Category D5C included two blastocysts evaluated as non-top quality			

Regarding the implantation rate, women aged 34 years old presented with a statistically significant decreased risk with respect to implantation failure in comparison to women aged 36 years old (RR: 0.62, 95% CI: 0.46-0.84) (Table 4). Furthermore, women aged 35 years old presented with a statistically significant decreased risk with respect to implantation failure in comparison to women aged 36 years old (RR: 0.62, 95% CI: 0.46-0.82) (Table 4). In regards to the above mentioned results, women aged 36 years old presented with a significantly lower implantation rate in comparison to the cycles for women aged 34 or 35 years old. Implantation rates for each age group are presented in Table 3.

Table 3. Implantation Rate for each age group.

	Age 34	**Age 35**	**Age 36**
Implanted	87 (64.9%)	70 (64.8%)	48 (43.2%)
Not Implanted	47 (35.1%)	38 (35.2%)	63 (56.8%)

Table 4. Risk Ratios for Risk of not achieving implantation status following a single cycle of IVF treatment Dependent on Patients' Age.

Patients' Age	**Risk Ratio (RR)**	**95% Confidence Interval (95% CI)**

Age 36	1	1
Age 35	0.62	0.46-0.82
Age 34	0.62	0.46-0.84
¶Reference group: women Age 36 years old		

Evaluating the clinical pregnancy rate, a similar trend to the implantation rate was observed. Women aged 35 years old presented with a statistically significant reduced risk with respect to clinical pregnancy failure in comparison to women aged 36 years old (RR: 0.74, 95% CI: 0.56-0.96) (Table 7). Women aged 34 years old presented with a reduced risk with respect to clinical pregnancy failure in comparison to women aged 36 years old, but a statistically significant difference could not been established marginally (RR: 0.79, 95% CI: 0.62-1.00) (Table 7). Clinical pregnancy rates for each age group are presented in Table 6. Implantation and clinical pregnancy rates are graphically represented in Figure 1.

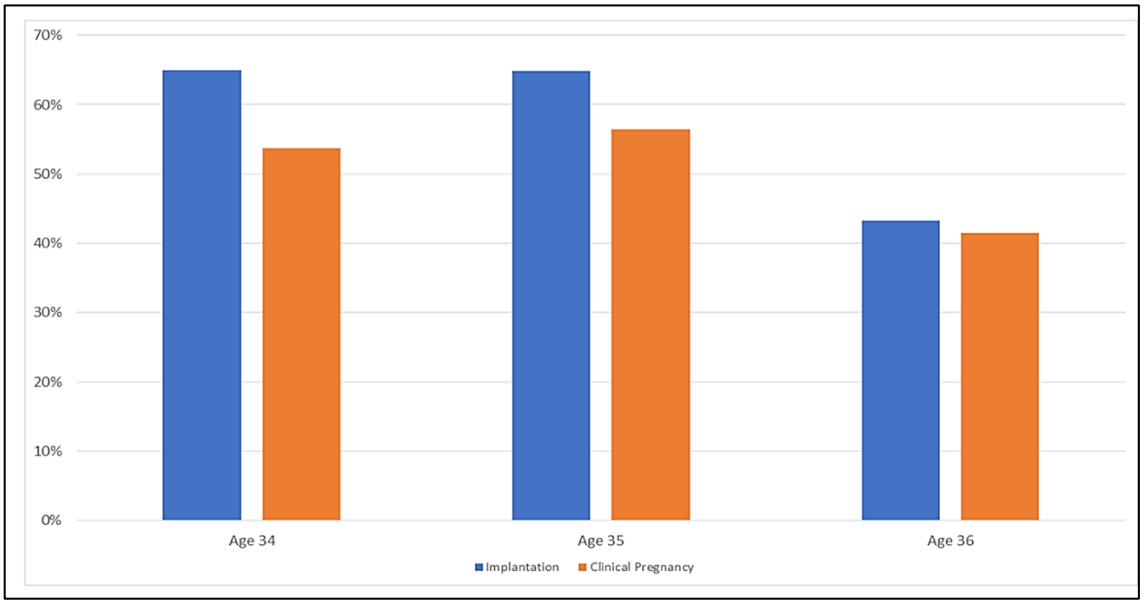
Table 5. Clinical Pregnancy rate for each age group.

	Age 34	**Age 35**	**Age 36**
Pregnant	72 (53.7%)	61 (56.5%)	46 (41.4%)
Not pregnant	62 (46.3%)	47 (43.5%)	65 (58.6%)

Table 6. Risk Ratios for Risk of not achieving clinical pregnancy status following a single cycle of IVF treatment Dependent on Patients' Age

Patients' Age	**Risk Ratio (RR)**	**95% Confidence Interval (95% CI)**
Age 36	1	1
Age 35	0.74	0.56-0.96
Age 34	0.79	0.62-1.00
¶Reference group: women Age 36 years old		

Figure 18. Chart of implantation and clinical pregnancy rate according to maternal age.



Chapter 5: Discussion

Chapter 5: Discussion

Over the past three decades delaying parenthood after the age of 35 years has emerged as a trend worldwide. As a result, a continuous increase in the mean age of women achieving their first pregnancy is reported. Maternal age and adverse pregnancy outcomes, as well as high miscarriage rates (Carolan et al., 2012; J Cleary-Goldman et al., 2005; Giri et al., 2013; Kenny et al., 2013b; Khalil et al., 2013; Samantha C. Lean et al., 2017; Martinelli et al., 2018; Salihu et al., 2008) are associated through a strong linear correlation (Spandorfer et al., 1998; Younis et al., 2015). Nowadays, this correlation is becoming more significant in the field of reproductive medicine, as clinicians are called to overcome the age-related challenges in treatment. Nevertheless, questions related to the management of AMA group women remain unanswered and thus concentrating on optimal management is more important than ever (Huang et al., 2008; Martin et al., 2018; Mills et al., 2011).

The literature is overwhelmed with large and well-designed cohort studies investigating the age limits associated to pregnancy potential. The majority of these studies compare cohorts including large numbers of individuals categorized in a wide range of age-frames described as "age boxes" at 30–35, 35–39 or >45 cohorts (Jane Cleary-Goldman et al., 2005; Delbaere et al., 2007b; Kenny et al., 2013c; Khalil et al., 2013; Martinelli et al., 2018; Wang et al., 2011b, 2008). However, in spite of the abundance in investigating this subject through systematic reviews and meta–analyses there is still lack of robust data leading to full proof, safe conclusions about the employment of specific age cut–off points in guiding and adjusting the management of this special group.

Several cohort studies report that age 35 should be considered as the appropriate age cut-off point to employ regarding several aspects of ART. Others suggest that age 40 is the crucial cut–off point (Andersen, 2000; S Cnattingius et al., 1992; Delbaere et al., 2007b). The rationale behind our research was to raise practitioners' awareness regarding the current and future use of age 35 as a cut–off point in assisted reproduction. The exact age of 35 is not only a theoretical transitional phase of women's fertility but concurrently, a point in time associated with specific guidelines regarding clinical practice in obstetrics and gynecology and respective patient management. Therefore,

the question we set–out to delineate is “Could this age categorization in obstetrics and gynecology practice extent to matters of infertility and assisted reproduction?” This study uniquely brings to literature reports on the associations between age evaluated in distinct calendar years 34, 35, 36 rather than larger interval age cohorts, and IVF outcome in good prognosis patients undergoing a first attempt in assisted reproduction.

In order to achieve this purpose, we set up a novel strategy of methodology relying on the comparison of IVF outcome between women of 34, 35 and 36 years of age. This strict categorization ensured that we do not address the general time-frame related to the “age 35 cut-off point. Instead, we opt to approach and examine-in a precise fashion-the true effect of “age” expressed in calendar years on fertility. The value of this report is heightened, as 34-36 is considered to be a time-sensitive period in a woman’s reproductive life span. This study originating from an Assisted Conception Unit does not report on information regarding live birth rate or obstetrical history and pregnancy final outcomes as patient follow up may be challenging. The retrospective nature of this study constitutes a limitation of this work. The reported number of patients included for analysis is attributed to the fact that this study dictated extensively strict criteria in recruiting patients, in order to isolate confounders and be able to highlight the true effect of age on IVF outcome.

Regarding the hormonal profile of the patients in all three groups of the study the levels of FSH, LH and AMH did not differ significantly. The number of oocytes collected was similar for all three groups. With respect to the number of normally fertilized oocytes, no differences are reported between the three age groups. Subgroup analysis regarding the quality of transferred embryos revealed that-as anticipated-the majority of blastocysts were of top quality in all age groups, and no statistical difference was presented between them. The comparison of implantation rates between the three age groups led to an important observation, namely that IVF cycles performed for women of 36 years of age presented with a significantly lower implantation potential in comparison to the other two age groups. A similar trend was also observed regarding the clinical pregnancy rate. Women aged 35 years old presented with a significant higher potential to achieve a clinical pregnancy following a single cycle of IVF treatment, in comparison to women aged 36 years old. Furthermore, women aged 34 years old presented with a higher clinical pregnancy rate in comparison to women of 36 years of age, however this trend was considered marginal and therefore not reaching

statistical significance. Possibly, a slightly larger sample size could establish a statistically significant difference, which observed between the groups of 35 and 36 years old women, regarding the clinical pregnancy rate.

Several studies demonstrate that application of different ovarian stimulation protocols in women over the age of 35 year may be associated with differences concerning the number and the quality of oocytes retrieved as well as the implantation and pregnancy rates. On the contrary, others suggest that there is no significant difference between the comparative protocols (Matorras et al., 2011; Ou et al., 2016; Vuong et al., 2017; Xu et al., 2014). Our results indicate that the number of oocytes collected following ovarian stimulation with GnRH – long protocol was similar for all three age groups, indicating a good ovarian response. Furthermore, regarding the number of normally fertilized oocytes, no differences were noted between the age groups. This observation indicates that the fertilization success might not be affected when maternal age increases from age 34 to age 35 or from age 35 to age 36.

The dilemmas practitioners encounter regarding management may be intensified when a patient is in transition from one age box to the next, especially if this age is thought to be a cut-off point. Questions such as "how many embryos can and should-according to respective legislation- be included in the transfer?" and "which is the appropriate COH protocol to employ?" depict only one of the many facets of the conundrum that patients aged 34-36 years present with in management. It is therefore well argued that, defining the absolute age is of paramount importance. The correct answers enable equally both the increased chances of a successful outcome in one hand and the avoidance of complications such as multiple pregnancies.

The observations documented herein could be useful for clinicians when called to decide regarding the ideal number of embryos included in the embryo transfer in a single IVF cycle. On the matter of the maximum number of embryos that should be transferred in one IVF cycle, the debate is constantly fueled. This is attributed to the fact that the existence of guidelines and different legal frameworks between different countries synergistically create lack of a universal consensus regarding the embryo transfer strategy ("Guidelines on number of embryos transferred," 2009; "Regulation and legislation in assisted reproduction," 2017). Our results indicate that women at the age of 36 years present with a significant lower implantation rate and clinical pregnancy

rate in comparison to that of women at the age of 35 years. This observation may in turn lead the authors to conclude that indeed this study supports that the age of 35 years may serve as a cut-off point regarding the number of embryos employed in embryo transfer. The authors refrain from making statements regarding the optimal number of embryos regarding patients aged 36 and whether including more embryos in the transfer would ensure a higher implantation rate especially in the era of elective single embryo transfer.

Our results are in line with the current American Society of Reproductive Medicine guidelines recommending elective single embryo transfer for patients under the age of 35 years, while no more than two cleavage-stage embryos should be transferred for patients with favorable prognosis aged 35-37 years old ("Guidelines on number of embryos transferred," 2009). The reduction in implantation and clinical pregnancy rate is principally attributed to increased age. It may be suggested that the possible underlying pathophysiological basis behind it, is placental dysfunction and abnormal placentation as both of these mechanisms have been reported to be implicated in both AMA and IVF treatment (Samantha C Lean et al., 2017; Silberstein et al., 2014).

In conclusion, our study refers to a strictly defined group of patients described as good prognosis IVF patients of good ovarian response on their first cycle of treatment aged 34-36 years old. According to the Human Fertilization and Embryology Authority the average age of an IVF patient was 35.5 years old in 2016 and has increased by a calendar year since 2000 (HFEA, 2018). The age range studied herein is admittedly the one surrounded by most controversy regarding the optimal number of embryos in embryo transfer. Regarding this particular cohort of women, our results support that the decision on the number of embryos to be transferred should be justly depended on accepting age 35 as the appropriate cut-off point, as implantation and clinical pregnancy rates presented to be higher reaching statistical significance for women aged 35 years old in comparison to women aged 36 years old. These observations could be useful for clinicians when called to make a decision about the ideal number of embryos included in the embryo transfer in a single IVF cycle, especially when patients are in transition from cut–off point age 35 to age 36. Robust data provided by larger prospective studies and meta-analyses would provide stronger evidence in order to address the benefits and delineate further the issue of the appropriate age cut–off points in IVF treatment. This

will assist towards implementation of a common universal protocol on the optimal age-related management in assisted reproductive medicine.

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