REPORT ON THE LIVE BIRTH RATES AND NEONATAL OUTCOMES OF ART PATIENTS AT AEVITAS FERTILITY CLINIC; IMPLICATIONS OF A FROZEN EMBRYO TRANSFER (FET) PROGRAM.

By

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DECLARATION

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SUMMARY

Background: The practice of Assisted Reproductive Technologies (ARTs) has become quite commercial in the last decade or two, since an increasing number of couple's face fertility challenges and nevertheless remain hopeful for a family of their own. Recent refinements in ART, especially in the area of embryo cryopreservation especially vitrification, has also increased its popularity. Despite the many benefits of ART, concerns about the health of children born following ART treatment is a relevant topic of discussion.

Pregnancy after infertility treatment is associated with negative neonatal and obstetric outcomes in comparison to spontaneous conceptions. Concerns about gestational age and birthweight in babies of patients of advanced maternal age have been voiced and recent evidence mentioned differences in neonatal outcomes between fresh embryo transfer (ET) cycles and frozen embryo transfer (FET) cycles. However, contradictory outcomes exist in the published literature on this subject. Some studies have hypothesized those inferior reproductive outcomes after ART may be due to parental characteristics, insemination method such as *in vitro* fertilization (IVF) an intracytoplasmic sperm injection (ICSI), embryo culture, cryopreservation and epigenetic modifications. It is therefore the professional responsibility of fertility specialists and scientists to be conscious of the risks of unfavourable outcomes in ART and to record and publish their results in peer reviewed publications.

Aims: The primary aim was to conduct a retrospective audit of the fresh and the frozen (vitrified/warmed) embryo transfer (FET) cycle success at Aevitas Fertility Clinic for the period of 2015-2017, by calculating the LBR and evaluating neonatal outcomes.

The secondary aim was to retrospectively investigate the possible effect of female age, number of ova retrieved at the time of oocyte pick up (OPU), number of embryos transferred at the time of embryo transfer (ET) and blastulation rate (BR) on the live birth rate (LBR) and neonatal outcomes in FET and fresh ET cycles.

Materials and Methods: Data was acquired from the standard, routine de-identified data files of the Aevitas Fertility Clinic, using medical/laboratory records ONLY for the period of 2015-2017. Patients were included in the study after exclusion and inclusion criteria were applied. The data was submitted for statistical analysis and p-values of <0.05 were considered statistically significant. Multiple regression analyses

compensated for cycle type (fresh ET/FET), female age, number of embryos transferred at the time of ET, and donor oocyte cycles.

Results:

The **LBR** (43,62% vs 45,15%), **birthweight** (2837,01g vs 2861,41g) and **gestational age** (36,29 weeks vs 36,53 weeks) of neonates were not significantly between the FET and the Fresh ET groups, respectively (p>0.05). Female age was significantly, negatively associated with the LBR (p=0.003) and neonatal birthweight (p=0.038) and oocyte donation cycles resulted in lower birthweight individuals (p=0.003). Blastulation rate was significantly different between the fresh ET and FET group (48, 18% and 65, 41%) (p=0.000).

Conclusion: This retrospective study indicated similar **LBRs** between the fresh ET and FET groups, which is in agreement with some published works. Contrary to some reports in the literature, reporting improved outcomes on following FETs, our study showed no difference in neonatal **birthweight** or **gestational age** between the fresh ET and FET groups. This is possibly due to the compilation of the patient population. The results of the study indicate that the ART and also the blastocyst vitrification programs at Aevitas Fertility Clinic follow good clinical laboratory practices. It is very successful and in this study cohort of patients, no adverse neonatal outcomes were evident. The audit analysis could be followed up including a larger sample size, additional confounders could be added to increase the power of the findings and subgroup analysis might also give more relevant information.

OPSOMMING

Agtergrond: Die toepassing van reproduktiewe biologie en *in vitro* bevrugtingsmetodes het die afgelope jare taamlik kommersieël geword, omdat 'n toenemende aantal mense uitdagings met infertiliteit moet oorkom, maar steeds hoopvol bly om hul begeertes van ouerskap te bevredig. Die toename in infertiliteitsbehandeling kan ook toegeskryf word aan verfynings in tegnologie en tegnieke in hierdie veld, veral op die gebied van die embryokriobewaring en meer spesifiek vitrifikasie. Genoegsame bewyse van suksesvolle behandeling is al gelewer. Daar is egter kommer oor die gesondheid van die kinders wat na infertiliteitsbehandeling gebore is. Swangerskappe na infertiliteitsbehandeling word geassosieer met negatiewe neonatale en verloskundige uitkomste in vergelyking met spontane swangerskappe. Vorige studies dui aan dat daar swakker resultate is met betrekking tot swangerskapouderdom en geboortegewig by neonatale individue by moeders van 'n meer gevorderde ouderdom. Onlangse bewyse het verder verskille in uitkomste getoon tussen **vars** embrio-terugplasings (ET) en **bevrore** embrio-terugplasing. Daar bestaan egter teenstrydige uitkomste in gepubliseerde literatuur oor hierdie onderwerp. Daar word veronderstel dat swakker uitkomste na infertiliteitsbehandeling moontlik te wyte is aan ouerlike eienskappe, inseminasiemetode soos *in vitro* bevrugting (IVB), 'n "intracytoplasmic sperm injection" (ICSI), embriokultuur, kriobewaring en epigenetiese modifikasies. Dit is dus die professionele verantwoordelikheid van die infertiliteitsspesialiste en wetenskaplikes om bewus te wees van die risiko's van ongunstige uitkomste van infertiliteitsbehandeling en om rekord te hou van die statistiek binne die onderskeie klinieke. Publikasie van hierdie data aan betrokke rolspelers in die bedryf is van kardinale belang.

Doelwitte: Die primêre doel is om 'n retrospektiewe oudit uit te voer van die sukses van die vars en bevrore embrioterugplasing-siklusse by Aevitas Infertiliteitskliniek vir die periode 2015-2017, deur die geboortesyfer te bereken en die neonatale uitkomste te evalueer.

Die sekondêre doel is om die effek van vroulike ouderdom, aantal oösiete by aspirasie, die aantal embrios wat teruggeplaas is en die blastoliseringstempo, op die geboortesyfer en neonatale uitkomste in vars en bevrore ET-siklusse te assesseer.

Materiale en Metodes: Data vir die projek is verkry uit die standaard, roetine, anonieme datalêers van die Aevitas Infertiliteitskliniek, slegs van mediese/laboratoriumrekords vir die periode 2015-2017. Data is gefiltreer volgens insluit- en uitsluitingskriteria. Die data is daarna aangebied vir statistiese analise en p-waardes van <0.05 is as statisties betekenisvol beskou. Meervoudige regressie-ontledings het die tipe siklus (vars ET / FET), vroulike ouderdom, aantal embrio's wat teruggeplaas is, en die skenker-oösiet-siklusse, in ag geneem.

Resultate: Die **geboortesyfer** (43,62% vs 45,15%), **geboortegewig** (2837,01g vs 2861,41g) en **swangerskapsouderdom** (36,29 weke vs 36,53 weke) van pasgeborenes het nie betekenisvol verskil tussen die vars en bevrore embrioterugplasings groepe nie. Die vroulike ouderdom is negatief geassosieer met die geboortesyfer (p = 0.003) en neonatale geboortegewig (p = 0.038), en die siklusse van oösietskenking het gelei tot pasgeborenes met 'n laer geboortegewig (p = 0.003). Verder, het die blastoliseringstempo het betekenisvol verskil tussen die twee groepe (48,18% en 65,41%) (p=0.000).

Gevolgtrekking: Hierdie studie het soortgelyke **geboortesyfers** aangetoon tussen die vars en bevrore ET-groepe. Ons studie het geen verskil getoon in neonatale **geboortegewig** of **swangerskapsouderdom** tussen vars en bevrore ET-groepe nie, wat teenstrydig is met resultate in die literatuur. Hierdie bevinding is moontlik te wyte aan die samestelling van die pasiëntpopulasie. Die resultate van die studie dui aan dat Aevitas-Infertiliteitskliniek goeie kliniese praktykstandaarde volg. Die uitkomste is baie suksesvol, en binne hierdie samestelling van pasiënte is geen ongunstige uitkomste waargeneem nie. Hierdie oudit kan moontlik opgevolg word met 'n groter steekproef, en addisionele veranderlikes kan in ag geneem word om die betekenisvolheid van die bevindings te bevorder en subgroup analises mag addisionele belangrike informasie verskaf.

DEDICATION

Dedicated to my wonderful parents and sister, Michael, Sonja and Tina.

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Table of Contents

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

The following section includes an assembly of background information, presented as a literature review, that may have an impact on the final outcomes of the study: outcomes in vitrified-warmed ET/frozen embryo transfer (FET) cycles versus fresh ET cycles, including neonatal outcomes.

In this literature review, various topics that form part of the principle theme of this retrospective study are discussed. This includes reports on the live birth rates (LBR) and neonatal outcomes in a frozen embryo transfer (FET) program in comparison to fresh embryo transfer cycles. The topics to be discussed include a general overview of infertility and Assisted Reproductive Technology **(ART)** and Controlled Ovarian Hyperstimulation **(COS)** – an important element of infertility treatment. Furthermore, the effect of **female factors** such as female age, ovarian reserve, Antral Follicle Count (AFC), Anti-Müllerian Hormone (AMH) and the number of ova retrieved at the time of oocyte pick up (OPU) on infertility outcomes is discussed. Additional factors such as genetic factors, environmental factors, lifestyle factors, medical factors such as endometriosis, ovarian surgery, chemotherapy and radiation are mentioned. **Extended embryo culture**, including information on culture media, culture duration and blastulation rate is included in this review. In addition, **embryo cryopreservation** will be described. Most importantly, outcomes in **vitrified-warmed ET/frozen embryo transfer (FET) cycles versus fresh ET cycles** are elaborated on. Finally, **neonatal outcomes** such as birthweight and gestational age will be discussed, as well as other maternal outcomes and delivery methods.

INFERTILITY AND ASSISTED REPRODUCTIVE TECHNOLOGY (ART)

Infertility has been characterized by the inability of a couple to achieve a pregnancy over an average period of 12 months of regular unprotected intercourse (Luke, 2017; Pfeifer *et al.*, 2017) Currently, infertility continues to be a common condition affecting people all over the globe (Inhorn and Patrizio, 2015). According to reports in various literature studies, infertility affects 8 to 12% of couples who are of procreative age, which is approximately 186 million people (Inhorn and Patrizio, 2015; Vander Borght and Wyns, 2018). Furthermore, the incidence of infertility may be more concentrated in some areas of the world, especially in developing countries where infertility treatment is often not available, easily accessible or affordable (Inhorn and Patrizio, 2015; Vander Borght and Wyns, 2018).

Evidence in the literature states that infertility can by categorized as primary or secondary infertility, the former referring to couples who have never been able to conceive and the latter referring to difficulty in establishing a pregnancy after having conceived (Vander Borght and Wyns, 2018). Various studies report that the most prominent factor that may negatively affect fertility is increasing female age, where fertility declines rapidly after the age of 35 (Vander Borght and Wyns, 2018).

In addition, causes of infertility include abnormalities regarding ovulation, tubal function and uterine factors in the female and sperm function and semen parameters in the male partner. (Child, 2013). It has been stated that approximately 35% of couples will have infertility originating from the female, 35% originating from the male and 30% of the causes of infertility is unexplained (Child, 2013; Jones and Lopez, 2013). Furthermore, lifestyle factors may also have a negative impact on reproductive health and success, such as delayed childbearing, obesity, restrained or excessive exercise, diet, smoking, stress, alcohol and caffeine consumption, and exposure to environmental pollutants and chemicals (Petraglia *et al.*, 2013).

However, hope is present for couples since 85-90% of infertility cases can be diagnosed, and approximately 50-60% can be treated successfully (Jones and Lopez, 2013).

Assisted Reproductive Technology (ART) refers to various specialized laboratory techniques and procedures that are implemented to treat infertility, with the aim of potentially establishing a pregnancy (Luke, 2017; Waynforth, 2018). These technologies include non-

in vitro fertilization treatments such as ovulation induction and artificial insemination, and *in vitro* ART treatments where oocytes are fertilized by spermatozoa in the laboratory and not in the female reproductive tract (Luke, 2017). *In vitro* ART treatment options include *In Vitro* Fertilization (IVF), Intracytoplasmic Sperm Injection (ICSI) and Gamete Intra-Fallopian transfer (GIFT), which are prescribed according to the specific aetiology of the patient (Child, Tim *et al.*, 2013). The field of ART has proliferated exponentially since the first triumphant live birth through IVF in 1978 (Maheshwari *et al.*, 2016b). At present, more than 1 million infertility treatment procedures such as IVF and ICSI are performed all over the world and have resulted in more than 6 million live births (Coward and Wells, 2013; Maheshwari *et al.*, 2016b).

The application of ART to treat infertility is continuing to increase at a remarkable pace, with various novel techniques being added to routine laboratory ART practices (Kushnir *et al.*, 2017). At present, advanced technological methods such as cryopreservation of oocytes and embryos and genetic testing of embryos by means of pre-implantation genetic testing (PGT), are applied regularly in ART laboratories (Sakkas *et al.*, 2018). Moreover, the population of patients have been a centre subject of change in recent years, with donor gamete programs and surrogacy becoming more apparent (Sakkas *et al.*, 2018). Subsequently, strict measures should be adopted to ensure that treatment protocols and standard operating procedures are adhered to and to minimize risks associated with unfavourable occurrences in ART (Sakkas *et al.*, 2018).

Although ART has proven to be a particularly successful modality for infertility treatment, the health of children conceived by ARTs has been subject to apprehension since the initial successful treatments (Spijkers *et al.*, 2017). Evidence in the literature states that the incidence of low birth weight (<2500g), very low birth weight (<1500g) and pre-term birth (babies born alive before 37 weeks of pregnancy are completed) is higher in children conceived through ART compared to spontaneous conceptions (Barsky *et al.*, 2016; Spijkers *et al.*, 2017; De Vos *et al.*, 2018; World Health Organization, 2018). Furthermore, ART using controlled ovarian hyper stimulation (COS) has evidently been characterized by low embryo implantation rates, and a greater probability of ovarian hyper stimulation syndrome (OHSS) (Aflatoonian *et al.*, 2016; Basirat *et al.*, 2016; Özgür *et al.*, 2015). Moreover, it has been reported that fresh embryo transfers following COS may lead to pregnancies with a higher probability of unfavorable perinatal, neonatal, and long-term health outcomes in comparison to natural conceptions (Özgür *et al.*, 2015). Conversely,

various studies have reported that an increased risk of adverse perinatal outcomes does not exist among children born following ART (Barsky *et al.*, 2016; Spijkers *et al.*, 2017). It should therefore be elucidated whether the risk of unfavorable reproductive outcomes is due to the specific ART procedure and associated processes, or the inherent infertility or other causes (Barsky *et al.*, 2016; Spijkers *et al.*, 2017).

CONTROLLED OVARIAN STIMULATION

It is common knowledge that the treatment of fertility may be intensely complicated, as it involves various steps and processes that require precise execution since mistakes may ultimately lead to failure to reach conception (Farquhar and Marjoribanks, 2018). The efficacy of infertility treatment partly relies on the acquisition of an adequate number of oocytes, to establish good quality embryos for subsequent embryo transfer, without subjecting the patient to risks of excessive ovarian stimulation (Gallos *et al.*, 2018) . Ovarian stimulation techniques - one of the cornerstones of successful ART - have been established with the aim to increase the simplicity and efficacy of ART (Youssry *et al.*, 2008).

The efficacy of COS relies on an unimpaired hypothalamic-pituitary-gonadal (HPG) axis (Maggi *et al.*, 2016). The principal components of the HPG axis are gonadotropin-releasing hormone (GnRH), gonadotropins such as luteinizing hormone (LH) and follicle stimulating hormone (FSH) and the gonads (Maggi *et al.*, 2016). The hypothalamus is responsible for the production and release of gonadotropins from the pituitary, which subsequently triggers the synthesis of steroid hormones from the gonads (Maggi *et al.*, 2016). It is possible to modify these functions in order to reach the desired outcome during ovarian stimulation, due to the intricate coordination of the HPG axis (Maggi *et al.*, 2016).

One rationale behind ovarian stimulation or controlled ovarian stimulation (COS) is to induce ovulation in women who experience anovulation (Luk and Patrizio, 2013). In addition, the ultimate result of COS is to induce the development of multiple ovarian follicles allowing the recovery of several mature oocytes and subsequently the potential production of many embryos, by means of exogenous gonadotropins, such as LH and FSH (Youssry *et* al., 2008; Luk and Patrizio, 2013; Gallos *et al.*, 2018). Gonadotropin-releasing hormone (GnRH) agonists or antagonists are administered in conjunction with exogenous gonadotropins to suppress the pituitary and to prevent premature ovulation (Gallos *et al.*, 2018). The final element of COS is the use of a drug to trigger final oocyte maturation, which is usually 36-

38 hours preceding OPU (Gallos *et al.*, 2018). The two drugs that are generally used for final oocyte maturation is human chorionic gonadotropin (HCG) or a GnRH agonist in an antagonist protocol (Gallos *et al.*, 2018).

Currently, there are several stimulation protocols that make use of different medication for ovarian stimulation which subsequently allows individualized care according to the specific aetiology of the patient's infertility (Nagy *et al.*, 2012). Other factors that determine the course of treatment medication is typically female age, basal FSH levels, Anti-Müllerian Hormone (AMH), estrogen levels and body weight. Stimulation regimes range from the use of clomiphene citrate (Clomid), aromatase inhibitors and human chorionic gonadotropin (hCG), to the use of GnRH analogues for ovulation induction and ovarian stimulation (Elder *et al.*, 2010).

Clomid is usually administered orally, which stimulates the release of endogenous LH and FSH from the pituitary due to a negative feedback effect from decreasing estrogen levels and stimulation outcomes or responses from previous COS treatments (Luk and Patrizio, 2013). Ovarian response is monitored continuously by ultrasonography and when the follicles reach a certain diameter, hCG is administered for final follicle maturation (Luk and Patrizio, 2013).

In other COS regimes, GnRH analogues such as GnRH agonists and GnRH antagonists are used to obstruct the release of endogenous LH and FSH by adhering to the receptors on the anterior pituitary (Elder *et al.*, 2010). GnRH agonists were generally used to prevent an LH surge, and are generally administered in a constant fashion, which causes gonadotropins (LH and FSH) to have a flare-up reaction (Elder *et al.*, 2010). Eventually, after approximately ten days, the endogenous gonadotropins are diminished. Desensitization of the pituitary is the subsequent result which inhibits the release of LH and FSH. Finally, the production of steroid hormones and follicular development is seized (Elder *et al.*, 2010). GnRH agonists may however lead to an untimely rise in LH levels, an estrogen deficiency and ovarian hyperstimulation syndrome (OHSS) in some patients (Al-Inany et al., 2011).

OHSS is the result of an excessive reaction to COS due to high hCG levels, which causes protein-rich fluid to partially relocate from the intravascular space to the abdominal cavity due to the expansion of the ovaries (Fatemi *et al.*, 2014). OHSS is the most severe condition that may result from COS during ART, which may lead to hospitalization and death in the

worst cases (Aflatoonian *et al.*, 2018; Fatemi *et al.*, 2014). Furthermore, COS may lead to excessive levels of female sex hormones which may influence the receptivity between the embryo and the endometrium (Aflatoonian *et al.*, 2016). This may subsequently lead to poor implantation, placentation and growth of the fetus (Basirat *et al.*, 2016). It has also been established that COS may affect certain neonatal outcomes, such as gestational age and birthweight (Maheshwari *et al.*, 2018). Pre-term births, low birthweight and small for gestational age babies were observed as a result of COS (Maheshwari et al., 2018; Jwa et al., 2019).

A strategy to improve endometrial receptivity and implantation, and to reduce the risk of OHSS, has been suggested by various authors. This strategy suggests the separation of the COS program and the embryo transfer (ET), whereby the ovarian stimulation and GnRH agonist trigger is performed and immediately followed by the cryopreservation of the total number of embryos for future transfers (Fatemi *et al.*, 2014; Li *et al.*, 2019). This strategy allows the transfer of embryos in a natural, unstimulated cycle during which the environment of the uterus is most favourable for embryo implantation (Fatemi *et al.*, 2014; Li *et al.*, 2019). Consequently, OHSS and the risks associated with COS may be eradicated, and the safety of ART may be improved as well as pregnancy, maternal and neonatal outcomes (Ernstad *et al.*, 2019; Fatemi *et al.*, 2014; Li *et al.*, 2019; Zhu *et al.*, 2018).

There are many additional factors that may have an influence on the success and outcomes of ART, such as female factors, extended embryo culture and embryo cryopreservation.

FEMALE FACTORS

<u>Female Age</u>

It is well established that female fertility declines progressively with increasing female age, however the decline becomes more significant after the age of 30 years and then rapid after the age of 35 years (Fleming *et al.*, 2015). During reproductive aging, the number and quality of oocytes decreases, and the oocytes do not regenerate (Practice Committee ASRM, 2012). Consequently, a higher proportion of abnormal oocytes remain as a result of a constant process of oocyte atresia (Practice Committee ASRM, 2015). The oocyte number peaks during fetal life at approximately 6-7 million, followed by a decrease to approximately 1-2 million oocytes present at birth and 300 000-500 000 at the start of puberty (Faddy *et*

al., 1992). Moreover, when women reach menopause at an average age of 51 years, the number of oocytes present is about 1000 (Practice Committee ASRM, 2015).

Furthermore, the expression of markers for ovarian activity changes with increasing female age, which is characterized by an increase in circulating Follicle-stimulating hormone (FSH) and a decline in circulating anti-Müllerian hormone (AMH) and inhibin B concentrations (Fleming *et al.*, 2015). It is however important to note that the pace of this apparent reproductive decline with female age, differs considerably among women of similar age (Te Velde and Pearson, 2002; Fleming *et al.*, 2015). It is therefore evident that factors other than female age should be considered during the recommendation of a treatment plan.

Ovarian Reserve

According to evidence in the literature, ovarian reserve comprises resting primordial follicles, which are inactive for several years before forming primary follicles (Baerwald *et al.*, 2012; Fleming *et al.*, 2015). The majority of these primordial follicles that may develop further will be subjected to atresia and therefore be lost. Following the selection of a primordial follicle for further growth, AMH will be expressed from the granulosa cells from the now primary follicle (Visser and Themmen, 2005; Fleming *et al.*, 2015). AMH expression will then continue until the antral stages of development and will arrest as the follicle becomes dependent on FSH for further growth (Fleming *et al.*, 2015).

Since large variations exist in oocyte reserve between individual women, ovarian reserve testing has been used to make a decision regarding ART treatment options (Fleming *et al.*, 2015). Currently, accessible tests for ovarian reserve may include biochemical markers such as FSH, estradiol, AMH and inhibin B, and ovarian ultrasound imaging such as antral follicle count (AFC) and ovarian volume (Committee on Gynecologic Practice, 2015; Practice committee ASRM, 2015). Recently, evidence has surfaced stating that AFC and AMH levels are the favoured methods for predicting ovarian reserve (Fleming *et al.*, 2015). It has been suggested that these tests may be used to predict the outcomes of IVF with regards to oocyte yield in response to ovarian stimulation and the pregnancy rate It has been described that women with a low ovarian reserve may respond poorly to ovarian stimulation with regards to follicular development (Fleming *et al.*, 2013). These women may have a diminished ovarian reserve (DOR), which is distinct from menopause or premature ovarian failure (Cooper *et al.*, 2011). Conversely, women with a high ovarian reserve may respond

excessively to ovarian stimulation which can lead to OHSS (Marca *et al.*, 2012; Fleming *et al.*, 2013; Committee on Gynecologic Practice, 2015). Both these responses may occur in up to 30% of *in vitro* fertilization (IVF) cycles and may be very harmful to patients and may lead to death in some cases (Fleming *et al.*, 2015).

Number of ova

It is well established that the aim of COS is to give rise to the development of numerous ovarian follicles, granting the potential retrieval of several mature oocytes which may ultimately lead to the development of multiple embryos. It has been stated that the number of oocytes recovered at oocyte pick up may provide information regarding the prognosis of the patient during ART treatment (Kamath *et al.*, 2018). Categories of a female patient's response to ovarian stimulation include poor (≤3 oocytes), normal/intermediate (8-12 oocytes), or high/excessive (>15 oocytes) (Sunkara *et al.*, 2015). The recovery of approximately 3 oocytes at oocyte pick up has been linked to a low live birth rate, and the retrieval of oocytes in the region of 15 are thought to optimize live birth rates during ART treatment (Kamath *et al.*, 2018).

It has been described that the risks of **pre-term birth (PTB)** and **low birth weight (LBW)** infants are higher in women who are of advanced age, which may possibly be due to vascular ageing and vascular endothelial dysfunction (Sunkara *et al.*, 2015). The latter vascular disease may cause a reduction in sex steroid hormones, which is the result of ovarian ageing (Sunkara *et al.*, 2015). It is consequently important to note whether patients who respond poorly to ovarian stimulation have a greater probability of deleterious obstetric outcomes during ART treatment (Sunkara *et al.*, 2015). Results from an observational study revealed that there is a definite a link between the number of oocytes recovered at oocyte pick up and unfavourable obstetric outcomes, more specifically PTB and LBW after ART treatment (Sunkara *et al.*, 2015). It was found that women from whom more than 20 oocytes are recovered, may have a greater probability of unfavourable obstetric results (Sunkara *et al.*, 2015). Poor responder patients (≤3 oocytes) were not associated with an increased risk of unfavourable obstetric outcomes (Sunkara *et al.*, 2015). However, additional studies are required to clarify these associations.

Additional Factors

The quantity and quality of female oocytes may also be influenced by genetic factors, the environment, lifestyle factors and medical matters such as endometriosis, ovarian surgery, chemotherapy and radiation (Committee on Gynecologic Practice, 2015; Practice committee ASRM, 2015). In addition, a worldwide rising trend in health issues such as obesity, which is defined as a body mass index (BMI) of >30 kg/m^2, is affecting several women and men of reproductive age (Flegal *et al.*, 2012; Ng *et al.*, 2014; Provost *et al.*, 2016). The negative effects of obesity on fertility, assisted reproductive technology (ART) success, and pregnancy and birth outcomes are well established, however the mechanism of these damaging effects remains to be elucidated (Provost *et al.*, 2016). Interestingly enough, limited data is available on the effect of having a very low BMI (<18.5 kg/m2) has on fertility (Kawwass *et al.*, 2016). Recently, it has been suggested that both extremities of BMI may be associated with an increased risk for miscarriage in the general population and in the population seeking ART treatment (Hahn *et al.*, 2014; Kawwass *et al.*, 2016).

EXTENDED EMBRYO CULTURE

Since COS during ART treatment may lead to the retrieval of several gametes from the human body, there is a necessity for an adequate environment for the development of multiple embryos in the ART laboratory. Therefore, it is the responsibility of the embryologists to attempt to recreate the environment in the uterus of the female reproductive tract in the laboratory to ensure optimal developmental potential of the preimplantation embryos (Coward and Wells, 2013). It is well known that the culture of embryos is an intricate process. There are various elements to consider that need to be in accordance to minimize stress for the gametes and embryos and to establish an optimal environment for embryo development (Coward and Wells, 2013). Such elements include the culture media, air quality, temperature and gas phase in the laboratory, the incubators and culture dishes that are used (Coward and Wells, 2013).

Culture Media

It has been described that the metabolic and physiological requirements of an embryo change as it reaches different stages of development (Nagy *et al.*, 2012). In addition, the environment and conditions are constantly changing during the embryo's journey within the female reproductive tract (Nagy *et al.*, 2012). Therefore, it is of utmost importance to use culture media which incorporates and fulfills all the changing requirements of the developing embryo in order to assure successful ART treatment (Nagy *et al.*, 2012).

The initial culture media that was formulated for human embryo culture was proposed in 1980, which did not necessarily provide for the specific stages of embryo development (Coward and Wells, 2013). Since then, several advancements have been made to ensure good quality preimplantation embryos, with culture media developing from simple salt solutions into highly complex defined media, specifically designed to reduce stress to the embryo and maintain high pregnancy rates (Mantikou *et al.*, 2013).

Currently, advances in the field of assisted reproduction led to expansions in the knowledge of *in vitro* culture conditions, with the development of stage-specific or sequential media, which was originally introduced by Gardner *et al.* in 1998 (Maheshwari *et al.*, 2016b). There are currently two approaches being employed for *in vitro* blastocyst culture, without any clear evidence stating which system is better: sequential media and one-step media (Cimadomo, Scarica, *et al.*, 2018).

The rationale behind the use of sequential media rests on a "back to nature" approach, which attempts to provide the embryo of nutrients according to its physiological and metabolic requirements during *in vivo* environmental changes to the blastocyst stage (Werner *et al.*, 2016). This medium consists of a two-step formulation which is used in succession to support the changes in embryo metabolism prior to and after the compaction stage of development (Werner *et al.*, 2016). With the sequential media approach, the composition of the medium used for culture from day 1 to 3 of development varies in concentration from the components in the medium used for subsequent culture from day 3 to the blastocyst stage. The first medium typically has a higher concentration of pyruvate, to promote the cleavage process and the initial stages of embryo development, where the last medium has a higher concentration of glucose to support increased energy-dependent blastocyst establishment (Peña *et al.*, 2018).

The one-step or continuous media relies on a "let the embryo choose" approach, which holds the ability to support embryonic growth through all stages of preimplantation development (Werner *et al.*, 2016). In this system, the composition and concentration of all the components remain consistent from day 1 to day 5 of development, with the aim to encourage the embryo to choose the required components based on the stage of development (Schneider *et al.*, 2009). Furthermore, this system does not require a change of medium on day 3, therefore the exposure of the embryo to stress due to possible

15

environmental changes is minimized (Sfontouris *et al.*, 2016). Moreover, the handling of embryos is reduced, which minimized potential risks associated with pipetting and the transfer of embryos into different dished and across the laboratory (Swain, 2019). However, due to the continuous nature of this system, there is a concern regarding a build-up of ammonium due to the processing of the amino acid, glutamine (Sfontouris *et al.*, 2016). Consequently, the establishment of an interrupted one-step culture system occurred, during which a medium-change takes place on day 3 (Sfontouris *et al.*, 2016). Fortunately, the stable dipeptide of glutamine was introduced, which now allows an uninterrupted one-step culture system without the concern of negative accumulation of components (Sfontouris *et al.*, 2016). Furthermore, one-step culture systems are suited for time-lapse imaging incubators, which allows evaluation of embryo morphokinetics and additional embryo characteristics, adding valuable information towards the embryo selection process for ET (Swain, 2019).

At present, there are various culture media brands that are commercially available, of which the composition varies between companies, but are based on either the sequential or one-step culture systems (Cimadomo, Scarica, *et al.*, 2018; Coward and Wells, 2013). Evidence in the literature reports inconsistent results when comparing sequential and continuous media systems in conventional incubators (Cimadomo, Scarica, *et al.*, 2018; Peña *et al.*, 2018). According to some studies, no significant differences were observed with regards to blastulation rate [blastocyst development from fertilized (2PN) mature metaphase II oocytes] and embryo morphology between the two approaches. Additionally, blastulation rate and ongoing pregnancy rates did not vary between the continuous culture systems, with or without medium replenishment on day 3, or the sequential culture systems (Cimadomo, Scarica, *et al.*, 2018; Macklon *et al.*, 2002). Interestingly, some studies found that the blastulation rate and morphology of the embryos were improved when one-step media was used rather than sequential media (Cimadomo, Scarica, *et al.*, 2018; Peña *et al.*, 2018; Sfontouris *et al.*, 2017). However, superior clinical outcomes were not observed (Cimadomo, Scarica, *et al.*, 2018; Dieamant *et al.*, 2016; Sfontouris *et al.*, 2017). Available data suggest that both types of media seem to provide adequate support to the developing embryo (Machtinger and Racowsky, 2012). However, the clinical efficiency and safety of one-step versus sequential media are still unclear. The studies that have been performed to date indicate the need for a review of the best available evidence to facilitate a stronger conclusion (Cimadomo, Scarica, *et al.*, 2018).

Other studies have reported potential effects of culture media on embryo and fetal development. It has been observed that the environment that the early embryos are exposed to could lead to alterations in embryonic growth, which may result in modifications in fetal growth patterns, neonatal birthweight, childhood growth and long term disease (Sunde *et al.*, 2016). One study found that culture media used during ART treatment affects neonatal birthweight (Kleijkers *et al.*, 2016). Moreover, several review articles have reported that singletons conceived through ART treatment are at an increased risk of unfavourable neonatal outcomes in comparison to children born from spontaneous conception, including pre-term delivery, lower birthweight, neonatal mortality and congenital abnormalities (Sunde *et al.*, 2016).

Culture Time / Embryo Stage

Preimplantation embryos are exposed to various occurrences and factors that might pose risks with regards to their growth and survival (Peña *et al.*, 2018). One such factor that has a great influence is the duration spent in culture media (Peña *et al.*, 2018). Previously, it was customary to use media that facilitated the culture of human zygotes for 2 to 3 days to reach the four- to eight-cell stage, at which time the embryos were replaced in the patient. However, the extended culture to day 5 or 6, when the embryos should be at the blastocyst stage, has recently attracted more attention (Gardner *et al.*, 2008; Maheshwari, Hamilton, *et al.*, 2016). The motivation for this interest was based on the argument that the extended culture of human embryos to the blastocyst stage may provide the ability to select more robust embryos for embryo transfer (ET) in the patient (Gardner *et al.*, 2008). Another rationale behind extended culture to the blastocyst stage is the potential enhanced physiological synchronization between embryo development and the endometrial environment (Glujovsky *et al.*, 2016).

The selection of superior embryos for ET is a pivotal stage during ART treatment (Peña *et al.*, 2018). At present, one of the most crucial factors influencing successful ART outcomes is multiple gestations due to the transfer of multiple embryos with the aim to increase the probability of pregnancy (Özgür *et al.*, 2015). However, the extension of the culture duration to the blastocyst stage may aid in this dilemma. It has been described that embryos that reach the blastocyst stage of development, have potentially been subjected to voluntary selection during culture, and are potentially of higher quality (Peña *et al.*, 2018). Additionally, embryos that develop into a blastocyst have undergone embryonic genome activation,

17

which is not necessarily guaranteed for cleavage-stage embryos on day 3 (Maheshwari *et al.*, 2016b). Therefore improved pregnancy outcomes were observed when blastocysts were transferred at ET rather than cleavage-stage embryos (Peña *et al.*, 2018; Robinson, 2018). Moreover, the number of embryos transferred may be reduced to minimize the risks associated with multiple pregnancies (Youssry *et al.*, 2008). According to Maheshwari *et al.*, (2016b), pregnancy rates per ET were notably improved after single blastocyst transfer, in comparison to cleavage stage transfer. In addition, extended culture has been the preferred culture method since blastocyst stage embryos may contribute to an improved synchrony with the endometrium, subsequently enhancing the probability of implantation (Maheshwari *et al.*, 2016b).

<u>Blastulation Rate</u>

Blastulation rate, or blastocyst development rate, has been described as the fraction of 2PN zygotes that have developed into blastocysts by Day 5 post embryo transfer, which should be approximately 116 hours (± 2 hours) after insemination (Vermeulen *et al.*, 2017). It was reported that the blastulation rate is an essential factor to consider in terms of the outcome of the ART treatment cycle, since it potentially provides an indication of the effectiveness of the entire embryo culture system (Vermeulen *et al.*, 2017). Generally, the blastulation rate is determined on Day 5 or Day 6, or an integration of Day 5/6, of which Day 5 is the preferred evaluation day, since fewer embryos are usually available on Day 6 (Vermeulen *et al.*, 2017). The standard values for blastulation rate on the fifth day of development should preferably be between 25-40% (competency rate) and 44-60% (benchmark rate) (Vermeulen *et al.*, 2017).

Furthermore, the quality of the blastocyst is usually based on three parameters: blastocoele expansion, the quality of the inner cell mass (ICM) and the quality of the trophectoderm (TE). All three of these factors have previously been associated with pregnancy and LBR, however more recent evidence in the literature states that only the TE is a significant predictive factor of pregnancy outcomes (Ahlström *et al.*, 2011). A good quality TE has been correlated to an improved hatching process and implantation rate (Ahlström *et al.*, 2011). However, current literature is leaning towards the blastocoele expansion, being the most predictive entity with regards to pregnancy outcomes, especially in FET cycles (Zhao *et al.*, 2019). Information in the literature is contradictory and more studies are needed to confirm these findings.

CRYOPRESERVATION

Following the development of cryopreservation technology, there is now also the option of cryopreserving the subsequent surplus embryos for future use after previously failed fresh ETs or to have another baby (Aflatoonian *et al.*, 2016; Bharracharya *et al.*, 2016; Maheshwari *et al.*, 2017). It is therefore essential for the ART clinic to have an adequate cryopreservation program for the storage of the surplus embryos and to improve cumulative pregnancy rates (Roy *et al.*, 2016).

There are currently two embryo cryopreservation methods used, slow-freezing and vitrification (Basirat *et al.*, 2016). Slow-freezing involves the cryopreservation of embryos at a relatively slow rate to allow sufficient dehydration of cells whilst decreasing intracellular ice formation (Rienzi *et al.*, 2017).This method of cryopreservation has been described to be a sufficiently safe method due to the use of low concentrations of cryoprotectants that might not lead to toxic and osmotic shock (Rezazadeh Valojerdi *et al.*, 2009). However, these low concentrations of cryoprotectants might not be adequate for avoiding the formation of intracellular ice crystals, and this method has also proved to be timely and costly due to the necessity of a freezing machine (Rezazadeh Valojerdi *et al.*, 2009). In addition, this method has been associated with low embryo post-warming survival rates, prompting questions of its application (Zhu *et al.*, 2015).

An alternative method of cryopreservation, vitrification, was introduced with the aim to improve outcomes. Vitrification allows for the solidification of the solution and cells in a glass-like state whilst completely avoiding ice crystal formation (Rienzi *et al.*, 2017). This method has been reported to be relatively simple and more cost-effective, since freezing machinery is not necessary (Tavukcuoglu *et al.,* 2012). Nevertheless, this method requires a relatively high cooling rate and high concentrations of cryoprotectants that have raised concerns regarding toxicity (Rezazadeh Valojerdi *et al.*, 2009). A high cooling rate may be achieved by direct plunging of the cryo-device into liquid nitrogen, which allows for short exposure of the cells to the cryoprotectant (Raju *et al.*, 2005). In addition, the effect of toxicity may be minimized by using a combination of different cryoprotectants (Kuwayama *et al.*, 2005; Raju *et al.,* 2005). It has been reported that the vitrification technique is the superior method, having improved post-warming survival rates, implantation rates and pregnancy rates, making it the method of choice in most ART clinics (Basirat *et al.*, 2016). Evidence in

the literature demonstrated that the survival of blastocysts after vitrification and subsequent warming were substantially higher than with the slow-freezing method and warming (Bernal *et al.*, 2008). The superior survival of blastocyst stage embryos after vitrification seem to be associated with potential higher implantation and pregnancy rates, however no statistical significance was achieved (Bernal *et al.*, 2008).

Embryos intended to be cryopreserved, may be vitrified at the pronuclear, cleavage, and/or blastocyst stage of development (Basirat *et al.*, 2016). Furthermore, vitrification involves the use of various cryoprotectants at high concentrations with the purpose of dehydrating the cells prior to rapid cooling. Subsequently, intracellular ice crystal formation is reduced which in turn minimizes cellular damage (Basirat *et al.*, 2016, Vidal *et al.*, 2017). Refinements in the vitrification protocol have resulted in improved embryo survival rates (especially for blastocyst stage embryos), higher transfer rates and higher delivery rates per embryo transferred (Basirat *et al.*, 2016; Richter *et al.*, 2016; Vidal *et al.*, 2017).

The utilization of cryopreserved-warmed embryos has increased rapidly since the first successful live birth through a frozen embryo transfer (FET) in 1984 and has become a noteworthy technique worldwide (Maheshwari *et al.*, 2016b; Bharracharya *et al.*, 2016; Spijkers *et al.*, 2017; Sha *et al.*, 2018). Moreover, the implementation of embryo cryopreservation and FETs is becoming a more prominent choice of ART due to adequate pregnancy and live birth rates (Spijkers *et al.*, 2017). FET has also been proven to be advantageous since cryopreserved embryos are typically warmed and transferred in a natural, non-stimulated cycle (Basirat *et al.*, 2016, Belva *et al.*, 2016, Aflatoonian *et al.*, 2016). The potential deleterious effects of COS due to supraphysiological estradiol and progesterone levels can potentially be avoided when embryos are cryopreserved for FET cycles (Ozgur *et al.*, 2015). During FET cycles, the physiological intrauterine state may have a favorable effect on the endometrial receptivity, early implantation, placentation, and ultimately fetal growth (Ozgur *et al.*, 2015; Basirat *et al.*, 2016, Aflatoonian *et al.*, 2016) and may reduce the risk of maternal and perinatal morbidity (Wirleitner *et al.*, 2016). In addition, the risk of OHSS is reduced when embryos are cryopreserved and subsequently transferred (Bharracharya *et al.*, 2016).

"Freeze-all embryos" policies are currently becoming the strategy of choice in ART clinics for patients at risk of OHSS and to limit poor endometrial receptivity resulting from COS (Bharracharya *et al.*, 2016; Wirleitner *et al.*, 2016; Sha *et al.*, 2018). It is important to note

that neonatal outcomes are not only influenced by the quality of the embryo transferred, but also by the endometrial environment. "Freeze-all" strategies and FETs could therefore be a feasible alternative to fresh ETs (Vidal *et al.*, 2017; Sha *et al.*, 2018).

NEONATAL HEALTH

It has become apparent that obstetrical and neonatal outcomes differ between cryopreserved and fresh embryo transfer cycles (Spijkers *et al.*, 2017). Recent improvements in embryo cryopreservation methods have reportedly led to more favorable neonatal outcomes such as gestational age and birthweight, in comparison to children born after fresh ET (Barsky *et al.*, 2016; Maheshwari *et al.*, 2016; Spijkers *et al.*, 2017; Sha *et al.*, 2018).

Gestational age

Gestational age is usually calculated from the day of conception until the day of birth (Benson & Doubilet, 2018). Previously, gestational age was determined from the day of the last menstrual period (LMP) since uncertainty was present as to when conception occurred (Benson & Doubilet, 2018). However, pregnancies established from ART treatment should be calculated in completed weeks according to embryo developmental stage, embryo transfer date and the neonatal individual's date of birth (Li *et al.*, 2014a).

Term gestational age may range from 38 weeks until 42 weeks of gestation (Benson & Doubilet, 2018). Individuals born prior to 37 completed weeks of gestation are regarded as pre-term and those delivered before 32 weeks are considered very pre-term (Maheshwari *et al.*, 2018). Furthermore, babies born after 42 completed weeks of gestation are considered post-term (Benson & Doubilet, 2018). The birth of pre-term babies places great strain on the health care professionals and infrastructure, since these individuals may be at risk for long term disabilities such as mental retardation, learning disabilities, behavior abnormalities, autism, cerebral palsy, diabetes, hypertension and heart complications (Walker, 2018). Therefore, the gestational age may be an important factor to consider when evaluating neonatal health risks.

Birthweight

Birthweight has been an essential measure used to determine the potential adversity of obstetric and neonatal outcomes and neonatal mortality and morbidity (Yao *et al.*, 2018).

Abnormal neonatal birthweight (high or low) may be an indicator of potential increased risks of diseases later in the child's life (Yao *et al.*, 2018). According to Hann *et al.* (2018), birthweight may be a guideline for fetal growth and may be a strong indicator of conditions such as cardiometabolic disease and obesity.

Birthweight percentiles are used to compare neonatal individuals to the general population (Yao *et al.*, 2018). A low birthweight (LBW) has been defined as a birthweight less than 2500 g and very low birthweight is a birthweight less than 1500 g (Maheshwari *et al.*, 2018). According to Castillo *et* al. (2019), low birthweight may be correlated to an increased predisposition to adult onset to disease such as insulin resistance, type II diabetes, kidney disease and cardiovascular disease. Individuals that are small for gestational age (SGA) have a birthweight less than two standard deviations of the mean for that gestation, or less than 22% of the anticipated mean birthweight, or less than the 10th percentile as stated by the birthweight percentile reference standards for the specific gestational age (Li *et al.*, 2014a; Maheshwari *et al.*, 2018; Yao *et al.*, 2018).

Conversely, babies with a high birthweight are those that have a birthweight higher than 4000 g (macrosomia) and very high birthweight has been defined as a birthweight higher than 4500 g (Castillo *et al.*, 2019; Maheshwari *et al.*, 2018). Individuals that are large for gestational age (LGA) have a birthweight higher than two standard deviations of the mean for that gestation, or higher than 22% of the anticipated mean birthweight, or higher than the 90th percentile for the gestational age with regards to reference standards (Li *et al.*, 2014a; Maheshwari *et al.*, 2018). Neonatal individuals that are LGA may be at risk for adulthood diseases such as chronic kidney disease, obesity, and arterial complications (Castillo *et al.*, 2019).

The identification of individuals with extreme birthweights following comparison to birthweight percentile charts, may aid in the recognition of high risk neonatal individuals and whether additional care is required (Li *et al.*, 2014a). It is important to note that these birthweight percentile charts are constructed according to the particular population and should be according to gender (Li *et al.*, 2014a).

Neonatal outcomes in ART

The rise of ART has granted the conception of countless children who in other respects would not have been part of this world (Roseboom, 2018). Due to the increasing number of children born after ART-treatment, it has been suggested that adequate numbers have been reached for researchers to potentially determine whether these technologies are associated with certain risks (Waynforth, 2018). Despite the fact that many ART children are born in good health, concern exists regarding certain perinatal (the period that extends from 22 weeks of gestation until one week after birth) and neonatal (the period that initiates at birth and extends to 28 days after birth) outcomes in children born after ART in comparison to naturally conceived children (Fang *et al.*, 2018; Zupan and Åhman, 2006). ART has also been correlated to possible detrimental maternal outcomes in terms of obstetric complications (Sunderam *et al.*, 2018). Several systematic reviews and meta-analyses confirm these observations and have exhibited an increased risk of **pre-term birth(PTB), very pre-term birth (VPTB), low birthweight (LBW), very low birthweight (VLBW), small for gestational age (SGA), birth defects, perinatal and neonatal mortality and admission of neonates to intensive care** in singletons conceived from ART treatment (Pandey *et al.*, 2012; Sunderam *et al.*, 2018).

There are several elements involved during the infertility treatment process and ART that may influence neonatal outcomes, from ovarian stimulation, to insemination strategy by means of IVF or ICSI, the constitution of the culture media and length of embryo culture, the cryopreservation and thawing process, and the number of embryos transferred at ET (Fang *et al.*, 2018).

ART treatments such as IVF involves an environment that varies from the environment of the female reproductive tract where natural fertilization and embryo development takes place (Castillo *et al.*, 2019). These variations may lead to prospective epigenetic modifications in the phenotype of the progeny and may have detrimental effects on their health in the long term (Castillo *et al.*, 2019). The fertilization process during IVF has been linked to a higher prevalence of **LBW** in comparison to ICSI (Castillo *et al.*, 2019). Furthermore, extended culture of the embryos to day 5 or 6 (blastocyst stage) has been correlated to an increased risk **of PTB and LGA** individuals (Castillo *et al.*, 2019). Interestingly, it has been shown that singletons born after FET are at lower risk of LBW, SGA, and preterm birth, but at higher risk of LGA in comparison to singletons born after fresh ET (Castillo *et al.*, 2019). Focus has also been placed on the effects of culture media on outcomes in terms of birth. When comparing different culture media for ART, it was found

that gene expression profiles of embryos vary, as well as **intrauterine growth patterns, placental weight, live birth rate and birth outcomes such as birthweight and growth** (Castillo *et al.*, 2019). One randomized controlled trial conducted by Kleijkers *et al.* (2016) observed a notable difference **in birthweight** when they compared two different ART culture media (Castillo *et al.*, 2019; Kleijkers *et al.*, 2016). It is yet to be elucidated which of ART treatment influences health of the children to a significant extent.

Certain adverse birth outcomes have been impelled by the increased incidence of **multiple pregnancies** and associated increased risk of **PTB** after ART treatment (Hwang *et al.*, 2018). An observable trend has become evident recently regarding the number of embryos transferred at embryo transfer. In the general practice of ART, the strategy has been to select the best-quality embryo or embryos for transfer in a fresh treatment cycle (Bhattacharya, 2016). It has become quite standard to transfer two or more embryos where IVF/ICSI treatments are rather costly and patients want to potentially enhance their chances of conception, even in younger patient groups (Qin *et al.*, 2015). In addition, another trend regarding the childbearing age of women has also had an increasing effect on the multiple status (Caserta *et al.*, 2014). Therefore, the frequency of multiple pregnancies has increased due to the rise in the demand of ART and the transfer of multiple embryos at the time of ET (Qin *et al.*, 2016).

It has been described that this increased multiple pregnancy rate is a complication of ART, since **maternal and neonatal outcomes** seem to be substandard in comparison to singleton pregnancies (Qin *et al.*, 2016). It has been expressed that obstetric outcomes are worse after ART treatment, mainly due to the increased rate of multiple pregnancies (Pandey *et al.*, 2012). However, it has been reported that pre-term birth as well as low birthweight have also been observed in singleton births from ART pregnancies (Hwang *et al.*, 2018). In addition, more recent evidence has shown comparable results between ART singletons and naturally conceived singletons (Pandey *et al.*, 2012). Yet, additional evidence in the literature contradicts the previous statement, presenting poorer outcomes in ART singletons (Luke, 2017; Pandey *et al.*, 2012).

Since the success of infertility treatment has previously been linked to the number of transferred embryos it has become common practice in ART laboratories to implement single embryo transfer (SET) protocols as a means to reduce the proportion of multiple pregnancies and to confine the concomitant risks regarding maternal and neonatal

outcomes (Martin *et al.*, 2017; Özgür *et al.*, 2015; Qin *et al.*, 2016). This practice has resulted in a significant drop in multiple pregnancies (Pandey *et al.*, 2012).

It is important to note there are two types of single embryo transfers – elective (eSET) and non-elective (non-eSET) (Martin *et al.*, 2017). eSET refers to the selection and transfer of one embryo from a group of good quality embryos and non-eSET is the transfer of one embryo since it is the only remaining embryo (Martin *et al.*, 2017). It is essential to distinguish between these two groups, since the eSET population group may represent women with the most favourable circumstances and the non-eSET group possibly represent a population of women with poor response to ovarian stimulation or lower embryo developmental potential. The latter group may have less embryos of sufficient quality for transfer and may therefore indicate unrevealed pathology that may render these women and children more susceptible to poorer outcomes (Martin *et al.*, 2017).

This distinction has not always been made clear within the literature, however it has been observed that outcomes in eSET groups may be inferior in terms of **gestational age and birthweight,** in comparison to naturally conceived children (Martin *et al.*, 2017). Observations from other studies found similar outcomes between eSET singletons and singletons conceived naturally (Martin *et al.*, 2017). Current information in the literature states that the unfavourable outcomes among ART singletons were restricted in cases of double embryo transfer (DET). This may most likely be due to the occurrence of a vanishing twin, which may have a negative effect on the implantation potential of the other twin as well as growth (Martin *et al.*, 2017). Similar results were reported in 2017 by Luke *et al.* (2017) in cases of fetal loss, there may be a higher risk of **LBW, PTB and SGA** outcomes in singleton and twin births. Moreover, it has been expressed that even when the number of fetal heartbeats and the number of babies born are equal, the transfer of multiple embryos may lead to growth restrictions in singleton and twin births (Luke, 2017). Therefore, the risk of substandard outcomes for singleton pregnancies may also be associated to the number of early fetal heartbeats, and not only to the number of embryos transferred, as mentioned before (Luke, 2017; Martin *et al.*, 2017).

Attempts to interpret these conflicting results between previous evidence and more recent results have raised awareness that procedures and protocols of ART treatment has evolved from the initial stages, with SET, blastocyst transfer and vitrified-warmed embryo transfers becoming more apparent (Pandey *et al.*, 2012). Furthermore, the intricacy of the ART

treatment process also adds to the complexity of resolving these inconsistencies. Therefore, these inconsistent results regarding the risks associated with ART treatment and multiple status elicits a necessity for additional studies to provide clarity on this matter, and to determine which facet or facets of the ART treatment process presents with the most risk (Pandey *et al.*, 2012). With this knowledge, the potential risks can ultimately be minimized (Pandey *et al.*, 2012).

Pregnancy and Neonatal outcomes in Fresh ET vs. Vitrified-warmed ET (FET)

In general practice during IVF or ICSI treatment, embryos of the highest quality are typically selected for transfer in a fresh cycle after 2-5 days in embryo culture, while the surplus embryos of good quality may be cryopreserved for potential use in the future. (Zhu *et al.*, 2013). Therefore, a standard IVF/ICSI cycle will consist of one fresh ET and one or multiple vitrified-warmed ETs (FETs) (Wong *et al.*, 2017). As mentioned previously, the vitrified embryos are normally warmed and transferred into the uterine cavity in an unstimulated-natural cycle or in a hormone modified cycle in instances where the fresh ET was unsuccessful or when patients return with the desire to expand their family further (Maheshwari *et al.*, 2018). FET cycles have proven to be advantageous for several patients to establish a pregnancy, most likely due to a more favourable uterine environment (Agha-Hosseini *et al.*, 2018).

A fundamental element in the journey to a successful ART-treatment cycle is the implantation of the embryo into the endometrium (Roque *et al.*, 2013). There are various factors that may have an influence on the embryo implantation, including the quality of the embryos, the receptivity of the endometrium and the connective interface between the embryo and the endometrium (Roque *et al.*, 2013). The most opportune time for implantation depends highly on the condition of the endometrium for embryo adherence, in terms of morphology and functionality (Roque *et al.*, 2013). Endometrial receptivity is consequently essential for conception achieved spontaneously and artificially (Roque *et al.*, 2013). It has been proposed that COS may potentially lead to deleterious conditions in the endometrium, which may subsequently affect the interaction between the embryo and endometrium during infertility treatment (Roque *et al.*, 2019). COS leads to supraphysiological oestradiol (E_2) and progesterone levels in the course of the follicular phase of the menstrual cycle, which modifies the morphologic and biochemical properties of endometrium resulting in a more superior endometrium (Roque *et al.*, 2013). It is possible that these modified physiological

properties and modified levels of hormones may influence the synchronization of the embryo and the endometrium, the implantation potential and ultimately, the prosperity of the treatment cycle (Roque *et al.*, 2013).

It has been described that the most successful cycle type may be a fresh oocyte donation cycle, in which an embryo transfer takes place in a milieu that was not exposed to any negative effects from supraphysiological hormone levels that arise during COS (Roque *et al.*, 2013; Shapiro *et al.*, 2009). However, it has been mentioned that improved implantation rates during oocyte donation cycles is likely due to the prime quality oocytes from younger oocyte donor patients (Shapiro *et al.*, 2016). Interestingly, it was found that pregnancy rates were substantially higher in the recipients than the oocyte donors after embryo transfers in cycles in which oocytes were shared (Roque *et al.*, 2013). It was stated that the motivation for the improved outcomes was likely associated with a higher-quality endometrium and subsequent receptivity (Roque *et al.*, 2013).

Comparably, in vitrified-warmed embryo transfer (FET) cycles, endometrial preparation may be reached with the use of oestradiol and progesterone with the goal to manage the endometrial development more accurately than during COS (Roque *et al.*, 2013). It is currently well established that the class of vitrified-warmed embryos and their potential to implant are on par with the quality of fresh embryos, when taking into account the impressive progress and improvement of cryopreservation methods to date (Roque *et al.*, 2013). The number of FET cycles has increased significantly over the years and there are three possible motivations for this trend: firstly, due to continuous development and refinement of vitrification techniques and improved post-warming survival rates in comparison to slow-freezing (Zhang *et al.*, 2018). Secondly, the increased application of eSET in conjunction with pre-implantation genetic diagnosis (PGD) or pre-implantation genetic screening (PGS) has elevated the number of potential embryos accessible to cryopreserve (Zhang *et al.*, 2018). Finally, evidence in the literature has revealed that improved perinatal and neonatal outcomes can be expected from FET cycles (Zhang *et al.*, 2018; Li *et al.*, 2019)

It has been demonstrated that the risk of **PTB, LBW, and being SGA** is reduced in infants born after FET compared to fresh ET (Belva *et al.*, 2016; Barsky *et al.*, 2016; Spijkers *et al.*, 2017; Vidal *et al.*, 2017; Maheshwari *et al.*, 2018). COS during fresh ET cycles may be responsible for the increased prevalence of PTB, LBW, and SGA infants due to reduced implantation and abnormal placentation (Maheshwari *et al.*, 2018). Other studies have

described that the risk of **post-term birth, macrosomia, being LGA**, perinatal mortality, and hypertensive disorders is higher after FET compared to fresh ET (Aflatoonian *et al.*, 2016; Spijkers *et al.*, 2017; Vidal *et al.*, 2017). According to Maheshwari *et al.* (2018), higher implantation potential after FET results in improved placentation and overgrowth of the fetus, accounting for the increased rate of infants being large for gestational age. In addition, it has been suggested that the freezing and warming process may also have an influence on the growth potential of the fetus (Maheshwari *et al.*, 2018). Table 1 shows a summary of obstetric and neonatal outcomes in FET and fresh ET cycles from singleton pregnancies published over the years (Maheshwari et al., 2018).

Table 1: Summary of obstetric and neonatal outcomes between FET and fresh ET cycles from a cumulative meta-analysis (Maheshwari et al., 2018)

Risk of outcome	Evidence	Evidence available by year	No further change in precision, magnitude or direction	More observational data needed
Small for gestational age	Lower in Frozen embryo transfer	2010	2014	No
Low birth weight	Lower in Frozen embryo transfer	1997	2014	No
Very low birth weight	Lower in Frozen embryo transfer	2013	2016	No
Large for gestational age	Higher in Frozen embryo transfer	2010	2014	No
High birth weight	Higher in Frozen embryo transfer	2014	2016	No
Very high birth weight	Higher in Frozen embryo transfer	2013	2014	No
Preterm delivery	Lower in Frozen embryo transfer	2005	2014	No
Very preterm delivery	Lower in Frozen embryo transfer	2016	2016	No
Antepartum haemorrhage	No difference	2010	2014	Yes
Admission to NICU	No difference	2012	2013	Yes
Congenital anomalies	No difference	2014	2016	Yes
Perinatal mortality	No difference	2014	2014	Yes
Hypertensive disorders of pregnancy	Higher in Frozen embryo transfer	2015	2015	Yes

As mentioned previously, some authors have reported improved neonatal outcomes, such as a reduced risk of **SGA, LBW and PTB** in infants born following FET cycles in comparison to fresh ETs (Acharya *et al.*, 2018; Maheshwari *et al.*, 2018; Sha *et al.*, 2018). It was however also noted that a higher risk of **LGA** and **hypertensive disorders of pregnancy** exists in FET cycles (Acharya *et al.*, 2018; Maheshwari *et al.*, 2018; Sha *et al.*, 2018). One meta-analysis found improved pregnancy outcomes after FET, such as a reduced risk of **LBW, VLBW, VPTB, SGA**, neonatal mortality in comparison to outcomes following fresh ETs (Sha *et al.*, 2018). Though, it was observed that pregnancies following FETs were correlated to an increased risk of **pregnancy induced hypertension (PIH), postpartum haemorrhage** and **LGA** in comparison to pregnancies following fresh ETs (Sha *et al.*, 2018). Refer to Table 2 for the associations between FET and fresh ET cycles and pregnancy and neonatal outcomes in IVF pregnancies (Sha *et al.*, 2018).

Table 2: Pregnancy and baby outcomes in IVF pregnancies in FET and fresh ET cycles (Sha. Clinical outcomes in pregnancies with FET. Fert Steril 2017)

Association between frozen-thawed embryo transfer and pregnancy-related complications and perinatal outcomes in in vitro fertilization pregnancies.

Outcome	No. of studies	RR (95% CI), fixed-effect models	RR (95% CI), random-effect models	P_Q[a] value	I^2 (%)	Sensitivity analysis[b]	P_B[c] value
Pregnancy-related complications							
Pregnancy-induced hypertension	6	1.47 (1.34–1.61)	1.44 (1.16–1.78)	.01	67.9	1.41 (1.27–1.65)	.62
Gestational diabetes mellitus	5	0.97 (0.82–1.15)	0.97 (0.82–1.15)	.75	0.0	0.95 (0.79–1.15)	.39
Postpartum hemorrhage	3	1.28 (1.14–1.44)	1.28 (1.14–1.44)	.12	51.2	1.23 (1.09–1.39)	.45
Placenta previa	6	0.75 (0.64–0.88)	0.61 (0.43–0.88)	.02	64.5	0.80 (0.68–0.95)	.06
Placental abruption	5	0.63 (0.47–0.85)	0.63 (0.474–0.85)	.95	0.0	0.62 (0.46–0.84)	.05
Premature rupture of membranes	3	0.92 (0.72–1.18)	0.92 (0.72–1.18)	.44	0.0	0.85 (0.62–1.15)	.68
Adverse perinatal outcomes							
Low birth weight	22	0.79 (0.77–0.81)	0.78 (0.73–0.83)	<.01	66.7	0.77 (0.72–0.82)	.87
Very low birth weight	15	0.78 (0.72–0.84)	0.79 (0.72–0.87)	.30	12.8	0.77 (0.71–0.83)	.69
Preterm birth	25	0.94 (0.91–0.96)	0.95 (0.90–1.01)	<.01	66.8	0.94 (0.89–1.00)	.34
Very preterm birth	14	0.81 (0.75–0.87)	0.83 (0.75–0.91)	.17	24.5	0.79 (0.73–0.87)	.27
Small for gestational age	11	0.63 (0.60–0.66)	0.63 (0.58–0.67)	.12	32.9	0.63 (0.60–0.67)	.74
Large for gestational age	6	1.52 (1.47–1.59)	1.58 (1.31–1.90)	<.01	90.6	1.44 (1.14–1.68)	.80
Perinatal mortality	17	0.88 (0.79–0.99)	0.90 (0.79–1.04)	.31	11.4	0.86 (0.77–0.97)	.20

Note: CI = confidence interval; RR = risk ratio.
[a] P_Q = significance of the Q statistic.
[b] Sensitivity analysis performed after excluding the most heterogeneous study.
[c] P_B = significance of the publication bias.

Sha. Clinical outcomes in pregnancies with FET. Fertil Steril 2017.

Clinical and Neonatal outcomes in a "freeze-all embryos" strategy

An alternative option to the fresh embryo transfer strategy, is the "freeze-all embryos" strategy, which suggests that only vitrified-warmed embryos are transferred, excluding fresh ETs (Wong *et al.*, 2017). Refinements in vitrification protocols have facilitated the elective vitrification of all available embryos after a stimulated cycle, followed by subsequent FETs in unstimulated cycles during a time that the uterine environment is perceived to be the most suitable for embryo implantation (Li *et al.*, 2019; Roque *et al.*, 2019). Consequently, the top-quality embryos are chosen for vitrified-warmed ET with concomitant superior endometrial receptivity and implantation (Li *et al.*, 2019). The "freeze-all" strategy has been made available to many patients receiving ART treatment and has recently become a subject of discussion whether this strategy improves birth outcomes in comparison to fresh ET cycles (Li *et al.*, 2019; Roque *et al.*, 2019).

The rationale behind a "freeze-all" strategy is to potentially reduce the risk of OHSS in patients who display hyper-response to stimulation (Li *et al.*, 2019). Furthermore, the "freeze-all" strategy has been practiced in PGD, PGS and PGT cycles during which blastocysts are biopsied and then vitrified to allow time for results to return from a genetic testing company, and finally to ensure an optimal endometrial profile unaffected by COS (Li *et al.*, 2019; Roque *et al.*, 2019). Some literature reports suggest that obstetric and neonatal

outcomes in individuals conceived from FETs surpass those conceived from fresh ETs (Maheshwari *et al.*, 2018; Li *et al.*, 2019; Roque *et al.*, 2019; Shi *et al.*, 2018; Spijkers *et al.*, 2017; Zhang *et al.*, 2018).

As might be expected, the "freeze-all embryos" strategy has become one of the most debated subjects recently, arguing whether it leads to more favourable clinical pregnancy rates and outcomes (Acharya *et al.*, 2018).

Many studies have compared the outcomes after performing COS and a fresh embryo transfer in the same cycle to the complete vitrification of all embryos and a delayed embryo transfer in an unstimulated cycle, reporting contradictory results (Acharya *et al.*, 2018). A Cochrane review by Wong *et al.* (2017) reported information from different randomized controlled trials, and found no significant difference in the **cumulative live birth rate** between FET and fresh ET cycles (Wong *et al.*, 2017; Acharya *et al.*, 2018). However, it was discovered that there was a reduced rate of OHSS in the FET group, as well as a reduced rate of spontaneous abortions (Wong *et al.*, 2017). It was important to note that the population of patients included in the previously mentioned Cochrane review by Wong *et al.* (2017) was constituted of high responders to ovarian stimulation, whom had approximately 13-20 oocytes recovered at oocyte pick (Wong *et al.*, 2017; Acharya *et al.*, 2018). Similarly, another study reported improved implantation rates and ongoing pregnancy rates from FETs in comparison to fresh ETs in patients who also fell into the category of high responders, having 21 oocytes retrieved on average (Wang *et al.*, 2017). One study further categorized patients according to the number of oocytes recovered at oocyte pick up, comprising a subgroup with 4-9 oocytes recovered and another with 10-15 oocytes recovered (Roque *et al.*, 2017). It was observed that improved outcomes with regards to implantation and ongoing pregnancy was reported by the subgroup having 10-15 oocytes recovered (high responders). During an analysis separating the two subgroups it was discovered that there was no clear difference in pregnancy rates in FET and fresh ET cycles (Roque *et al.*, 2017). Therefore, it was expressed by several study groups that the "freeze-all" strategy won't necessarily be advantageous to all patients undergoing ART treatment, since this strategy is mainly applied to high responder patients (Basile and Garcia-Velasco, 2016; Blockeel *et al.*, 2016). These reports emphasized that the advantages of a "freeze-all" strategy for low or intermediate responders is yet to be elucidated (Basile and Garcia-Velasco, 2016; Blockeel *et al.*, 2016).

More recent literature review studies have reported that the **live birth rate** did not differ between FET and fresh ET cycles in ovulatory women who respond to ovarian stimulation to an intermediate extent (Shi *et al.*, 2018a). One study conducted in China, which sub divided high responder patients into four groups depending on the number of oocytes retrieved (15-18; 19-21; 22-24; >25 oocytes), observed that the clinical pregnancy rate and live birth rate was notably higher following FET when more than 20 oocytes were retrieved in comparison to fresh ETs. However, no difference was detected in **pregnancy rates** between FET and fresh ET cycles when less than 20 oocytes were retrieved at oocyte pick up (Xu *et al.*, 2017). Another study in 2018 reported higher **pregnancy rates** following an FET from a "freeze-all" cycle in women who displayed high response to stimulation (>15 oocytes) undergoing their first IVF cycle, and poorer pregnancy outcomes in low and intermediate responders in comparison to fresh ET cycles (Acharya *et al.*, 2018). A population-based study concluded that the **cumulative live birth rate** was comparable for high responder patients between the FET group and fresh ET group (Li *et al.*, 2019). Although, the probability of a **live birth** was reduced in the "freeze-all" FET group for normal responders, in comparison to the fresh ET group (Li *et al.*, 2019). Refer to Table 3 for outcomes in FET cycles and fresh ET cycles by number of oocytes retrieved.

Table 3: IVF and pregnancy outcomes following fresh ET and FET by number of oocytes retrieved (Acharya. Fresh vs. FET by ovarian response to IVF. *Fertil Steril* 2018)

Patient and in vitro fertilization cycle characteristics in fresh and frozen embryo transfer by number of retrieved oocytes.

Characteristic	Oocytes 1–5		Oocytes 6–14		Oocytes 15+	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
No. of patients	10,791	2,064	34,137	4,432	24,174	7,337
Patient age (y)	36.8 ± 4.6	38.2 ± 4.7	34.4 ± 4.5	34.8 ± 4.5	32.7 ± 4.2	32.2 ± 4.1
BMI (kg/m^2)	26.3 ± 6.4	25.8 ± 6.2	26.1 ± 6.0	25.7 ± 5.9	26 ± 5.9	25.3 ± 5.6
Missing BMI, n (%)	2,107 (19.5)	612 (29.7)	6,054 (17.7)	1,023 (23.1)	4,006 (16.6)	1,533 (20.9)
Basal FSH, mIU/mL	9.7 ± 6.2	12.1 ± 11.5	7.7 ± 3.6	8.1 ± 3.6	6.8 ± 2.4	6.7 ± 2.3
Missing FSH, n (%)	3,187 (29.5)	562 (27.2)	10,197 (29.9)	1,398 (31.5)	7,616 (31.5)	2,421 (33.0)
Total FSH dose (IU)	4,059 ± 1,909	4,127 ± 2,388	3,264 ± 1,539	4,200 ± 1,956	2,582 ± 1,168	3,475 ± 1,740
Oocytes retrieved	3.6 ± 1.3	3.1 ± 1.6	9.9 ± 2.5	10.1 ± 2.6	21.2 ± 6.4	25.2 ± 9.2
No. of fertilized oocytes	2.4 ± 1.2	1.5 ± 1.2	6 ± 2.6	4.7 ± 2.6	12.6 ± 22.8	10.9 ± 5.5
No. of embryos transferred	1.7 ± 0.7	1.5 ± 0.7	1.9 ± 0.7	1.7 ± 0.7	1.7 ± 0.6	1.6 ± 0.6
Blastocyst transfer (%)	26.9	24.3	58.4	65.3	78.1	87.7

Note: Values are mean ± standard deviation unless otherwise indicated. BMI = body mass index; FSH = follicle-stimulating hormone.

Acharya. Fresh vs. FET by ovarian response to IVF. Fertil Steril 2018.

Moreover, a **higher birthweight** was observed in neonatal individuals (singletons) born following FET from intermediate and high responder patients, and a reduced risk of **LBW** following FET in comparison to fresh ET neonates across all stimulation-response subgroups (Acharya *et al.*, 2018). This study concluded that the "freeze-all" strategy does not necessarily prove to be advantageous to all patients, irrespective of the ovarian stimulation response (Acharya *et al.*, 2018). In addition, it is important to remember the

possible negative effects of the vitrification and thawing process such as damage to or loss of the embryos, the additional financial burden, the potential emotional trauma due to a delayed transfer, the higher risks of **LGA** and **hypertensive disorders of pregnancy** (Li *et al.*, 2019).

Maternal outcomes

It has been published that the essential maternal attributes vary between women who conceive naturally and women undergoing ART treatment (Li *et al.,* 2014a). Study observations claim that women in need of fertility treatment constitute a population with a larger proportion of women who are 40 years or older, in comparison to women who conceive spontaneously (Li *et al.*, 2014a). Furthermore, there is a higher probability that the pregnancy achieved after ART treatment is the first pregnancy (Li *et al.*, 2014a). It has also come to light that suboptimal perinatal and neonatal outcomes might potentially be due to maternal attributes of women following ART treatment and possibly certain facets of the infertility treatment process itself (Li *et al.*, 2014a).

In a longitudinal cohort study conducted by Luke *et al.* (2017), which included women who gave birth to singletons in Massachusetts over a six-year period that were grouped by their fertility status as *in vitro* fertilization, subfertile and fertile, found that the **subfertile and *in vitro* fertilization-treated groups** were generally older, in comparison to the fertile group (Luke *et al.*, 2017). Moreover, it was observed that these two groups also had a higher probability of having conditions such as diabetes and hypertension and were at greater risk of developing gestational diabetes and pregnancy related hypertension. In addition, these two groups were also more likely to experience detrimental pregnancy outcomes, such as complications with regards to the placenta and bleeding from the uterus (Luke *et al.*, 2017). It was concluded that these increased risks in the *in vitro* fertilization-treated group may potentially indicate an increased severity of infertility and unrevealed pathology that may affect pregnancy outcomes (Luke *et al.*, 2017).

A cumulative meta-analysis considered unfavourable maternal outcomes such as the risk of antepartum hemorrhage, placenta previa, placental abruption and reported no significant difference between FET and fresh ET cycles from singleton pregnancies (Maheshwari *et al.*, 2018). A more recent meta-analysis published more favourable outcomes with regards to placenta previa, placental abruption following FET cycles in comparison to fresh ET cycles

(Sha *et al.*, 2018). However, the latter meta-analysis found a slight increased risk for **pregnancy induced hypertension (PIH)** and **postpartum hemorrhage** (Sha *et al.*, 2018). A higher risk of **hypertensive disorders of pregnancy** was also observed in singleton pregnancies following FET cycles, according to Maheshwari *et al.* (2018).

Method of delivery

A study carried out by Stern *et al.* in 2018, which used the same Massachusetts patient population mentioned in the previous section, established a correlation between infertility treatment and an increased rate of caesarean delivery (Stern *et al.*, 2018). Previous studies have asserted that this may likely be due to the previously mentioned unrevealed obstetric and medical elements in ART-treated and subfertile women (Stern *et al.*, 2018). It was declared, from this population of women, that an increased proportion of babies that had a low birthweight and that were born pre-term in the ART-treated group and subfertile group, were delivered via caesarean section.

In cases of obstructed labour and additional emergency obstetrical situations, the caesarean section is a precautionary measure with the aim to save lives (Molina *et al.*, 2015). There has been an upward trend with the application of caesarean sections even in the absence of medical indication, due to social, cultural and financial factors, increase in multiple pregnancies, as well as an increasingly older population of primiparous women (Keag *et al.*, 2018; Mylonas and Friese, 2015).

Although this method of delivery currently poses near to no risks, it is important to note that the caesarean section remains a surgical procedure with a number of possible maternal and neonatal complications that may arise (Mylonas and Friese, 2015). Short term risks include complications that may occur during the procedure, such as injuries to the organs, the necessity of a blood transfusion, infection and issues with regards to anesthesia (Khunpradit *et al.*, 2005; Mylonas and Friese, 2015). In addition, side effects may surface after the procedure such as the obstruction of blood vessels due to blood clots (thromboembolism) (Mylonas and Friese, 2015). Long term risks include circumstances of subsequent pregnancies later in life following a previous caesarean section that may be at increased risk for uterine rupture, placental abnormalities and infertility (Mylonas and Friese, 2015). Interestingly, potential negative outcomes have also been observed in neonates after elective caesarean delivery, such as modified immune development, lower diversity

microbiome in the gastrointestinal tract, allergic rhinitis, the development of type I diabetes mellitus, bronchial asthma and obesity (Khunpradit *et al.*, 2005; Mylonas and Friese, 2015).

However, these risks have been subject to controversy due to inconsistencies within the literature. Some articles agreed with the previously mentioned risks associated with elective caesarean deliveries and added increased mortality in comparison to vaginal deliveries (Mylonas and Friese, 2015; Signore and Klebanoff, 2008). Other studies observed no significant difference in outcomes of neonates delivered vaginally or via caesarean section (Lavender *et al.,* 2012).

According to the World Health Organization (WHO), caesarean section is linked to an increased risk of maternal and neonatal morbidity in comparison to vaginal delivery. A literature update conducted by the National Institute for Health and Care Excellence in the United Kingdom compared results of elective vaginal deliveries and elective caesarean deliveries and discovered that vaginal deliveries were correlated to a reduced risk of longer hospitalization, hysterectomy in cases of postpartum haemorrhage and cardiac arrest relative to elective caesarean section (Sandall *et al.*, 2018). Conversely, it was found that elective caesarean section was linked to minimized risks of vaginal trauma, pain in the abdomen and perineal area at birth and after delivery and obstetric shock in comparison to elective vaginal deliveries (Sandall *et al.*, 2018). According to Sandall *et al.* (2018), mortality and morbidity in mothers after caesarean section is increased, in comparison to mothers who proceeded with vaginal deliveries. Additionally, caesarean section has also been correlated to higher probability of ectopic pregnancy, stillbirth and pre-term delivery (Sandall *et al.*, 2018).

It has been mentioned that the method of delivery, whether it be spontaneous, induced, supplemented or assisted vaginal delivery as opposed to caesarean section, has an effect on the development of neonatal individuals and their health later in life (Tribe *et al.*, 2018). It has been established that the clinical results of children are influenced by certain biological systems that accompany the specific delivery method (Tribe *et al.*, 2018).

The first system is the conveyance of the maternal microbiome to the offspring, which has suggested to be inadequate after caesarean delivery and leads to deficient settlement of microflora in the intestinal tract of the child and subsequent modified immunological development (Jakobsson *et al.*, 2014). Despite the fact that the constitution of the

34

microbiome of the child is only partly molded by the method of delivery, it remains an important factor during the initial stages of the child's life (Jakobsson *et al.*, 2014). It has been suggested that these early immune consequences may remain until adulthood, which may influence their predisposition to certain diseases (Jakobsson *et al.*, 2014). However, evidence to support these findings is lacking. Some literature studies have pointed out that there might be a relationship between delivery by caesarean section and elements of metabolic syndrome in the child's life, such as asthma, obesity, and the development of type I diabetes (as mentioned previously), as well as high blood pressure, modification of liver functionality, issues with regards to the immune system, neurological problems, stress-linked issues and autoimmune disorders specific to the gastrointestinal tract (Sandall *et al.*, 2018). Other studies observed that these issues only remain until the age of 12, or even earlier (Keag *et al.*, 2018).

Another biological system that may aid in the explanation of how the delivery method may influence the clinical results of the children, is the mechanical force and stress hormones that are simply diverted during caesarean section deliveries. This diversion prevents the establishment of crucial physiological stimuli that occurs during vaginal delivery, as well as essential developmental signals necessary after birth (Sandall *et al.*, 2018). During vaginal delivery, high levels of stress hormones are released at a certain point which is partially responsible for the establishment of the hypothalamic-pituitary-adrenal axis, which is the central stress response system in the human body. These hormones may also potentially signal further progression of the immune system, certain organs and formation of nervous system cells such as neurons (Tribe *et al.*, 2018). If these important signals are missed in babies born after caesarean section during this critical period of development, it may further be influenced by the generally compacted gestational time as a result of elective caesarean sections that usually occur before 40 weeks of pregnancy (Tribe *et al.*, 2018).

The last biological system which is referenced to is varying epigenetic modification that occurs during each method of delivery which ultimately determines gene expression (Dahlen *et al.*, 2013). This may have an effect on the epigenome of neonatal individuals and fundamentally on the health of the children, however further research is required to support this theory (Dahlen *et al.*, 2013; Sandall *et al.*, 2018).

With regards to the mode of delivery in fresh ET cycles versus FET cycles, it was found that the proportion of vaginal deliveries and caesarean were approximately equivalent for

singleton pregnancies (Pereira *et al.*, 2016). FET cycles have been associated with an **extended gestation** and **macrosomia** within the literature, which may potentially promote an increased risk of caesarean section, especially in a cycle including hormone replacement (Saito *et al.*, 2017).

It was mentioned that the data and information within the literature with regards to caesarean section delivery is intricate and the quality may deem the evidence unreliable, therefore further investigation should be conducted to optimize information for longer term outcomes and to confirm causation. For now, it has been recommended by the WHO that caesarean section should exclusively be performed if substantial benefits are anticipated for the mother and child (Keag *et al.*, 2018; Sandall *et al.*, 2018).

RESEARCH QUESTION

Are live birth rates comparable between fresh embryo transfer (ET) and frozen embryo transfers (FETs) and are neonatal outcomes more favourable after FETs than fresh ETs.

HYPOTHESIS

We hypothesize that the live birth rates (LBRs) are similar between fresh ETs and FETs and that neonatal outcomes (gestational age, birthweight, and multiple status) are more favourable after FETs than fresh ETs at Aevitas Fertility Clinic.

OBJECTIVES

PRIMARY:
To implement a retrospective audit of the fresh and the vitrified-warmed embryo transfer (FET) cycle success at Aevitas Fertility Clinic - using medical/laboratory records ONLY for the period of 2015-2017, by calculating the LBR and evaluating the neonatal outcomes.

SECONDARY:
To retrospectively investigate (using medical/laboratory records ONLY) the effect of female age, number of ova retrieved at the time of oocyte pick up (OPU), number of embryos transferred at the time of embryo transfer (ET) and blastulation rate (BR) on the live birth rate (LBR) and neonatal outcomes in FET and fresh ET cycles.

CHAPTER 2

MATERIALS AND METHODS

Study Population

The patient population included all those individuals who were subject to a fresh ET and those individuals that granted consent for blastocyst vitrification and received subsequent vitrified-warmed blastocyst transfers (FETs) during 2015-2017. Furthermore, patients with available live birth data were exclusively included in the retrospective analysis of live birth rate (LBR) outcomes. Outcomes of patients in same-sex relationships and who required a surrogate were included in this study. Patients were excluded in cases of no embryo transfer, missing outcome data (lost to follow up), PGD/PGS/PGT- embryo testing, or if patients were HIV positive. According to the laws and legislation of the country with regards to gamete donation, all gamete donors are tested for HIV and infectious diseases and are not accepted as donors when they test positive. In a small number of cases, donor gametes are used for HIV positive recipients. The effect of HIV and HIV treatment on pregnancy outcome is not clear – and can add a confounding factor to results. Standard operating procedures for HIV-positive patients differ from those for HIV-negative patients, especially with regards to semen preparation. In addition, some studies have reported that certain antiretroviral treatments may have a negative effect on neonatal outcomes, including low birthweight and pre-term birth (Stringer *et al.*, 2018). HIV recipient cycles were therefore excluded. Furthermore, PGD/PGS/PGT-A cycles were excluded from this study to eliminate additional variables that could have an influence on the outcomes, since the effect thereof is yet to be elucidated. Furthermore, patients that did not receive an embryo transfer or patients with no outcome data (lost to follow-up) were also excluded from data collection and analysis.

It is important to note that the data used in this study was assembled solely from patient medical records from 2015-2017 from the Aevitas Fertility Clinic. The assembly, examination and analysis of the data was performed in a retrospective fashion.

A total of 1231 fresh ET cycles and 423 vitrified-warmed ET cycles were performed in this patient population.

Study Design

A retrospective analysis of:
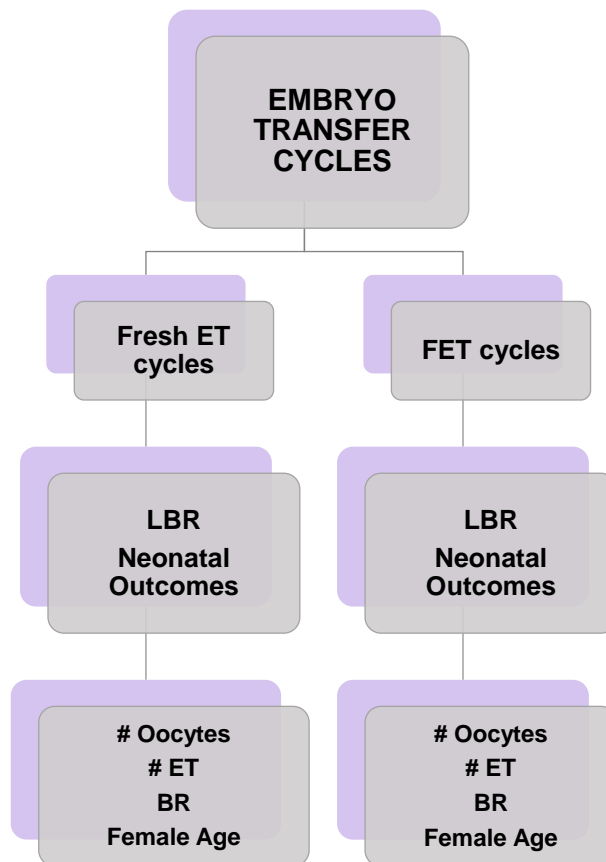
Data Management and Statistical Analysis

Data for the project was acquired from the standard, routine data files of the Aevitas Fertility Clinic. Relevant data was transferred to a Microsoft Excel spreadsheet specifically designed for the analyses.

A statistician from the Division of Epidemiology and Biostatistics of Stellenbosch University used appropriate statistical methods to analyze the data. Descriptive statistics of means, standard deviations and proportions were calculated from the data and are presented by fresh and FET transfer cycle groups. The comparison of means and proportions between the two groups took the clustering of cycles within the participants into account. For the live birth proportions, a binominal regression model was used with standard errors adjusted for clustering. The mean difference between the proportions was estimated using this model as well as 95% confidence intervals. For neonatal outcomes; birth weight and gestational age, a linear regression model was used to test for a significant difference and to estimate the

mean difference with standard errors adjusted for clustering. The difference in means was reported with 95% confidence intervals. Detailed statistical analysis can be found in Appendix O.

Ethical Aspects

Due to the retrospective nature of the study, patients were not subjected to any additional risks (other than the normal risks of the ART procedures and pregnancy), injury or pain. This study evaluated retrospective data of the medical/laboratory records ONLY of Drs. Aevitas Fertility Clinic on completed procedures. Data was obtained with the clinic's consent to do so – they also gave consent to disclose the Clinic's name (Appendix M). During the collection of the data on the password protected spreadsheet, no patient identity was entered. Each patient's folder was allocated a number and therefore remained anonymous. The patient's identity and folder were only available to the investigators who had access to the files in the normal course of treatment. Loss of patient confidentiality remained a risk but was minimized by anonymizing and aggregating the generated data. Additionally, the study specific number was not linked to the patient folder/identifier.

Ethical aspects regarding oocyte donor cycles:

Oocyte donors (from a SASREG [Southern African Society of Reproductive Medicine and Gynaecological Endoscopy] accredited oocyte donor agency) donate oocytes to receiving couples. Donor oocytes are fertilized with the spermatozoa of the male partner of the receiving couple and resultant supernumerary blastocysts are cryopreserved. There is a legal contract between the donor and the receiving couple, and the donor has no legal claim to the embryos.

Oocyte donors give consent to donate their oocytes to a recipient and the recipient gives consent to vitrify the resultant blastocysts after fertilization, not the donor.
The research that was done produced valuable information that is likely to improve patient care in the future.

Waiver of consent
This study evaluated retrospective data of the medical/laboratory records ONLY of Aevitas Fertility Clinic on completed procedures and for this reason the Health Research Ethics

Committee waived informed consent when submitted for ethical approval (HREC Reference number: S18/05/092). A progress report for the current study was submitted and approval was granted by the HREC (Appendix Q).

Methods

Ovarian stimulation protocols

Standard ovarian stimulation protocols according to the standard operating procedures (SOP) of the Aevitas Fertility Clinic was used (Appendix A). Ovarian stimulation occurs with the administration of medication that stimulate the ovaries to produce multiple oocytes via follicular development.

Oocyte retrieval

A standard oocyte retrieval procedure according to the standard operating procedures (SOP) of the Aevitas Fertility Clinic was used (Appendix A). Briefly, follicular fluid is aspirated, using a vaginal sonar guided method and examined for the presence of oocyte-corona-complexes (OCC). Subsequently, the oocytes will be graded (GV, MI and MII) and finally transferred to fertilization Medium [Quinn's Advantage ® Protein Plus Fertilization Medium].

Semen preparation

Semen was processed using standard, routine protocols. Both the Swim-up (Appendix B) or Density Gradient (Appendix B) centrifugation preparation methods were performed according to the semen characteristics.

Fertilization/insemination process

Mature MII oocytes were fertilized using standard protocols for IVF (Appendix C), Intracytoplasmic Sperm Injection (ICSI) (Appendix D), Physiological Intracytoplasmic Sperm Injection (PICSI) (Appendix E) and Intracytoplasmic Morphologically selected Sperm Injection (IMSI) (Appendix F).

Embryo culture

Standard sequential embryo culture methods were used (Appendix G). Following denuding of oocytes, the oocytes were cultured in Quinn's Advantage ® Protein Plus Fertilization Medium, which was covered by Sage ® Oil for Tissue Culture. After checking for fertilization,

2PN cells were transferred to pre-incubated Quinn's Advantage ® Protein Plus Cleavage

Medium drops were covered with Sage ® Oil for Tissue Culture. Following cell stage/blastocyst morphology checks on the inverted microscope with heated stage, embryos/blastocysts were transferred to pre-incubated Quinn's Advantage ® Protein Plus Blastocyst Medium drops covered with Sage ® Oil for Tissue Culture

Embryo evaluation – specifically blastocyst stage

Standard embryo and blastocyst evaluation for quality and morphology was followed.

Embryos are graded according to the number of cells present and the quality

thereof, using a modified Veeck *et al.* classification (Appendix H). Blastocysts are graded

according to expansion, inner cell mass (ICM) and trophectoderm morphology

(Appendix H).

Blastocyst vitrification/warming

A standard blastocyst vitrification and warming procedure was followed (Appendix I). The

procedure is modified from the Fertipro™ Vitrithaw kit method and using the Cryotop™

from Kitazato ® as storage device. For this study blastocysts were warmed specifically for

the purpose of transferring it into the uterus and all surviving blastocysts were

either transferred or re – vitrified. No embryos were destroyed or discarded.

Embryo transfer

A standard embryo transfer procedure was followed (Appendix J). In general,

one to three embryos or blastocysts were transferred into the uterus using a

standard embryo transfer method, using sonar guidance. Blastocysts were transferred

with the patient having been informed to present with a full bladder.

Pregnancy

Patients were continuously monitored throughout their pregnancy to determine the

outcome. During pregnancy evaluation, two consecutive Beta-human Chorionic

Gonadotrophin (βHCG) blood serum values on day 10 and 14 after embryo transfer

as well as a 7-8-week sonar was done to determine pregnancy outcome. Refer

to Appendix K for the acquisition of Human Chorionic Gonadotrophin (HCG) levels.

According to ICMART (The International Committee for Monitoring Assisted Reproductive

Technology) pregnancy is defined as follows (Zegers-Hochschild et al., 2009):

Pregnancy	Definition
Biochemical pregnancy	A positive βHCG blood serum value on day 10 or 14 after embryo transfer – but no fetal sac or heartbeat.
Clinical pregnancy	Any product of conception 7 weeks (including a gestational sac, a positive heartbeat and ectopic pregnancy) post embryo.
Ongoing pregnancy	A positive fetal heartbeat after 12 weeks of gestation.
Ectopic pregnancy	A pregnancy where implantation occurs outside of the uterine cavity.
Perinatal death	Death of the fetus after 20 weeks of gestation.
Live Birth	The complete expulsion or extraction from its mother of a product of fertilization, irrespective of the duration of the pregnancy, which, after such separation, breathes or shows any other evidence of life such as heartbeat, umbilical cord pulsation, or definite movement of voluntary muscles, irrespective of whether the umbilical cord has been cut or the placenta is attached (Zegers-Hochschild et al., 2009).
Blighted ovum	The occurrence of the implantation of a fertilized egg, with the absence of any further embryonic development. A gestational sac is formed and develops, without embryo development. A blighted ovum is also referred to as an anembryonic pregnancy and contributes greatly to miscarriages (Chaudhry and Siccardi, 2019).

Clinical pregnancy rate (CPR) expressed/ embryo transfer

Ongoing pregnancy rate (OPR) expressed/ embryo transfer

Live birth rate (LBR) expressed/ embryo transfer

Miscarriage

Miscarriage is defined as the premature loss of an intrauterine pregnancy before 20-24 completed weeks of gestation (WHO, RCOG and NICE). A miscarriage will have three different sub-categories:

- If the miscarriage occurred in the first seven weeks

- If the miscarriage occurred between 7 – 14 weeks

- If the miscarriage occurred after 14 weeks

Birth Weight

When the birth weight of an infant was evaluated, **normal** birth weight was a mass greater than 2500g (>2500g), **low** birth weight was a mass lower than 2500g (<2500g), and **very low** birth weight a mass lower than 1500g (<1500g) (De Vos *et al.*, 2017).

Gestational Age

Gestational age (in weeks) was calculated from the day of oocyte retrieval, defined as Day 14 of the menstrual cycle (Tunón *et al.*, 2000).

Pre-term birth

Pre-term birth was the delivery before 37 weeks of gestation, and very pre-term birth the delivery before 32 weeks of gestation (De Vos *et al.*, 2017).

Donor oocyte cycles

Donor oocyte cycles were also included in the retrospective analysis. Oocyte donors [from a SASREG accredited oocyte donor agency] donated oocytes to receiving couples – oocytes were fertilized with the spermatozoa of the male partner of the receiving couple and resultant supernumerary blastocysts were cryopreserved. There is a legal contract between the donor and the receiving couple, and the donor has no legal claim to the embryos. For clinic consent forms, see Appendix L.

The appropriate data was entered onto a study specific spreadsheet in order to analyse the data. An example of the spreadsheet headings is presented in Appendix M.

CHAPTER 3

RESULTS

The results of the study are presented in Figures 1-13.

Patient Population

The data that was utilized in this study was recorded exclusively from patient medical records acquired from Aevitas Fertility Clinic over a time interval of 2015 – 2017. This data was assembled and examined retrospectively in this study. Patients that were considered for this study were those who received fresh embryo transfers and patients who gave consent for blastocyst vitrification and received subsequent vitrified-warmed blastocyst transfers. In addition, only patients with known live birth data was analyzed retrospectively for live birth rate (LBR) outcomes.

The patient population included 1231 fresh embryo transfer (ET) cycles and 423 vitrified-warmed embryo transfer (FET) cycles. Fresh ETs included Day 3 and Day 5 embryos, where FETs only included Day 5 embryos.

For the LBR outcomes between the fresh ET cycle group and FET cycle group, a binominal regression model was used with standard errors adjusted for clustering. The mean difference between the two groups will be estimated using this model as well as 95% confidence intervals.

For the neonatal birthweight and gestational age, a linear regression model was used to test for a significant difference between the two previously mentioned groups and to estimate the mean difference with standard errors adjusted for clustering. The difference in means will be reported with 95% confidence intervals.

Data Analysis

As mentioned previously, 1231 fresh ET cycles and 423 FET cycles were included in this study. A total of 2578 embryos were transferred in a fresh ET cycle and 771 blastocysts were warmed for ensuing FET.

Descriptive Data and statistical analysis

1. Cycle Numbers

Figure 1 depicts the frequency of fresh ET cycles and FET cycles in the years 2015, 2016 and 2017 respectively. The incidence of fresh ET cycles in 2015 was 78,22% and 21,78% for FET cycles. In 2016, the proportion of fresh ET cycles was 75,24% and 24,76% for FET cycles. During 2017, the proportion of FET cycles increased to 35,38%. From this chart, it can be discerned that the incidence of FET cycles increased significantly over time.
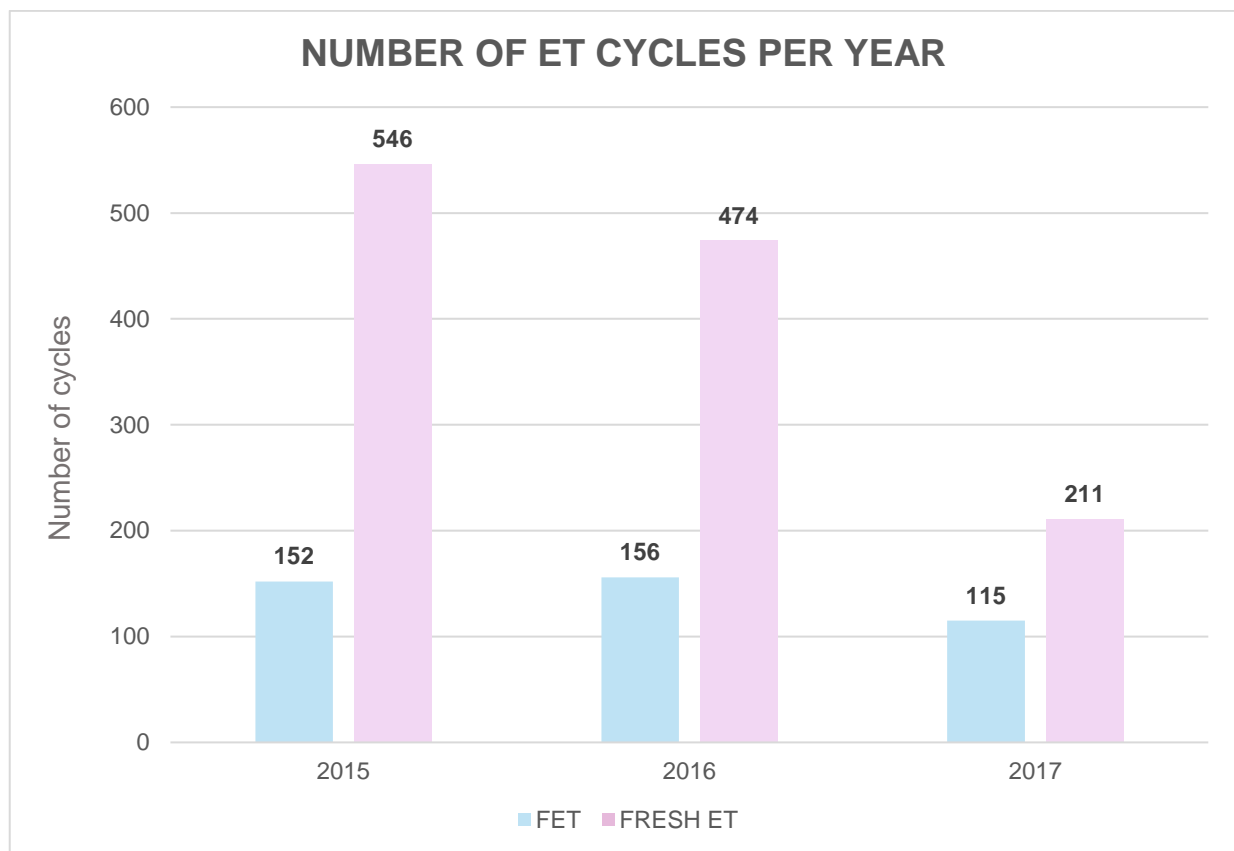
Figure 1: A clustered column chart displaying the number of fresh ET cycles and FET cycles per year (2015-2017).

2. Donor Cycles

The prevalence of donor cycles used in both groups (vitrified-warmed FET group and fresh ET group) is illustrated in the column chart below (Figure 2). From the chart it is clear that cycles analyzed were predominantly autologous. However, the FET group included significantly more donor oocytes in comparison to the fresh ET group.

46
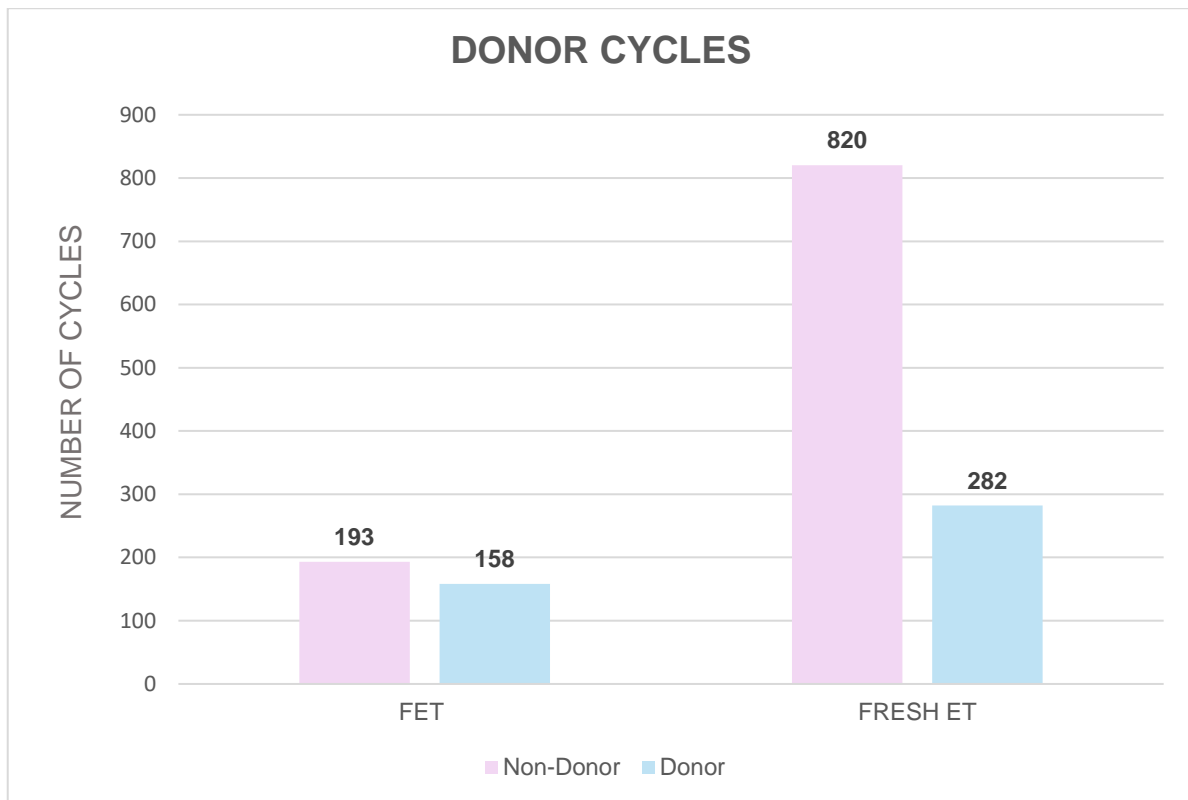
Figure 2: Clustered column chart exhibiting the proportion of donor cycles utilized (blue) in FET cycles as well as fresh ET cycles. The proportion of non-donor (pink) cycles are also shown for both groups.

3. <u>Ova Age</u>

The age of the ova denotes the age of the female patient from whom the ova are extracted preceding an ART cycle. In cycle types that include the use of donor gametes (ova), the age of the ova is represented by the age of the female donor at the time of oocyte retrieval. In non-donor (autologous) cycles, the age of the ova refers to the age of the female recipient. A clustered column chart below (Figure 3) depicts the average female age of the ova (donor and autologous ova included) in FET cycles and fresh ET cycles during 2015-2017. The average age of the ova in FET cycles was 30,43 (SD = 0,28). For fresh ET cycles, the average age of the ova was 33,19 (SD = 0,17). The average age of the ova differed significantly between the two groups (p = 0.0000). The average age of the ova was significantly less in the FET cycle group in comparison to the fresh ET group.

47
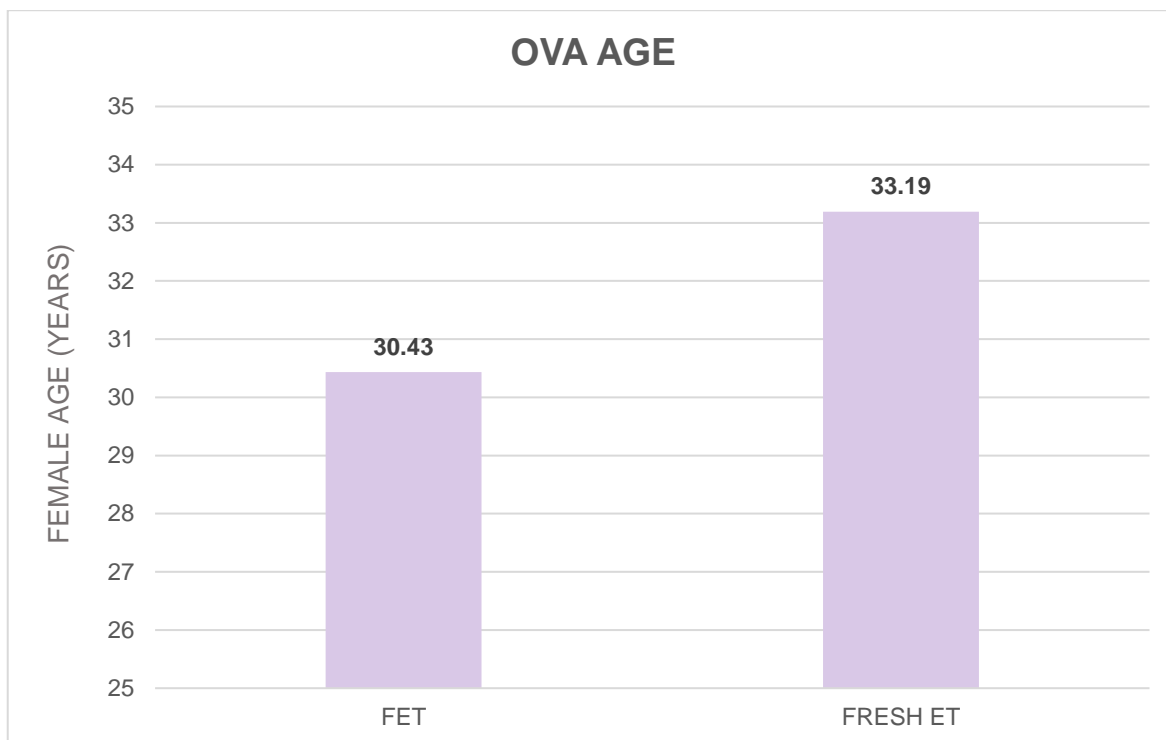
Figure 3: A clustered column chart of the average female age of the ova (including donor cycles) for both the FET (n= 423) cycle group and the fresh (n=1231) ET group.

4. Number of Ova at Aspiration

The average number of oocytes retrieved at aspiration for the FET group was 17,77 and 9,70 for the fresh ET group (Figure 4). A t-test was executed, and it was found that the difference (8,07) in the number of ova at the time aspiration between the two groups was significant. The number of ova retrieved was significantly less in the fresh ET group (p = 0.000).
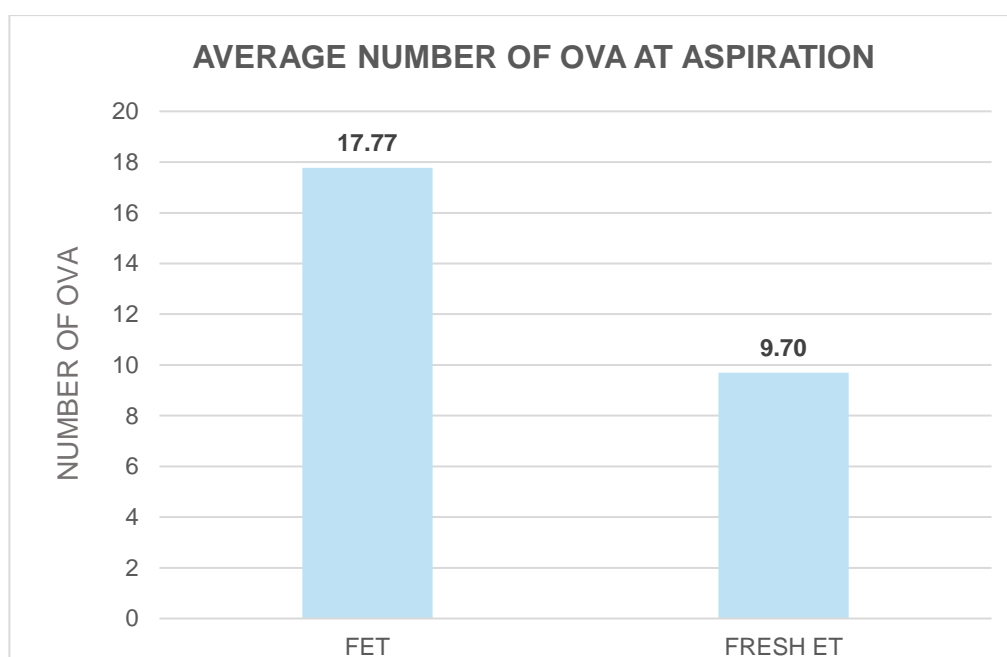
Figure 4: A clustered column chart displaying the average number of ova aspirated from female patients at the time of oocyte retrieval in the FET (n= 423) and fresh ET (n=1231) groups.

5. <u>Number of embryos transferred at embryo transfer (ET)</u>

The number of embryos transferred (1, 2, 3 or 4 embryos) for each patient in the FET group and fresh ET group is illustrated in the pie chart below (Figure 5). Single embryo transfers accounted for 25,3% of FETs and 15,43% of fresh ETs. Double embryo transfers were performed in 67,85% of FET cycles and 66,45% of fresh ET cycles. Three embryos were transferred in 6,15% of cases in the FET group and 12,75% in the fresh ET group. Embryo transfers that included the replacement of 4 embryos were 0,71% of FET cycles and 4,55% of fresh ET cycles. From these figures and from the pie charts, it can be discerned that the embryo transfer profile (number of embryos that were replaced at the time of the embryo transfer) differed significantly between the two groups (p = 0.000). There was a substantial difference in the proportion of single embryo transfers, and also when three or four embryos were transferred between the two groups. There was however no significant difference between the number double embryo transfers in the two groups (67,85% vs 66,45%).
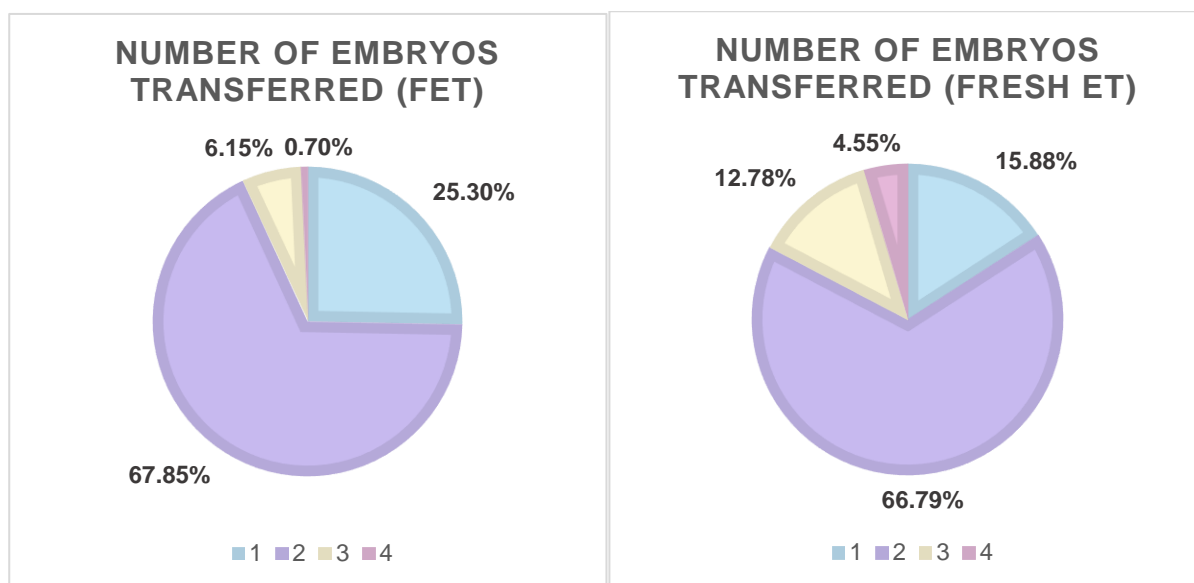
Figure 5: Pie charts depicting the embryo transfer profile for the FET (n= 423) group and fresh ET (n=1231) group respectively. One, two, three or four embryos were transferred at ET.

6. <u>Blastulation Rate</u>

The blastulation rate was calculated from the proportion of blastocysts formed from fertilized (2PN) metaphase II ova. The blastulation rate for FET cycles was 65,42% (n=180) (SD = 1,49) and 48,18% (n=908) (SD = 1,05) for fresh ET cycles (Figure 6 and Figure 7). The

blastulation rate varied by 17,24% between the two groups, which is a significant difference (p < 0.001).
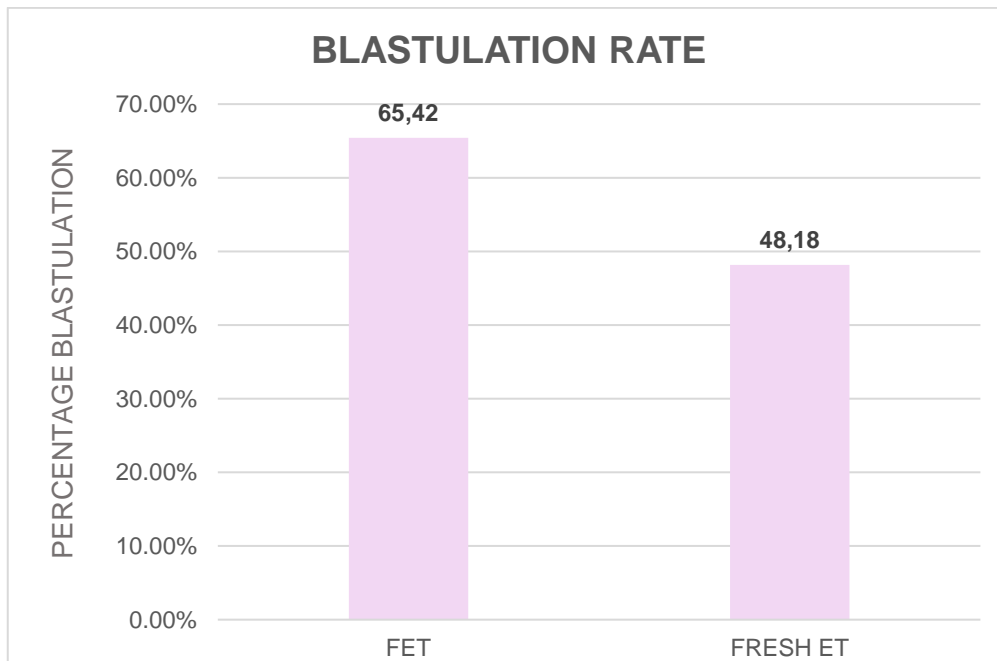
Figure 6: A clustered column chart displaying the blastulation rate for the FET and fresh ET groups.

7. <u>Number of babies born (Singletons and Multiples)</u>

The total number of babies born from both groups between 2015 and 2017 was 728 (Figure 7). Only singleton, twin and triplet births were taken into account. Of the 728, 109 and 306 were singleton births from the FET (31,98%) and fresh ET (29,38%) groups respectively. Thirty-eight births (11,05%) in the FET group and 112 births (10,81%) in the fresh ET group were twin births. Finally, there were 2 cases of triplet births in both of the groups. The proportion of multiple pregnancies did not differ among the two groups (the number of babies born per patient) (p = 0.490).
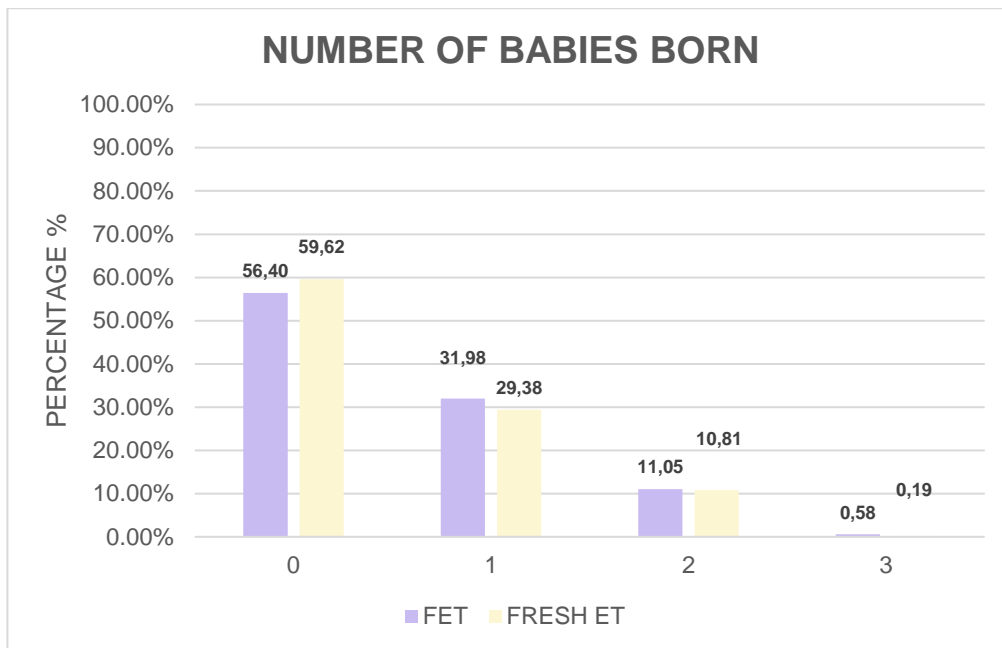
Figure 7: A clustered column chart of the number of babies born (multiple status) from the FET (n= 192) group and fresh ET (n=539) group.

8. Pregnancy outcomes

Pregnancy (%) was calculated per ET.

Various pregnancy outcomes for both fresh ETs and FETs are presented in the following column chart (Figure 8). The live birth rate was 45,15% (191/423) in the FET group and 43,62% (537/1231) for the fresh ET group. The prevalence of biochemical pregnancies in this study was 5,44% (23/423) for the FET group and 4,87% (60/1231) for the fresh ET group. Furthermore, the occurrence of blighted ovum (anembryonic pregnancy) was 0,47% (2/423) for the FET group and 0,65% (8/1231) in the fresh ET group. The miscarriage rate was 8,04% (34/423) and 8,69% (107/1231) for the FET group and fresh ET group respectively. Ectopic pregnancies accounted for 0,71% (3/423) and 0,16% (2/1231) in the FET and fresh ET groups respectively. Perinatal deaths occurred in 1,18% (5/423) and 0,65% (8/1231) of cases in the FET and fresh ET group respectively. Lastly, no fetal hearts were observed in 1,42% (6/423) the FET group and 1,30% (16/1231) in the fresh ET group. With this, the difference in pregnancy outcomes between the two groups was not significant (p = 0.579).
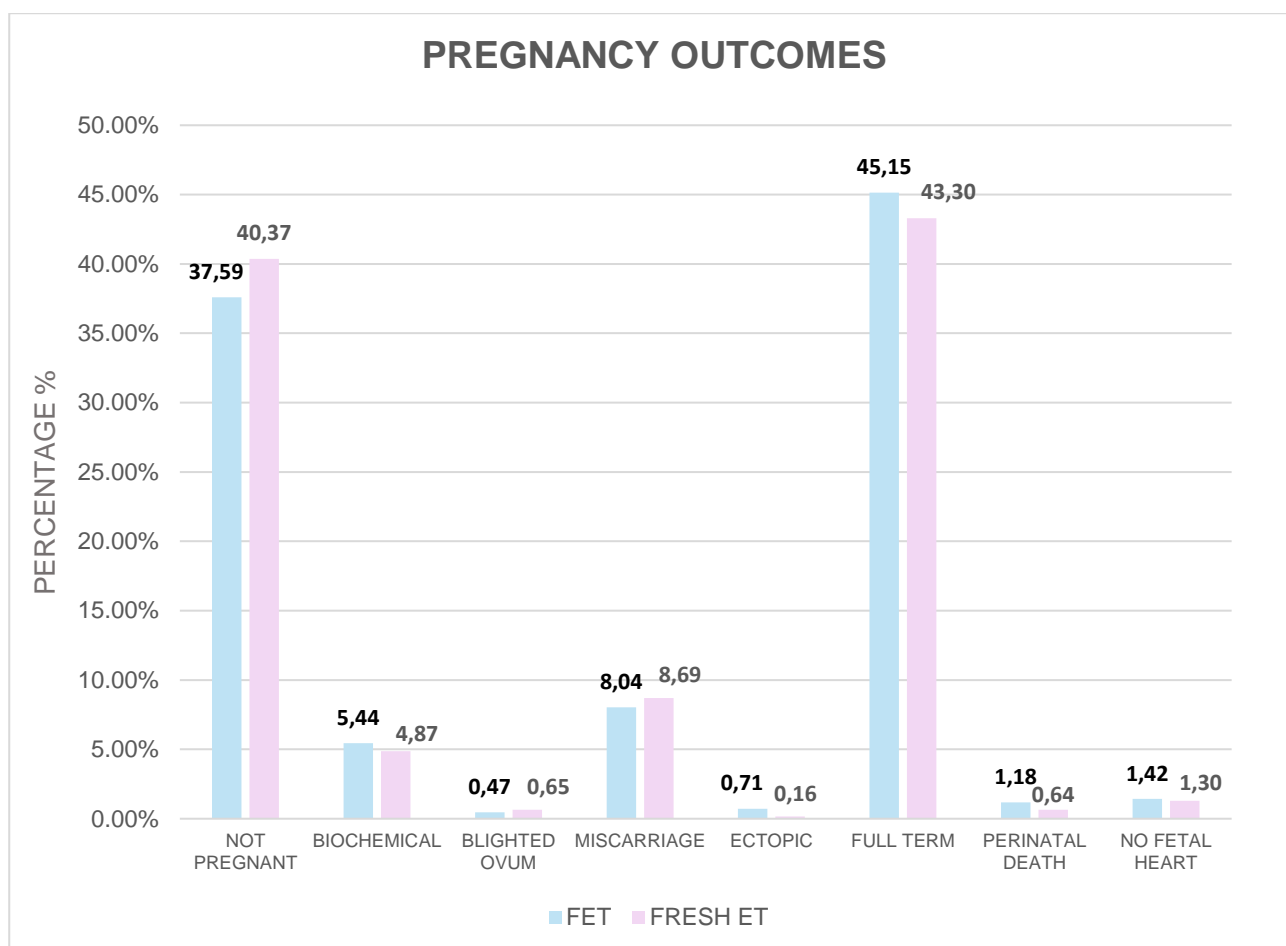
Figure 8: A clustered column chart showing the various pregnancy outcomes for both FET (n=423) and fresh ET (n=1231) groups.

9. Live Birth Rate (LBR)

LBR was calculated as births per embryo transfer.

A **Chi-squared test** was performed to determine whether the difference in the LBR between the two groups was significant. The average live birth rate was 45,15% (191/423) for the FET group and 43,62% (537/1231) for the fresh ET group, as seen on the column chart below (Figure 9). This difference was however not significant (p = 0.584).

In addition, a **binomial regression analysis** was performed to confirm whether the difference in LBR between the two groups was significant. The estimated difference between the FET and fresh ET group was 1,5% (95% confidence interval: -4,0 to 6,0%). Since the confidence interval included zero, the difference was not statistically significant between the two groups (p = 0.585).
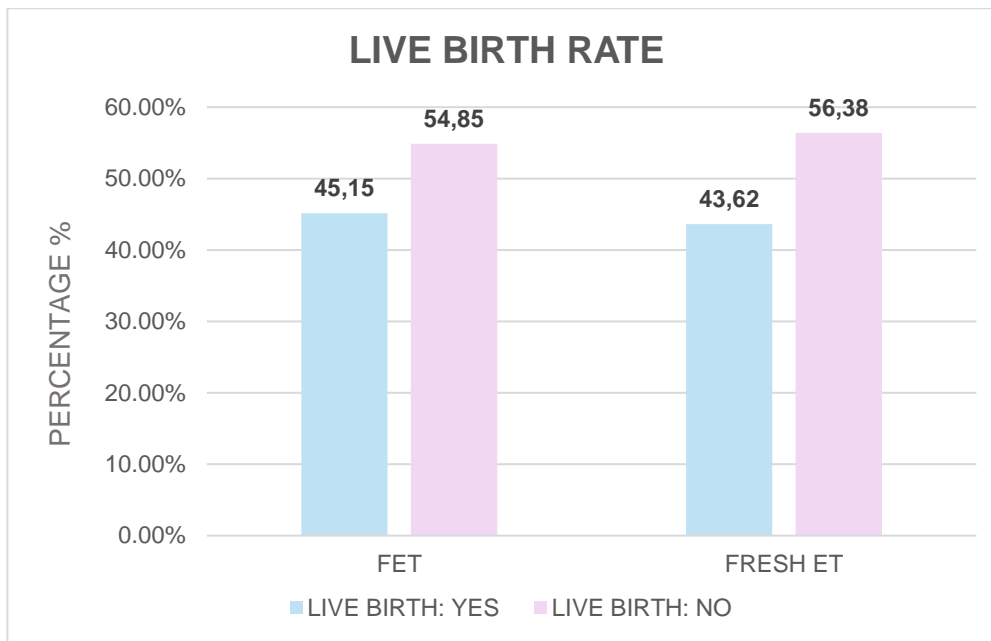
Figure 9: A clustered column chart showing the live birth rate of the FET (n=191) group and fresh ET (n=533) group respectively.

A **multiple regression model** was then utilized to analyze differences in the live birth rate (LBR) outcomes in fresh ET and in FET groups, taking confounding factors into account. Adjustment for an assortment of covariates such as, the age of the ova, the cycle type (fresh ET vs. FET), number of embryos transferred and whether donor ova were used, was applied. Due to the non-randomized design of this study, it was important to consider these covariates in order to acknowledge potential differences in the treatment groups. This model exhibited no significant correlation between the LBR and the type of cycle ($p = 0.320$). There was however a significant relationship between the number of embryos transferred at ET ($p = 0.000$) and the LBR, as well as the age of the ova ($p = 0.003$) and the LBR. It was shown that there was a significant positive correlation between the LBR and when 2 embryos or 4 embryos were transferred at ET, however no association was observed for 1 or 3 embryos.

Additionally, the age of the ova was inversely associated with the LBR, a higher oocyte age corresponded with a lower LBR.

10. Gestational Age

The gestational age of a neonatal individual refers to the time period from conception until birth.

53

To establish whether there was a difference in the average gestational age between the two groups (FET and fresh ET), a **two-sample t-test with equal variances** was conducted. It can be discerned from the clustered column chart below (Figure 10) that there is no significant difference in the average completed weeks of gestation for FET and fresh ET groups. The average gestational age of neonatal individuals born after FET (n=154) was 36,53 weeks (SD = 0,25) and 36,29 weeks (SD = 0,16) after fresh ET (n=420) (p = 0.4069).
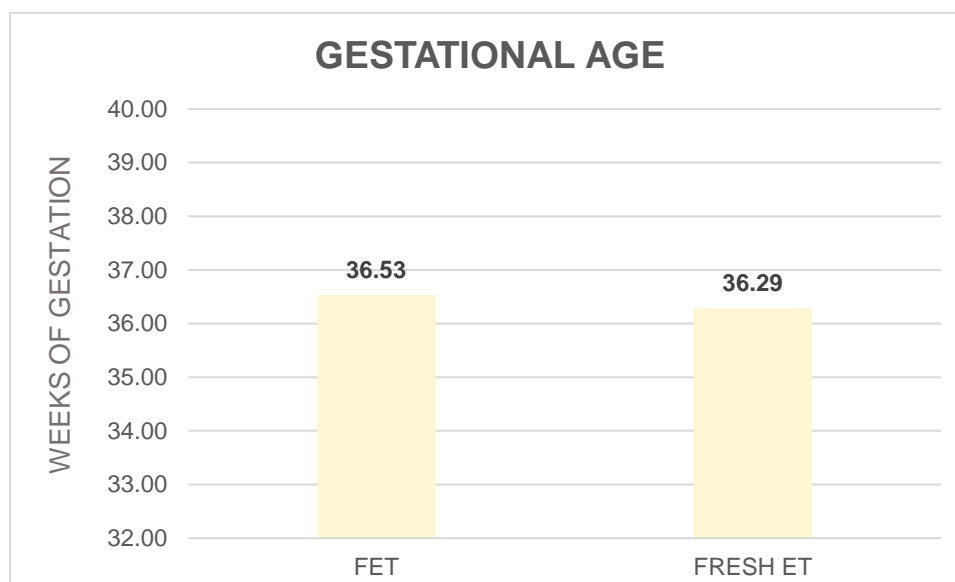
Figure 10: A clustered column chart of the completed weeks of gestation (gestational age) of live born babies from the FET (n = 154) and fresh ET (420) groups, respectively.

A Multiple Regression Model was then applied to reveal the difference in gestational age outcomes in the fresh ET and the FET groups. This model was adjusted for covariates such as the age of the ova, the cycle type (fresh ET vs. FET), the number of embryos transferred and whether donor ova were used. From the data, no significant difference was noted between the FET group and fresh ET group for gestational age. Moreover, there was no significant relationship between the gestational age of the neonatal individuals and the age of the ova (p=0.800), cycle type, number of embryos transferred or donor cycles and the gestational age (p = 0.1423).

11. Birthweight

A normal birthweight of a neonatal individual ranges between 2500 g and 4000 g. A birthweight lower than 2500 g is considered small for gestational age (SGA) and a birthweight higher than 4000 g is deemed large for gestational age (LGA). A **two-sample t-test with equal variances** was performed to determine whether the average birthweight

was different for the FET group and fresh ET group. The resultant weights are presented in Figure 11. For this study, the average birthweight of individuals born from FET cycles (n=137) was 2861,41 g and 2837,08 g for individuals born after fresh ET (n=349) cycles. There was no significant difference in the birthweight of individuals born from the two groups (p = 0.3725).
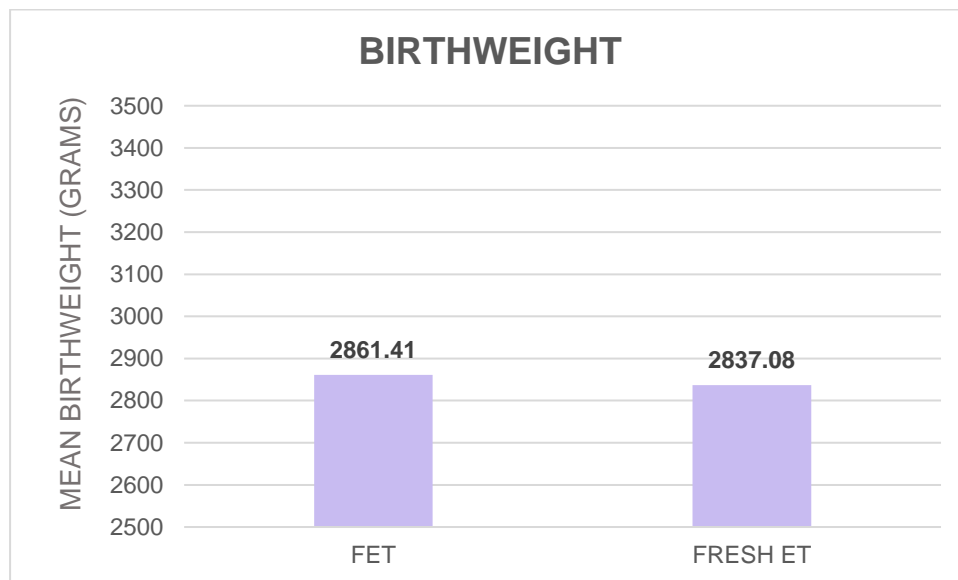
Figure 11: A clustered column chart of the average birthweight (g) of neonatal individuals born in the FET (n=137) or fresh ET (n=349) groups.

A Multiple Regression Model was then applied to determine the birthweight outcomes between the FET group and fresh ET group. The age of the ova, cycle type, number of embryos transferred and whether donor ova were used were the covariates that were taken into account during this analysis. No significant difference was noted between the FET and fresh ET groups cycles for neonatal birthweight. However, it was found that the age of the ova was negatively associated with the neonatal birthweight – an older age was correlated to a lower birthweight (-25,4 g per year). This association was significant (p = 0.038). Interestingly enough, a significant negative association was observed between the donor cycles and the birthweight of the neonatal individuals (-448 g). Neonates born from donor cycles had a lower birthweight in comparison to their counterparts in autologous (non-donor) cycles (p = 0.003). The number of embryos transferred had no significant effect on the birthweight (p = 0.0762).

12. Delivery Method

Three different methods of delivery were taken into account in this study: vaginal delivery, elective caesarean section, and emergency caesarean section. Emergency caesarean sections are usually performed when complications arise that will negatively impact the mother and child. LBR was calculated for each delivery method and reported for all cycles, not separating FET cycles and fresh ET cycles. In this study, it was found that the majority of babies were delivered via an elective caesarean section (87,07%) (505/580) (Figure 12). Only 6,21% (36/580) and 6,72% (39/580) of babies were born by means of emergency caesarean section or vaginal delivery or respectively.
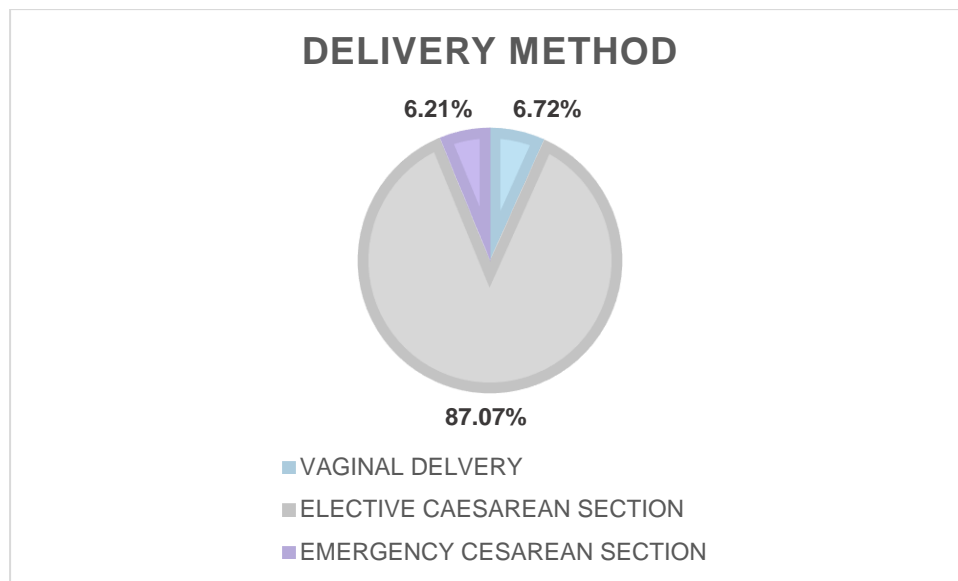
Figure 12: Pie chart displaying the delivery method profile for the study group (FET and fresh ET).

In addition (Figure 13), the percentage of live babies per delivery between the three delivery methods did not vary significantly (p = 0.242).

PERCENTAGE LIVE BABIES PER BIRTH
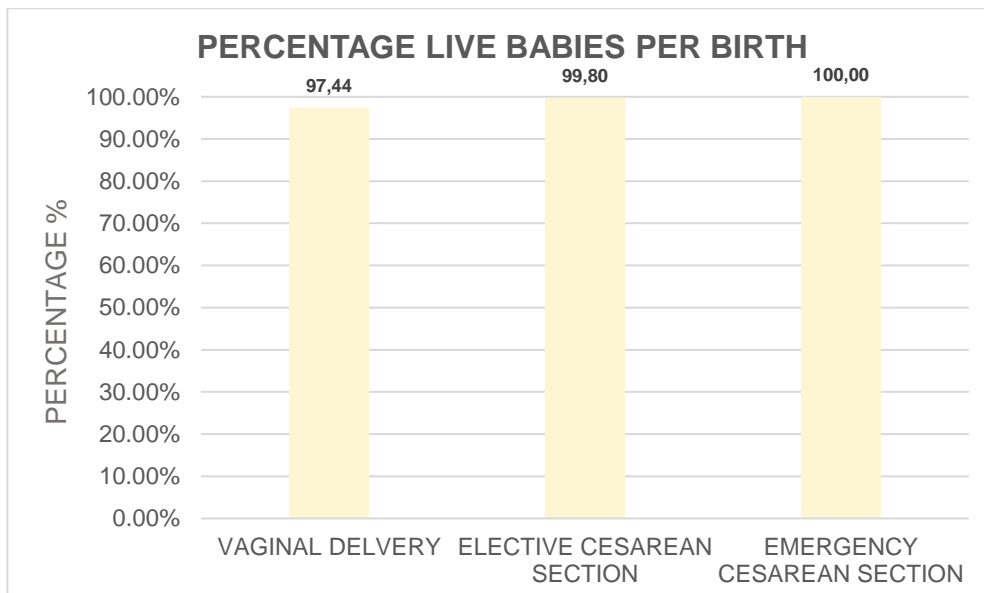
Figure 13: A clustered column chart of the percentage of live babies per birth for the different delivery methods.

CHAPTER 4

DISCUSSION

The application of innovative Assisted Reproductive Technologies (ART), such as IVF, ICSI with fresh or frozen embryos to overcome infertility has become extremely popular since the initial successful live birth, as well as the number of infertility clinics providing these services. In addition, the frequency of FET cycles has increased impressively in the past couple of years, likely owing to extraordinary improvements in the cryopreservation procedure in the laboratory, novel vitrification techniques and more than adequate post-warming survival rates (Zhang *et al.*, 2018). Although ART has shown beyond doubt to be an effective strategy in an attempt to overcome infertility, concern exists due to the complex nature of the entire infertility treatment process and the increased probability of adverse outcomes in children conceived following ART treatment. The factual grounds for poorer reproductive outcomes of children born from ARTs remains unclear, other than the effect of parental attributes. Though, proof within the literature is increasing, suggesting that procedures during infertility treatment such as ovarian stimulation, method of insemination and cryopreservation may have an effect on the outcomes (Pereira *et al.*, 2016; Zhang *et al.*, 2018). Additionally, the composition and length of embryo culture has also been cited as a possible cause of unfavourable outcomes after ART treatment (Zhang *et al.*, 2018).

Current evidence in the literature raises concerns regarding neonatal outcomes and pregnancy rates while comparing fresh ET cycles with FET cycles. It is therefore the moral responsibility of the fertility specialists – clinicians and scientists – to monitor, document and publish the outcomes of the children conceived from ART as well as outcomes from FET cycles. The rationale behind this study was to evaluate available data on FET cycles versus fresh ET cycles at Aevitas Fertility Clinic, since the practice of embryo vitrification and subsequent FETs have become more prevalent at this clinic – especially in donor oocyte cycles.

This study also aimed to investigate the effect of female age on the LBR and neonatal outcomes, as well as the number of embryos transferred at the time of embryo transfer, the number of oocytes recovered at OPU between fresh ET cycles and FET cycles, and to compare the number of babies born (multiple status) and blastulation rate between fresh ET cycles and FET cycles. It was the initial aim to compare the abovementioned covariates

within singletons and multiple births, however the small dataset did not allow for this sub-analysis and data was pooled and reported as a whole.

Patient Demographics & General Outcomes – FET vs. Fresh ET

The results of the current study showed that the two patient groups were not similar in terms of their demographics. This was expected since patients with embryos available for vitrification are usually better prognosis patients. The two groups differed in terms of ova age, number of oocytes aspirated, number of embryos transferred and also in blastulation rate. In the final statistical analysis these confounding factors were accounted for.

The above-mentioned factors as well as multiple births, that can influence the outcomes (LBR, neonatal outcomes) of the current study are discussed in the section below.

Ova Age

It was found that there was a significant difference in the age profile between the two groups of the study. The FET group was constituted by younger patients in comparison to the fresh ET group. Interestingly, it was found that the proportion of donor oocyte cycles was higher in the FET group. Oocyte donation first became known in the early 1980's and since then, has proliferated as a treatment option to those patients who suffer from poor ovarian reserve and to patients of advanced maternal age (Savasi *et al.*, 2016). Evidence in the literature suggests that oocyte donation is one of the most successful treatment options to treat infertility, especially in cases of advance maternal age (Yeh *et al.*, 2014; Savasi *et al.*, 2016). Oocyte donors are generally of a younger age since they are currently recruited in cases of advanced maternal age and for this reason, accounting for the significant younger age in the FET group. Furthermore, the increasing number of oocyte donation cycles required by the older patient population have prompted deliberation regarding the association between the oocyte recipient age and clinical outcomes. It was found that the pregnancy rates are not linked to the age of the recipient, until the age of 40 years, but then pregnancy rates begin to decline (Yeh *et al.*, 2014). Another study found that the implantation rate is dependent on the oocyte age, and not the age of the recipient (Savasi *et al.*, 2016).

In addition, the younger age in the FET group may also be explained by the fact that the assembly of patients included in this study was a relatively specialized group. Generally, patients who have more oocytes retrieved at OPU and more blastocysts available for cryopreservation are of a younger age and may potentially be better prognosis patients. According to Thomas *et al.* (2010), blastocyst formation is decreased with increasing female age (Thomas *et al.*, 2010), which is in agreement with what was found in our study.

Since the ova age was significantly different between the fresh ET and FET groups, statistical methods that corrected for the effect of female age was used to minimize the effect of this confounding factor on the results.

It is well known that there is an age-related downward trend in the reproductive potential of women as a result of a decrease in ovarian reserve (quantity) and a decreased oocyte or embryo developmental capability (quality) (La Marca *et al.*, 2017). Significant numbers of women have delayed the age at which they decide to bear children, due to a myriad of economic and social factors (Chamani and Keefe, 2019). Human reproductive success, whether it be natural or through ART treatment, rests largely on the age of the female (Cimadomo, Fabozzi, *et al.*, 2018). It has also been reported that the success of ART treatment may, to some degree, be predicted by the age of the female (Cimadomo, Fabozzi, *et al.*, 2018). Advanced maternal age, which is 35 years and older, evidently leads to a decline in the ability to conceive, a higher prevalence of aneuploidy among oocytes and unfavourable pregnancy outcomes (Chamani and Keefe, 2019). Advanced maternal age has also been implicated indicated for severe effect on the prevalence of aneuploidy among blastocysts (Cimadomo, Fabozzi, *et al.*, 2018). Currently, the precise physiological mechanisms associated with infertility due to advanced maternal age and the effect it has on oocyte and embryo competence is uncertain (Cimadomo, Fabozzi, *et al.*, 2018). The decline in oocyte and embryo quality has been linked to complications during energy generating pathways, metabolism, gene expression, and meiosis (Santonocito *et al.*, 2013;(Capalbo *et al.*, 2017).

To date, no remedy exists to counter the effects of advanced maternal age on the quantity and quality of oocytes and embryos. However, there are treatment options available to aid patients of advanced age to conceive and start a family. The first option is fertility preservation by means of oocyte cryopreservation – preferably at a younger age (<35 years) to limit the effects of oocyte ageing. Furthermore, specialized COS regimes could be applied to optimize the number of oocytes retrieved from the patient, such as double ovarian stimulation and oocyte and embryo collection from multiple cycles (Cimadomo, Fabozzi, *et al.*, 2018). An increased number of ova retrieved per cycle has been described to increase the likelihood of a successful ART treatment outcome (Drakopoulos *et al.*, 2016). Another option is advanced embryo selection methods by means of genetic testing (PGD/PGS/PGT) in order to avoid the transfer of aneuploid embryos that may likely affect the mother and

baby negatively (Franasiak *et al.*, 2014). As soon as an euploid embryo is recognized, the concern of the effect of female age on implantation may be disregarded, to an extent, and in conjunction with an eSET policy, the risks related to multiple gestations may be reduced and the treatment outcome can be optimized (Cimadomo, Fabozzi, *et al.*, 2018). Lastly, the use of donor oocytes can be considered to potentially compensate for the decline in infertility associated with advanced maternal age.

Number of Ova

Our study found that the oocyte yield was significantly greater in the FET group in comparison to the fresh ET group is possibly due to the compilation of patients in each group. The fresh ET group consisted of an older patient population, therefore the lower oocyte yield is likely due to the fact that an increased age has been associated to a decline in the quantity of oocytes at the time of OPU, due to poorer response to COS and poor ovarian reserve (La Marca *et al.*, 2017; Zhou *et al.*, 2017). The FET group consisted of younger patients, from whom more oocytes were retrieved at OPU. A higher oocyte yield typically gives rise to a higher blastulation rate – and subsequently a larger number of blastocysts available for cryopreservation.

Current literature reports that the number of oocytes retrieved after COS for ART treatment may potentially influence the outcome of the treatment cycle with regards to live birth (Magnusson *et al.*, 2018; Malchau *et al.*, 2019). It was suggested that between 6 and 15 oocytes maximizes LBR outcomes in patients younger than 35 years in fresh ET cycles and that LBR decreased when a lower number of oocytes were retrieved (Magnusson *et al.*, 2018). When an oocyte number higher than 15 were recovered during a treatment cycle, there was a tendency of the LBR to decrease (Magnusson *et al.*, 2018). High oocyte number have also been associated with an elevated risk of OHSS, which could range from mild to severe (Magnusson *et al.*, 2018). With regards to outcomes in FET cycles, it was found that an increased number of oocytes retrieved at aspiration is associated with an increase cumulative live birth rate (Magnusson *et al.*, 2018). This advantage was however not applicable for patients of advanced maternal age, in some cases (Venetis *et al.*, 2019).

Number of embryos transferred

Our study reports that the number of embryos transferred in the fresh ET group was significantly different form the FET numbers. The prevalence of eSET was higher in the FET

group in comparison to the fresh ET group. This finding was most probably due to the fact that eSET are typically performed in younger patients with an improved prognosis and the FET group had a significantly younger patient population. At Drs. Aevitas Fertility Clinic, the fertility specialists and embryologists opt for double embryo transfers in patients of increased maternal age, which may account for the lower prevalence of eSETs in the fresh ET group, which represented an older patient population. Double embryo transfers were the most popular approach in this study, dominating the charts with 67,85% in the FET group and 66,79% in the fresh ET group. The objective of multiple embryo transfers, primarily in advanced age groups, is to maximize the probability of pregnancy, whilst being aware of the risks related to multiple gestations (Dyer and Kruger, 2012).

In agreement with our study, Dyer and Kruger (2012) reported that the number of embryos transferred was inversely associated to the age of the female (Dyer and Kruger, 2012). It was established that the majority of females who were 40 years and older, had three embryos replaced at the time of ET and females of a younger age mostly received double embryo transfers (Dyer and Kruger, 2012).

The debate regarding the number of embryos to be transferred at the time of ET, is ongoing. In the beginning stages of ART, when the success rates were very modest and techniques not as advanced as it is to date, it was general practice of IVF specialists and embryologists to transfer multiple embryos at ET, with the aim to increase the chances of conception (Kemper *et al.*, 2019). Consequently, the rate of multiple pregnancies increased which has raised concerns due to pregnancy complications and unfavourable outcomes in neonatal individuals (Kemper *et al.*, 2019). Therefore, many clinics have opted to transfer fewer embryos and there is an increasing trend in the number of eSET policies (Kemper *et al.*, 2019).

Elements that are usually taken into account when more than one embryo is transferred is poor maternal characteristics such as increased age, substandard quality of oocytes and embryos, poor prognosis and failed previous ART treatment cycles, among other reasons (Luke *et al.*, 2015).

Number of Babies (singletons and multiples)

Our study reports a relatively low multiple pregnancy rate, and the proportion of multiple gestations did not vary significantly between the fresh ET and FET group. Some studies indicate increased clinical pregnancy rates after double embryo transfer in comparison to SETs, and as expected, a higher rate of fraternal twin pregnancies (Abuzeid *et al.*, 2017). It should be noted that our finding may have been influenced by a small sample size.

The desired outcome of ART treatment is the birth of a healthy singleton baby and many strategies have been implemented to limit the prevalence of multiple gestations and to encourage singleton pregnancies (Penzias *et al.*, 2017). It is well-established that many risks are concomitant with multiple gestations, such as PTB, LBW, morbidity, mortality and unfavourable obstetric outcomes (Bhandari *et al.*, 2017; Hwang *et al.*, 2018).

Blastulation Rate

Our study observed a significant increased blastulation rate in the FET group in comparison to the fresh ET group (65,42% vs 48,18%). Both values fall within the standard benchmark values for blastulation rate, according to the Vienna Consensus Workshop Report (Vermeulen *et al.*, 2017). The significant difference in blastocyst formation between the two groups was most likely the result of the difference in female age profile between the two groups. A younger female age are generally better prognosis patients, usually associated with a higher number of oocytes and subsequent higher blastocyst development rate. This is in concordance with findings in various studies, where female age was shown to be an essential predictor of blastocyst formation, where females with an increased age had a reduced quantity of blastocysts available for embryo transfer from fertilized oocytes than females of a younger age (Shapiro *et al.*, 2002; Porter *et al.*, 2009; Thomas *et al.*, 2010). A more recent study also agreed with these observations and showed that the age of the woman influences the number of euploid blastocysts, with a higher number of oocytes and younger age having a positive effect on the formation of euploid blastocysts (La Marca *et al.*, 2017).

It is important to note that patient numbers were limited in this study and that important covariates were not always compensated for, such as male and female diagnoses and insemination method. Therefore, the results should be interpreted cautiously, and future endeavours should include a larger sample size.

Due to the increasing implementation of single embryo transfers in ART laboratories worldwide in an attempt to decrease the incidence of multiple pregnancies, additional strategies are required for the selection of viable embryos for transfer (Ahlström *et al.*, 2011). One such strategy includes the extended embryo culture to the blastocyst stage and subsequent transfer. This strategy has proven to be more successful in terms of synchronicity between the pre-implantation embryo and the endometrium, implantation rate and live birth rate in comparison to cleavage stage embryos (Ahlström *et al.*, 2011). Advancements in *in vitro* culture systems to date have resulted in an greater prevalence of blastocyst development, therefore providing several blastocysts to select from for embryo transfer (Ahlström *et al.*, 2011). Subsequently, excess blastocysts are routinely cryopreserved by means of vitrification for use in future FET cycles (Zhao *et al.*, 2019).

It was reported that the blastulation rate is an essential factor to consider in terms of the outcome of the ART treatment cycle, since it potentially provides an indication of the effectiveness of the entire embryo culture system and also embryo viability (Vermeulen *et al.*, 2017).

The rate of blastocyst development may vary, since there are many factors that may influence the outcome of embryo development, such as the conditions of the ART laboratory, *in vitro* culture system and the compilation of patients (Thomas *et al.*, 2010). One such patient factor is female age. Some studies have established trends in relation to a decreased blastocyst development rate with increasing female age (Thomas *et al.*, 2010). Larger studies have shown that an increased female age had a deleterious effect on blastocyst formation (Thomas *et al.*, 2010).

Neonatal Outcomes and Concerns in the literature

Over the past 40 years, ART has been revolutionized from being beyond far-fetched to a well-established practice carried out routinely for infertility patients all over the world (Qin *et al.*, 2016). To date, millions of children have been born as a result of ART treatment , and therefore the health of these children have been a subject of concern due to potential risks related to certain techniques during ART treatment (Berntsen *et al.*, 2019). It is paramount to take these risks into consideration in order to limit adverse outcomes in succeeding ART-conceived generations (Berntsen *et al.*, 2019).

It is the general consensus within the literature that pregnancies as a product of ART treatment may be at an increased risk of unfavourable maternal and neonatal outcomes, in comparison to individuals born from spontaneous conception (Berntsen *et al.*, 2019). Previously, these unfavourable results have been connected to the elevated incidence of **multiple gestations**, due to increased demands to transfer more than one embryo to enhance pregnancy rates (Qin et al., 2016; Berntsen *et al.*, 2019). However, the increase global application of eSETs to reduce the incidence of multiple pregnancies have resulted in a decrease in unfavourable risks and improvement in the health of children conceived through ART (Berntsen *et al.*, 2019). Despite all the effort, children born following ART treatment at present time remain at a substantial risk for poor neonatal outcomes, even in singleton pregnancies (Kamath et al., 2018; Berntsen *et al.*, 2019). According to some authors, unfavourable reproductive outcomes from ART have been associated with underlying causes of infertility, the method of insemination, ART techniques and processes such as, COS, manipulation of *in vitro* reproductive material, and embryo culture (Kamath *et al.*, 2018). Clarification is therefore required to determine whether unfavourable reproductive outcomes and health risks are caused by innate parental factors associated to infertility or to the ART procedures itself (Berntsen *et al.*, 2019).

ART Singleton pregnancies have been linked to **SGA, LBW, PTB, perinatal mortality, antepartum hemorrhage, pregnancy induced hypertension (PIH), premature rupture of membranes and gestational diabetes mellitus** and **congenital malformations** in comparison to naturally conceived children (Qin *et al.*, 2016).

ART conceived Multiple gestations have been associated with **PIH, placenta previa, gestational diabetes mellitus, premature rupture of membranes,** and **anemia** in the

course of pregnancy when compared to naturally conceived individuals (Qin *et al.*, 2016). Additionally, multiple pregnancies have also been linked to an increased rate of caesarean deliveries (Qin *et al.*, 2016). However, contradictory results exist in the literature regarding maternal outcomes, with other studies reporting similar outcomes between ART and naturally conceived children (Qin *et al.*, 2016)

It has been described that the short term risk of **SGA, LBW and PTB** is increased in singletons born following <u>fresh ET cycles</u>, whilst singletons from <u>FET cycles</u> are posed to increased risk of being **LGA** and mothers may be predisposed to **pre-eclampsia** (Maheshwari *et al.*, 2018; Berntsen *et al.*, 2019; Ernstad *et al.*, 2019). One study found no difference in the risk of **perinatal mortality** between fresh and FET cycles (Berntsen *et al.*, 2019). Furthermore, it was observed that the **miscarriage** rate did not vary substantially among fresh ET and FET cycles (Maheshwari *et al.*, 2018). The type of insemination method has also displayed effects on neonatal outcomes – ICSI has been correlated to an elevated risk of **birth defects, inheritance of substandard semen quality** to male offspring, and oocyte donation has been correlated to **SGA and pre-eclampsia** risks (Maheshwari *et al.*, 2018; Berntsen *et al.*, 2019; Ernstad *et al.*, 2019). Other concerns include a higher risk of **stillbirth** and **neonatal/perinatal mortality** (Kirby, 2018).

Information relating to the long term risks in infants conceived through ART treatment is sparse, however it was found that these children are predisposed to modified **blood pressure** and **cardiovascular function** (Berntsen *et al.*, 2019). In addition, information on malignant **cancer** is promising, while evidence regarding **neuro-developmental** aspects is yet to be clarified, with a potential correlation between **cerebral palsy** and infertility treatment (Berntsen *et al.*, 2019). Adverse outcomes such as cerebral palsy, very pre-term birth and very low birthweight are potentially disastrous due to emotional torment, expensive financial responsibilities and intensive care in severe cases (Davies *et al.*, 2018).

The various techniques and methods used in the ART laboratory may also be an influencing factor concerning neonatal outcomes, as it has been observed that versatile embryo culture media has been linked to alterations in **birthweight** and **growth** in infants, while **PTB** has been correlated to extended embryo culture to the blastocyst stage (Berntsen *et al.*, 2019). Moreover, **epigenetic modifications** have been shown to differ among children born from ART treatment, which could be a principle area to investigate to enlarge the knowledge regarding unfavourable outcomes in ART conceived children (Berntsen *et al.*, 2019).

Outcomes in the current study

Live Birth Rate (LBR)

Our study found no significant difference in the LBR between the FET group and fresh ET group (45,15% vs 43,62%) using the Chi-squared test and confirmed with a binomial regression analysis. Furthermore, a multiple regression analysis was done to compensate for covariates such as cycle type (fresh ET vs FET), female age (age of the ova), number of embryos transferred and donor cycles. There was a significant relationship between the number of embryos transferred at ET and the LBR, as well as the age of the ova and the LBR.

In spite of a significant difference in **female age** and **number of embryos transferred at ET** between the two groups, the LBR was still not significantly different between groups. Cycle type and donor cycles as covariates were not different between the two groups.

Some studies agree with this finding, reporting similar LBRs between FET and fresh ETs (Wong *et al.*, 2017). One study also states that the LBR was similar between FET and Fresh ET groups in normal responders to ovarian stimulation (Shi *et al.*, 2018b). Other studies reported increased pregnancy rates in "freeze-all" FET groups in high responder patients in comparison to fresh ET groups, but was reduced in the FET group in normal and poor responder patients (Acharya *et al.*, 2018). One more study reported an increased LBR in female patients suffering from PCOS after FETs in comparison to fresh ETs (Vuong *et al.*, 2018).

An increased **ova age** was associated with a lower LBR (in the combined group- FET and fresh) in this retrospective study, displaying an inverse relationship. This finding can possibly be attributed to the fact that older patients present with a poorer prognosis due to reduced oocyte number and quality. This is in agreement with various literature studies, which emphasize the reducing effect advanced maternal age has on live birth rate outcomes in ART treatment cycles (Sunkara et al., 2011; La Marca *et al.*, 2017; Hogan *et al.*, 2019). Information from cycle-based studies described that the LBR is subject to the age of the oocytes (Hogan *et al.*, 2019). According to Wang et al. (2012), it has become common knowledge that women of younger ages have improved outcomes in terms of pregnancy and LBR in comparison to females of advanced ages in ART and normal conception (Wang *et al.*, 2012). An increasing age does not exclusively result in reduced fertility due to poorer

oocyte quantity and quality, impaired endometrial receptivity and reduced hormones (Wang *et al.*, 2012). The reduced infertility may in addition be due to underlying persistent conditions regarding health, such as hypertension and diabetes, which may lead to problematic pregnancies (Wang *et al.*, 2012). Therefore, it has been suggested that female age may be a forecasting entity with regards to LBR and pregnancy outcomes during ART (Çiray *et al.*, 2004; Wang *et al.*, 2008; Wang *et al.*, 2012). Despite the fact that outcomes of ART have successfully progressed, it was not necessarily observed for older patients in autologous cycles (Çiray *et al.*, 2004; Wang *et al.*, 2012). The reason for this is possibly the reduced quality of the oocytes, and it has been proposed that the quality of the oocytes may dictate the outcome of the ART treatment cycle and ensuing LBR and pregnancy outcomes (Wang *et al.*, 2012). Oocyte donation has become a more popular option as a therapeutic strategy to confine the risks associated with advanced maternal age (Wang *et al.*, 2012).

Another confounding factor observed during this study was the **number of embryos transferred at ET**. The embryo transfer profiles differed significantly between the FET and fresh ET group. The use of SETs was significantly higher in the FET group in comparison to the fresh ET group (25,30% vs 15,88%). And the transfer of >3 embryos at ET was more prevalent in the fresh ET group. This might possibly be due to the fact that the FET group consisted of a younger age group and SET are usually implemented for these patients at Aevitas Fertility Clinic. The application of double embryo transfers did not differ among the two groups.

The transfer of a one good quality blastocyst has been endorsed by several recommendations and guidelines in the world, but this approach has however not been practiced by all ART clinics (Bhandari *et al.*, 2017). In certain cases, such as recurrent cycle failure, or in cases where the selection of the best embryo for transfer becomes challenging, multiple embryos are transferred intentionally (Bhandari *et al.*, 2017). As such, the multiple gestation rate is elevated, although singleton pregnancies may also emerge (Bhandari *et al.*, 2017). Consequently, varying embryo transfer policies have led to increased rates of multiple pregnancies (Bhandari *et al.*, 2017).

According to the guidelines set out by the American Society of Reproductive Medicine (ASRM) (2017) for **good prognosis** patients and who fall within the ages of <35-37 years, SET is recommended (Penzias *et al.*, 2017). Since the FET group in our study potentially

had a greater proportion of good prognosis patients due to the younger patient population, this could account for the increased application of SET in this group.

In cases of **poorer prognosis**, extra embryos may be transferred depending on the general health status or potential underlying pathology of the patient (Penzias *et al.*, 2017). Furthermore, in cases of recurrent cycle failures an extra embryo should be transferred. Patients who have existing health issues that may be negatively influenced by multiple pregnancies, should not have more than one embryo transferred. Inadequate evidence exists to propose a certain number of embryos to be transferred in women who exceed the age of 43 years using autologous oocytes, and these patients should be made aware about the increased risk of multiple gestations in older women (Penzias *et al.*, 2017). The older patient population in the fresh ET group in our study most likely accounted for the transfer of multiple embryos at the time of transfer.

Donor cycles had no significant effect on the LBR in our study. Evidence in the literature state contradicting results, and have shown in multiple studies that the LBR during a donor oocyte cycle is determined by the age of the donor (Hogan *et al.*, 2019). It was also observed that the LBR was increased in recipients who received younger donor oocytes, in comparison the recipients who received older donor oocytes (Hogan *et al.*, 2019). Information has also surfaced expressing that an association exists between the uterine receptivity of the recipients of older ages and reproductive outcomes (Hogan *et al.*, 2019). Poorer outcomes in ART in recipient patients older than 45 and 50 years was reported and the conclusion was that a correlation exists between the reproductive outcomes after ART, oocyte age of donors, and endometrial receptivity in females of very advanced age (>45 years) (Hogan *et al.*, 2019). Although our study had significantly more donor cycles in the FET group than the fresh ET group, the recipient age of the patients in the FET group could have influenced the results and could account for the absence of variation between the LBR between the FET and fresh ET group.

Gestational Age

Our study found no significant difference in terms of the gestational age between the fresh ET and FET group. Both groups displayed normal gestational ages for babies born in both groups, with the average gestational age in completed weeks being approximately 37 weeks in both groups. A multiple regression analysis was also done to compensate for covariates

such as cycle type (fresh ET vs FET), female age (age of the ova), number of embryos transferred and donor cycles. Including the potential confounding factors in the statistical analysis still resulted in no significant difference in gestational age between the two groups.

It was interesting to observe no substantial prematurity among infants born from fresh ET cycles, since consensus is quite evident within the literature regarding this matter. Most studies have reported PTB and even VPTB among infants born following fresh ETs in comparison to FETs (Barsky *et al.*, 2016; Spijkers *et al.*, 2017; Maheshwari *et al.*, 2018; Sha *et al.*, 2018). It was also observed that the risk of PTB was higher in cases of high oocyte numbers in fresh ET cycles than FET cycles. (Sunkara *et al.*, 2015). Even though the exact rationale behind improved neonatal outcomes following FET is yet to be completely elucidated, it has become clear in the literature that a possible reason for superior outcomes are due to ramification with regards to COS on the receptive capacity of the endometrium when comparing FET and fresh ETs (Pereira *et al.*, 2016; Sha *et al.*, 2018).

Ovarian stimulation during ART treatment has been linked to a condition of hyperestrogeinism during fresh ET cycles (Maheshwari *et al.*, 2018). This is thought to result in an irregular process of angiogenesis in the endometrium, consequently causing a **decrease in implantation potential** and **irregular formation of the placenta** (Maheshwari *et al.*, 2018). The supraphysiological levels of progesterone, oestrodiol, and hormones related to stress as a result of COS have been associated with compromised uterine receptivity and embryo development during fresh ET cycles (Sha *et al.*, 2018). As a result, the rate of implantation is decreased, subsequently causing unfavourable neonatal outcomes (Maheshwari *et al.*, 2018). In addition, the risk of OHSS is increased due to these elevated hormone levels resulting from COS during fresh ET cycles, which may be very severe in some cases (Maheshwari *et al.*, 2018). During FET cycles, the endometrial/uterine environment is suggested to be less hostile since the timing of the procedure is delayed, allowing the detrimental effects of COS to decrease, granting a more natural environment for the pre-implantation embryo to be transferred into, conferring improved outcomes in terms of gestational age (Maheshwari *et al.*, 2018).

However, one study found no difference in the prevalence of term of delivery of pre-term delivery between fresh ET cycles and FET cycles in singleton pregnancies (Pereira *et al.*, 2016), which is in agreement with our study.

Our study also noted no significance with regards to the relationship between ova age and gestational age (p=0.800). This finding is contradictory to evidence in the literature, which report increased risks of pre-term deliveries in women of advanced maternal age (Bayrampour and Heaman, 2010; Goisis *et al.*, 2017). It has been observed that children born pre-term are predisposed to heart deficiencies, lung defects, cerebral palsy and postponed development (Goisis *et al.*, 2017).

Our study observed no significant effect of the number of embryos transferred and the gestational age. It is well established that more than one embryo are transferred in order to increase pregnancy rates, which has a negative effect due to the higher prevalence of multiple gestations (Bhandari *et al.*, 2017). It is consequently of utmost importance to gain understanding regarding the implantation of these embryos and the parameters influencing multiple rates (Bhandari *et al.*, 2017). In some instances where multiple embryos are transferred, one embryo may be reabsorbed, due to embryo crowding at implantation, which is referred to as the vanishing twin phenomenon (Bhandari *et al.*, 2017) A lower implantation rate due to this phenomenon may cause a lower gestational age (Bhandari *et al.*, 2017). This study by Bhandari et al. (2017) concluded that an association exists between the number of embryos transferred and the gestational age (Bhandari *et al.*, 2017). In a study reported by Sunkara *et al.* (2015) it was found that transfer of multiple embryos did not have a noteworthy effect on pregnancy outcomes, a multiple pregnancy eventually ending in a singleton live birth, had a significant increased risk of PTB increased significantly (Sunkara *et al.*, 2015).

Our study also found no correlation between oocyte donation cycles and gestational age. This finding corresponded with two studies, that published no difference in preterm deliveries in women receiving donor or autologous oocyte cycle treatment (Krieg *et al.*, 2008; Stoop *et al.*, 2012). Yet, one study described that within oocyte donation cycles, the risk of pre-term deliveries was elevated (Yu *et al.*, 2018), which was not in concordance with our study results.

Birthweight

Our study found no significant difference in the birthweight of infants born from fresh ET and FET groups. The average birthweight of the neonatal individuals were approximately 2800 grams in both groups, falling within the normal parameters for birthweight, which should be

< 2500 grams and < 4000 grams. When other covariates were compensated for in a multiple regression analysis [cycle type (fresh ET vs FET), female age (age of the ova), number of embryos transferred and donor cycles], still no significant difference was observed between the fresh ET group and FET group. However, female age had a negative association with the birthweight. No significant effect was observed for the number of embryos transferred or the utilization of oocyte donation.

Published literature regarding the birthweight outcomes of neonatal individuals following ART treatment have suggested that infants born after fresh ETs have a lower birthweight and are predisposed to being SGA. One possible rationale might be negative consequences resulting from hormonal stimulation of the ovaries during infertility treatment prior to fresh ETs (Vermeulen *et* al., 2017, Maheshwari *et al.*, 2018; Sha *et al.*, 2018; Zhang *et al.*, 2018). It was postulated that the excessive supraphysiological measure of oestrodiol evidently compromises the receptivity of the endometrium, rendering the uterine environment relatively unfavourable for embryo adherence, which may increase the potential risk of irregular placentation and growth of the embryo (Maheshwari *et al.*, 2018; Zhang *et al.*, 2018). The subsequent result has been observed to be lower birthweight and SGA infants (Maheshwari *et al.*, 2018; Zhang *et al.*, 2018). Our study however, found different results. The literature regarding equivalence in birthweight outcomes between fresh ETs and FETs is limited, however, one retrospective study found that the risk of LBW and VLBW was comparable between the two groups (Pereira *et al.*, 2016).

Furthermore, with regards to high birthweight, several literature studies have demonstrated an increased birthweight and cases of LGA following FETs (Maheshwari *et al.*, 2018; Sha *et al.*, 2018; Zhang *et al.*, 2018). According to Maheshwari *et al.* (2018), the endometrial environment is less hostile and reflects more natural conditions (Maheshwari *et al.*, 2018). A possible explanation might be due to an improved implantation potential associated with a more natural uterine environment during FET cycles, leading to enhanced placenta formation and excessive growth of the implanted embryo (Maheshwari *et al.*, 2018).

To date, there have been no clear elucidation regarding the incidence of LGA babies (Maheshwari *et al.*, 2018).

Some studies have proposed that the vitrification and warming process might lead to higher birthweights, possibly due to gene expression alterations at early stages of embryo development, during which time growth patterns are modifiable (Maheshwari *et al.*, 2018;

Sha *et al.*, 2018). In certain fertility clinics, the vitrification process requires additional embryo culture duration in comparison to fresh ET culture, where are evaluated and cultured for 20-24 hours after thawing (Spijkers *et al.*, 2017). It has been suggested that in vitro culture might also have an effect on the health of the individuals born from FETs, in the long term (Spijkers *et al.*, 2017). However more evidence is required as to why babies are prone to be LGA after vitrification and subsequent FETs (Spijkers *et al.*, 2017). These findings are however, of clinical importance, since LGA babies are predisposed to adverse obstetric conditions such as trauma and distress, as well as a higher risk of caesarean section births, stillbirths, and metabolic disruptions (Spijkers *et al.*, 2017). Our study did not agree with these findings, as higher birthweight or LGA was not recorded for FETs, when compared to fresh ET cycles. A study conducted by Pereira *et al.* in 2016, found equivalent birthweights between FET and fresh ET groups (Pereira *et al.*, 2016).

An increased female age was associated with a lower birthweight in this study. This observation concurs with certain information in the literature. Evidence regarding this subject is contradictory, due to heterogeneity that exists with regards to patient populations, since certain covariate are not compensated for. However, women of increased female age have been shown to be more prone to established, possibly underlying illnesses or defective health conditions, such as substandard cardiovascular reserve, which may lead to irregular placentation and LBW (Restrepo-Méndez *et al.*, 2015). Moreover, females of advanced age, who are also first time mothers, have been linked to a higher risk of SGA (Kahveci *et al.*, 2018). The exact rationale behind the increased risk of lower birthweight at advanced maternal ages is—currently remains inconclusive, however it has been proposed that defective oxygen exchange may be the inherent element at play (Kahveci *et al.*, 2018).

Our study also observed a negative association between birthweight and donor cycles. It was found that donor cycles gave rise to neonatal individuals with a lower birthweight. Oocyte donation is generally suggested in cases of advanced maternal age and donors are typically young good prognosis patients. However, it has been observed in some literature articles that substandard endometrial receptivity may have a hindering effect on the ability of the embryo to implant and subsequent pregnancy, despite the quantity and quality of oocytes (Shapiro *et al.*, 2002). Therefore, reports of reduction in uterine receptivity parallel to increasing female age emerged (Shapiro *et al.*, 2002), potentially implicating recipient age as an essential factor influencing the outcome of conception (Shapiro *et al.*, 2002). The endometrial environment and uterine receptivity might therefore be a greater influencing

factor with regards to neonatal outcomes, instead of the oocyte *per se* (Baker *et al.*, 2015). Therefore, the lower birthweight in donor oocyte cycles was likely due to recipient factors. It is important to note that the sample size was rather small and the patient population rather specialized, which might have had an influence on the results.

Delivery Method

Our study found that the majority of babies in both groups were born via an elective caesarean section (87,07%). A small percentage of births took place via vaginal delivery (6,72%) and emergency caesarean section (6,21%). The LBR was also calculated for each delivery method, which was 97,44% LBR for vaginal deliveries and 99,89% for elective caesarean section deliveries.

As mentioned previously in this study, women who seek infertility treatment usually account for a cohort of patients of an older age, in contrast to women who have established a pregnancy naturally (Li *et al.*, 2014b). In addition, these patients are more likely to become first time mothers (Li *et al.*, 2014b). Therefore, these pregnancies are regarded as high risk pregnancies, also due to reports of negative neonatal and obstetric outcomes from ART pregnancies (Benli *et al.*, 2015) and are at an increased risk for caesarean section deliveries (Bayrampour and Heaman, 2010).

According to various observations within the literature, an increased female age may be linked to several unfavourable pregnancy outcomes, such as chromosomal irregularities, multiple gestations, PTB and LBW and are as a result, destined to a higher rate of caesarean sections (Bayrampour and Heaman, 2010). Hesitancy exist regarding the rationale behind the increased rate of caesarean section deliveries among women of advanced maternal age (Bayrampour and Heaman, 2010). It was described that the possible increase in the prevalence of caesarean section might be due to long-term, underlying diseases and conditions such as hypertensive disorders of pregnancy and gestational diabetes in older females (Bayrampour and Heaman, 2010). Another possible reason for an increased rate of caesarean sections is due to the decision of the obstetrician or medical professional in charge of the birth, since many obstetricians regard pregnancies in older women as risky and prefer to perform caesarean sections (Bell *et al.*, 2001). In addition, many mothers request this method of delivery and therefore might contribute to the increased incidence of caesarean section deliveries (Lin *et al.*, 2004).

Publications have also reported increased caesarean section rates in FET cycles, which may be as a result high birthweight and macrosomia (Vidal *et al.*, 2017).

Limitations

The study has a number of limitations.

- This was a **retrospective** study, which has a number of disadvantages. This type of study can only identify associations and cannot determine causation.
- Furthermore, the study is subject to various **influencing factors**, which were not identified or measured. The patient diagnosis [male and female] was for instance not included for evaluation as an influencing factor on the outcomes, which may have affected the results.
- In addition, the **sample size** also might have affected the validity of the results of the study. The number or patients analyzed were significantly reduced due to the exclusion criteria to minimize potential influencing factors. Larger sample sizes may have optimized the study design.
- One of the aims of this retrospective study was to investigate all the major neonatal outcomes in singleton and **multiple pregnancies** separately - however the small sample size did not allow for this analysis.
- Moreover, differences in stimulation protocols, embryo/blastocyst quality and ART procedures (method of insemination) for fresh and FET cycles were not specified in this study.

Conclusion and summary of results

Based on the results of the current study, it can be concluded that the **LBR and neonatal outcomes** including gestational age and birthweight of the neonatal individuals receiving fresh ET and FET ART treatment at Drs. Aevitas Fertility Clinic, was within normal parameters and **were not significantly different** from each other.

The **blastulation rate, number of ova aspirated, and ova age** was significantly higher in the FET group possibly due to a younger patient population and more donor cycles included. The **number of embryos transferred** was higher in the fresh ET group, primarily because the patient profile was different in terms prognosis.

Future research

Future research should include a larger sample size to detect differences in patient groups with regards to potential influencing factors, and to ensure sufficient accuracy of results and outcomes. Measured outcomes should also include stimulation protocols, male and female diagnosis, cycle number and insemination method (ICSI/IVF). Studies to follow should preferably include randomization of patients and prospective data. In addition, separate analyses should be performed for multiple pregnancies with regards to neonatal outcomes. Lastly, more information should be included on miscarriages.

CHAPTER 6

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CHAPTER 7

APPENDICES

Appendix A - Ovarian Stimulation & Oocyte Retrieval Protocol

Ovarian Stimulation

The standard antagonist stimulation protocol consists of daily gonadotropins (225 IU FSH in a step-down fashion to 150 IU) for 5 days beginning on day 3 of the menstrual cycle. Adjustments to the gonadotropin dosage is determined by ultrasound monitoring; 0.25 mg of Cetrorelix® (GnRH antagonist) is added as a subcutaneous injection when the leading follicle measures 14 mm or more. 10,000 IU hCG is given SC or IM when the lead follicle was ≥ 18 mm and at least two other follicles were ≥ 16 mm in size. Oocyte retrieval is performed within 36 hours after hCG administration (The Practice Committee of the American Society for Reproductive Medicine, 2013).

Oocyte Retrieval

Oocyte pickup requires the aspiration of follicular fluid using a long 16-gauge aspiration needle into sterile tubes which is sent through to the laboratory. The fluid is poured into large petri dishes under the stereomicroscope fitted with a heated stage. The examination of the follicular fluid must take place immediately after follicular aspiration and at 37 ºC for later examination as red and white cells are prone to attach strongly to the cumulus cells. If the cumulus cells are excessively stained with blood, then those areas of cumulus can be removed with sterile needles because it is well established that the blood interferes with the fertilization rate and subsequent embryo quality. Then, the cumulus-oocyte-complexes (COC) are identified, graded (MI vs MII) and collected with a sterile, rounded, wide-bore glass pipette. The COC are transferred into test tubes (MI and MII) into approximately 2.0 ml fresh, warmed flushing medium to wash them of excess blood. The OCC are then transferred into a Greiner dish with 2.0 ml gassed fertilization medium at 37 ºC and incubated until denuding or insemination time.

Ovum Pick up

Check suction pump (100-120 mmHg)
Prepare glass polished pipettes for pick up
Place pick-up tubes in heated block
Place aspirated follicular fluids in heated block and examine for oocyte-cumulus complexes using a large Petri dish on a heated stage (37-40ºC) of a dissection microscope

Note obvious abnormal features and maturities

Put the complexes (with as little as possible blood and medium) in the pick-up tube

When done, rinse all complexes in small Petri dish with gassed fertilization medium – check number obtained.

Appendix B - Semen Preparation

Semen Preparation

The sperm preparation method is determined by the quality of the sample produced; therefore, the visual/microscopic analysis of the sample is extremely important. Factors that may influence the decision are; percentage motile sperm, rate of forward progression, concentration (total count) and the number of other cells in the semen sample. In addition, the assisted reproduction procedure being followed will also determine the procedure. Whereas with in vitro fertilization [IVF], gamete intrafallopian tube transfer [GIFT] and Intrauterine insemination [IUI] procedures you may need 100 000 to 1x106 cells/oocyte, you only need 1 sperm/oocyte for an intracytoplasmic sperm injection [ICSI] procedure.

Two basic procedures (wash and swim-up and continuous gradient centrifugation) and modifications of these, are used for the majority of sperm preparation procedures. The standard wash and swim-up procedure, however, remains the most commonly used procedure for sperm preparation, even for ICSI. The reasons; no foreign particles are introduced into the sample; the sample is free of other cells and the percentage of motile sperm is high. Gradient centrifugation has however been shown to yield sperm with less DNA damage due to eliminating reactive oxygen species [ROS] early on in the preparation method.

The medium used for all the sperm preparation procedures is Quinn's™ Sperm Washing Medium [SAGE]

All tubes are labeled with the patient's surname and all lids and tubes also with a colour sticker. The final tube should show both partners surnames and initials.

Wash and swim-up [IUI and ICSI/PICSI/IMSI/IVF]

Three 15 mL round bottomed tubes are labelled correctly with the patent's surname and initials. 5 mL sperm prep medium in one of the tubes is warmed to37ºC

On the completion of liquefaction the semen sample- produced by masturbation in a accurately labelled semen container - is diluted 1:2 (semen:medium) in a test tube and centrifuged at 350 to 400xg for 10 minutes

The supernatant is aspirated after centrifugation and the pellet resuspended with 2mL of medium and re-centrifuged at 350 to 400xg for 10 minutes

After the 2nd centrifugation the supernatant is aspirated as close as possible to the pellet and the pellet then carefully overlayed with 0.5mL of medium taking care not to disturb the pellet

The test tube is placed at an approximate 45o angle at 37ºC for 30 - 60 minutes

After the swim-up period the top 2/3rds of the medium with motile sperm is carefully aspirated and placed in a clean correctly labeled test tube and stored at 37ºC until used

Swim-up samples should be used within 1-hour, post preparation

When faced with a problem sample the above procedure can be modified as follows; the number of test tubes the sample is divided into can be increased with a subsequent decrease in the volume of medium overlayed on each pellet. Different test tube shapes can also be employed. In cases of very low initial concentrations the standard flat-bottomed tubes can be replaced with conical tubes

The procedure for IVF is slightly modified: the final 0.5mL medium for the swim-up step is replaced with equilibrated Quinn's Advantage™ mL Protein Plus Fertilization (HTF) Medium (equilibrated in a CO_2 incubator to reach a pH of 7.2). The 30-60-minute swim-up step is also done in a CO2 incubator.

Gradient centrifugation SilSelect (FertiPro)

Masturbation samples
This technique is mainly used for samples with low concentrations, poor motility, viscous samples, samples with high concentrations of other cells/debris and testis biopsy samples.
A Stock solution of SilSelect (100%) is used and different gradients created by adding
Quinn's™ Sperm Washing Medium [SWM]

Gradients (in 15 mL round bottomed tubes) – prepared fresh weekly.

90%: 9 mL of stock solution plus 1.0mL of SWM
70%: 7 mL of stock solution plus 3 mL of SWM
45%: 4.5 mL of stock solution plus5.5 mL of SWM

For a 3-layer gradient, the 3 solutions are carefully layered on each other in a conical tube, starting with the 90% solution at the bottom and making sure they are not mixing.
The gradient is then allowed to equilibrate at 37oC for 15 minutes (Figure1)
For a 2-layer gradient, the 905 and 45% is used.

The volumes of the solution for the gradient depends on the fertilization procedure and the semen sample.

In general, I mL is used for IVF and IUI, 0.5 mL for ICSI/PICSI/IMISI and 0.3 mL for a testis biopsy sample

The gradient should be used within 2 hours of preparation

Two 15 mL round bottomed tubes and 2 conical tubes are labelled correctly with the patent's surname and initials. 5 mL sperm prep medium in one of the round bottomed tubes is warmed to 37ºC and one of the conical tubes prepared with the gradient.

On the completion of liquefaction the semen sample - produced by masturbation in a accurately labelled semen container - is diluted 1:2 (semen:medium) in a test tube and centrifuged at 350 to 400xg for 10 minutes. Washing is performed to concentrate all possible sperm into a smaller volume t use in the gradient method. Ideally an unwashed sample should be overlaid on the gradient and can be done in samples with adequate concentration and motility.

The pellet is re-suspended in 0.5mL of medium and carefully overlaid on the gradient
The gradient is centrifuged for 15 minutes at 300xg

After centrifugation, the top layer/s are carefully aspirated and the pellet with ± 0.5 mL medium remaining, placed into a clean conical tube. The pellet is then washed twice with 2 mL medium by centrifugation 9400- 450xg) for 10 minutes each.

The resulting pellet is re-suspended in 0.5mL of sperm prep medium

Frozen samples [I mL final volume – 2 straws] are overlayed directly onto the gradient

When faced with a problem sample the volumes of the gradient can decreased (0.3mL; mini-gradient, Figure 1), the number of gradients can be increased, and a two-step gradient can be used

The concentrations for a two-step gradient are: 90% and 45% (Figure 1). The latter procedure results in a higher final sperm concentration, but a decreased percentage of motile sperm – often used for IUI

Testis biopsy samples
The method of preparation is similar to that of masturbated semen:

96

An aliquot (± 0.3mL) of the fresh testis biopsy tissue/liquid containing the sperm is overlaid on the 3 layer "mini gradient". The mini gradient is three 0.3mL layers [90%, 70%, and 45% - Figure 1]. (the rest of the sample is frozen)

The gradient is then centrifuged for 20 minutes at 400g

After centrifugation, the top layer/s are carefully aspirated and the pellet with ± 0.3 mL medium remaining, placed into a clean conical tube. The pellet is then washed twice with 2 mL medium by centrifugation (450xg) for 10 minutes each.

The resulting pellet is resuspended in 0.2-0.3 mL of sperm prep medium and kept at ROOM TEMPERATURE until use

It is preferable to use a thin glass pipette for aspirations to facilitate very fine and accurate removal of the supernatants.

Appendix C - Insemination process: In Vitro Fertilization (IVF)

In Vitro Fertilization (IVF)

IVF procedure

Make sure that all forms and documents are prepared

Check patient's file and record to eliminate all possible uncertainties/queries

- After the aspiration, transfer COC's to a 4 well NUNC dish – maturities separate and not more than 5 complexes per well

- Incubate in the CO2 incubator until insemination

- Complete all forms

Semen preparation

See section on semen preparation methods [Appendix B]

Insemination

- Inseminate complexes with the correct number/volume of prepared sperm

    - Morphology   ≤ 4 % - up to 2 x 106 sperm/ovum
    - Morphology   > 4, ≤ 14 % – 500 000 sperm/ovum
    - Morphology   > 14 % – 100 000 sperm/ovum

        - (*work out the correct volume*)

- Do insemination ± 40 hours post HCG administration if at all possible

- Incubate overnight at 37 ºC, 6% CO_2

Appendix D - Insemination process: Intracytoplasmic Sperm Injection (ICSI)

Intracytoplasmic Sperm Injection (ICSI)

ICSI procedure

Make sure that all forms and documents are prepared

Check patient's file and record to eliminate all possible uncertainties/queries

Semen preparation

      See section on semen preparation methods [Appendix B]

Aspiration and Ovum Pick up – see Appendix A

When done, rinse all complexes in small Petri dish with gassed fertilization medium – check number obtained

Leave in fertilization medium in CO_2 incubator until time for denuding of oocytes

Try to do denuding ±38 hours post HCG injection

Denuding of oocytes

Prepare pipettes for the process: fire polished glass Pasteur pipettes, hand drawn glass pipettes and the Cook stripper (Marcus Medical) pipette

Prepare a 4 well NUNC dish for denuding: [USE ONE DISH FOR EVERY 8 OOCYTES]

In well 2, 3 and 4, place ± 0.7 – 0.8 mL warmed HEPES buffered flushing medium (Quinn's, Cooper Surgical)

In well 1 place 0.6 mL HEPES buffered flushing medium and add 0.3 mL hyaluranidase [80 UI/mL] (Quinn's, Cooper Surgical)

Place in the incubator [without CO_2] at 37ºC for ± 10 minutes to reach 37ºC

Place a predetermined number of oocyte/cumulus complexes in well 1, wait for ± 30 seconds

Gently flush the complexes with a standard fire polished pipette until all cumulus cells are digested (oocytes with corona cells and small number of cumulus cells form "fluffy balls")

If complexes stay intact use two hypodermic needles to "tease" oocytes from the complexes

Use the same fire polished pipette and transfer the oocytes with as little as possible hyaluronidase solution to well 2

Now flush oocytes individually with a big [170-200um] pulled glass pipette to remove some of the loose cumulus cells and transfer to well 3

In well 3 start the stripping using the plastic Cook denuding pipette [130um] (Marcus Medical) – try to remove all corona cells to allow for evaluation of oocyte maturity

Make sure that the pipette works correctly before oocytes are aspirated

If oocytes seem stuck in pipette – blow out into one of the wells using a syringe and adapter

Hyaluronidase solution

HEPES buffered medium
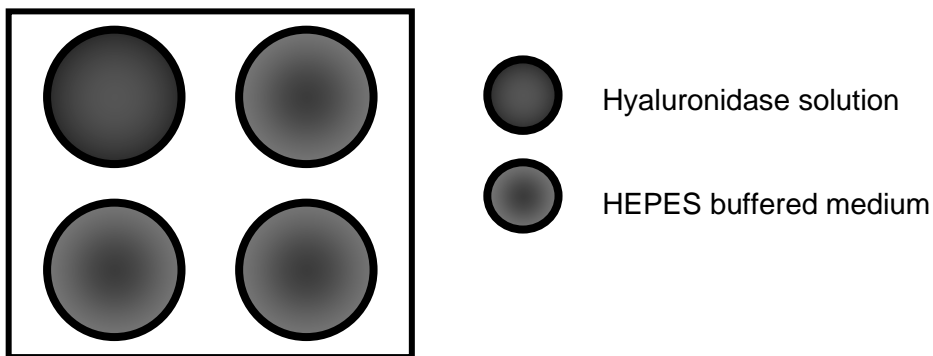
Place the denuded oocytes into a pre-prepared holding or "rugby ball" dish, into the left elliptical fertilization wash medium drop and transfer then to the right sided one

[this dish is prepared the previous day and cultured at 6% CO_2/37ºC – drops are covered with oil – Quinn's- Cooper Surgical)

Cleavage medium

Fertilization medium
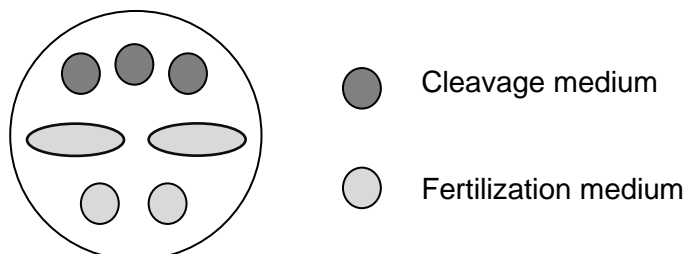
Now determine the maturity of the oocytes and transfer to the clean fertilization drops – all MII oocytes to the left drop and all MI and GV to the right drop

Culture until injection

Complete all forms

Set up the inverted microscope for ICSI [heated stage, holding and injection pipettes, manipulators]

Injection procedure

For ICSI with **ejaculated semen**, prepared the following injection dish:

Sperm preparation medium with sperm cells

PVP

HEPES buffered medium
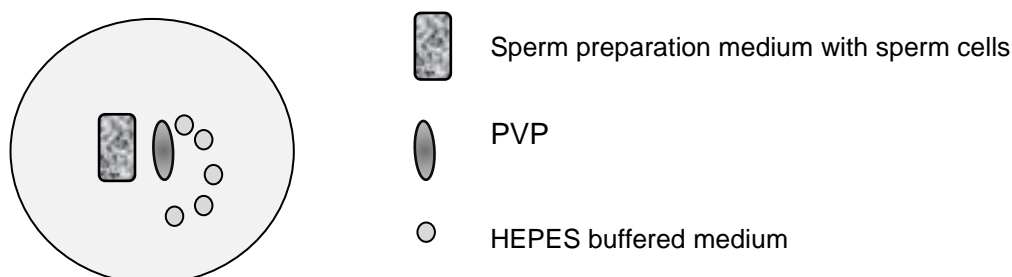
Incubate for ±30 minutes at 37ºC [no CO_2]

For ICSI with **testicular or severe oligiosoospermia semen**, prepare the following injection dish:

100

Testis biopsy sperm

Sperm prep

PVP

HEPES buffered medium
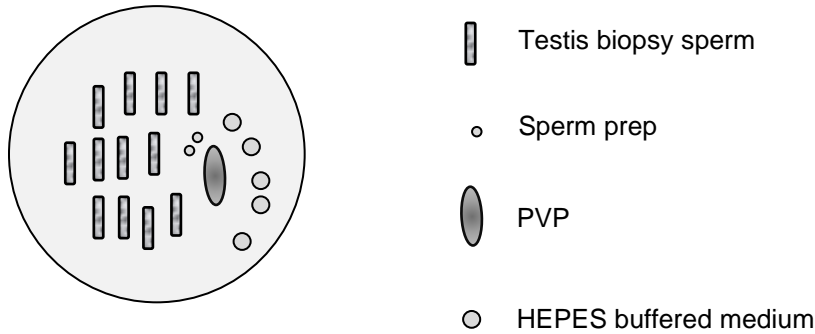
- Incubate for ±30 minutes at room temperature [no CO_2]

Sperm Immobilization – ejaculated sperm

- Add sperm cells to the sperm preparation drop

- Select motile, morphological normal spermatozoa from the drop with the ICSI injection pipette and immobilize the sperm cell

    - Collect enough sperm cells for the injection procedure.

Sperm Immobilization – testicular or severe oligiosoospermia semen

- Use a "testisbiopsy" pipette – with inner diameter of ± 15 μm – and fill with PVP

- Find a moving or twitching sperm, expel a little bit or PVP around the chosen sperm and aspirate

- Deposit in the small Sperm prep drop and repeat until enough sperm have been collected [ideally 2X more that the number of oocytes to be injected]

- Now heat the dish to 37ºC before injection

Sperm injection

- Place MII oocytes to be injected in the oocyte drops (2 -3 per drop)

- Select an immobilized sperm cell and carefully inject oocyte

- Continue until all oocytes are injected

- Transfer back into the left elliptical drop (fertilization) to wash and then to the cleavage drops for overnight culture (6% CO_2/37ºC)

- Do injection ± 40 hours post HCG administration if at all possible

Appendix E: Insemination process: Physiological Intracytoplasmic Sperm Injection (PICSI)

PICSI procedure

Make sure that all forms and documents are prepared

Check patient's file and record to eliminate all possible uncertainties/queries

Semen preparation, oocyte aspiration, denuding of oocytes, injection procedure/technique, embryo evaluation, embryo transfer and cryopreservation is exactly as for ICSI [SOP 10]

PICSI® dish preparation for use

- Hydrate the hyaluronan microdots by placing single 10-µL elliptical droplets of Sperm preparation medium [SAGE] at the end of each locating line covering the area where the microdot is situated

- Also add a drop polyvinylpyrrolidone (PVP) and HEPES buffered drop and carefully flood the dish with tissue culture oil

- Leave for ± 5minutes and add a small volume [10µL] of prepared sperm to the drop

  -Touch the tip of the micropipette containing the sperm to the edge of
    the hydrating drop at the bottom of the dish under the oil and expel the sperm
  -By delivering the sperm in a volume equal to the hydrating fluid, immediate
    mixing and delivery of sperm to the vicinity of the microdot is assured
  -If the sperm are delivered in a smaller volume at the edge of the drop, greater
    than 30 minutes may be required for them to swim through the hydrating fluid
    to the microdot

- Alternatively, the sperm suspension can be added directly to the dry microdot.

- Sperm binding begin normally in 5 minutes or less

- Some microdots may require 30 minutes or more to reach full binding capability

Therefore, whenever marginal sperm binding is observed, pre-hydrate for 30minutes or more, or allow sperm to incubate on the dot for 30 minutes or more before selecting sperm

Sperm preparation medium with selected sperm cells

PVP

HEPES buffered medium
Hyaluron drop with sperm
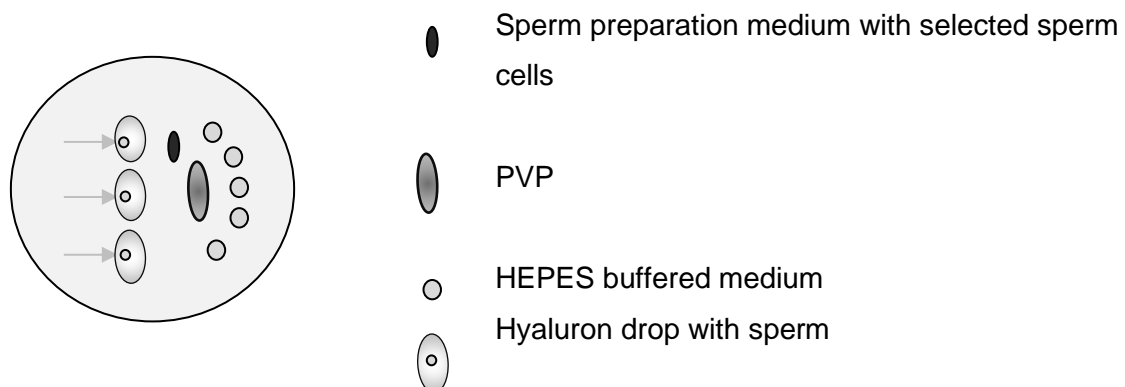
Sperm Selection for injection

- Once bound, hyaluronan bound sperm are easily identified: they exhibit no progressive migration despite vigorous tail beating

- Factors governing sperm binding: To rapidly populate the microdot with bound sperm, place approximately 100,000 hyaluronan-binding sperm per mL (approximately 1,000-2,000 total sperm in 10-20 µL volume) over the microdot

Sperm Location Selection

- The wall of the hyaluronan microdot is a physical barrier to which many sperm will bind since this is usually the first point of contact

- It is sometimes difficult to distinguish whether the sperm are bound or simply swimming against the edge of the microdot. You may be sure of selecting bound sperm by selecting them from the interior of the microdot

- Obtaining a good density of bound sperm: If the density of bound sperm is too high or too low for good sperm selection, dilute or concentrate the prepared sperm sample and use the adjusted sperm sample to seed the next microdot

- Three microdots are provided on each PICSI® Sperm Selection Device to give a sufficient opportunity

Sperm collection

- To collect a bound sperm, position the tip of the ICSI micropipette next to the sperm and gently suck fluid into the pipette, drawing in the sperm

- Place in the small reservoir drop of sperm prep

- Continue collecting until 20-50 sperm are captured

- Aspirate a single selected sperm cell and place in the PVP and immobilize

- Proceed to do the standard ICSI injection [SOP 10]

Temperature

- Sperm bind best to hyaluronan hydrogel at temperatures below 30°C

- At temperatures above 30°C, sperm swimming vigour increases and the swimming force may overcome the binding force

- The result is that about one-third of sperm bound at room temperature will show some progressive migration at 37°C and may be deemed not bound and therefore immature

- PICSI® Sperm Selection Device dishes placed on a 37°C heated stage will come to about 33°C and then remain at that temperature

    - Therefore, select bound sperm at room temperature – store in Sperm prep drop and warm to 37ºC before final injection

Technique considerations

- Microdot shape: The PICSI® Sperm Selection Device hyaluronan microdot is crater shaped. The edge of the microdot is a raised wall of hydrogel surrounding a low, flat interior layer. The wall is flexible and may be irregular in shape due to uneven hydration of the hydrogel. The hydrogel wall can be pierced and torn by an ICSI micropipette driven directly into it. It is best to position the elevated micropipette tip over the microdot interior and lower it to the microdot surface for recovery of sperm.

- Microdot caves: During manufacture, uneven hydration may cause segments of the microdot wall to create small "caves" that open toward the inside edge of the wall. Sperm that swim into a cave are trapped, not bound. Trapped sperm usually all face away from the centre of the microdot and show vigorously beating tails, often in clusters. The heads of trapped sperm can move laterally and sometimes back and forth within the walls of the cave. Trapped sperm should not be selected since their binding status is unclear.

- Microdot stability: If a part of the wall separates from the polystyrene, the same forces that create caves can cause the microdot wall to progressively detach from the dish and coil up like a spring.

105

When this occurs, some or all of the wall will separate from the microdot. However, the microdot interior hyaluronan layer will remain intact. The interior hyaluronan layer is stable for hours, it collects, and houses bound sperm that may be used for ICSI. Sperm bound to the curled-up wall remnant should not be used for sperm selection and isolation.

Appendix F: Insemination process: Morphologically Selected Sperm Injection (IMSI)

IMSI procedure

Make sure that all forms and documents are prepared

Check the patient's file and record to eliminate all possible uncertainties/queries

Semen preparation, oocyte aspiration, denuding of oocytes, injection procedure/technique, embryo evaluation, embryo transfer and cryopreservation is exactly as for ICSI [SOP 10]

IMSI Dish preparation for use

- For IMSI with ejaculated semen, prepare the following injection dish (glass bottom dish):
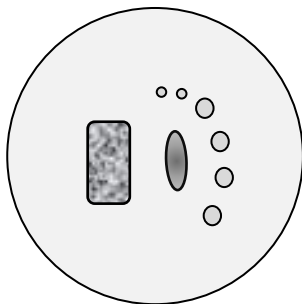
Spermatozoa

Sperm preparation medium

PVP

HEPES buffered medium

- Incubate for ±30 minutes at 37ºC [no CO_2]

Sperm selection and immobilization

- Add sperm cells to the sperm preparation medium

- Select motile, morphological normal spermatozoa from the drop with the

- ICSI injection pipette using the 20x objective

- Place the selected sperm into the left sperm preparation drop and focus on

- the edge of the drop

- Change the heated stage - metal one with a hole - the dish needs to come into contact with the objective

- Put the 100x objective in place and place a small drop of oil onto the objective

- Place the glass bottom dish containing the sperm onto the oil-covered objective

- The left sperm preparation drop should be in the center of the objective.

    - Use the 100X magnification to focus on the sperm preparation drop edge

    - Bring the needle down and make an indentation in the drop edge

        - The sperm will swim into the indentation

- Select morphologically normal sperm without any vacuoles and move them to the sperm preparation drop to the right

- After selecting enough sperm, change the heated stage again and proceed with the normal ICSI protocol [SOP 10]

Collect enough sperm cells for the injection procedure.

Appendix G - Embryo Culture and Evaluation

Embryo culture and pre-blastocyst grading used at Drs Aevitas Institute for Reproductive Medicine

Following oocyte aspiration and retrieval, and prior to insemination, the oocytes are denuded in those patients undergoing ICSI treatment. The oocytes are then cultured in Quinn's Advantage ® Protein Plus Fertilization Medium, which is covered by Sage ® Oil for Tissue Culture. Insemination will then occur, followed by overnight incubation at 37°C, 5% CO_2.

- Day 1: Fertilization Check

  (In the case of IVF patients, the oocytes are cleaned using denuding pipettes and rinsed well before checking for fertilization.)

  Check for PN and PB number on the inverted microscope fitted with heated stage. (Take note whether PN numbers are abnormal at > or < 2, or any other anomalies). Fertilization is indicated by the presence of 2PN. Transfer to preincubated Quinn's Advantage ® Protein Plus Cleavage Medium drops covered with Sage ® Oil for Tissue Culture in a greiner dish and incubate overnight at 37°C, 5% CO_2.

- Embryos are graded according to the number of cells present and the quality thereof. A scale ranging from 1-5 is used to grade the quality. 1 being of worst quality and 5 being of the best. Cells are ideally equal in size with no fragments.

- Day 2: 2-4 cell

  On day 2 the embryos should have cleaved to a 2-4 cell stage and are graded accordingly. Slow dividers are usually evident of genetic abnormalities.

- Day 3: 6-8 cell

  By Day 3 the embryos should have cleaved to a 6-8 cell stage and are graded accordingly.

- Day 4: The cells within the embryo continue to grow and begin to tightly align themselves against each other to form a compact ball of cells, known as a morula or compacting embryo. The cells are no longer distinguishable from each other.

- Day 5: Blastocyst Stage

Appendix H - Blastocyst Evaluation

Blastocyst grading: Drs Aevitas Institute of Reproductive Medicine

Blastocyst Grading

Degree of Expansion and hatching status

1 Early blastocyst, the blastocoel filling more than half the volume of conceptus, but
   no expansion in overall size as compared to early cleavage stage embryos

2 Blastocyst, the blastocoel filling more than half of the volume of conceptus, with
   slight expansion in overall size and notable thinning of the zona pellucida.

3 Full blastocyst, a blastocoel more than 50% of the conceptus volume and overall size
   fully enlarged with a very thin zona pellucida

4 Hatching blastocyst, non-preimplantation genetic diagnosis. The trophectoderm has started
   to herniate

5 Fully hatched blastocyst, non-preimplantation genetic diagnosis. Free blastocyst fully
   removed from the zona pellucida.

6 Hatching or hatched blastocyst, preimplantation genetic diagnosis.

Inner cell mass (ICM) grading

A Tightly packed, compacted cells

B Larger loose cells

C No ICM distinguishable

D Cells of ICM appear degenerative

Trophectoderm grading

A Many healthy cells forming a cohesive epithelium

B Few, but healthy cells, large in size

C Poor, very large or unevenly distributed cells, may appear as few cells squeezed to the side

D Cells of the trophectoderm appear degenerative

Appendix I - Blastocyst Vitrification & Warming

Blastocyst Vitrification procedure

Make sure that all forms and documents are prepared
Make sure the patients are aware of extra cost and have signed the consent form
Check patient's file and record to eliminate all possible uncertainties/queries

Modified from the Fertipro™ Vitrifreeze kit method and using the Cryotop™ from Kitazato® as carrier/storage device

Medium Preparation and labeling

Remove an aliquot of the vitrification mediums [Fertipro™]and place into eppendorf tubes and label

Pre-incubation medium   **[P] or 1**

Vitrification medium 1 **[E] or 2**

Vitrification medium 2 **[V] or 3**

Allow to reach room temperature

Use finely drawn glass pipettes

- o   *Use whatever pipette and suction device you find you have the best control with*

Label cryotops in the correct manner with a non-toxic permanent marker pen

- o   Female partner surname and initials

- o   DOB or Id number

- o   Date of vitrification

Complete and duplicate all applicable vitrification forms

Find a suitable LN_2 storage place

☐   Check the availability of LN_2

☐   Get LN_2 vitri container ready

Place 300µL of P medium in a well of a 4 well dish

Use a big petri dish for 100 µL the medium drops of E and V respectively – make these drops just before use

Get the LN2 ready and put the cryotop cover straws in the LN2

Artificial collapsing of blastocoel cavity

Do artificial collapsing of expanded blastocysts – all 2 and 3 gradings of expansion

Use a drop of flushing medium covered with oil (37ºC)

Hold blastocyst with holding pipette at ICM side

Push collapsing pipette through trophectoderm

If blast does not collapse by itself, perform gentle suction to collapse blast

Method

Place blastocysts (maximum 3) in the P medium in the w 4 well dish well.

Leave for 5-7 minutes

During this time make two 50µL drops of E medium in a petri dish lid

Set a timer for 2 minutes

Place the blastocysts in the E medium – using as little as possible of the P medium

Empty the pipette of all excess medium

Move blastocysts gently around the drop to different areas

Set a timer for 30 seconds

After 1 minute transfer to second E drop

During this time make two 50µL drops of V medium in the petri dish lid

After another 1 minute [total time in E - 2 minutes] move to the V medium drop- using as little as possible of the E medium

Blastocysts will float – so make sure to find them and place them at the bottom of the drops

Empty the pipette of all excess medium

Within 30 seconds move from 1st to second V medium drop and place on Cryotop™ tip in the correct manner

ALWAYS EMPTY THE PIPETTE BETWEEN TRANSFERS TO MINIMIZE DILUTION OF DROPS

Aspirate blastocysts in a VERY small amount of V medium and place on tip of cryotop – remove most of the V medium while ensuring to keep the blastocysts on the cryotop

Insert the cryotop tip into the LN_2 and swirl around for a few seconds

Using a metal clamp to hold the cover straw, and place the tip into the cover straw – NEVER TAKE THE TIP/DEVICE OUT OF THE LN_2

Immerge the whole device into the LN_2

While keeping the cryotop unit under LN_2 at all times, place into a goblet and place in storage tank in the correct place

Finalize all the forms and carefully note the storage identification
Write all the details about the cryopreservation into the patient file

Blastocyst warming procedure

Make sure that all forms and documents are prepared
Make sure the patients are aware of extra cost
Make sure the correct blastocysts are warmed
Make sure how many blastocysts should be warmed Check patient's file and record to eliminate all possible uncertainties/queries

Modified from the Fertipro™ Vitrithaw kit method and using the Cryotop™ from Kitazato® as carrier/storage device

Medium preparation

☐ Prepare a transfer dish one day before the transfer

- o 4 well dish with blastocyst medium in well 1 and 3 and blastocyst transfer medium in well 2 and 4 (37ºC, 6% CO_2)

☐ Remove an aliquot of the warming mediums [Fertipro™] and place into an eppendorf tube and label (to reach 37ºC)

- ▪ Thaw medium 1 (± 1 ml in centre well dish) **[1]**
- ▪ Thaw medium 2 **[2]**
- ▪ Thaw medium 3 **[3]**
- ▪ Thaw medium 4 **[4]**

☐ **ALL MEDIUMS MUST BE AT 37ºC**

☐ Use finely drawn glass pipettes

- o *Use whatever pipette and suction device you find you have the best control with*

☐ Check names, initials and storage place

☐ Use a big petri dish for the 100 µLmedium drops (Thaw 2-4)

☐ Get all paperwork in place

Method

Remove CryoTop™ from the LN_2 storage tank and place into the LN_2 in container without exposing CryoTop™ to the air and take to lab

☐ Remove cover straw very carefully

☐ Insert CryoTop™ tip with embryos directly into the 1ml thaw medium 1 at 37ºC

☐ Stir for a few seconds and dislodge embryos with a pipette if necessary – keep this part of the step as short as possible

☐ Incubate the embryos **3 minutes** in thaw medium 1 (37ºC)

- o During this time make 100µL drops each of thaw solution 2,3 and 4 in a big petri dish

☐ Transfer the embryos to the thaw solution 2 drop and incubate for **2 minutes** (37ºC)

☐ Transfer the embryos to the thaw solution 3 drop and incubate for **2 minutes** (37ºC)

☐ Transfer the embryos to the thaw solution 4 drop and incubate for **at least 1 minute** (37ºC)

☐ Transfer now to ± 0.7 ml equilibrated blastocyst medium in a 4 well dish (well 1), and wash once more in the second well of the 4 well dish at 37ºC, 6% CO_2

- ☐ Note survival, expansion and blastocyst grading/quality after 2 hours
- ☐ Confirm number to be transferred
- ☐ Transfer to ET well [usually 4] just before ET

  - ☐ Fill in all applicable forms correctly

Embryo transfer is done according to Appendix J.

Appendix J - Embryo transfer

Embryo transfer method

<u>Preparation</u>

Decide which and how many embryos will be transferred after consultation with the patients and the clinician

Transfer to the transfer dish [4 well NUNC]- prepared the previous day or 6 hours prior to ET

0.8 mL cleavage/blastocyst in well 2 and 3 and 2 ml medium in the middle

Place sterile instruments [forceps, speculum, valsellum], sterile gauze, and the transfer catheter and stylet on a sterile green cloth

A stylet is placed into the cannula of the soft catheter, ready for the clinician to use

Keep 5 mL warm sterile rinsing medium ready

The procedure and what to expect is discussed with couple and pictures of similar types of embryos also shown to them

The patient is positioned on the bed so that she is comfortable, and the clinician has good access and vision to the vagina and cervix

The procedure starts by placing a speculum in the vagina to visualize the cervix, which is rinsed with sterile medium. The cervix is the cleaned to remove an old blood and mucus

It is important that the patient's bladder is full before the transfer occurs as this ensures that the endometrial cavity can be accessed easily and automatically, and embryos transferred easily and exactly at the right place

The procedure starts by placing a speculum in the vagina to visualize the cervix, which is rinsed with sterile medium. The cervix is the cleaned to remove all old blood and mucus

<u>Embryo Catheter loading</u>

Once the clinician is ready for the transfer – with good sonar vision of the uterus cavity and confirmation that the cannula can be visualized in the correct place, the embryo/s are loaded into the catheter:

<u>Method:</u>

Aspirate medium [blast or cleavage] from well 2 into a nontoxic 1 ml syringe

Connect the soft catheter and expel the whole volume back into well 2

115

Make an air space of about 1 cm at the tip of the catheter

Visualize the embryos

Aspirate ± 10 µL of medium [± 4 cm] into the catheter and then the embryos until a total of ± 20µL in total has been aspirated

Take to the clinician

Catheter tip

air 20µL medium with embryos

Transfer Procedure

A soft transfer catheter, which contains the embryos to be transferred, is inserted through the cannula into uterine cavity.

After insertion of the catheter, the media containing the embryos are deposited into the uterine cavity. It is important that the embryos be placed in the correct position and that care is taken NOT to touch the fundus

After the deposit of the embryos, the embryologist checks the catheter immediately to ensure that the embryos did not remain inside the catheter – if they did they can just be transferred against.

The transfer is guided with an ultrasound (abdominal ultrasound) to ensure correct placement in the uterine cavity.

Anesthesia is not required when performing an embryo transfer.

It is recommended that the patient remains lying down for 15 minutes before she gets up to empty her bladder

It is also recommended that the couple abstain from sex until the pregnancy test, the female partner avoid strenuous exercise, alcohol, caffeine, medication and cigarette smoke

Progesterone support is given until the pregnancy test – a blood 10 days after the transfer.

Appendix K - Acquisition of Human Chorionic Gonadotrophin (HCG) levels

Protocol for obtaining Human Chorionic Gonadotrophin (HCG) levels

Human Chorionic Gonadotropin (hCG) is a glycoprotein hormone comprising 2 subunits, namely alpha and beta, which are joined non-covalently. The corpus luteum is responsible for the production of progesterone during the luteal phase of the menstrual cycle. In the absence of fertilization, the corpus luteum undergoes the degradation process known as luteolysis, and, as a consequence, progesterone levels decline (Richard et al., 2001). In the event of fertilization, the hormone hCG is secreted by the implanting embryo and interacts with the Leutenizing Hormone Choriogonadropin Receptor to maintain the corpus luteum and consequently secrete progesterone. The progesterone enriches the uterus with a thick lining of blood vessels and capillaries so that it is able to sustain the developing foetus. Due to its highly negative charge, hCG may repel the immune cells of the mother, protecting the fetus during the first trimester.

Although the timing from fertilization to the initial appearance of hCG can differ between assisted and unassisted pregnancies, the amounts and rate of increase per day of hCG levels are similar (Lenton et al., 1982). Implantation in patients usually occurs within 10 days after embryo transfer (Liu et al., 1995) and is the day on which the first hCG measurement is obtained. The maternal plasma levels of hCG continue to double about every 2 days in a normally developing intrauterine pregnancy (Seeber, 2006), and peak at 6–8 weeks after conception, when production of progesterone is taken over by the placenta (Braunstein, 1996). Failed pregnancies have been correlated with a low amount of hCG (France et al., 1996) and with a slow doubling time during pregnancy (Kratzer and Taylor., 1990). Comparisons show that hCG mean levels are several times higher in successful pregnancies than in unsuccessful pregnancies (Seeber, 2006; Bjerke et al., 1999).

The Vacutainer© needle has a sharp point at both ends, covered by a rubber sheath, with one end being shorter than the other. The long end of the needle (distal) is used for penetrating the vein, the shorter end (proximal) is used to pierce the rubber stopper of the vacuum tube. The sheath makes it possible to draw several tubes of blood by preventing leakage of blood as tubes are changed, which is called a multi-draw.

As the name implies, there is a vacuum in the collection tube. The vein is punctured with the distal hypodermic needle and when the sheathed proximal needle pierces the colour-coded rubber stopper, blood is sucked into the tube. That assumes that the distal end of the needle has successfully pierced the vein.

A positive βhCG recording is considered to be when the first reading (day 10) is >10 and the second reading (day 14) is at least double the amount recorded in the first reading.

Appendix L – Consent Forms

Egg Donation and Embryo Replacement

AEVITAS
Clinic

Dr Victor Hulme	**Prof Thinus Kruger**	**Prof Igno Siebert**
MBChB, M.Med	MBChB, MPharMed, MMed (O+G)	MBChB, MMed (O+G)
(O+G) (Stell)	FCOG (SA), FRCOG (London), MD	LKOG / FCOG (SA)

FertiliteitsSpesialiste&Ginekoloë / Fertility Specialists &Gynaecologists
PR No 0626546 / 7700539   VAT 4190266280

CONSENT TO TREATMENT INVOLVING EGG DONATION AND
EMBRYO REPLACEMENT AS AN EGG RECIPIENT

....
(Full name of husband / partner 1)

AND

....
(Full name of wife / partner 2)

OF THE FOLLOWING ADDRESS:

....

....

....

We, being husband/partner and wife/female partner of the above address hereby state:

1. We authorise **Prof. T.F. Kruger / Prof. T.I. Siebert / Dr. V.A. Hulme** (here after referred to as "the doctor") or suitable trained member of AEVITAS Clinic's staff to inseminate donor oocytes and transfer the obtained embryos to endeavour to cause the wife to become pregnant.

2. We authorise the doctor to obtain oocytes donated by:

   o   an anonymous egg donor. Code...................Agency......................

   o   a known egg donor. Name...

Vincent Pallotti Hospital
Park Road, Pinelands, 7405
Tel: 021 531 6999 • Fax: 021 531 7919
PO Box 112, Howard Place, 7405 • Posbus 112, Howard Place, 7405

Email: medici@aevitas.co.za

2.

3. The sperm to be used to endeavour to make the wife/female partner/surrogate pregnant will be that of:
   o   the husband/male partner

   o   an anonymous sperm donor. Code....................................

   o   a known sperm donor. Name...................................

4. The doctor has explained to us the nature and implications of the procedure and we understand that, even though the oocyte donation may be repeated as often as recommended by the doctor, there is no guarantee on his part that a pregnancy or full term pregnancy will result.

5. We understand that should we be married at the time of artificial fertilisation of the wife, we will be considered the parent/s of the child/children and will have full parental rights and responsibilities in respect of the child/children. Both the husband and the wife agree to maintain the child/children born as a result of the embryo transfer as if it was their own. Should we be unmarried at the time of artificial fertilisation of the female partner (recipient), the recipient will be considered the parent of the child/children.

6. We agree to rely upon the discretion of the doctor as to the suitability of the donor and we agree that we shall not be entitled at any stage to be informed as to the identity of an anonymous donor.

7. Fertilisation of fresh or frozen thawed eggs: We understand that the eggs in a fresh cycle will be fertilised immediately after retrieval. However we understand that eggs may be frozen after retrieval for fertilisation as a later specified date.

8. Cycle cancellation. We understand that failed fertilisation of eggs and failure to culture embryos is possible complication of IVF (*In Vitro Fertilisation*) treatment. We understand that there is also a rare risk of incubator failure, laboratory technical problems or problems with culture medium, which can affect our embryo culture. We understand that there is no guarantee that eggs will be retrieved. We accept these risks of IVF treatment and will not hold Aevitas Clinic or any of its staff responsible for these complications. We understand that we would not be responsible for all the costs of the treatment in these circumstances although we will be liable to pay the costs up to the point of cancellation of the cycle.

120

3.

9. We also understand that there might be problems with the egg donor not adhering strictly to instructions resulting in cycle cancellation or the harvest of fewer eggs than expected. We accept that Aevitas Clinic cannot be held responsible for any problems due to donor non-compliance.

10. We understand that more than one embryo may be transferred, and this may increase the risk of a multiple pregnancy. We understand that multiple pregnancies have an increased risk of miscarriage, premature labour and an increased financial and emotional cost. We also understand that it is possible for an embryo to split leading to a multiple pregnancy.

11. We acknowledge that no more than two zygotes or embryos may be transferred to the wife during an embryo transfer procedure, unless there is a specific medical indication requiring the contrary.

12. We understand that if pregnancy results there is a possibility, as with any pregnancy, of complications of childbirth and delivery, or the birth of an abnormal child, or undesirable hereditary tendencies of such a child, or other adverse consequences, and we hereby waive any legal action which we may have against the doctor or any member of his staff or the donor in respect of such adverse results that may in any way have been caused by oocyte donation. Although the vast majority of children conceived with ART (Artificial Reproductive Therapy) are healthy, pregnancy after IVF is altered as evidenced by risk of preterm delivery, low birth weight among infants, and an altered prevalence of preeclampsia. In men with very low sperm count, utilizing ICSI (*Intra Cytoplasmic Sperm Injection*) treatment, there may be an increased prevalence of low sperm count in male offspring.

13. Any dispute or claim arising out of or in relation to this agreement or any breach thereof or any alleged derelict relating to the donor, shall be referred to confidential arbitration for decision by a single arbitrator to be agreed upon between the parties within seven (7) days of a dispute having been declared in writing. Should no such agreement be reached as to who shall act as an arbitrator, an advocate of no less than 10 (ten) years practical experience shall be selected to act as an arbitrator. The arbitrator shall act informally and shall not necessarily be bound by the provisions of the Arbitration Act No. 42 of 1965. Arbitration shall be held in Cape Town and finalized within thirty (30) days after his / her appointment.

14. The liability of the doctor for any and all claims in term of this Agreement shall be limited to a maximum amount of 2.5 million South African rand (ZAR).

Vincent Pallotti Hospital
Park Road, Pinelands, 7405
Tel: 021 531 6999 • Fax: 021 531 7919
PO Box 112, Howard Place, 7405 • Posbus 112, Howard Place, 7405

Email: medici@aevitas.co.za

4.

15. We will have the benefit of a maximum of 15 eggs from our donor. Any eggs retrieved in addition to that number may be vitrified (frozen) and stored in an egg bank. We understand that if no eggs are retrieved from our chosen donor, we may have the choice of receiving frozen eggs from the egg bank, if available, at no additional charge.

16. We will not engage in any financial arrangements with the egg donor except directly through Aevitas Clinic.

17. We understand that we need to complete the necessary AEVITAS Clinic forms should we wish to:
17.1 have our embryo/s stored for a further period for the purpose of subsequent embryo transfer to the wife/recipient; and/of
17.2 have our embryo/s for transfer to another specific recipient; and/or
17.3 have our embryo/s for a purpose, other than embryo transfer, which purpose must be stated at the time that our consent is given.

18. We agree to store our vitrified (frozen) embryos according to the protocols of Aevitas Clinic and acknowledge that Aevitas Clinic discard any embryos that are unclaimed for a period of **five (5) years**.

19. I, the wife, consent to:
20.1 a physical examination and questioning by the doctor; and
20.2 my particulars being made available to the Central Data Bank.

20. We confirm that we have / have not (please select) previously undergone artificial fertilisation and embryo transfer at _____(place) on the _____(date).

21. I, the wife, confirm that I will notify the doctor, in writing and within **30 (thirty)** days, should I give birth as a result of the artificial fertilisation. Furthermore I confirm that I will immediately notify AEVITAS Clinic should I become aware that my child may suffer from any mental illness or disorder.

5.

SIGNED AT.....................................ON ....DAY OF..............................20 ....

1. HUSBAND / PARTNER: ....

2. WIFE / PARTNER: ....

3. WITNESS: (1)..................................

    (2) ....

Letter of Consent by the Donor

LETTER OF CONSENT BY THE DONOR

I hereby declare that the above given information is correct and that I will inform the unit of any change(s) in the above mentioned questions/statements. I also give my consent to the following:

1. a medical examination, which shall include a physical examination, by a competent person, if necessary;

2. questioning by a competent person related to my ova donation;

3. the collection of blood samples when necessary;

4. the removal of and use of my ova in the donor ova programme;

5. the testing and analysing of my ova and/or any other processing deemed necessary by a competent person;

6. the registration of my identification number with the Directorate of Health Services and the Central Data Bank;

7. my particulars, including my date of birth, age, height, mass, eye colour, hair colour, complexion, population group, nationality, sex, religion, occupation, highest educational qualification, and fields of interest, but excluding my identity, being made available to the parents/recipient and the Central Data Bank; and

8. providing details of my family history, especially with regard to any possible genetic condition or carrier status and mental illness in respect of any member of my family.

Note:
Blood samples collected will be used to identify any blood related diseases, i.e. HIV, Hepatitis B sAg & C Ab, RPR & TPHA, Chlamydia Trachomatis and Blood Group, AMH & Cystic Fibrosis.

I confirm that I have not given birth to more than 6 (six) children through artificial fertilisation using my ovum. Once I have had six live births, as contemplated herein, I consent to the further use of my ovum to achieve pregnancy by parents who have previously used my ova to achieve pregnancy and wish to have further children with the use of my ova. I understand that this will only be possible with the consent of the Minister. I further consent to my ovum being destroyed should six live births have occurred as the result of the use of my ovum.

I understand that the parents will be the owners of any embryos that are created with the use of my ova. I confirm that I am aware that they may donate their embryos to other recipients or for other purposes (other than embryo transfer). I confirm that a competent person may destroy embryos, created with the use of my ova if a parent/recipient has not claimed the embryo for 10 (ten) years.

The intention of donating ova is to achieve pregnancies.

I confirm that I have/ have not (please select) previously donated my ova.

I confirm that I donated my ova at _____(place) on the _____(date).

Signature(Donor): _____

124

Signature(Spouse): _____

125

Signature(Witness): _____

Signature(Witness):_____

Date: _____

Consent for the Vitrification of Human Embryos

AEVITAS
Fertility Clinic

VITRIFICATION OF HUMAN EMBRYOS
INFORMATION & CONSENT

- Vitrification is a process similar to freezing and is a standard and routine method used all over the world to successfully preserve and store human embryos, especially blastocyst stage embryos.
- Only embryos/blastocysts of top quality can be vitrified and warmed (thawed) successfully.
- Human embryos/blastocysts can be stored in vitrified state for a number of years.
- Some embryos/ blastocysts can be affected by the vitrification-thawing process and may therefore not survive and not be available for transfer. There is a 60% post-thaw survival rate in our clinic.
- A thawed embryo/blastocyst cannot be vitrified a second time.
- The transfer of surviving, thawed embryos/blastocysts does NOT guarantee a pregnancy.

We need your consent to treat your supernumerary embryos if vitrification is possible.

We, _____ (Husband/Partner) [ID / Passport No._____]

and _____ (Wife/Partner) [ID / Passport No._____]

referred by _____ (Doctor), request Aevitas Fertility Clinic to vitrify and store our embryos, according to the provisions stated below:

1. An **initial fee of R2365** is payable to Aevitas Fertility Clinic for the vitrification. This includes one storage straw and the fee for one year storage.
An **annual storage fee of R700** is payable to the Aevitas Fertility Clinic. **Failure to pay the annual fee within 3 months after the account has been issued, will result in the stored embryo/s being thawed.**

2. Embryo's can be stored individually or together (up to 3 embryos) per storage straw. (Note that if multiple embryos are stored on one straw they will all be thawed together and we cannot re-freeze thawed embryos). **A single straw costs R720** and for every **additional straw** used during freezing this fee is payable.

   The final decision as to exactly which embryos are vitrified and how many embryos will be stored on a straw will be made by the scientist.

   Taking into account that there is a 60% post-thaw survival rate, that usually at least two embryos would be thawed to ensure at least one embryo to transfer and that re-freezing embryos is not possible, we request Aevitas Fertility Clinic to:
   (Please select ONE option below and sign)   **Sign**

Vitrify (freeze) embryos individually on separate straws	
Vitrify (freeze) up to 2 embryos per straw	
Vitrify (freeze) up to 3 embryos per straw	

3. Aevitas Fertility Clinic must be informed of any **changes in personal details** (eg. postal address; telephone numbers). If patients cannot be contacted using the latest personal details on record after a period of 1 year, the stored embryos will be thawed.

AEVITAS _____ Fertility Clinic

Embryo vitrification & storage

126

4. We agree to indemnify Aevitas Fertility Clinic and/or its employees from any liability or claims for damages or loss of whatsoever nature flowing directly or indirectly from the use of the clinic/facility.

5. We understand and consent to our embryos being stored for a maximum period of 3 years for non-pregnant cycles and stored for a maximum period of 5 years for pregnant cycles. Should we wish our embryos to be stored for a further period we confirm that we will contact Aevitas Fertility Clinic and consent to the further storage of the embryos. We understand that Aevitas Fertility Clinic requires our permission for the further storage of our embryos and that, should we fail to contact them, our embryos will be thawed and discarded.

6. Vitrified – stored embryos must be warmed and/or transferred before a new stimulation and oocyte aspiration cycle will be attempted.

   ❖ We understand that the vitrified-stored embryos may not survive the thawing process in preparation for embryo transfer, and that surviving, transferred embryos might not result in a pregnancy.

   ❖ We understand the necessity of selecting embryos of sufficient quality for vitrification and give the doctor and laboratory staff the authority to make this decision.

   ❖ We acknowledge that if, at any time, we wish to have our vitrified embryo/s thawed, we both must give written consent to Aevitas Fertility Clinic by means of the form which is available from the clinic.

8. In the event of the death of one or both husband and wife, the stored embryos must be:

PLEASE CONSIDER EACH SCENARIO BELOW AND, FOR EACH ONE, SELECT ONE OPTION AND SIGN:

I _____ (wife/partner) request that in the event of my death I wish to have the following done with my vitrified embryos:	SIGN
1. Discarded	
2. Used for scientific research	
3. Offered for donation to a couple	
4. Assigned to the care of my husband/partner	
Husband's / partner's name: ID / Passport No.	

I _____ (husband/partner) request that in the event of my death I wish to have the following done with my vitrified embryos:	SIGN
1. Discarded	
2. Used for scientific research	
3. Offered for donation to a couple	
4. Assigned to the care of my wife/partner	
Wife's /partner's name: ID / Passport No.	

In the event of both of our deaths we wish to have the following done with our vitrified embryos: BOTH SIGN		
discarded		
used for scientific research		
offered for donation to a couple		

Signed at _____ on ____ / ____ / _____ (date)

Husband / Partner _____ (print name) _____(signature)

Wife / Partner _____ (print name) _____(signature)

AEVITAS _____ **Fertility Clinic** _____
Embryo vitrification & storage

127

Witness 1 _____ (print name) _____ (signature)

Witness 2 _____ (print name) _____ (signature)

AEVITAS   **Fertility Clinic**
Embryo vitrification & storage

Consent to Medical Procedure

AEVITAS
Fertility Clinic

CONSENT TO MEDICAL
PROCEDURE / EXAMINATION

	Print Name		I have explained the nature, risks and possible consequences of the medical procedure to the undersigned patient or person legally competent to give consent.
NAME OF DOCTOR	Signature	Date	

MEANS USED TO EXPLAIN THE PROCEDURE	*Personally*	*Via interpreter*	CIRCLE whichever is applicable.

NATURE OF PROCEDURE ....
....
....
....
....

ANAESTHETIC	*Local*	*Deep sedation*	CIRCLE whichever is applicable.

CONSENT TO USE OF BLOOD AND/OR BLOOD PRODUCTS			Granting or withholding of consent by the undersigned patient to the use of blood and/or blood products should it become necessary during the procedure. CIRCLE whichever is applicable.
	Granted	*Withheld*	

I agree that a sample of my blood will be taken and tested for Hepatitis B and the Human Immunodeficiency Virus should an incident of contamination of a health care worker by bodily fluids occur during the procedure.

FULL NAME OF PATIENT		I, the undersigned, hereby consent to the performance of, and understand the nature, risks and possible consequences of the above procedure. The doctors who perform the above may increase the reasonable scope thereof or carry out additional or alternative measure (including general anaesthesia) if considered necessary.
Signature/Thumb-print of patient _____ Date _____		

PERSON LEGALLY COMPETENT TO GIVE CONSENT	Print Name _____ Signature _____ Date _____ Capacity or relationship to patient _____ Means by which consent was given: *Personally* *Telephonically* *Telegraphically*	This section to be filled in if consent is given by a person other than the patient.

WITNESS 1 **WITNESS 2**	Print Name_____ Signature_____ Print Name_____ Signature _____ Date _____	Names and signatures of witnesses to the signing of this document by the patient or a person legally competent to give consent on behalf of the patient.

129

Appendix M – Disclosure for use of Clinic's name

TO WHOM IT MAY CONCERN
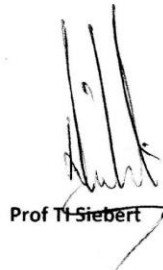
We, the partners of the Aevitas Fertility Clinic, Life Vincent Pallotti Hospital, Alexandra Road, Cape Town, herby give consent that Miss Elizabeth Meiring (student number: 18289045) can use de-identified routine medical records of patients treated at the clinic for her MSc Reproductive Biology research study titled: Report on the live birth rates and neonatal outcomes of ART patients at Drs. Aevitas Fertility Clinic; Implications of a frozen embryo transfer [FET] program.

We also give consent that the clinic's name may be used in the final thesis document.

Kind regards

Prof TF Kruger   Prof TI Siebert   Dr VA Hulme

Date: 24/04/2018

130

Appendix N – Data Sheet

DATE	Patient Code	PROC	DONOR	SURR	AGE(V)	AGE(D)	OVA AGE	PREGNANT	ABORT WEEK

B1	B2	SAC	FH	IMPLANT	FRESH VS FROZEN	# OVA	# FERT	# BLASTOCYSTS	EMBRYO/ET

DAY ET	BR	LIVE BIRTH	# BABIES BORN	SEX	DELIVERY	GESTATION	WEIGHT	COMMENTS

Proc – Procedure (IVF/ICSI/PICSI)

Surr: Surrogate (Yes/No)

Age (V): Age of the Recipient)

Age (D): Age of the Donor

B1: First βHCG blood test value on day 10 (post ET)

B2: Second βHCG blood test value on day 14 (post ET)

FH: Fetal Heart

Ova: Number of oocytes retrieved at OPU

Fert: Number of ova that fertilized

Embryo/ET: Number of embryos transferred at ET

Day ET: Embryo developmental stage at which the ET takes place

BR: Blastulation Rate

Appendix 0 – Statistical Analysis Results (Results from Statistician)

PRIMARY

<u>Outcomes in Fresh embryo transfer (fresh ET)</u> vs <u>vitrified-warmed embryo transfer (FET) cycles</u>

Calculate the **LBR** (live birth rate) for each (Fresh ET vs FET)

Calculate the average **birthweight** for each

Calculate the average **gestational age** for each

Calculate the **multiple status** for each

Determine the effect of the **number of ova** at aspiration on LBR for each.

Determine the effect of the **number of embryos transferred** on the LBR for each

Determine the effect of the **blastulation rate** on the LBR for each

Determine the effect of **female age** of the LBR

Calculate the LBR & average birthweight & average gestational age for elective and emergency c/section deliveries

Calculate the LBR & average birthweight & average gestational age for vaginal deliveries

SECONDARY

<u>Outcomes in single and multiple pregnancies</u>

Calculate the **LBR** for each (single pregnancies vs multiple pregnancies) within fresh and vitrified-warmed ET groups

<mark>Cjl: this does not make sense since there is no indicators of multiple or single pregnancies – only the number born live</mark>

Calculate the average **birthweight** for each within fresh and vitrified-warmed ET groups

Calculate the average **gestational age** for each within fresh and vitrified-warmed ET groups

147

Determine the effect of the **number of ova** at aspiration on LBR for each.

Determine the effect of the **number of embryos transferred** on the LBR for each

Determine the effect of the **blastulation rate** on the LBR for each

Determine the effect of **female age** of the LBR

Statistical Analysis

FRESH VS FROZEN CYCLE NUMBERS OVER TIME

```
. tab date fresh , row col chi2

+------------------+
| Key              |
|------------------|
|     frequency    |
|  row percentage  |
| column percentage |
+------------------+

          |        FRESH
   DATE |      FET      FRESH |     Total
----------+----------------------+----------
   2015 |      152        546 |       698
          |    21.78      78.22 |    100.00
          |    35.93      44.35 |     42.20
----------+----------------------+----------
   2016 |      156        474 |       630
          |    24.76      75.24 |    100.00
          |    36.88      38.51 |     38.09
----------+----------------------+----------
   2017 |      115        211 |       326
          |    35.28      64.72 |    100.00
          |    27.19      17.14 |     19.71
----------+----------------------+----------
  Total |      423      1,231 |     1,654
          |    25.57      74.43 |    100.00
          |   100.00     100.00 |    100.00

     Pearson chi2(2) =  21.6287   Pr = 0.000
```
- FET increasing with time

NUMBER OF FRESH VS FROZEN ETS IN DONOR VS NON-DONOR CYCLES

```
. tab donor fresh, row chi2

+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+

          |        FRESH
  DONOR |      FET      FRESH |     Total
----------+----------------------+----------
      0 |      193        820 |     1,013
          |    19.05      80.95 |    100.00
----------+----------------------+----------
      1 |      158        282 |       440
          |    35.91      64.09 |    100.00
----------+----------------------+----------
  Total |      351      1,102 |     1,453
          |    24.16      75.84 |    100.00

     Pearson chi2(1) =  47.5760   Pr = 0.000
```

148

```
. tab proc fresh, col row chi2

+-------------------+
| Key               |
|-------------------|
|     frequency     |
|  row percentage   |
| column percentage |
+-------------------+

           |        FRESH
      PROC |       FET      FRESH |     Total
-----------+----------------------+----------
         1 |         0         17 |        17
           |      0.00     100.00 |    100.00
           |      0.00       1.38 |      1.03
-----------+----------------------+----------
         2 |         0        239 |       239
           |      0.00     100.00 |    100.00
           |      0.00      19.42 |     14.46
-----------+----------------------+----------
         3 |         4        546 |       550
           |      0.73      99.27 |    100.00
           |      0.95      44.35 |     33.27
-----------+----------------------+----------
         4 |         0         51 |        51
           |      0.00     100.00 |    100.00
           |      0.00       4.14 |      3.09
-----------+----------------------+----------
         5 |         1        146 |       147
           |      0.68      99.32 |    100.00
           |      0.24      11.86 |      8.89
-----------+----------------------+----------
         6 |         0        145 |       145
           |      0.00     100.00 |    100.00
           |      0.00      11.78 |      8.77
-----------+----------------------+----------
         7 |       300         33 |       333
           |     90.09       9.91 |    100.00
           |     71.09       2.68 |     20.15
-----------+----------------------+----------
         8 |         0          2 |         2
           |      0.00     100.00 |    100.00
           |      0.00       0.16 |      0.12
-----------+----------------------+----------
         9 |       114         17 |       131
           |     87.02      12.98 |    100.00
           |     27.01       1.38 |      7.92
-----------+----------------------+----------
        10 |         0         14 |        14
           |      0.00     100.00 |    100.00
           |      0.00       1.14 |      0.85
-----------+----------------------+----------
        11 |         0         15 |        15
           |      0.00     100.00 |    100.00
           |      0.00       1.22 |      0.91
-----------+----------------------+----------
        12 |         0          3 |         3
           |      0.00     100.00 |    100.00
           |      0.00       0.24 |      0.18
-----------+----------------------+----------
        13 |         0          3 |         3
           |      0.00     100.00 |    100.00
           |      0.00       0.24 |      0.18
-----------+----------------------+----------
        16 |         1          0 |         1
           |    100.00       0.00 |    100.00
           |      0.24       0.00 |      0.06
-----------+----------------------+----------
        17 |         2          0 |         2
           |    100.00       0.00 |    100.00
           |      0.47       0.00 |      0.12
-----------+----------------------+----------
     Total |       422      1,231 |     1,653
           |     25.53      74.47 |    100.00
           |    100.00     100.00 |    100.00
```

149

```
        Pearson chi2(14) =  1.4e+03   Pr = 0.000


. tab surr fresh , row chi2

+---------------+
| Key           |
|---------------|
|   frequency   |
| row percentage |
+---------------+

           |        FRESH
    SURR |      FET      FRESH |     Total
-----------+----------------------+----------
       0 |      291        998 |     1,289
           |    22.58      77.42 |    100.00
-----------+----------------------+----------
       1 |       23         23 |        46
           |    50.00      50.00 |    100.00
-----------+----------------------+----------
   Total |      314      1,021 |     1,335
           |    23.52      76.48 |    100.00

        Pearson chi2(1) =  18.5699   Pr = 0.000
```

COMPARISON OF THE OVA AGE IN FRESH VS FROZEN GROUPS

```
. ttest ovaage , by(fresh)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.    Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
     FET |     423    30.43262   .2789874    5.737924    29.88425      30.981
   FRESH |   1,231    33.19253   .1731392    6.074696    32.85285    33.53221
---------+--------------------------------------------------------------------
combined |   1,654    32.4867    .1502008    6.108576     32.1921     32.7813
---------+--------------------------------------------------------------------
    diff |            -2.759902   .3376209               -3.422112   -2.097692
------------------------------------------------------------------------------
    diff = mean(FET) - mean(FRESH)                          t =  -8.1746
Ho: diff = 0                                degrees of freedom =     1652

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
Pr(T < t) = 0.0000       Pr(|T| > |t|) = 0.0000       Pr(T > t) = 1.0000.
```

PREGNANCY OUTCOMES IN FRESH VS FROZEN ET GROUPS

```
. tab pregnant fresh, row col chi2

+-------------------+
| Key               |
|-------------------|
|     frequency     |
|  row percentage   |
| column percentage |
+-------------------+

            |        FRESH
PREGNANT |      FET      FRESH |     Total
-----------+----------------------+----------
       0 |      159        497 |       656
           |    24.24      75.76 |    100.00  NOT PREGNANT
           |    37.59      40.37 |     39.66
-----------+----------------------+----------
       1 |       23         60 |        83
           |    27.71      72.29 |    100.00  BIOCHEMICAL PREGNANCY
           |     5.44       4.87 |      5.02
-----------+----------------------+----------
       2 |        2          8 |        10
           |    20.00      80.00 |    100.00  BLIGHTED OVUM
           |     0.47       0.65 |      0.60
```

```
-----------+--------------------+----------
         3 |       34       107 |      141
           |    24.11     75.89 |   100.00  MISCARRIAGE
           |     8.04      8.69 |     8.52
-----------+--------------------+----------
         4 |        3         2 |        5
           |    60.00     40.00 |   100.00  ECTOPIC
           |     0.71      0.16 |     0.30
-----------+--------------------+----------
         6 |      191       533 |      724
           |    26.38     73.62 |   100.00  FULL TERM
           |    45.15     43.30 |    43.77
-----------+--------------------+----------
         7 |        5         8 |       13
           |    38.46     61.54 |   100.00  PERINATAL DEATH
           |     1.18      0.65 |     0.79
-----------+--------------------+----------
         9 |        6        16 |       22
           |    27.27     72.73 |   100.00  NO FETAL HEART
           |     1.42      1.30 |     1.33
-----------+--------------------+----------
     Total |      423     1,231 |    1,654
           |    25.57     74.43 |   100.00
           |   100.00    100.00 |   100.00

        Pearson chi2(7) =   5.6645   Pr = 0.579


. tab abortweek fresh, row col chi2

+------------------+
| Key              |
|------------------|
|     frequency    |
|  row percentage  |
| column percentage |
+------------------+


           |        FRESH
ABORT WEEK |     FET     FRESH |    Total
-----------+--------------------+----------
         1 |       20        46 |       66
           |    30.30     69.70 |   100.00
           |    58.82     42.99 |    46.81
-----------+--------------------+----------
         2 |       11        56 |       67
           |    16.42     83.58 |   100.00
           |    32.35     52.34 |    47.52
-----------+--------------------+----------
         3 |        3         5 |        8
           |    37.50     62.50 |   100.00
           |     8.82      4.67 |     5.67
-----------+--------------------+----------
     Total |       34       107 |      141
           |    24.11     75.89 |   100.00
           |   100.00    100.00 |   100.00

        Pearson chi2(2) =   4.3336   Pr = 0.115
```

LIVE BIRTH RATE FOR FRESH VS FROZEN CYCLES

```
. tab fresh livebirth, row chi2

+----------------+
| Key            |
|----------------|
|    frequency   |
| row percentage |
+----------------+


           |      LIVE BIRTH
     FRESH |        0         1 |    Total
-----------+--------------------+----------
```

```
     FET |        232          191 |          423
         |      54.85        45.15 |       100.00
-----------+--------------------+----------
   FRESH |        694          537 |        1,231
         |      56.38        43.62 |       100.00
-----------+--------------------+----------
   Total |        926          728 |        1,654
         |      55.99        44.01 |       100.00

      Pearson chi2(1) =    0.2993   Pr = 0.584
```

- No difference in live birth rate between the two methods p=.584

```
     FET |        232          191 |          423
         |      54.85        45.15 |       100.00
-----------+--------------------+----------
   FRESH |        694          537 |        1,231
         |      56.38        43.62 |       100.00

      Pearson chi2(1) =    0.2993   Pr = 0.584
```

ESTIMATED DIFFERENCE IN LIVEBIRTHS

```
. binreg livebirth i.fresh_1, rd

Iteration 1:   deviance =  2268.872
Iteration 2:   deviance =  2268.872

Generalized linear models                    No. of obs      =      1,654
Optimization     : MQL Fisher scoring        Residual df     =      1,652
                   (IRLS EIM)                Scale parameter =          1
Deviance         =  2268.872433             (1/df) Deviance  =   1.373409
Pearson          =          1654            (1/df) Pearson   =   1.001211

Variance function: V(u) = u*(1-u)                [Bernoulli]
Link function    : g(u) = u                      [Identity]

                                             BIC             =   -9974.02

------------------------------------------------------------------------------
             |                EIM
   livebirth | Risk Diff.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  1.fresh_1 |  .0153059    .0280223     0.55   0.585    -.0396167    .0702286 difference
      _cons |  .4362307    .0141345    30.86   0.000     .4085276    .4639338 Fresh
------------------------------------------------------------------------------
```

- Estimated difference between FET and Fresh is 1.5% (95%CI: -4.0 to 6.0%)
- Since the interval includes zero the is no difference

GESTATIONAL AGE IN FRESH VS FROZEN CYCLE GROUPS

```
. ttest gestation, by(fresh_2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     154    36.53312    .2456602    3.048562    36.04779    37.01844
       1 |     420     36.2869    .1554228     3.18522     35.9814    36.59241
---------+--------------------------------------------------------------------
combined |     574    36.35296    .1314114    3.148392    36.09485    36.61107
---------+--------------------------------------------------------------------
    diff |             .2462121    .2966728               -.3364888    .8289131
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                 t =    0.8299
Ho: diff = 0                                 degrees of freedom =       572

   Ha: diff < 0                 Ha: diff != 0                  Ha: diff > 0
Pr(T < t) = 0.7965        Pr(|T| > |t|) = 0.4069          Pr(T > t) = 0.2035
```


BIRTHWEIGHT IN FRESH VS FROZEN CYCLE GROUPS

```
. ttest weight, by(fresh_2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     137    2861.409    67.10928     785.494    2728.696    2994.121
       1 |     349    2837.077    38.76263    724.1457    2760.839    2913.316
---------+--------------------------------------------------------------------
combined |     486    2843.936    33.62208    741.2125    2777.873    2909.999
---------+--------------------------------------------------------------------
    diff |              24.3314    74.79776                -122.637    171.2998
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                 t =    0.3253
Ho: diff = 0                                 degrees of freedom =       484

   Ha: diff < 0                 Ha: diff != 0                  Ha: diff > 0
 Pr(T < t) = 0.6275          Pr(T > t) = 0.3725
```

- No difference between groups for weight and gestation

153

- Subgroup – only those with live births included

BOX PLOT OF THE BIRTHWEIGHT (GRAMS) OF NEONATES IN FROZEN (0) AND FRESH (1) ET GROUPS

. graph box weight, over(fresh_2)
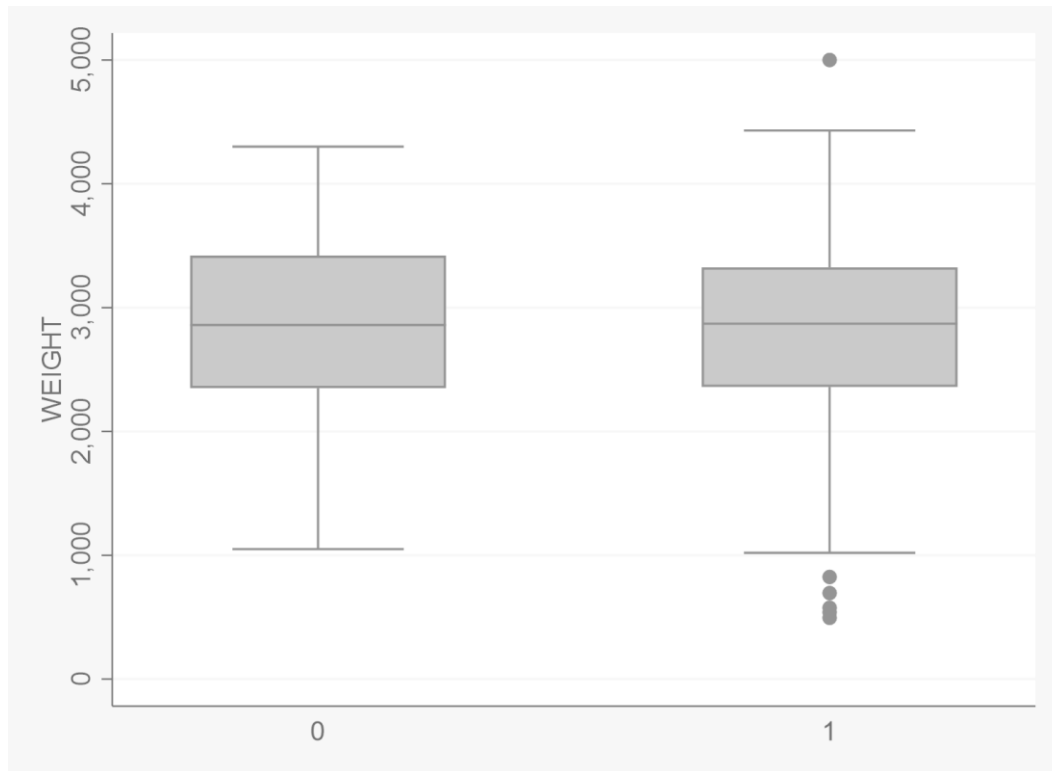
BOX PLOT OF THE GESTATIONAL AGE (WEEKS) OF NEONATES IN FROZEN (0) AND FRESH (1) ET GROUPS

Gestation

154
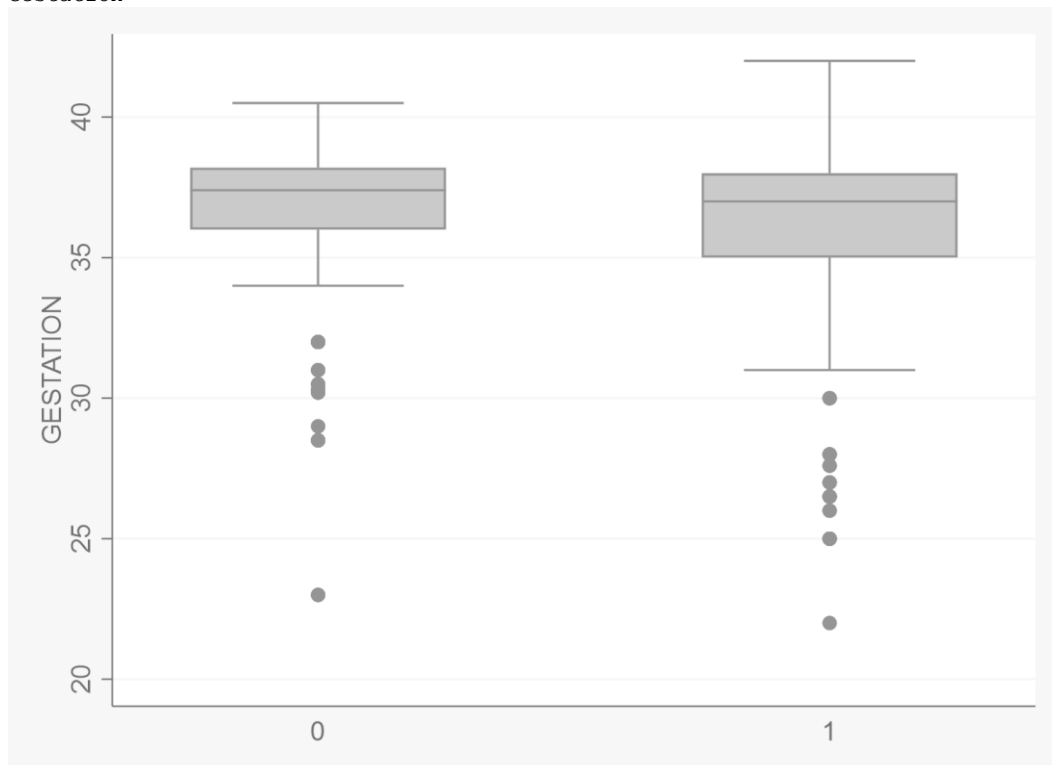
NUMBER OF BABIES BORN IN FRESH VS FROZEN ET GROUPS

```
. tab fresh n_babiesborn , row chi2

+---------------+
| Key           |
|---------------|
|   frequency   |
| row percentage|
+---------------+

           |              n_BABIES BORN
     FRESH |       0         1         2         3 |     Total
-----------+-------------------------------------------+----------
       FET |     194       110        38         2 |       344
           |   56.40     31.98     11.05      0.58 |    100.00
-----------+-------------------------------------------+----------
     FRESH |     623       307       113         2 |     1,045
           |   59.62     29.38     10.81      0.19 |    100.00
-----------+-------------------------------------------+----------
     Total |     817       417       151         4 |     1,389
           |   58.82     30.02     10.87      0.29 |    100.00

        Pearson chi2(3) =   2.4189   Pr = 0.490
```

- Number profile the same p=.490

NUMBER OF OVA AT ASPIRATION IN FRESH VS FROZEN ET GROUPS

```
. ttest n_ova, by(fresh_2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     181    17.77348    .6743961    9.073071    16.44274    19.10422 fet
       1 |   1,227    9.709046    .1817782    6.367429    9.352416    10.06568 fresh
---------+--------------------------------------------------------------------
combined |   1,408    10.74574    .1942911     7.29045    10.36461    11.12687
---------+--------------------------------------------------------------------
    diff |            8.064434    .5393995                 7.00632    9.122549
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                    t =  14.9508
Ho: diff = 0                                   degrees of freedom =      1406

    Ha: diff < 0                 Ha: diff != 0                  Ha: diff > 0
 Pr(T < t) = 1.0000       Pr(|T| > |t|) = 0.0000          Pr(T > t) = 0.0000
```

- Number of ova less in fresh group

```
. tab fresh emb_et, row chi2
```

NUMBER OF EMBRYOS TRANSFERRED AT ET IN FRESH AND FROZEN ET CYCLES

```
+---------------+
| Key           |
|---------------|
|   frequency   |
| row percentage|
+---------------+

           |                        EMB_ET
     FRESH |       1         2         3         4 |       5         6 |     Total
-----------+-------------------------------------------+-------------------+---------
       FET |     107       287        26         3 |       0         0 |       423
           |   25.30     67.85      6.15      0.71 |    0.00      0.00 |    100.00
-----------+-------------------------------------------+-------------------+---------
     FRESH |     190       818       157        56 |       5         4 |     1,231
           |   15.43     66.45     12.75      4.55 |    0.41      0.32 |    100.00
```

155

```
----------+------------------------------------------------------------------+----------
   Total |     297     1,105       183        59         5         4 |     1,654
         |   17.96     66.81     11.06      3.57      0.30      0.24 |    100.00
```

```
         |   EMB_ET
   FRESH |       8 |     Total
----------+----------+----------
   FET   |       0 |       423
         |    0.00 |    100.00
----------+----------+----------
   FRESH |       1 |     1,231
         |    0.08 |    100.00
----------+----------+----------
   Total |       1 |     1,654
         |    0.06 |    100.00

         Pearson chi2(6) =   46.0120   Pr = 0.000
```

- Et profile differs significantly

NUMBER OF EMBRYOS TRANSFERRED AT ET FOR FET VS. FRESH (%)
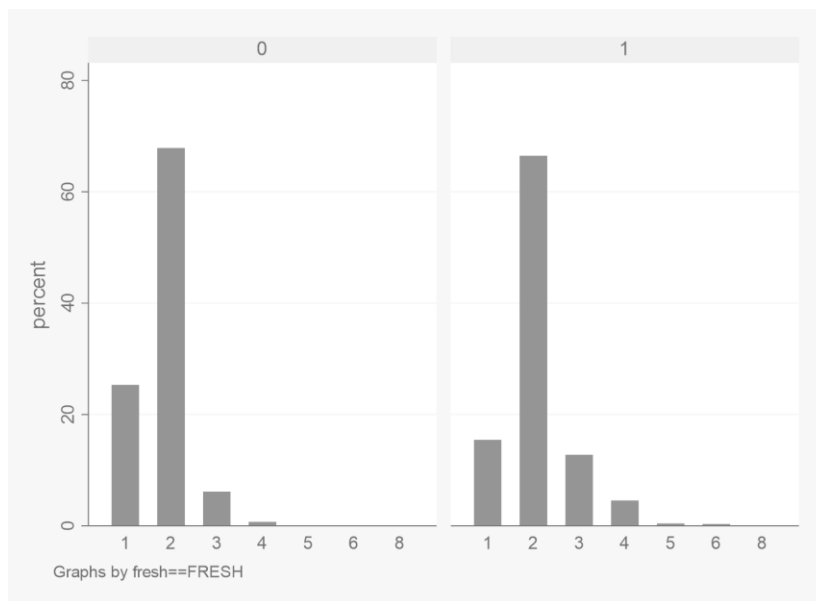
graph bar, over(emb_et) by(fresh_2)

BLASTULATION RATE: FRESH VS. FROZEN

```
. generate br_rate= n_blasts/ n_fert
(567 missing values generated)

. ttest br_rate , by(fresh_2)

Two-sample t test with equal variances
------------------------------------------------------------------------------
   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       0 |     180    .6541724    .0149145    .2000997    .6247414    .6836033  fet
       1 |     908     .481786    .0104508    .3149139    .4612755    .5022965  fresh
---------+--------------------------------------------------------------------
combined |   1,088    .5103058    .0092677    .3056944    .4921212    .5284905
---------+--------------------------------------------------------------------
    diff |             .1723864    .0243985                .1245128    .2202599
------------------------------------------------------------------------------
    diff = mean(0) - mean(1)                                      t =   7.0654
Ho: diff = 0                                     degrees of freedom =     1086

   Ha: diff < 0                  Ha: diff != 0                   Ha: diff > 0
```

Pr(T < t) = 1.0000   Pr(|T| > |t|) = 0.0000   Pr(T > t) = 0.0000

- Blastocyst rate significantly higher in fet, 65% versus 48% in fresh p<.001
- Difference of 17.3% in br rate
- Missing data quite high

BOX PLOT OF THE BLASTULATION RATE IN FRESH AND FROZEN ET GROUPS
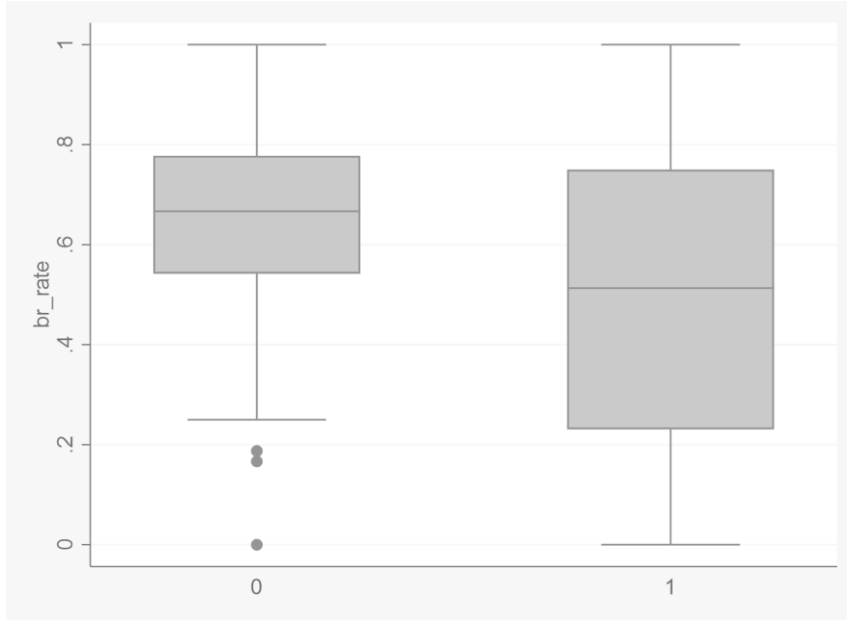
graph box br_rate, over(fresh_2)

RECIPIENT AGE IN FRESH CYCLES VS FROZEN CYCLES
. ttest age_v , by(fresh_2)

Two-sample t test with equal variances
```
--------------------------------------------------------------------------------
  Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
--------+-----------------------------------------------------------------------
      0 |     421     36.87886    .2992168    6.139416    36.29071    37.46701
      1 |   1,231     37.10073    .1479643    5.191418    36.81044    37.39102
--------+-----------------------------------------------------------------------
combined|   1,652     37.04419    .1340296    5.447607     36.7813    37.30707
--------+-----------------------------------------------------------------------
   diff |            -.2218713    .3076124                -.8252231    .3814805
--------------------------------------------------------------------------------
   diff = mean(0) - mean(1)                                    t =   -0.7213
Ho: diff = 0                                   degrees of freedom =     1650

   Ha: diff < 0                   Ha: diff != 0                  Ha: diff > 0
Pr(T < t) = 0.2354         Pr(|T| > |t|) = 0.4708          Pr(T > t) = 0.7646
```

?

- No difference in women's age – mean age 37 years in both groups

TYPE OF DELIVERY IN FRESH VS FROZEN CYCLES

. tab delivery livebirth , row exact

```
+----------------+
| Key            |
|----------------|
|   frequency    |
| row percentage |
+----------------+


          |     LIVE BIRTH
 DELIVERY |      0          1 |     Total
----------+----------------------+----------
        1 |      1         38 |        39  vag (Vaginal Delivery)
```

157

```
          |     2.56      97.44 |     100.00
----------+---------------------+----------
        2 |        1        504 |        505  cs  (Elective Cesarean Section)
          |     0.20      99.80 |     100.00
----------+---------------------+----------
        3 |        0         36 |         36  ecs (Emergency Cesarean Section)
          |     0.00     100.00 |     100.00
----------+---------------------+----------
    Total |        2        578 |        580
          |     0.34      99.66 |     100.00


          Fisher's exact =                 0.242
```

- LBR not different for different delivery modalities p=.242


```
. by delivery, sort: summarize gestation weight age_v

------------------------------------------------------------------------------------------
-> delivery = 1

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
   gestation |        38    38.36053    1.791804        31       40.6
      weight |        36    3199.306    404.0017      2200       4135
       age_v |        39    35.10256    4.739503        23         46

------------------------------------------------------------------------------------------
-> delivery = 2

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
   gestation |       466    36.48691    2.626699        25         42
      weight |       402    2835.993    735.1172       495       5000
       age_v |       505     36.9604    5.538682        21         54

------------------------------------------------------------------------------------------
-> delivery = 3

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
   gestation |        36    33.54722    4.623325      26.5       40.5
      weight |        31    2265.645    885.1363       540       3600
       age_v |        36    36.69444    4.125319        29         46

------------------------------------------------------------------------------------------
-> delivery = .

    Variable |       Obs        Mean    Std. Dev.       Min        Max
-------------+--------------------------------------------------------
   gestation |        34    35.24412    5.820194        22         40
      weight |        17    3333.765    308.5759      2720       3950
       age_v |     1,072    37.16604    5.458822        22         56
```

MULTIPLE REGRESSION MODEL OF LIVE BIRTH RATE

```
Binomial regression model
Covariates used : date , age women, fresh, number transferred, donor ova used

. xi: binreg livebirth i.date age_v fresh_2  i.emb_et i.donor, rd
i.date          _Idate_2015-2017    (naturally coded; _Idate_2015 omitted)
i.emb_et        _Iemb_et_1-8        (naturally coded; _Iemb_et_1 omitted)
i.donor         _Idonor_0-1         (naturally coded; _Idonor_0 omitted)

Generalized linear models                      No. of obs      =      1,451
Optimization    : MQL Fisher scoring           Residual df     =      1,441
                  (IRLS EIM)                    Scale parameter =          1
Deviance        = 1986.756988                   (1/df) Deviance =   1.378735
Pearson         = 500001438.5                   (1/df) Pearson  =   346982.3

Variance function: V(u) = u*(1-u)              [Bernoulli]
Link function    : g(u) = u                    [Identity]

                                               BIC             =  -8503.735
```

158

```
------------------------------------------------------------------------------
             |                    EIM
   livebirth | Risk Diff.  Std. Err.      z    P>|z|    [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Idate_2016 |  .0386343   .0254685     1.52   0.129    -.0112831    .0885516
 _Idate_2017 |  .2508098   .0494786     5.07   0.000     .1538335    .3477861
       age_v | -.0097439   .0028176    -3.46   0.001    -.0152663   -.0042215
     fresh_2 |  .0236005    .028773     0.82   0.412    -.0327934    .0799945
  _Iemb_et_2 |  .2079909   .0302099     6.88   0.000     .1487805    .2672013
  _Iemb_et_3 |  .0587581    .041733     1.41   0.159    -.0230371    .1405532
  _Iemb_et_4 |  .1652187   .0683825     2.42   0.016     .0311916    .2992459
  _Iemb_et_5 |  .0799748   .2120258     0.38   0.706    -.3355882    .4955378
   _Idonor_1 |  .1856799   .0382461     4.85   0.000     .1107189     .260641
       _cons |  .5005719   .1055795     4.74   0.000       .29364    .7075038
------------------------------------------------------------------------------
Post tests

. test _Idate_2016 _Idate_2017

 ( 1)  _Idate_2016 = 0
 ( 2)  _Idate_2017 = 0

          chi2( 2) =   25.72
        Prob > chi2 =    0.0000   overall date (year) a significant factor

. test _Iemb_et_2 _Iemb_et_3 _Iemb_et_4 _Iemb_et_5 _Iemb_et_6 _Iemb_et_8

 ( 1)  _Iemb_et_2 = 0
 ( 2)  _Iemb_et_3 = 0
 ( 3)  _Iemb_et_4 = 0
 ( 4)  _Iemb_et_5 = 0

          chi2( 4) =   52.50
        Prob > chi2 =    0.0000   number of emb a significant factor
```

- Year and number emt and donor are significant factors
- Age of the women inversely associated with live birth
- Fresh status not associated adjusted for the other factors p=.412

Also ran a model with an interaction term between year and fresh status. This interaction is not significant p=.8207. Hence the model and results presented above is fine.

Summary

- Other factors are important in determining a live birth, but fresh status is not a factor that should be considered.

EXTRA MODELS REQUIRED (RESULTS PLUS)

MULTIPLE REGRESSION MODEL OF LBR – USING Both age and ova age

```
. xi: binreg livebirth i.date age_v ovaage fresh_2  i.emb_et i.donor, rd
i.date           _Idate_2015-2017   (naturally coded; _Idate_2015 omitted)
i.emb_et         _Iemb_et_1-8       (naturally coded; _Iemb_et_1 omitted)
i.donor          _Idonor_0-1        (naturally coded; _Idonor_0 omitted)

Generalized linear models                      No. of obs      =      1,451
Optimization     : MQL Fisher scoring          Residual df     =      1,440
                   (IRLS EIM)                  Scale parameter =          1
Deviance       =  1985.766486                  (1/df) Deviance =   1.379005
Pearson        =  500001438.5                  (1/df) Pearson  =   347223.2

Variance function: V(u) = u*(1-u)              [Bernoulli]
Link function    : g(u) = u                    [Identity]

                                               BIC             =  -8497.445

-----------------------------------------------------------------------------
             |               EIM
   livebirth | Risk Diff.   Std. Err.     z    P>|z|     [95% Conf. Interval]
-------------+---------------------------------------------------------------
 _Idate_2016 |  .0388908    .0253855    1.53   0.126    -.0108638    .0886454
 _Idate_2017 |  .2530454    .049504     5.11   0.000     .1560194    .3500714
       age_v | -.0077162    .0037029   -2.08   0.037    -.0149737   -.0004587
      ovaage | -.0038576    .0039212   -0.98   0.325    -.011543     .0038277
     fresh_2 |  .0247536    .0287801    0.86   0.390    -.0316543    .0811615
   _Iemb_et_2 | .2080037    .0301108    6.91   0.000     .1489876    .2670198
   _Iemb_et_3 | .0659823    .0417399    1.58   0.114    -.0158264    .1477909
   _Iemb_et_4 | .175466     .0685005    2.56   0.010     .0412074    .3097245
   _Iemb_et_5 | .0899128    .2107053    0.43   0.670    -.3230619    .5028875
    _Idonor_1 | .1339218    .0668739    2.00   0.045     .0028514    .2649921
        _cons |  .5623037   .1181827    4.76   0.000     .3306699    .7939376
-----------------------------------------------------------------------------
```

MULTIPLE REGRESSION MODEL OF LBR – USING Ovaage only

```
. xi: binreg livebirth i.date ovaage fresh_2  i.emb_et i.donor, rd
i.date           _Idate_2015-2017   (naturally coded; _Idate_2015 omitted)
i.emb_et         _Iemb_et_1-8       (naturally coded; _Iemb_et_1 omitted)
i.donor          _Idonor_0-1        (naturally coded; _Idonor_0 omitted)

Generalized linear models                      No. of obs      =      1,453
Optimization     : MQL Fisher scoring          Residual df     =      1,443
                   (IRLS EIM)                  Scale parameter =          1
Deviance       =  1994.273651                  (1/df) Deviance =   1.382033
Pearson        =  500001440.5                  (1/df) Pearson  =   346501.3

Variance function: V(u) = u*(1-u)              [Bernoulli]
Link function    : g(u) = u                    [Identity]

                                               BIC             =  -8512.766

-----------------------------------------------------------------------------
             |               EIM
   livebirth | Risk Diff.   Std. Err.     z    P>|z|     [95% Conf. Interval]
-------------+---------------------------------------------------------------
 _Idate_2016 |  .038496     .0255097    1.51   0.131    -.0115021    .0884941
 _Idate_2017 |  .2463984    .0496575    4.96   0.000     .1490714    .3437254
      ovaage | -.0087958    .0029972   -2.93   0.003    -.0146701   -.0029214
     fresh_2 |  .0286424    .0288007    0.99   0.320    -.0278059    .0850907
   _Iemb_et_2 | .208862     .030238     6.91   0.000     .1495965    .2681275
   _Iemb_et_3 | .0622815    .0422819    1.47   0.141    -.0205896    .1451526
   _Iemb_et_4 | .1614189    .068815     2.35   0.019     .0265439    .2962939
   _Iemb_et_5 | .0752075    .2127718    0.35   0.724    -.3418176    .4922326
    _Idonor_1 | .0283606    .0425955    0.67   0.506    -.055125     .1118461
        _cons |  .4625563   .1105748    4.18   0.000     .2458337    .6792789
-----------------------------------------------------------------------------

. test _Iemb_et_2 _Iemb_et_3 _Iemb_et_4 _Iemb_et_5

 ( 1)  _Iemb_et_2 = 0
```
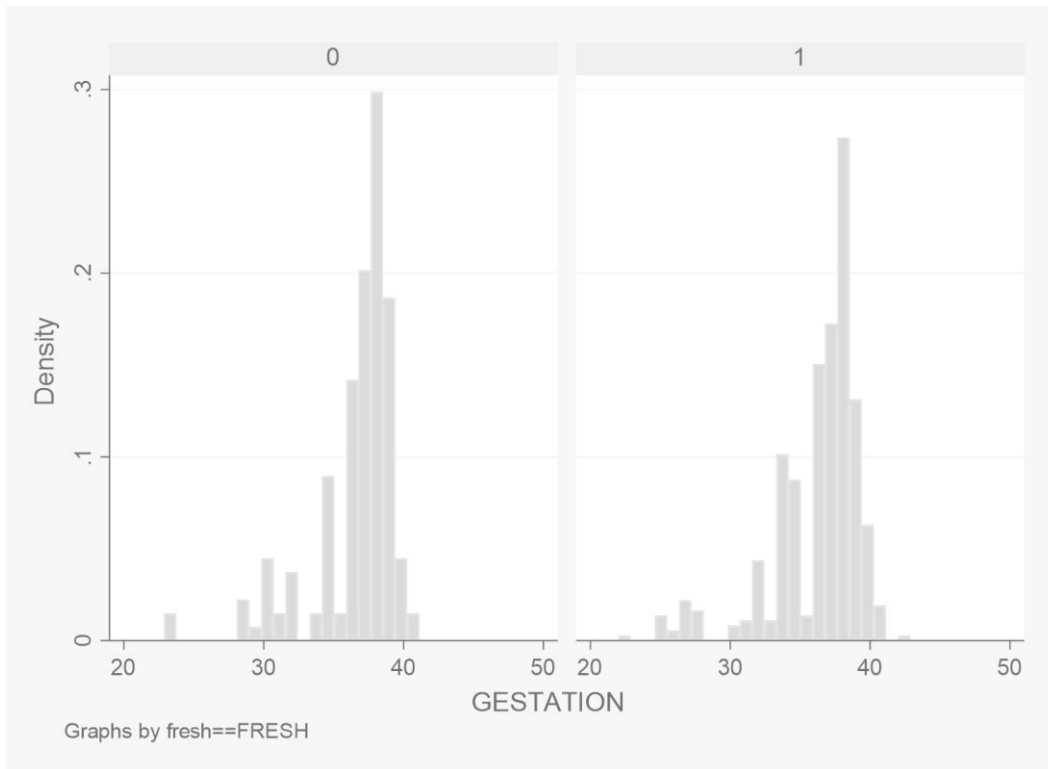
160

```
( 2)  _Iemb_et_3 = 0
( 3)  _Iemb_et_4 = 0
( 4)  _Iemb_et_5 = 0

        chi2(  4) =   52.05
      Prob > chi2 =   0.0000  number of embryos a significant factor
```
- Ova age significantly associated

MULTIPLE REGRESSION MODEL OF GESTATIONAL AGE

Used quantile regression (median) since the gestational age negatively skewed (fresh=1)

Graphs by fresh==FRESH

Gestation

```
xi: qreg gestation i.date ovaage fresh_2  i.emb_et i.donor,
i.date            _Idate_2015-2017    (naturally coded; _Idate_2015 omitted)
i.emb_et          _Iemb_et_1-8        (naturally coded; _Iemb_et_1 omitted)
i.donor           _Idonor_0-1         (naturally coded; _Idonor_0 omitted)

Median regression                          Number of obs =       456
  Raw sum of deviations    478.4 (about 37)
  Min sum of deviations 468.4868             Pseudo R2     =     0.0207

------------------------------------------------------------------------------
  gestation |     Coef.   Std. Err.      t    P>|t|    [95% Conf. Interval]
------------+-----------------------------------------------------------------
_Idate_2016 |  .2842116   .3448468     0.82   0.410    -.3935148    .9619381
_Idate_2017 |  .2421062   .5323918     0.45   0.650    -.8042018    1.288414
     ovaage | -.0105264   .0416241    -0.25   0.800      -.09233    .0712773
    fresh_2 | -.0315791   .3626137    -0.09   0.931    -.7442228    .6810646
 _Iemb_et_2 | -.8842101   .5014527    -1.76   0.079    -1.869714    .1012935
 _Iemb_et_3 | -1.894736   .7600082    -2.49   0.013    -3.388378   -.4010945
 _Iemb_et_4 | -1.468421   1.017061    -1.44   0.150    -3.467247    .5304055
 _Iemb_et_5 |  .8315783   3.334086     0.25   0.803    -5.720891    7.384048
  _Idonor_1 | -.4421069   .5067888    -0.87   0.383    -1.438097    .5538836
       _cons |  38.33684   1.486012    25.80   0.000     35.41639     41.2573
------------------------------------------------------------------------------

. test _Iemb_et_2 _Iemb_et_3 _Iemb_et_4 _Iemb_et_5

 ( 1)  _Iemb_et_2 = 0
```

161

```
( 2)  _Iemb_et_3 = 0
( 3)  _Iemb_et_4 = 0
( 4)  _Iemb_et_5 = 0

      F(  4,   446) =    1.73
           Prob > F =    0.1423   number of embryos not significant factor
```

- Nothing associated.
- Note that this analysis is only done in 456 women. This is a small subgroup

MULTIPLE REGRESSION MODEL OF BIRTHWEIGHT

```
. xi: qreg weight i.date ovaage fresh_2  i.emb_et i.donor,
i.date            _Idate_2015-2017    (naturally coded; _Idate_2015 omitted)
i.emb_et          _Iemb_et_1-8        (naturally coded; _Iemb_et_1 omitted)
i.donor           _Idonor_0-1         (naturally coded; _Idonor_0 omitted)

Median regression                          Number of obs =         390
  Raw sum of deviations   115007 (about 2830)
  Min sum of deviations 109862.5           Pseudo R2     =      0.0447

------------------------------------------------------------------------------
      weight |    Coef.    Std. Err.      t     P>|t|    [95% Conf. Interval]
-------------+----------------------------------------------------------------
 _Idate_2016 |  149.5455   100.7705     1.48    0.139    -48.59046    347.6814
 _Idate_2017 |       275   158.6771     1.73    0.084    -36.99243    586.9924
      ovaage | -25.45455   12.23923    -2.08    0.038    -49.51944   -1.389653
     fresh_2 |  116.8182   106.9346     1.09    0.275    -93.43775    327.0741
  _Iemb_et_2 | -326.8182   140.1102    -2.33    0.020    -602.3042   -51.33221
  _Iemb_et_3 | -216.3636   230.6318    -0.94    0.349    -669.8341    237.1068
  _Iemb_et_4 | -567.2727   282.4498    -2.01    0.045    -1122.628   -11.91709
    _Idonor_1 | -448.1818  148.9225    -3.01    0.003    -740.9946    -155.369
       _cons |  3868.636   428.0668     9.04    0.000    3026.967    4710.305
------------------------------------------------------------------------------

. test _Iemb_et_2 _Iemb_et_3 _Iemb_et_4

( 1)  _Iemb_et_2 = 0
( 2)  _Iemb_et_3 = 0
( 3)  _Iemb_et_4 = 0

      F(  3,   381) =    2.31
           Prob > F =    0.0762   number of embryos not a significant factor
```

- NB n=390 thus small subgroup analysis
- Ova age negatively associated -25.4 gm per year
- Donor material leads to a lower birthweight -448gm

Appendix P: Plagiarism Report

163

E. Meiring MSc Thesis

by Elizabeth Meiring

Submission date: 01-Dec-2019 10:01AM (UTC+0200)
Submission ID: 1224213767
File name: 49949_Elizabeth_Meiring_E._Meiring_MSc_Thesis_79786_1804273106.docx (12.21M)
Word count: 23614
Character count: 128754

E. Meiring MSc Thesis

ORIGINALITY REPORT

16%	**6**%	**13**%	**8**%
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Appendix Q: HREC Approval Letter & Progress Report

Health Research Ethics Committee (HREC)

Approval Notice

New Application

08/06/2018

Project ID : 6949

HREC Reference #: S18/05/092

Title: Report on the live birth rates and neonatal outcomes of ART patients at Drs. Aevitas Fertility Clinic; Implications of a frozen embryo transfer (FET) program

Dear Miss Elizabeth Meiring,

The **New Application** received on 21/05/2018 14:18 was reviewed by members of **Health Research Ethics Committee 2 (HREC2)** via **expedited** review procedures on 08/06/2018 and was approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: **This project has approval for 12 months from the date of this letter.**

Please remember to use your **Project ID [6949]** on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see *Forms and Instructions* on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Departement of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website https://applyethics.sun.ac.za/ProjectView/Index/6949

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Francis Masiye ,

HREC Coordinator,

Health Research Ethics Committee 2 (HREC2).

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY
Approval Letter
Progress Report

01/07/2019

Project ID: 6949

Ethics Reference No: S18/05/092

Title: Report on the live birth rates and neonatal outcomes of ART patients at Drs. Aevitas Fertility Clinic; Implications of a frozen embryo transfer [FET] program

Dear Miss Elizabeth Meiring

Your request for extension/annual renewal of ethics approval dated 07/06/2019 11:36 refers.

The Health Research Ethics Committee reviewed and approved the annual progress report you submitted through an expedited review process.

The approval of this project is extended for a further year.

Approval date: 01 July 2019

Expiry date: 30 June 2020

Kindly be reminded to submit progress reports two (2) months before expiry date.

Where to submit any documentation

Kindly note that the HREC uses an electronic ethics review management system, *Infonetica*, to manage ethics applications and ethics review process. To submit any documentation to HREC, please click on the following link: https://applyethics.sun.ac.za.

Please remember to use your Project Id 6949 and ethics reference number S18/05/092 on any documents or correspondence with the HREC concerning your research protocol.

Yours sincerely,

Ms Elvira Rohland
Health Research Ethics Committee 2 (HREC2)